An increase in suppressive dendritic cells and T cells is a hallmark of healthy aging, a phenomenon exacerbated by cancer and modulated by chemo- and immunotherapy

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

**Human Ethics** The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number #HR102_2012

**Animal Ethics** The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number #AEC-2012-21

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Abstract

Dendritic cell (DC) and T cell function changes throughout life, and their age-related changes may contribute to the increased incidence of cancer in the elderly. However, the effects of healthy aging on human and murine DC function, and DC/T cell interactions are not well-characterised, with studies reporting conflicting results. Even less is known about the combined effects of aging and cancer on DC and T cell function. Thus, this thesis aimed to: (i) examine the effects of aging on blood DCs from healthy young (21-33 years of age) and elderly (60-77 years of age) human volunteers, and blood and lymphoid tissue DCs from healthy young (2-5 months of age; equivalent to 16-26 human years) and elderly mice (20-27 months of age; equivalent to 60-80 human years); examine the effects of mesothelioma (a cancer caused by asbestos exposure) on (ii) young and elderly DCs in vitro, and (iii) in vivo in mice; and (iv) determine the effects of chemotherapy and immunotherapy on young and elderly murine DCs and T cells in vivo.

This study showed that regardless of age: (i) mesothelioma reduced cross-presenting human blood-derived myeloid (m)DC2s, and murine CD8α⁺CD11b⁻ conventional DCs, and increased suppressive plasmacytoid DC proportions in murine tumour-draining lymph nodes (TDLNs) and spleens; (ii) mesothelioma induced semi-mature human monocyte-derived DCs (characterised by increased CD40, IL-12 and tumour necrosis factor (TNF)-α, and decreased CD1a and interferon (IFN)-γ), and murine TDLN DCs (characterised by increased MHC class II and CD80, and reduced CD40, TNF-α and IL-12); and (iii) mesothelioma-exposed DCs showed decreased responses to lipopolysaccharide (LPS)/IFN-γ and IL-2/CD40 stimulation, and up-regulated expression of inhibitory molecules, including the adenosine-producing enzymes CD39 and CD73, the adenosine-binding A2A and A2B receptors, and the suppressive cytokine transforming growth factor (TGF)-β after chemotherapy. This suggests that anti-mesothelioma immune responses may be impaired, contributing to tumour progression, and reducing the efficacy of chemotherapeutic and immunotherapy.

With aging: (i) the mesothelioma-induced semi-mature DC status was exacerbated;
(ii) elderly murine lymph node and splenic DCs and T cells were skewed towards a suppressive/regulatory status, characterised by increased expression of multiple inhibitory molecules, including CD39, CD73, A2A and A2B receptors, programmed cell death ligand-1 (PD-L1), programmed cell death protein-1 (PD-1), cytotoxic T lymphocyte-associated protein-4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), IL-10 and/or TGF-β; and (iii) chemotherapy and IL-2/CD40 exacerbated the suppressive status of elderly DCs and T cells. These results suggest that antimesothelioma immune responses may be further compromised in the elderly, and that combining chemotherapy and IL-2/CD40 with checkpoint blockade of inhibitory molecules is required to improve the efficacy of anti-cancer therapies in elderly patients with mesothelioma.
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<tbody>
<tr>
<td>A2AR</td>
<td>Adenosine-binding A2A receptor</td>
</tr>
<tr>
<td>A2BR</td>
<td>Adenosine-binding A2B receptor</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BATF3</td>
<td>Basic leucine zipper transcription factor ATF-like 3</td>
</tr>
<tr>
<td>BDCA</td>
<td>Blood dendritic cell antigen</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BST-2</td>
<td>Bone marrow stromal antigen-2</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
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<tr>
<td>cDC</td>
<td>Conventional dendritic cell</td>
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<tr>
<td>CDP</td>
<td>Common dendritic cell precursor</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CLIP</td>
<td>Class II invariant chain peptide</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte-associated protein-4</td>
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<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DLN</td>
<td>Draining lymph node</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELC</td>
<td>EBI1 ligand chemokine</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>Abbreviation</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<td>Killer-cell lectin-like receptor G1</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte activation gene-3</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cell</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>mDC1</td>
<td>Myeloid dendritic cell 1</td>
</tr>
<tr>
<td>mDC2</td>
<td>Myeloid dendritic cell 2</td>
</tr>
<tr>
<td>MDP</td>
<td>Monocyte, macrophage and dendritic cell precursor</td>
</tr>
</tbody>
</table>
MDSC  Myeloid-derived suppressor cell
MHC  Major histocompatibility complex
MIP  Macrophage inflammatory protein
MLR  Mixed lymphocyte reaction
MoDC  Monocyte-derived dendritic cell
NCS  Normal calf serum
NFκB  Nuclear factor kappa B
NK cell  Natural killer cell
NLR  Nucleotide-binding oligomerisation domain-like receptor
NLRP3  Nucleotide-binding oligomerisation domain-like receptor family pyrin domain containing-3
PAMP  Pathogen-associated molecular pattern
PBMC  Peripheral blood mononuclear cell
PBS  Phosphate buffered saline
PD-1  Programmed cell death protein-1
pDC  Plasmacytoid dendritic cell
PD-L1  Programmed cell death ligand-1
PD-L2  Programmed cell death ligand-2
Pre-cDC  Pre-conventional dendritic cell
PRR  Pattern recognition receptor
SLC  Secondary lymphoid tissue chemokine
SMAC  Supra-molecular activation complex
STAT  Signal transducer and activator of transcription
TAP  Transporter associated with antigen processing
TCM  Tumour cell-conditioned media
TCR  T cell receptor
TDLN  Tumour-draining lymph node
TGF-β  Transforming growth factor-beta
Th  T helper
TIM-3  T cell immunoglobulin and mucin-domain containing-3
TipDC  Tumour necrosis factor and inducible nitric oxide synthase-producing dendritic cell
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter 1  Introduction

1.1  Dendritic cells and aging

Most of our current understanding of dendritic cells (DCs), specialised antigen-presenting cells (APCs) first described as stellate cells in mouse spleens (1, 2), comes from studies using young adult humans and murine models. We now know that DCs are a heterogeneous population comprised of several subsets, which can be broadly divided into conventional/myeloid DCs (cDCs/mDCs) and plasmacytoid DCs (pDCs; 3, 4). DCs link innate and adaptive immunity and play a key role in the generation of antigen-specific T cell immune responses (3, 5, 6). In addition, DCs play an important role in the induction and maintenance of immune tolerance (7, 8). DCs are highly responsive to local signals that influence their differentiation into immunogenic or tolerogenic cells (5, 6, 9).

Similar to our bodies, immune cells undergo chronological development, starting after conception with changes continuing throughout life, and these changes impact immune responses to infection and cancer. Fetal/neonatal immune cells are quantitatively and qualitatively different to their young adult counterparts (10-13). The fetal immune system is skewed away from pro-inflammatory function and displays more features of anti-inflammatory/tolerogenic function (e.g. increased levels of suppressive mediators in plasma) to reduce the risk of potentially harmful alloimmune reactions between the fetus and mother (13-16). This profile is reflected in human neonatal DCs which are skewed away from pro-inflammatory function (17-20), leaving the neonate susceptible to infections and with a reduced capacity to respond to vaccinations (13, 21-24). However, studies examining murine and human neonatal DCs report conflicting results and a consensus has not yet been reached. DC function continues to develop and change over the first years of life (14, 17, 25). In general, this is characterised by a reduction in anti-inflammatory cytokines and a simultaneous gradual increase in the ability to mount pro-inflammatory immune responses beginning around three months of age and continuing across the first two years of life (26, 27), with some components of DC function taking longer than 12 years to reach adult levels (28). This may explain why
children remain more susceptible to infections, compared to adults (14, 17, 25).

Immune cells, including DCs, continue to change as the age of the individual increases beyond young adulthood, and age-associated modulation of the immune system at the other end of the age spectrum is referred to as immunosenescence (29-31). Immunosenescence is a complex process that is not yet fully understood. It can involve seemingly contradictory changes, for example, increases in concentrations of circulating pro-inflammatory cytokines; a phenomenon termed “inflamm-aging” (32-34), yet impaired immune function, and increases in anti-inflammatory cytokines and suppressive activity (29, 35-38). There is a general consensus that adaptive immune function declines with age (36, 39), whilst changes to innate immunity, including DCs, are less well-defined (30, 35, 37, 40, 41). Overall, the result seems to be disruption of the balance between immune activation and tolerance, and the outcomes are increased susceptibility of elderly individuals to infections, cancer and autoimmunity, and reduced responses to vaccinations (31, 42-45). Similar to our understanding of immune function, particularly innate immune function, during the early years of life, little is known about the later years of life with contradictory results reported for DCs and the consequences of DC/T cell interactions. Thus, this project aimed to examine how immunosenescent changes to DCs during healthy aging are modulated by the presence of a cancer, mesothelioma, and whether anti-cancer treatment improves or degrades elderly DC function.

1.1.1 Development of dendritic cells from bone marrow precursors

Studies in young adult mice show that the majority of DC subsets arise from a distinct haemopoietic lineage in the bone marrow (BM), and that tissue DCs are continually replenished by BM precursors (3, 46, 47). Murine BM haemopoietic stem cells sequentially give rise to myeloid precursors, granulocyte-macrophage precursors (GMPs), monocyte, macrophage and DC precursors (MDPs), which in turn produce monocytes and common DC precursors (CDPs; Figure 1.1; 46, 48).
Figure 1.1 Development of human and murine DCs

Abbreviations used: haemopoietic stem cells (HSCs), granulocyte-macrophage precursor (GMP), monocyte, macrophage and DC precursor (MDP), common DC precursor (CDP), pre-conventional dendritic cell (pre-cDC), plasmacytoid dendritic cell (pDC), multi-lymphoid precursor (MLP), dendritic cell (DC). Growth factors, cytokines and transcription factors required for each step of the developmental pathway are shown in italics. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (49) © 2011.

Plasmacytoid DCs and pre-conventional DCs (pre-cDCs) arise from CDPs and travel via the blood to seed peripheral tissues (50-52). Plasmacytoid DCs are fully differentiated once they leave the BM and do not undergo further development in peripheral tissues (53). In contrast, pre-cDCs differentiate into cDC subsets in peripheral lymphoid and non-lymphoid tissues (Figure 1.1; 46, 50, 51). In humans, DCs originate from BM GMPs and multi-lymphoid precursors (Figure 1.1; 54, 55). Recent studies have identified that, similar to mice, human GMPs sequentially give rise to MDPs and CDPs, which differentiate into the three major human DC subsets; CD1c+ DCs, CD141+ DCs and pDCs (56, 57).

With aging, numbers of haemopoietic precursor cells increase in the BM of elderly
humans and mice, and demonstrate increased myeloid differentiation potential (58-63). However, aged BM precursors display defects, such as DNA and oxidative damage (58, 64, 65), and reduced self-renewal and homing capacity (58, 59), which may impact on their ability to differentiate into functional immune cells. A single study has reported that the capacity of elderly murine BM precursors to differentiate into DCs in vitro is reduced with age (66). However, most studies to-date have reported an intact ability of elderly human monocytes and murine BM precursors to differentiate into DCs in vitro (67-71), and one study observed an improved ability of aged murine BM precursors to reconstitute cDCs in vivo (72).

1.1.2 Murine dendritic cell subsets

Murine DCs have been typically identified based on their stellate morphology, high efficiency at presenting antigens to, and activating, naïve T cells, and surface phenotype, particularly expression of the integrin, CD11c, and the antigen-presenting molecule, major histocompatibility complex (MHC) class II (2, 3, 73-75). With advances in technology, such as polychromatic flow cytometry, mass cytometry and transcriptional profiling, the characteristics used to identify and classify DC subsets are expanding (3, 76, 77). Furthermore, recent studies have shown that features typically used to identify DCs are shared with other cell types (3, 77). For example, CD11c is expressed by monocytes/macrophages (78-81), B cells (82, 83), activated T cells (84-86), and natural killer (NK) cells (87). This has led to debate about DCs and macrophages representing the same, or different, cell types, as they share other overlapping features (77, 79, 88, 89). DCs can express markers used to identify monocytes/macrophages, such as CD14; lipopolysaccharide (LPS) co-receptor; a pattern recognition receptor (90) and F4/80; a member of the epidermal growth factor-seven transmembrane receptor family (91-93), and monocytes/macrophages prime naïve T cells, although not as efficiently as DCs (94-96). Nonetheless, there is strong evidence that DCs and monocytes/macrophages are distinct cell types, as recent ontogenic studies reported that DCs have a distinct developmental lineage (46, 73, 97, 98), and transcriptional studies revealed that genetic profiles for DC subsets cluster away
from macrophages (3, 99-102). Thus, CD11c could be considered a marker of APCs, with CD11c+ DCs being the most efficient APCs (4, 74).

Characterisation of DC subsets is complex and represents ongoing research (3, 73). Murine DCs can be broadly classified into cDCs and pDCs. Conventional DCs can be further separated into non-migratory lymphoid-tissue resident cDCs which differentiate within lymphoid tissues and remain there for their lifespan, and migratory cDCs which reside in non-lymphoid peripheral tissues where they capture antigens and subsequently transport them to draining lymph nodes (DLNs; 3).

1.1.2.1 Murine lymphoid-tissue resident conventional dendritic cells

Lymphoid-tissue resident cDCs can be divided into two main subsets based on expression of CD8α (α chain of the CD8+ T cell co-receptor) and CD11b (complement receptor 3, an integrin): CD8α+CD11b+ cDCs and CD11b+CD8α- cDCs, which are present in spleens, lymph nodes (LN), thymus and mucosa-associated lymphoid tissues (3, 4, 103). These two subsets have distinct transcriptional profiles (3, 99, 102, 104-106) and functional specialisations (3, 107).

CD8α+CD11b+ cDCs comprise 20-40% of LN and splenic cDCs, and the majority of thymic cDCs (3, 108-110). CD8α+CD11b+ cDC development requires the growth factor fms-related tyrosine kinase-3 ligand (FLT3L) and the transcription factors inhibitor of DNA binding protein 2 (ID2), basic leucine zipper transcription factor ATF-like 3 (BATF3) and interferon regulatory factor (IRF)-8 (3, 111, 112). CD8α+CD11b+ cDCs have specialised antigen uptake and presentation pathways (3) and are highly efficient at capturing antigens from dead and necrotic cells due to expression of the scavenger receptor CD36, the endocytic receptor CD205/DEC-205, and the lectin CLEC9A (113-119). CD8α+CD11b+ cDCs have a superior ability to cross-present antigens to CD8+ T cells (3, 111, 118, 120-123), and their deletion abrogates the generation of antigen-specific effector CD8+ T cells in murine viral infection and tumour models, highlighting the critical role they play (124-128). Several features of CD8α+CD11b+ cDCs confer their superior ability to prime CD8+ T cells, including greater expression of genes in the MHC class I pathway (120), secretion of cytokines involved in CD8+ T cell differentiation (IL-12 and IL-15; 129, 130-133), antigen
capture via CLEC9A favouring antigen diversion into the MHC class I pathway (114-116, 134), and expression of the chemokine receptor XCR1, which binds its ligand (XCL1) produced by CD8+ T cells, facilitating more stable DC/T cell interactions and T cell activation (135). Whilst their main role is priming CD8+ T cells, CD8α+CD11b− cDCs can also present antigens to CD4+ T cells, and together with secretion of IL-12, activate T helper (Th)-1 responses (132, 136). Thus, CD8α+CD11b− cDCs are key mediators of anti-viral and anti-tumour immunity (3, 111). In addition, CD8α+CD11b− cDCs contribute to tolerance by promoting deletion of self-reactive T cells in the thymus, spleen and LNs (3, 111, 137-139), and inducing regulatory T cell (Treg) development (140).

The other main lymphoid tissue-resident cDC subset, CD11b+CD8α− cDCs, comprises approximately 75% of total DCs in spleens and 20-40% of total DCs in LNs (4). Splenic CD11b+CD8α− cDCs can be divided into CD4+ (CD4+ T cell co-receptor) and CD4− subsets, which represent 55% and 20% of total splenic DCs, respectively (109), however, these two subsets have similar gene expression profiles (99, 106) and functions (3). The main function of CD11b+CD8α− cDCs is priming CD4+ T cells, particularly Th2 responses, thus mediating defence against extracellular pathogens and allergic responses (109, 120, 132, 136, 141, 142). CD11b+CD8α− cDCs are more efficient than CD8α+CD11b− cDCs in priming CD4+ T cells, due to higher expression of genes involved in the MHC class II pathway (120, 143). In general, CD11b+CD8α− cDCs are poor antigen cross-presenters (123), but can cross-present under certain conditions, for example, after activation through Fcγ receptors (144), or when tumours (145) or pathogens are present (146).

1.1.2.2 Murine migratory conventional dendritic cells

Migratory cDCs can be divided into two major subsets: CD103+CD11b− cDCs and CD11b+ cDCs (3), and are located in skin, lungs, liver, small intestine, pancreas and Peyer’s patches, as well as in LNs draining these sites, but not in spleen (3, 112, 147-149). Migratory cDCs also include specialised subsets, such as Langerhans cells (LCs) in the epidermis (150). Migratory cDCs comprise 1-5% of tissue cells, depending on the organ (3).
In mice, CD103+ (integrin αE) cDCs are the migratory counterpart for CD8α+CD11b- cDCs, based on: similar transcriptional profiles (99, 102, 104, 112, 135, 147, 149, 151), similar transcription factors required for development (112, 127, 152), expression of the surface receptors XCR1 and CLEC9A (101, 153-156), and functional specialisation, i.e. capturing antigens from dead/necrotic cells, antigen cross-presentation to CD8+ T cells, and activating Th1 responses (147, 157-160). In the healthy steady state, CD103+ cDCs are involved in the induction and maintenance of peripheral tolerance by promoting Treg development (147, 158, 160-162).

Migratory CD11b+ cDCs are transcriptionally and functionally similar to their lymphoid-resident CD11b+ cDC counterparts (3, 99, 107, 158), and mediate responses against extracellular pathogens by preferentially priming CD4+ T cells (160, 163-165) and inducing Th2 (166-171) and Th17 responses (143, 172-174). In the periphery, CD11b+ cDCs play a role in stimulation of memory CD8+ T cells (175). CD11b+ cDCs contribute to tolerance by inducing Treg development via retinoic acid (162, 165, 176-178), and inducing T cell anergy by presenting self-antigens after migrating to LNs (179, 180).

1.1.2.3 Murine plasmacytoid dendritic cells

Plasmacytoid DCs are found in lymphoid tissues, where they represent 0.3-0.5% of cells (181), and many non-lymphoid tissues, where their proportions vary depending on tissue site (182). Plasmacytoid DCs require a unique transcription factor (E2-2) for their development (183, 184), and transcriptional analyses show that pDCs are related to the other DC subsets, but cluster separately from cDCs (99, 102, 104, 185). Murine pDCs are identified by low to intermediate expression of CD11c, and expression of MHC class II, SiglecH (a sialic acid binding lectin; 186), bone marrow stromal antigen-2 (BST2), B220/CD45RA (a member of the protein tyrosine phosphatase family), Ly6C/GR-1 (a glycoposphatidylinositol-linked protein), CD4 and CD8 (181, 183, 187). A defining characteristic of pDCs is their high expression of Toll-like receptors (TLRs) 7 and 9, which allow them to sense viral nucleic acids, and respond by producing large amounts of type I interferons (IFN-α and β); pDCs are the most potent producers of these cytokines (181, 187-190). This
and their production of IFN-λ (IL-28/29) upon virus encounter (191) makes pDCs important mediators of anti-viral immunity. TLR-mediated production of type I IFNs, and other pro-inflammatory cytokines (IL-12, tumour necrosis factor (TNF)-α and IL-6) by pDCs exert immunostimulatory effects on other immune cells, such as cDCs, NK cells and macrophages (181, 182, 187, 189, 190, 192, 193). Although naïve pDCs are poor APCs (182), TLR-activated pDCs acquire the ability to prime T cells by up-regulating antigen-presenting and co-stimulatory molecules (182, 194, 195). Activated pDCs are capable of priming CD4+ Th1 cells (196-198) and CD8+ T cells (199), and cross-presenting antigens to CD8+ T cells (194), although not as efficiently as cDCs (182, 183). In addition to their immunostimulatory functions, pDCs have been implicated as mediators of tolerance, particularly due to their ability to induce Treg development (181, 197, 200-205).

1.1.2.4 Murine inflammatory dendritic cells

During inflammation (such as infection or active autoimmunity), inflammatory DCs emerge (206, 207). Inflammatory DCs are transiently formed and their phenotype is determined by the stimuli that induced them (103, 206, 207). The precursors for inflammatory DCs are usually circulating monocytes, however there is evidence that they can be derived from haemopoietic precursors (103, 206, 208, 209). In mice, Ly6Chi monocytes differentiate into inflammatory DCs in peripheral tissues after exposure to pathogenic stimuli (207, 210-213), and during non-pathogenic inflammation (207, 214, 215). Most murine inflammatory DCs are characterised by expression of CD11c, CD11b, MHC class II and Ly6C, and can express additional markers depending on the stimuli encountered (206, 207, 213). Similar to cDCs, inflammatory DCs can migrate to LNs and present/cross-present antigens to naïve CD8+ and CD4+ T cells (211, 216-218). A well-described subset of inflammatory DCs is TNF and inducible nitric oxide synthase-producing DCs (TipDCs), first observed in spleens of mice infected with *Listeria monocytogenes* (219); these DCs are important for clearing infections, and cause host tissue damage if not regulated (210, 219-221).
1.1.2.5  Murine in vitro dendritic cell models

Two main in vitro models are used to study murine DCs (3). The first involves culturing BM precursor cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) +/- IL-4 to generate CD11c⁺CD11b⁺ DCs that lack CD8α, CD4 and B220 expression, and are proposed to resemble inflammatory DCs (71, 222-224). This method does not generate pDCs (222). The second method, in which BM precursor cells are cultured with FLT3L, generates three DC populations (CD11b<sup>high</sup>, CD11b<sup>low</sup>, and pDCs) similar to in vivo splenic DC subsets (225-227). In this thesis, the GM-CSF/IL-4 bone marrow-derived DC (BMDC) model was used to extend our previous studies (228) into the context of aging.

1.1.2.6  Murine dendritic cells and aging

Only a few studies have examined DCs in middle-aged mice, 8-14 months of age, equivalent to approximately 34-47 human years (229, 230), and reported conflicting results (see Table 1.1), nonetheless they support the idea that DCs are different in later life, compared to young adult life.

Table 1.1 Age-related changes in DCs in middle-aged mice

<table>
<thead>
<tr>
<th>DC subset</th>
<th>Stimuli</th>
<th>Middle-aged (8-14 months) DC phenotype/function compared to young adults</th>
<th>Refs</th>
</tr>
</thead>
</table>
| Splenic CD11c⁺ or CD11c⁺CD11b⁺ DCs | Non-activated/ healthy | Increased: CD11c⁺ DCs  
Comparable: CD11c⁺CD11b⁺ DCs | (231) |
| Splenic CD8α⁺ cDCs, CD8α⁺ cDCs and pDCs | Non-activated/ healthy | Comparable: CD8α⁺ cDCs and pDCs, MHC-II, CD40, CD80, CD86 on cDCs  
Reduced: CD8α⁺ cDCs  
Increased: MHC-II, CD40, CD80, CD86 on pDCs | (232) |
| Splenic cDCs | In vivo E. cuniculi infection | Reduced: MHC-II, CD86, induction of IFN-γ⁺ CD8⁺ T cells  
Comparable: IL-12, T cell priming, induction of cytotoxic activity | (233)  
(234) |
| Splenic pDCs | In vivo E. cuniculi infection | Reduced: MHC-II  
Comparable: CD86  
Increased: PD-L1  
Elderly splenic pDCs suppress cDCs’ ability to induce CD25⁺IFN-γ⁺CD8⁺ T cells | (233) |
| LN DCs | In vivo ovalbumin + | Reduced: antigen uptake  
Comparable: CD11c⁺ DC numbers, MHC- | (235) |
Modulation of murine DCs continues into old age, but similar to the neonatal field this is an under-studied area. There are conflicting reports regarding changes to murine DC numbers/proportions in the healthy steady state (see Table 1.2).

**Table 1.2 Age-related changes in DC numbers/proportions in elderly healthy mice**

<table>
<thead>
<tr>
<th>DC subset</th>
<th>Stimuli</th>
<th>Middle-aged (8-14 months) DC phenotype/function compared to young adults</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN DCs</td>
<td>In vivo viral infection</td>
<td>Increased: pDCs Reduced: CD8α-cDCs</td>
<td>(235)</td>
</tr>
<tr>
<td>LN DCs</td>
<td>In vivo E. cuniculi infection</td>
<td>Reduced: CD80, CD86, IL-12, induction of T cell proliferation and cytotoxicity Comparable: MHC-II and CD40</td>
<td>(234)</td>
</tr>
<tr>
<td>Blood CD11c⁺ or CD11c⁺CD11b⁺ DCs</td>
<td>Non-activated/healthy</td>
<td>Increased proportions</td>
<td>(231)</td>
</tr>
<tr>
<td>Lung CD11c⁺ DCs</td>
<td>In vivo influenza and respiratory viral infections</td>
<td>Reduced: migration, CCR7 expression Comparable: CD40 and CD86</td>
<td>(236)</td>
</tr>
<tr>
<td>Thymic CD8α⁺ cDCs, CD8α⁺ cDCs and pDCs</td>
<td>Non-activated/healthy</td>
<td>Comparable: cDC proportions, MHC-II, CD40, CD80 and CD86 Reduced: pDC numbers</td>
<td>(232)</td>
</tr>
<tr>
<td>Peyer’s patches CD11c⁺ DCs</td>
<td>Non-activated/healthy</td>
<td>Reduced CD11c⁺ DC numbers</td>
<td>(237, 238)</td>
</tr>
</tbody>
</table>

**Table 1.2 Age-related changes in DC numbers/proportions in elderly healthy mice**

<table>
<thead>
<tr>
<th>DC subset</th>
<th>Tissue and change in elderly (&gt;16 months) DC proportions/numbers compared to young adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD11c⁺ MHC-II⁺ DCs or CD11c⁺ DCs</td>
<td>Spleen</td>
</tr>
<tr>
<td></td>
<td>Comparable (72, 239) ↑ (240, 241) ↓ (242, 243)</td>
</tr>
<tr>
<td>CD11c⁺CD11b⁺ cDCs</td>
<td>Comparable (241, 242, 245) ↑ (72) ↓ (231)</td>
</tr>
<tr>
<td>CD8α⁺ cDCs</td>
<td>Comparable (240-243, 245, 249) ↓ (72, 232, 250, 251) ↑ (240)</td>
</tr>
<tr>
<td>CD8α⁺ cDCs</td>
<td>Comparable (249, 250) ↑ (245, 251) ↓ (232, 240)</td>
</tr>
<tr>
<td>DC subset</td>
<td>Spleen</td>
</tr>
<tr>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>pDCs</td>
<td>Comparable (241, 242, 245, 249, 252) ↓ (232, 240, 250, 251)</td>
</tr>
<tr>
<td>Langerhans cells</td>
<td>↓ (253-257)</td>
</tr>
</tbody>
</table>

One reason for these conflicting reports may be due to differences in definitions of elderly mice and the ages of mice used. The majority of studies classify elderly mice as greater than 18 months of age; equivalent to approximately 56 human years (229, 230), although a few studies consider 16-17 month-old mice; approximately 51-55 human years (229, 230) to be elderly (232). Differences in mouse strains, tissues examined and markers used to identify DC subsets may also contribute to the conflicting reports. Further studies examining the effects of aging on murine DCs are required, as age-related changes in DC numbers/proportions may affect the generation of immune responses in the elderly. Changes in elderly murine DC function will be discussed in section 1.3, in relation to the DC lifecycle.

### 1.1.3 Human dendritic cell subsets

The identification and characterisation of human DC subsets is challenging, due to the rarity of DCs and limited access to human tissues. Early characterisation of human DCs came from blood and skin (3, 107, 258). Advances in technologies have assisted in identifying equivalent human and murine DC subsets (107, 185, 258).

Blood DCs comprising 0.1-1% of peripheral blood mononuclear cells (PBMCs; 259) are typically described as human leukocyte antigen (HLA)-DR (MHC class II)* lineagenegative cells, as they lack lineage markers that define other immune cell types; i.e. CD3 (T cell receptor (TCR), expressed on T cells), CD19/CD20 (component of the B cell co-receptor/a phosphoprotein, respectively, both expressed on B cells), CD14 (monocytes) and CD56 (neural cell adhesion molecule, expressed on NK cells; 3,
There are three major subsets in human blood: CD1c⁺ (blood dendritic cell antigen-1; BDCA-1; a molecule that presents lipid antigens to T cells) myeloid DC1 (mDC1), CD141⁺ (BDCA-3, thrombomodulin) myeloid DC2 (mDC2) and pDCs (260). A minor population of lineage negative CD16⁺ (low affinity IgG receptor III) DCs has been described, however transcriptional studies suggest they are more closely related to monocytes (104). CD1c⁺ mDC1s, CD141⁺ mDC2s and pDCs also exist in human lymphoid tissues (spleen, LNs and tonsils) as resident subsets (262-269), and can be found in non-lymphoid tissues, including skin, liver, lungs and intestine, as migratory cells (101, 154, 270-276). Blood DCs may be precursor forms of tissue-resident DCs in transit to peripheral tissues (101, 260, 263), as the former have an immature status and require activation in order to cross-present antigens, whilst the latter are more mature and capable of presenting antigens in vitro without prior activation (262). Additional DC subsets are present in human skin: LCs and CD14⁺ dermal DCs (277, 278). Similar to mice, human inflammatory DCs emerge during inflammation/infection (207, 279-282).

### 1.1.3.1 Human CD1c⁺ myeloid DC1

CD1c⁺ mDC1s are the major myeloid subset in human blood, comprising approximately 1% of PBMCs (107), and 19% of the HLA-DR⁺ lineage negative population (283), and are the predominant DC subset in non-lymphoid tissues (107). Genome expression profiling has shown that human CD1c⁺ mDC1s and murine lymphoid-resident and migratory CD11b⁺ cDCs are equivalents (104, 185). Like their murine counterparts, CD1c⁺ mDC1s express the integrin CD11b (264, 284) and are specialised in antigen presentation and CD4⁺ T cell activation (155, 266, 285, 286), particularly Th17 cells (173). In contrast to their murine equivalent, CD1c⁺ mDC1s secrete IL-12 and can therefore activate Th1 responses (173, 264). There are conflicting reports regarding the cross-presenting ability of CD1c⁺ mDC1s, with some studies showing they are inferior cross-presenters compared to CD141⁺ mDC2s (101, 155, 266, 285), and others showing that all human blood DC subsets are equally efficient at cross-presentation (262-264).
1.1.3.2 Human CD141+ myeloid DC

CD141+ mDC2s represent the rarest DC subset in human blood, comprising approximately 10% of blood DCs (284), and 0.01-0.02% of PBMCs (101, 284). CD141+ mDC2s are the human equivalent of murine CD8α+CD11b- cDCs and CD103+ cDCs, based on similarities in their transcriptomes (104, 185, 270), requirement for FLT3L and BATF3 for development (152, 154), expression of CD205/DEC-205, XCR1 and CLEC9A (116, 135, 154, 264, 266, 267, 287), and their identical functions, i.e. a superior ability to take up necrotic cells (116, 155), high efficiency of antigen cross-presentation to CD8+ T cells (101, 155, 266, 267), and IL-12 production which allows them to promote Th1 responses (266, 267).

1.1.3.3 Human plasmacytoid dendritic cells

Human pDCs are equivalent to murine pDCs (181, 185, 187), and represent approximately 1% of PBMCs and 50% of blood DCs (261). Human pDCs are characterised by expression of the surface molecules CD123 (IL-3 receptor), CD303 (the lectin CLEC4C; BDCA-2) and CD304 (neuropilin; BDCA-4), and low levels of CD11c (260, 284), and can be further classified based on CD2 (lymphocyte function-associated antigen-2, an adhesion molecule) expression (288). Similar to their murine counterparts, development of human pDCs requires the transcription factor E2-2 (289). Human pDCs exhibit the same functions as murine pDCs: (i) mediate anti-viral immunity due to high expression of TLRs 7 and 9 and secretion of large amounts of IFN-α and IFN-λ upon virus-induced activation (191, 290-292), (ii) acquisition of antigen-presenting function following activation, promoting their ability to activate Th1 and Th2 CD4+ T cells (198, 292-297), and cross-present antigens to CD8+ T cells (298-300), and (iii) mediate tolerance by inducing CD4+ T cell anergy (301) and promoting Treg development (202, 294, 302).

1.1.3.4 Human inflammatory dendritic cells

Human inflammatory DCs have been observed in chronic inflammatory conditions (e.g. rheumatoid arthritis, psoriasis, dermatitis) and tumour ascites (279, 280, 303, 304). Inflammatory DCs promote pro-inflammatory Th1 and Th17 responses (279,
and generally express CD11c, CD11b, MHC class II, and CD1a (a lipid antigen-presenting molecule), but the exact phenotypes can vary depending on local stimuli (207). A human equivalent of murine TipDCs has been identified which contributes to autoimmune pathogenesis (282, 305, 306). Further characterisation of human inflammatory DCs is required, particularly with regards to their origin; one study has shown that they can be derived directly from monocytes (306), this is supported by another study demonstrating that inflammatory DCs and in vitro monocyte-derived DCs (MoDCs) have similar transcriptional profiles (279). The classification of inflammatory DCs is also under debate, due to their transcriptional and functional overlap with monocytes/macrophages (207, 280, 281).

1.1.3.5 Human in vitro dendritic cell models

The isolation of human DC subsets is difficult due to the rarity of blood DCs and limited availability of other tissues, thus in vitro models are often employed. One method involves culturing CD34+ haemopoietic precursors with GM-CSF and TNF-α (307). A more common method is culturing peripheral blood CD14+ monocytes with GM-CSF and IL-4 (308, 309). The resulting MoDCs are CD11c+CD1a+ and CD14 negative, and display typical DC characteristics: in their immature state, they are capable of antigen uptake, and respond to maturation stimuli by up-regulating expression of antigen-presenting and co-stimulatory molecules and pro-inflammatory cytokines, and acquire the ability to prime naïve T cells (308-310). An advantage of this model is that it provides a convenient way to generate large numbers of DCs, and has been widely used to study DC biology and in clinical applications (reviewed by 311). However, the physiologic relevance of in vitro MoDCs is unclear, as their in vivo counterpart has yet to be identified (272, 312). Transcriptional analyses have shown that in vitro MoDCs most closely resemble tonsil mDC1 and mDC2 subsets (313), CD14+ dermal DCs (272) and inflammatory DCs in tumour ascites (279). MoDCs also express several different immune transcripts to in vivo DC subsets (272, 313), and the gene expression profiles of in vitro MoDCs may share more similarity with monocytes (104). Despite this, MoDCs are still a useful model to study DC biology (313).
1.1.3.6 Human dendritic cells and aging

There are relatively few studies looking at DCs in elderly humans (30, 40, 314, 315). The majority have reported that blood mDC numbers/proportions are unaltered during healthy aging, with the exception of two studies that observed declining mDC numbers in the elderly (Table 1.3). There is a consensus that LC and thymic DC proportions decline with age (Table 1.3). Most, but not all, studies have shown that blood pDCs decline with age (Table 1.3).

<table>
<thead>
<tr>
<th>DC subset</th>
<th>Change in elderly DC proportions/numbers compared to young adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood mDCs</td>
<td>Unaltered (68, 259, 316-319) Decreased (320, 321)</td>
</tr>
<tr>
<td>Peripheral blood pDCs</td>
<td>Unaltered (68, 320) Decreased (259, 316-319, 321-324)</td>
</tr>
<tr>
<td>Langerhans cells</td>
<td>Decreased (325-332)</td>
</tr>
<tr>
<td>Thymic DCs</td>
<td>Decreased (333, 334)</td>
</tr>
</tbody>
</table>

Several factors may contribute to the conflicting reports on human DCs. Similar to murine studies these include differences in the age ranges of volunteers used, and markers used to identify DC subsets. Other factors include: the genetic heterogeneity of humans; differences in exposure to antigens and environmental factors throughout life; and differences in criteria used to define healthy volunteers (30, 35, 41, 335). Changes to human DC function during aging will be discussed in section 1.3, in relation to the DC lifecycle.

1.2 T cells and aging

Dendritic cells play a pivotal role in driving T cell responses (5, 6). Young adults mount T cell responses against a wide variety of antigens, as they possess a highly diverse naïve T cell pool, where each T cell expresses a TCR with a unique antigen specificity (336, 337). T cells can be broadly classified into cytotoxic CD8+ T cells and CD4+ T helper cells, which are effector cells that mediate anti-pathogen and anti-tumour responses, and Tregs, which are suppressive cells that play a key role in the induction and maintenance of immune tolerance (338-340).
Similar to DCs and other cells, T cells undergo continuous chronological changes throughout life. Fetal/neonatal life is the period in which production of naïve T cells in the thymus is most active (341-343). T cell development in the thymus has been well-characterised using fetal/neonatal murine models, and involves negative selection, i.e. deletion of T cells displaying a high affinity for self-antigens via apoptosis, and positive selection of T cells that display a low to intermediate affinity for self-antigens (137, 344). Positively selected, self-tolerant naïve CD4+ and CD8+ T cells subsequently migrate from the thymus to peripheral lymphoid tissues, where they can interact with APCs (344, 345).

The functional profile of fetal/neonatal T cells reflects the overall skewing of the fetal/neonatal immune system away from pro-inflammatory, and towards anti-inflammatory activity (21, 346, 347). In particular, fetuses have high numbers of functional, suppressive Tregs in order to prevent the fetus from mounting an immune response against the mother (348-350). The anti-inflammatory, tolerogenic status of T cells leaves the fetus/neonate highly susceptible to infections (13, 347).

T cell function is also compromised at the other end of the age spectrum. It is well-known that adaptive immunity, including T cell function, declines during healthy aging, leading to reduced anti-pathogen and anti-tumour immune responses, increased susceptibility to infections and cancer and reduced vaccination responses in the elderly (36, 37, 351-353). However, changes to T cell function in the context of aging and cancer are less well-characterised, thus, this project aimed to determine how age-related changes to T cells may be further impacted by the presence of a cancer and during anti-cancer treatment.

1.2.1 CD8+ T cells

CD8+ T cells are cytotoxic effector cells that recognise antigens presented via MHC class I molecules (339). Stimulatory signals from IL-2, IL-12 and IFN-α/β during DC/CD8+ T cell interactions activates full cytotoxic T cell effector function (354-357). When the TCR of a CD8+ T cell recognises its specific MHC class I/peptide complex on a target cell, an immunological synapse forms between the CD8+ T cell and its
target cell (358). CD8+ T cells release cytoplasmic granules containing perforin and granzymes at the immunological synapse (358-360). Perforin polymerises to form a pore in the target cell membrane resulting in target cell death via osmotic lysis (359, 361), and permits entry of granzymes (a group of proteases) into target cells which induce DNA fragmentation leading to apoptosis (359, 362-364). CD8+ T cells also induce receptor-mediated cytolysis by up-regulating Fas ligand, which binds Fas on target cells, triggering apoptosis in the latter (359, 360). Additionally, CD8+ T cells secrete pro-inflammatory cytokines, including IFN-γ and TNF-α, which have direct anti-viral and/or cytotoxic effects, and activate other immune cells, such as macrophages (339, 365-367). Cytotoxic CD8+ T cells play key roles in the clearance of viruses and other intracellular pathogens (368, 369), and are important effectors of anti-tumour immunity (370, 371).

**1.2.2 CD4+ T cells**

CD4+ T cells recognise antigens presented in MHC class II molecules on DCs/APCs, and cytokines present during antigen presentation determine the type of CD4+ Th response generated (6, 372, 373); summarised in Table 1.4.

<table>
<thead>
<tr>
<th>Th subset</th>
<th>Cytokines required for polarisation</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IFN-γ, IL-12, type I IFNs, IL-1, IL-18</td>
<td>(338, 354, 374, 375)</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-2, IL-4, IL-6, IL-10, IL-11</td>
<td>(338, 374, 375)</td>
</tr>
<tr>
<td>Th9</td>
<td>TGF-β, IL-4</td>
<td>(338, 376)</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-1 β, IL-6, IL-21, IL-23, TGF-β</td>
<td>(377, 378)</td>
</tr>
<tr>
<td>Th22</td>
<td>IL-6, TNF-α</td>
<td>(379)</td>
</tr>
<tr>
<td>Follicular Th</td>
<td>IL-6, IL-21</td>
<td>(380, 381)</td>
</tr>
</tbody>
</table>

This study focused on the classical Th1 and Th2 subsets. Th1 cells secrete pro-inflammatory cytokines and chemokines, such as IFN-γ, lymphotoxin-α, IL-2, TNF-α, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α, and mediate responses against intracellular pathogens and anti-tumour immunity, but can also contribute to the pathology of autoimmune diseases (338, 374, 382). In contrast, Th2 cells produce the anti-inflammatory cytokines IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin, and mediate immunity against
extracellular pathogens and allergic responses (338, 374, 382, 383).

In regards to the other Th subsets, Th17 cells produce pro-inflammatory IL-17A, IL-17F, IL-21 and IL-22 (378), and mediate responses against extracellular bacteria and fungi (384-387), and contribute to the induction and pathogenesis of autoimmune conditions (378). Similar to Th17 cells, IL-22-producing Th22 cells play a role in autoimmunity and chronic inflammatory conditions (379). Th9 cells produce IL-9, which mediates allergic responses by inducing mucin production in epithelial cells (388). Lastly, follicular Th cells located in germinal centres of peripheral lymphoid tissues promote B cell antibody secretion and memory B cell development (389).

Cytotoxic CD4+ T cells have also been described, and most often exhibit a Th1-like phenotype due to IFN-γ, IL-2 and TNF-α production (390-393). Cytotoxic CD4+ T cells use the same effector mechanisms as their CD8+ counterparts, i.e. perforin and granzyme-mediated cytolysis (390, 394-396) and Fas/Fas ligand-mediated apoptosis of target cells (397-399), and mediate anti-viral and anti-tumour immunity (400-407).

**1.2.3 CD8+ and CD4+ T cells and aging**

CD8+ and CD4+ T cells display marked changes with increasing age. A major factor is thymic involution, characterised by a gradual reduction in the size of the thymus, and replacement of functional thymic tissue with fat (342, 408-410), resulting in reduced thymic output of naïve CD8+ and CD4+ T cells in elderly humans (411-414) and mice (231, 409, 415, 416). Homeostatic expansion of antigen-experienced effector and memory T cells in the periphery compensates for reduced thymic production of naïve T cells; this occurs in CD8+ (417-424) and CD4+ T cells (425-428), but is more pronounced for CD8+ T cells (417, 426). However, these expansions are oligoclonal, resulting in narrowing of TCR diversity and a reduction in the ability of elderly individuals to respond to new, previously unencountered antigens (242, 412, 417, 421-423, 429, 430). In humans, a major driving factor for age-related narrowing of the TCR repertoire (especially CD8+ T cells) is the influence of previous infections and chronic antigenic stimulation as a result of latent infections,
particularcyctomegalovirus (CMV), Epstein-Barr virus (EBV) and herpes simplex virus (HSV; 418, 431, 432-434). As a result, oligoclonally expanded T cells are mostly virus (CMV)-specific (418, 419, 435-438), and are dysfunctional, due to loss of co-stimulatory molecule expression (such as CD28), impaired cytokine secretion (such as IL-2 and IFN-γ) and cytotoxic activity, increased expression of inhibitory/senescence markers (such as killer-cell lectin-like receptor G1 (KLRG-1) and CD57; the enzyme galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1), and limited proliferative capacity (414, 419, 426, 433, 435-437, 439-446). The age-related decline in naïve T cell numbers and function contributes to reduced anti-pathogen and anti-tumour immunity in the elderly (37, 44, 431, 447-449).

1.2.4  Regulatory T cells

Regulatory T cells can be CD4+ or CD8+ cells; the former are more abundant and better characterised (340, 450-453). Regulatory T cells can arise in the thymus (naturally occurring Tregs), or develop in the periphery (inducible Tregs; 340, 454). Thymic Treg development requires expression of the transcription factor forkhead box P3 (FoxP3; 455, 456-461), and IL-2, IL-7 and IL-15 (462-464). In the periphery, CD4+FoxP3+ Tregs are induced by factors such as IL-10 (465-468), transforming growth factor (TGF)-β (162, 469-472), and retinoic acid (162, 178, 473-476). Additionally, FoxP3 negative T cells with regulatory activity have been described, such as Tr1 cells, which develop from naïve CD4+ T cells under the influence of IL-10 and IL-27 (477, 478), and exert suppression via IL-10 secretion (479), and Th3 cells, which are induced by IL-4, IL-10 and TGF-β, and suppress effector T cells and promote FoxP3+ Treg development via TGF-β (480, 481). Like their CD4+ counterparts, CD8+ Tregs can be thymus-derived or induced in the periphery (452, 453, 482).

Identification of murine Tregs is well-established, and they are typically identified as CD3+CD4+CD25+FoxP3+ cells (340, 454, 457, 460). In contrast, identification of human Tregs is more difficult, as markers typically used to identify Tregs, CD25 and FoxP3, are up-regulated on activated, non-regulatory human T cells (456, 483-487).
Human Tregs have been shown to express low levels of CD127 (the IL-7 receptor), thus, one approach is to identify human Tregs as CD3+CD4+CD25+CD127low cells; this has been validated as low CD127 expression correlates with high FoxP3 expression (488, 489). The identification of markers to distinguish human Tregs is ongoing (454, 490).

Tregs are crucial mediators of immune tolerance, preventing autoimmunity in the healthy steady state, and attenuating immune responses to prevent host tissue damage (340, 454, 491, 492). However, the regulatory/tolerogenic functions of Tregs can be exploited by tumours to evade the immune response (493-495). Tregs inhibit effector T cells, and other immune cells (DCs, macrophages, NK cells, NK T cells and B cells) through a variety of mechanisms (340, 491, 492). One mechanism is secretion of the anti-inflammatory cytokines IL-10, IL-35 and TGF-β (450, 454, 491), which inhibit: effector CD8+ and CD4+ T cell activation; proliferation and production of IL-2, IL-4, IL-5, IFN-γ, TNF-α, perforin and granzyme; development of memory T cells; and induce T cell apoptosis and promote T cell anergy (496-511). Treg-derived IL-10 and TGF-β act on DCs to induce exhaustion, promote regulatory function, as well as inhibiting DC maturation, expression of antigen-presenting and co-stimulatory molecules and pro-inflammatory cytokines, thus reducing the ability of DCs to activate effector T cells and increasing their potential to promote T cell anergy (496, 501, 503, 512-517). Additionally, IL-10 and TGF-β act in an autocrine manner to further promote Treg differentiation (467, 468, 472, 477, 496, 500, 501, 513, 518-524). Tregs also exert suppressive activity via inhibitory molecules, such as cytotoxic T lymphocyte-associated protein-4 (CTLA-4) and lymphocyte activation gene-3 (LAG-3; 450, 454, 491). CTLA-4 on Tregs can bind to the co-stimulatory molecules CD80 and CD86 on: (i) effector T cells, terminating their effector function (525), and (ii) DCs, leading to down-regulation of CD80/CD86 (526-529) and secretion of suppressive indoleamine 2,3-dioxygenase (IDO; 203, 530, 531-535), whilst binding of LAG-3 on Tregs to MHC class II on DCs inhibits DC maturation (536).

A major Treg suppressive mechanism is production of suppressive adenosine via the enzymes CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1; converts
adenosine triphosphate (ATP) to adenosine diphosphate (ADP), then adenosine monophosphate (AMP)) and CD73 (ecto-5’-nucleotidase; converts AMP to adenosine) (537-544). T cells respond to adenosine through adenosine-binding receptors, in particular, the A2A receptor which is expressed to a greater extent on T cells compared to the A1, A2B and A3 adenosine receptors (539, 545-551). Binding adenosine to the A2A receptor leads to multiple inhibitory effects on effector CD8+ and CD4+ T cells, including: blockade of TCR signalling, T cell differentiation and proliferation; reduced stability of IFN-γ, TNF-α, IL-2 and IL-4 mRNA, leading to reduced synthesis; decreased CD25 (IL-2 receptor α chain) expression; reduced CD8+ T cell cytotoxic activity and induction of T cell anergy (543, 544, 547, 550-556). Adenosine acts in an autocrine manner on Tregs via the A2A receptor and promotes their expansion (554, 557-561). Adenosine may also act through A2B and A3 receptors on T cells, inhibiting IL-2 production, preventing cytotoxic effector function, and inducing Treg development (557, 562-565). Treg-derived adenosine negatively affects APCs, specifically: (i) by binding the A2A and A2B receptors on DCs to inhibit IL-12 production, maturation, and their ability to stimulate T cells, whilst promoting IL-10 and TGF-β secretion (566-574), and (ii) binding the A2B receptor on macrophages, thereby polarising pro-inflammatory M1 macrophages into anti-inflammatory/suppressive M2 macrophages (575-580).

Other mechanisms by which Tregs suppress effector T cells include: (i) inhibition of effector T cell IL-2 production and/or consumption of IL-2 via CD25, which limits effector T cell expansion (581-583), (ii) cytolysis of effector T cells via granzyme A in human Tregs (584) and perforin and/or granzyme B in murine Tregs (585, 586), and (iii) inducing effector T cell apoptosis via Fas/Fas ligand interactions (587).

1.2.5 Regulatory T cells and aging

The effects of aging on human and murine Tregs are relatively less well-characterised (588, 589). Several studies have reported that numbers/proportions of CD4+ and CD8+ Tregs increase with aging in human blood (590-596), and murine spleens, LNs and blood (246, 590, 597-602). Yet conflicting studies show that numbers of human (603-606) and murine (246, 607) Tregs remain steady in these
tissues with aging. Numbers of thymic Tregs are reduced with aging, likely due to a reduction in thymic output (246, 601).

There is also no consensus as to how Treg function changes with age. Several studies demonstrated that the capacity of Tregs to suppress effector CD4+ T cell proliferation and/or secretion of IFN-γ, TNF-α, IL-2 and IL-6 is retained (591, 594, 601, 603, 607), or even increased (590, 592, 598) with aging. The consequences of increased Treg suppression are: inhibition of anti-pathogen immune responses, which could contribute to re-activation of latent infections and more severe pathology of infections in the elderly (590); and reduced anti-tumour immune responses (602, 608), which may lead to increased occurrence of spontaneous tumours in aged mice (600). In contrast, three studies reported that Treg suppressive activity declines with age (601, 609, 610), which may contribute to increased autoimmunity in the elderly (601, 609). Given the conflicting reports, further studies examining age-related changes in Tregs are required.

1.3 The dendritic cell life cycle and aging

DCs have a distinct life cycle which enables them to activate antigen-specific T cell responses (6); thus, age-related changes in DC function are likely to impact upon the generation of T cell immunity in the elderly. However, the effects of aging on human and murine DC function are not well-characterised, and the limited studies performed to-date have reported conflicting results (reviewed by 30, 40, 41, 314).

1.3.1 Antigen uptake by immature DCs

One of the first stages of the DC lifecycle occurs in peripheral tissues, where DCs have an immature status, characterised by low co-stimulatory molecule expression, and high antigen uptake and processing capacity (6, 611). Immature DCs continuously sample their local environment and capture antigens from a variety of sources (for example, self, pathogen and tumour antigens) through different mechanisms (612). DCs can take up exogenous antigens via: (i) phagocytosis involving engulfment of particles such as apoptotic cells and microbes, that may be assisted by ligand capture on endocytic receptors, (ii) micropinocytosis involving
uptake of extracellular fluid containing soluble antigens, and (iii) receptor-mediated endocytosis, where antigens are captured on cell surface receptors (such as scavenger, complement, lipid and Fcγ receptors, and lectins), and subsequently internalised (6, 611, 612). As described in sections 1.1.2 and 1.1.3, different DC subsets have different specialisations for antigen capture. Once taken up, antigens are processed into peptide fragments for later presentation to T cells (612, 613).

With aging, several studies reported that elderly murine splenic and lung cDCs, and BMDCs retain their capacity to take up tumour cells and/or peptides (66, 71, 236, 250, 614, 615). In contrast, three studies observed that antigen uptake is compromised with aging, shown by reduced uptake of bacteria by elderly murine splenic CD8α+ cDCs (249), reduced endocytosis of irradiated cells by elderly murine splenic DCs (241), and reduced antigen phagocytosis and pinocytosis by elderly human MoDCs (68). Defective endocytosis by splenic DCs was associated with an age-related decline in mitochondrial activity and accumulation of reactive oxygen species (241), whilst decreased MoDC antigen uptake was due to an age-related reduction in PI3 kinase signalling (68). Given the conflicting reports, further studies are required.

1.3.2 Antigen processing pathways

DCs process protein antigens via two classical pathways (Figure 1.2). In the classical MHC class I pathway, endogenous antigens, i.e. antigens originating within the cell, for example, viral or self-antigens, are proteolytically cleaved into peptides by the proteasome in the cytosol and transported to the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) molecules for loading onto MHC class I molecules (Figure 1.2; 612, 613, 616). Processing of self-antigens occurs continuously in the cytosol (612, 613) likely to induce/maintain self-tolerance. In the classical MHC class II pathway, endosomes containing exogenous proteins fuse with lysosomes, where the acidic pH and action of proteolytic enzymes cleaves proteins to produce peptides. These antigen-containing compartments fuse with endosomes containing MHC class II molecules, and the MHC-encoded glycoprotein HLA-DM releases the class II invariant chain peptide (CLIP) from MHC class II molecules,
allowing peptide loading onto MHC class II molecules (Figure 1.2; 612, 613).

**Figure 1.2 MHC class I and II antigen processing pathways**
The classical MHC class I pathway is shown in the left panel (A), the classical MHC class II pathway is shown in the right panel (B), and the diversion of exogenous antigens into the MHC class I pathway (cross-presentation) is shown in the centre (C). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (617) © 2001.

There is overlap between the two pathways, as exogenous antigens can be processed and presented in the MHC class I pathway, in a process called cross-presentation (Figure 1.2; 618, 619). How exogenous antigens gain access to the MHC class I pathway is not yet clear, but possible mechanisms include: (i) transfer of antigens internalised in endosomes to the cytosol for proteasome degradation, for example, via channels or transport molecules in endosomal membranes, or fusion of endosomes with the ER (618, 620-623), and (ii) antigen degradation within endosomes/lysosomes which fuse with endosomes containing MHC class I molecules recycled from the cell surface (618, 624-627). The cross-presentation pathway is important for generation of immune responses against tumours and intracellular pathogens (618). There is also evidence that endogenous antigens can be presented on MHC class II molecules, and this may occur via transfer of antigens from the cytosol into lysosomes, or during autophagy (612, 628-630). As outlined in sections 1.1.2 and 1.1.3, different DC subsets are specialised for particular antigen presentation pathways.
Age-related changes in DC antigen processing are poorly characterised and contradictory. In one study, elderly murine lung CD11b+ and CD103+ cDCs maintained their ability to process fluorescently labelled ovalbumin (236), and elderly murine BMDCs retained expression of MHC class I/peptide complexes, suggesting the MHC class I pathway remains intact with aging (614). In contrast, another study demonstrated persistence of ovalbumin following internalisation by elderly murine splenic cDCs, suggesting impaired antigen degradation (250). Decreased exogenous antigen processing by elderly murine splenic and LN CD11c+ cells due to accumulation of oxidised proteins within endosomes has also been reported (631), and may result in reduced expression of MHC class II/peptide complexes on the surface of elderly DCs (244), leading to impaired antigen presentation in the elderly.

1.3.3 Dendritic cell activation/maturation

Antigen uptake accompanied by a stimulus, or danger signal, triggers DC activation and maturation (5). Danger signals may be derived from pathogens, termed pathogen-associated molecular patterns (PAMPs), and include molecules such as bacterial LPS, flagellin, and bacterial and viral nucleic acids (5, 632). Damaged or necrotic host cells may also release danger signals called damage-associated molecular patterns (DAMPs) or alarmins, and examples include ATP, the nuclear protein high mobility group box protein-1 (HMGB-1), and heat shock proteins (5, 633-635). DCs express pattern recognition receptors (PRRs) allowing them to respond to danger signals from PAMPs and DAMPs (5, 636). Examples of PRRs include TLRs, C-type lectin receptors and receptors of the nucleotide-binding oligomerisation domain-like receptor (NLR) family (5). Signalling through TLRs, located on the cell surface or intracellularly, plays an important role in activation of DCs and other innate immune cells (5, 636-638). Examples of PRRs expressed on DCs, and the PAMPs and DAMPs they recognise are shown in Table 1.5. DCs can be stimulated through multiple PRRs simultaneously which has synergistic effects on DC activation (639).
Table 1.5 PRRs expressed on DCs, and the PAMPs and DAMPs recognised

<table>
<thead>
<tr>
<th>PRR</th>
<th>PRR location</th>
<th>PAMPs/DAMPs recognised</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-1, 2 and 6</td>
<td>Cell surface</td>
<td>Bacterial lipopeptides Host HMGB-1 (TLR-2)</td>
<td>(637, 638, 640)</td>
</tr>
<tr>
<td>TLR-3</td>
<td>Intracellular</td>
<td>Double-stranded viral RNA</td>
<td>(637, 638)</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Cell surface</td>
<td>Bacterial LPS Host heat-shock proteins Host fibrinogen Host HMGB-1</td>
<td>(637, 638, 640)</td>
</tr>
<tr>
<td>TLR-5</td>
<td>Cell surface</td>
<td>Bacterial flagellin</td>
<td>(637, 638)</td>
</tr>
<tr>
<td>TLR-7/8</td>
<td>Intracellular</td>
<td>Single-stranded viral RNA</td>
<td>(637, 641-643)</td>
</tr>
<tr>
<td>TLR-9</td>
<td>Intracellular</td>
<td>Bacterial CpG DNA</td>
<td>(637, 638, 644)</td>
</tr>
</tbody>
</table>

Other receptors

| Purinergic P2X and P2Y receptors | Cell surface | ATP | (645-647) |

DC activation is influenced by a balance between pro- and anti-inflammatory cytokines (6, 9). Pro-inflammatory cytokines released by local immune cells during inflammation, such as IFN-γ from macrophages and NK cells, TNF-α from macrophages and mast cells (648, 649), and IFN-α from pDCs (650-652) promote DC activation (6, 9). Anti-inflammatory cytokines, such as IL-10 and TGF-β, induce different functional profiles in DCs (9), discussed in section 1.3.6. The mature DC phenotype depends on the type, combination, strength and duration of stimulation received (5, 6, 653).

Stimulation activates signalling pathways within DCs, particularly the nuclear factor kappa B (NFkB) and IFN pathways, altering gene expression (654-658), and initiating a series of co-ordinated events that shift DCs from an immature to a mature profile (6). During maturation DCs lose their capacity for antigen uptake due to down-regulation of phagocytic and endocytic receptors (6). DCs also down-regulate receptors that respond to immunosuppressive signals, such as the IL-10 receptor, to prevent inhibition of DC maturation (9).

Maturing DCs up-regulate molecules involved in antigen presentation (MHC class I and II), co-stimulatory molecules (e.g. CD40, CD80 and CD86), adhesion molecules (e.g. CD54/intercellular adhesion molecule (ICAM)-1) and pro-inflammatory cytokines (e.g. IL-1β, IL-12, IL-6, IFN-γ and TNF-α) and chemokines (e.g. IL-8, MIP-1α...
and β, and MCP-1) (5, 6, 659), several were examined in this study. Expression of certain pro-inflammatory cytokines (e.g. IL-1β and TNF-α) and chemokines (e.g. IL-8, MIP-1α and β) up-regulate rapidly (e.g. 2-4 hours following DC activation) to recruit more effector cells, such as macrophages, neutrophils, and NK cells to the tissue site (6, 517, 659). Up-regulation of antigen-presenting and co-stimulatory molecules and other pro-inflammatory cytokines/chemokines occurs within the first 24-48 hours following activation, whilst DCs migrate to LNs (5, 6).

In regards to age-related changes in the response of DCs to activation stimuli, there is a consensus that elderly human pDCs, PBMCs and MoDCs, and murine pDCs have a reduced ability to produce the anti-viral cytokines IFN-α and IFN-λ following stimulation with viruses (influenza or HSV) or TLR-7/9 ligands (252, 316, 317, 323, 324, 660, 661), with the exception of one study showing comparable IFN-α secretion by young and elderly murine splenic pDCs (251). Impaired production of anti-viral cytokines by elderly pDCs could be due to reduced TLR-7 (316, 317) and/or TLR-9 (317) expression, although a few studies reported no age-related differences in TLR-7 (661) and TLR-9 (252, 316, 661). Alternatively, defects in transcription factors involved in IFN signalling pathways (67, 252, 661), histone modifications leading to reduced transcription of IFN genes (660), and/or down-regulation of genes involved in anti-viral responses (662) represent possible underlying mechanisms. Reduced pDC anti-viral activity likely contributes to the high susceptibility of elderly individuals to viral infections (67, 317, 661, 663).

Inconsistent results have been reported regarding the response of human mDCs and MoDCs, and murine cDCs and BMDCs to maturation-inducing stimuli, summarised in Table 1.6. Several studies demonstrated that surface expression of TLRs 2, 3 and 4 is maintained with age (68, 251, 316, 317, 664), which may explain the comparable TLR responses observed in some studies (Table 1.6). However, several studies described defects in elderly DCs which may lead to reduced TLR responses (Table 1.6), including: reduced expression of TLRs 1, 3 and 8 (316), alterations in NFκB and signal transducer and activator of transcription (STAT) signalling pathways (250, 251, 665, 666), altered expression and transcription of genes required for DC activation (662, 667), and impaired NLR family pyrin domain
containing-3 (NLRP3) inflammasome activation (668). Further studies are required to characterise the ability of elderly DCs to respond to maturation stimuli, as this is a crucial part of the DC lifecycle to ensure appropriate activation of T cell responses.

Table 1.6 Responses of elderly human and murine DCs to maturation stimuli

<table>
<thead>
<tr>
<th>DC subset</th>
<th>Stimuli</th>
<th>Elderly DC response compared to young adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human blood mDCs</td>
<td>TLR-1/2 agonist (Pam3CSK4)</td>
<td>Reduced TNF-α, IL-6, IL-12/23p40 (316)</td>
</tr>
<tr>
<td></td>
<td>TLR-2/6 agonist (lipoteichoic acid)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TLR-5 agonist (flagellin) or TLR7/8 agonist (R848)</td>
<td></td>
</tr>
<tr>
<td>Human blood mDCs</td>
<td>TLR-3 agonist (poly I:C)</td>
<td>Comparable: IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α (317)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced: TNF-α, IL-6, IL-12/23p40 (316)</td>
</tr>
<tr>
<td>Human blood mDCs</td>
<td>TLR-4 agonist (LPS)</td>
<td>Comparable/increased IL-6, TNF-α (669)</td>
</tr>
<tr>
<td>Human MoDCs</td>
<td>LPS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poly I:C or Viruses</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stimuli</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MHC-I/II, CD40, CD80, CD86 (68, 69, 320, 660, 670)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-1β, IL-12 and/or TNF-α (68, 670)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-10 (68, 320)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stimulation of CD4+ T cell proliferation and T cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IFN-γ (69)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α, IL-6 and/or IL-18 (68, 670)</td>
<td></td>
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<tr>
<td></td>
<td>Reduced:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD80, CD86 (67)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-12 (320)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IFN-α, β and λ (67, 660)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-10 (670)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Induction of T cell IFN-γ (671)</td>
<td></td>
</tr>
<tr>
<td>Human MoDCs</td>
<td>Human DNA or apoptotic cells</td>
<td>Increased CD80, CD86, IL-6, IFN-α and/or TNF-α (665, 672)</td>
</tr>
<tr>
<td>Human DCs from low-density</td>
<td>LPS</td>
<td></td>
</tr>
<tr>
<td>PBMC fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murine splenic CD11c+ DCs</td>
<td>TLR-2/6 agonist (peptidoglycan)</td>
<td>Comparable: CD40, CD80, CD86, IL-12, IL-10, TGF-β (673, 674)</td>
</tr>
<tr>
<td></td>
<td>LPS or Poly I:C</td>
<td>Reduced: IFN-γ, TNF-α, stimulation of T cell proliferation (673, 674)</td>
</tr>
<tr>
<td>Murine splenic CD8α+ and CD8α cDCs</td>
<td>TLR-7 agonist (polyU)</td>
<td>Increased: IL-10 (250)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced: MHC-II, CD40, CD80, PD-L1, TNF-α, IL-6, IL-12 (251)</td>
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<td></td>
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<tr>
<td>DC subset</td>
<td>Stimuli</td>
<td>Elderly DC response compared to young adults</td>
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</tr>
<tr>
<td>Murine splenic CD8α⁺ cDCs</td>
<td>LPS</td>
<td>Comparable MHC-II, CD40, CD86, stimulation of T cell proliferation (249)</td>
</tr>
<tr>
<td>Murine splenic CD8α⁺ cDCs</td>
<td>In vivo <em>Listeria monocytogenes</em> infection</td>
<td>Reduced MHC-II, CD40, CD86 (249)</td>
</tr>
<tr>
<td>Murine LN CD11c⁺CD11b⁺ cDCs</td>
<td>Pam3CSK4, Poly l:C or TLR-9 agonist (CpG)</td>
<td>Comparable MHC-II, CD40, CD86 (676)</td>
</tr>
<tr>
<td>Murine LN CD11c⁺ cDCs</td>
<td>LPS or Peptide</td>
<td><em>Comparable:</em> CD86 (244)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Reduced:</em> MHC-II/peptide, CD40, IFN-γ, TNF-α, IL-6, stimulation of CD4⁺ T cell proliferation, induction of T cell IFN-γ (244)</td>
</tr>
<tr>
<td>Murine lung CD11c⁺ DCs</td>
<td>In vivo influenza and respiratory viral infections</td>
<td><em>Comparable:</em> MHC-II (247)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD40, CD80 and/or CD86 (72, 236)</td>
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<tr>
<td></td>
<td></td>
<td>Stimulation of CD8⁺ T cell proliferation (236)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Reduced:</em> CD40 (247), CD86 (72)</td>
</tr>
<tr>
<td>Murine BMDCs (GM-CSF/IL-4)</td>
<td>LPS</td>
<td><em>Comparable:</em> MHC-II, CD40, CD54, CD86, IFN-γ, IL-12 (71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Increased:</em> MHC-I, CD80, IL-10 (71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α, IL-12 and/or nitric oxide (66, 239)</td>
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<tr>
<td></td>
<td></td>
<td><em>Reduced:</em> MHC-II, CD40, CD86 (66, 675)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α, IL-6 (71)</td>
</tr>
<tr>
<td>Murine BMDCs (GM-CSF)</td>
<td>Pam3CSK4, Peptidoglycan, Poly l:C, LPS, Flagellin or CpG</td>
<td>Comparable: CD40, CD80, CD86, IL-6, IL-12, TNF-α, induction of T cell proliferation (664, 676)</td>
</tr>
<tr>
<td>Murine BMDCs (GM-CSF)</td>
<td>LPS +/- R848</td>
<td><em>Comparable:</em> CD40, CD80, CD86 (677)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Increased:</em> IL-23, TGF-β (667, 677)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Reduced:</em> IL-10 (677)</td>
</tr>
<tr>
<td>Murine BMDCs (GM-CSF)</td>
<td>Influenza virus</td>
<td>Reduced IL-1β, IL-18 (668)</td>
</tr>
</tbody>
</table>

1.3.4 Dendritic cell migration

Following activation, DCs undergo several changes that facilitate their migration from peripheral tissues to DLNs. These include morphological changes and reorganisation of the cytoskeleton, to increase DC motility (6, 678). Expression of the chemokine receptor CCR7 is up-regulated (679-681) to engage with EBI1 ligand chemokine (ELC, MIP-3β) and secondary lymphoid tissue chemokine (SLC), facilitating DC migration via lymphatic vessels to T cell zones in LNs (6, 679-683).
DCs start arriving in LNs or spleens 2-8 hours following stimulation, peaking at 24-48 hours, shown using murine in vivo infection models (684-689).

Four studies have shown that migration by elderly DCs is impaired (68, 236, 614, 690), due to defects in PI3 kinase (68) and CCR7 signalling (614), reduced CCR7 expression (236), and/or down-regulation of genes involved in migration (662). In contrast, one study reported that migration of human blood DCs is maintained with age (691), whilst another showed that elderly murine peritoneal APCs have increased migratory ability (615); these differences may be due to differences in the types of DCs and models used. However, the majority of studies to-date have shown that DC migration is impaired with aging, which may lead to reduced trafficking of DCs to DLNs, and reduced generation of immune responses.

1.3.5 Antigen presentation and DC/T cell interactions

Activated DCs arriving at LNs from the periphery have a mature status, and present antigens to naïve T cells (5, 6). Debate continues over the type of DC (migratory versus lymphoid-resident) responsible for antigen presentation within lymphoid tissues (692). In vivo murine skin and lung infection models report that migratory DCs are the dominant antigen-presenting DCs in LNs (148, 693, 694). Conflicting studies demonstrate that lymphoid-resident DCs are the main contributors to antigen presentation (685, 695-697); these DCs may acquire antigens via lymphatic circulation (698, 699), or by antigen transfer from migratory DCs (685, 692). It is likely that migratory and lymphoid-resident DCs play a role as both DC types have been shown to co-operate to present antigens to T cells, and/or are involved in presenting antigens to specific T cell subsets (148, 684, 700-703).

Mature DCs interact with naïve T cells at the immunological synapse (Figure 1.3; 704). Formation of the immunological synapse involves changes in cell morphology and cytoskeletal structure, and movement of cell surface molecules into organised areas called supra-molecular activation complexes (SMACs; 704, 705, 706).
Figure 1.3 DC/T cell interactions at the immunological synapse
(A) Molecular interactions between DCs and T cells at the immunological synapse. (B) The three regions of the immunological synapse: the inner central supra-molecular activation complex (SMAC; brown circle), the peripheral SMAC (green circle), and the distal SMAC (grey circle). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (704) © 2003.

The outer region of the SMAC, termed peripheral SMAC, contains adhesion molecules (e.g. CD54/ICAM-1 on DCs binding lymphocyte function-associated antigen-1 (LFA-1) on T cells), which help stabilise DC/T cell interactions (Figure 1.3; 704, 705, 706). Larger molecules are excluded from the inner SMAC to a region outside the peripheral SMAC, known as the distal SMAC (Figure 1.3; 704, 705, 706). The inner central SMAC contains MHC/peptide complexes, the TCR, co-stimulatory molecules, and associated signalling molecules, which provide the three signals necessary for effector T cell activation (Figure 1.3; 5, 6, 704, 705, 706).

Signal one is engagement of MHC/peptide complexes on DCs with antigen-specific TCRs on T cells, with accompanying signals determining the nature of the T cell response (5, 6). Activation of effector T cells occurs when the number of positive signals outweighs the number of negative signals (7, 707, 708). Positive interactions between co-stimulatory molecules on DCs and T cells forms the second signal required for T cell activation (Figure 1.4; 5, 6, 9, 709). T cells require a third signal to develop full effector function and form memory cells, and this is provided by pro-inflammatory cytokines secreted by DCs (354). Effector T cells migrate from LNs to
peripheral tissues where they encounter antigen-specific targets and exert effector function such as protective immune responses against pathogens (5, 6).

Figure 1.4 Co-stimulatory and co-inhibitory interactions during DC/T cell cross-talk
Uptake of pathogen-derived antigens, accompanied by recognition of bacterial/viral PAMPs by TLRs, activates DCs into potent APCs which stimulate cytotoxic CD8+ T cells and Th1 cells for clearance of viruses and intracellular bacteria, or Th2 and Th17 cells to mediate elimination of extracellular pathogens (339, 373-375, 378).

Once an immune response has occurred and the initiating agent (e.g. a pathogen) eliminated, immune attenuation must occur to prevent damage to host tissues (9, 707, 710). Following activation, DCs and T cells up-regulate inhibitory molecules and anti-inflammatory cytokines (Figure 1.4; 9, 707, 710, 711). Inhibitory molecules are usually up-regulated at later time points after DC and T cell activation (e.g. at least 24 hours later), likely to allow sufficient time for an immune response to occur, before attenuation (9, 710, 711). During the attenuation phase, simultaneous up-regulation of several inhibitory molecules and their localisation within the central SMAC results in a greater summation of negative signals occurring during DC/T cell cross-talk, leading to T cell inhibition (Figure 1.4; 707, 712). Thus, T cell activation or suppression is determined by the overall summation of positive and negative signals delivered during DC/T cell cross-talk (7, 707, 708, 713).

There is no consensus regarding the effects of aging on the ability of DCs to stimulate T cells. Several studies reported that elderly human and murine DCs stimulate comparable levels of CD8+ and/or CD4+ T cell proliferation, compared to young DCs (69, 70, 72, 236, 242, 249, 251, 615, 661, 664, 676, 714, 715), with two studies reporting an increased ability of elderly murine DCs to stimulate T cell proliferation (239, 716). Yet conflicting studies show a reduced ability of elderly human and murine DCs to promote T cell proliferation, particularly CD4+ T cell proliferation (71, 244, 614, 665, 673, 674, 714). The impaired generation of T cell responses with aging could be due to defects in elderly DCs (discussed in the preceding sections), and is supported by studies showing that transfer of elderly DCs into young mice results in weak CD8+ T cell responses (71, 614), and transfer of young T cells into aged mice results in poor antigen-specific T cell expansion (244). Age-related defects in T cells may also contribute, as elderly T cells show reduced proliferation when stimulated by young DCs (664, 673, 715). Furthermore, elderly T cells, particularly CD4+ T cells, demonstrate diminished signalling through key
pathways associated with T cell activation, such as the TCR, CD28 and IL-2 receptor (717-721), and an impaired ability to form immunological synapses with APCs (722-726). Taken together, this suggests that reduced activation of T cell responses in the elderly is likely to be due to age-related changes in both DCs and T cells.

Age-related changes in DC cross-presenting capacity are also poorly characterised. This function may be retained (72), or reduced (241, 250) in elderly murine splenic DCs; these differences could be due to examination of healthy non-activated DCs (72) versus TLR-stimulated DCs (250).

An important outcome of DC/T cell interactions is the functional status of the T cells generated. The limited number of studies examining effector T cells stimulated by elderly DCs report conflicting findings. Induction of T cell effector molecules/cytokines (such as perforin, granzymes, IL-2 and IFN-γ) and/or CD8+ T cell cytotoxic activity by elderly DCs is comparable (69, 251, 661, 715), increased (239), or decreased (244, 250, 614, 615, 671), compared to young DCs. The lack of consensus suggests that more in-depth characterisation of T cell responses following elderly DC/T cell cross-talk is required. Additionally, those studies focused mainly on the activation phase of DC/T cell interactions, and little is known about the influence of aging on the attenuation phase.

Several factors may contribute to the conflicting reports on age-related changes in the DC lifecycle/function. These include differences: between humans and mice; in the relative ages of human volunteers and mice used; in the types of DCs examined (in vivo subsets versus in vitro-derived DCs, and differences in protocols used to generate the latter); and in the types of stimuli and exposure times. In summary, further characterisation of the effects of aging on the DC lifecycle/function are required.

1.3.6 Dendritic cells, T cells and tolerance

Another important function of DCs is the induction and maintenance of tolerance (7, 727). In the thymus, DCs contribute to central tolerance by: (i) presenting self-antigens to developing T cells, with self-reactive T cells subsequently eliminated by
clonal deletion (728-734); and (ii) inducing Treg development (729, 735). Migratory DC subsets play a key role in peripheral tolerance (7, 727) particularly in the healthy steady state (162, 176, 179, 617, 736-741). Immature DCs in peripheral tissues capture self- and harmless environmental antigens in the absence of maturation stimuli, and maintain their immature status upon migration to LNs. As a result, antigens are presented to T cells in the absence of co-stimulatory signals, leading to T cell anergy (antigen unresponsiveness) and/or deletion (7, 8, 138, 139, 727, 741-746).

Another mechanism by which DCs mediate peripheral tolerance is by inducing Tregs via IL-10, TGF-β and/or retinoic acid (140, 162, 165, 177, 178, 747-750), and Tregs are key mediators of immune tolerance as they exert suppressive function through the mechanisms discussed in section 1.2.4. In particular, pDCs have been implicated as key inducers of CD4+ and CD8+ Tregs (203, 294, 751) in the thymus (302, 752) and periphery (200, 204, 294). One mechanism is by expression of the enzyme IDO which mediates tryptophan catabolism, depletion of tryptophan results in effector T cell apoptosis, and tryptophan catabolites promote Treg differentiation (203, 531, 533, 753-758). This pathway may be amplified by ligation of CTLA-4 on Tregs with CD80/CD86 on pDCs, which leads to further IDO production by pDCs (530, 532, 533). Another mechanism is binding inducible T cell co-stimulator (ICOS) on CD4+ T cells to ICOS ligand (ICOSL) on pDCs, which generates IL-10-producing Tregs (202, 759-761). Additionally, pDCs can regulate effector T cell responses by inducing clonal deletion (201), and inhibiting T cell proliferation via molecules such as programmed cell death ligand-1 (PD-L1; 762) and granzyme B (763).

If DCs are alternatively activated by anti-inflammatory cytokines, particularly IL-10 and TGF-β, they develop into regulatory DCs with tolerogenic function (764-768). Other stimuli can induce regulatory DCs, such as vasoactive intestinal peptide (769, 770), vitamin D3 (771, 772), and thymic stromal lymphopoietin (752, 773). Regulatory DCs can have a mature or semi-mature status; the latter is characterised by high expression of certain antigen-presenting and co-stimulatory molecules (e.g. MHC class II, CD80 and CD86), but low expression of others (e.g. CD40), and low to no secretion of pro-inflammatory cytokines, especially IL-12 (516, 774-779).
Regardless of their maturation status, regulatory DCs have high expression of IL-10 and other suppressive molecules, such as TGF-β, PD-L1, and IDO (516, 774-780), and are unresponsive towards subsequent activation stimuli (781-784). Due to their low/partial expression of immune-activating molecules, and high expression of inhibitory molecules, regulatory DCs induce T cell anergy and apoptosis, inhibit T cell proliferation, and promote Tregs (516, 774-780) (764, 767, 769, 785). Regulatory DCs develop in healthy tissues, where they maintain tolerance and prevent unwanted immune activation and autoimmunity (776, 777, 779, 786), but can also be induced in pathological contexts, such as cancer, where they inhibit anti-tumour immune responses (513, 774, 787).

1.3.7 Molecules in DC/T cell cross-talk leading to activation or attenuation of T cells

Very few studies have examined age-related changes in expression of inhibitory markers and DC regulatory function (72, 233, 245, 250, 673, 674, 677, 788). Changes to DCs during aging, particularly those which compromise their ability to stimulate effector T cell responses, are likely to contribute to reduced immune responses against pathogens and tumours in aged mice and humans. The section below examines key activation and inhibitory molecules expressed by DCs.

1.3.7.1 MHC class I/II, TCR and LAG-3

MHC class I and II are classical antigen-presenting molecules encoded in the MHC region of the genome (613). MHC class I is comprised of a single heavy chain which non-covalently associates with the protein β2-microglobulin, and MHC class II is formed from an α chain and a β chain (613, 789). Antigens displayed on MHC class I molecules are presented/cross-presented to CD8+ T cells, whilst antigens displayed on MHC class II molecules are presented to CD4+ T cells (5, 6). At the immunological synapse, MHC/peptide complexes on DCs bind to antigen-specific TCRs on T cells, triggering TCR signalling, which promotes further organisation and stabilisation of the immunological synapse, T cell proliferation and IL-2 production (704). The T cell co-receptors (CD8 and CD4) bind to the non-polymorphic regions of MHC molecules.
increasing cell adhesion and augmenting TCR signalling (790, 791).

In contrast, LAG-3 (a CD4-related molecule) expressed on activated CD4+ T cells binds MHC class II molecules on DCs with a higher affinity compared to CD4, and negatively regulates T cell activation (792-796). LAG-3/MHC class II interactions inhibit TCR signalling, preventing T cell proliferation, IL-2 production and Th1 polarisation (792-794, 797). Activated CD8+ T cells also express LAG-3, and this is associated with functional exhaustion and anergy (798-801). However, the exact mechanisms and ligand for LAG-3 expressed on CD8+ T cells remain to be determined (794, 800); a possible ligand may be the lectin LSECTin (802). Ligation of LAG-3 may interfere with CD8 co-receptor signalling, as it has been shown to associate with CD8 and the TCR complex on activated CD8+ T cells (794, 803).

Most studies observed that expression of MHC class I and II is maintained on elderly DCs (Table 1.7), with a few exceptions reporting increased (240) or decreased expression (239, 240, 691), depending on the DC subset and/or tissue site examined (Table 1.7). Overall, this suggests that DC antigen-presenting capacity is likely to be retained with aging. Two studies described an age-related increase in LAG-3 on elderly CD8+ T cells in mouse blood and spleen (804), and human blood (439), suggesting increased potential for T cell inhibition.

<table>
<thead>
<tr>
<th>DC subset</th>
<th>Elderly DC molecule expression compared to young adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human blood mDCs or total DCs</td>
<td>Comparative: MHC-I/II, CD40, CD80, CD86, IL-12 and/or IFN-γ (320, 691, 805) Increased: CD83, CD86 (320), TNF-α, IL-6, IL-12/23p40 (316) Reduced: MHC-II (691), TNF-α, IL-6 (669)</td>
</tr>
<tr>
<td>Human blood pDCs</td>
<td>Increased TNF-α, IFN-α (316)</td>
</tr>
<tr>
<td>Human MoDCs</td>
<td>Comparative: MHC-I/II, CD40, CD80 and/or CD86 (67-70, 317, 660, 670) IL-1β, IL-6, IL-8, IL-12 and/or TNF-α (69, 660) Increased: TNF-α and IL-6 (806)</td>
</tr>
<tr>
<td>Human DCs from low-density PBMC fraction</td>
<td>Comparative: CD40, CD80, CD86, TNF-α (673, 674) Increased: IFN-γ (673, 674)</td>
</tr>
<tr>
<td>Murine splenic CD11c+ DCs</td>
<td>Comparative: MHC-II, CD40, CD54, CD80 and/or CD86 (72, 242, 251, 675, 715) Increased: CD86 (72)</td>
</tr>
<tr>
<td>Murine splenic CD8α+ cDCs</td>
<td>Comparative: MHC-I/II, CD40, CD80 and/or CD86 (232, 240, 249)</td>
</tr>
<tr>
<td>DC subset</td>
<td>Elderly DC molecule expression compared to young adults</td>
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</tr>
<tr>
<td>Murine splenic CD8α⁺ cDCs</td>
<td><strong>Reduced:</strong> MHC-II, CD40 (240, 249)</td>
</tr>
<tr>
<td></td>
<td><strong>Comparable:</strong> MHC-II, CD40, CD80, CD86 (232, 240)</td>
</tr>
<tr>
<td></td>
<td><strong>Increased:</strong> MHC-I (240)</td>
</tr>
<tr>
<td>Murine splenic pDCs</td>
<td><strong>Comparable:</strong> MHC-I/II (240)</td>
</tr>
<tr>
<td></td>
<td><strong>Increased:</strong> MHC-II, CD40, CD80, CD86 (232)</td>
</tr>
<tr>
<td></td>
<td><strong>Reduced:</strong> CD40 (240)</td>
</tr>
<tr>
<td>Murine LN CD11c⁺CD11b⁺ or CD11c⁺MHC-II⁺ cDCs</td>
<td><strong>Comparable:</strong> CD40, CD80, CD86, IFN-γ, TNF-α, IL-6, IL-12p70 and/or MCP-1 (72, 244)</td>
</tr>
<tr>
<td></td>
<td><strong>Reduced:</strong> CD40 and/or CD86 (72, 246)</td>
</tr>
<tr>
<td>Murine LN CD8α⁺ cDCs</td>
<td><strong>Depending on LN site:</strong> Increased/comparable MHC-I, CD40 (240)</td>
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<tr>
<td></td>
<td><strong>Reduced/comparable MHC-II (240)</strong></td>
</tr>
<tr>
<td>Murine LN CD8α⁺ cDCs</td>
<td><strong>Comparable:</strong> MHC-II (240)</td>
</tr>
<tr>
<td></td>
<td><strong>Depending on LN site:</strong> reduced/comparable MHC-I, CD40 (240)</td>
</tr>
<tr>
<td>Murine LN pDCs</td>
<td><strong>Comparable:</strong> MHC-I (240)</td>
</tr>
<tr>
<td></td>
<td><strong>Increased:</strong> MHC-I (240)</td>
</tr>
<tr>
<td></td>
<td><strong>Reduced:</strong> MHC-II (240)</td>
</tr>
<tr>
<td>Murine thymic CD8α⁺ cDCs, CD8α⁺ cDCs and pDCs</td>
<td>Comparable MHC-II, CD40, CD80, CD86 (232)</td>
</tr>
<tr>
<td>Lung CD11c⁺ DCs</td>
<td>Comparable CD40, CD80, CD86 (72)</td>
</tr>
<tr>
<td>Murine CD11c⁺ APCs from peritoneal exudate cells</td>
<td>Comparable MHC class I/II, CD80, CD86 (615)</td>
</tr>
<tr>
<td>Murine BMDCs (derived in vitro with GM-CSF +/- IL-4)</td>
<td><strong>Comparable:</strong> MHC-I/II, CD40, CD54, CD80 and/or CD86 (71, 239, 614)</td>
</tr>
<tr>
<td></td>
<td><strong>Increased:</strong> TNF-α (66)</td>
</tr>
<tr>
<td></td>
<td><strong>Reduced:</strong> MHC-II (239)</td>
</tr>
</tbody>
</table>

1.3.7.2 CD40 and CD40 ligand

Ligation of the co-stimulatory molecule CD40 on DCs with CD40 ligand (CD40L) expressed on antigen-specific CD4⁺ T cells provides a strong maturation stimulus that drives DCs to become potent APCs; termed “licensing” (807, 808). CD40-activated DCs up-regulate antigen-presenting, co-stimulatory and adhesion molecules, and pro-inflammatory cytokines (809, 810). IL-12 secretion by CD40-activated DCs is important, as it promotes activation of CD4⁺ T helper cells, CD8⁺ T cells and NK cells (809, 811-813). DCs may first receive CD40/CD40L help by interacting with antigen-specific CD4⁺ T cells in LNs which is critical for optimal priming of CD8⁺ cytotoxic T cells (617, 814-823). CD40/CD40L interactions have reciprocal effects on CD4⁺ T cells by inducing their activation and effector function (824, 825).
With aging, several studies have reported comparable CD40 expression on elderly, relative to young, DCs (Table 1.7). However, certain DC subsets demonstrate an age-related decrease in CD40 expression, specifically: murine splenic CD8α+ cDCs and pDCs (240), and murine LN CD11c+ DCs (246) and CD8α- cDCs (240), suggesting an impaired capacity for these subsets to undergo licensing. The capacity of elderly CD4+ T cells to license DCs may also be compromised with aging, as three studies described reduced up-regulation of CD40L on stimulated T cells (826-828). In contrast, another study reported increased expression of CD40L on activated elderly human CD4+ T cells (829); this may have been due to differences in the stimuli used.

1.3.7.3 CD80, CD86, CD28 and CTLA-4

The co-stimulatory molecules CD80 and CD86 expressed on DCs have two ligands on T cells: the co-stimulatory molecule CD28 and the negative regulatory molecule CTLA-4 (830, 831). CD28 is constitutively expressed on T cells, and its interaction with CD80/CD86 on DCs leads to enhanced T cell activation, proliferation and cytokine production (830-833). Signalling through CD28 stabilises IL-2 mRNA, facilitating increased IL-2 production which is important for T cell expansion and survival (9, 834). DC/T cell interactions between CD80/CD86 and CD28 exert effects on DCs, specifically, IL-6 secretion which prevents generation of suppressive IDO (835).

In contrast, CTLA-4 is expressed on activated, but not resting, T cells (830, 836). There are two mechanisms by which CTLA-4 negatively regulates T cells: (i) by competing with CD28 for CD80/CD86 binding (837), and (ii) by ligation to CD80/CD86 to deliver negative signals which inhibit T cell IL-2 production, IL-2 receptor expression and cell cycle progression, leading to T cell anergy and tolerance (837-841). Interactions between CD80/CD86 and CTLA-4 promote DCs to produce suppressive IDO, which can (i) inhibit effector T cells by depletion of tryptophan, leading to arrest of T cell growth and induction of apoptosis, and (ii) promote Treg development (203, 530-535). Binding CTLA-4 to CD80 also depletes CD80 from the DC surface, thereby preventing subsequent interactions with CD28 (526, 528, 842).
CD80 and CD86 have a stronger affinity for CTLA-4, compared to CD28 (830, 831, 843), and CD80 binds more strongly to CD28 and CTLA-4, compared to CD86 (830, 831, 833, 843-845). The significance of the differences in binding kinetics and ligand affinity remain unclear, although CD86 may be more involved in T cell activation via interactions with CD28, on account of earlier, more rapid induction and greater magnitude of up-regulation compared to CD80 (830, 831, 846, 847). CD80 may be important for attenuation of T cell responses (830, 831), due to later up-regulation coinciding with CTLA-4 up-regulation on T cells (848), stronger affinity for CTLA-4 binding (830, 843) and longer interaction time with CTLA-4 (833).

CD80 and CD86 expression on DCs is reported to be retained (Table 1.7) or increased (72, 320) during aging, with the exception of elderly murine LN CD11c+ DCs, which have reduced CD86 (72, 246). This suggests that elderly DCs retain their co-stimulatory capacity with aging. CD28 expression on human CD8+ and CD4+ T cells declines during aging (440, 828, 849-853), and this is paralleled by increased CTLA-4 expression on elderly human blood CD8+ and CD4+ T cells (854), as well as on murine splenic, LN and blood CD4+ T cells (855, 856). Human and murine Tregs also maintain or increase their CTLA-4 levels (590, 603, 856). Taken together, the reduction in CD28 and concomitant increase in CTLA-4 suggests that CD80/CD86-CTLA-4 interactions during DC/T cell cross-talk may be favoured, leading to increased T cell suppression in elderly hosts.

1.3.7.4 ICOS and ICOS ligand

ICOS, a co-stimulatory molecule expressed on activated T cells (857-859), binds ICOSL on DCs (858, 860-862). ICOS/ICOSL interactions can have immunogenic and tolerogenic effects (9). Production of IL-2 stimulated by CD80/CD86-CD28 interactions potentiates up-regulation of ICOS on T cells (9, 857, 863). The strong positive signals from CD80/CD86-CD28 interactions overcome tolerogenic ICOS function, such that ICOS/ICOSL signalling co-operates with CD28 signalling to promote differentiation, proliferation and cytokine production (IL-4, IL-5, IL-10, IL-13, IFN-γ and TNF-α) by effector T cells (9, 857, 860, 863-866). ICOS/ICOSL interactions mainly promote Th2 cells by inducing production of Th2 cytokines, such
as IL-4, IL-5 and IL-10 (863, 865, 867-869), although they can contribute to Th1 (870-872) and CD8+ T cell effector responses (873-875). In the absence of CD28 stimulation, ICOS/ICOSL signals are tolerogenic and stabilise expression of the IL-10 receptor on T cells (9). T cells can then respond to IL-10 secreted by DCs, which induces T cell anergy and drives the development of suppressive IL-10-secreting Tregs (202, 749, 876-880).

There are conflicting reports regarding the effects of aging on ICOS expression. Two studies have shown that ICOS expression is retained on elderly murine splenic CD8+ and CD4+ T cells (788), and human blood CD8+ T cells (854). An age-related decline in ICOS expression has been reported for human CD4+ T cells (854), and murine splenic CD11c+ DCs (788). In contrast, one study observed increased numbers of ICOS+ Tregs in elderly murine spleens (856); ICOS+ Tregs have potent suppressive activity (879, 881), suggesting that Treg-mediated suppression may increase with age. No studies have yet examined the effects of aging on ICOSL expression on DCs and T cells.

1.3.7.5 PD-L1 and PD-1

Interactions between DCs and T cells via the PD-L1/programmed cell death protein-1 (PD-1) pathway leads to inhibition of effector CD8+ and CD4+ T cells (882). PD-1 can also bind to PD-L2, another ligand from the same family as PD-L1, resulting in similar inhibitory effects (883-885). DC/T cell cross-talk via this pathway leads to attenuation of TCR signalling (886), inhibition of T cell IFN-γ, TNF-α and IL-2 production (882, 886), decreased T cell proliferation (882, 886), cell cycle arrest (887), reduced generation of survival factors, leading to apoptosis (883, 888, 889), and induction of anti-inflammatory cytokines, such as IL-10 (890). One study has shown that CD8+ T cells are more susceptible to PD-L1/PD-1-mediated inhibition than CD4+ T cells, demonstrated by greater inhibition of CD8+ T cell proliferation in vitro (882). Additionally, the PD-L1/PD-1 pathway plays a role in the induction of T cell anergy (743, 891, 892), and promotion of Treg development (893, 894). PD-L1/PD-1 signalling has reciprocal negative effects on DCs: (i) inhibition of DC activation, and (ii) generation of suppressive, IL-10-producing DCs (895).
Furthermore, PD-L1 expressed on T cells can bind CD80 expressed on DCs, leading to inhibition of T cell activation and cytokine production (896, 897), and promotion of T cell anergy and tolerance (898).

PD-1 has been shown to increase with aging on murine CD8+ and CD4+ T cells in spleens, LNs and/or blood (245, 804, 855, 856, 899-903), and on human blood CD8+ T cells (439). Increased expression of PD-L1 has also been reported on elderly murine splenic CD8+ T cells (788). Two conflicting studies showed no age-related changes in PD-1 expression on human blood and murine splenic CD8+ and CD4+ T cells (788, 854).

The effects of aging on PD-L1/2 expression on DCs are less clear, with one study reporting increased expression of PD-L1 and PD-L2 on elderly murine splenic and lung DCs (245), and two contrasting studies showing no age-related differences in PD-L1 and PD-L2 in splenic, LN and lung DCs (72, 788). Further studies are required to examine age-related changes in the PD-1/PD-L1 pathway, particularly on DCs.

1.3.7.6 Galectin-9 and TIM-3

A model for the role of the galectin-9 (GAL-9)/T cell immunoglobulin and mucin-domain containing-3 (TIM-3) pathway in attenuation of T cell responses has been proposed (904, 905) in which activated, terminally differentiated CD4+ Th1 and CD8+ T cells secrete IFN-γ as part of their effector function, and up-regulate TIM-3 expression. IFN-γ promotes GAL-9 expression on APCs (906-908). DCs/APCs expressing GAL-9 interact with TIM-3-expressing effector T cells, triggering T cell apoptosis, thereby terminating adaptive immune responses and/or inducing tolerance (800, 904-906, 909-916). Furthermore, GAL-9/TIM-3 interactions promote expansion of suppressive Tregs (906, 911, 917).

The two studies which examined age-related changes in TIM-3 on T cells report conflicting results, with TIM-3 increasing on elderly murine splenic CD8+ T cells (899), remaining unaltered on elderly non-activated human CD8+ and CD4+ T cells (854), and decreasing on elderly human CD8+ T cells following mitogen stimulation (854). The effects of aging on GAL-9 expression on DCs have not yet been reported.
1.3.7.7  **CD39, CD73, adenosine and adenosine receptors**

DCs express the enzymes CD39 and CD73 (918, 919), and can generate adenosine, which exerts suppressive effects on effector CD8+ and CD4+ T cells, DCs and other APCs, and promotes Tregs, as described in section 1.2.4. Only two studies have examined the adenosine pathway and elderly T cells, and report conflicting results (920, 921). One showed an age-related decline in CD73 activity in human lymphocytes (921). In contrast, the other demonstrated that elderly human T cells activated in vitro via TCR stimulation produced higher quantities of adenosine compared to young T cells, although expression of CD73 and the A2A receptor were comparable between the two age groups (920). No studies have reported age-related changes in molecules of the adenosine pathway on DCs.

1.3.7.8  **Pro-inflammatory cytokines**

Activated DCs produce pro-inflammatory cytokines (6) including IL-6, IL-12, IFN-γ and TNF-α which act in combination with each other, and other pro- and anti-inflammatory cytokines to influence the development of effector CD8+ and CD4+ T cells in LNs (6, 372, 373). IL-12 and IFN-α/β activate cytotoxic CD8+ T cells (354, 356, 357). The types and combinations of cytokines secreted by DCs during cross-talk with CD4+ T cells directs the type of Th response generated, summarised in Table 1.4 (372, 373). In addition, IL-6 and TNF-α provide important co-stimulatory, activation and survival signals to differentiating T cells (922-927).

As well as influencing T cell responses, pro-inflammatory cytokines produced by DCs have several other effects. For example, IL-12, IFN-γ and TNF-α can act in an autocrine manner on DCs to amplify DC activation, maturation and survival (309, 928-930). However, IFN-γ may play a role in attenuating immune responses, as it up-regulates PD-L1 expression by DCs/APCs (9, 886, 931).

Production of pro-inflammatory cytokines by DCs has been reported to be increased, decreased, or maintained with aging, during the healthy steady state (Table 1.7) and following immune stimulation (Table 1.6). The contradictory results may be due to differences in the types of DCs and/or stimuli used. The lack of
consensus suggests that further investigation of aged DC pro-inflammatory cytokine production is required.

1.3.7.9 Anti-inflammatory cytokines

DCs secrete the anti-inflammatory cytokines IL-10 and TGF-β (517, 519, 932-934), which inhibit effector CD8+ and CD4+ T cells and DCs, and induce Treg development, as discussed in section 1.2.4. Several studies have shown that there are no age-related changes in the production of IL-10 (244, 320, 660, 673, 674, 805) and TGF-β (673, 674) by human and murine healthy steady state DCs. However, the effects of aging on DC IL-10 secretion following stimulation (Table 1.6), have been reported to be maintained (68, 317, 320), increased (71, 250), or impaired (251, 670, 677). Only one study has examined TGF-β production following stimulation, and reported an increase in TGF-β mRNA in elderly murine BMDCs stimulated with LPS and a TLR-7 agonist (677).

Negative regulatory molecules represent natural mechanisms of maintaining immune tolerance and preventing autoimmunity by attenuating immune responses (707, 710, 711, 935), however, these and other tolerogenic pathways can be exploited by tumours as a means of subverting anti-tumour immune responses (709, 936-938), and this is addressed in the studies in this thesis in the context of aging.

1.4 Dendritic cells, T cells and cancer

DCs are important mediators of anti-tumour immunity due to their ability to activate tumour-specific cytotoxic CD8+ T cells and Th1 cells (939, 940). Whilst antigen presentation and tumour-specific T cell activation can occur in tumours or in tumour-associated lymphoid structures (941-944), the major site DCs initiate tumour-specific T cell responses is tumour-DLNs (TDLNs; 945). Ideally, tumour-associated DCs capture tumour antigens and are activated by DAMPs such as HMGB-1, ATP and heat shock proteins released from tumour cells undergoing immunogenic or necrotic cell death (946-952). Activated tumour-associated DCs migrate to TDLNs, where they present tumour antigens and/or transfer antigens to
lymphoid-resident DCs, for priming and activation of tumour-specific effector T cells (939, 945, 953, 954). The most important DCs for initiating anti-tumour immunity are cross-presenting DCs (murine CD8α⁺ and CD103⁺ cDCs, and human CD141⁺ mDC2s), which activate tumour-specific CD8⁺ T cells (124, 125, 127, 955, 956). Lymphoid-resident CD8α⁻ cDCs can also cross-present tumour antigens and activate CD8⁺ T cells (145). Although pDCs have been ascribed a predominantly suppressive role in cancer, there is evidence that production of IFN-α by appropriately activated pDCs can inhibit: tumour growth and angiogenesis (957-961); the generation of suppressive tumour-associated macrophages (962); as well as enhance NK cell cytotoxic activity (963-965), and promote survival, maturation and antigen-presenting function of pDCs and cDCs (650, 965-967).

Tumour-specific cytotoxic CD8⁺ T cells are important effectors of anti-tumour immunity as they induce antigen-specific cytolysis of tumour cells via perforin and granzymes (370, 968-974); cytotoxic CD4⁺ T cells mediate tumour cell destruction using the same mechanisms (400, 401, 403, 405-407). Th1 cells also contribute to anti-tumour immunity by: providing T cell help via CD40-mediated licensing of DCs (975-978); secretion of cytokines (e.g. IFN-γ and IL-2) to promote CD8⁺ T cell activation (978, 979); and secretion of chemokines (e.g. MCP-1 and MIP-1α) to recruit anti-tumour macrophages (979-981). The importance of cytotoxic CD8⁺ T cells and Th1 cells is highlighted by studies showing that the presence of these cells in tumours is associated with good prognosis (982-984). Th2 cells can promote anti-tumour immunity via IL-4, IL-5, IL-13 and eotaxin, which activate anti-tumour eosinophils and neutrophils and/or have direct anti-tumour effects (981, 985-991). However, Th2 cells play a dual role, as they have also been shown to inhibit anti-tumour immunity and promote tumour growth (383).

Studies in murine tumour models have shown that although DCs are able to activate functional tumour-specific CD8⁺ T cells, DCs and T cells are disabled by tumours, resulting in tumour escape from immune destruction and tumour progression (992-998). Furthermore, as tumours progress, DCs are skewed towards a dysfunctional/regulatory phenotype, preventing the generation of effective anti-tumour immune responses (999, 1000). Tumour cells and suppressive tumour-
associated immune cells, such as M2 macrophages, myeloid-derived suppressor cells (MDSCs) and Tregs produce a wide range of suppressive factors, including vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF), GM-CSF, IL-6, IL-10, TGF-β, gangliosides, prostanoids and adenosine, which interfere with and impair all aspects of the DC lifecycle/function, summarised in Table 1.8 (954, 1001, 1002).

### Table 1.8 Mechanisms/factors that mediate tumour-induced DC dysfunction

<table>
<thead>
<tr>
<th>Tumour factors/ mechanisms</th>
<th>Effects on DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF</strong></td>
<td>Skews DC precursors to immature myeloid cells/MDSCs (1005, 1009)</td>
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<tr>
<td></td>
<td>Reduces co-stimulatory molecules (1010)</td>
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<tr>
<td></td>
<td>Impairs antigen-cross-presenting capacity (1003, 1010)</td>
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<tr>
<td></td>
<td>Induces pro-angiogenic function (1011)</td>
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<tr>
<td><strong>GM-CSF</strong></td>
<td>Skews myeloid cell differentiation away from DCs and towards immature, suppressive myeloid cells (1012-1015)</td>
</tr>
<tr>
<td><strong>M-CSF</strong></td>
<td>Inhibits differentiation (1016-1018)</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>Skews monocytes towards macrophage development (1022, 1023)</td>
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<tr>
<td></td>
<td>Impairs maturation (1024)</td>
</tr>
<tr>
<td></td>
<td>Reduces capacity to stimulate T cells (1024)</td>
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<tr>
<td></td>
<td>Promotes tolerogenic/regulatory DCs (1025, 1026)</td>
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<tr>
<td><strong>IL-10</strong></td>
<td>Inhibits maturation (1024, 1027-1031)</td>
</tr>
<tr>
<td></td>
<td>Reduces capacity to stimulate T cells (1024, 1027, 1032)</td>
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<tr>
<td></td>
<td>Induces regulatory/tolerogenic DCs (768)</td>
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<tr>
<td><strong>TGF-β</strong></td>
<td>Reduces co-stimulatory molecules, impairs maturation (1010, 1024, 1030, 1033)</td>
</tr>
<tr>
<td></td>
<td>Impairs antigen cross-presenting capacity (1010)</td>
</tr>
<tr>
<td></td>
<td>Impairs migration (1034)</td>
</tr>
<tr>
<td></td>
<td>Promotes regulatory DCs (999, 1033, 1035)</td>
</tr>
<tr>
<td></td>
<td>Induces apoptosis (1036)</td>
</tr>
<tr>
<td><strong>Prostaglandin E2/prostanoids</strong></td>
<td>Inhibits differentiation (1037, 1038)</td>
</tr>
<tr>
<td></td>
<td>Promotes regulatory DCs (999, 1035, 1039, 1040)</td>
</tr>
<tr>
<td><strong>Gangliosides</strong></td>
<td>Impairs differentiation (1041, 1042)</td>
</tr>
<tr>
<td></td>
<td>Induces apoptosis (1041, 1043)</td>
</tr>
<tr>
<td><strong>Adenosine</strong></td>
<td><strong>Binds A2A receptor:</strong> promotes localization of DCs and Tregs, leading to DC suppression (1044)</td>
</tr>
<tr>
<td></td>
<td><strong>Binds A2B receptor:</strong> Induces tolerogenic DCs (571)</td>
</tr>
<tr>
<td></td>
<td>Promotes DCs that induce Th17 responses (1045)</td>
</tr>
<tr>
<td></td>
<td>Blocks DC maturation (566, 569)</td>
</tr>
<tr>
<td><strong>Hypoxia</strong></td>
<td>Impairs antigen uptake and migration (1046)</td>
</tr>
<tr>
<td></td>
<td>Polarises DCs to a Th2-stimulating phenotype (568)</td>
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<tr>
<td></td>
<td>Induces A2B receptor expression (568)</td>
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<tr>
<td><strong>Lipid acquisition</strong></td>
<td>Reduces DC antigen uptake and processing (228, 1047)</td>
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<tr>
<td></td>
<td>Impairs antigen presentation and T cell activation (1047, 1048)</td>
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</table>
The effects of tumour factors are not limited to the tumour, and can impair the maturation and T cell-stimulatory capacity of resident splenic and LN DCs (1049-1051). As summarised in Table 1.8, tumour factors impair DC differentiation and induce apoptosis, which may contribute to reduced numbers of circulating DCs in cancer patients, relative to healthy controls (322, 1005, 1052-1058), and reduced DC numbers in spleens, LNs and/or tumours of tumour-bearing mice (228, 1059, 1060), all of which lead to diminished activation of anti-tumour T cell responses (954, 1002).

Moreover, tumours skew DCs towards tolerogenic activity by inducing immature or semi-mature DCs as seen circulating in cancer patients (1005, 1054, 1056, 1058, 1061), within tumour tissue (1030, 1051, 1062-1066), and following in vitro exposure of DCs to tumour factors (228, 1019, 1067-1070). Immature/semi-mature DCs are characterised by minimal up-regulation of antigen-presenting and co-stimulatory molecules, low secretion of pro-inflammatory cytokines (particularly IL-12), and often secrete IL-10 (516, 1002, 1071); they promote T cell anergy (516, 954, 1002) and engage in tumour-promoting activities, such as stimulating angiogenesis (1011, 1073-1076).

Tumours may induce regulatory DCs as a means of subverting anti-tumour immunity (1002, 1071), this is mainly associated with CD11b+ cDCs (1035, 1077, 1078) and pDCs (1079, 1080). Tumour-induced regulatory DCs inhibit tumour-specific CD8+ and CD4+ Th cell proliferation and effector function, induce anergy and/or apoptosis, and drive Treg expansion via PD-L1 (1000, 1033, 1076), IL-10 (748, 787) and/or TGF-β (519, 932, 933). Tumour-modulated DCs may also produce: arginase, which inhibits T cell proliferation via depletion of arginine (1035, 1078, 1081), and blocks CD8+ T cell IFN-γ secretion (1078); and adenosine, which inhibits T cell proliferation (918). Suppressive pDCs in tumour-bearing hosts induce CD4+ and CD8+ Tregs (751, 1079, 1080) due to low IFN-α secretion (1062, 1082-1084), IDO production (203, 533, 1085), expression of ICOSL (202, 759-761), and LAG-3 (1086). Thus, tumours modulate DCs away from anti-tumour activity, and towards suppressive, tumour-promoting functions using a variety of mechanisms.
Several tumour factors in Table 1.8, such as IL-10, TGF-β, VEGF, IDO and adenosine also suppress effector T cells and induce T cell anergy and exhaustion (933, 1087-1100). T cell exhaustion, which also occurs during chronic infections, is a state of T cell dysfunction characterised by loss of effector functions (e.g. proliferative and cytotoxic activity and secretion of IL-2, IFN-γ, and TNF-α), and co-expression of high levels of multiple inhibitory molecules, including several examined in this thesis: PD-1, CTLA-4, LAG-3, TIM-3 and CD39 (799, 1101-1105). As a result, exhausted T cells are unable to mediate tumour destruction (798, 936, 1106-1120). In addition, tumour cells express several ligands for the inhibitory molecules up-regulated on exhausted T cells, such as PD-L1, PD-L2 and GAL-9, resulting in termination of tumour-specific T cells (1121-1130). Tumour cells can also evade recognition by CD8+ T cells by down-regulating tumour antigens and MHC class I molecules (1100, 1131-1133).

Additionally, tumours promote Treg expansion via factors such as IL-10, TGF-β, and adenosine (1134, 1135), and the tolerogenic activity of Tregs is hijacked by tumours as a means of suppressing DCs and effector CD8+ T cells and Th1 cells (203, 751, 760, 761, 1134-1150). Tumour-associated Th2 cells can also adopt a suppressive role during cancer (383), via secretion of IL-4 and IL-13, which promote the development of pro-tumourigenic M2 macrophages (1151, 1152), and suppress CD8+ T cell cytotoxic activity (1153). Thus, tumours skew T cell responses away from effector function, and towards regulatory function; tumour-induced changes in DCs and T cells may be further modulated by aging.

### 1.4.1 Dendritic cells, T cells, cancer and aging

Given that DCs and T cells are important mediators of anti-tumour immunity (939, 940, 953), age-associated changes in DC and T cell function are likely to affect anti-tumour immune responses, and contribute to the increased incidence of cancer in the elderly (315, 614, 1154). Few studies have examined the combined effects of aging and cancer on immune function (43, 353, 1155). Most studies examining blood DCs in cancer patients examined patients across a wide range of ages, for example, 30-80 years of age (322, 1005, 1022, 1028, 1052-1058, 1061, 1156-1160).
Some studies compared cancer patients with age-matched healthy volunteers to identify only the effects of cancer (322, 1022, 1028, 1052-1057, 1159, 1160), however, others did not specify the ages of the patients (1065, 1161) or healthy controls (1005, 1058, 1061, 1156-1158). Additionally, in vitro studies examining the effects of tumour-derived factors on human DC function did not address the effects of aging, as blood samples or buffy coats from young donors were used as the source of MoDCs (228, 1019, 1067-1070). Studies examining T cells in elderly cancer patients are scarce, with one study observing similar proportions of tumour-infiltrating T cells expressing CD3, CD4, CD8 or FoxP3 in lung cancer patients aged <65 years, 66-79 years and >80 years (1162), and another study showing that the presence of functional, tumour-specific CD8+ T cells was associated with improved 5-year survival rates in breast cancer patients aged 65-87 years (1163), although these two studies did not examine age-related differences in T cells in elderly versus younger patients.

Furthermore, the majority of studies to-date using murine tumour models have been performed using young mice (1164-1167). There is evidence to suggest that elderly DCs have reduced anti-tumour function, as injection of elderly DCs into young tumour-bearing mice resulted in weaker tumour-specific cytotoxic CD8+ T cell (71, 614) and NK cell (1168) responses, and an inability to eradicate tumours, compared to tumour-bearing mice that received young DCs (71, 614, 1168). Administration of DC vaccines to elderly tumour-bearing mice leads to generation of weak cytotoxic T cell activity, and does not slow tumour growth, resulting in shorter survival time (1169, 1170). Similarly, age-related defects in murine CD8+ and CD4+ T cell anti-tumour function have been reported, including reduced T cell infiltration in tumours, impaired CD8+ T cell cytotoxic activity, and decreased production of effector cytokines, such as IFN-γ and IL-2, resulting in an inability to control tumour progression and reject tumours (614, 1171-1178). In contrast, one study reported that the ability of APCs to generate tumour-specific CD8+ T cells, and CD8+ T cell cytotoxic activity is intact in elderly tumour-bearing mice (1179). There is insufficient information regarding DC and T cell function during tumour progression in elderly mice and this area requires further investigation, as it may also impact on
therapies that can involve DC and T cell anti-tumour function, such as chemotherapy and immunotherapy.

1.5 Dendritic cells, T cells and chemotherapy

We now know from studies performed in young adult mouse cancer models that the immune system plays an important role in mediating the anti-cancer effects of some chemotherapeutic agents (1180-1183). Several chemotherapeutic agents induce ER stress and autophagy leading to immunogenic tumour cell death (ICD), characterised by: (i) pre-apoptotic exposure of calreticulin on the cell surface and release of other intracellular molecules, such as heat shock proteins; (ii) secretion of ATP, and (iii) release of HMGB-1 (948, 951, 1184-1189). Whilst most chemotherapies induce tumour cell apoptosis involving at least one of these features, only anthracyclines and oxaliplatin induce full ICD (1184, 1188, 1190). ICD leads to release of tumour antigens (1191-1193), whilst calreticulin stimulates DCs/APCs to phagocytose tumour cells, leading to processing and presentation of tumour antigens to T cells (1186, 1188, 1189). Chemotherapy-induced HMGB-1 binds TLR-4 on DCs promoting pro-IL-1β (an important step in the production of active IL-1β), as well as preventing lysosomal degradation of tumour antigens allowing their transfer into the cross-presentation pathway (951). ATP released from tumour cell ICD binds purinergic P2RX7 receptors on DCs, leading to activation of the NLRP3 inflammasome and IL-1β secretion (947, 948, 1194). DC-derived IL-1β activates IFN-γ-producing tumour-specific CD8+ T cells, and recruits IL-17-secreting γδ T cells into tumours; the latter recruit IFN-γ’CD8+ effector T cells into tumours (947, 948, 1194-1196). ATP also recruits myeloid cells into tumours and stimulates their differentiation into CD11c'CD11b'Ly6Chi inflammatory DCs which stimulate tumour-specific T cell responses (1197).

Chemotherapeutic agents can directly increase DC numbers (1198), promote DC differentiation/maturation leading to increased expression of co-stimulatory molecules (e.g. CD40 and CD80), promote DC migration, and enhance their T cell-stimulatory capacity (1199-1203). Moreover, chemotherapy can enhance anti-tumour CD8+ and CD4+ T cell responses by promoting T cell proliferation (1204,
1205) and secretion of effector cytokines, such as IL-2 and IFN-γ (1204, 1206-1208), increasing effector T cell trafficking into tumours (1204, 1207, 1209, 1210), and polarising CD4+ T cells towards Th1 and away from Th2 and Treg responses (1211-1216). Additionally, chemotherapeutic agents can render tumour cells susceptible to T cell-mediated destruction by: increasing expression of death receptors (e.g. Fas), leading to apoptosis (1217); sensitising tumour cells to TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis (1218); making tumour cells more permeable to granzymes (1219); increasing expression of MHC class I, leading to increased recognition by cytotoxic CD8+ T cells (1220, 1221); and broadening the range of tumour antigens exposed to the immune system, and the repertoire of tumour-specific T cells generated (1222). Furthermore, chemotherapy can deplete and/or reduce the suppressive activity of regulatory cells, such as Tregs (1223-1227) and MDSCs (1207, 1217, 1228-1233), thereby relieving suppression of DCs and effector T cells and enabling anti-tumour immune responses to occur (1180, 1182).

Chemotherapy is a double-edged sword as it can also inhibit and/or kill effector cells with certain agents inducing DC and T cell apoptosis, reducing circulating DC numbers in patients, and inhibiting DC maturation and T cell stimulatory capacity, and T cell proliferation (1182, 1234-1240). In addition, non-immunogenic tumour cell apoptosis can occur with some agents, leading to immune tolerance (1182). Moreover, some chemotherapies activate MDSCs (1241) and B cells (1242) that suppress anti-tumour immunity. The immunostimulatory effects of ATP released during chemotherapy-induced ICD may be undermined by CD39 and CD73 on tumour cells and tumour-associated immune cells, which convert ATP into adenosine to exert immunosuppressive effects on DCs and T cells (described in section 1.2.4), abrogating the positive effects of chemotherapy (1095, 1184, 1185, 1197, 1243, 1244). This may contribute to the tumour outgrowth seen once chemotherapy ceases in murine tumour models (1193, 1233, 1245-1247).

1.5.1 Dendritic cells, T cells and gemcitabine

One of the chemotherapies used in this study is gemcitabine, a synthetic analogue of the nucleoside cytidine, which incorporates into DNA, arresting DNA synthesis
and preventing DNA repair, leading to apoptosis (1248, 1249). Additionally, gemcitabine depletes the deoxyribonucleotide pool, thereby promoting its incorporation into DNA (1248, 1249). Gemcitabine is used to treat a variety of solid cancers in human patients, and demonstrates anti-tumour activity in pre-clinical murine tumour models when used alone or in combination with other chemo- and immunotherapies (1193, 1205, 1222, 1233, 1246, 1247, 1250-1255).

Studies using young adult mice have shown that gemcitabine exerts several positive immunostimulatory effects (1182), including ATP and HMGB-1 release from tumour cells (1190) and NFκB activation (1256, 1257) leading to DC maturation. In vivo studies show that gemcitabine increases expression of MHC class I and II, and co-stimulatory molecules on tumour-associated DCs (1217), and improves DC tumour antigen presentation/cross-presentation, leading to activation of tumour-specific effector CD8+ and CD4+ T cells (1193, 1258). Gemcitabine also expands the effector CD8+ T cell response to subdominant tumour antigens (1222), and increases expression of MHC class I on tumour cells, increasing recognition by CD8+ T cells (1220). In vitro exposure of DCs to supernatants from gemcitabine-treated tumour cells enhances their ability to stimulate tumour-specific T cell proliferation (1220). Gemcitabine also increases circulating DC numbers in cancer patients (1198), selectively eliminates suppressive MDSCs (1217, 1228, 1230-1233) and Tregs (1223, 1224, 1259), and inhibits tumour-specific suppressive B cell proliferation and antibody production (1205). This is likely to alleviate tumour-mediated immune suppression of DCs and T cells.

Despite the immunostimulatory effects of gemcitabine, studies in murine tumour models have observed tumour outgrowth upon treatment cessation (1193, 1233, 1245-1247). This may be because gemcitabine does not remove all suppressive immune cell subsets, as suppressive M2 macrophages were maintained in tumours and TDLNs in a murine mesothelioma model (1245). Moreover, gemcitabine may activate suppressive MDSCs that limit anti-tumour immunity by promoting IL-17-secreting CD4+ T cells (1241). Taken together, these studies show that gemcitabine exerts dual effects on anti-tumour immunity. Gemcitabine may initially exert positive effects on DCs and T cells, leading to anti-tumour immunity and tumour
regression, with the suppressive effects of gemcitabine occurring later and contributing to tumour outgrowth (1182). Further investigation of the effects of gemcitabine on DCs and T cells is required.

1.5.2 Dendritic cells, T cells and cisplatin

The other chemotherapy used in this study is cisplatin, a platinum-based compound that binds and cross-links DNA, inhibiting DNA replication and transcription, triggering a DNA-damage response that results in cell apoptosis (1260-1262). Cisplatin is used to treat a variety of human cancers, usually in combination with other chemotherapies, radiotherapy or immunotherapies (1252, 1260, 1263-1268). However, cisplatin is associated with toxicity, which has prompted the development of other, less-toxic platinum-based compounds (1269). Cisplatin also demonstrates anti-tumour activity in animal cancer models (1222, 1270), and this has been shown to involve the immune system (1222, 1260, 1271).

Like gemcitabine, cisplatin induces ATP and HMGB-1 release from tumour cells, which may activate DCs (1187, 1190, 1194, 1272, 1273). An in vitro study showed that cisplatin increases the capacity of DCs to stimulate T cells to proliferate and secrete IL-2 and IFN-γ, and reduces the likelihood of T cell inhibition by reducing PD-L2 expression on DCs and tumour cells (1264). In vivo, cisplatin increases DC proportions and enhances CD8+ T cell cytotoxic function and migration into murine tumours (1271), decreases Treg numbers in cancer patients (1259) and MDSCs in murine tumours (1271), alleviating suppression of DCs and T cells in tumour-bearing hosts. In addition, cisplatin increases tumour cell permeability to granzyme B, thereby increasing susceptibility to cytolysis (1219). Yet conflicting studies show that cisplatin exerts negative effects on DCs and T cells. One study reported that exposure of DCs to cisplatin-treated tumour cells in vitro failed to enhance tumour antigen uptake, IL-12 secretion and antigen presentation by DCs (1191). Others demonstrated that cisplatin promotes DC apoptosis (1234), maintains DCs in an immature state by down-regulating MHC class I and II, CD80 and CD86, whilst up-regulating their endocytic capacity, and promoting tolerogenic IL-10-secreting DCs that induce Th2 and Treg responses (1274-1276). Cisplatin can suppress T cells by
reducing their proliferation, IL-2 secretion and IL-2 receptor expression (1239, 1240). These conflicting results may be due to differences in cancer types and disease models, in vivo versus in vitro studies, use of human versus murine DCs and T cells, different dosages of cisplatin, and different treatment schedules/exposure times. Further studies are required as nothing is known about cisplatin’s effects on DCs and T cells in elderly hosts with cancer.

1.5.3 Chemotherapy, aging, dendritic cells and T cells

The combined effects of aging and cancer on immune function may affect responses to chemotherapy in the elderly, given that chemotherapeutic agents modulate immune cells, and successful responses to some chemotherapies require a functioning immune system (1180-1183). Despite this, the effects of aging on anti-cancer immune responses during chemotherapy have not been studied in depth (31). Elderly patients are often excluded from clinical trials due to increased risk of adverse responses and concerns regarding toxicity (1277-1280). Studies examining anti-cancer therapies in pre-clinical murine models often use young mice, due to the difficulties and expense associated with using aged mice (353, 1166, 1167, 1281, 1282). The use of young murine pre-clinical models to evaluate anti-cancer therapies may not accurately reflect the immune system of elderly cancer patients (353, 1166, 1167, 1281, 1282). One recent study reported that treatment of elderly (70-80 years of age) breast cancer patients with docetaxel and cyclophosphamide did not significantly alter biomarkers of aging, such as circulating IL-6 levels (1283). No studies have yet reported the effects of aging on DC and T cell function during treatment with chemotherapy.

1.6 Dendritic cells, T cells and immunotherapy

Cancer immunotherapy aims to promote anti-tumour immune responses using strategies to activate innate and adaptive effector cells, and/or strategies to alleviate immune suppression (709, 1284-1286). Many strategies focus on beneficially modulating DC and T cell function (953, 1284, 1285) and are tested in pre-clinical murine models that inevitably use young adult mice (1166, 1167, 1281).
Immunotherapeutic approaches involving DCs include DC vaccines, whereby DCs generated ex vivo from blood monocytes or haemopoietic precursors are loaded with tumour antigens, and injected into tumour-bearing hosts to stimulate tumour-specific effector CD8+ and CD4+ T cells (649, 940, 953). DCs can also be expanded in vivo using growth factors, such as FLT3 ligand (1287), and tumour antigens targeted to DCs in vivo by conjugating tumour peptides to antibodies specific for receptors involved in DC antigen uptake, such as the lectins DEC-205 (1288-1290) and CLEC9A (1291, 1292). As tumours can maintain DCs in an immature state, another approach is to induce DC maturation, for example, using TLR ligands (940, 1293-1295), cytokines, such as GM-CSF and IFN-α (1296), and/or agonist antibodies directed against DC co-stimulatory molecules, such as CD40 (940, 1297).

One of the main approaches involving T cells is adoptive cell therapy, which involves in vitro expansion of tumour-specific T cells, which can be host T cells with natural tumour antigen specificity, or host T cells that have been genetically engineered to express a tumour-specific TCR or chimeric antigen receptor, and re-administration of these T cells to the tumour-bearing host (1284, 1298). Cytokines (such as IL-2), and agonist antibodies directed against T cell co-stimulatory molecules (such as 4-1BB, OX40 and glucocorticoid-induced TNF receptor-related protein) are often co-administered to stimulate effector T cells (1296, 1299-1306).

Strategies aimed at relieving tumour-induced immunosuppression represent another way of enhancing DC and T cell anti-tumour activity (940, 953, 1307). One strategy is checkpoint blockade, which is the use of antagonistic antibodies directed against checkpoint regulatory molecules to inhibit negative signalling (709). Anti-CTLA-4, anti-PD-1 and anti-PD-L1 antibodies are currently used to treat cancer patients (709, 1286, 1308, 1309); blockade of PD-L1, which is expressed by DCs, aims to reduce DC/T cell interactions via the PD-1/PD-L1 pathway, allowing DCs to activate tumour-specific effector T cells (1310-1314), whilst blockade of PD-1 and CTLA-4 prevents T cells from responding to negative signals and permits them to perform their effector function (709). Blockade of other regulatory molecules, such as TIM-3 (1100, 1118, 1315, 1316), LAG-3 (798, 801, 1317), CD39 (1318-1320), CD73 (1097, 1243, 1319, 1321-1324), and the A2A and A2B receptors (545, 566, 1097,
1325, 1326) have been shown to improve anti-tumour immune responses in vitro and/or in murine cancer models. As DCs and/or T cells express these molecules, it is possible their blockade reduces DC and T cell inhibition and suppressive function. Depletion and/or inhibition of suppressive immune cells, such as Tregs (493, 1135), MDSCs (1327-1329), and M2 macrophages (1330, 1331), may also reduce tumour-mediated DC and T cell suppression (1307). Different immunotherapies can be combined with other anti-cancer treatments, such as surgery, chemotherapy, and radiotherapy to produce synergistic effects, leading to improved anti-cancer immune responses and efficacy (709, 1286, 1332). Thus, several immunotherapeutic approaches can augment DC and T cell anti-tumour activity.

### 1.6.1 Immunotherapy, aging, dendritic cells and T cells

To-date, most pre-clinical animal studies have used young adult mouse models to evaluate cancer immunotherapies, and human clinical studies often exclude elderly patients (1166, 1167, 1281, 1282, 1333). As many immunotherapies target DCs and T cells, age-associated modulation of DC and T cell function may impact on their efficacy (1154, 1166). Of the few murine studies that considered aging, several showed reduced efficacy of immunotherapy in elderly tumour-bearing mice (1166, 1167, 1334). Vaccinating elderly tumour-bearing mice with tumour antigens/peptides, tumour cells, or peptide-pulsed DCs derived from young mice, or combining DC vaccines with IL-2 is ineffective, demonstrated by reduced tumour-specific cytotoxic CD8+ T cell generation, impaired tumour regression and shorter survival times, compared to young mice (614, 1169, 1170, 1335-1340). Administration of certain TLR ligands, such as LPS (1341), poly I:C (1342), and CpG (1343) promotes tumour rejection in young, but not elderly, mice. Agonist anti-OX40 antibody alone was unable to induce tumour regression in elderly mice, and this was only partially improved by co-administration of IL-12 (608, 1344). Whilst immunisation with IL-2-secreting tumour cells (1340), or tumour cells expressing the co-stimulatory molecule CD80 (1345), or administration of agonist anti-OX40 or anti-4-1BB antibodies (1345) induced tumour rejection in elderly mice, these mice failed to develop protective memory responses. The reduced efficacy of these
strategies in elderly mice could be attributed to age-associated defects in DCs and T cells (1154, 1166).

Nonetheless, if appropriate co-stimulatory signals are provided, immunotherapies involving DCs and T cells can be effective in the elderly. Combining agonist anti-OX40 or anti-4-1BB antibodies with: (i) a DC vaccine inhibited tumour growth in young and elderly mice (1169); and (ii) immunisation with CD80-expressing tumour cells led to tumour rejection and memory development in elderly mice (1345). Neutralising IL-6 also improved tumour cell/peptide vaccines and DC vaccines in elderly mice, resulting in improved slowing of tumour growth (1335, 1336). In contrast to the study by Dominguez and Lustgarten (1343), two studies showed that the TLR ligand CpG is equally effective at promoting tumour rejection and inducing memory responses in young and elderly mice (602, 1342); the conflicting results may be due to differences in strains of mice and tumour models used.

Depletion/inhibition of suppressive Tregs is another potential strategy, although the studies performed to-date have reported conflicting results. One study demonstrated that Treg depletion in elderly tumour-bearing mice led to tumour rejection via restoration of cytotoxic T cell activity, and induced memory responses (602). In contrast, others have shown that Treg depletion alone (1346), or in combination with CpG (1343) is unable to induce complete tumour regression in elderly mice. Yet another study showed that Treg depletion increased immunosuppression by promoting MDSC expansion, however combining Treg and MDSC depletion improved tumour rejection in elderly mice (1347). Differences in mouse strains, ages and tumour models may account for the conflicting results. Taken together, these studies suggest that aging influences the efficacy of immunotherapy, and designing specific strategies to target elderly immune cells, including DCs and T cells, could improve outcomes for elderly patients with cancer.

1.6.2 Dendritic cells, T cells and IL-2/agonist anti-CD40 antibody immunotherapy

The studies in this thesis extend previous studies from our laboratory using an IL-2
and agonist anti-CD40 antibody combination immunotherapy (IL-2/CD40) that induces permanent, curative regression of tumours in young mice (1245, 1348-1351).

IL-2 is a cytokine produced mainly by activated CD4+ T cells, and to a lesser extent, by activated CD8+ T cells, NK cells and DCs (1352). It acts via the IL-2 receptor, which contains three subunits: α chain (CD25), β chain (CD122), and the common γ chain (CD132; 1352). The β and γ chains form the low-affinity IL-2 receptor, whilst the combination of all three chains forms the high affinity IL-2 receptor, with CD25 playing an important role in high-affinity IL-2 binding (1352). IL-2 is mainly used as a cancer immunotherapy due to its T cell-promoting activity, specifically, promoting the survival, effector function, proliferation and differentiation of CD8+ and CD4+ T cells (1301, 1302, 1353). Other immune cells, including DCs, express CD25 (1354-1356) and IL-2 enhances the ability of DCs to prime T cells (1357, 1358), and increases CD40 expression, augmenting its T cell activation capacity (1359). IL-2 can promote anti-tumour activity in other immune cells, such as activating macrophages with cytotoxic function (1360-1363), and stimulating TNF-α and nitric oxide production by neutrophils, enabling them to lyse tumour cells (1364, 1365). A limitation of IL-2 is that it may induce Treg expansion, due to constitutive expression of high levels of the IL-2 receptor on Tregs (1366-1369).

Agonist anti-CD40 antibody targets the co-stimulatory molecule CD40 expressed on DCs and other APCs (macrophages and B cells), as well as several non-immune cells (1297, 1370). The primary rationale lies in its ability to activate/license DCs via CD40 into potent APCs; licensed DCs then activate tumour-specific CD8+ T cells, bypassing CD4+ T cell help, which is usually required (811, 822, 1370-1378). In addition to stimulating effector CD8+ T cells, CD40-activated DCs secrete IL-12, which activates NK cells with anti-tumour cytolytic function (1379). The DC subsets involved in anti-tumour immune responses stimulated by agonist anti-CD40 antibody are yet to be identified (1297).

In addition to activating DCs, agonist anti-CD40 antibody has effects on other cell types. It promotes antigen-presenting function (810, 1380, 1381) and tumouricidal
activity via secretion of TNF-α, IFN-γ and/or nitric oxide by macrophages (1382-1386). Similarly, it promotes development of B cells into APCs (1387-1393), and induces secretion of tumour-specific antibodies which mediate tumour cell death (1350). Ligation of CD40 on CD8+ T cells has been shown to play a role in generation of memory (1394). Anti-CD40 antibody directly targets CD40+ tumour cells, leading to antibody-dependent cellular cytotoxicity and tumour cell apoptosis, which releases tumour antigens for presentation by APCs (1395-1399). Anti-CD40 exerts dual effects on CD40+ endothelial cells lining tumour blood vessels: one study has shown that CD40-activated tumour vessel endothelia facilitate the access and accumulation of effector immune cells into tumours (1400), whilst others have reported that activation of tumour endothelia via CD40 promotes angiogenesis (1401-1403).

Several studies have tested the IL-2/CD40 combination in young adult murine cancer models. Studies in murine renal cell carcinoma, lung cancer, lymphoma and melanoma models showed that systemic administration of IL-2/CD40 is non-toxic and mediates regression of primary tumours and metastases, increases survival and confers protection against tumour re-challenge; these effects were not observed with either monotherapy, suggesting that IL-2/CD40 acts synergistically (1359, 1404-1406). In these studies, the IL-2/CD40-induced response involved several immune cells, with IFN-γ+CD8+ T cells playing an important role (1359, 1405, 1407). IL-2/CD40 alleviated suppression of effector T cells by reducing numbers of Tregs and MDSCs, and arginase-mediated inhibitory activity of the latter, within tumours (1405, 1408). The role of DCs was less clear. Whilst IL-2/CD40 increased DC numbers in spleens, which was associated with increased CD8+ T cell activation and expansion (1359), a later study by the same group observed that IL-2/CD40 increased expression of PD-L1 on splenic CD11c+ cells, reduced splenic CD4+ Th cells and induced defective secondary immune responses, which was speculated to be due to increased PD-L1/PD-1 interactions between splenic DCs and T cells, leading to loss of CD4+ T cells (1407, 1409).

In contrast, our previous studies showed that systemic administration of IL-2/CD40 to young mesothelioma or lung carcinoma-bearing mice was highly toxic, but this
could be overcome by targeted, intra-tumoural delivery of IL-2/CD40 (1351). The IL-2/CD40 combination was synergistic and induced permanent regression of large tumours, with cured mice remaining tumour-free for the remainder of their natural lives (1348, 1349, 1351). T cells, neutrophils (1351), B cells (1350) and pro-inflammatory M1 macrophages (1245) play an important role in the IL-2/CD40-mediated anti-tumour response, whilst CD8+ and CD4+ T cells and NK cells promoted memory responses following IL-2/CD40 treatment (1348, 1349). Anti-CD40 antibody alone induced a transient increase in DC numbers/proportions mid-way through treatment that declined in TDLNs and tumours by the end of treatment (1350). However, our previous studies did not examine the effects of IL-2/CD40 on DC function.

### 1.6.3 Dendritic cells, T cells, IL-2/agonist anti-CD40 antibody and aging

Few studies have examined use of IL-2 and/or agonist anti-CD40 antibody in elderly tumour-bearing hosts. There are conflicting studies regarding the efficacy of IL-2 with aging. It has been reported that elderly cancer patients (aged 70 years and above) can tolerate IL-2, and have similar overall survival and progression-free survival rates as young patients (1410, 1411). Two murine studies also reported positive effects, demonstrating that IL-2 restores the anti-tumour function of elderly T cells, and increases the survival time of elderly tumour-bearing mice (1174, 1412). In contrast, others have shown that administration of IL-2 to elderly tumour-bearing mice cannot inhibit tumour growth (1413), and does not restore CD8+ T cell cytotoxic activity, but instead induces suppressive T cell function (1414). Combining IL-2 with other agents (indomethacin or cyclophosphamide) did not slow tumour growth and prolong survival in elderly mice (1413, 1415). The effects of IL-2 on elderly DCs were not examined in these studies.

Activation of DCs via CD40 may be a promising translational strategy for elderly cancer patients, as one study reported that administration of a tumour antigen vaccine conjugated to CD40L improved T cell anti-tumour responses in elderly tumour-bearing mice (1416).
Two studies have shown that systemic administration of IL-2 plus agonist anti-CD40 antibody to elderly mice resulted in a systemic, lethal toxicity mediated by macrophages secreting pro-inflammatory IFN-γ, TNF-α and IL-6 causing a cytokine storm (1417, 1418). Targeted, intra-tumoural delivery of IL-2/CD40 may be better tolerated in elderly mice, as our laboratory has demonstrated that this approach is non-toxic in young mice (1351). Our studies have also shown that healthy and tumour-exposed elderly murine macrophages treated with IL-2/CD40 in vitro restore IFN-γ and perforin production by elderly T cells, suggesting that IL-2/CD40 may be a promising therapy for elderly tumour-bearing hosts by beneficially modulating APCs (1419, 1420). The effects of IL-2/CD40 on DCs and T cells during aging and cancer have not yet been examined.

1.7 Mesothelioma

This study focused on mesothelioma, a cancer prevalent in elderly populations (1421, 1422) that affects serosal membranes of the pleura, peritoneum, pericardium, vagina and ovaries, with the majority (80-85%) of cases occurring in the pleura (1422, 1423). Mesothelioma has a poor prognosis, with an average survival time of 9-12 months from the time of diagnosis (1422, 1424). The principal carcinogen for mesothelioma is asbestos (1423). Inhalation of asbestos fibres leads to deposition in the lungs, where they can induce tumour development by: (i) penetrating the lung and causing pleural irritation; (ii) damaging chromosomes by piercing the mitotic spindle; (iii) promoting local inflammation, leading to the generation of reactive oxygen species which damage DNA; and (iv) activating proto-oncogenes (1423, 1424).

There is a long latency period, at least 20 years, but can extend up to 70 years, between the time of asbestos exposure and clinically detectable mesothelioma (1422, 1425). As a result, mesothelioma affects elderly populations, as most cases occur in individuals aged 60 years or greater (1421, 1422), with the highest incidences occurring in individuals aged 75-85 years (1421). Interestingly, young hosts exposed to asbestos fibres have a lower risk of developing mesothelioma compared to elderly hosts (1426-1428). It is possible that the lower risk of
mesothelioma in young hosts is due to their more efficient immune system (1426, 1428), and the long latency period may be related to immunosenescence and failing tumour immunosurveillance (43).

1.7.1 Mesothelioma, dendritic cells and T cells

The immune system can recognise mesothelioma (1429), as suggested by reports of spontaneous regressions in mesothelioma patients (1430-1432), which were associated with tumour-infiltrating lymphocytes (1431). The presence of lymphocytes and CD8+ T cells in tumours has also been associated with better prognosis and survival (1433-1435). A previous study from our laboratory showed that increased numbers of circulating mDC1s, and maintenance of the ability of patient-derived MoDCs to mature in response to in vitro stimulation with LPS +/- IFN-γ or CD40L is also associated with increased survival (322).

There is evidence of DC and T cell dysfunction in mesothelioma patients. DCs are reported to be absent from human mesothelioma tumours (1436), and circulating mDC1, mDC2 and pDC numbers are reduced in patients (aged 47-84 years), relative to age-matched healthy controls (aged 48-84 years; 322). MoDCs derived from patient monocytes are dysfunctional due to reduced CD40 expression, antigen uptake capacity, and impaired maturation in response to stimulation, relative to age-matched healthy controls (322). Another of our studies observed that in vitro exposure of immature MoDCs from young donors to a human mesothelioma cell line promoted increased MoDC lipid content, which was associated with impaired antigen processing ability, partial DC maturation (reduced CD1a and increased CD86), and secretion of IL-10, suggesting tolerogenic function (228). These studies did not observe defects in the ability of patient MoDCs or tumour-exposed young MoDCs to stimulate proliferation of allogeneic young T cells (228, 322), and peripheral blood lymphocytes from mesothelioma patients are able to proliferate after stimulation by allogeneic PBMCs (1437). However, the quality of the T cell response may be altered, due to reduced perforin expression on patient CD8+ T cells (1438), and reduced expression of IFN-γ and the chemokine receptor CXCR3 on patient CD4+ T cells (1439), suggesting impaired effector and migratory function.
Similarly, studies in young adult murine mesothelioma models have shown that anti-mesothelioma immune responses are ineffective (996, 1440-1442). Whilst there is evidence that DCs can present/cross-present tumour antigens, with both CD8α+ and CD8α- cDCs playing a role in tumour antigen cross-presentation (145), and activate functional, tumour-specific cytotoxic CD8+ T cells in TDLNs, tumour regression does not occur (145, 1193, 1349, 1440, 1441, 1443, 1444). In one study, the defective T cell response was attributed to impaired migration of effector CD8+ T cells into tumours (1441). In contrast, our previous studies showed that effector CD8+ T cells trafficked to tumours, and it was speculated that their function was thwarted by suppressive mechanisms within tumours (996, 1440). DCs and T cells may become increasingly dysfunctional as tumour burden increases, as suggested by a study from our laboratory showing that increasing tumour burden was associated with: reduced proportions of cross-presenting CD8α+ cDCs in tumours and TDLNs; decreased tumour antigen-specific T cell proliferation in TDLNs; as well as increased lipid content of tumour-associated DCs (228) implying DC dysfunction (1047). Another study demonstrated that tumour-associated DCs co-express antigen-presenting and co-stimulatory molecules (MHC class I and II, CD40, CD80 and CD86), and inhibitory PD-L1 and PD-L2; the latter implies an increased potential to suppress effector T cells, which was supported by the observation that these DCs were unable to cross-present tumour antigens and activate T cells (1258).

The possible mechanisms by which mesothelioma disables DC and T cell anti-tumour function are the same as those described in section 1.4 and include secretion of suppressive cytokines/factors, such as as TGF-β, VEGF, IL-6, IL-10 and prostaglandin E2, all of which have been detected in pleural effusions from mesothelioma patients (1436, 1445, 1446), and human and murine mesothelioma cell lines secrete TGF-β, VEGF and/or IL-6 (1436, 1447-1450). Human and murine mesothelioma tumours also express PD-L1 (1451, 1452), which may inhibit effector T cells, as CD8+ and CD4+ T cells in mesothelioma-bearing mice express PD-1 (1452). Mesothelioma also promotes suppressive immune cells, as mesothelioma factors polarise human and murine macrophages towards suppressive M2 cells in vitro (1245, 1419, 1453), and suppressive macrophages comprise a large proportion of
murine tumours (1245, 1440) that increase with tumour burden (1245). Tregs may mediate suppression, as Tregs have been observed in patient tumour specimens (1436) and pleural effusions (1445), and their presence is associated with shorter survival times (1434). Tumour-associated Tregs have been reported to increase with tumour burden in murine mesothelioma (1454-1456), and their depletion slows tumour growth and improves survival (1436, 1455, 1456). Our laboratory has shown that Tregs only play a role when tumour burden is minimal and that Treg depletion in animals with large tumours did not restore CD8+ T cell cytotoxic activity (1440). However, most studies in murine mesothelioma models used young adult mice that are 6-8 weeks of age, equivalent to approximately 14-16 human years (230), with a limited number of studies using mice aged up to 11 months, equivalent to approximately 40 human years (1457-1460), and this may not accurately reflect the immune system of elderly mesothelioma patients, and may influence responses to anti-cancer therapies.

### 1.7.2 Mesothelioma, chemotherapy and immunotherapy

Current treatment options for mesothelioma patients are surgery, radiotherapy and chemotherapy, or a combination of these approaches (1263). The two chemotherapies used in this study, gemcitabine and cisplatin, were used in combination for mesothelioma, and cisplatin is currently part of the first line chemotherapy for mesothelioma, in combination with pemetrexed (1263, 1461, 1462). None of these therapies are curative, and extend survival time by only a few months (1263, 1463). Therefore, other approaches need to be explored, and immunotherapy is promising (1422, 1463). Immunotherapies that have been, or are currently being tested alone or in combination with chemotherapy, include DC vaccines (1464), cytokines such as IL-2, IL-12, GM-CSF, IFN-α and IFN-γ (1463, 1465, 1466), CD40-activating antibodies (1467), VEGF inhibitors (1263, 1468), and CTLA-4, PD-1 and PD-L1 blockade (1263, 1469-1471) However, the median age of mesothelioma patients in clinical trials is often between 60-70 years, with low proportions of patients in higher age brackets (1462, 1467, 1470, 1472-1475). Given that the highest incidences of mesothelioma occur in people older than 70 years
(1421), these age groups need to be better represented in clinical trials (1472).

The rarity of human mesothelioma makes it difficult to investigate the effects of chemo- and immunotherapy on immune cells, thus, murine models are often used (1463). A variety of chemo- and immunotherapies, including combination approaches, have been tested in young adult mice and show promising results (1193, 1205, 1251, 1258, 1349, 1463, 1467). Examples include DC vaccines (1476), tumour cell vaccines (1477), TLR agonists (1294, 1478), agonist anti-CD40 antibody (1247, 1251, 1348-1351, 1467, 1478, 1479), cytokines such as IL-2 (1348, 1349, 1351, 1444), gene therapy (1480-1483), checkpoint blockade (1246, 1452), and depletion of suppressive immune cells, such as Tregs and macrophages (1245, 1436, 1455, 1456).

Treatment of mesothelioma-bearing mice with gemcitabine enhances DC tumour antigen presentation/cross-presentation (1193, 1258), inhibits tumour-specific B cell proliferation and antibody production (1205), and expands effector CD8+ T cell responses to sub-dominant tumour antigens (1222) leading to tumour regression in young mice. Additionally, gemcitabine synergises with agonist anti-CD40 antibody (1251), surgical de-bulking and agonist anti-CD40 antibody (1247), and CTLA-4 blockade (1246); the latter is also synergistic with cisplatin (1484). Despite this, tumour outgrowth occurs after chemotherapy ceases (1193, 1245-1247), which may be due to failure to remove all suppressive cell types (1245).

Our previous studies examined intra-tumoural IL-2 and agonist anti-CD40 antibody. IL-2 alone induces complete regression of small tumours, mediated by CD8+ and CD4+ T cells, but is ineffective against larger tumours (1444). Similarly, agonist anti-CD40 antibody could only induce regression of small tumours, mediated by follicular B cells and CD8+ T cells (1350). The combination of IL-2/CD40 is synergistic, and induces permanent, curative regression of large tumours, mediated by CD8+ T cells, granulocytes, and M1 macrophages (1245, 1348, 1349, 1351), and eradicates distal tumours, mediated by CD8+ and CD4+ T cells (1349, 1351). Tumours did not recur (1349, 1351), and mice were protected from tumour re-challenge due to memory responses mediated by CD8+ and CD4+ T cells (1348). These studies were in young
adult mice, and further studies are required to examine chemo- and immunotherapy in elderly murine models to better represent the elderly immune system of mesothelioma patients.

1.8 Project aims

DCs and T cells change continuously throughout life, and their age-related changes may contribute to the increased susceptibility of elderly individuals to cancer. However, changes to human and murine DCs in the contexts of healthy aging and cancer have not been well-characterised to-date and report conflicting findings.

Thus, this thesis aimed to:

1. Examine changes to the functional status of DC subsets during healthy aging by comparing:
   a) Blood samples from healthy young (21-33 years of age) and elderly (60-77 years of age) human volunteers;
   b) Blood and lymphoid tissue samples from healthy young mice, aged 2-5 months (equivalent to 16-26 human years) and elderly mice, aged 20-27 months (equivalent to 60-80 human years).
2. Determine the effect of mesothelioma factors on young and elderly human DCs using in vitro studies.
3. Determine the effect of mesothelioma on young and elderly murine DCs and T cells using in vivo studies.
4. Determine the effect of chemotherapy and immunotherapy on young and elderly murine DCs and T cells using in vivo studies.
Chapter 2  
Materials and methods

2.1  Cell culture

2.1.1  Cell culture media and maintenance

All cell lines and primary cells were cultured in complete medium, consisting of RPMI 1640 media (Invitrogen, California, USA), supplemented with 10% fetal calf serum (FCS; ThermoScientific, Victoria, Australia), 100 units/ml of penicillin and 100 μg/ml streptomycin (Penicillin-Streptomycin; Life Technologies, Victoria, Australia), 2 mM L-glutamax (Life Technologies), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Missouri, USA). All cells were cultured at 37°C in a 5% CO2 atmosphere.

2.1.2  Tumour cell lines and tumour cell-conditioned media

JU77 is a human malignant mesothelioma cell line established from the pleural effusion of a patient with confirmed disease diagnosis (1485). AE17 is a murine malignant mesothelioma cell line derived from the peritoneal cavity of C57BL/6J mice injected with asbestos fibres, as previously described (1444).

For the generation of tumour cell-conditioned media (TCM), tumour cell lines were cultured in serum-free medium (Invitrogen). After 48-72 hours, conditioned media from tumour cell cultures were centrifuged and cell-free supernatants collected and stored at -80°C until use.

2.1.3  Passaging tumour cell lines

JU77 and AE17 tumour cell lines were grown to approximately 80-90% confluence before passaging. For passaging, medium was removed from confluent cells and cells were washed using phosphate buffered saline (PBS; Life Technologies). AE17 cells were detached from tissue culture flasks using 0.25% trypsin (Life Technologies). JU77 cells were detached from tissue culture flasks using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Life Technologies). Trypsin is a proteolytic enzyme which cleaves adhesion proteins that mediate cell attachment.
(1486), and EDTA chelates calcium ions (1487), which are required by integrins to mediate cell-to-cell and cell-to-extracellular matrix binding (1488). Once cells were detached, complete medium was added to neutralise trypsin/trypsin-EDTA, due to the presence of trypsin-inactivating enzymes, such as α1-antitrypsin, in serum (1489). Cells were centrifuged at 1,200 rpm for 5 minutes, resuspended in complete medium and seeded into new tissue culture flasks or plates (Beckton Dickinson, California, USA).

2.1.4 Freezing and thawing cells

For cell freezing, cells were resuspended at 1-2 x 10⁶ cells/ml in freezing solution, consisting of 90% FCS and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich), a cryopreservative agent (1490). One ml of cell suspension was aliquoted per cryovial (Nunc/ThermoScientific). Cryovials containing cells were placed in a Mr. Frosty (Nalgene/ThermoScientific) or styrofoam rack at -80°C overnight for gradual freezing, then transferred for storage at -80°C.

Cells stored at -80°C in 90% FCS and 10% DMSO were thawed in a 37°C water bath and transferred to 15 ml tubes containing complete medium, by drop-wise addition of the cell suspension. Cells were centrifuged at 1,200 rpm for 5 minutes to remove DMSO, resuspended in complete medium, transferred to tissue culture flasks or plates and cultured at 37°C in a 5% CO₂ atmosphere. Primary cells were cultured overnight, to allow recovery time, before being used in assays.

2.2 Human studies

2.2.1 Human ethics approval

This study was approved by the Curtin University Human Research Ethics Committee (approval number HR102_2012) and the Australian Red Cross Blood Service Ethics Committee (approval number 13-03WA-19).

2.2.2 Volunteer recruitment

Healthy young (21-33 years of age) and elderly (60-77 years of age) volunteers were
recruited via: (i) radio advertising, (ii) poster advertising at Curtin University and rotary clubs and (iii) word of mouth within Curtin University. All volunteers gave written consent prior to study participation and their health status was assessed via a questionnaire detailing current and past medical conditions, current medications, family disease history, smoking status and asbestos exposure status (Appendix A).

### 2.2.3 Blood sample collection

Whole blood samples (50 ml) were collected from healthy young and elderly volunteers via venipuncture into five 10 ml K$_2$EDTA vacutainers (Beckton Dickinson) and transported to the laboratory for immediate processing. EDTA acts as an anticoagulant by chelating calcium ions, which are required for enzymatic reactions in the coagulation cascade (1487). Three buffy coat samples from healthy male donors aged 23-31 years were collected by the Australian Red Cross Blood Service and obtained the following day for laboratory processing.

### 2.2.4 Analysis of whole blood parameters

Full blood counts were performed on fresh, anti-coagulated whole blood samples (100 µl) on a Mindray BC-2800Vet automated haematology analyser (Mindray, Shenzhen, China), belonging to Dr. Pat Metharom from the School of Biomedical Sciences, Curtin University.

### 2.2.5 Human plasma isolation and storage

Anti-coagulated whole blood samples were centrifuged at 200 g, 20°C for 10 minutes with deceleration set at 1. The plasma layer was removed and transferred to a 15 ml tube, then centrifuged at 800 g, 20°C for 15 minutes with deceleration set at 9, to remove platelets. The platelet-poor plasma layer was removed, divided into 1 ml aliquots, and stored at -80°C.

### 2.2.6 PBMC isolation via density gradient centrifugation

Following plasma separation, the remaining buffy coat and red blood cell fractions were aliquoted as follows: (i) into one 50 ml tube for volumes less than 25 ml or (ii)
divided evenly between two 50 ml tubes for volumes between 25-45 ml. To each tube, PBS containing 2 mM EDTA (PBS/EDTA; Sigma-Aldrich) was added to give a total volume of 35 ml per tube. Each 35 ml volume of diluted blood was overlaid onto 15 ml of Ficoll-Paque™ Plus (GE Healthcare, New South Wales, Australia) for density gradient centrifugation at 400 g, 20°C for 40 minutes without acceleration and brake. Following centrifugation, the PBMC layer was collected, and washed three times: for the first wash, PBMCs were resuspended in 50 ml of PBS/EDTA and centrifuged at 300 g, 20°C for 10 minutes with acceleration and brake, for the second and third washes, PBMCs were resuspended in 50 ml of PBS/EDTA and centrifuged at 200 g, 20°C for 10 minutes with acceleration and brake. PBMCs from healthy young and elderly volunteers were resuspended in complete medium and aliquoted for (i) PBMC/blood DC studies, as per section 2.2.7, and (ii) further processing to isolate monocytes for monocyte-derived DC (MoDC) studies, as per section 2.2.8. For the latter, PBMCs were seeded into 25 cm² BD Falcon flasks (5 ml/flask; Beckton Dickinson) or BD Falcon 6 well plates (3 ml/well; Beckton Dickinson), incubated for 2 hours at 37°C, following which media containing non-adherent lymphocytes was removed, leaving behind adherent monocytes in the flask or plate. Lymphocyte fractions were frozen and stored at -80°C for future use, as per section 2.1.4, whilst monocytes were used to generate MoDCs, as per section 2.2.8. PBMCs isolated from Red Cross buffy coat samples were frozen and stored at -80°C, as per section 2.1.4, for future use in mixed lymphocyte reactions.

2.2.7 PBMC/blood DC cultures: stimulation with LPS/IFN-γ and/or exposure to JU77 TCM

Young and elderly PBMCs were cultured in 96-well BD Falcon plates (Beckton Dickinson), in a volume of 200 μl per well, under the following conditions: (i) complete medium only (unstimulated/non-tumour-exposed control), (ii) complete medium supplemented with the DC activation stimuli 1 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) and 20 ng/ml recombinant human IFN-γ (Biolegend, California, USA), (iii) a mixture of 50% complete medium and 50% JU77 TCM, or (iv) a mixture of 50% complete medium and 50% JU77 TCM supplemented with 1 μg/ml LPS and
20 ng/ml recombinant human IFN-γ. After 24 hours, PBMC suspensions were stained for blood DC subsets and analysed via flow cytometry, as per section 2.4.1. PBMC culture supernatants were also collected and stored at -20°C for cytokine bead array analysis, described in section 2.4.2.

2.2.8 Generation of MoDCs and MoDC cultures: stimulation with LPS/IFN-γ and/or exposure to JU77 cells

Monocytes (isolated as per section 2.2.6) were differentiated into immature MoDCs as per the protocols described by Romani et al. (1491) and Sallusto et al. (309). Monocytes were cultured for 7 days in BD Falcon 6-well plates (3 ml culture volume per well) in complete medium supplemented with 80 ng/ml human granulocyte-macrophage colony-stimulating factor (GM-CSF; Shenandoah Biotechnology, Pennsylvania, USA), 10 ng/ml recombinant human IL-4 (Shenandoah Biotechnology) and 10 µg/ml Polymixin B (Sigma-Aldrich) to neutralize LPS and prevent premature MoDC activation (309, 1491, 1492). Cultures were supplemented with GM-CSF, IL-4 and Polymixin B on days 0 and 4. On day 7, immature MoDCs were either: (i) supplemented with GM-CSF, IL-4 and Polymixin B (unstimulated/non-tumour-exposed controls), or (ii) supplemented with GM-CSF and IL-4 and stimulated with 1 µg/ml LPS and 20 ng/ml recombinant human IFN-γ for a further 2 days.

For MoDC co-cultures with JU77 tumour cells, JU77 cells were added on day 0 at a ratio of 1 tumour cell: 1 monocyte. The presence of JU77 tumour cells was maintained throughout the 7 day differentiation period. On day 7, MoDC/JU77 tumour cell co-cultures were either stimulated with LPS/IFN-γ (as above) or cultured without further stimulation for another 2 days.

On day 9: (i) MoDCs were stained with fluorescently labelled antibodies and their functional phenotype analysed via flow cytometry, as per section 2.4.1, (ii) MoDCs were co-cultured with allogeneic T cells in a mixed lymphocyte reaction to test their ability to stimulate functional T cell proliferation, described in section 2.2.9, and (iii) supernatants from MoDC cultures were collected and stored at -20°C for analysis using a cytokine bead array, described in section 2.4.2.
2.2.9 Allogeneic mixed lymphocyte reaction (MLR)

Young responder T cells were obtained from healthy young volunteers and Red Cross buffy coat samples, and elderly responder T cells were obtained from healthy elderly volunteers. T cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen), a fluorescent dye that covalently binds to amine groups on molecules located in the cytoplasm (1493, 1494). T cells were resuspended at 2 x 10^7 cells per ml in RPMI 1640 media containing 2.5 μM CFSE and no FCS, and incubated at room temperature for 10 minutes, protected from light. Cells were washed three times, each time resuspending in complete medium with an FCS underlay and centrifuging at 1,200 rpm for 5 minutes. Young and elderly MoDCs were co-cultured with CFSE-labelled allogeneic young or elderly T cells at a ratio of 1 MoDC: 5 T cells for 5 days in 200 μl of complete medium at 37°C. Young and elderly T cells cultured with 1 μg/ml Concanavalin A (ConA; Sigma-Aldrich), a lectin which triggers T cell activation by cross-linking the T cell receptor (1495, 1496), were used as positive controls. At day 5, cells were stained with fluorescently labelled antibodies to identify CD8⁺ and CD4⁺ T cells, and assess T cell functional phenotypes, as described in section 2.4.1. As T cells proliferate, CFSE segregates equally between each daughter population; in flow cytometric analysis each round of proliferation is seen as sequential halving of CFSE staining intensity (1494). The percent of T cell proliferation was calculated based on the loss of staining intensity of the parent peak.

2.3 Murine studies

2.3.1 Mice, tumour growth and treatment with chemotherapy and immunotherapy

This project was approved by the Curtin University Animal Ethics Committee (approval number AEC-2012-21), and all experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes. Young (2-5 months) and elderly (20-27 months) female C57BL/6J mice were obtained from the Animal Resources Centre (Western Australia, Australia) and
maintained under specific pathogen-free conditions in the Curtin University animal facility. Tissue samples from elderly female C57BL/6J mice were also obtained from collaborators: Professor Miranda Grounds, University of Western Australia, and Dr. Danielle Dye, Curtin University.

For studies involving healthy mice, any mice that had a palpable mass, enlarged lymph nodes, enlarged spleen or enlarged liver were excluded to ensure that only healthy, non-tumour-bearing mice were examined.

Dr. Connie Jackaman, Curtin University, performed tumour inoculation, measurement of tumour growth and administration of therapies. For tumour inoculation, mice were injected subcutaneously in the right flank with $5 \times 10^5$ AE17 tumour cells per mouse, in 100 µl of PBS. Tumour growth was monitored and tumour size was measured daily using microcallipers. The maximum tumour size allowed was 140 mm$^2$ in accordance with ethics approval from the Curtin University Animal Ethics Committee.

Chemotherapeutic agents (gemcitabine or cisplatin) and the diluent control (PBS) were administered intra-peritoneally to AE17 tumour-bearing mice. Mice were treated with one-third of the usual schedule for both chemotherapies, i.e. one dose of gemcitabine (120 µg/gram bodyweight in PBS; Eli Lilly, Indianapolis, USA) or PBS (100 µl/dose) every 3 days for 2 doses in total, as previously described (1251). For cisplatin treatment, one dose of cisplatin (6 µg/gram bodyweight in PBS; Bristol Myers-Squibb, New York, USA) or PBS (100 µl/dose) was administered, as previously described (1222).

For immunotherapy, AE17 tumour-bearing mice were treated intra-tumourally with one-third of the usual treatment schedule, i.e. once every 3 days with PBS (100 µl/dose) or interleukin-2 (IL-2, 20 µg/dose; Cetus Corporation, California, USA) and agonist anti-CD40 antibody (FGK45, 40 µg/dose; Absolutions, Western Australia, Australia) as previously described (1351), for 2 doses in total. Sample collection occurred 2-3 days after the last dose of chemotherapy/immunotherapy.
2.3.2 Collection and processing of murine tissues

Mice were euthanized using methoxyflurane (Medical Developments International, Victoria, Australia). Spleens, lymph nodes, blood and/or bone marrow were collected from healthy (non-tumour-bearing) mice, and tumours, spleens, tumour-draining lymph nodes and blood were collected from AE17 tumour-bearing mice for flow cytometric analysis. Spleens, lymph nodes and tumours were disaggregated into single-cell suspensions by gentle dispersion between two frosted glass slides. Bone marrow was flushed from femurs and tibiae using fluorescence activated cell sorting (FACS) buffer (1x PBS/1% normal calf serum (NCS)/1% bovine serum albumin (BSA); NCS sourced from ThermoScientific, BSA sourced from Sigma-Aldrich) in a 0.5 ml insulin syringe. Whole blood was collected via cardiac puncture into tubes containing EDTA (Sigma-Aldrich). Red blood cells were lysed by adding 2 ml of 1x red cell lysis buffer (Biolegend) per 100 µl of whole blood, gently vortexing, then incubating for 15 minutes at room temperature, protected from light. Samples were centrifuged at 350 g for 5 minutes, supernatants removed, cells resuspended in FACS buffer and washed once by centrifuging at 1,200 rpm for 5 minutes.

2.3.3 Generation of murine bone marrow-derived DCs (BMDCs)

DCs were generated from young and elderly murine bone marrow haemopoietic precursor cells using a procedure adapted from Lutz et al. (223). Bone marrow was flushed from the femurs and tibiae of healthy young and elderly mice using complete medium in a 0.5 ml insulin syringe, and cells were seeded in bacteriological grade Optilux petri dishes (Beckton Dickinson), at 1 x 10^6 cells per dish. Cultures were supplemented with 20 ng/ml recombinant mouse GM-CSF (eBiosciences), 20 ng/ml recombinant mouse IL-4 (eBiosciences) and 10 µg/ml Polymixin B on days 0, 3, 6 and 8. At day 8, BMDCs were collected for phenotypic analysis by flow cytometry, described in section 2.4.1, or co-culture with allogeneic splenocytes in an MLR assay to determine their ability to stimulate T cell proliferation, described in section 2.3.4.
2.3.4 MLR co-culture of murine BMDCs with allogeneic splenocytes

To obtain responder T cells, spleens from young BALB/c mice (2-3 months of age) were disaggregated into single cell suspensions, resuspended in complete medium, seeded into 96-well tissue cultures plates (Becton Dickinson), incubated for 1-2 hours 37°C, and the non-adherent cell fraction (containing T cells) was removed. Splenocyte-derived T cells were labelled with CFSE, as per section 2.2.9, then co-cultured with young and elderly C57BL/6J BMDCs (from section 2.3.3) at BMDC: T cell ratios of 1:5, 1:20, 1:50, 1:100 and 1:200, for 5 days. ConA-stimulated T cells were included as a positive control. At day 5, co-cultures were stained for CD8+ and CD4+ T cells, and T cell proliferation measured by loss of CFSE staining intensity using flow cytometry, as per section 2.4.1.

2.4 Flow cytometry

2.4.1 Staining human and murine samples for FACS analysis

The antigen specificities, fluorochromes, isotypes, clones, dilutions and suppliers of anti-human antibodies used are shown in Table 2.1, and details of anti-mouse antibodies used are shown in Table 2.2. Secondary reagents and viability dyes are shown in Table 2.3. Isotype control antibodies used are shown in Table 2.4; each isotype control was used at the same concentration as the corresponding anti-human or anti-mouse antibody.

<table>
<thead>
<tr>
<th>Table 2.1 Anti-human antibodies used in this study</th>
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**Table 2.2 Anti-mouse antibodies used in this study**

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<th>Isotype</th>
<th>Clone</th>
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<td>Supplier</td>
<td>Dilution</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------</td>
<td>---------</td>
<td>-------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>CD73</td>
<td>PE</td>
<td>Rat IgG1</td>
<td>TY/11.8</td>
<td>Biolegend</td>
<td>1:500</td>
</tr>
<tr>
<td>CD73</td>
<td>PerCP-Cy5.5</td>
<td>Rat IgG1</td>
<td>TY/11.8</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
<tr>
<td>CD80</td>
<td>BV421</td>
<td>Hamster IgG2</td>
<td>16-10A1</td>
<td>Beckton Dickinson</td>
<td>1:200</td>
</tr>
<tr>
<td>CD86</td>
<td>FITC</td>
<td>Rat IgG2a</td>
<td>GL1</td>
<td>Biolegend</td>
<td>1:200</td>
</tr>
<tr>
<td>GR-1 (Ly6C and Ly6G)</td>
<td>PE</td>
<td>Rat IgG2b</td>
<td>RB6-8C5</td>
<td>Beckton Dickinson</td>
<td>1:200</td>
</tr>
<tr>
<td>ICOS (CD278)</td>
<td>Pacific Blue</td>
<td>Hamster IgG</td>
<td>C398.4A</td>
<td>Biolegend</td>
<td>1:200</td>
</tr>
<tr>
<td>LAG-3 (CD223)</td>
<td>PerCP-Cy5.5</td>
<td>Rat IgG1</td>
<td>C9B7W</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
<tr>
<td>MHC class I (H-2Kb)</td>
<td>PerCP-Cy5.5</td>
<td>Mouse IgG2a</td>
<td>AF6-88.5</td>
<td>Biolegend</td>
<td>1:500</td>
</tr>
<tr>
<td>MHC class II (I-A/I-E)</td>
<td>APC-Cy7</td>
<td>Rat IgG2b</td>
<td>M5/114.15.2</td>
<td>Biolegend</td>
<td>1:500</td>
</tr>
<tr>
<td>PD-1 (CD279)</td>
<td>Biotin</td>
<td>Rat IgG2b</td>
<td>RMP1-30</td>
<td>Biolegend</td>
<td>1:200</td>
</tr>
<tr>
<td>PD-L1 (CD274, B7-H1)</td>
<td>PE-Cy7</td>
<td>Rat IgG2b</td>
<td>10F.9G2</td>
<td>Biolegend</td>
<td>1:500</td>
</tr>
</tbody>
</table>

**Intracellular antigens/markers**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorochrome/Conjugate</th>
<th>Isotype</th>
<th>Clone</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine A2A receptor</td>
<td>PerCP-Cy5.5</td>
<td>Mouse IgG2a</td>
<td>7F6-G5-A2</td>
<td>Santa Cruz</td>
<td>1:50</td>
</tr>
<tr>
<td>CTLA-4 (CD152)</td>
<td>BV421</td>
<td>Hamster IgG</td>
<td>UC10-4B9</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
<tr>
<td>FoxP3</td>
<td>AF647</td>
<td>Mouse IgG1</td>
<td>15D</td>
<td>Biolegend</td>
<td>1 μl/test</td>
</tr>
<tr>
<td>Galectin-9</td>
<td>PerCP-Cy5.5</td>
<td>Rat IgG2a</td>
<td>RG9-35</td>
<td>Biolegend</td>
<td>1:50</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>PE-Cy7</td>
<td>Rat IgG1</td>
<td>XMG1.2</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
<tr>
<td>IL-10</td>
<td>BV421</td>
<td>Rat IgG2b</td>
<td>JES5-16E3</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
<tr>
<td>IL-12 (p40/p70)</td>
<td>PE</td>
<td>Rat IgG1</td>
<td>C15.6</td>
<td>Beckton Dickinson</td>
<td>1:200</td>
</tr>
<tr>
<td>Perforin</td>
<td>APC</td>
<td>Rat IgG2a</td>
<td>eBio0MAK-D</td>
<td>eBioscience</td>
<td>1:50</td>
</tr>
<tr>
<td>TGF-β1 latency-associated peptide (LAP)</td>
<td>Biotin</td>
<td>Mouse IgG1</td>
<td>TW7-16B4</td>
<td>Biolegend</td>
<td>1:100</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AF647</td>
<td>Rat IgG1</td>
<td>MP6-XT22</td>
<td>Biolegend</td>
<td>1:100</td>
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</table>

**Table 2.3 Secondary reagents and viability dyes used in this study**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Fluorochrome/Conjugate</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-rabbit IgG</td>
<td>AF488</td>
<td>Invitrogen</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG</td>
<td>AF647</td>
<td>Invitrogen</td>
<td>1:500</td>
</tr>
<tr>
<td>Reagent</td>
<td>Fluorochrome/Conjugate</td>
<td>Supplier</td>
<td>Dilution</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------</td>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>Streptavidin V500</td>
<td>Beckton Dickinson</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>Zombie green N/A</td>
<td>Biolegend</td>
<td>1:400</td>
<td></td>
</tr>
<tr>
<td>Zombie NIR N/A</td>
<td>Biolegend</td>
<td>1:400</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 Isotype control antibodies used in this study

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Fluorochrome/Conjugate</th>
<th>Clone</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster IgG1 APC</td>
<td>A19-3</td>
<td>Beckton Dickinson</td>
<td></td>
</tr>
<tr>
<td>Hamster IgG1 FITC</td>
<td>A19-3</td>
<td>Beckton Dickinson</td>
<td></td>
</tr>
<tr>
<td>Hamster IgG2 BV421</td>
<td>Ha4/8</td>
<td>Beckton Dickinson</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 AF647</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 APC</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 APC-Cy7</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 APC-eFluor780</td>
<td>P3.6.2.8.1</td>
<td>eBioscience</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 Biotin</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 BV421</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 BV510</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 FITC</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 PE</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 PE-Cy7</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG1 PerCP-Cy5.5</td>
<td>MOPC-21</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG2a APC-Cy7</td>
<td>MOPC-173</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG2a PerCP-Cy5.5</td>
<td>MOPC-173</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG2b BV510</td>
<td>MPC-11</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG2b PE-Cy7</td>
<td>MPC-11</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rabbit IgG Purified</td>
<td>Not provided</td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>Rat IgG1 BV421</td>
<td>RTK2071</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rat IgG1 PE</td>
<td>RTK2071</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rat IgG1 PE-Cy7</td>
<td>RTK2071</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rat IgG1 PerCP-Cy5.5</td>
<td>RTK2071</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rat IgG2a APC-Cy7</td>
<td>RTK2758</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rat IgG2a Biotin</td>
<td>RTK2758</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rat IgG2a FITC</td>
<td>R35-95</td>
<td>Beckton Dickinson</td>
<td></td>
</tr>
<tr>
<td>Rat IgG2a PE</td>
<td>R35-95</td>
<td>Beckton Dickinson</td>
<td></td>
</tr>
<tr>
<td>Rat IgG2a PerCP-Cy5.5</td>
<td>RTK2758</td>
<td>Biolegend</td>
<td></td>
</tr>
<tr>
<td>Rat IgG2b APC-eFluor780</td>
<td>eB149/10H5</td>
<td>eBioscience</td>
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</tr>
<tr>
<td>Rat IgG2b BV421</td>
<td>RTK4530</td>
<td>Biolegend</td>
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<tr>
<td>Rat IgG2b PE</td>
<td>A95-1</td>
<td>Beckton Dickinson</td>
<td></td>
</tr>
<tr>
<td>Rat IgG2b PE-Cy7</td>
<td>A95-1</td>
<td>Beckton Dickinson</td>
<td></td>
</tr>
</tbody>
</table>

Human PBMC cultures were stained with the panels shown in Table 2.5 to identify and characterise blood DC subsets. Human MoDC phenotyping was according to the panels in Table 2.6, and human T cells from MLR experiments were stained with the
panels in Table 2.7. For murine in vitro studies, the panels in Table 2.8 were used to assess BMDC functional phenotype and T cell proliferation in the MLR assay. Staining panels used to characterise murine DCs and T cells from in vivo studies are shown in Tables 2.9 and 2.10, respectively. The expression and functions of molecules used to identify DCs and T cells, and activation and regulatory markers analysed on DCs and T cells are summarised in Table 2.11.

### Table 2.5 Staining panels to identify and characterise human blood DC subsets

<table>
<thead>
<tr>
<th>Human blood DC Panel 1</th>
<th>Human blood DC Panel 2</th>
<th>Human blood DC Panel 3</th>
<th>Human blood DC Panel 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC subset markers</td>
<td>DC subset markers</td>
<td>DC subset markers</td>
<td>DC subset markers</td>
</tr>
<tr>
<td>Lineage cocktail-FITC</td>
<td>Lineage cocktail-FITC</td>
<td>Lineage cocktail-FITC</td>
<td>Lineage cocktail-FITC</td>
</tr>
<tr>
<td>CD1c-AF647</td>
<td>CD1c-AF647</td>
<td>CD1c-AF647</td>
<td>CD1c-AF647</td>
</tr>
<tr>
<td>CD123-PerCP-Cy5.5</td>
<td>CD123-PerCP-Cy5.5</td>
<td>CD123-PerCP-Cy5.5</td>
<td>CD123-PerCP-Cy5.5</td>
</tr>
<tr>
<td>CD141-BV421</td>
<td>CD141-BV421</td>
<td>CD141-BV421</td>
<td>CD141-BV421</td>
</tr>
<tr>
<td>CD303-PerCP-Cy5.5</td>
<td>CD303-PerCP-Cy5.5</td>
<td>CD303-PerCP-Cy5.5</td>
<td>CD303-PerCP-Cy5.5</td>
</tr>
<tr>
<td>Viability dye</td>
<td>Viability dye</td>
<td>Viability dye</td>
<td>Viability dye</td>
</tr>
<tr>
<td>Zombie green</td>
<td>Zombie green</td>
<td>Zombie green</td>
<td>Zombie green</td>
</tr>
<tr>
<td>Antigen-presenting marker</td>
<td>Inhibitory markers</td>
<td>Inhibitory marker</td>
<td>Co-stimulatory molecule</td>
</tr>
<tr>
<td>MHC class I-APC-Cy7</td>
<td>A2A receptor-PE</td>
<td>Galectin-9-PE</td>
<td>CD80-biotin + streptavidin-V500</td>
</tr>
<tr>
<td>Co-stimulatory molecules</td>
<td>A2B receptor + anti-rabbit AF647</td>
<td>Cytokines</td>
<td>Cytokines</td>
</tr>
<tr>
<td>CD40-PE</td>
<td>CD39-BV510</td>
<td>IFN-γ-BV510</td>
<td>IL-6-PE</td>
</tr>
<tr>
<td>CD86-BV510</td>
<td>CD73-PE-Cy7</td>
<td>TGF-β-PE-Cy7</td>
<td>IL-10-BV421</td>
</tr>
<tr>
<td>Inhibitory marker</td>
<td></td>
<td>TNF-α-APC-Cy7</td>
<td>IL-12-APC</td>
</tr>
<tr>
<td>PD-L1-PE-Cy7</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.6 Human MoDC staining panels

<table>
<thead>
<tr>
<th>Human MoDC Panel 1</th>
<th>Human MoDC Panel 2</th>
<th>Human MoDC Panel 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoDC markers</td>
<td>MoDC marker</td>
<td>MoDC marker</td>
</tr>
<tr>
<td>CD1a-PE-Cy7</td>
<td>CD11c-APC</td>
<td>CD11c-APC PerCP-Cy5.5</td>
</tr>
<tr>
<td>CD11c-APC</td>
<td>Viability dye</td>
<td>Cytokines</td>
</tr>
<tr>
<td>CD14-FITC</td>
<td>Zombie NIR</td>
<td>IFN-γ-BV510</td>
</tr>
<tr>
<td>Antigen-presenting marker</td>
<td>Inhibitory markers</td>
<td>IL-6-PE</td>
</tr>
<tr>
<td>MHC class I-APC-Cy7</td>
<td>A2A receptor-PerCP-Cy5.5</td>
<td>IL-10-BV421</td>
</tr>
<tr>
<td>Co-stimulatory molecules</td>
<td>A2B receptor + anti-rabbit</td>
<td>IL-12-APC</td>
</tr>
<tr>
<td>CD40-PE</td>
<td>AF488</td>
<td>TGF-β-PE-Cy7</td>
</tr>
<tr>
<td>CD80-BV421</td>
<td>CD39-BV510</td>
<td>TNF-α-APC-Cy7</td>
</tr>
<tr>
<td>CD86-BV510</td>
<td>CD73-BV421</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.7 Staining panels to characterise human T cells in MLR studies

<table>
<thead>
<tr>
<th>Human MLR Panel 1</th>
<th>Human MLR Panel 2</th>
<th>Human MLR Panel 3</th>
<th>Human MLR Panel 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell markers</td>
<td>T cell markers</td>
<td>T cell markers</td>
<td>T cell markers</td>
</tr>
<tr>
<td>CD3-APC-eFluor780</td>
<td>CD3-APC-eFluor780</td>
<td>CD3-APC-eFluor780</td>
<td>CD3-APC-eFluor780</td>
</tr>
<tr>
<td>CD4-BV510</td>
<td>CD4-PerCP-Cy5.5</td>
<td>CD4-PerCP-Cy5.5</td>
<td>CD4-BV510</td>
</tr>
<tr>
<td>CD8-AF647</td>
<td>CD8-AF647</td>
<td>CD8-AF647</td>
<td>CD8-AF647</td>
</tr>
<tr>
<td>CD25-Pe-Cy7</td>
<td>CD25-Pe-Cy7</td>
<td>CD25-Pe-Cy7</td>
<td>CD25-Pe-Cy7</td>
</tr>
<tr>
<td>CD127-BV421</td>
<td>CD127-BV421</td>
<td>CD127-BV421</td>
<td>CD127-BV421</td>
</tr>
<tr>
<td>Proliferation marker</td>
<td>CFSE</td>
<td>CFSE</td>
<td>CFSE</td>
</tr>
<tr>
<td>Inhibitory markers</td>
<td>CTLA-4-PE-Cy7</td>
<td>CTLA-4-PE-Cy7</td>
<td>CTLA-4-PE-Cy7</td>
</tr>
<tr>
<td>PD-1-BV510</td>
<td>PD-1-BV510</td>
<td>PD-1-BV510</td>
<td>PD-1-BV510</td>
</tr>
<tr>
<td>TIM-3-BV421</td>
<td>TIM-3-BV421</td>
<td>TIM-3-BV421</td>
<td>TIM-3-BV421</td>
</tr>
</tbody>
</table>

Table 2.8 Staining panels to assess murine BMDCs and T cells in MLRs

<table>
<thead>
<tr>
<th>Mouse BMDC Panel</th>
<th>Mouse MLR Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c-APC</td>
<td>T cell markers</td>
</tr>
<tr>
<td>Antigen-presenting markers</td>
<td>CD8-AF405</td>
</tr>
<tr>
<td>MHC class I-PerCP-Cy5.5</td>
<td>CD4-APC-Cy7</td>
</tr>
<tr>
<td>MHC class II-APC-Cy7</td>
<td>Proliferation marker</td>
</tr>
<tr>
<td>Co-stimulatory molecules</td>
<td>CFSE</td>
</tr>
<tr>
<td>CD40-PE</td>
<td></td>
</tr>
</tbody>
</table>

81
<table>
<thead>
<tr>
<th>Mouse BMDC Panel</th>
<th>Mouse MLR Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80-BV421</td>
<td></td>
</tr>
<tr>
<td>CD86-FITC</td>
<td></td>
</tr>
<tr>
<td>Inhibitory marker</td>
<td></td>
</tr>
<tr>
<td>PD-L1-PE-Cy7</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.9 Staining panels to identify and characterise murine DCs in vivo

<table>
<thead>
<tr>
<th>Mouse DC Panel 1</th>
<th>Mouse DC Panel 2</th>
<th>Mouse DC Panel 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC subset markers</td>
<td>CD11c-APC</td>
<td>CD11c-FITC</td>
</tr>
<tr>
<td>CD11c-APC</td>
<td>Antigen-presenting marker</td>
<td>Viability dye</td>
</tr>
<tr>
<td>CD11b-PE-Cy7</td>
<td>MHC class II-APC-Cy7</td>
<td>Zombie NIR</td>
</tr>
<tr>
<td>CD4-APC-Cy7</td>
<td>Co-stimulatory molecules</td>
<td>Inhibitory marker</td>
</tr>
<tr>
<td>CD8-BV421</td>
<td>CD40-PE</td>
<td>Galectin-9-PerCP-Cy5.5</td>
</tr>
<tr>
<td>B220-biotin + streptavidin-V500</td>
<td>CD80-BV421</td>
<td>Cytokines</td>
</tr>
<tr>
<td>GR-1-PE</td>
<td>CD86-FITC</td>
<td>IL-10-BV421</td>
</tr>
<tr>
<td>Antigen-presenting marker</td>
<td></td>
<td>IL-12-PE</td>
</tr>
<tr>
<td>MHC class I-PerCP-Cy5.5</td>
<td></td>
<td>IFN-γ-PE-Cy7</td>
</tr>
</tbody>
</table>

Table 2.10 Staining panels to identify and characterise murine T cells in vivo

<table>
<thead>
<tr>
<th>Mouse T cell Panel 1</th>
<th>Mouse T cell Panel 2</th>
<th>Mouse T cell Panel 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell markers</td>
<td>T cell markers</td>
<td>T cell markers</td>
</tr>
<tr>
<td>CD3-APC-eFluor780</td>
<td>CD3-PE</td>
<td>CD3-APC-eFluor780</td>
</tr>
<tr>
<td>CD4-PerCP-Cy5.5</td>
<td>CD4-APC-Cy7</td>
<td>CD4-PerCP-Cy5.5</td>
</tr>
<tr>
<td>CD8-FITC</td>
<td>CD8-AF647</td>
<td>CD8-FITC</td>
</tr>
<tr>
<td>Regulatory T cell markers</td>
<td>Viability dye</td>
<td>Cytokines</td>
</tr>
<tr>
<td>CD25-PE-Cy7</td>
<td>Zombie green</td>
<td>IFN-γ-PE-Cy7</td>
</tr>
<tr>
<td>FoxP3-AP647</td>
<td></td>
<td>IL-10-BV421</td>
</tr>
<tr>
<td>Inhibitory markers</td>
<td>Inhibitory markers</td>
<td>TGF-β-biotin + streptavidin-V500</td>
</tr>
<tr>
<td>CD39-biotin + streptavidin-V500</td>
<td>PD-1-PE-Cy7</td>
<td>Marker of T cell effector function</td>
</tr>
<tr>
<td>CD73-PE</td>
<td>ICOS-BV421</td>
<td>Perforin-APC</td>
</tr>
<tr>
<td>CTLA-4-BV421</td>
<td>LAG-3-PerCP-Cy5.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.11 Molecules examined in this study, their expression and functions

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Other names</th>
<th>Expression and functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2A receptor</td>
<td>ADORA2A</td>
<td>DCs, macrophages, T cells, Tregs, B cells, neutrophils, NK cells, MDSCs, thymocytes, endothelial cells, platelets, spleen, thymus, brain, myocardium, lung, blood vessels,</td>
</tr>
<tr>
<td>Molecule</td>
<td>Other names</td>
<td>Expression and functions</td>
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<tr>
<td></td>
<td></td>
<td>kidney, liver, muscle, colon</td>
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<tr>
<td></td>
<td></td>
<td>Binds adenosine, leading to: negative immune cell regulation, inhibition of pro-inflammatory activity, promotion of anti-inflammatory immune responses, tolerance and Tregs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mediates vasodilation, inhibits platelet aggregation, modulates brain function, regulates cardiac physiology (545, 546, 548, 554, 1497-1501)</td>
</tr>
<tr>
<td>A2B receptor</td>
<td>ADORA2B</td>
<td>DCs, macrophages, T cells, Tregs, mast cells, endothelial cells, fibroblasts, epithelial cells, muscle cells, brain, intestine, bladder, lung, skin, blood vessels, eye, adipose tissue, kidney, liver, adrenal gland</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Binds adenosine, leading to: negative regulation of T cells, induction of pro- or anti-inflammatory innate immune cells, depending on the context</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mediates vasodilation, mast cell degranulation and allergic responses, regulates cardiac physiology (1497, 1498, 1502-1505)</td>
</tr>
<tr>
<td>B220</td>
<td>CD45R</td>
<td>B cells, activated T cells, NK cells, pDCs, thymocytes, BM precursors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymphocyte proliferation, differentiation and activation (87, 187, 1506-1512)</td>
</tr>
<tr>
<td>CD1a</td>
<td>T6 R4</td>
<td>DCs, LCs, thymocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presents lipid antigens to T cells (1513-1516)</td>
</tr>
<tr>
<td>CD1c</td>
<td>Blood dendritic cell antigen-1 (BDCA-1) R7, M241</td>
<td>DCs, LCs, thymocytes, B cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Presents lipid antigens to T cells (1514, 1517, 1518)</td>
</tr>
<tr>
<td>CD3</td>
<td>T3 complex</td>
<td>T cells, thymocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T cell co-receptor, associates with TCR, promotes antigen recognition and T cell activation (1519, 1520)</td>
</tr>
<tr>
<td>CD4</td>
<td>T4 L3T4</td>
<td>T helper cells, thymocytes, monocytes, macrophages, DCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T cell co-receptor, binds MHC class II, assists TCR antigen recognition and T cell activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Binds IL-16, leading to Th1 cell migration (790, 1521-1529)</td>
</tr>
<tr>
<td>CD8α</td>
<td>T8 Lyt2 Ly-2</td>
<td>Cytotoxic T cells, NK cells, DCs, thymocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T cell co-receptor, binds MHC class I, assists TCR antigen recognition and T cell activation Function on DCs is unknown (344, 1526, 1528-1532)</td>
</tr>
<tr>
<td>Molecule</td>
<td>Other names</td>
<td>Expression and functions</td>
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</tbody>
</table>
| CD11b    | Integran αM Integrin αM Macrophage-1 antigen (Mac-1) Complement receptor 3 (CR3) | Monocytes, macrophages, DCs, granulocytes, NK cells, T cells, B cells  
Associates with CD18 (β2 integrin), binds ICAM-1, 2, and 4, mediates cell migration and adhesion, phagocytosis of particles opsonized with complement component iC3b, binds fibrinogen, neutrophil cytotoxicity (4, 1533-1540) |
| CD11c    | Integran αX subunit Complement receptor 4 p150 | DCs, monocytes, macrophages, NK cells, neutrophils, T cells, B cells  
Associates with CD18 (β2 integrin), binds ICAM-1 and 4 to mediate adhesion, phagocytosis of particles opsonized with complement component iC3b, mediates monocyte migration, binds fibrinogen and LPS, activating immune cells (82, 83, 87, 1541-1553) |
| CD25     | IL-2 receptor α chain Tac Ly-43 p55 | Lymphocyte precursors, activated T and B cells, Tregs, NK cells, monocytes, macrophages, DCs, thymocytes, tumour cells  
Associates with IL-2 receptor β and γ chains to form high affinity IL-2 receptor complex, binds IL-2, promoting T cell proliferation (483, 1354-1356, 1554-1565) |
| CD39     | Ecto-nucleoside triphosphate diphosphohydrolase-1 NTPDase-1 | Activated T and B cells, Tregs, DCs, LCs, monocytes, macrophages, granulocytes, NK cells, endothelial cells, blood vessels, spleen, thymus, lung, placenta, blood (soluble form), tumour cells  
Hydrolyses ATP and ADP to generate AMP, leukocyte trafficking and adhesion, cell-to-cell contact and signalling, recruitment, activation and polarization of naïve T cells by DCs, regulates platelet and endothelial cell activation and thrombosis (542, 1566-1573) |
| CD40     | BP50 TNFRSF5 | DCs, monocytes, macrophages, T and B cells, endothelial cells, epithelial cells, platelets, tumour cells  
Co-stimulatory molecule: on DCs binds CD40L on CD4+ T cells, leading to DC activation, on B cells binds CD40L on CD4+ T cells, promoting B cell survival, proliferation and antibody production (808-810, 1381, 1394, 1400, 1574-1580) |
| CD73     | Ecto-5'-nucleotidase | T and B cells, neutrophils, monocytes, macrophages, DCs, endothelial cells, epithelial cells, spleen, lymph nodes, thymus, bone marrow, colon, brain, kidney, liver, lung, heart, tumour cells  
Dephosphorylates extracellular AMP to adenosine,
<table>
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<tr>
<th>Molecule</th>
<th>Other names</th>
<th>Expression and functions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>mediates adhesion between follicular DCs and germinal centre B cells, regulates leukocyte trafficking and adhesion to endothelia, co-stimulatory molecule for lymphocyte proliferation and activation via binding to CD2 and CD3 (543, 1095, 1566, 1569, 1581-1589)</td>
</tr>
<tr>
<td>CD80</td>
<td>B7-1 B7 BB1</td>
<td>DCs, monocytes, macrophages, T and B cells, NK cells Co-stimulatory molecule: binds CD28 on T cells, leading to T cell activation, proliferation and cytokine production, binds CTLA-4 and PD-L1 on T cells, inhibiting T cells (830-833, 896-898, 1590-1596)</td>
</tr>
<tr>
<td>CD86</td>
<td>B7-2 B70</td>
<td>DCs, monocytes, macrophages, T and B cells, NK cells Co-stimulatory molecule: binds CD28 on T cells, leading to T cell activation, proliferation and cytokine production, binds CTLA-4 on T cells, leading to T cell inhibition (830-833, 898, 1590-1596)</td>
</tr>
<tr>
<td>CD123</td>
<td>IL-3 receptor α chain</td>
<td>BM precursors, macrophages, DCs, basophils, mast cells, B cells, tumour cells Associates with IL-3 receptor common β chain, binds IL-3, promotes BM cell proliferation and differentiation (1597-1602)</td>
</tr>
<tr>
<td>CD127</td>
<td>IL-7 receptor α chain</td>
<td>T and B cells, thymocytes, BM stromal cells Complexes with common γ chain, binds IL-7, promoting development, proliferation and activation of T and B cells (488, 489, 1603-1606)</td>
</tr>
<tr>
<td>CD141</td>
<td>Thrombomodulin BDCA-3</td>
<td>Endothelial cells, myeloid DCs, monocytes, macrophages Co-factor for thrombin, activates Protein C and anticoagulant activity (264, 266, 1607-1609)</td>
</tr>
<tr>
<td>CD303</td>
<td>CLEC-4C BDCA-2</td>
<td>Plasmacytoid DCs Antigen capture, processing and presentation, inhibits cytotoxic activity and TLR-9-induced IFN-α/β production (284, 1610-1613)</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>CD152 Ly-56</td>
<td>Activated T and B cells, monocytes, DCs, MDSCs, tumour cells Binds CD80 and CD86: negatively regulates T cell activation, induces IDO production by DCs (838, 841, 1614-1625)</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box P3 Scurfin</td>
<td>Tregs, activated T cells (transiently expressed) DNA-binding transcription factor, Treg differentiation, inhibits TCR signalling and effector T cell activation (450, 457, 460, 484, 486, 1626-1629)</td>
</tr>
<tr>
<td>Galectin-9</td>
<td>Ecalectin</td>
<td>DCs, macrophages, T cells, Tregs, endothelial and epithelial</td>
</tr>
<tr>
<td>Molecule</td>
<td>Other names</td>
<td>Expression and functions</td>
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</tr>
<tr>
<td>Molecule</td>
<td>Other names</td>
<td>Expression and functions</td>
</tr>
<tr>
<td>Lectin which binds β-galactosides, binds TIM-3 on CD8+ T cells and CD4+ Th1 cells, inducing apoptosis, and promotes Tregs and MDSCs. Regulates signal transduction, apoptosis and gene expression, mediates cell-cell adhesion, cell-extracellular matrix interactions, migration, proliferation and apoptosis, brain development and angiogenesis, pathogenesis of autoimmune conditions and cancer, binds microbial carbohydrates (904, 906, 908, 910-914, 917, 1130, 1630-1634).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR-1</td>
<td>Ly6C/Ly6G</td>
<td>Monocytes, macrophages, granulocytes, MDSCs, pDCs, BM cells. Ly6C mediates adhesion and homing of CD8+ T cells. Function of Ly6G is unclear, may play a role in neutrophil migration (187, 1635-1643).</td>
</tr>
<tr>
<td>LAG-3</td>
<td>CD223</td>
<td>Activated T and NK cells, Tregs, B cells, pDCs. Binds MHC class II molecules, leading to: negative regulation of CD4+ T cell proliferation and activation, promotion of suppressive pDCs, inhibition of DC maturation and T cell-stimulatory capacity, amplifies Treg suppressive activity. Binds an alternate ligand, inhibiting effector CD8+ T cells (536, 792-797, 800, 802, 1086, 1644-1651).</td>
</tr>
<tr>
<td>MHC class I</td>
<td>Humans: HLA-A, B, C Mice: H2-D, K, L</td>
<td>All nucleated cells. Antigen-presenting molecule, presents peptides to CD8+ TCR (613, 1652).</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Humans: HLA-DR, DQ, DP Mice: I-A, I-E</td>
<td>DCs, macrophage, B cells. Antigen-presenting molecule, presents peptides to CD4+ TCR (613, 1652).</td>
</tr>
<tr>
<td>IL-6</td>
<td>B cell stimulatory factor 2 IFN-β2</td>
<td>DCs, monocytes, macrophages, T and B cells, endothelial cells, fibroblasts, keratinocytes, adipocytes, tumour cells. Pro-inflammatory cytokine, pleiotropic effects: regulates immune responses, inflammation and haemopoiesis, pathogenesis of cancer, autoimmune and chronic inflammatory diseases, promotes CD4+ T cell survival/activation, polarises Th2 phenotype, inhibits Treg development, acts with TGF-β to promote Th17 cells, promotes B cell differentiation and antibody production, blocks DC maturation, promotes tumour growth (6, 924-926, 1020, 1653-1661).</td>
</tr>
<tr>
<td>Molecule</td>
<td>Other names</td>
<td>Expression and functions</td>
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</tr>
<tr>
<td>IL-10</td>
<td>B cell-derived T cell growth factor&lt;br&gt;Cytokine synthesis inhibitory factor&lt;br&gt;T cell growth inhibitory factor</td>
<td>DCs, monocytes, macrophages, T and B cells, Tregs, NK cells, mast cells, granulocytes, tumour cells&lt;br&gt;Anti-inflammatory cytokine, pleiotropic effects: inhibits APC activation, antigen-presenting ability and T cell stimulatory capacity, promotes regulatory APCs, suppresses effector CD4+ Th cells, dual effects on effector CD8+ T cells: promotes or suppresses their activity, promotes Tregs and tolerance, inhibits granulocyte and mast cell pro-inflammatory activity, enhances B cell proliferation, differentiation and class switching, and prevents apoptosis, stimulates NK cell cytotoxic activity (503, 505, 1031, 1662-1664)</td>
</tr>
<tr>
<td>IL-12</td>
<td>IL-12p70</td>
<td>Activated DCs, macrophages, T and B cells, neutrophils&lt;br&gt;Pro-inflammatory cytokine, bioactive IL-12p70 is composed of p35 and p40 chain&lt;br&gt;Th1 cell differentiation/activation, promotes IFN-γ secretion by CD8+ and CD4+ T cells and NK cells (812, 813, 1665, 1666)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Type II IFN Macrophage-activating factor</td>
<td>CD4+ Th1 cells, CD8+ T cells, NK cells, DCs, macrophages, B cells, NK T cells&lt;br&gt;Pro-inflammatory cytokine, induces cytotoxic activity in CD8+ T cells and NK cells, promotes Th1, inhibits Th2 cells, promotes DC maturation, activates M1 macrophages, inhibits viral replication, promotes immune cell trafficking Immune attenuating effects: promotes activation-induced cell death in CD4+ T cells, up-regulates PD-L1 on DCs/APCs and tumour cells (9, 1667-1674)</td>
</tr>
<tr>
<td>PD-1</td>
<td>CD279</td>
<td>Activated T and B cells, Tregs, NK T cells, monocytes, myeloid DCs, thymocytes, MDSCs&lt;br&gt;Binds PD-L1 and PD-L2, leading to inhibition of T cell proliferation and cytokine production, and apoptosis&lt;br&gt;Function on monocytes and DCs is unclear (883, 888, 1000, 1675-1682)</td>
</tr>
<tr>
<td>PD-L1</td>
<td>CD274&lt;br&gt;B7-H1</td>
<td>DCs, monocytes, macrophages, activated T cells, B cells, granulocytes, mast cells, endothelial cells, keratinocytes, lung, vascular endothelium, liver, placenta, muscle, pancreas, tumour cells&lt;br&gt;Binds PD-1 on T cells, negatively regulates T cell proliferation and cytokine production&lt;br&gt;PD-L1 on T cells binds CD80 on DCs, inhibiting T cells&lt;br&gt;Binds unknown T cell ligand, co-stimulates T cell proliferation and cytokine production in an IL-2-dependent, PD-1-independent manner (883, 889, 890, 896, 897, 1124, 1313, 1679, 1683-1689)</td>
</tr>
<tr>
<td>Molecule</td>
<td>Other names</td>
<td>Expression and functions</td>
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</tbody>
</table>
| TGF-β | TGF-β1 | Produced by most cell types, including: DCs, monocytes, macrophages, T cells, Tregs, NK cells, granulocytes, mast cells, endothelial cells, epithelial cells, neuronal cells, connective tissue cells, tumour cells  
Latent form: mature TGF-β molecule non-covalently bound to latency-associated peptide (LAP), LAP cleavage releases active TGF-β  
Anti-inflammatory cytokine, pleiotropic effects: cell proliferation, differentiation, migration and survival, role in embryonic development, angiogenesis, wound healing, fibrosis and carcinogenesis, protects from autoimmunity, maintenance of immune tolerance, inhibits effector T and NK cells, inhibits DC and macrophage activation, promotes Tregs, leukocyte recruitment, B cell isotype switching (496, 500, 501, 1690-1692) |
| TIM-3 | CD366  
Hepatitis virus cellular receptor 2 | CD4+ Th1 cells, CD8+ T cells, Tregs, DCs, monocytes, macrophages, NK cells, mast cells, endothelial cells, tumour cells  
Binds GAL-9 on CD8+ T cells and CD4+ Th1 cells, inducing apoptosis, synergises with TLR signalling to promote DC activation, clearance of apoptotic cells (800, 904, 913, 1316, 1693-1698) |
| TNF-α | TNFSF2  
Cachectin  
Necrosin  
Macrophage cytotoxic factor | Activated DCs, monocytes, macrophages, T and B cells, NK cells, neutrophils, endothelial cells, fibroblasts, tumour cells  
Pro-inflammatory cytokine, pleiotropic effects: mediates immune responses, regulates haemopoiesis and tissue development, pathogenesis of cancer, chronic inflammatory conditions and metabolic disorders, promotes DC maturation/survival, stimulates pro-inflammatory innate immune cells, T cell co-stimulation/activation (short-term exposure), T cell inhibition (chronic exposure), induces Tregs and tolerance (309, 922, 923, 927-930, 1699-1707) |

For murine samples that required intracellular cytokine staining, murine tissues were collected into FACS buffer (1x PBS/1% NCS/1% BSA) + 2.5 μg/ml brefeldin A (Sigma), and incubated for 4 hours prior to staining. For human samples requiring intracellular cytokine staining, brefeldin A was added to human primary cell cultures at a final concentration of 10 μg/ml, and cells incubated for 4 hours at 37°C prior to staining. Brefeldin A inhibits protein/cytokine trafficking and secretion by reversibly disrupting the Golgi complex and preventing vesicle formation (1708-1710).
Single cell suspensions were incubated with surface primary antibodies (diluted in FACS buffer + 2.5 µg/ml brefeldin A) for 30 minutes at 4°C in the dark, and then washed twice in FACS buffer + 2.5 µg/ml brefeldin A by centrifuging at 1,200 rpm for 2 minutes. Samples stained with unconjugated or biotin-conjugated primary antibodies were incubated with the appropriate secondary antibody (diluted in FACS buffer + 2.5 µg/ml brefeldin A), as specified in Tables 2.5-2.10, for 30 minutes at 4°C in the dark, washed twice in FACS buffer + 2.5 µg/ml brefeldin A, followed by two PBS washes. Cells were incubated with Zombie green or Zombie NIR viability dyes (both from Biolegend) for 15 minutes at 4°C in the dark, washed twice in FACS buffer, then twice in PBS. Cells were fixed by resuspending in 1% paraformaldehyde (Sigma) diluted in PBS, incubating for 20 minutes at 4°C in the dark, then washing twice with FACS buffer. Paraformaldehyde acts by cross-linking proteins, thereby preserving cellular structure (1711, 1712). Permeabilisation for intracellular staining was performed by incubating cells with FACS buffer + 0.1% saponin (Sigma) for 15 minutes at 4°C in the dark, then centrifuging at 1,200 rpm for 2 minutes and removing supernatants; saponin permeabilises cells by removing membrane cholesterol (1713). Samples were stained with intracellular primary antibodies (diluted in FACS buffer + 0.1% saponin) for 30 minutes at 4°C in the dark, washed twice in FACS buffer + 0.1% saponin, and where required, incubated with secondary antibodies (diluted in FACS buffer + 0.1% saponin), as specified in Tables 2.5-2.10, for a further 30 minutes at 4°C in the dark, then cells were washed twice in FACS buffer and resuspended in 200 µl of FACS buffer per well. Samples were analysed immediately, or stored overnight at 4°C in the dark (up to a maximum of 1 week) before analysis on a FACSCanto II using FACSDiva software (Beckton Dickinson) or FlowJo software (TreeStar, Oregon, USA).

2.4.2 Cytokine bead array

Concentrations of the cytokines and chemokines IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33, and monocyte chemoattractant protein-1 (MCP-1) were measured in: (i) young and elderly healthy human plasma samples, (ii) young and elderly human PBMC culture supernatants, and (iii) young
and elderly human MoDC culture supernatants using a LEGENDplex Human Inflammation Panel cytokine bead array (Biolegend). The assay was optimised so that each test could be performed using half of the manufacturer’s recommended volumes for samples and reagents. All other aspects of the assay were carried out in accordance with the manufacturer’s instructions. Plasma samples were diluted 1:4 and cell culture supernatant samples were diluted 1:2; both sample types were diluted in assay buffer. Samples and cytokine standards were incubated with capture beads and detection antibodies in a 96-well plate for 2 hours at room temperature, protected from light, at 600 rpm on a plate shaker. This was followed by incubation with streptavidin-PE for 30 minutes, at room temperature, protected from light, at 600 rpm on a plate shaker. Samples/standards were centrifuged at 1,000 g for 5 minutes and resuspended in 200 μl of wash buffer per well. These two steps were repeated once, followed by immediate analysis on a FACSCanto II using FACSDiva software (Beckton Dickinson) and LEGENDplex software (Biolegend).

2.5 Data analysis

Statistical significance was calculated using GraphPad PRISM 6 (GraphPad Software Inc, California, USA). Student’s t-test and Mann-Whitney U-test were used to determine differences between two populations. Spearman’s rank correlation test was used to assess the correlation between tumour size and (i) DC and T cell proportions, and (ii) marker expression on DCs and T cells. P-values of < 0.05 were considered statistically significant.
Chapter 3  The effect of healthy aging on human dendritic cells

3.1 Introduction

Aging is associated with alterations in immune function, termed immunosenescence, that are associated with increased susceptibility to infections, cancer and autoimmunity, as well as decreased responses to vaccinations in elderly individuals (42). Whilst many studies have shown that adaptive immunity declines with age (36, 37), fewer recent studies have shown that cells of the innate immune system, including DCs, are also affected by aging (30, 35, 37, 40, 42).

Dendritic cells play a key role in initiating and modulating T cell responses (6). To do this, immature DCs capture and process antigens and undergo maturation in response to immune stimuli (including pathogen-derived molecules such as LPS, and pro-inflammatory cytokines such as IFN-γ), during which they up-regulate co-stimulatory molecules (such as CD40, CD80 and CD86) and pro-inflammatory cytokines (such as IFN-γ, TNF-α and IL-12). During this process, DCs migrate to LNs, where they present antigens to naïve T cells, and together with co-stimulatory interactions, for example, CD80/CD86 on DCs binding to CD28 on T cells, and CD40 on DCs binding to CD40L on T cells (807, 830, 833) stimulate effector T cells (5, 6).

DCs can also limit T cell responses via up-regulation of checkpoint inhibitory molecules, such as PD-L1, and secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β (9, 1714). Interactions between DCs and T cells, such as CD80/CD86 on DCs binding to CTLA-4 on T cells, and PD-L1 on DCs binding to PD-1 on T cells, leads to attenuation of effector T cells (830, 833, 883). Expression of inhibitory molecules also allows DCs to maintain immune tolerance under healthy steady state conditions (8, 9, 1715). However, the effect of aging on DC function is not yet fully known.

Three major DC subsets have been described in human blood: two conventional DC subsets: CD1c+ mDC1s and CD141+ mDC2s, which present antigens and stimulate T cell responses (49, 260), and CD123+CD303+ pDCs, which mediate anti-viral
immunity via IFN-α secretion (260, 292). Most studies showed that during healthy aging the numbers/proportions of myeloid DCs are unaltered, with the exception of two which reported reduced myeloid DC numbers in the elderly (summarised in Chapter 1, Table 1.3). However, most studies, except one (319), examined myeloid DCs as a single subset and did not examine mDC1s and mDC2s separately. Numbers/proportions of pDCs have been shown to be reduced with healthy aging in several studies, however there are conflicting studies reporting no change in pDC numbers with aging (summarised in Chapter 1, Table 1.3). Differences in these observations may be accounted for by differences in the age ranges of the volunteers, differences in cut-off points for defining young and elderly age groups and the markers used to identify DC subsets.

Similarly, the few studies that examined the impact of aging on the functional status of healthy blood DCs described conflicting results, as summarised in Chapter 1, section 1.3 and Table 1.7. Expression of antigen-presenting molecules (HLA-DR), co-stimulatory molecules (CD40, CD80 and CD86) and pro-inflammatory cytokines (TNF-α, IL-6 and IL-12) have been reported to be increased, decreased or unaltered with aging (see Chapter 1, Table 1.7). Due to the rarity of blood DC subsets, several studies examined the impact of aging on the functional status of in vitro human MoDCs, summarised in Chapter 1, section 1.3 and Table 1.7. Most studies showed that elderly unstimulated MoDCs (proposed to represent healthy steady state DCs) express similar levels of antigen-presenting molecules (MHC class I and II), and co-stimulatory molecules (CD40, CD80 and CD86), produce similar levels of pro-inflammatory cytokines (such as IL-12 and TNF-α), and demonstrate an equivalent ability to stimulate T cell proliferation to their younger counterparts (see Chapter 1, Table 1.7). However, contradictory studies reported that secretion of pro-inflammatory cytokines (TNF-α and IL-6) increases with age (316, 806), whilst phagocytic and migratory capacity and the ability to stimulate CD4+ T cells declines with age (68, 673, 674). Differences in the age range of volunteers used, use of the entire blood DC population versus specific DC subsets, analysis of DCs in whole blood versus culturing DCs prior to analysis, as well as differences in protocols used to generate MoDCs may account for the contradictory observations.
An important function of DCs is their ability to respond to pathogen-associated stimulation. There is a consensus that elderly pDCs have a reduced ability to secrete the anti-viral cytokines IFN-α and IFN-λ, and reduced T cell stimulatory capacity following stimulation with viruses or TLR7/9 ligands (316, 317, 323, 324, 660, 661). There are conflicting reports regarding the response of myeloid DCs and MoDCs to maturation-inducing stimuli, as expression of co-stimulatory molecules and pro-inflammatory cytokines have been reported to be increased, decreased or maintained in elderly DCs following stimulation with viruses, LPS or other TLR ligands, summarised in Chapter 1, Table 1.6.

To-date, there is no consensus regarding the effects of aging on human DCs and their functional status in several contexts, including during healthy aging and infection. The few studies performed described conflicting results, and mostly examined the effects of age on DC activation markers and pro-inflammatory cytokines, and little is known about the effects of aging on expression of regulatory molecules on DCs. Moreover, most studies grouped mDC1s and mDC2s together, and have not examined the effects of aging on these two subsets separately.

The pilot studies in this chapter assessed the effects of aging on DC function from young (21-33 years of age) and elderly (60-77 years of age) healthy volunteers. The specific aims of these studies were to examine the effects of aging on human blood DC subsets and MoDCs in the contexts of: (i) healthy aging, and (ii) immune stimulation with LPS/IFN-γ. The functional status of blood DC subsets and MoDCs, as well as the ability of MoDCs to stimulate T cell proliferation and the functional status of the daughter T cells generated was examined.
3.2 Results

3.2.1 Characteristics of study volunteers

This study was approved by the Curtin University Human Research Ethics Committee (approval number HR102_2012) and the Australian Red Cross Blood Service Ethics Committee (approval number 13-03WA-19). This study aimed to recruit 65 young and 65 elderly volunteers in order to achieve statistically meaningful data. The proposed sample size was calculated using a power analysis, where a change of 10% in the numbers and/or function in any cell subset from baseline was considered to have potential biological significance; 65 volunteers per group were required to detect this change with $\sigma=1.1$, $\alpha=0.05$ and $\beta=0.8$. However, due to time constraints, the proposed sample size was not met, and 20 young and 20 elderly volunteers were recruited in total. Volunteers were recruited via: (i) radio advertising, (ii) poster advertising at Curtin University and rotary clubs and (iii) word of mouth within Curtin University. Volunteers were classified as young volunteers (21-33 years of age) and elderly volunteers (60-77 years of age), shown in Table 3.1. All volunteers gave written consent prior to study participation and completed a questionnaire detailing current and past medical conditions, current medications, family disease history, smoking status and asbestos exposure status (summarised in Table 3.1, further details shown in Appendix B: Table B.1). Volunteers were excluded from the study if they previously or currently had cancer or currently had disorders known to affect the immune system, such as autoimmune disease.

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<th>Table 3.1 Age and health status of study volunteers</th>
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3.2.2 Whole blood profiles of young and elderly volunteers

3.2.2.1 Healthy young and elderly volunteers have similar whole blood profiles

The first experiments compared full blood counts between young volunteers (n=15) and elderly volunteers (n=10). Absolute numbers of total white blood cells, lymphocytes, monocytes and granulocytes were similar between the age groups (Figure 3.1A). There was a significant increase in the percentage of monocytes with increasing age (Figure 3.1B; p = 0.02). No age-related differences were seen between percentages of lymphocytes and granulocytes (Figure 3.1B) or absolute numbers of red blood cells (Figure 3.1C) and platelets (Figure 3.1D).

![Figure 3.1](image)

**Figure 3.1** Healthy young and elderly volunteers have similar whole blood profiles

Whole blood samples from healthy young and elderly volunteers were analysed on a haematology analyser for white blood cell (WBC) counts, expressed as absolute numbers of white blood cells (A), and percentages of white blood cell subsets (B), red blood cell (RBC) counts (C) and platelet counts (D). Data are shown as mean ± SEM, n = 15 young volunteers, n = 10 elderly volunteers, * = p<0.05 comparing young to elderly volunteers using an unpaired t test.
3.2.2.2 Healthy young and elderly volunteers have similar plasma cytokine profiles

Human aging has been associated with increased levels of circulating pro-inflammatory cytokines (33). Therefore, pro-inflammatory cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-12p70, IL-17A, IL-18, IL-23 and IL-33), chemokines (monocyte chemoattractant protein-1 (MCP-1) and IL-8) and the anti-inflammatory cytokine IL-10 were measured in plasma from young (n=15) and elderly (n=10) volunteers. No age-related differences were observed (Figure 3.2).

![Figure 3.2 Plasma cytokine concentrations are similar with age](image)

Figure 3.2 Plasma cytokine concentrations are similar with age
Concentrations of pro-inflammatory cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-12p70, IL-17A, IL-18, IL-23 and IL-33), chemokines (monocyte chemoattractant protein-1 (MCP-1) and IL-8) and the anti-inflammatory cytokine IL-10 were measured in plasma from young and elderly healthy volunteers using a cytokine bead array. Data are shown as mean ± SEM and were compared using an unpaired t test, n = 15 young volunteers, n = 10 elderly volunteers.

3.2.3 Comparing young and elderly healthy DCs

3.2.3.1 Blood DC proportions do not differ with healthy aging

Currently, there are conflicting studies regarding the effects of healthy aging on human blood DCs. Therefore, to determine whether the proportions and phenotypes of the major human blood DC subsets differed with age, PBMCs from young (n=13) and elderly (n=11) volunteers were stained for mDC1s: lineage negative, CD1c⁺CD123⁻CD303⁻ (260), mDC2s: lineage negative, CD141⁺CD123⁻CD303⁻
(260), and pDCs: lineage negative, CD123+CD303+CD1c–CD141– (260) and analysed by flow cytometry (gating strategy shown in Figures 3.3A-3.3G). No age-related differences were seen in the proportions of circulating mDC1s, mDC2s and pDCs when expressed as percentages of total PBMCs (Figure 3.3H).

**Figure 3.3 Proportions of blood DC subsets are similar with healthy aging**

PBMCs from young and elderly volunteers were stained with a lineage cocktail (containing CD3, CD14, CD16, CD19, CD20 and CD56), and markers of blood DC subsets (CD1c, CD141, CD123 and CD303), and analysed by flow cytometry. Within PBMC (A), single cells (B) and viable lineage negative cells (C) gates, blood DC subsets were gated as: mDC1s (CD1c–CD123–CD303; D), mDC2s (CD141–CD123–CD303; E) and pDCs (CD123+CD303–CD1c–; D or CD123+CD303+CD141; E). Expression of activation and regulatory markers were measured on blood DCs using percentage of cells positive (representative graph shown in F) and geometric mean fluorescence intensity (MFI) expression levels (representative graph shown in G). Blood DC subsets as percentages of total PBMCs (H) are shown as mean ± SEM and compared using an unpaired t test, n = 13 young volunteers, n = 11 elderly volunteers.
3.2.3.2 Elderly mDC2s express higher levels of suppressive A2A receptor and TGF-β

As there are conflicting studies regarding age-related changes in activation markers, and changes in multiple regulatory markers have not yet been reported, young and elderly blood DC subsets were examined for expression of molecules associated with activation/antigen presentation (i.e. surface MHC class I and the co-stimulatory molecules CD40, CD80 and CD86, as well as intracellular IFN-γ, TNF-α, IL-6 and IL-12) and regulation (i.e. CD39, CD73, A2A receptor, A2B receptor, PD-L1 and galectin-9 (GAL-9), as well as intracellular IL-10 and TGF-β latency-associated peptide) using flow cytometry (representative graphs shown in Figures 3.3F and 3.3G).

No significant age-related differences were seen for mDC1s (Figures 3.4A and 3.4B), however there was a trend towards reduced IL-12 with increased age (p = 0.08; Figure 3.4A).

![Figure 3.4](image)

Figure 3.4 Young and elderly healthy mDC1s have similar phenotypes
Young and elderly PBMCs were stained for CD1c+ mDC1s, activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1 and galectin-9 (GAL-9), and intracellular IL-10 and TGF-β latency-associated peptide) for flow cytometric analysis. The percentages of mDC1s positive for activation markers (A) and regulatory markers (B) were measured. Data are shown as mean ± SEM and were compared using an unpaired t test, n = 13 young volunteers, n = 11 elderly volunteers.
Young and elderly mDC2s had similar expression of most activation markers, i.e. CD40, CD80 and CD86, and the pro-inflammatory cytokines (Figure 3.5A), and regulatory markers (Figure 3.5B). However, elderly mDC2s demonstrated a significantly lower percentage of MHC class I+ cells compared to their younger counterparts (p = 0.04; Figure 3.5C). At the same time, a significantly higher percentage of elderly mDC2s expressed the A2A receptor (p = 0.02; Figure 3.5D) in association with a trend towards a higher percentage of TGF-β+ cells (p = 0.07; Figure 3.5B), relative to their younger counterparts.

![Figure 3.5 Elderly mDC2s have reduced MHC-I and increased A2A receptor expression](image)

Young and elderly PBMCs were stained for CD141+ mDC2s, activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1 and galectin-9 (GAL-9), and intracellular IL-10 and TGF-β) for flow cytometric analysis. Percentages of mDC2s positive for activation markers (A), regulatory markers (B), MHC class I (C), and A2A receptor (D) were measured. Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, * = p<0.05 comparing young to elderly volunteers using an unpaired t test.
3.2.3.3  Elderly pDCs have lower levels of regulatory CD73

There were no age-related differences in the percentages of pDCs positive for the activation markers assessed (Figure 3.6A). In contrast, a significantly lower percentage of elderly pDCs expressed CD73 compared to young pDCs ($p = 0.02$; Figure 3.6B). Elderly pDCs also showed a trend towards a decreased percentage of PD-L1$^+$ cells ($p = 0.07$; Figure 3.6C) relative to young pDCs. Expression of other regulatory markers on pDCs did not differ with age (Figure 3.6C).

![Figure 3.6](image_url)

**Figure 3.6** Elderly pDCs express lower levels of CD73
Young and elderly PBMCs were stained for CD123$^+$CD303$^+$ pDCs, activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1 and galectin-9 (GAL-9), and intracellular IL-10 and TGF-β) for flow cytometric analysis. Percentages of pDCs positive for activation markers (A), CD73 (B), and other regulatory markers (C) were measured. Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, $* = p<0.05$ comparing young to elderly volunteers using an unpaired t test.
3.2.3.4 PBMC pro-inflammatory cytokine secretion does not differ with age

As cytokine secretion is a powerful indicator of DC function, pro-inflammatory cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-12p70, IL-17A, IL-18, IL-23 and IL-33), chemokines (MCP-1 and IL-8) and the anti-inflammatory cytokine, IL-10, were measured to identify any age-related differences. Due to low numbers and the difficulty associated with isolating blood DC subsets, concentrations of secreted cytokines were measured in supernatants from cultured PBMC suspensions. Young and elderly PBMCs had similar pro-inflammatory cytokine secretion profiles, with both age groups producing MCP-1 and IL-8, whilst none of the other cytokines were detected in the majority of young and elderly volunteers (Figure 3.7).

![Figure 3.7 PBMC cytokine secretion profiles are similar with age](image)

Figure 3.7 PBMC cytokine secretion profiles are similar with age
Concentrations of pro-inflammatory cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-12p70, IL-17A, IL-18, IL-23 and IL-33), chemokines (monocyte chemoattractant protein-1 (MCP-1) and IL-8) and the anti-inflammatory cytokine IL-10 were measured in culture supernatants from young and elderly unstimulated PBMCs using a cytokine bead array. Data are shown as mean ± SEM and were compared using an unpaired t test, n = 10 young volunteers, n = 10 elderly volunteers.

3.2.3.5 Elderly monocytes can differentiate into functional immature MoDCs

MoDCs represent another DC subset. They arise from circulating monocytes, migrate into peripheral tissues and differentiate into DCs when exposed to factors such as GM-CSF and IL-4 (211). Due to the rarity of blood DC subsets, MoDCs provide a useful in vitro model for studying human DC function (3). Therefore, the next series of studies investigated whether there were age-related differences in: (i)
the ability of monocytes to differentiate into MoDCs in vitro, and (ii) the MoDC phenotype and functional status. Peripheral blood CD14+ monocytes from young (n=12-16) and elderly (n=11-15) volunteers were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days and MoDCs analysed using flow cytometry (Figures 3.8A-3.8D). No age-related differences were seen in the proportions of immature CD11c+CD14+ MoDCs generated from young and elderly monocytes (Figure 3.8E).

![Flow cytometry images](image)

**Figure 3.8 Young and elderly monocytes can differentiate into immature MoDCs**
Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and then stained for MoDC markers (CD11c and CD14) for flow cytometric analysis. MoDCs were identified within viable cells (A), large cells (B) and single cells (C) gates as CD11c+CD14+ cells (D). CD11c+CD14+ MoDCs as a percentage of viable single cells (E) are shown as mean ± SEM and were compared using an unpaired t test, n = 12 young volunteers, n = 11 elderly volunteers.

To assess whether elderly immature MoDCs were functional, expression of antigen-presenting/activation markers and regulatory markers were measured using flow cytometry (Figures 3.9A-3.9C). No age-related differences were seen in any of the activation and regulatory markers examined (Figures 3.9D and 3.9E).

Concentrations of pro-inflammatory cytokines secreted by young and elderly MoDCs were also assessed. Young and elderly MoDCs secreted similar levels of IFN-γ, TNF-α, MCP-1, IL-6, and IL-8, with IL-10, IFN-α and IL-17A detected in some but not other volunteers regardless of age (Figure 3.10).
Young and elderly immature MoDCs have similar functional phenotypes.

Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and then stained for CD11c^+CD14^‐ MoDCs, activation markers (MHC class I, CD1a, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1 and galectin-9 (GAL-9), and intracellular IL-10 and TGF-β) and analysed by flow cytometry. Marker expression on CD11c^+CD14^‐ MoDCs (A) was measured using percentage of cells positive (representative graph shown in B) and expression levels via geometric mean fluorescence intensity (MFI; representative graph shown in C). Percentages of immature MoDCs positive for activation (D) and regulatory markers (E) are shown as mean ± SEM and were compared using an unpaired t test, n = 12 young volunteers, n = 11 elderly volunteers.
**3.2.3.6 Elderly immature MoDCs stimulate T cell proliferation**

A key role of DCs is activation of effector T cell responses. With aging, the generation of effector T cell responses declines. This could be due to age-related changes in the ability of DCs to stimulate T cells and/or age-related defects in the ability of T cells to respond to stimulation. Therefore, the influence of aging on the ability of MoDCs to stimulate T cell proliferation, and the ability of T cells to respond to stimulation by MoDCs was examined using an allogeneic mixed lymphocyte reaction (MLR).

Immature DCs, which are poor stimulators of T cell activation, were first studied in case of age-related changes to this function. Young (n=12) and elderly (n=11) immature MoDCs were co-cultured with allogeneic, CFSE-labelled T cells from young volunteers, at a ratio of 1 MoDC: 5 T cells, for 5 days, to determine if elderly MoDCs are defective in stimulating T cells. Young responder T cells were obtained from two healthy volunteers (aged 22-24 years) and three buffy coat samples from the Australian Red Cross Blood Service (donors aged between 23-31 years).
Young and elderly immature MoDCs were also co-cultured with allogeneic, CFSE-labelled T cells from elderly volunteers (aged 60-74 years, n=6), at a ratio of 1 MoDC: 5 T cells, for 5 days, to determine: (i) if elderly T cells are defective or if they are capable of responding to stimulation by DCs from young individuals, and (ii) whether elderly T cell responses can be stimulated by elderly DCs. CD8+ T cell, CD4+ T cell and CD4+CD25+CD127low Treg proliferation was measured using flow cytometry (Figures 3.11A-3.11F), and the percentages of daughter T cells (that had undergone at least one round of division/proliferation; Figure 3.11F) compared.

Elderly immature MoDCs induced similar levels of young CD8+ T cell, CD4+ T cell and Treg cell proliferation to their younger counterparts (Figure 3.11G). Moreover, elderly T cell proliferative responses were similar when stimulated by young and elderly immature MoDCs (Figure 3.11H).

3.2.3.7 Young and elderly immature MoDCs induce young T cells with similar phenotypes

Measuring T cell proliferation does not reveal the functional quality of activated T cells. Therefore, T cells were stained for markers of effector function/activation (CD25 and intracellular IFN-γ, IL-12 and perforin), as well as regulatory markers (CD39, CD73, A2A receptor, CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β), and analysed using flow cytometry. Expression of all markers were initially examined using 3-5 volunteers per age group, and markers showing trends for age-related differences examined in additional volunteers, (n=10-12 volunteers per age group). Changes in marker expression from parent to daughter T cell populations, induced by young versus elderly immature MoDCs, were compared.

Young and elderly immature MoDCs induced similar changes in the functional phenotype of young CD8+ T cells. This included up-regulation of the activation markers CD25, IFN-γ and IL-12 (Figure 3.12A), as well as increased expression of the regulatory markers CD39, CTLA-4, ICOS, TIM-3, PD-1, IL-10 and TGF-β (Figure 3.12B), on daughter, compared to parent, young CD8+ T cells.
Figure 3.11 Young and elderly immature MoDCs stimulate similar levels of T cell proliferation

Young and elderly immature MoDCs were co-cultured with allogeneic, CFSE-labelled (i) young T cells, and (ii) elderly T cells, at a ratio of 1 DC: 5 T cells, for 5 days. Samples were stained with CD3, CD4, CD8, CD25 and CD127 and analysed by flow cytometry. Viable cells (A), single cells (B), then CD3+ T cells (C) were gated. Within the CD3+ gate, CD8+ and CD4+ T cells were identified (D). CD25+CD127low regulatory T cells (Tregs) were gated within the CD4+ gate (E). In each of the CD8+, CD4+ and Treg cell gates, parent and daughter T cells were identified based on CFSE staining intensity (F). The percentage of T cell proliferation (measured by the percentage of divided cells, which corresponds to the daughter cells gate) was calculated based on loss of staining intensity of the parent peak (F). Percentages of young (G) and elderly (H) CD8+ T cell, CD4+ T cell and Treg cell proliferation are shown as mean ± SEM and were compared using an unpaired t test, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs.
Young and elderly immature MoDCs induce young CD8+ T cells with similar phenotypes

Young CD8+ T cells were stimulated by young and elderly immature MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD8+ T cells were compared for samples stimulated by young versus elderly immature MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing parent to daughter T cells using a paired t test.

Similarly, young and elderly MoDCs induced increased expression of a mixture of activation markers (CD25 and IL-12; Appendix B: Figure 1A) and regulatory markers (CD39, A2A receptor, CTLA-4, ICOS, TIM-3, IL-10 and TGF-β; Appendix B: Figure 1B)
on young CD4+ daughter T cells, relative to parent T cells, and the extent of T cell marker up-regulation induced was similar for MoDCs from both age groups.

3.2.3.8 Immature young and elderly MoDCs induce elderly T cells with similar phenotypes

As elderly T cells did proliferate in response to young and elderly MoDCs, the next step was to determine whether these T cells were functional. Elderly-derived CD8+ daughter T cells increased IL-12, CD73, A2A receptor, TIM-3 and TGF-β expression, in response to interactions with young and elderly immature MoDCs (Appendix B: Figures 2A and 2B). Young and elderly MoDCs also induced increases in IL-12, ICOS and TGF-β expression on elderly CD4+ daughter relative to parent T cells (Appendix B: Figures 3A and 3B). Young and elderly MoDCs induced a similar extent of marker up-regulation on elderly T cells. This suggests that, similar to young T cells, elderly T cells adopt a mixed phenotype, characterised by up-regulation of activation and regulatory markers, following stimulation by young and elderly immature MoDCs.

These data suggest that in healthy aging: (i) elderly mDC1s may have a reduced ability to activate T cells due to lower IL-12; (ii) elderly mDC2s may not be able to activate CD8+ T cells due to lower MHC class I, and increased A2A receptor and TGF-β expression; (iii) elderly pDCs may have a better T cell stimulatory effect, due to reduced CD73 and PD-L1 expression; (iv) elderly PBMCs may retain their ability to recruit immune cells via the chemokines MCP-1 and IL-8; and (v) elderly monocytes differentiate into immature MoDCs with a similar functional status as their younger counterparts.

3.2.4 Do young and elderly DCs respond similarly to LPS/IFN-γ?

3.2.4.1 After LPS/IFN-γ elderly mDC1s express increased suppressive TGF-β

An important part of the DC lifecycle is their ability to mature into potent antigen-presenting cells in response to activation stimuli. During an infection, DC maturation is triggered by danger signals from pathogen-derived molecules, such as LPS, and activation signals from other molecules, such as IFN-γ, produced by immune cells at
the site of infection. DC maturation involves up-regulation of antigen-presenting molecules, co-stimulatory molecules and pro-inflammatory cytokines, such that mature DCs arriving at lymph nodes activate antigen-specific effector T cells (6). Therefore, the next studies investigated whether elderly blood DCs responded to LPS/IFN-γ stimulation to the same extent as DCs from young volunteers. PBMC suspensions from young (n=10) and elderly (n=10) volunteers were stimulated with LPS/IFN-γ for 24 hours, then mDC1s, mDC2s and pDCs analysed by flow cytometry (Figures 3.3A-3.3G). Responses to LPS/IFN-γ were compared to unstimulated age-matched controls. No age-related changes were seen regarding the proportions of pDCs yielded post-stimulation, although LPS/IFN-γ-stimulation led to reduced proportions of young and elderly mDC1s and mDC2s relative to their age-matched unstimulated controls (Figure 3.13).

![Figure 3.13 Reduced proportions of mDC1s and mDC2s following LPS/IFN-γ stimulation, regardless of age](image)

Young and elderly PBMCs were stimulated with LPS/IFN-γ for 24 hours, then stained for CD1c⁺ mDC1s, CD141⁺ mDC2s and CD123⁺CD303⁺ pDCs, and analysed via flow cytometry. Proportions of mDC1s, mDC2s and pDCs in young and elderly LPS/IFN-γ-stimulated samples were compared to age-matched unstimulated samples and expressed as percentages of total PBMCs. Data are shown as mean ± SEM, n = 10-13 young volunteers, n = 10-11 elderly volunteers, ** = p<0.005 comparing LPS/IFN-γ-stimulated samples to age-matched unstimulated controls using a paired t test.

A comparison of LPS/IFN-γ-stimulated versus unstimulated mDC1s within each volunteer showed that young and elderly mDC1s experienced similar changes in
antigen-presenting/activation markers following LPS/IFN-γ stimulation; i.e. MHC class I and CD86 expression decreased, whilst CD40 and CD80 expression increased (Appendix B: Figure 4A). There were no age-related differences in pro-inflammatory intracellular cytokine levels, as mDC1s from both age groups increased IFN-γ, TNF-α and IL-6, following LPS/IFN-γ stimulation (Appendix B: Figure 4A).

In both age groups, PD-L1 expression increased, whilst CD39 and A2B receptor expression decreased following LPS/IFN-γ-stimulation, with no other consistent results seen (Figure 3.14A).

The percentage of IL-10+ cells and intracellular IL-10 increased in young, but not elderly, LPS/IFN-γ-stimulated mDC1s (p = 0.02; Appendix B: Figure 4B; p = 0.03; Appendix B: Figure 4C), compared to controls. In contrast, elderly, but not young, LPS/IFN-γ-stimulated mDC1s significantly up-regulated the percentage of TGF-β+ cells as well as intracellular TGF-β levels (p = 0.03; Figure 3.14B; p = 0.03; Figure 3.14C), relative to controls.

3.2.4.2 Elderly LPS/IFN-γ-stimulated mDC2s demonstrated variable responses

Young LPS/IFN-γ-stimulated mDC2s significantly down-regulated surface MHC class I expression levels leading to lower percentages of MHC class I+ cells (p = 0.02; Figure 3.15A; p = 0.02; Figure 3.15B), relative to unstimulated controls. This was associated with significantly increased intracellular IFN-γ levels as well as an increased percentage of IFN-γ+ cells (p = 0.0002; Figure 3.15C and p = 0.009; Figure 3.15D, respectively) in young LPS/IFN-γ-stimulated mDC2s. In contrast, MHC class I (Figures 3.15A and 3.15B), and IFN-γ expression (Figures 3.15C and 3.15D) on elderly mDC2s was variable. No other age-related differences in activation markers were observed (Appendix B: Figure 5A).

Elderly mDC2s also showed variable responses in CD73 expression, whilst CD73 levels significantly increased on young mDC2s following LPS/IFN-γ stimulation (p = 0.03; Figure 3.15E). No other age-related differences in regulatory markers were seen (Appendix B: Figure 5B).
Young and elderly PBMCs were stimulated with LPS/IFN-γ for 24 hours, then regulatory markers (CD39, CD73, A2AR, A2BR, PD-L1, GAL-9, and intracellular TGF-β) were analysed on mDC1s via flow cytometry. Percentages of mDC1s positive for regulatory markers (A) and TGF-β (B), and expression levels (measured as geometric mean fluorescence intensity; MFI) of TGF-β (C) were measured. Each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing LPS/IFN-γ-mDC1s to unstimulated mDC1s from the same volunteer using a paired t test.
Young and elderly PBMCs were stimulated with LPS/IFN-γ for 24 hours, then stained for CD141⁺ mDC2s, MHC class I, intracellular IFN-γ, and CD73 for flow cytometric analysis. Expression levels (shown as geometric mean fluorescence intensity; MFI) of MHC class I (A), IFN-γ (C) and CD73 (E), and percentages of mDC2s positive for MHC class I (B) and IFN-γ (D) were measured. Each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing LPS/IFN-γ-mDC2s to unstimulated mDC2s from the same volunteer using a paired t test.

3.2.4.3 Elderly LPS/IFN-γ stimulated pDCs express increased PD-L1

The only age-related difference in the response of pDCs to LPS/IFN-γ stimulation was a significant up-regulation of the percentage of PD-L1⁺ cells (p = 0.004; Figure 3.16A) and PD-L1 expression levels (p = 0.008; Figure 3.16B) when comparing each
elderly volunteer’s LPS/IFN-γ-stimulated sample to their unstimulated control, whilst young pDCs showed variable responses. Both young and elderly pDCs demonstrated similar changes in activation marker expression (Appendix B: Figure 6A), as well as expression of the other regulatory markers examined (Appendix B: Figure 6B), in response to LPS/IFN-γ stimulation.

In order to properly activate effector T cells, mature DCs must up-regulate secretion of pro-inflammatory cytokines. Young and elderly PBMCs responded similarly to LPS/IFN-γ stimulation by up-regulating secretion of the pro-inflammatory cytokines IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-12p70, IL-18 and IL-23, the chemokines MCP-1 and IL-8, and the anti-inflammatory cytokine IL-10, relative to unstimulated controls (Appendix B: Figures 7A and 7B).

3.2.4.4 LPS/IFN-γ-stimulated MoDCs express similar activation and regulatory markers, regardless of age

MoDCs that differentiate and mature at an inflammatory site play a key role in generating antigen-specific effector T cell responses (211). Thus, the ability of young and elderly MoDCs to mature in response to LPS/IFN-γ stimulation was compared. Monocytes from young (n=10) and elderly (n=10) volunteers were differentiated
into immature MoDCs, stimulated with LPS/IFN-γ, and analysed using flow cytometry, as per Figures 3.9A-3.9C. No age-related differences were seen in the proportions of CD11c⁺CD14⁻ MoDCs post-LPS/IFN-γ stimulation (Figure 3.17A).

Young and elderly MoDCs responded similarly to LPS/IFN-γ; i.e. percentages of cells positive for MHC class I, CD40, CD80 and CD86, as well as intracellular IL-6, were up-regulated, relative to unstimulated controls (Figure 3.17B). The suppressive molecules CD39 and PD-L1 also increased, regardless of age (Appendix B: Figure 8), whilst changes in the other markers varied (Figures 3.17B and Appendix B: Figure 8).

**Figure 3.17 Young and elderly LPS/IFN-γ-MoDCs have similar activation marker expression**

Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, then stimulated with LPS/IFN-γ for a further 2 days, then stained for CD11c⁺CD14⁻ MoDCs and activation markers (MHC class I, CD1a, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and analysed by flow cytometry. CD11c⁺CD14⁻ MoDCs as a percentage of viable single cells in LPS/IFN-γ-stimulated samples were compared to age-matched unstimulated controls (A). Percentages of CD11c⁺CD14⁻ MoDCs positive for activation markers were measured (B); each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers. * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing LPS/IFN-γ-MoDCs to unstimulated MoDCs from the same volunteer using a paired t test.
3.2.4.5 Elderly LPS/IFN-γ-MoDCs have variable MCP-1, IL-8, IL-6 and IL-23 secretion

Young LPS/IFN-γ-MoDCs significantly up-regulated secretion of MCP-1 ($p = 0.03$; Figure 3.18A), IL-6 ($p = 0.03$; Figure 3.18B), IL-8 ($p = 0.001$; Figure 3.18C) and IL-23 ($p = 0.02$; Figure 3.18D), compared to unstimulated controls, whilst inconsistent changes were seen for elderly LPS/IFN-γ-MoDCs (Figures 3.18A-3.18D). No other differences in MoDC cytokine secretion were observed (Appendix B: Figure 9).

![Figure 3.18 Elderly LPS/IFN-γ-MoDCs have variable changes in MCP-1, IL-6, IL-8 and IL-23 secretion](image)

Concentrations of MCP-1 (A), IL-6 (B), IL-8 (C), and IL-23 (D) were measured in culture supernatants from LPS/IFN-γ-stimulated young and elderly MoDCs via cytokine bead array. Each line in graphs A-D represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, $n = 10$ young volunteers, $n = 10$ elderly volunteers, $* = p<0.05$ comparing LPS/IFN-γ-MoDCs to unstimulated MoDCs from the same volunteer using a paired $t$ test.
3.2.4.6 Elderly LPS/IFN-γ-MoDCs stimulate lower levels of young CD4⁺ T cell proliferation

A key function of mature/activated DCs is the activation of antigen-specific effector T cells. Thus, the next experiments assessed whether elderly LPS/IFN-γ-stimulated MoDCs could stimulate allogeneic T cell proliferation. To determine whether elderly LPS/IFN-γ-stimulated MoDCs are defective in stimulating T cells, young (n=10) and elderly (n=10) LPS/IFN-γ-stimulated MoDCs were co-cultured with allogeneic, CFSE-labelled T cells from young volunteers (aged 22-31 years, n=5). To determine if elderly T cells are defective, young and elderly LPS/IFN-γ-stimulated MoDCs were co-cultured with allogeneic, CFSE-labelled T cells from elderly volunteers (aged 60-74 years, n=6); CD8⁺ T cells, CD4⁺ T cells, CD4⁺CD25⁺CD127low Tregs, and T cell proliferation was measured as shown in Figures 3.11A-3.11F.

Young and elderly LPS/IFN-γ-stimulated MoDCs induced significantly higher levels of young CD8⁺ T cell proliferation compared to age-matched, unstimulated controls, (Figure 3.19); however, a significant increase in CD4⁺ T cell proliferation was only induced by young, but not elderly, LPS/IFN-γ-MoDCs (p = 0.001; Figure 3.19).

Figure 3.19 Elderly LPS/IFN-γ-MoDCs stimulate lower levels of young CD4⁺ T cell proliferation

Young and elderly LPS/IFN-γ-MoDCs were co-cultured with allogeneic, CFSE-labelled young T cells at a ratio of 1 DC: 5 T cells, for 5 days. Samples were stained for CD8⁺ T cells, CD4⁺ T cells, and CD4⁺CD25⁺CD127low Tregs and analysed by flow cytometry as per Figures 3.11A-3.11F. The percentage of T cell proliferation was calculated based on loss of staining intensity of the parent peak. Percentages of young CD8⁺ T cell, CD4⁺ T cell and Treg cell proliferation stimulated by LPS/IFN-γ-MoDCs were compared to those stimulated by age-matched unstimulated MoDCs. Data are shown as mean ± SEM, n = 10 young volunteers’ MoDCs, n = 10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005 comparing LPS/IFN-γ-MoDCs to age-matched unstimulated MoDCs using a paired t test.
To determine whether elderly LPS/IFN-γ-MoDCs stimulate functional T cells, markers of T cell activation/effector and regulatory function were examined. There were no age-related differences on daughter compared to parent young CD8⁺ T cells following interactions with young and elderly LPS/IFN-γ-MoDCs: i.e. CD25, IL-12, CD39, A2A receptor, ICOS, IL-10 and TGF-β increased, CD73 decreased, and all other markers varied (Appendix B: Figures 10A and 10B). Young daughter CD4⁺ T cells increased most markers, except IFN-γ, perforin and CD73, relative to the corresponding parent populations, in response to stimulation by young and elderly LPS/IFN-γ-MoDCs (Appendix B: Figures 11A and 11B).

Young and elderly LPS/IFN-γ-MoDCs stimulated similar levels of elderly CD8⁺ and CD4⁺ T cell proliferation that were increased relative to age-matched, unstimulated MoDC controls (Appendix B: Figure 12). There were no age-related differences in changes in marker expression on elderly parent versus daughter T cells; elderly daughter CD8⁺ T cells had increased IL-12, CD73 and TGF-β, and variable changes in the other markers (Appendix B: Figures 13A and 13B), whilst elderly daughter CD4⁺ T cells had increased IL-12 and TGF-β (Appendix B: Figures 14A and 14B).

In summary: (i) elderly LPS/IFN-γ-stimulated mDC1s retain their ability to activate T cells via up-regulation of CD40, CD80, IFN-γ, TNF-α and IL-6, but increase regulatory potential mediated by TGF-β; (ii) elderly mDC2s show variable responses to LPS/IFN-γ-stimulation; (iii) elderly LPS/IFN-γ-stimulated pDCs are more suppressive due to increased PD-L1; and (iv) elderly LPS/IFN-γ-stimulated PBMCs may activate T cell responses via IFN-γ, TNF-α and IL-12p70. Furthermore, regardless of age, LPS/IFN-γ-stimulated MoDCs acquire increased antigen-presenting and co-stimulatory capacity due to increased MHC class I, CD40, CD80 and CD86, concomitant with increased regulatory potential through CD39 and PD-L1.
3.3 Discussion

Changes in human DCs during healthy aging have not been well-characterised to-date, and there are several conflicting reports. The pilot studies in this chapter examined changes to DC function during healthy aging.

The results from this study did not find age-related differences in blood mDC1, mDC2 and pDC proportions. The findings for the two myeloid DC subsets agree with most other studies (68, 259, 316-319) but contrast with two studies showing that myeloid DC numbers decrease with age (320, 321). Reasons for this difference could be that Della Bella et al. (320) and Vuckovic et al. (321) used different age ranges compared to this study (i.e. 20-92 years and 0-60 years, respectively); differences in markers used to identify myeloid DCs; the relatively small sample size of this study; and whether analysis was performed on whole blood or on PBMCs cultured overnight. The findings for pDCs in this study agree with two other studies showing no change in pDC proportions with age (68, 320), but contrast with the majority of studies to-date, which report an age-associated decline in pDCs (259, 316-319, 321-324). A possible reason could be that in this study, pDCs were cultured for 24 hours before analysis, whilst most other studies analysed fresh whole blood samples, and furthermore, the small sample size may have been insufficient to detect any age-related differences.

Data from this study suggests that mDC2s develop increased regulatory activity during healthy aging on account of elevated A2A receptor expression, meaning that they may have increased potential to respond to microenvironmental immunosuppressive adenosine. Activation of DCs via this pathway results in a shift away from pro-inflammatory cytokine production and towards anti-inflammatory cytokine production, which could skew immune responses away from Th1 and towards Th2 responses in the elderly (539, 571, 573, 1716). In addition, elderly mDC2s may have increased immunosuppressive activity through TGF-β, as suggested by the observed increase in the TGF-β latency-associated peptide, which non-covalently associates with the latent form of TGF-β (1717, 1718). Further studies are required to confirm whether the active form of TGF-β also increases
with aging. The possible effects of age-associated increases in TGF-β include reduction of T cell proliferation (1719), inhibition of T cell effector function (502, 1720), and promotion of regulatory immune cells, such as Tregs (472, 518, 519, 1721), which could lead to suppression, rather than activation, of T cell responses in the elderly.

Reductions in antigen-presenting/activation markers on blood DCs during healthy aging may further compromise the generation of T cell responses in the elderly. Myeloid DC2s are the most efficient DC subset at cross-presentation of antigens (155, 266, 267), and the reduction of MHC class I seen in elderly mDC2s may compromise their ability to cross-present viral (617, 1722) and tumour antigens to CD8+ T cells (145). Effective anti-viral and anti-tumour immune responses require generation of Th1 effector cells, which may also be compromised in the elderly due to reduced IL-12 production by elderly mDC1s. Another study reported decreased MHC class II expression on elderly blood myeloid DCs (317), which could also contribute to impaired antigen presentation and generation of Th1 responses in the elderly. Thus, reduced cross-presenting ability and capacity to generate effector T cells may contribute to the increased susceptibility of elderly individuals to infections and cancer. Due to the rarity of blood DC subsets and difficulties in their isolation, this study could not examine how age-related changes in blood mDC1 and mDC2 function impact upon generation of T cell responses, and further studies are required.

This study examined the impact of age-related changes in DCs on T cell responses using MoDCs. Elderly immature MoDCs were as capable as their younger counterparts at stimulating CD4+ and CD8+ T cell proliferation, which agrees with other studies (69, 70, 661). However, and more importantly, elderly LPS/IFN-γ-MoDCs were unable to induce young CD4+ T cells to proliferate to the same levels as young LPS/IFN-γ-MoDCs. Reductions in the ability of elderly DCs to stimulate CD4+ T cells was observed in another study (714), and suggests that generation of CD4+ T helper responses may be impaired in the elderly. However, the mechanisms leading to this defect in elderly DCs are not yet clear; possible reasons include reduced MHC class II expression with age, as shown by others (317, 691), or age-associated
defects in the MHC class II/endosomal antigen processing pathway (631). Furthermore, another two studies showed that elderly DCs were less efficient than their younger counterparts at stimulating proliferation of T cells from both young and elderly subjects, and that elderly T cells showed poor responses to stimulation by young DCs (673, 674). This contrasts with results from this study, which showed that elderly DCs retained their ability to stimulate CD8+ T cells, and also that elderly T cells were as capable as young T cells at proliferating in response to interactions with young and elderly MoDCs. Possible reasons for these differences include differences in the types of DCs used: MoDCs in this study, versus DCs isolated from a low-density PBMC fraction (673, 674), different types of DC stimuli, and differences in experimental procedures for obtaining responder T cells. This study used young responder T cells from buffy coats, which were stored overnight before T cell isolation, which could have impacted on the functional capacity of the T cells.

Results from this study also showed that elderly MoDCs induced functional T cell proliferation. Elderly MoDCs induced increases in molecules associated with T cell effector function (CD25, IFN-γ, and IL-12) on young and elderly T cells to similar levels as those induced by young MoDCs. These observations agree with others showing that elderly MoDCs induced IFN-γ, TNF-α and/or IL-12 in activated T cells (661, 673, 674), suggesting that elderly DCs retain the capacity to stimulate functional effector T cells. Yet, both young and elderly MoDCs simultaneously induced increased expression of several inhibitory molecules on young and elderly T cells (CD39, A2A receptor, CTLA-4, ICOS, TIM-3, IL-10 and/or TGF-β latency-associated peptide). Simultaneous up-regulation of pro-inflammatory (IL-12) and anti-inflammatory cytokines (IL-10 and TGF-β) on proliferating T cells was observed in another study (673). These results suggest that elderly DCs promote a suppressive and/or exhausted phenotype in proliferating T cells (1104, 1105). These changes may be related to the time point of analysis (5 days post-stimulation), as T cells may have undergone their initial activation phase before up-regulating checkpoint inhibitory molecules in order to terminate an effector T cell response that is no longer required (707). Analysis at earlier time points may reveal differences in the kinetics of T cell activation induced by young versus elderly DCs,
for example, whether elderly DCs promote earlier and/or faster up-regulation of checkpoint inhibitory molecules on effector T cells.

This study suggests that pDCs have improved T cell activating potential during healthy aging due to a trend for reduced expression of PD-L1 on healthy (non-activated) pDCs, meaning they may be less likely to inhibit T cell activation via the PD-L1/PD-1 pathway. Also, lower expression of CD73 on elderly pDCs may mean that their ability to participate in production of immunosuppressive adenosine is reduced (1723). As adenosine exerts suppressive effects on T cells, reduced adenosine may create an environment more permissive to T cell activation and/or allow maintenance of activated T cells (551, 920). Whilst these changes may be beneficial in the context of an immune response, they may also affect maintenance of tolerance. As pDCs play a key role in inducing and maintaining tolerance (181, 294, 302), reductions in their suppressive activity with aging may contribute to a breakdown of immune tolerance, which may be a contributing factor to the increased incidence of autoimmune conditions in the elderly (42).

Possible reasons for changes in DC function during healthy aging could be attributed to intrinsic changes in DCs, or to the influence of other changes occurring in the aging microenvironment, for example, changes in circulating cytokine levels. Plasma levels of the anti-inflammatory cytokine TGF-β are reported to increase with age (38, 1724), and TGF-β can inhibit DC maturation and promote tolerogenic/suppressive DCs (1725, 1726), which may explain why elderly mDC2s develop a suppressive phenotype. However, aging is also associated with inflammaging, which is characterised by elevated basal levels of pro-inflammatory cytokines in plasma, particularly TNF-α, IL-6 and IL-1β (33, 34, 38, 1727). Exposure to elevated levels of pro-inflammatory cytokines might suggest development of pro-inflammatory DCs, however this was not observed in this study. Furthermore, no differences were observed in plasma pro-inflammatory cytokine levels with age, which contrasts with other studies (33, 34, 38, 1727). This may be due to the ages of the elderly volunteers, as other studies used elderly volunteers in a higher age bracket, for example, 80-100 years of age (33, 34, 38), or other factors such as genetics, which have been reported to influence cytokine production (1728, 1729),
or the small sample size of this study.

This study also showed that whilst elderly DCs maintained their ability to respond to LPS/IFN-γ stimulation, they seemed to develop a more suppressive phenotype. Elderly mDC1s, pDCs and MoDCs up-regulated co-stimulatory molecules (CD40, CD80 and CD86) and pro-inflammatory cytokines (IFN-γ and TNF-α) to similar levels as their younger counterparts, which agrees with other studies showing that elderly blood myeloid DCs and MoDCs demonstrate comparable responses to stimulation with LPS or other TLR ligands as young DCs (68, 69, 317, 320, 660, 669, 670, 673, 1730). This suggests that mDC1s, and MoDCs that migrate into tissue sites of infection, and pDCs, which can become antigen-presenting cells under inflammatory conditions (182, 198, 199, 298), may retain their ability to stimulate effector T cell responses against infectious agents. However, elderly mDC1s, pDCs and MoDCs simultaneously up-regulated PD-L1, CD39 and/or TGF-β latency-associated peptide following LPS/IFN-γ stimulation, suggesting that they have increased potential to inhibit T cell responses.

In this study, PD-L1 was elevated on elderly DCs, and, in agreement with others, its ligand, PD-1, increased on elderly T cells following interactions with DCs (439, 854, 856, 902), which may result in inhibition of effector T cells (883). In addition, CD80, which was up-regulated on elderly LPS/IFN-γ-DCs, may act as a negative regulator of T cell activation by engaging with CTLA-4 on T cells (833, 837, 844); in this study, CTLA-4 increased on T cells following DC/T cell interactions, and others have observed increased CTLA-4 on elderly T cells (854, 1731). Thus, there is an increased likelihood of CD80/CTLA-4 interactions, leading to attenuation of effector T cells in the elderly. Elevated CD39 on elderly DCs suggests increased potential for adenosine production, particularly if CD73+ cells are present in the aged microenvironment; CD73 is the other enzyme required for adenosine production. The source of CD73 could be DCs themselves, as shown in this study, or neighbouring cells, such as Tregs, which can express CD73 (538, 543), and are increased in numbers during aging (590, 592-596, 598). It is possible that elderly DCs and Tregs act together to generate adenosine, to decrease T cell proliferation (543) and their production of effector molecules such as IFN-γ (920, 1566). Elevated
TGF-β expression by elderly DCs may directly suppress effector T cells and/or induce Treg generation (472, 502, 1719-1721), although further studies are required to confirm that the active form of TGF-β is increased with aging. Thus, the overall outcome of LPS/IFN-γ-activated elderly DCs polarised towards a suppressive phenotype may be inhibition of T cells, leading to reduced immune responses to infectious agents.

The up-regulation of checkpoint inhibitory molecules, particularly PD-L1, by DCs seen in this study may reflect normal DC activation kinetics in response to stimulation by LPS, IFN-γ and other activation stimuli (9, 710, 886, 890, 931, 1314, 1732), this has been shown to occur one day post-stimulation with LPS (1732). Binding PD-L1 to its ligand, PD-1 (which is up-regulated on activated T cells) sends inhibitory signals to T cells to regulate T cell activation (742, 886, 1679), a response that is essential once an infectious agent has been cleared to prevent tissue damage (742, 883, 1679). Future studies should determine if there are age-related differences in DC activation kinetics.

In contrast, elderly mDC2 function may be impaired by reduced responsiveness to LPS/IFN-γ stimulation. In this study, mDC2 responses to LPS/IFN-γ varied between elderly individuals with some unable to up-regulate MHC class I and IFN-γ implying that their ability to cross-present antigens and activate full effector function in CD8+ T cells is compromised (155, 266, 267), which could contribute to diminished immune responses against pathogens.

Further studies are required to extend the pilot studies presented in this chapter. A major limitation of these studies is the small sample size and a larger cohort is needed to strengthen the data. The small sample size may be one reason why discrepancies were seen between the results of this study and the results of other studies. Other factors, such as gender, genetic heterogeneity and the influence of exposure to Australian-specific environmental factors throughout life may have a further confounding influence on the results of this study. Additionally, CMV serostatus should be taken into consideration, as this is known to influence both DC (669) and T cell function (418, 434) during aging.
In summary, the pilot studies in this chapter suggest that during healthy aging, DCs retain several aspects of their T cell co-stimulatory/activating function, but display increased capacity for immune suppression. This is due to up-regulation of checkpoint inhibitory molecules with aging (increased A2A receptor and TGF-β on mDC2s) and following stimulation with LPS/IFN-γ (increased PD-L1, CD39 and/or TGF-β on mDC1s, pDCs and MoDCs). Further studies using a larger cohort of volunteers are required to confirm these data. The development of a regulatory phenotype by elderly DCs may lead to suppression of immune responses to infection and cancer, the latter is examined in the next chapter.
Chapter 4  The effect of aging and cancer on human dendritic cells

4.1 Introduction

The studies in Chapter 3 showed that DCs from elderly healthy volunteers are similar to DCs from young healthy volunteers in regards to expression of co-stimulatory molecules, production of pro-inflammatory cytokines, and T cell stimulation, however they also increased expression of PD-L1, CD39, A2A receptor and TGF-β implying increased regulatory function. As DCs play a key role in activating tumour-specific cytotoxic CD8+ T cells (939, 940), age-related changes in DC function may diminish the generation of effective anti-tumour immunity in the elderly. This may contribute to the increased incidence of cancer in the elderly, and could also affect tumour progression in elderly patients (31, 1164).

The combined impact of aging and cancer on DC function in humans has not been sufficiently well studied. Cancer patients have been shown to have reduced numbers of circulating DCs that demonstrate reduced function, due to decreased expression of antigen-presenting (HLA-DR) and co-stimulatory (CD40, CD80 and CD86) molecules, reduced ability to stimulate T cell proliferation and increased expression of anti-inflammatory cytokines, such as IL-10 (322, 1005, 1022, 1028, 1053-1058, 1061, 1158). Most studies included patients across a wide range of ages (for example, 30-80 years of age), and whilst some compared cancer patients to age-matched healthy controls to take into account the effects of cancer (322, 1022, 1028, 1052-1057, 1159, 1160), others did not specify the ages of the patients or healthy controls, did not dichotomise their cohorts into different age groups and did not compare young versus older cancer patients and young versus older healthy controls (1005, 1058, 1061, 1065, 1156-1158, 1161).

In addition, in vitro studies examining the effects of tumour factors on DC function have not examined the potential influence of age. Exposure of MoDCs from healthy donors to conditioned media from tumour cells has been shown to impair DC differentiation and maturation, reduce T cell stimulatory capacity, skew DCs
towards production of anti-inflammatory cytokines and induce DC apoptosis (228, 1019, 1067-1070). However, these studies used buffy coats or blood samples from young donors as sources of MoDCs.

Several strategies have been used to restore DC function in cancer, including stimulating DCs with CD40L, LPS and TLR agonists to improve antigen presentation and co-stimulatory capacity (29, 1054, 1056, 1156, 1159), or blocking regulatory molecules on DCs, such as PD-L1, to reduce suppression (1313, 1314). Only a few studies have asked if DCs from aging hosts can be restored. One study found that probiotic bacteria enhanced elderly DC cytokine production (TNF-α and TGF-β) as well as their ability to stimulate young-derived T cells (674). Other strategies include: cytokines (IL-7), growth factors and growth hormones to rejuvenate thymic function and T cell output (1733-1735); pharmacologic agents and antioxidants to target molecular changes in aging immune cells; nutritional modulation, for example lipid and vitamin intake, to reduce inflammation and improve T cell function; and adjuvants to improve responses to vaccines (31, 1736, 1737). However, most studies were performed in animal models and testing in elderly humans is ongoing. Importantly, further studies are required to determine ways to rejuvenate aged human DC function, particularly in the context of cancer.

Cornwall et al. (322) from our laboratory showed that age impacts on DC function, and that these effects are further exacerbated in mesothelioma patients. Monocytes from mesothelioma patients are unable to differentiate into functional immature MoDCs, as they have reduced antigen processing capacity and expression of the co-stimulatory molecule CD40, as well as an impaired ability to mature in response to stimulation with LPS+/−IFN-γ, compared to MoDCs from healthy, age-matched counterparts. Furthermore, stimulation with CD40L was unable to restore MoDCs from mesothelioma patients (322); this forerunner study formed the basis for this project. Whilst the aim of the study by Cornwall et al. (322) was to examine DC function in mesothelioma patients, the main focus of this study was to examine the influence of aging on tumour-induced alterations in DC function, and determine whether tumour-exposed elderly DCs can still respond to immune stimulation. This study also performed in-depth analysis of the functional phenotypes of young and
elderly tumour-exposed blood DCs, as well as in-depth analysis to determine the functional activity of T cells stimulated by elderly tumour-exposed DCs, which was not investigated by Cornwall et al. (322).

As there is currently little information regarding the combined effects of aging and cancer on human DCs, the pilot studies in this chapter compared the functional status of blood DCs and MoDCs from young (21-33 years of age) and elderly (60-77 years of age) healthy volunteers following in vitro exposure to tumour cells and/or tumour-derived factors. For these studies, a mesothelioma model was used, as mesothelioma is a cancer that affects elderly populations, with most patients being older than 60 years of age (1421, 1422). This study also investigated whether elderly mesothelioma-exposed DCs were capable of responding to immune stimulation with LPS/IFN-γ.
4.2 Results

4.2.1 Comparing the effects of tumours on young and elderly DCs

4.2.1.1 Tumour factors reduce mDC1 and mDC2 proportions regardless of age

Few studies have examined the combined effects of aging and cancer on DC function. A previous study from our laboratory has shown that mesothelioma patients have reduced numbers of circulating mDC1s, mDC2s and pDCs (322). However, the effects of mesothelioma on the functional phenotype of elderly blood DC subsets, and how this compares to mesothelioma-induced changes in blood DCs from young individuals, have not yet been characterised. Thus, the first series of studies examined whether there were age-related differences in the response of blood DCs to exposure to mesothelioma tumour-derived factors. To simulate in vivo conditions in which blood DCs may be exposed to tumour-derived factors in circulation, PBMC suspensions from young (n=13) and elderly (n=11) healthy volunteers were exposed to conditioned media from cultured JU77 human mesothelioma cells (tumour cell-conditioned media; TCM) for 24 hours, then analysed by flow cytometry for proportions of mDC1s, mDC2s and pDCs, as well as expression of activation and regulatory markers (as per the gating strategy in Figures 3.3A-3.3G). Responses to JU77 TCM were compared to non-tumour-exposed (i.e. media only) age-matched controls. No age-related differences were seen when comparing proportions of tumour-exposed DC subsets (Figure 4.1). Exposure to JU77 TCM induced reduced of percentages of young and elderly mDC1s and mDC2s, whilst young or elderly tumour-exposed pDC proportions did not change relative to non-tumour-exposed controls (Figure 4.1).
Reduced proportions of mDC1s and mDC2s following exposure to JU77 TCM, regardless of age

Young and elderly PBMCs were exposed to conditioned media from the human JU77 mesothelioma cell line (JU77 tumour-conditioned media; TCM) for 24 hours, then stained for CD1c+ mDC1s, CD141+ mDCs and CD123+ CD303+ pDCs, and analysed via flow cytometry, as described in Figures 3.3A-3.3G. Proportions of mDC1s, mDC2s and pDCs in young and elderly tumour-exposed samples were compared to age-matched non-tumour-exposed samples and expressed as percentages of total PBMCs. Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, * = p<0.05 comparing tumour-exposed samples to age-matched non-tumour-exposed controls using a paired t test.

4.2.1.2 Tumours increase CD80, TNF-α and IL-6 on young, but not elderly, mDC1s

To assess the impact of mesothelioma factors on young versus elderly blood DC functional phenotypes, changes in expression of activation and regulatory markers were examined. A comparison of non-tumour-exposed versus tumour-exposed mDC1s from the same person showed that young, but not elderly, tumour-exposed mDC1s significantly up-regulated CD80 expression (p = 0.009; Figure 4.2A). The percentages of mDC1s producing intracellular TNF-α (p = 0.04; Figure 4.2B) and IL-6 (p = 0.01; Figure 4.2C), as well the levels of intracellular IL-6 (p = 0.002; Figure 4.2D) also significantly increased in young tumour-exposed mDC1s, with variable changes seen in elderly mDC1s (Figures 4.2B-4.2D). Regardless of age, surface CD40 and intracellular IL-12 expression increased, MHC class I expression decreased, and CD86 and IFN-γ expression varied on tumour-exposed mDC1s (Appendix C: Figure 1A).
In both age groups, PD-L1 expression increased, whilst expression of other regulatory markers varied on tumour-exposed mDC1s relative to controls (Appendix C: Figure 1B).

**Figure 4.2** Tumours increase CD80, TNF-α and IL-6 on young, but not elderly, mDC1s

Young and elderly PBMCs were exposed to conditioned media from the human JU77 mesothelioma cell line (JU77 tumour-conditioned media; TCM) for 24 hours, then stained for CD1c+ mDC1s, CD80, and intracellular TNF-α and IL-6, and analysed via flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of CD80 (A) and IL-6 (D), and percentages of mDC1s positive for TNF-α (B) and IL-6 (C) were measured. Each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed to non-tumour-exposed mDC1s from the same volunteer using a paired t test.

### 4.2.1.3 Elderly tumour-exposed mDC2s express reduced CD40, and do not up-regulate IL-6 and IL-12

Whilst mDC2s from both age groups up-regulated CD40 expression in response to tumour factors (Appendix C: Figure 2A), a significantly lower percentage of elderly tumour-exposed mDC2s expressed CD40, compared to their younger counterparts (p = 0.047; Figure 4.3A). Again, comparing mDC2s from the same person showed that tumour factors induced a significant increase in intracellular IL-12 (p = 0.047;
Figure 4.3B) and IL-6 levels ($p = 0.008$; Figure 4.3C), with the latter associated with an increased percentage of IL-6$^+$ cells ($p = 0.02$; Figure 4.3D) in young mDC2s; variable results were seen for the same cytokines in elderly mDC2s (Figures 4.3B-4.3D). No age-related differences were seen in the other activation markers (Appendix C: Figure 2A).

Young, but not elderly, tumour-exposed mDC2s significantly up-regulated PD-L1 ($p = 0.009$; Appendix C: Figure 2B), relative to controls, whilst changes in the other regulatory markers were variable in both age groups (Appendix C: Figure 2B).

**Figure 4.3 Elderly tumour-exposed mDC2s express reduced CD40, whilst tumours increase IL-6 and IL-12 on young, but not elderly, mDC2s**

Young and elderly PBMCs were exposed to conditioned media from the human JU77 mesothelioma cell line (JU77 tumour-conditioned media; TCM) for 24 hours, then stained for CD141$^+$ mDC2s, CD40, and the intracellular cytokines IL-6 and IL-12, and analysed via flow cytometry. Percentages of CD40$^+$ mDC2s were compared in tumour-exposed young and elderly samples; each point represents one volunteer (A). Expression levels (measured as geometric mean fluorescence intensity; MFI) of IL-12 (B) and IL-6 (C), and percentages of mDC2s positive for IL-6 (D) were measured; in graphs (B-D), each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, $n = 13$ young volunteers, $n = 11$ elderly volunteers, $* = p<0.05$, $** = p<0.005$ comparing young to elderly volunteers in (A) using an unpaired t test, or comparing tumour-exposed to non-tumour-exposed mDC2s from the same volunteer in (B-D) using a paired t test.
4.2.1.4 Elderly tumour-exposed pDCs have decreased MHC class I and TNF-α

A significantly reduced percentage of elderly tumour-exposed pDCs expressed MHC class I compared to their younger counterparts (p = 0.04; Figure 4.4A). In addition, when comparing pDCs within the same individual, a significant decrease in intracellular TNF-α was seen in pDCs from elderly, but not young, volunteers following tumour exposure (p = 0.02; Figure 4.4B). The other activation markers examined did not show age-related differences (Appendix C: Figure 3A).

Examination of regulatory markers showed that elderly tumour-exposed pDCs had significantly lower percentages of PD-L1+ (p = 0.004; Figure 4.4A) and TGF-β+ cells (p = 0.02; Figure 4.4A), and a trend towards a lower percentage of CD73+ cells (p = 0.055; Figure 4.4A) compared to their younger counterparts. Comparisons of pDCs from the same person showed that young, but not elderly, tumour-exposed pDCs significantly up-regulated PD-L1 (p = 0.03; Appendix C: Figure 3B) and CD73 (p = 0.005; Appendix C: Figure 3B). No further age-related differences in regulatory markers were seen (Appendix C: Figure 3B).

Figure 4.4 Elderly tumour-pDCs have reduced MHC-I, TNF-α, CD73, PD-L1 and TGF-β
Young and elderly PBMCs were exposed to JU77 TCM for 24 hours, then stained for CD123+CD303+pDCs, MHC class I, CD73, PD-L1, and the intracellular cytokines TNF-α and TGF-β, and analysed via flow cytometry. Percentages of pDCs positive for MHC-I, PD-L1, CD73 and TGF-β were compared in tumour-exposed young and elderly samples (A). Expression levels of TNF-α were measured as geometric MFI; each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control (B). Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, * = p<0.05 comparing young to elderly volunteers in (A) using an unpaired t test, or comparing tumour-exposed to non-tumour-exposed pDCs from the same volunteer in (B) using a paired t test.
4.2.1.5 Young and elderly tumour-exposed PBMCs have similar cytokine profiles

As cytokine secretion is an indicator of DC function, culture supernatants from young and elderly PBMCs exposed to JU77 TCM were tested for pro-inflammatory cytokines to determine if tumour factors modulated cytokine secretion in an age-dependent manner. TCM sample testing showed that JU77 tumour cells secrete TNF-α, MCP-1, IL-6 and IL-8 (Appendix C: Figure 4), with lower concentrations of IL-1β, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33, whilst IFN-α and IFN-γ were not detected (Appendix C: Figure 4). Concentrations of cytokines measured in PBMC/JU77 TCM co-cultures were adjusted by subtracting the average concentration of each cytokine measured in JU77 TCM only.

No age-related differences were seen in PBMC pro-inflammatory cytokine secretion following exposure to JU77 TCM. Comparisons of PBMCs from the same person showed that, regardless of age, IL-8 production increased, IFN-α, IL-17A and IL-33 were not detected, and variable changes were observed in the other cytokines (Appendix C: Figures 5A and 5B).

4.2.1.6 Exposure to tumour cells increased CD73 on elderly MoDCs

Monocytes migrating into a tumour site may be affected by tumour factors which determine whether they can differentiate into DCs. Therefore, the next series of studies examined the impact of exposure to JU77 mesothelioma tumour cells on monocytes as they differentiate into MoDCs and whether this was affected by age. Young (n=12) and elderly (n=11) MoDCs were co-cultured with JU77 tumour cells for the entire 7 day differentiation period, and analysed using flow cytometry as per Figures 3.8A-3.8D. Tumour-exposed MoDCs were compared to age-matched non-tumour-exposed (i.e. media only) controls. Approximately two-thirds of elderly tumour-exposed monocytes were unable to develop into CD11c^+CD14^− MoDCs (p = 0.01; Figure 4.5A), whilst the majority of tumour-exposed young monocytes developed into CD11c^+CD14^− MoDCs (Figure 4.5A).

To determine whether young versus elderly tumour-exposed MoDCs were
functional, expression of markers of activation and regulatory function were measured, as per Figures 3.9A-3.9C. Changes in marker expression in each volunteer’s tumour-exposed sample were compared to their non-tumour-exposed control. There were no age-related differences in changes in activation markers following exposure to JU77 tumour cells; percentages of MoDCs positive for CD40 and intracellular IL-12 increased, whilst percentages of CD1a+ cells decreased, with the remaining markers showing variable changes (Appendix C: Figure 6A).

Comparisons within each individual showed that an increased percentage of elderly tumour-exposed MoDCs expressed CD73 (p = 0.02; Figure 4.5B), concomitant with increased CD73 expression levels (p = 0.006; Figure 4.5C); variable changes were seen in young CD73 levels (Figures 4.5B and 4.5C). Regardless of age, exposure to JU77 tumour cells induced increased expression of the A2B receptor and IL-10, and mixed changes in the other regulatory markers (Appendix C: Figure 6B).

**Figure 4.5 Tumours significantly increase CD73 on elderly, but not young, MoDCs**
Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and during this period, MoDCs were exposed to human JU77 mesothelioma cells. On day 7, samples were stained for CD11c⁺CD14⁺ MoDCs and the regulatory marker CD73, and analysed using flow cytometry. Percentages of CD11c⁺CD14⁺ MoDCs in samples exposed to JU77 tumour cells were compared to age-matched non-tumour-exposed controls (A). Percentages of CD11c⁺CD14⁺ MoDCs positive for CD73 (B), and CD73 expression levels (shown as geometric MFI; C) were measured. In graphs (B and C), each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 12 young volunteers, n = 11 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed to age-matched non-tumour-exposed samples in (A), or comparing tumour-exposed to non-tumour-exposed MoDCs from the same volunteer in (B and C) using a paired t test.
Young and elderly tumour-exposed MoDCs also showed similar cytokine secretion profiles, with increased production of TNF-α, MCP-1, IL-6, IL-8, IL-10 and IL-18, and decreased IFN-γ, IL-12p70, IL-23 and IL-33 in comparison to non-tumour-exposed controls (Appendix C: Figures 7A and 7B).

4.2.1.7 Tumour-exposed MoDCs induce suppressive young and elderly T cells

DCs from cancer patients have been shown to have a reduced T cell stimulatory capacity (1005, 1054, 1058, 1061), and some studies have also shown that in vitro exposure to tumour factors can impair the ability of young DCs to stimulate T cells (1019, 1068-1070). However, the ability of elderly tumour-exposed DCs to stimulate functional T cell proliferation has not been well-studied and was investigated in the next series of experiments. Tumour-exposed MoDCs from young (n=12) and elderly (n=11) volunteers were co-cultured with allogeneic, CFSE-labelled T cells from young volunteers (aged 22-31 years, n=5) to determine if elderly tumour-exposed MoDCs had an impaired ability to stimulate T cells. Young and elderly tumour-exposed MoDCs were also co-cultured with allogeneic, CFSE-labelled T cells from elderly volunteers (aged 60-74 years, n=6) to determine if elderly T cells are able to respond to stimulation by tumour-exposed young versus elderly MoDCs. CD8+ T cells, CD4+ T cells, and CD4+CD25+CD127\textsuperscript{low} Tregs, and T cell proliferative responses were measured using flow cytometry, as per Figures 3.11A-3.11F. No defects in the ability of elderly tumour-exposed MoDCs to stimulate young (Figure 4.6) or elderly (Appendix C: Figure 8) CD8+ and CD4+ T cells and Tregs to proliferate were seen.

To determine whether the proliferating T cells stimulated by elderly tumour-exposed MoDCs were functional, markers of T cell effector/activation (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory (CD39, CD73, A2A receptor, CTLA-4, ICOS, TIM-3, PD-1 and intracellular IL-10 and TGF-β) function were examined using flow cytometry. Changes in marker expression from parent to daughter T cells induced by young versus elderly tumour-exposed MoDCs were compared.
Figure 4.6 Young and elderly tumour-exposed MoDCs stimulate similar levels of young T cell proliferation
Young and elderly MoDCs exposed to JU77 tumour cells were co-cultured with allogeneic, CFSE-labelled young T cells at a ratio of 1 DC: 5 T cells, for 5 days. Samples were stained for CD8+ T cells, CD4+ T cells, and CD4+CD25+CD127low Tregs and analysed by flow cytometry as per Figures 3.11A-3.11F. The percentage of T cell proliferation was calculated based on loss of staining intensity of the parent peak. Percentages of young CD8+ T cell, CD4+ T cell and Treg cell proliferation stimulated by tumour-exposed MoDCs were compared to those stimulated by age-matched non-tumour-exposed MoDCs using a paired t test. Data are shown as mean ± SEM, n = 12 young volunteers’ MoDCs, n = 11 elderly volunteers’ MoDCs.

Regardless of age, tumour-exposed MoDCs stimulated increased CD25 and/or IL-12 on young (Figure 4.7A) and elderly (Appendix C: Figure 9A) daughter CD8+ T cells, compared to parent T cells. However, young and elderly tumour-exposed MoDCs seemed to promote a more regulatory phenotype in proliferating CD8+ T cells, due to increased expression of most regulatory markers (including CD39, A2A receptor, CTLA-4, ICOS, TIM-3, PD-1, IL-10 and/or TGF-β) on daughter young (Figure 4.7B) and elderly (Appendix C: Figure 9B) CD8+ T cells.

Although young and elderly tumour-exposed MoDCs induced increased expression of activation markers (CD25 and/or IL-12) on young (Appendix C: Figure 10A) and elderly (Appendix C: Figure 11A) CD4+ T cells, MoDCs from both age groups skewed young (Appendix C: Figure 10B) and elderly (Appendix C: Figure 11B) CD4+ T cells towards a more regulatory phenotype, due to increases in most inhibitory markers, including CD39, CD73, A2A receptor, CTLA-4, TIM-3, PD-1 and/or TGF-β.
Young and elderly tumour-exposed MoDCs induce young CD8$^+$ T cells with a regulatory phenotype

Young CD8$^+$ T cells were stimulated by young and elderly tumour-exposed MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8$^+$ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD8$^+$ T cells were compared for samples stimulated by young versus elderly tumour-exposed MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing parent to daughter T cells using a paired t test.

In summary, tumour-derived factors may: (i) reduce the T cell activation capacity of elderly mDC1s as they do not up-regulate CD80, TNF-α and IL-6, and have increased PD-L1; (ii) reduce T cell licensing capacity of elderly mDC2s due to decreased CD40, and reduce co-stimulatory capacity via reduced IL-6 and IL-12, and (iii) compromise
the ability of elderly pDCs to present antigens on MHC class I molecules and activate T cells through reduced TNF-α. Irrespective of age, tumour-exposed PBMCs appear to retain the ability to recruit polymorphonuclear leukocytes via increased IL-8. Moreover, young and elderly tumour-exposed MoDCs display a partially activated phenotype due to increased CD40 and IL-12, but may have a reduced ability to present tumour-associated lipid antigens due to reduced CD1a. Furthermore, exposure to tumour cells induced elderly MoDCs to up-regulate CD73 suggesting increased suppressive potential. However, no defects were seen in the ability of elderly tumour-exposed MoDCs to stimulate T cell proliferation, with young and elderly tumour-exposed MoDCs promoting a suppressive/exhausted phenotype in young as well as elderly T cells.

4.2.2 Can elderly tumour-exposed DCs respond to LPS/IFN-γ?

4.2.2.1 LPS/IFN-γ increases IL-10 in elderly tumour-exposed mDC1s

The next series of studies investigated whether tumour-exposed blood DC subsets could respond to LPS/IFN-γ stimulation and if their responses were influenced by aging. PBMC suspensions from young (n=10) and elderly (n=10) volunteers were exposed to JU77 TCM and simultaneously stimulated with LPS/IFN-γ before flow cytometric analysis for DC subsets as per Figures 3.3A-3.3G. In both age groups, mDC1 (but not mDC2 or pDC) proportions were reduced in tumour-exposed-LPS/IFN-γ samples, compared to age-matched LPS/IFN-γ and tumour-exposed controls (Figure 4.8).

Changes in expression of activation and regulatory markers were assessed by comparing each volunteer’s tumour-exposed-LPS/IFN-γ sample to their tumour-exposed and LPS/IFN-γ only controls. Young and elderly tumour-exposed mDC1s responded to LPS/IFN-γ by up-regulating CD80, IFN-γ and IL-6, relative to tumour-exposed controls (Figures 4.9A and 4.9B). Unexpectedly, for most volunteers, CD80, IFN-γ and IL-6 levels were also higher than LPS/IFN-γ controls (Figures 4.9A and 4.9B). In contrast, regardless of age, tumour-exposed-LPS/IFN-γ-stimulated mDC1s had reduced CD86 and IL-12 levels compared to tumour-exposed controls, which
were similar to LPS/IFN-γ controls (Appendix C: Figure 12A). There were no age-related differences in MHC class I, CD40 and TNF-α, and changes were variable compared to LPS/IFN-γ controls (Appendix C: Figure 12A).

LPS/IFN-γ increased IL-10 (p = 0.001; Figure 4.9C; p = 0.002; Figure 4.9D) in elderly tumour-exposed mDC1s relative to the tumour-exposed and LPS/IFN-γ controls (Figures 4.9C and 4.9D). Mixed responses were seen in their younger counterparts (Figures 4.9C and 4.9D). There were no age-related differences in the other regulatory markers examined (Appendix C: Figures 12B and 12C).

**Figure 4.8 LPS/IFN-γ reduces proportions of tumour-exposed mDC1s**
Young and elderly PBMCs were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ for 24 hours, stained for mDC1s, mDC2s and pDCs, and analysed using flow cytometry, as per Figures 3.3A-3.3G. Proportions of mDC1s, mDC2s and pDCs in tumour-exposed-LPS/IFN-γ-stimulated samples were compared to age-matched (i) tumour-exposed controls, and (ii) LPS/IFN-γ controls. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to age-matched (i) tumour-exposed controls or (ii) LPS/IFN-γ controls using a paired t test.
**Figure 4.9** LPS/IFN-γ increases IL-10 in elderly tumour-exposed mDC1s

Young and elderly PBMCs were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ for 24 hours, stained for CD1c⁺ mDC1s, activation markers (CD80 and intracellular IFN-γ and IL-6), and intracellular IL-10, and analysed by flow cytometry. Percentages of mDC1s positive for activation markers (A) and IL-10 (C), and expression levels (shown as geometric mean fluorescence intensity; MFI) of activation markers (B) and IL-10 (D) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.

**4.2.2.2 LPS/IFN-γ does not restore CD40 in elderly tumour-exposed mDC2s**

LPS/IFN-γ did not restore CD40 expression in elderly tumour-exposed mDC2s, as comparisons within the same person showed that elderly tumour-exposed-LPS/IFN-
γ mDC2s had significantly lower CD40, relative to LPS/IFN-γ (non-tumour-exposed) controls (p = 0.02; Figure 4.10A; p = 0.03; Figure 4.10B); this was not seen for young mDC2s (Figures 4.10A and 4.10B). Age did not affect the ability of tumour-exposed mDC2s to up-regulate other activation markers in response to LPS/IFN-γ. In both age groups, CD80, IFN-γ, TNF-α and IL-6 increased in tumour-exposed-LPS/IFN-γ mDC2s compared to tumour-exposed controls, and reached levels similar or greater than those seen in LPS/IFN-γ controls (Figure 4.10C). Variable changes were seen in the other activation markers examined (Appendix C: Figure 13A).

Elderly tumour-exposed mDC2s also responded to LPS/IFN-γ by significantly up-regulating PD-L1 (p = 0.003; Figure 4.10D; p = 0.007; Figure 4.10E), relative to tumour-exposed controls; reaching at least equivalent levels to the LPS/IFN-γ controls (Figures 4.10D and 4.10E). In contrast, young tumour-exposed-LPS/IFN-γ mDC2s showed variable changes in PD-L1 (Figures 4.10D and 4.10E). Both age groups showed similar changes in other regulatory markers (Appendix C: Figures 13B and 13C).

**4.2.2.3 Elderly tumour-exposed-LPS/IFN-γ-stimulated pDCs have increased CD40, CD80, CD86 and PD-L1**

LPS/IFN-γ significantly increased CD40, CD80 and CD86 expression in young and elderly tumour-exposed pDCs relative to tumour-exposed controls; the levels achieved were equal to or greater than the levels seen in the LPS/IFN-γ controls, importantly no age-related differences were seen (Figures 4.11A and 4.11B). Variable changes were seen in levels of other activation markers in both age groups, relative to controls (Appendix C: Figure 14A).

Following LPS/IFN-γ stimulation, elderly tumour-exposed pDCs also significantly up-regulated PD-L1 (p = 0.006, Figure 4.11C; p = 0.0049, Figure 4.11D), relative to tumour-exposed controls, to levels that were equal to or greater than those in the LPS/IFN-γ controls. In contrast, the percentage of young PD-L1+ pDCs was reduced compared to LPS/IFN-γ controls (p = 0.04; Figure 4.11C). No other significant changes in regulatory markers were seen (Appendix C: Figures 14B and 14C).
Figure 4.10 LPS/IFN-γ does not restore CD40, but increases PD-L1 on elderly tumour-exposed mDC2s

Young and elderly PBMCs were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ for 24 hours, stained for CD141+ mDC2s, activation markers (CD40, CD80 and intracellular IFN-γ, TNF-α and IL-6), and PD-L1, and analysed by flow cytometry. Expression levels (shown as geometric mean fluorescence intensity; MFI) of CD40 (A) and PD-L1 (D), and percentages of mDC2s positive for CD40 (B), activation markers (C) and PD-L1 (E) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.
Figure 4.11 Elderly tumour-exposed-LPS/IFN-γ pDCs have increased CD40, CD80, CD86 and PD-L1

Young and elderly PBMCs were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ for 24 hours, stained for CD123+CD303+ pDCs, activation markers (CD40, CD80 and CD86), and PD-L1, and analysed by flow cytometry. Percentages of pDCs positive for activation markers (A) and PD-L1 (C), and expression levels (shown as geometric mean fluorescence intensity; MFI) of activation markers (B) and PD-L1 (D) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.

Culture supernatants from young and elderly tumour-exposed-LPS/IFN-γ PBMCs were tested for pro-inflammatory cytokines. No age-related differences were observed, as young and elderly tumour-exposed PBMCs up-regulated most
cytokines following LPS/IFN-γ stimulation, relative to tumour-exposed controls (Figures 4.12A-4.12C). Secretion of IFN-γ, MCP-1, IL-6, IL-10 and IL-18 achieved similar or higher levels than those in LPS/IFN-γ controls (Figure 4.12A), whilst TNF-α, IL-1β, IL-8, IL-12p70 and IL-23 levels were barely elevated (Figure 4.12B).

Figure 4.12 No age-related differences in cytokine secretion by tumour-exposed-LPS/IFN-γ PBMCs
Concentrations of IFN-γ, MCP-1, IL-6, IL-10 and IL-18 (A), TNF-α, IL-1β, IL-8, IL-12p70 and IL-23 (B), and IFN-α, IL-17A and IL-33 (C) were measured in culture supernatants from young and elderly PBMCs that were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ, using a cytokine bead array. Cytokine concentrations were adjusted by subtracting the average concentration of each cytokine measured in JU77 TCM only. In (A-C), each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.
4.2.2.4 LPS/IFN-γ partially restores CD40, CD80 and CD86 on tumour-exposed MoDCs

Monocytes that differentiate into MoDCs within the tumour microenvironment may have an altered ability to respond to activation/maturation stimuli, and this could be further affected by aging. Therefore, the influence of age on the ability of tumour-exposed MoDCs to respond to LPS/IFN-γ stimulation was investigated by exposing young (n=10) and elderly (n=10) monocytes to JU77 tumour cells during differentiation into MoDCs, followed by stimulation with LPS/IFN-γ before flow cytometric analysis for CD11c⁺CD14⁻ MoDCs (as per Figures 3.8A-3.8D) and expression of activation and regulatory markers (as per Figures 3.9A-3.9C). Regardless of age, LPS/IFN-γ did not restore CD11c⁺CD14⁻ MoDCs proportions in tumour-exposed samples (Figure 4.13).

LPS/IFN-γ induced increased expression of MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, IL-6 and IL-12 on young and elderly tumour-exposed MoDCs, relative to tumour-exposed only controls (Figures 4.14A and 4.14B, Figures 4.15A and 4.15B). MHC class I, IFN-γ, IL-6 and IL-12 levels were higher than the LPS/IFN-γ controls (Figures 4.14A and 4.14B), whilst CD40, CD80 and CD86 expression did not
reach the levels seen in the LPS/IFN-γ controls (Figures 4.15A and 4.15B). CD1a and TNF-α on tumour-exposed-LPS/IFN-γ young and elderly MoDCs decreased compared to tumour-exposed and LPS/IFN-γ controls (Appendix C: Figure 15A).

Figure 4.14 LPS/IFN-γ improves MHC class I, IFN-γ, IL-6 and IL-12 on young and elderly tumour-exposed MoDCs

Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and during this period, some MoDCs were exposed to human JU77 mesothelioma cells. On day 7, tumour-exposed MoDCs were stimulated with LPS/IFN-γ for a further 2 days. On day 9, samples were stained for CD11c^-CD14^- MoDCs and activation markers (MHC class I, and intracellular IFN-γ, IL-6 and IL-12), and analysed using flow cytometry. Percentages of MoDCs positive for activation markers (A), and expression levels (shown as geometric mean fluorescence intensity; MFI; B) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.
Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and during this period, some MoDCs were exposed to human JU77 mesothelioma cells. On day 7, tumour-exposed MoDCs were stimulated with LPS/IFN-γ for a further 2 days. On day 9, samples were stained for CD11c⁺CD14⁻ MoDCs and co-stimulatory markers (CD40, CD80 and CD86), and analysed using flow cytometry. Percentages of MoDCs positive for co-stimulatory markers (A), and expression levels (shown as geometric mean fluorescence intensity; MFI; B) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.

Young and elderly tumour-exposed MoDCs up-regulated CD39, CD73, PD-L1 and IL-10 following LPS/IFN-γ stimulation, relative to tumour-exposed and LPS/IFN-γ controls (Figures 4.16A and 4.16B). Expression of TGF-β was lower than tumour-
Exposed and LPS/IFN-γ controls, whilst other regulatory markers showed variable changes (Appendix C: Figure 15B).

**Figure 4.16 LPS/IFN-γ up-regulates CD39, CD73, PD-L1 and IL-10 on young and elderly tumour-exposed MoDCs**

Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and during this period, some MoDCs were exposed to human JU77 mesothelioma cells. On day 7, tumour-exposed MoDCs were stimulated with LPS/IFN-γ for a further 2 days. On day 9, samples were stained for CD11c+CD14+ MoDCs and regulatory markers (CD39, CD73, PD-L1 and intracellular IL-10), and analysed using flow cytometry. Percentages of MoDCs positive for regulatory markers (A), and expression levels (shown as geometric mean fluorescence intensity; MFI; B) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.
Tumour-exposed-LPS/IFN-γ-stimulated MoDCs increased secretion of most pro-inflammatory cytokines measured (except IL-17A and IL-33), with their concentrations higher than those produced by LPS/IFN-γ-MoDC controls in both age groups (Figures 4.17A-4.17C).

Figure 4.17 LPS/IFN-γ improves cytokine secretion by young and elderly tumour-exposed MoDCs
Concentrations of IFN-α, IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1) and IL-1β (A), IL-6, IL-8, IL-10, IL-12p70 and IL-17A (B), and IL-18, IL-23 and IL-33 (C) were measured in culture supernatants from young and elderly MoDCs that were exposed to JU77 tumour cells and stimulated with LPS/IFN-γ, using a cytokine bead array. Cytokine concentrations were adjusted by subtracting the average concentration of each cytokine measured in JU77 TCM only. In (A-C), each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer using a paired t test.
4.2.2.5 Elderly tumour-exposed-LPS/IFN-γ MoDCs have an improved ability to stimulate young CD8⁺ T cell proliferation

The next experiments investigated whether LPS/IFN-γ improved the ability of tumour-exposed elderly MoDCs to stimulate T cell proliferation and generate functional T cells. To determine if elderly MoDCs were defective in stimulating T cells, tumour-exposed-LPS/IFN-γ MoDCs from young (n=10) and elderly (n=10) volunteers were co-cultured with allogeneic T cells from young volunteers (22-31 years, n=5). Young and elderly tumour-exposed-LPS/IFN-γ MoDCs were also co-cultured with allogeneic T cells from elderly volunteers (60-74 years, n=6) to determine if elderly T cells are impaired. CD8⁺ T cells, CD4⁺ T cells, and CD4⁺CD25⁺CD127low Tregs, and T cell proliferative responses were measured using flow cytometry, as per Figures 3.11A-3.11F.

Elderly, but not young, tumour-exposed-LPS/IFN-γ-stimulated MoDCs increased young CD8⁺ T cell proliferation, relative to tumour-exposed MoDCs (for elderly p = 0.01; Figure 4.18; for young p = 0.08; Figure 4.18); the T cell proliferation levels achieved were similar to those induced by LPS/IFN-γ-MoDCs (Figure 4.18). In contrast, tumour-exposed-LPS/IFN-γ-activated MoDCs could not increase young CD4⁺ T cell proliferation to the levels seen in LPS/IFN-γ controls; this difference was significant for young (p = 0.035; Figure 4.18), but not elderly, MoDCs (Figure 4.18). No age-related differences in young Treg proliferation were observed (Figure 4.18).

To determine if tumour-exposed-LPS/IFN-γ MoDCs could stimulate functional T cells, markers of T cell effector/activation and regulatory function were examined using flow cytometry. Both young and elderly tumour-exposed-LPS/IFN-γ MoDCs induced simultaneous increases in activation (CD25, IFN-γ, and IL-12; Appendix C: Figure 16A) and regulatory (CD39, A2A receptor, CTLA-4, ICOS, TIM-3, IL-10 and TGF-β; Appendix C: Figure 16B) markers on young daughter CD8⁺ T cells, implying a suppressive phenotype. Similarly, young and elderly tumour-exposed-LPS/IFN-γ MoDCs seemed to induce a more suppressive phenotype in proliferating young CD4⁺ T cells as expression of CD25 (Appendix C: Figure 17A), CD39, A2A receptor, CTLA-4, ICOS, TIM-3, IL-10 and TGF-β (Appendix C: Figure 17B) increased.
Figure 4.18 Elderly tumour-exposed-LPS/IFN-γ MoDCs stimulate increased young CD8⁺ T cell proliferation

Young and elderly MoDCs exposed to JU77 tumour cells and stimulated with LPS/IFN-γ were co-cultured with allogeneic, CFSE-labelled young T cells at a ratio of 1 DC: 5 T cells, for 5 days. Samples were stained for CD8⁺ T cells, CD4⁺ T cells, and CD4⁺CD25⁺CD127low Tregs and analysed by flow cytometry as per Figures 3.11A-3.11F. The percentage of T cell proliferation was calculated based on loss of staining intensity of the parent peak. Percentages of young CD8⁺ T cell, CD4⁺ T cell and Treg cell proliferation stimulated by tumour-exposed-LPS/IFN-γ MoDCs were compared to those stimulated by age-matched (i) tumour-exposed MoDC controls, and (ii) LPS/IFN-γ MoDC controls. Data are shown as mean ± SEM, n = 10 young volunteers’ MoDCs, n = 10 elderly volunteers’ MoDCs, * = p<0.05 comparing tumour-exposed-LPS/IFN-γ MoDCs to age-matched (i) tumour-exposed MoDCs or (ii) LPS/IFN-γ MoDCs using a paired t test.

4.2.2.6 Elderly tumour-exposed-LPS/IFN-γ MoDCs reduce elderly CD4⁺ T cell proliferation

Unlike their younger controls, elderly tumour-exposed-LPS/IFN-γ-activated MoDCs could not stimulate elderly CD4⁺ T cells to proliferate at the same levels as their LPS/IFN-γ age-matched counterparts (p = 0.03; Figure 4.19). In contrast, no age-related differences were observed for elderly CD8⁺ T cell and Treg cell proliferation in response to tumour-exposed-LPS/IFN-γ-activated MoDCs (Figure 4.19).

Regardless of age, tumour-exposed-LPS/IFN-γ MoDCs promoted up-regulation of activation and suppressive markers on proliferating elderly T cells: i.e. IFN-γ and IL-
increased on elderly daughter CD8+ (Appendix C: Figure 18A) and CD4+ T cells (Appendix C: Figure 19A), however, expression of the A2A receptor, CTLA-4, ICOS, TIM-3, PD-1, IL-10 and TGF-β also increased on elderly CD8+ (Appendix C: Figure 18B) and CD4+ (Appendix C: Figure 19B) daughter T cells.

Figure 4.19 Elderly tumour-exposed-LPS/IFN-γ MoDCs stimulate reduced elderly CD4+ T cell proliferation
Young and elderly MoDCs exposed to JU77 tumour cells and stimulated with LPS/IFN-γ were co-cultured with allogeneic, CFSE-labelled elderly T cells at a ratio of 1 DC: 5 T cells, for 5 days. Samples were stained for CD8+ T cells, CD4+ T cells, and CD4+CD25+CD127low Tregs and analysed by flow cytometry as per Figures 3.11A-3.11F. The percentage of T cell proliferation was calculated based on loss of staining intensity of the parent peak. Percentages of elderly CD8+ T cell, CD4+ T cell and Treg cell proliferation stimulated by tumour-exposed-LPS/IFN-γ MoDCs were compared to those stimulated by age-matched (i) tumour-exposed MoDC controls, and (ii) LPS/IFN-γ MoDC controls. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05 comparing tumour-exposed-LPS/IFN-γ MoDCs to age-matched (i) tumour-exposed MoDCs or (ii) LPS/IFN-γ MoDCs using a paired t test.

In summary, the data shows that: (i) elderly tumour-exposed mDC1s can respond to LPS/IFN-γ and stimulate T cells through increased CD80, IFN-γ and IL-6, which may be confounded by increased IL-10; (ii) elderly tumour-exposed mDC2s can respond to LPS/IFN-γ and activate T cells through increased CD80, IFN-γ, TNF-α and IL-6, however their T cell licensing capacity may not be improved as CD40 levels were not restored and PD-L1 increased; (iii) elderly tumour-exposed pDCs can respond to
LPS/IFN-γ and have increased T cell co-stimulatory capacity due to up-regulation of CD40, CD80 and CD86, which may be confounded via increased PD-L1; (iv) elderly tumour-exposed MoDCs respond to LPS/IFN-γ by up-regulating their antigen-presenting capacity to CD8+ T cells via increased MHC class I, and increasing IFN-γ, IL-6 and IL-12, this was confirmed as they stimulated proliferation of young CD8+ but not CD4+ T cells. However, tumour-exposed-LPS/IFN-γ young and elderly MoDCs stimulated a predominantly regulatory phenotype in T cells due to up-regulation CD39, A2A receptor, CTLA-4, ICOS, TIM-3, PD-1, IL-10 and/or TGF-β.
4.3 Discussion

The pilot studies in this chapter investigated whether there were age-related differences in the effects of exposure to mesothelioma tumour cells/factors on young and elderly DCs, and whether elderly tumour-exposed DCs were able to respond to immune stimulation.

There were several mechanisms by which mesothelioma appeared to modulate DC function, regardless of age. Results from this study suggest that mesothelioma may impair mDC1s, mDC2s and MoDCs by reducing their numerical proportions. These results agree with a previous study in our laboratory showing that mesothelioma patients have reduced circulating numbers of mDC1s and mDC2s, compared to age-matched healthy controls (322), and also agree with studies showing that blood DC numbers are reduced in patients with other types of cancer (1005, 1052-1056, 1058).

Mesothelioma may also promote partial activation in tumour-infiltrating DCs, irrespective of age. MoDCs, which may represent DCs that have differentiated from monocytes within the mesothelioma microenvironment, displayed a partially activated phenotype characterised by increased expression of some activation markers (CD40 and intracellular IL-12) and secretion of some pro-inflammatory cytokines/chemokines (TNF-α, MCP-1, IL-6, IL-8 and IL-18). However, mesothelioma further modulates DC function, possibly by increasing their suppressive activity. Both young and elderly mesothelioma-exposed mDC1s up-regulated the inhibitory molecule PD-L1, and mesothelioma-exposed MoDCs induced increased PD-1 on T cells; this DC/T cell interaction is likely to lead to T cell suppression and termination of anti-tumour cytotoxic T cell responses (882, 1738). As a result, DCs that differentiate within the tumour site and subsequently migrate to lymph nodes may be unable to provide all of the necessary co-stimulatory signals to T cells, and their expression of inhibitory molecules could prevent activation of tumour-specific effector T cells and instead lead to T cell tolerance (5, 6). Tumour-induced development of semi-mature DCs is in agreement with other studies which exposed MoDCs from young donors to tumour cells/factors in vitro (228, 1019, 1067-1070).
Moreover, tumour-infiltrating CD8+ T cells in other cancers, such as melanoma and colorectal cancer, have been shown to express high levels of PD-1 (1110, 1113, 1118, 1739). Furthermore, PD-1 expression on T cells increases with aging (245, 899), which suggests that the likelihood of PD-L1/PD-1 interactions occurring between DCs and CD8+ T cells in the mesothelioma microenvironment may be further increased by aging.

Regardless of age, mesothelioma may also compromise anti-tumour T cell responses due to induction of suppressive/exhausted T cells by tumour-exposed DCs. Data from this study showed that whilst young and elderly tumour-exposed MoDCs could stimulate young and elderly T cell proliferation, the resulting T cells had increased expression of several inhibitory molecules, such as CTLA-4, ICOS, TIM-3 and PD-1, which is indicative of T cell exhaustion (1105, 1740, 1741). Exhausted T cells have been described in cancer (1108, 1119, 1316), and these T cells are unable to proliferate and have functional defects, including impaired production of IL-2 and IFN-γ, and reduced cytotoxic activity (1102, 1105). Furthermore, tumour-exposed DCs may also promote T cells with increased suppressive activity via IL-10 and TGF-β; these cytokines mediate suppressive effects by inhibiting the cytotoxic activity of CD8+ T cells (502, 1720, 1742) and promoting the development of Tregs (467, 472, 477, 519, 520, 1721, 1743). Thus, these factors may contribute to reduced effector T cell responses and increased immunosuppression within the tumour microenvironment, regardless of age. However, it is possible that the T cell phenotype observed may be related to the time point of analysis, and further studies are required to confirm that tumour-exposed DCs do indeed promote T cell exhaustion.

There were also age-specific mechanisms by which mesothelioma modulated DC function. One mechanism may be through reduced blood DC activation. Elderly tumour-exposed mDC2s and pDCs had reduced expression of antigen-presenting molecules, co-stimulatory molecules and/or pro-inflammatory cytokines (MHC class I, CD40 and/or TNF-α), suggesting that these DCs may less able to present antigens and activate tumour-specific effector T cells. Furthermore, unlike their younger counterparts, elderly mDC1s and mDC2s did not up-regulate certain co-stimulatory
molecules and pro-inflammatory cytokines (CD80, TNF-α, IL-6 and/or IL-12) in response to exposure to tumour factors, which provides further support that mesothelioma may prevent elderly DCs from developing full co-stimulatory function. These findings agree with others showing that blood DCs from cancer patients have reduced expression of antigen-presenting and co-stimulatory molecules (1005, 1054, 1055, 1057, 1058, 1061). As blood DC subsets are proposed to be immature DCs in transit to peripheral tissues (260), circulating DCs exposed to mesothelioma factors may migrate to lymph nodes where they are unable to activate tumour-specific effector T cells. Alternatively, they may migrate into the tumour microenvironment where they are further disabled prior to trafficking to lymph nodes. This may reduce or even prevent the generation of anti-mesothelioma immune responses in elderly hosts as well as disable effector T cells in tumours.

The tumour-induced changes in blood DC subsets could be due to direct effects of tumour factors. A previous study has shown that JU77 cells secrete VEGF and TGF-β (228). Tumour-derived VEGF and TGF-β exert direct suppressive effects on DCs, such as preventing up-regulation of co-stimulatory molecules, blocking DC maturation and impairing their T cell-stimulating capacity (1005, 1007, 1030, 1059). However, in this study, blood DCs were exposed to mesothelioma factors together with other cells in PBMC suspensions, including monocytes and lymphocytes. It is possible that the changes in blood DCs may be a result of mesothelioma factors inducing changes in other cell types within the PBMC suspension which then modulate DCs. For example, tumour factors can induce Tregs (1744) and monocytic MDSCs (1745, 1746), which suppress DC function by down-regulating their expression of co-stimulatory molecules and pro-inflammatory cytokines (1747, 1748). Age-related changes in other PBMCs are also likely to have an impact. Further studies are required to determine the mechanisms of tumour-induced changes in blood DCs, and how these are affected by aging.

Elderly MoDCs that differentiate within tumours may generate an even more suppressive milieu through increased adenosine production via CD73. Elderly DCs also expressed CD39, the other enzyme required for adenosine production, thus
they are capable of generating adenosine. However, as CD39 expression on DCs did not increase with aging, CD39 expressed on neighbouring cells such as Tregs or tumour cells (538, 542, 1097, 1749, 1750) could collaborate with elderly DCs to generate increased adenosine (538). Adenosine concentrations have been reported to be elevated in tumours from young hosts (1751, 1752), and this could be exacerbated with age due to the increased potential of elderly MoDCs to produce adenosine. Adenosine can exert multiple suppressive effects on several immune cell types within tumours, for example, decreasing the proliferative capacity, expression of co-stimulatory molecules and IFN-γ production by effector T cells (543, 551, 920, 1753). This study showed that tumour-exposed MoDCs induced increased expression of the A2A receptor on T cells, suggesting that if these T cells migrate to tumours, they would have an increased capacity to respond to adenosine, resulting in the aforementioned effects. Adenosine can also exert suppressive effects on DCs, such as inhibiting DC production of IL-12 and TNF-α, as well as reducing their capacity to stimulate T cells (570, 573). Adenosine also promotes expansion of suppressive cells, such as Tregs (559) and M2 macrophages (575). The overall outcome could be reduced effectiveness of anti-mesothelioma immunity in the elderly. Thus, elderly DCs may not only be unable to fully activate tumour-specific effector T cells, but they could also have increased potential to inhibit anti-mesothelioma immune responses. All of the changes described may impair generation of effective anti-tumour immune responses in mesothelioma patients.

The results from this pilot study suggest that, regardless of age, strategies aimed at activating DCs may be required to overcome mesothelioma-induced changes in DCs. However, LPS/IFN-γ may not be optimal for restoring tumour-induced impairments in DC numbers, as LPS/IFN-γ was unable to restore numerical proportions of young and elderly mDC1s and MoDCs following tumour exposure. Also, regardless of age, LPS/IFN-γ may not be the best strategy for restoring the function of MoDCs that differentiate within tumours, as this study showed that LPS/IFN-γ was unable to fully restore expression of co-stimulatory molecules (CD40, CD80 and CD86), and promoted increased expression of inhibitory molecules (CD39, CD73, PD-L1 and IL-10) on young and elderly tumour-exposed MoDCs. This suggests that, regardless of
age, tumour-exposed-LPS/IFN-γ-activated MoDCs may be skewed towards suppressive function, and this is supported by the observation that these MoDCs promoted development of a suppressive/exhausted phenotype in young and elderly T cells. Thus, other DC activation stimuli should be considered.

DC-activating strategies may also be required to overcome the combined age- and tumour-induced reduction in DC activation status in mesothelioma. In order for these strategies to be successful, it is important to determine whether elderly tumour-exposed DCs are capable of responding to stimulation. Stimulation with LPS/IFN-γ revealed that aged mDC1s, pDCs and MoDCs are responsive to activating stimuli. However, LPS/IFN-γ may not be optimal for rejuvenating blood DC function in elderly cancer patients, as LPS/IFN-γ did not fully restore specific aspects of tumour-induced elderly DC dysfunction, such as the CD40 differences observed in young versus elderly tumour-exposed mDC2s. Furthermore, LPS/IFN-γ seemed to skew elderly mDC2s and pDCs towards a suppressive phenotype, characterised by increased PD-L1, and elderly mDC1s were skewed towards an exhausted DC phenotype, characterised by increased IL-10 and decreased IL-12 expression; exhausted DCs may then promote Th2 responses or tolerance, instead of anti-tumour Th1 responses (517). Again, other DC activation stimuli should be considered for restoring aged DC function in mesothelioma.

Other approaches to activate DCs to overcome age- and tumour-induced dysfunction may include CD40-targeting strategies; CD40-activating therapies are currently in clinical trials to treat mesothelioma (1467), or use of TLR agonists, which have been shown to improve DC antigen-presenting and co-stimulatory function in cancer (1054, 1056, 1156, 1159). Approaches used to rejuvenate other immune cells in healthy aging may also be applicable in cancer, examples include growth factors/hormones and cytokines, such as growth hormone, keratinocyte growth factor and IL-7 to attempt to restore thymic function and T cell output (1733, 1734). Use of pharmacologic agents to target signalling pathways and/or transcription factors that may be altered with aging has been shown to rejuvenate aged haemopoietic precursor cells (1754) and T cells (1755, 1756). Similar approaches could be used to restore aged DC function. Dietary/nutritional
interventions, such as treating DCs with probiotic bacteria have been shown to improve their cytokine production and capacity to stimulate T cells (674). Modulating lipid and vitamin intakes are other possible strategies to rejuvenate elderly immune function, as fatty acids and vitamin E have been shown to improve elderly T cell function (1757-1759). As lipids and vitamins are known to modulate DCs (1760-1762), there is the potential to use them to restore DC function in the elderly.

Other strategies to restore DC function in elderly cancer patients may be blockade of checkpoint inhibitory molecules. Results from this study suggest that, in mesothelioma patients, regardless of age, circulating mDC1s may require PD-L1 blockade. However, elderly, but not young, tumour-infiltrating DCs may require blockade of CD73. Blockade of PD-L1 (1312, 1763, 1764) or CD73 (1097, 1321) has been shown to restore anti-tumour immune function in cancer patients, in vitro tumour models and animal models; PD-L1 blockade has also been shown to specifically restore DC function in cancer (1313, 1314). However, these studies were performed in young hosts and the impact of aging needs to be considered.

In summary, results from this study suggest that there are several mechanisms by which mesothelioma disables DC function, irrespective of age: (i) reduced DC numerical proportions, (ii) promoting increased suppressive function in mDC1s, and (iii) partial MoDC activation, with tumour-exposed MoDCs inducing suppressive/exhausted T cells. There were additional age-associated mechanisms by which mesothelioma modulated elderly DCs: (i) reduced activation of elderly mDC1s, mDC2s and pDCs, and (ii) increased potential for elderly MoDCs to generate immunosuppressive adenosine. In addition, LPS/IFN-γ skewed elderly tumour-exposed DCs towards a more suppressive/exhausted phenotype, which could impede the generation of anti-mesothelioma immune responses. Overall, these changes may contribute to increased cancer progression in the elderly, as well as impact on the responses of elderly patients to anti-cancer therapies that involve the immune system, such as chemotherapy and immunotherapy. Due to the limitations of this human study, studies of the effects of aging on DCs were further investigated using a murine model, which will be discussed in the following chapters.
Chapter 5  Investigating the effects of healthy aging on murine dendritic cells and T cells

5.1 Introduction

The studies in Chapter 3 showed that during healthy aging human DCs retain antigen-presenting and T cell co-stimulatory functions but adopt a more suppressive phenotype. However, these studies were limited to blood samples, confounding effects of genetic and environmental differences between volunteers and a small sample size. Thus, murine studies examining the effects of aging on DCs and T cells were conducted which also allows for examination of several tissue sites.

Murine lymphoid tissues contain three main tissue-resident cDC subsets: CD8α+CD11b- cDCs, CD11b+CD8αCD4+ cDCs and CD11b+CD8αCD4- cDCs (4, 103, 109). CD8α+CD11b- cDCs are potent cross-presenting DCs and play an important role in activating CD8+ cytotoxic T cells (121, 123, 142), and Th1 responses (132). In contrast, the two CD8α- cDC subsets exhibit poor cross-presenting ability and are mainly involved in presentation of extracellular antigens to CD4+ T cells and promotion of Th2 responses (123, 132, 142). Murine CD11c+B220-GR-1+ pDCs are also found in lymphoid tissues and mediate anti-viral immunity and play a role in immune tolerance (187, 205, 1765).

There is a lack of consensus regarding changes to total DC proportions/numbers during healthy aging, as total DCs have been reported to remain similar, increase or decrease in spleens, LNs, lungs and/or bone marrow of elderly mice, as summarised in Chapter 1, Table 1.2. Similarly, there are conflicting reports describing the effects of aging on murine DC subsets, as summarised in Chapter 1, Table 1.2. For example, in spleens and LNs, CD8α+ cDCs decrease or do not change with age; elderly CD8α- cDCs increase, decrease, or remain steady; and pDCs are similar, or reduced with age (Chapter 1, Table 1.2).

There are also conflicting reports regarding the effects of healthy aging on murine DC function, as summarised in Chapter 1, section 1.3 and Table 1.7. Expression of
co-stimulatory molecules (such as CD40, CD80 and CD86) and pro-inflammatory cytokines (such as IL-6 and TNF-α), and antigen presentation, including cross-presentation, has been reported to be retained, improved, or reduced with aging; summarised in Chapter 1, section 1.3 and Table 1.7. Reasons for these differences include varying definitions of aged mice, different mouse strains, different tissues and/or different markers to identify DC subsets, use of different stimuli to activate DCs, as well as whether in vivo or in vitro-derived DCs were examined, and for the latter, differences in the methods used to generate DCs.

Changes in regulatory markers on murine DCs during healthy aging have not been well-studied. One study showed that expression of PD-L1 and PD-L2 is increased on elderly murine DCs in spleens and lungs (245), whilst two other studies have reported no change in PD-L1 and PD-L2 in splenic, LN and lung DCs with age (72, 788). A wider spectrum of regulatory markers needs to be considered and enzymes involved in adenosine production (CD39 and CD73) and adenosine receptors (A2A and A2B receptors) have not yet been studied in the context of healthy aging.

Changes in T cells during healthy aging have also been reported. Naïve CD8^+ and CD4^+ T cells decline in numbers, which is attributed to thymic involution (231, 246, 409, 415, 416, 1766, 1767). Age-related reductions in T cell function may be due to intrinsic changes in T cells and/or defective function in APCs with age. These changes include reduced CD8^+ T cell cytotoxic effector function (1768), reduced CD4^+ T helper function (826, 1769), reduced proliferative capacity (690), and reduced cytokine production (690, 1767, 1770). The majority of studies have shown that suppressive Tregs increase in murine spleens, LNs and blood with aging (246, 590, 597-602), except for two studies reporting that Treg proportions are unaltered in elderly spleens and blood (246, 607). Elderly T cells adopt an exhausted phenotype characterised by increased expression of checkpoint inhibitory molecules, such as PD-1, ICOS, TIM-3 and CTLA-4 (245, 804, 855, 856, 899-903). However, there is little information regarding age-related changes in expression of other checkpoint inhibitory molecules on T cells, such as those involved in the adenosine pathway.
As there is currently no consensus regarding the effects of healthy aging on murine DCs, the aim of this study was to compare the functional phenotypes of DCs and T cells in young and elderly healthy C57BL/6J mice. This study used the Jackson laboratory definition of age, which defines mice aged 18-24 months as elderly and equivalent to humans aged 56-70 years, and mice aged 24-27 months as geriatric and equivalent to humans aged 70-80 years (229). These definitions reflect similarities in senescent changes in several biomarkers in both species (229) and are supported by data showing that levels of sarcopenia (age-related loss of muscle mass) in mice aged 24 months and 27-29 months corresponds to those seen in humans aged 70 years and greater than 80 years, respectively (1771). To reduce environmental influences, these studies were performed in a controlled, specific pathogen-free environment.

This study compared DCs and T cells in blood, LNs, spleens, and BM of healthy young mice, aged 2-5 months; equivalent to 16-26 human years (229) and elderly mice, aged 20-27 months; equivalent to 60-80 human years (229, 1771). Changes in the proportions and functional phenotypes of (i) CD11c+ cells and DC subsets, and (ii) CD8+ T cells, CD4+ T cells and Tregs were examined.
5.2 Results

5.2.1 Examining effects of healthy aging on murine DCs

5.2.1.1 CD11c⁺ cell proportions increase in spleens and BM with healthy aging

The first series of studies investigated the influence of healthy aging on total CD11c⁺ cell proportions in LNs, spleens, blood and BM of healthy young (2-5 months; n=13-35; Figure 5.1A) and elderly (20-27 months; n=11-38; Figure 5.1B) female mice using flow cytometry (Figures 5.1C-5.1E). CD11c⁺ cell proportions significantly increased in elderly-derived spleens (p = 0.0003; Figure 5.1F) and BM (p < 0.0001; Figure 5.1F) compared to young mice, whilst similar proportions of CD11c⁺ cells were observed in LNs and blood with age (Figure 5.1F).

Figure 5.1 CD11c⁺ cell proportions increase in spleens and BM with healthy aging
Spleens, lymph nodes (LNs), bone marrow (BM) and blood from young C57BL/6J mice (aged 2-5 months; equivalent to 16-26 human years; A) and elderly C57BL/6J mice (aged 20-27 months; equivalent to 60-80 human years; B) were stained for viable cells and total CD11c⁺ cells and analysed by flow cytometry. Within viable cells (C) and single cells (D) gates, CD11c⁺ cells were identified (E). Proportions of CD11c⁺ cells within blood, LNs, spleens and BM (F) are shown as mean ± SEM, n = 13-35 young and n = 11-38 elderly mice, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.
Changes in cell proportions may be due to alterations in organ cellularity with age. Therefore, absolute numbers of CD11c+ cells were also calculated per spleen and per ml of blood. Absolute numbers for LNs and BM were not calculated due to difficulties in weighing these tissues. A significant increase in the total number of CD11c+ cells per spleen \( (p = 0.035; \text{Appendix D: Figure 1A}) \) and absolute numbers of CD11c+ cells per ml of blood \( (p = 0.02; \text{Appendix D: Figure 1B}) \) was seen with age.

### 5.2.1.2 CD11b+CD8α+CD4+ cDCs decrease whilst pDCs increase in LNs with aging

Changes to CD8α+CD11b− cDCs, CD11b+CD8α+CD4+ cDCs, CD11b+CD8α+CD4− cDCs and B220+GR-1+ pDCs in LNs, spleens, blood and BM during healthy aging were examined in young \( (n=13-32) \) and elderly \( (n=11-35) \) mice using flow cytometry (gating strategy in Figures 5.2A-5.2D). Some early experiments did not include CD11b to identify cDC subsets (gating strategy in Appendix D: Figures 2A-2E), however, as similar percentages of CD8α+ cDCs, CD8α+CD4+ cDCs and CD8α+CD4− cDCs were obtained with the two gating strategies (Figures 5.2A-5.2D and Appendix D: Figures 2D and 2E), the data were grouped together.

Proportions of pDCs significantly increased \( (p = 0.02; \text{Figure 5.2E}) \), whilst CD11b+CD8α+CD4+ cDC proportions decreased \( (p = 0.054; \text{Figure 5.2E}) \) in LNs of healthy elderly mice, relative to young mice. CD8α+CD11b− cDC and CD11b+CD8α+CD4− cDC proportions remained similar with age in LNs (Figure 5.2E). CD11b+CD8α+CD4+ cDCs and CD11b+CD8α+CD4− cDCs were the dominant subsets in young LNs, whilst CD11b+CD8α+CD4− cDCs, followed by pDCs, were the main subsets in elderly LNs (Figure 5.2E).

Splenic CD8α+CD11b− cDC \( (p = 0.001; \text{Figure 5.2F}) \) and CD11b+CD8α+CD4+ cDC \( (p = 0.005; \text{Figure 5.2F}) \) proportions significantly decreased with age. In contrast, CD11b+CD8α+CD4− cDCs significantly increased in elderly, compared to young spleens \( (p = 0.03; \text{Figure 5.2F}) \). No age-related differences were observed in splenic pDC proportions (Figure 5.2F). In both age groups, CD11b+CD8α+CD4− cDCs were the predominant DC subset found in spleens (Figure 5.2F). Absolute numbers of splenic CD11b+CD8α+CD4− cDCs also significantly increased with age \( (p = 0.03; \text{Appendix D:} \).
Figure 3A). No other age-related differences were seen in absolute numbers of splenic DC subsets (Appendix D: Figure 3A).

Figure 5.2 CD11b⁺CD8α⁺CD4⁺ cDCs decrease in LNs and spleens, whilst pDCs increase in LNs with aging

Spleens and lymph nodes (LNs) from young and elderly mice were stained for markers of DC subsets (CD11c, B220, GR-1, CD11b, CD8α and CD4) and analysed by flow cytometry. Within the CD11c⁺ cells gate (A), plasmacytoid DCs (pDCs) were gated as B220⁺GR-1⁻ cells (B). Within the pDC exclusion gate (i.e. cells that are not B220 and GR-1 double positive; B), CD8α⁺CD11b⁻ conventional DCs (cDCs) and CD11b⁺CD8α⁻ cDCs were gated (C), and CD11b⁺CD8α⁺ cDCs were further distinguished into CD4⁺ and CD4⁻ subsets (D). Proportions of DC subsets in LNs (E) and spleens (F) are shown as mean ± SEM, n = 13-32 young and n = 11-35 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing young to elderly mice.
CD11b⁺CD8α⁺CD4⁺ cDCs (p = 0.07; Figure 5.3A) and pDC proportions (p = 0.008; Figure 5.3A) decreased in BM of elderly mice, compared to young mice. In contrast, CD11b⁺CD8α⁻CD4⁺ cDCs significantly increased with age (p < 0.0001; Figure 5.3A), and these DCs were the dominating DC subset in young and elderly BM. BM CD8α⁻CD11b⁻ cDCs remained constant with age (Figure 5.3A).

Similar to spleen and BM, CD11b⁺CD8α⁻CD4⁺ cDCs were the main DC subset present in blood, irrespective of age (Figure 5.3B). No age-related differences in blood DC subset proportions (Figure 5.3B) or absolute numbers (Appendix D: Figure 3B) were observed.

![Figure 5.3](image.png)

**Figure 5.3 CD11b⁺CD8α⁻CD4⁺ cDCs and pDCs decrease in BM with aging**
Bone marrow (BM) and blood from young and elderly mice were stained for B220⁺GR-1⁺ pDCs, CD8α⁺CD11b⁻ cDCs, CD11b⁺CD8α⁺CD4⁻ cDCs, and CD11b⁺CD8α⁻CD4⁺ cDCs, and analysed by flow cytometry. Proportions of DC subsets in BM (A) and blood (B) are shown as mean ± SEM, n = 13-16 young and n = 11-19 elderly mice, ** = p<0.005, **** = p<0.0001 comparing young to elderly mice.
5.2.1.3 Elderly LN CD11c+ cells have increased MHC class I, CD80, CD73 and PD-L1

To investigate whether antigen-presenting cell function is altered during aging, markers of antigen presentation (MHC class I and II), activation (CD40, CD80, CD86, intracellular IFN-γ, TNF-α and IL-12) and regulation (CD39, CD73, A2A receptor, A2B receptor, PD-L1, galectin-9 (GAL-9), intracellular IL-10 and TGF-β latency-associated peptide) were compared on CD11c+ cells from young (n=3-20) and elderly (n=3-24) healthy mice using flow cytometry. Marker expression was measured using percentage of cells positive for a marker and expression levels.

Elderly LN CD11c+ cells appeared to retain, or even improve, expression of antigen-presenting/activation markers; i.e. significantly increased percentages of MHC class I+ cells (p = 0.006; Figure 5.4A) that were associated with increased MHC class I expression levels (p = 0.04; Figure 5.4B), and increased percentages and expression levels of CD80+ cells (p = 0.05; Figure 5.4C, p = 0.04; Figure 5.4D). No age-related differences in expression of other antigen-presenting and activation markers, or pro-inflammatory cytokines on LN CD11c+ cells were seen (Figure 5.4E).

Examination of regulatory markers revealed that elderly LN CD11c+ cells had significantly increased percentages of PD-L1+ (p = 0.004; Figure 5.5A) and CD73+ cells (p = 0.03; Figure 5.5B), which were associated with increased CD73 expression levels (p = 0.03; Figure 5.5C). Percentages of CD11c+ cells positive for the other regulatory markers were similar in young and elderly LNs (Figure 5.5D).

5.2.1.4 PD-L1 increases with aging on LN DC subsets

To gain further insight into changes occurring in DC subsets, expression of the antigen-presenting/activation markers MHC class I, CD40, CD80 and CD86, and the regulatory marker PD-L1 were examined on LN DC subsets.

Elevated expression of PD-L1 on total CD11c+ cells in elderly LNs could be attributed to increases in PD-L1 on most DC subsets: i.e. CD8α+CD11b- cDCs (p = 0.002; Figure 5.6A, p = 0.02; Figure 5.6B), CD11b+CD8α+CD4+ cDCs (p = 0.0004; Figure 5.6C, p <
Figure 5.4 Elderly LN CD11c+ cells have increased MHC class I and CD80

Lymph nodes (LNs) from young and elderly mice were stained for CD11c+ cells and activation markers (MHC class I, MHC class II, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α and IL-12), and analysed via flow cytometry. Percentages of CD11c+ cells positive for MHC class I (A), CD80 (C), and other activation markers (E), and expression levels (measured as geometric mean fluorescence intensity; MFI) of MHC class I (B) and CD80 (D) were measured. Data are shown as mean ± SEM, n = 10-20 young and n = 11-24 elderly mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Figure 5.5 Elderly LN CD11c+ cells have increased CD73 and PD-L1
Lymph nodes (LNs) from young and elderly mice were stained for CD11c+ cells and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1, galectin-9 (GAL-9), and intracellular IL-10 and TGF-β), and analysed via flow cytometry. Percentages of CD11c+ cells positive for PD-L1 (A), and CD73 (B), expression levels of CD73 (measured as geometric mean fluorescence intensity; MFI; C), and percentages of CD11c+ cells positive for other regulatory markers (D) are shown as mean ± SEM, n = 3-20 young and n = 3-24 elderly mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Figure 5.6 PD-L1 increases with aging on LN DC subsets
Lymph nodes (LNs) were stained for B220^GR-1^ pDCs, CD8α^CD11b^- cDCs, CD11b^CD8α^ CD4^- cDCs and PD-L1 and analysed by flow cytometry. PD-L1 expression levels (shown as geometric mean fluorescence intensity; MFI) on CD8α^CD11b^- cDCs (A), CD11b^CD8α^CD4^- cDCs (C), and pDCs (E), and percentages of PD-L1^+^ CD8α^CD11b^- cDCs (B) and CD11b^CD8α^- cDCs (D) were measured. Data are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.

Examination of activation markers showed that elderly CD8α^CD11b^- cDCs, and CD11b^CD8α^- cDCs had significantly reduced percentages and expression levels of CD40 (p = 0.0048; Figure 5.7A, and p = 0.03; Figure 5.7B; p = 0.0005; Figure 5.7C, and p = 0.0006; Figure 5.7D, respectively). No age-related differences in the other markers were seen for elderly LN pDCs (Appendix D: Figure 4A), CD8α^CD11b^- cDCs (Appendix D: Figure 4B) and CD11b^CD8α^- cDCs (Appendix D: Figure 4C).
Lymph nodes (LNs) were stained for CD8α+CD11b− cDCs, CD11b+CD8α+CD4− cDCs, and CD40 and analysed by flow cytometry. Percentages of CD40+ CD8α+CD11b− cDCs (A) and CD11b+CD8α+CD4− cDCs (B), and CD40 expression levels (measured as geometric mean fluorescence intensity; MFI) on CD8α+CD11b− cDCs (C) and CD11b+CD8α+CD4− cDCs (D) are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing young to elderly mice.

In contrast, elderly CD11b+CD8α+CD4− cDCs had elevated expression of all activation markers examined, as percentages of cells positive for MHC class I (p < 0.0001), CD40 (p = 0.004), CD80 (p = 0.01) and CD86 (p = 0.001) increased (Figure 5.8A), as did their expression levels (p < 0.0001 for MHC class I, p = 0.003 for CD40, p = 0.03 for CD80 and p = 0.02 for CD86; Figure 5.8B), relative to DCs from young mice.

5.2.1.5 Splenic CD11c+ cells increase expression of CD73, A2B receptor and PD-L1 with age

Elderly splenic CD11c+ cells followed a similar pattern to those seen in elderly LNs, as they retained expression of all activation markers examined (Appendix D: Figure 5A), yet up-regulated several inhibitory markers. Specifically, expression of CD73 (p
= 0.002; Figure 5.9A, p = 0.03; Figure 5.9B), A2B receptor (p < 0.0001; Figure 5.9C, p = 0.0002; Figure 5.9D) and PD-L1 (p = 0.01; Figure 5.9E, p = 0.058; Figure 5.9F) significantly increased with age. The only other age-related difference was a reduction in the percentage of A2A receptor+ CD11c+ cells in elderly, compared to young, spleens (p = 0.02; Appendix D: Figure 5B).

**Figure 5.8 Elderly CD11b+CD8α+CD4+ cDCs have increased MHC-I, CD40, CD80 and CD86**

Lymph nodes (LNs) were stained for CD11b+CD8α+CD4+ cDCs, and activation markers (MHC class I, CD40, CD80 and CD86) and analysed by flow cytometry. Percentages of CD11b+CD8α+CD4+ cDCs positive for activation markers (A) and expression levels (measured as geometric mean fluorescence intensity; MFI; B) were measured. Data are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing young to elderly mice.
Figure 5.9 Splenic CD11c+ cells have increased CD73, A2B receptor and PD-L1 with age
Young and elderly spleens were stained for CD11c+ cells, CD73, A2B receptor (A2BR) and PD-L1, and analysed by flow cytometry. Percentages of CD11c+ cells positive for CD73 (A), A2BR (C) and PD-L1 (E), and geometric mean fluorescence intensity (MFI) expression levels of CD73 (B), A2BR (D) and PD-L1 (F) were measured. Data are shown as mean ± SEM, n = 3-15 young and n = 3-15 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.

Elderly blood CD11c+ cells were also skewed towards a more regulatory phenotype, due to increased percentages of circulating CD73+ CD11c+ cells in elderly mice, relative to young mice (p = 0.0095; Appendix D: Figure 6A), whilst expression of other activation and regulatory markers remained similar with age on blood CD11c+ cells (Appendix D: Figures 6B and 6C).
5.2.1.6 Elderly splenic pDCs and CD8α⁺CD11b⁻ cDCs express lower MHC class I, CD40, CD80 and CD86

Age-related differences were also examined on splenic DC subsets. Although expression of activation markers appeared to be similar with age on total splenic CD11c⁺ cells, this was not reflected by all DC subsets. Elderly splenic pDCs had significantly decreased expression levels of MHC class I (p = 0.008; Figure 5.10A), compared to young pDCs. This was accompanied by reduced percentages and expression levels of CD80 (p < 0.0001; Figure 5.10B, p < 0.0001; Figure 5.10C), and CD86 (p = 0.046; Figure 5.10D, p < 0.0001; Figure 5.10E) on pDCs with age.

![Figure 5.10](image)

Figure 5.10 Elderly splenic pDCs have lower MHC class I, CD80 and CD86
Young and elderly spleens were stained for B220⁺GR-1⁺ pDCs, MHC class I, CD80 and CD86 for analysis by flow cytometry. Expression levels (measured via geometric mean fluorescence intensity; MFI) of MHC class I (A), CD80 (C), and CD86 (E), and percentages of CD80⁺ cells (B) and CD86⁺ cells (D) were measured. Data are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing young to elderly mice.
Elderly CD8α⁺CD11b⁻ cDCs also displayed a reduced activation status, characterised by significantly decreased expression of MHC class I (p = 0.001; Figure 5.11A), CD40 (p = 0.0001; Figure 5.11B, p = 0.0004; Figure 5.11C) and CD80 (p < 0.0001; Figure 5.11D, p = 0.0007; Figure 5.11E). No age-related differences were seen for the other activation markers on pDCs (Appendix D: Figure 7A) and CD8α⁺CD11b⁻ cDCs (Appendix D: Figure 7B).

Young and elderly spleens were stained for CD8α⁺CD11b⁻ cDCs, MHC class I, CD40 and CD80 for analysis by flow cytometry. Expression levels (measured via geometric mean fluorescence intensity; MFI) of MHC class I (A), CD40 (B), and CD80 (D), and percentages of CD40⁺ cells (C) and CD80⁺ cells (E) were measured. Data are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.

In contrast, splenic CD11b⁺CD8α⁺CD4⁺ cDCs had significantly increased expression levels of all activation markers examined; p = 0.03 for MHC class I, p = 0.0002 for CD40, p = 0.002 for CD80, and p = 0.0007 for CD86 (Figure 5.12A). In addition, percentages of CD40⁺ (p = 0.04; Figure 5.12B), CD80⁺ (p = 0.01; Figure 5.12B) and
CD86⁺ (p = 0.001; Figure 5.12B) CD11b⁺CD8α⁺CD4⁺ cDCs increased in elderly spleens. Similar changes were seen in CD11b⁺CD8α⁺CD4⁺ cDCs, with increased expression of CD40 (p = 0.0001; Appendix D: Figure 8A, p = 0.008; Appendix D: Figure 8B), CD80 (p = 0.04; Appendix D: Figure 8A, p = 0.0007; Appendix D: Figure 8B), and CD86 (p = 0.002; Appendix D: Figure 8A, p < 0.0001; Appendix D: Figure 8B) with age.

Figure 5.12 Elderly splenic CD11b⁺CD8α⁺CD4⁺ cDCs have increased activation markers and PD-L1

Young and elderly spleens were stained for CD11b⁺CD8α⁺CD4⁺ cDCs, activation markers (MHC class I, CD40, CD80 and CD86), and the regulatory marker PD-L1 for flow cytometric analysis. Geometric mean fluorescence intensity (MFI) expression levels of activation markers (A) and PD-L1 (D), and percentages of cells positive for activation markers (B) and PD-L1 (C) were analysed. Data are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing young to elderly mice.
The increase in PD-L1 levels on elderly splenic CD11c+ cells could be attributed to increased percentages of PD-L1+ CD11b+CD8α+CD4+ cDCs (p = 0.04; Figure 5.12C), and PD-L1+ CD11b+CD8α+CD4+ cDCs (p = 0.002; Appendix D: Figure 8C), associated with elevated PD-L1 expression (p = 0.01; Figure 5.12D, p = 0.002; Appendix D: Figure 8D, respectively). In contrast, pDCs had reduced percentages of PD-L1+ cells (p < 0.0001; Appendix D: Figure 7A), whilst PD-L1 did not change on CD8α+CD11b+ cDCs in elderly relative to young spleens (Appendix D: Figure 7B).

5.2.1.7 Elderly BM precursors can differentiate into CD11c+ cells

Preliminary studies were conducted to determine whether there were age-related differences in the ability of BM precursors to differentiate into functional CD11c+ cells. BM cells from young and elderly mice (n=5 BM samples per age group, each pooled from 3-5 mice) were differentiated in vitro using GM-CSF and IL-4 for 8 days, to produce CD11c+ cells, using a protocol adapted from Lutz et al. (223). Expression of MHC class I and II, CD40, CD80, CD86, and PD-L1 was examined on BM-derived CD11c+ cells using flow cytometry; no age-related or statistically significant differences were seen (data not shown).

To assess the ability of BM-derived CD11c+ cells to stimulate T cell proliferation, young and elderly BM-derived CD11c+ cells were co-cultured with CFSE-labelled, allogeneic splenocyte-derived T cells from young BALB/c mice (2-3 months of age), at CD11c+ cell: T cell ratios of 1:5, 1:20, 1:50, 1:100 and 1:200, for 5 days. Co-cultures were analysed for percentages of CD8+ and CD4+ T cells that had undergone proliferation, using flow cytometry. No age-related or statistically significant differences in the ability of young and elderly BM-derived CD11c+ cells to stimulate T cell proliferation were seen (data not shown). The low sample numbers, staining issues, and variable data rendered the results inconclusive.

In summary, these data show increased CD11c+ cells in spleens, blood and BM with age. Changes in DC subset proportions were tissue-specific: (i) in elderly LNs, increased pDCs suggest improved anti-viral immune responses and/or increased potential for immune tolerance, whilst reduced CD11b+CD8α+CD4+ cDCs suggests compromised activation of Th1 responses; and (ii) antigen cross-presentation and
CD8⁺ T cell activation may be reduced in elderly spleens, due to reduced proportions of CD8α⁺CD11b⁻ cDCs.

The data also suggest that elderly LN, spleen and blood CD11c⁺ cells may retain or improve their antigen-presenting and co-stimulatory capacities, due to similar or higher expression of MHC class I, CD40, CD80 and CD86 than their younger counterparts. Nonetheless, elderly CD11c⁺ cells could have increased regulatory activity due to up-regulation of inhibitory markers, such as CD73, the A2B receptor and PD-L1. Examination of DC subsets showed that: (i) LN pDCs, CD8α⁺CD11b⁻ cDCs and CD11b⁺CD8α⁺CD4⁺ cDCs may become more suppressive with aging via up-regulated PD-L1; (ii) elderly LN CD8α⁺CD11b⁻ and CD11b⁺CD8α⁺CD4⁺ cDCs’ co-stimulatory/licensing capacity could be compromised due to reduced CD40; (iii) elderly splenic pDCs and CD8α⁺CD11b⁻ cDCs may have a reduced ability to present antigens and stimulate effector T cells, due to reduced expression of MHC class I, CD40, CD80 and/or CD86; and (iv) whilst elderly splenic CD11b⁺CD8α⁺CD4⁺ cDCs and CD11b⁺CD8α⁺CD4⁺ cDCs may have improved antigen-presenting and co-stimulatory functions, they simultaneously could have increased regulatory potential via PD-L1.

### 5.2.2 Examining effects of healthy aging on murine T cells

#### 5.2.2.1 T cell proportions are reduced in LNs and spleens with healthy aging

This study also investigated changes in T cells during healthy aging, using young (2-5 months; n=10-22) and elderly (20-27 months; n=11-21) female C57BL/6J mice. Spleens and LNs were stained for CD3⁺CD8⁺ T cells, CD3⁺CD4⁺ T cells and CD3⁺CD4⁺CD25⁺FoxP3⁺ Tregs and analysed via flow cytometry (Figures 5.13A-5.13E).

CD8⁺ T cells, CD4⁺ T cells and Treg proportions were decreased in LNs (p < 0.0001 for CD8⁺ T cells; p < 0.0001 for CD4⁺ T cells; p = 0.001 for Tregs Figure 5.13F) and spleens (p = 0.03 for CD8⁺ T cells; p = 0.0005 for CD4⁺ T cells; p = 0.0501 for Tregs Figure 5.13G) in elderly, relative to young, mice.
Figure 5.13 Proportions of T cells are reduced in elderly LNs and spleens
Spleens and lymph nodes (LNs) from young and elderly mice were stained for CD3⁺CD8⁺ T cells, CD3⁺CD4⁺ T cells and CD3⁺CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) and analysed using flow cytometry. Viable cells (A), single cells (B), then CD3⁺ T cells (C) were gated. Within the CD3⁺ T cells gate, CD8⁺ and CD4⁺ T cells were identified (D). Within the CD4⁺ T cells gate, CD4⁺CD25⁺FoxP3⁺ Tregs were identified (E). Proportions of CD3⁺CD8⁺ T cells, CD3⁺CD4⁺ T cells and CD3⁺CD4⁺CD25⁺FoxP3⁺ Tregs within LNs (F) and spleens (G) were measured. Data are shown as mean ± SEM, n = 10-22 young and n = 11-21 elderly mice, * = p<0.05, ** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.

5.2.2.2 Elderly LN T cells have increased inhibitory CD73, A2B receptor, CTLA-4, PD-1, ICOS, LAG-3 and IL-10

To investigate whether T cell function is altered during healthy aging, CD8⁺ and CD4⁺ T cells in LNs and spleens of young (n=5-11) and elderly (n=5-12) mice were stained for markers of T cell activation/effector function (CD25, intracellular IFN-γ and perforin), checkpoint inhibitory molecules (CTLA-4, PD-1, ICOS and LAG-3), intracellular anti-inflammatory cytokines (IL-10 and TGF-β latency-associated
peptide), and regulatory markers in the adenosine pathway (CD39, CD73, the A2A receptor and the A2B receptor), and analysed via flow cytometry.

Elderly CD8+ T cells in LNs demonstrated increased expression of several regulatory markers, including CD73 (p = 0.0009; Figure 5.14A, p = 0.0055; Figure 5.14B), the A2B receptor (p = 0.004; Figure 5.14A, p = 0.0002; Figure 5.14B), PD-1 (p = 0.009; Figure 5.14A, p = 0.004; Figure 5.14B), ICOS (p = 0.008; Figure 5.14A, p = 0.007; Figure 5.14B) and LAG-3 (p = 0.006; Figure 5.14A), whilst percentages of IL-10+ cells showed an increasing trend (p = 0.06; Figure 5.14A), relative to young CD8+ T cells. No age-related differences in the other regulatory markers (Appendix D: Figure 9A) or the activation/effector markers (Appendix D: Figure 9B) were observed.

Figure 5.14 Elderly LN CD8+ T cells have increased CD73, A2B receptor, PD-1, ICOS, LAG-3 and IL-10
Young and elderly lymph nodes (LNs) were stained for CD8+ T cells and the regulatory markers CD73, A2B receptor (A2BR), PD-1, ICOS, LAG-3, and intracellular IL-10 for flow cytometric analysis. Percentages of CD8+ T cells positive for regulatory markers (A), and expression levels of regulatory markers (shown as geometric mean fluorescence intensity; MFI; B) were analysed. Data are shown as mean ± SEM, n = 6-9 young and n = 6-9 elderly mice, ** = p<0.005, *** = p<0.0005 comparing young to elderly mice.
Elderly LN CD4⁺ T cells up-regulated CD25 (p = 0.01; Figure 5.15A, p = 0.001; Figure 5.15B) relative to young CD4⁺ T cells. Expression of most regulatory markers examined also increased with age on CD4⁺ T cells in LNs including CD73 (p < 0.0001; Figure 5.15C, p < 0.0001; Figure 5.15D), A2B receptor (p = 0.003; Figure 5.15C, p = 0.01; Figure 5.15D), CTLA-4 (p = 0.03; Figure 5.15C, p = 0.053; Figure 5.15D), PD-1 (p = 0.007; Figure 5.15C, p = 0.002; Figure 5.15D), ICOS (p < 0.0001; Figure 5.15C, p < 0.0001; Figure 5.15D), LAG-3 (p = 0.001; Figure 5.15C, p = 0.02; Figure 5.15D), and IL-10 (p = 0.009; Figure 5.15C, p = 0.006, Figure 5.15D). No age-related changes were seen for IFN-γ and perforin (Appendix D: Figure 10A) or CD39, A2A receptor and TGF-β (Appendix D: Figure 10B) on LN CD4⁺ T cells.

### 5.2.2.3 Elderly splenic T cells may be more suppressive due to increased CD39, CD73, CTLA-4, PD-1, ICOS, LAG-3, IL-10 and TGF-β

Splenic CD8⁺ and CD4⁺ T cells were also examined. Elderly splenic CD8⁺ T cells up-regulated CD25 (p = 0.002; Figure 5.16A, p = 0.0008; Figure 5.16B), as well as CD73 (p = 0.0005; Figure 5.16C, p = 0.001; Figure 5.16D), CTLA-4 (p = 0.02; Figure 5.16C), and TGF-β (p = 0.005; Figure 5.16C, p = 0.046; Figure 5.16D). This was in conjunction with higher expression levels of CD39 (p = 0.02; Figure 5.16D) and a trend for increased IL-10 (p = 0.052; Figure 5.16C, p = 0.06; Figure 5.16D). No age-related differences were seen in the other activation (Appendix D: Figure 11A) and regulatory markers (Appendix D: Figure 11B) examined on splenic CD8⁺ T cells.

Similarly, most regulatory markers were significantly increased with age on splenic CD4⁺ T cells including CD73 (p = 0.0001; Figure 5.17A, p < 0.0001; Figure 5.17B), CTLA-4 (p = 0.003; Figure 5.17A, p= 0.002; Figure 5.17B), PD-1 (p = 0.02; Figure 5.17A), ICOS (p < 0.0001; Figure 5.17A, p < 0.0001; Figure 5.17B), LAG-3 (p = 0.001; Figure 5.17A, p = 0.007; Figure 5.17B), and IL-10 (p = 0.006; Figure 5.17A). This was associated with a trend for increased TGF-β by elderly CD4⁺ T cells (p = 0.07; Figure 5.17B). No other age-related changes were seen (Appendix D: Figures 12A and 12B) on splenic CD4⁺ T cells.

In summary, during healthy aging T cell function in LNs and spleens may be compromised due to reduced proportions of CD8⁺ T cells and CD4⁺ T cells, and
skewing towards a predominantly regulatory or exhausted T cell phenotype, due to increased expression of CD39, CD73, A2B receptor, CTLA-4, PD-1, ICOS, LAG-3, IL-10 and TGF-β.

Figure 5.15 Elderly LN CD4+ T cells have increased regulatory markers
Young and elderly lymph nodes (LNs) were stained for CD4+ T cells, CD25, and the regulatory markers CD73, A2B receptor (A2BR), CTLA-4, PD-1, ICOS, LAG-3 and intracellular IL-10. Percentages of CD4+ T cells positive for CD25 (A) and regulatory markers (C), and expression levels (measured as geometric mean fluorescence intensity; MFI) of CD25 (B) and regulatory markers (D) were analysed. Data are shown as mean ± SEM, n = 6-19 young and n = 6-20 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.
Figure 5.16 Regulatory markers are increased on CD8+ T cells in elderly spleens

Young and elderly spleens were stained for CD8+ T cells, CD25, and regulatory markers (CD39, CD73, CTLA-4, and intracellular IL-10 and TGF-β), and analysed via flow cytometry. Percentages of CD8+ T cells positive for CD25 (A) and regulatory markers (C), and expression levels (measured as geometric mean fluorescence intensity; MFI) of CD25 (B) and regulatory markers (D) were measured. Data are shown as mean ± SEM, n = 5-8 young and n = 6-9 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing young to elderly mice.
Figure 5.17 Regulatory markers are increased on CD4+ T cells in elderly spleens
Young and elderly spleens were stained for CD4+ T cells, and the regulatory markers CD73, CTLA-4, PD-1, ICOS, LAG-3, and intracellular IL-10 and TGF-β, and analysed via flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI; A) and percentages of CD4+ T cells positive for regulatory markers (B) were measured. Data are shown as mean ± SEM, n = 5-13 young and n = 6-14 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.
5.3 Discussion

The effects of healthy aging on murine DCs are not yet clear, as there are several conflicting studies. Therefore, the studies in this chapter examined changes in murine DCs and T cells in lymphoid tissues during healthy aging, and the results suggest that CD11c+ cells and T cells adopt a regulatory phenotype with age.

The outcome of a T cell response depends on the summation of positive and negative signals it receives (708). LN pDCs and CD11b+CD8α+CD4+ cDCs, and splenic CD11b+CD8α+ cDCs demonstrated simultaneous increases in antigen-presenting, co-stimulatory and regulatory molecules with age. Therefore, whilst antigen presentation to T cells may be maintained with aging, it is accompanied by an increased breadth of negative signals. The strength of a signal delivered by a molecule is determined by interaction with its ligand, and although CD80 expression increased on elderly DCs, others have reported that expression of its ligand, CD28, is reduced on elderly T cells (440, 442, 446, 849, 850, 853), suggesting reduced T cell activation in the elderly. In this scenario, CD80 may instead play a role in inhibiting T cell responses during aging. This study, and others (855, 856), showed increased CTLA-4 expression on elderly T cells; ligation of CD80 and CTLA-4 negatively regulates T cell activation (1624). Furthermore, CD80 on DCs can also bind PD-L1 on T cells, leading to T cell inhibition (896, 897), and others have shown that PD-L1 expression is increased on aged CD8+ T cells (788). Thus, elevated CD80 on elderly DC subsets may lead to T cell suppression, rather than activation. The PD-L1/PD-1 pathway represents another mechanism by which T cell responses may be suppressed during healthy aging. In agreement with others, this study observed increased PD-L1 expression on elderly DC subsets (245), and increased PD-1 expression on elderly T cells (245, 804, 856, 899-903), suggesting that the likelihood of inhibitory PD-L1/PD-1 interactions is increased with aging, the outcomes being inhibition of T cell proliferation, reduced effector T cell differentiation, reduced cytokine production and cytotoxic function, as well as increased T cell apoptosis (882, 1677, 1772, 1773). In addition, the age-related increase in LAG-3 expression on T cells, also observed in two other studies (439, 804), may lead to increased
negative signalling through the MHC class II/TCR complex (792-794), and could contribute to the impaired ability of elderly DCs to prime CD4+ T cells and Th1 responses, described by others (71, 235, 244). Thus, with aging, negative signals may outweigh positive signals during DC/T cell interactions leading to suppression, rather than activation of effector T cells. Further studies are required to assess age-related changes in other regulatory markers and cytokines expressed by DC subsets, and their potential influences on elderly T cell responses.

Due to limitations in flow cytometer capability, this study could not examine a broad range of activation and regulatory markers on DC subsets, and instead analysed CD11c+ cells. CD11c is expressed predominantly on antigen-presenting cells including murine DCs (4), monocytes/macrophages (79, 80), B cells (82, 83) and NK cells (87) in LNs and spleens. Therefore, changes in CD11c+ cells could be considered representative of changes to antigen-presenting cells during aging. In elderly LNs, changes in CD11c+ cells reflected pDCs and CD11b+CD8α-CD4+ cDCs, whilst changes in elderly splenic CD11c+ cells reflected CD11b+CD8α- cDCs, with these cells simultaneously up-regulating activation and regulatory markers. Changes in the functional phenotype of CD11c+ cells further reinforce the idea that antigen-presenting cells provide more negative signals to T cells, thus skewing T cell responses towards suppression during aging. Elevated CD80 and PD-L1 expression on elderly CD11c+ cells may lead to T cell inhibition as discussed above. Increased CD73 on elderly CD11c+ cells represents another mechanism of T cell suppression as it indicates increased capacity to participate in production of immunosuppressive adenosine, particularly if neighbouring cells express CD39 (1566), such as the concomitant increase in CD39 expression on elderly splenic T cells. Elderly T cells also had increased CD73, suggesting they contribute to the generation of adenosine. Moreover, elderly T cells in LNs and spleens have an increased capacity to respond to adenosine due to elevated expression of the adenosine-binding A2B receptor. Adenosine-mediated signalling deactivates T cells by impairing IL-2 production (563, 565) and induces Tregs (557, 562). Elderly CD11c+ cells/APCs also expressed increased A2B receptor suggesting they are more responsive to adenosine; the consequences include induction of tolerogenic/inhibitory DCs (569-
571) and polarisation of pro-inflammatory M1 macrophages into anti-inflammatory/suppressive M2 macrophages (575-580). This is supported by previous studies in our laboratory showing that spleens and BM of elderly mice contain increased suppressive IL-10-producing M2 macrophages (1419, 1420), and other studies showing increased suppressive macrophage subsets in elderly mice (1774, 1775). Thus, elderly CD11c+ cells have a greater capacity to transmit inhibitory signals to T cells, resulting in suppression of effector T cells, leading to diminished immune responses in the elderly.

The concept that elderly DCs promote T cell suppression is supported by the observation that elderly T cells developed a suppressed/exhausted phenotype. In agreement with others, elderly CD8+ and CD4+ T cells had increased PD-1, CTLA-4, ICOS and LAG-3 expression (245, 804, 855, 856, 899-903). The simultaneous up-regulation of several checkpoint inhibitory markers (particularly PD-1, CTLA-4, LAG-3, TIM-3 and CD39) is a hallmark of an exhausted T cell phenotype (1101, 1105, 1740, 1741). T cell exhaustion has been described in chronic viral infections (799, 1104, 1740, 1741, 1776) and cancer (1108, 1119, 1316) and is a state in which T cells are unable to proliferate, have reduced production of effector cytokines such as IL-2 and IFN-γ, have diminished cytotoxic activity and effector function, and cannot generate memory responses (1102, 1104, 1105, 1777). The development of an exhausted phenotype in aged T cells is supported by studies showing that PD-1-expressing elderly T cells are dysfunctional, as they have reduced proliferative capacity (245, 899, 902, 903). The consequences of T cell exhaustion in the elderly could be compromised immune responses against pathogens and tumours.

Comparisons between the effects of healthy aging on murine DC subsets in this chapter, and equivalent human DC subsets in Chapter 3 revealed similarities. Murine CD8α+CD11b+ cDCs, which are functionally equivalent to human CD141+ mDC2s, on account of their high efficiency at cross-presenting antigens (155, 266, 267), demonstrated similar age-related changes; i.e. reduced expression of antigen-presenting/activation markers in association with increased regulatory markers. This suggests increased potential for suppression of effector CD8+ T cells with age. Both human blood pDCs and murine splenic pDCs demonstrated reduced PD-L1
expression, suggesting a reduced capacity to maintain tolerance. Whilst human CD1c+ mDC1s maintained expression of most activation and regulatory molecules with age, their murine equivalent, CD11b+CD8α+CD4+ cDCs, up-regulated expression of antigen-presenting, co-stimulatory and regulatory molecules. Reasons for these differences could include species differences and examination of different tissue sites (murine spleens and LNs versus human blood).

Results from the studies in this chapter suggest that aging induces compartmentalised changes in murine DCs, as age-related changes to DC phenotype can vary depending on the subset examined and tissue site. The studies in this chapter have also contributed knowledge regarding age-related changes in regulatory marker expression on DCs/CD11c+ cells. However, a consensus on age-related changes to DC phenotype and function is yet to be reached in the literature.

In summary, this chapter shows that an immunoregulatory microenvironment develops in LNs and spleens with aging, characterised by up-regulation of several inhibitory molecules on DC subsets, CD11c+ cells and T cells. This could result in increased inhibitory interactions between DCs/APCs and T cells and contribute to the development of exhausted elderly T cells, resulting in significant T cell dysfunction leading to diminished responses against pathogens and tumours in the elderly. The next chapter examines the combined effects of aging and cancer on murine DCs and T cells.
Chapter 6  
Examining the effects of aging and cancer on murine dendritic cells and T cells

6.1  
Introduction

The studies in the previous chapter showed that during healthy aging, murine DCs and T cells are skewed towards a more suppressive and/or exhausted phenotype, which may impair the generation of effective anti-tumour immune responses in elderly hosts. DCs are important mediators of anti-tumour immunity, on account of their ability to activate tumour-specific cytotoxic CD8⁺ T cells, which can directly kill tumour cells and mediate tumour regression (371, 953). However, tumours can thwart anti-tumour immunity by secreting suppressive factors, such as VEGF, TGF-β and IL-10, which impair DC differentiation and maturation as well as their ability to activate T cells, and promote tolerogenic/regulatory DCs, as summarised in Chapter 1, section 1.4 and Table 1.8. The same tumour-derived factors also impair effector T cell cytotoxic function and pro-inflammatory cytokine secretion, induce T cell exhaustion and promote Treg development (371, 1135). However, the majority of studies to-date have been performed in young mice, and few studies have considered the combined effects of aging and cancer on DC and T cell function (315, 1164, 1165).

There is limited evidence to suggest that aging influences DC anti-tumour function. DCs from elderly mice were shown to have reduced ability to stimulate tumour-specific cytotoxic CD8⁺ T cells (71, 614), and NK cell-mediated anti-tumour responses in vivo (1168). Furthermore, DC vaccines have reduced efficacy in elderly tumour-bearing mice, as the cytotoxic T cell responses generated are insufficient, and do not slow tumour growth (1169, 1170). In contrast, one study has shown that the function of APCs is intact in elderly tumour-bearing mice (1179).

Age-related defects in murine T cell anti-tumour function have also been reported, these include; reduced numbers of tumour-antigen-specific T cells, decreased proliferative capacity, impaired cytotoxic activity and reduced production of effector cytokines, such as IFN-γ and IL-2, in elderly tumour-bearing mice (608,
1171, 1174-1178, 1339, 1340). However, one study has shown that tumour-specific
CD8+ T cells in elderly mice retain their proliferative ability and cytotoxic function
(1179). Further studies are required to help resolve these conflicting results which
may be due to differences in mouse strain, age, and tumour models.

Previous studies in our laboratory using the AE17 murine mesothelioma model,
which was induced by the relevant human carcinogen, asbestos, and presents a
similar histology to human mesothelioma (1444), have shown that in young mice
(aged 1.5-2 months), as tumour burden increases, tumour-associated CD11c+ DC
proportions decrease, whilst their intracellular lipid content increases, suggesting
DC dysfunction (228). Activated CD8+ and CD4+ T cells are also present in AE17
tumours (1440). Furthermore, whilst presentation of tumour antigens occurs in
TDLNs, leading to proliferation and activation of functional cytotoxic CD8+ T cells
which traffic to tumours, the function of these T cells is compromised within the
tumour (996, 1440-1442, 1444). However, the possible effects of aging on
mesothelioma-induced modulation of DCs and T cells have not yet been examined
in a murine system.

The aim of the studies in this chapter was to examine the combined effects of aging
and mesothelioma on DC and T cell function. Proportions and functional
phenotypes of DC subsets, CD11c+ cells and T cells in TDLNs, spleens, and tumours
from young and elderly AE17 mesothelioma tumour-bearing mice were compared.
6.2 Results

6.2.1 Examining the effects of aging and cancer on murine dendritic cells

6.2.1.1 Tumour growth rates are similar in young and elderly mice

The first experiments investigated whether aging influenced AE17 mesothelioma tumour growth in young (2-5 months; n=13-15) and elderly (20-24 months; n=16) C57BL/6J mice. Tumours grew at similar rates in young and elderly mice (Figure 6.1A). Mice were collected for analysis 14-16 days after tumour cell inoculation, and at this time point, the range of tumour sizes was 60-160 mm² (Figure 6.1B), and the range of tumour weights was 85-640 mg (Figure 6.1C), no age-related differences in tumour sizes or weights were seen (Figures 6.1B and 6.1C).

![Graph showing tumour growth rates in young and elderly mice](image)

**Figure 6.1** Tumour growth rates are similar in young and elderly mice

Young and elderly mice were inoculated subcutaneously with $5 \times 10^5$ AE17 tumour cells per mouse, and tumour size (mm²) was measured daily to determine tumour growth rates (A). At the experimental end points (14-16 days after tumour cell inoculation), tumour size (mm²; B) and weight (mg; C) were measured. Data are shown as mean ± SEM, n = 13-15 young and n = 16 elderly tumour-bearing mice.
6.2.1.2  Mesothelioma increases pDCs and reduces CD8α⁺CD11b⁻ cDCs and CD11b⁺CD8α⁻CD4⁻ cDCs in TDLNs, regardless of age

The effect of aging and cancer on CD11c⁺ cells and DC subset proportions in TDLNs of young (2-5 months; n=11-14) and elderly (20-24 months; n=12-16) AE17 mesothelioma-bearing C57BL/6J mice was investigated using flow cytometry (as per Figures 5.1C-5.1E and Figures 5.2A-5.2D). CD11c⁺ cell and DC subset proportions in TDLNs from tumour-bearing mice were compared to LNs from age-matched healthy mice. To assess the influence of tumour size, correlations between cell proportions and tumour weight were also analysed.

Neither age nor the presence of a tumour significantly affected TDLN/LN CD11c⁺ cell proportions in mice (Figure 6.2A). Nonetheless, TDLN CD11c⁺ cell proportions appeared to increase with increasing tumour size (r = 0.6629, p = 0.01) in young (Figure 6.2B), but not elderly, tumour-bearing mice (Figure 6.2C).

![Figure 6.2 Proportions of TDLN CD11c⁺ cells increase with tumour size in young mice](image-url)

Young and elderly mice were inoculated subcutaneously with 5 x 10⁵ AE17 tumour cells per mouse, and tumours allowed to grow. Analysis occurred 14-16 days after tumour cell inoculation. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c⁺ cells, and analysed by flow cytometry. Proportions of CD11c⁺ cells in LNs (A) and the correlation between proportions of TDLN CD11c⁺ cells and tumour weight in young mice (B) and elderly mice (C) were measured. Data in (A) are shown as mean ± SEM, n = 14 young and n = 16 elderly tumour-bearing mice, n = 32-35 young and n = 35-38 elderly healthy mice.
Mesothelioma tumours significantly decreased CD8α+CD11b− cDC (young p = 0.01, elderly p = 0.0047; Figure 6.3A) and CD11b+CD8α+CD4− cDC proportions (young p = 0.001, elderly p = 0.0008; Figure 6.3B) in TDLNs compared to LNs of age-matched healthy mice, with age-related differences seen only for the latter subset in tumour-bearing mice (p = 0.003; Figure 6.3B). In contrast, pDC proportions already increased in healthy elderly LNs were further elevated in the presence of a tumour (young p = 0.07, elderly p = 0.005; p = 0.0008; Figure 6.3C). No age-related or tumour-induced changes in CD11b+CD8αCD4+ cDC proportions were observed (Figure 6.3D). There was no correlation between tumour size and proportions of any of the DC subsets examined (data not shown).

Figure 6.3 Increased pDCs and reduced CD8α+CD11b− cDCs and CD11b+CD8α+CD4− cDCs in TDLNs, regardless of age
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for DC subsets and analysed by flow cytometry. Proportions of CD8α+CD11b− cDCs (A), CD11b+CD8α+CD4− cDCs (B), pDCs (C) and CD11b+CD8α+CD4+ cDCs (D) are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 32 young and n = 35 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.
6.2.1.3 Tumours elevate MHC class II and CD80, and reduce CD40, TNF-α and IL-12 on TDLN CD11c⁺ cells

To investigate the influence of aging and cancer on CD11c⁺ cell function, the same markers of antigen presentation, activation and regulation described for chapter 5 were examined on CD11c⁺ cells from LNs of young (n=3-11) and elderly (n=3-12) tumour-bearing mice and age-matched healthy mice using flow cytometry.

Regardless of age, increased percentages of TDLN CD11c⁺ cells expressed MHC class II (young p = 0.001, elderly p = 0.01; Figure 6.4A) and CD80 (young p = 0.003, elderly p = 0.03; Figure 6.4B), compared to age-matched healthy LNs. However, expression levels of MHC class II and CD80 on CD11c⁺ cells did not differ between tumour-bearing mice and age-matched healthy mice (Appendix E: Figures 1A and 1B).

![Figure 6.4 Increased MHC class II⁺ and CD80⁺ CD11c⁺ cells in TDLNs](image)

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c⁺ cells, MHC class II and CD80, and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for MHC class II (A) and CD80 (B) were measured. Data are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 18-20 young and n = 18-25 elderly healthy mice, * = p<0.05, ** = p<0.005 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.

In both age groups, the presence of a tumour reduced CD40 expression levels on TDLN CD11c⁺ cells compared to healthy LNs (young p = 0.01, elderly p = 0.02; Figure 6.5A), whilst the percentage of CD40⁺ cells remained unchanged (Appendix E: Figure 1C). Tumours reduced the percentages of TNF-α⁺ (young p = 0.04, elderly p = 0.08;
Figure 6.5B) and IL-12* (young p = 0.0008, elderly p = 0.01; Figure 6.5C) CD11c* cells in TDLNs, relative to age-matched healthy LNs. This was associated with reduced expression levels of TNF-α (young p = 0.04, elderly p = 0.02; Appendix E: Figure 2A) and IL-12 (young p < 0.0001, elderly p = 0.02; Appendix E: Figure 2B) on CD11c* cells from TDLNs, compared to healthy LNs. Note that the percentage of IL-12* CD11c* cells (p = 0.03; Figure 6.5C), and IL-12 expression levels (p = 0.051; Appendix E: Figure 2B) remained higher in elderly TDLNs, relative to young TDLNs. For the remainder of this chapter, where the percentage of cells positive for a marker and MFI expression levels of that same marker show similar changes, only the percentage of cells positive data will be shown.

Figure 6.5 Reduced CD40, TNF-α and IL-12 on CD11c* cells in TDLNs
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c* cells, CD40, and intracellular TNF-α and IL-12, and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of CD40 (A), and percentages of CD11c* cells positive for TNF-α (B) and IL-12 (C) were measured. Data are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 10-18 young and n = 11-18 elderly healthy mice, * = p<0.05, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) tumour-bearing mouse to age-matched healthy mice.
Young, but not elderly, tumour-bearing mice had reduced percentages of IFN-γ⁺ CD11c⁺ cells in TDLNs, relative to their healthy counterparts (p = 0.0008; Figure 6.6A). Additionally, percentages of IFN-γ⁺ CD11c⁺ cells (r = -0.8667, p = 0.002; Figure 6.6B) and IFN-γ expression levels (r = -0.7212, p = 0.02; data not shown) significantly decreased with increasing tumour size in young, but not elderly, tumour-bearing mice (Figure 6.6C). This may have contributed to the significantly higher percentage of IFN-γ⁺ CD11c⁺ cells seen in elderly TDLNs (p = 0.03; Figure 6.6A), compared to their young counterparts.

![Figure 6.6 Reduced IFN-γ⁺ CD11c⁺ cells in TDLNs of young mice with increasing tumour size](image)

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c⁺ cells and intracellular IFN-γ, and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for IFN-γ (A), and the correlation between proportions of TDLN IFN-γ⁺ CD11c⁺ cells and tumour weight in young (B) and elderly mice (C) were measured. Data in (A) are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 10 young and n = 11 elderly healthy mice, * = p<0.05 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.
Tumours did not affect other markers on TDLN CD11c+ cells that had demonstrated age-related differences in healthy mice, e.g. MHC class I, CD73 and TGF-β (Appendix E: Figures 3A-3C). No age-related differences were seen in the remaining markers examined (Appendix E: Figures 1D, 4A and 4B), and there was no correlation between the other markers and tumour size.

6.2.1.4 Tumours increase splenic pDCs and reduce CD11b+CD8αCD4+ cDCs, regardless of age

CD11c+ cells and DC subsets were also examined in spleens from young and elderly AE17 mesothelioma-bearing mice. Tumours did affect splenic CD11c+ cells compared to healthy controls (Figure 6.7A). In both age groups, pDC proportions significantly increased (young p = 0.02, elderly p = 0.006; Figure 6.7B) whilst CD11b+CD8αCD4+ cDCs significantly decreased (young p = 0.005, elderly p = 0.004; Figure 6.7C) in spleens of tumour-bearing mice compared to age-matched healthy mice; nonetheless, pDC (p = 0.04; Figure 6.7B) and CD11b+CD8αCD4+ cDC proportions (p = 0.0003; Figure 6.7C) remained higher in elderly, compared to young, tumour-bearing mice. The age-related reduction of splenic CD8α+CD11b- cDC proportions was maintained in tumour-bearing mice (p = 0.02; Figure 6.7D). Young, but not elderly, mice demonstrated a reduction in splenic CD11b+CD8αCD4+ cDC proportions with increasing tumour size (r = -0.8118, p = 0.03; Figures 6.8A and 6.8B). Despite this, splenic CD11b+CD8αCD4+ cDC proportions were still lower in elderly tumour-bearing mice (p = 0.007; Figure 6.8C).

6.2.1.5 Tumours reduce MHC class I/II and CD40, and increase CD80 and A2A receptor on splenic CD11c+ cells

Tumours reduced MHC class I (young p = 0.02, elderly p = 0.0004; Figure 6.9A) and CD40 levels (young p = 0.0004, elderly p = 0.01; Figure 6.9B) on splenic CD11c+ cells from young and elderly mice relative to their healthy counterparts, although percentages of MHC class I+ and CD40+ CD11c+ cells remained similar (Appendix E: Figures 5A and 5B). Both age groups also had reduced percentages of splenic MHC class II+ CD11c+ cells (young p = 0.003, elderly p = 0.001, Figure 6.9C), in association with reduced MHC class II levels (data not shown) in tumour-bearing, compared to
healthy mice. Expression of MHC class I, MHC class II and CD40 remained similar with age in spleens of tumour-bearing mice (Figures 6.9A-6.9C).

Figure 6.7 Increased splenic pDCs and reduced CD11b⁺CD8α⁻CD4⁻ cDCs in tumour-bearing mice
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Spleens from tumour-bearing mice and age-matched healthy mice were stained for CD11c⁺ cells and DC subsets and analysed by flow cytometry. Proportions of CD11c⁺ cells (A), pDCs (B), CD11b⁺CD8α⁻CD4⁻ cDCs (C), and CD8α⁺CD11b⁻ cDCs (D) are shown as mean ± SEM, n = 8-11 young and n = 8-11 elderly tumour-bearing mice, n = 29-32 young and n = 32-35 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.
Figure 6.8 Reduced splenic CD11b+CD8αCD4+ cDCs with increasing tumour size in young mice

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Spleens from tumour-bearing mice and age-matched healthy mice were stained for CD11b+CD8αCD4+ cDCs and analysed by flow cytometry. The correlation between proportions of splenic CD11b+CD8αCD4+ cDCs and tumour weight in young (A) and elderly mice (B), and proportions of CD11b+CD8αCD4+ cDCs in spleens (C) were measured. Data in (C) are shown as mean ± SEM, n = 7-8 young and n = 8 elderly tumour-bearing mice, n = 29 young and n = 32 elderly healthy mice, ** = p<0.005 comparing young to elderly mice.
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Spleens from tumour-bearing mice and age-matched healthy mice were stained for CD11c+ cells, MHC class I, MHC class II and CD40, and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of MHC class I (A) and CD40 (B), and percentages of CD11c+ cells positive for MHC class II (C) were measured. Data are shown as mean ± SEM, n = 8 young and n = 8 elderly tumour-bearing mice, n = 15-17 young and n = 15-21 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing tumour-bearing mice to age-matched healthy mice.

In contrast, tumours increased the percentages of splenic CD11c+ cells that were CD80+ (young p = 0.005, elderly p = 0.006; Figure 6.10A) and A2A receptor+ (young p = 0.001, elderly p = 0.01; Figure 6.10B) relative to healthy controls; this was associated with increased expression levels of both markers (data not shown). However, expression of the A2A receptor remained lower on splenic CD11c+ cells from elderly, compared to young, tumour-bearing mice (p = 0.008; Figure 6.10B). Elderly tumour-bearing mice demonstrated reduced percentages of CD86+ (p = 0.04; Appendix E: Figure 6A) and TGF-β+ (p = 0.04; Appendix E: Figure 6B) splenic CD11c+.
cells, compared to their younger counterparts; expression levels of these markers were also reduced with age (data not shown). Age-related differences in other markers seen in healthy, non-tumour-bearing mice were maintained in tumour-bearing mouse: i.e. increased CD73 and PD-L1 on elderly splenic CD11c⁺ cells (Appendix E: Figures 6C and 6D). No age-related differences were seen in the other markers examined (Appendix E: Figures 5A-5C). There was also no correlation between marker expression on splenic CD11c⁺ cells and tumour size.

**Figure 6.10 Increased CD80 and A2AR on splenic CD11c⁺ cells from tumour-bearing mice**
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Spleens from tumour-bearing mice and age-matched healthy mice were stained for CD11c⁺ cells, CD80, and A2A receptor (A2AR), and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for CD80 (A) and A2AR (B) were measured. Data are shown as mean ± SEM, n = 3-8 young and n = 4-8 elderly tumour-bearing mice, n = 3-15 young and n = 3-15 elderly healthy mice. * = p<0.05, ** = p<0.005 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.

6.2.1.6 CD11c⁺ cells in elderly tumours have reduced MHC class I and II, CD80, CD86, CD39, PD-L1 and TGF-β, and increased TNF-α and IL-10

Elderly tumours had a significantly lower proportion of CD11b⁺CD8α⁺CD4⁺ cDCs, compared to young tumours (p = 0.02; Figure 6.11A). No age-related differences in the other three DC subsets (Figures 6.11B-6.11D) or in total CD11c⁺ cells (Figure 6.11E) were observed in tumours. There was also no correlation seen between tumour-associated DC subsets and tumour size. In both age groups, pDCs and CD11b⁺CD8α⁺CD4⁻ cDCs were the main subsets in tumours, and CD8α⁺CD11b⁻ cDCs were present in low proportions (Figures 6.11B-6.11D).
Figure 6.11 Reduced proportions of CD11b<sup>+</CD8α·CD4</sup> cDCs in elderly tumours

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD11c<sup>+</CD11b·cDCs</sup>, and analysed by flow cytometry. Proportions of CD11b<sup>+</CD8α·CD4</sup> cDCs (A), CD8α<sup>+</CD11b·cDCs</sup> (B), CD11b<sup>+</CD8α·CD4</sup> cDCs (C), pDCs (D) and CD11c<sup>+</CD11c·cDCs</sup> (E) are shown as mean ± SEM, n = 11-14 young and n = 12-16 elderly tumour-bearing mice, * = p<0.05 comparing young to elderly mice.

Elderly tumour-associated CD11c<sup>+</CD11c·cDCs</sup> demonstrated significantly reduced percentages of MHC class II<sup>+</CD11c·cDCs</sup> (p = 0.004; Figure 6.12A), as well as trends for reduced percentages of MHC class I<sup>+</CD11c·cDCs</sup> (p = 0.08; Figure 6.12A) and CD80<sup>+</CD11c·cDCs</sup> (p = 0.08; Figure 6.12A), relative to their younger counterparts; expression levels of these markers showed similar age-related differences (data not shown). Furthermore, percentages of CD86<sup>+</CD11c·cDCs</sup> cells were reduced in elderly tumours.
with increasing tumour size \( (r = -0.6996, p = 0.02; \text{Figure 6.12B}) \), but no correlation was seen between CD86+ CD11c+ cells and tumour size in young mice (Figure 6.12C). In contrast, percentages of TNF-\(\alpha\)+ CD11c+ cells \( (p = 0.048; \text{Figure 6.12A}) \), as well as TNF-\(\alpha\) expression levels (data not shown) were increased in elderly, compared to young tumours.

![Graph](image)

**Figure 6.12** Elderly tumour CD11c+ cells have reduced MHC-I/II, CD80 and CD86, and increased TNF-\(\alpha\).

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD11c+ cells, MHC class I, MHC class II, CD80, CD86 and intracellular TNF-\(\alpha\), and analysed by flow cytometry. Percentages of CD11c+ cells positive for MHC class I, MHC class II, CD80 and TNF-\(\alpha\) (A), and the correlation between percentages of CD86+ CD11c+ cells and tumour weight in elderly (B) and young mice (C) were measured. Data in (A) are shown as mean \pm SEM, \( n = 11 \) young and \( n = 11-12 \) elderly tumour-bearing mice, * = \( p<0.05 \), ** = \( p<0.005 \) comparing young to elderly mice.
Examination of regulatory markers showed that percentages of IL-10+CD11c+ cells increased (p = 0.04; Figure 6.13), whilst percentages of CD39+ (p = 0.006; Figure 6.13), PD-L1+ (p = 0.001; Figure 6.13) and TGF-β+ CD11c+ cells (p = 0.04; Figure 6.13) decreased in elderly, relative to young tumours. Age-related changes in expression levels of IL-10, CD39, PD-L1 and TGF-β on tumour-associated CD11c+ cells reflected the changes seen in percentages of cells positive for these markers (data not shown). No further age-related differences in the other markers were seen (Appendix E: Figures 7A and 7B), and none of the remaining markers examined showed any correlation with tumour size.

![Figure 6.13 Elderly tumour CD11c+ cells have reduced CD39, PD-L1 and TGF-β, and increased IL-10](image)

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD11c+ cells, CD39, PD-L1, and intracellular IL-10 and TGF-β, and analysed by flow cytometry. Data are shown as mean ± SEM, n = 11 young and n = 11-12 elderly tumour-bearing mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.

In summary, regardless of age, tumours led to: (i) reduced CD8α+CD11b- cDCs and CD11b+CD8αCD4+ cDCs in TDLNs and/or spleens, suggesting compromised stimulation of tumour-specific CD8+ T cells and Th2 responses, (ii) increased pDCs in TDLNs and spleens suggesting increased suppressive activity, (iii) increased MHC class II and CD80 on CD11c+ cells in TDLNs, suggesting improved antigen-presentation and co-stimulatory capacities, but reduced potential to activate T cells via CD40, TNF-α and IL-12, and (iv) reduced MHC class I/II and CD40 expression on splenic CD11c+ cells suggesting reduced T cell antigen-presenting and co-stimulatory capacity, and increased potential to respond to inhibitory adenosine due to higher A2A receptor.
Maintained age-related differences in CD11c+ cells and DC subsets in tumour-bearing mice include: (i) increased pDCs and CD11b+CD8αCD4− cDCs in elderly TDLNs and spleens suggesting increased suppressive activity and Th2 responses, (ii) reduced elderly splenic CD8α+CD11b− cDCs and CD11b+CD8α+CD4+ cDCs suggesting impaired cross-presentation of tumour antigens to CD8+ T cells and activation of Th1 responses with age, (iii) increased MHC class I and IL-12 on elderly TDLN CD11c+ cells leading to improved antigen-presenting ability to CD8+ T cells and T cell stimulatory capacity, yet increased potential to inhibit T cells through increased CD73 and TGF-β, and (iv) elderly splenic CD11c+ cells may have increased regulatory function through CD73 and PD-L1. Furthermore, in elderly tumours: (i) stimulation of Th1 responses may be reduced due to declining CD11b+CD8α+CD4+ cDCs, and (ii) elderly tumour-associated CD11c+ cells may have impaired antigen-presenting and co-stimulatory functions due to reduced MHC class I, MHC class II, CD80 and CD86, but their regulatory potential via IL-10 may be increased.

6.2.2 Examining the effects of aging and cancer on murine T cells

6.2.2.1 Tumours increase CD25 expression on elderly TDLN CD8+ and CD4+ T cells

The next studies examined the effects of aging and cancer on CD8+ T cells, CD4+ T cells and Tregs in TDLNs, spleens and tumours of young (2-5 months; n=11-14) and elderly (20-24 months; n=12-16) AE17 mesothelioma tumour-bearing mice.

The presence of a tumour was associated with reduced TDLN CD4+ T cell proportions in both age groups (young p = 0.0002, elderly p = 0.0075; Figure 6.14A), compared to healthy LNs, with CD4+ T cell proportions remaining lower in elderly TDLNs (p < 0.0001; Figure 6.14A). Tumours did not affect TDLN CD8+ T cell and Treg proportions (Appendix E: Figures 8A and 8B). There was no correlation between TDLN T cell subset proportions and tumour size.

Significantly more CD8+ T cells in elderly, but not young, TDLNs expressed CD25 (p = 0.03; Figure 6.14B) at higher expression levels (data not shown) compared to PBS controls.
 Figure 6.14 Reduced proportions of CD4+ T cells and increased CD25 expression on T cells in TDLNs
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice and lymph nodes (LNs) from age-matched healthy mice were stained for CD8+ T cells, CD4+ T cells and CD25, and analysed by flow cytometry. Proportions of CD4+ T cells in LNs (A), and percentages of CD25+ CD8+ T cells (B) and percentages of total CD4+ T cells positive for CD25 (C) were measured. Data are shown as mean ± SEM, n = 14 young and n = 16 elderly tumour-bearing mice, n = 11-21 young and n = 12-21 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.

Tumours exerted significant effects on regulatory markers on CD8+ T cells from young mice including increased percentages of cells expressing ICOS (p = 0.004; Figure 6.15), LAG-3 (p = 0.0046; Figure 6.15) and TGF-β (p = 0.02; Figure 6.15), relative to healthy controls; similar changes were seen for their expression levels (data not shown). Nonetheless, most regulatory markers were still expressed at higher levels in TDLNs from elderly, relative to young, tumour-bearing mice.
including ICOS, LAG-3, TGF-β, CD39, CD73, CTLA-4, PD-1 and perforin (Figure 6.15, Appendix E: Figures 9A and 9B). The remaining markers examined did not show age-related differences (Appendix E: Figures 9B and 9C).

**Figure 6.15** Tumours increase ICOS, LAG-3 and TGF-β on CD8+ T cells in young TDLNs

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice and lymph nodes (LNs) from age-matched healthy mice were stained for CD8+ T cells, and regulatory markers (ICOS, LAG-3, and intracellular TGF-β), and analysed by flow cytometry. Percentages of CD8+ T cells positive for ICOS, LAG-3 and TGF-β were measured. Data are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 8 young and n = 9 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.

The presence of a tumour increased the percentages of TDLN CD4+ T cells expressing CD25 in young and elderly mice (young p = 0.001, elderly p = 0.01; Figure 6.14C), similar results were seen for CD25 expression levels (data not shown), relative to LNs from age-matched healthy mice, with higher levels still seen in elderly mice (p = 0.02; Figure 6.14C). No other changes were observed for TDLN CD4+ T cells (Appendix E: Figures 10A, 10B, 11A and 11B). There was no correlation between marker expression on TDLN CD8+ and CD4+ T cells and tumour size.

**6.2.2.2 Tumours reduce perforin in elderly splenic CD4+ T cells**

Young, but not elderly, tumour-bearing mice demonstrated reduced proportions of splenic CD4+ T cells, relative to controls (p = 0.0004; Figure 6.16A), removing the age-related differential (Figure 6.16A). No tumour-induced changes in splenic CD8+
T cell or Treg proportions were seen (Appendix E: Figures 12A and 12B), and the age-related reduction in splenic CD8+ T cell proportions remained (Appendix E: Figure 12A).

Tumours reduced the percentage of elderly splenic CD4+ T cells expressing perforin compared to healthy mice (p = 0.01; Figure 6.16B) and young tumour-bearing mice (p = 0.008; Figure 6.16B). No other changes in expression of activation or regulatory markers on splenic CD4+ and CD8+ T cells were observed (data not shown) and there was no correlation between splenic T cell proportions or marker expression on splenic CD8+ and CD4+ T cells and tumour size.

**Figure 6.16 Reduced young CD4+ T cell proportions and reduced perforin on elderly CD4+ T cells in spleens of tumour-bearing mice**

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Splens from tumour-bearing mice and age-matched healthy mice were stained for CD4+ T cells and intracellular perforin, and analysed by flow cytometry. Proportions of CD4+ T cells in spleens (A) and percentages of perforin+ CD4+ T cells (B) were measured. Data are shown as mean ± SEM, n = 8-11 young and n = 8-12 elderly tumour-bearing mice, n =5-18 young and n = 6-19 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.

**6.2.2.3 Elderly CD8+ T cells in tumours have reduced IFN-γ and TGF-β, and increased CD73 expression**

An investigation of tumour-infiltrating T cells showed that CD4+ T cell (p = 0.0002; Figure 6.17A) and Treg (p = 0.0005; Figure 6.17A) proportions decreased in elderly hosts, whilst CD8+ T cells were similar with age (Figure 6.17A). Examination of the relationship between T cell proportions and tumour size showed that CD8+ T cell
proportions decreased in young tumours with increasing tumour size \( (r = -0.7702, p = 0.002; \) Figure 6.17B); this was not seen in elderly tumours (Figure 6.17C). There was no correlation between tumour-associated CD4\(^+\) T cells and Tregs with tumour size.

Figure 6.17 Reduced proportions of CD4\(^+\) T cells and Tregs in elderly tumours, and reduced CD8\(^+\) T cell proportions in young tumours with increasing tumour size

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD8\(^+\) T cells, CD4\(^+\) T cells and CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Tregs, and analysed by flow cytometry. Proportions of CD8\(^+\) T cells, CD4\(^+\) T cells and Tregs in tumours (A), and the correlation between proportions of tumour-associated CD8\(^+\) T cells and tumour weight in young (B) and elderly mice (C) were measured. Data in (A) are shown as mean ± SEM, \( n = 14 \) young and \( n = 16 \) elderly tumour-bearing mice, *** = \( p<0.0005 \) comparing young to elderly mice.

A reduced percentage of IFN-γ\(^+\) and TGF-β\(^+\) CD8\(^+\) T cells infiltrating elderly tumours \( (p = 0.005; \) Figure 6.18A; \( p = 0.03; \) Figure 6.18B) was associated with increased
percentages of CD73+ CD8+ T cells ($p = 0.04$; Figure 6.18C), compared to young tumours; the same results were seen for expression levels of these markers (data not shown). No differences were observed for the other markers examined on tumour-associated CD8+ T cells (Appendix E: Figures 13A and 13B), nor was there any correlation between marker expression and tumour size.

**Figure 6.18** Elderly tumour-associated CD8+ T cells have reduced IFN-γ and TGF-β, and increased CD73

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD8+ T cells, CD73, and intracellular IFN-γ and TGF-β, and analysed by flow cytometry. Percentages of CD8+ T cells positive for IFN-γ (A), TGF-β (B), and CD73 (C) were measured. Data are shown as mean ± SEM, $n = 11$ young and $n = 12$ elderly tumour-bearing mice, * = $p<0.05$, ** = $p<0.005$ comparing young to elderly mice.

**6.2.2.4** Elderly CD4+ T cells in tumours have reduced IFN-γ, ICOS and TGF-β expression

CD4+ T cells infiltrating elderly tumours expressed lower levels of IFN-γ compared to their younger counterparts ($p = 0.02$; Figure 6.19A), despite percentages of IFN-γ+ CD4+ T cells in tumours being similar with age (Appendix E: Figure 14A). However,
there were reduced percentages of ICOS+ (p = 0.03; Figure 6.19B) and TGF-β+ (p = 0.03; Figure 6.19C) elderly tumour-infiltrating CD4+ T cells with reduced expression levels (data not shown), relative to their young controls. No further age-related differences were observed on tumour-associated CD4+ T cells (Appendix E: Figures 14A and 14B), nor was there any correlation between marker expression on CD4+ T cells and tumour size.

Figure 6.19 Elderly tumour-associated CD4+ T cells have reduced IFN-γ, ICOS and TGF-β
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD4+ T cells, ICOS, and intracellular IFN-γ and TGF-β, and analysed by flow cytometry. Expression levels of IFN-γ on CD4+ T cells (measured as geometric mean fluorescence intensity; MFI; A), and percentages of CD4+ T cells positive for ICOS (B), and TGF-β (C) were measured. Data are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, * = p<0.05 comparing young to elderly mice.

In summary, in both age groups: (i) tumours may reduce the generation of CD4+ T cell responses due to their declining proportions in TDLNs, and (ii) CD4+ T cells in TDLNs may be more activated or could be transitioning to Tregs due to increased CD25.
With aging and tumours, (i) reduced CD8+ and CD4+ T cells in elderly TDLNs and reduced CD8+ T cells in elderly spleens relative to their younger counterparts were maintained, suggesting compromised CD8+ and/or CD4+ T cell responses, (ii) elderly splenic CD4+ T cells may have decreased cytotoxic function due to reduced perforin, (iii) the age-associated suppressive/exhausted phenotype of CD8+ and CD4+ T cells is maintained in TDLNs and spleens of elderly tumour-bearing mice, (iv) CD4+ T cell and Treg activity may be reduced in elderly tumours due their declining proportions, and (v) elderly tumour-associated CD8+ and CD4+ T cells have decreased effector activity due to decreased IFN-γ.
6.3 Discussion

The studies in this chapter examined changes in CD11c⁺ cells, DC subsets and T cells in TDLNs, spleens and tumours of mesothelioma-bearing mice, and whether this was influenced by aging. The results show that whilst tumours drove significant changes, aging also impacted on immune cell function in tumour-bearing mice.

The data show that mesothelioma tumours further skew DCs towards suppressive function, regardless of age. The dominating DC subsets in tumours were pDCs and CD11b⁺CD8α⁺CD4⁻ cDCs, and both have been associated with immune suppression. Within tumours, pDCs often co-localise with Tregs (760, 761, 1082, 1778) and promote Treg expansion via (i) secretion of the enzyme IDO (203, 533, 1085), and/or (ii) expression of ICOSL, which binds to ICOS expressed on Tregs, leading to Treg activation and IL-10 secretion (202, 759-761). Although this study did not examine ICOS expression on Tregs, it did show that up to 30% of tumour-associated CD4⁺ T cells expressed ICOS, suggesting that they could interact with ICOSL⁺ pDCs. Plasmacytoid DCs can induce Th1 cell anergy and apoptosis via IDO (1085), and production of granzyme B by pDCs inhibits Th1 cell proliferation (763). CD11b⁺CD8α⁺ CD4⁻ cDCs stimulate anti-inflammatory Th2 cells (132, 142), which inhibit cytotoxic CD8⁺ T cell function via IL-10 (1087, 1141, 1779) and IL-13 (1153, 1780), they also induce tolerogenic DCs via IL-10 (767, 1087), and promote pro-tumourigenic macrophages via IL-4 and IL-13, all leading to enhanced tumour growth (1152, 1781). Furthermore, tumour-infiltrating CD11c⁺CD11b⁺ DCs act as regulatory DCs that inhibit CD8⁺ T cell effector function by metabolising L-arginine which suppresses CD8⁺ T cell priming and expansion (1078) and by expressing PD-L1, which binds PD-1 on CD8⁺ T cells, leading to T cell inhibition (1000). Thus, the main DC subsets present within mesothelioma tumours are likely to promote suppression of T cells.

This study was unable to examine the phenotype of tumour-associated DC subsets, due to limitations in flow cytometer capability, but instead examined the phenotype of CD11c⁺ cells, which represent APCs, including DCs (4, 79, 80). The majority of tumour-associated CD11c⁺ cells co-expressed the antigen-presenting molecules
MHC class I and II, the co-stimulatory molecule CD80, and the pro-inflammatory cytokine IFN-γ, in association with several regulatory markers (CD39, CD73, A2A receptor, A2B receptor, galectin-9, PD-L1 and TGF-β). The summation of positive and negative signals delivered by DCs to T cells during DC/T cell interactions determines the T cell response (708). Data from this study, and others, shows that tumour-infiltrating CD8⁺ and CD4⁺ T cells express several ligands for the regulatory molecules expressed by CD11c⁺ cells, such as PD-1 and CTLA-4 (1110, 1117, 1119, 1316, 1739, 1782, 1783) and TIM-3 (1119, 1316, 1784). This suggests a high likelihood of negative interactions, for example via the PD-L1/PD-1, CD80/CTLA-4 and galectin-9/TIM-3 pathways within tumours, leading to inhibition and/or apoptosis of effector CD8⁺ and CD4⁺ T cells (831, 883, 913, 1000, 1033, 1785).

Additionally, CD11c⁺ cells may contribute to production of intra-tumoural adenosine via CD39 and CD73; this is supported by a recent study showing that tumour-infiltrating DCs expressing CD39 generated adenosine, resulting in suppression of T cell proliferation and IFN-γ secretion (918). Tumour-associated CD8⁺ and CD4⁺ T cells in mesothelioma could respond to adenosine due to expression of the A2A and A2B receptors, leading to inhibition of T cell proliferation, activation and effector function (543, 552, 1095, 1097). DC/CD11c⁺ cell-mediated suppression of infiltrating tumour antigen-specific T cells previously activated in TDLNs could enable tumour progression (996, 998, 1376).

An important part of the DC lifecycle is migration of tumour-antigen bearing DCs from tumours to TDLNs (6). If tumour-associated CD11c⁺ cells expressing several regulatory markers did migrate to TDLNs, they could promote T cell suppression in this site. However, others have shown that DC migration is impaired in tumour-bearing hosts (1006, 1786-1790). This study showed reduced CD8α⁺CD11b⁻ cDCs in TDLNs, which could be due to reduced numbers trafficking from tumours, and/or reductions in LN tissue-resident CD8α⁺CD11b⁻ cDCs. As CD8α⁺CD11b⁻ cDCs play a crucial role in cross-presenting tumour antigens and activating CD8⁺ T cells (124, 125, 127), this suggests that generation of tumour-specific cytotoxic CD8⁺ T cells may be reduced in TDLNs of mesothelioma-bearing mice. Cross-presentation of tumour antigens in TDLNs and spleens may be further reduced due to declining
proportions of CD11b<sup>+</sup>CD8α<sup>+</sup>CD4<sup>+</sup> cDCs, as CD8α<sup>+</sup> cDCs can also cross-present tumour antigens and generate anti-tumour immunity (145); this is supported by a study from our laboratory showing decreasing tumour antigen-specific CD8<sup>+</sup> T cell proliferation with increasing tumour burden (228). This suggests that larger tumours are likely to be more suppressive, and is supported by data from this study showing that with increasing tumour burden, young TDLN CD11c<sup>+</sup> cells and elderly tumour-associated CD11c<sup>+</sup> cells may have reduced T cell-activating capacity (via IFN-γ and CD86, respectively), and that infiltration of effector CD8<sup>+</sup> T cells into tumours is reduced in young hosts. It is further supported by another study showing that as murine ovarian tumours progress, the T cell-activating ability of tumour-associated DCs declines, and their suppressive function increases (999). Further studies are required to: (i) examine the migratory capacity of CD11c<sup>+</sup> cells/DC subsets in the AE17 mesothelioma model, and (ii) distinguish migratory from tissue-resident DCs in TDLNs and spleens.

Although this study could not examine functional changes in DC subsets, changes in CD11c<sup>+</sup> cells suggest reduced potential for T cell activation in lymphoid tissues of tumour-bearing mice. CD11c<sup>+</sup> cells in TDLNs displayed a semi-mature phenotype, characterised by increased MHC class II and CD80, but decreased CD40, TNF-α and IL-12. This suggests that whilst CD11c<sup>+</sup> cells in TDLNs may have an improved ability to present antigens to CD4<sup>+</sup> T cells, they may be unable to fully activate tumour-specific Th1 effector cells (1050, 1051, 1791), and may instead induce T cell anergy or tolerance (516, 1792). In addition, reduced proportions of CD4<sup>+</sup> T cells in TDLNs may further compromise generation of anti-tumour Th1 responses. A reduction in Th1 cells may reduce the generation of cytotoxic CD8<sup>+</sup> T cell responses, as Th1 cells play an important role in augmenting CD8<sup>+</sup> T cells, via cytokine secretion (1793, 1794) and CD40L/CD40 interactions with LN-resident DCs, including CD8α<sup>+</sup>CD11b<sup>−</sup> cDCs (822, 1790, 1795, 1796). In this study, DC licensing may be impaired due to decreased CD40 on TDLN CD11c<sup>+</sup> cells. Thus, there appears to be reduced potential for T cell activation in TDLNs of mesothelioma-bearing mice; this is supported by other studies demonstrating inefficient generation of tumour-specific T cells in TDLNs (1051, 1790, 1797). Generation of anti-tumour immunity may also be
compromised in spleens, as antigen presentation to CD8+ and CD4+ T cells may be impaired due to reduced MHC class I/II and CD40 on splenic CD11c+ cells.

The TDLN and splenic microenvironments may be further skewed towards immune suppression due to increased pDCs. As discussed earlier, pDCs promote expansion of Tregs via IDO (203, 1798) and ICOSL/ICOS interactions (202, 759-761). Tregs are the main mediators of suppression within TDLNs, as they inhibit effector CD8+ and CD4+ T cells, and promote regulatory function in DCs (945). One study has shown that IDO-expressing pDCs in TDLNs induce anergy in effector T cells responding to antigens presented by non-suppressive APCs (1798). A subset of partially activated LAG-3+ pDCs has been described in human TDLNs; these pDCs induce Tregs, and promote secretion of chemokines that facilitate MDSC recruitment to tumours (1086). As discussed earlier, pDCs can also inhibit effector Th1 cells via molecules such as IDO and granzyme B (763, 1085). Thus, mesothelioma skews CD11c+ cells/DCs in lymphoid tissues towards immune suppression, which may impair the generation of anti-tumour effector T cells, and allow tumours to evade immune-mediated destruction.

Mesothelioma-induced changes in CD11c+ cells and T cells may be mediated by tumour-derived factors and their effects may be more powerful within tumours, due to higher concentration gradients and/or direct cell-to-cell contact between tumour cells and immune cells (1002, 1799). For example, AE17 cells secrete TGF-β (1447), and mesothelioma tumour cells have been shown to secrete VEGF and IL-6 (1436, 1800, 1801), which inhibit DC differentiation, maturation, migration and T cell-activating capacity (1005, 1007, 1008, 1026, 1030, 1787). Mesothelioma tumours also contain suppressive immune cells, such as Tregs (1436), M2 and M3 macrophages and MDSCs (1245, 1440), which can inhibit DCs and effector T cells (1081, 1135, 1802-1808).

Additional changes were seen with aging which could further impair anti-tumour immune responses within elderly tumours. CD11c+ cells in elderly tumours have reduced ability to present antigens to CD8+ and CD4+ T cells, on account of decreased MHC class I and II, and their co-stimulatory capacity may also be
compromised due to decreased CD80 and CD86. These changes may be more pronounced with increasing tumour burden, as data from this study also showed that the co-stimulatory capacity of elderly tumour-associated CD11c⁺ cells via CD86 was further reduced with increasing tumour size. A reduction in the ability of elderly CD11c⁺ cells to stimulate anti-tumour effector T cells is consistent with other studies showing that elderly BMDCs are less efficient in stimulating tumour antigen-specific CD8⁺ and CD4⁺ T cells and mediating tumour regression (71, 614), and that administration of DC vaccines to elderly mice results in weak cytotoxic CD8⁺ T cell activity and does not slow tumour growth (1169, 1170). Generation of Th1 responses within elderly tumours may be further compromised due to reduced CD11b⁺CD8α⁺CD4⁺ cDCs, whose main role is activation of Th1 responses (132, 142), and reduced CD4⁺ T cells. In addition, elderly tumour-associated CD11c⁺ cells may be further skewed towards suppressive function due to increased IL-10 and TNF-α. Production of IL-10 by DCs can lead to T cell inhibition and induction of Tregs (465, 748, 1809), and TNF has recently been shown to promote CD4⁺ T cell exhaustion during chronic viral infections (1810). Elderly tumour-associated CD11c⁺ cells demonstrated decreased expression of TGF-β latency-associated peptide, which could reflect increased secretion of active TGF-β, leading to inhibition of T cell proliferation, activation and cytotoxic function, and induction of suppressive immune cells, such as Tregs and M2 macrophages (1691); further studies examining age-related changes in active TGF-β are required.

Changes in elderly tumour-associated CD11c⁺ cells suggest that cross-talk between CD11c⁺ cells and T cells in tumours is likely to lead to T cell suppression. This is supported by data from this study showing that elderly tumour-associated CD8⁺ and CD4⁺ T cells had reduced IFN-γ, implying reduced effector activity, moreover others have shown that T cells in elderly tumour-bearing hosts are dysfunctional due to impaired cytotoxic activity and reduced production of IFN-γ and IL-2 (608, 1171, 1174-1178, 1339, 1340). Further studies are required to determine whether CD8⁺ T cell cytotoxic activity is also impaired in elderly mesothelioma-bearing mice. Results from this study suggest that elderly tumour-associated CD8⁺ T cells are skewed towards suppressive function, due to increased CD73, suggesting increased
potential to generate adenosine. Both CD8+ and CD4+ T cells may also have increased inhibitory function via secretion of TGF-β, as suggested by reduced expression of TGF-β latency-associated peptide; again further studies are required to confirm this. Thus, with aging, there appear to be additional mechanisms by which anti-tumour immune responses may be disabled in elderly tumours, which could contribute to tumour progression in the elderly.

Results from this study suggest that if elderly tumour-associated CD11c+ cells migrate to TDLNs, the outcomes are likely to be T cell tolerance and/or generation of suppressive T cells. However, one study has shown that impaired generation of anti-tumour immunity in elderly mice can be attributed to defects in DC migration (614), and DC migration is impaired in tumour-bearing hosts (1006, 1786-1790), and in elderly non-tumour-bearing hosts (68, 236, 690). In this study, CD11c+ cell proportions in TDLNs increased with tumour progression in young, but not elderly mice, which could be explained by impaired DC migration to TDLNs in elderly hosts; further studies are required to investigate this.

The regulatory environment established in lymphoid tissues during aging appears to be increased in elderly tumour-bearing mice. Proportions of pDCs remained elevated in elderly TDLNs and spleens, compared to younger hosts, suggesting pDC-mediated immunosuppression may be increased in elderly hosts. As discussed in Chapter 5, CD11c+ cells in elderly TDLNs and spleens displayed increases in activation and regulatory markers, showing they can deliver positive and negative signals to T cells. The suppressive/exhausted phenotype of elderly T cells in these tissues suggests they are more likely to respond to negative signals from CD11c+ cells/DCs, leading to T cell suppression. The data from this study, plus other studies from our laboratory (1419, 1420) suggest that the regulatory environment within elderly lymphoid tissues may further impair generation of effective anti-mesothelioma immunity in the elderly. Thus, age-related changes in immune cells may exacerbate the tumour-mediated effects on the immune system described earlier.

It is possible that changes in DC phenotype in two different directions at the same
time, for example, simultaneous up-regulation of MHC class I and IL-12 (which may be advantageous for anti-tumour immunity) and CD73 and TGF-β (which may be disadvantageous for anti-tumour immunity) on elderly TDLN CD11c⁺ cells, may mean that overall tumour growth is not affected, and may explain why tumours grew at similar rates in young and elderly mice. Future studies should investigate if depleting DCs and T cells, or blocking regulatory molecules affects tumour growth, and whether this differs with age.

In summary, mesothelioma promotes immune suppression in tumours due to high proportions of potentially suppressive pDCs and CD11b⁺CD8α⁺CD4⁻ cDCs, and CD11c⁺ cells and T cells with regulatory phenotypes. The generation of CD8⁺ cytotoxic T cell responses may be compromised in TDLNs and spleens due to declining proportions of CD8α⁺CD11b⁻ cDCs, semi-mature CD11c⁺ cells that are unable to fully activate tumour-specific T cells due to decreased CD40, TNF-α and/or IL-12, and elevated pDC proportions. This may prevent the generation of effective anti-mesothelioma immune responses. With aging, tumour-associated CD11c⁺ cells have a reduced ability to present antigens to, and activate T cells, and are more likely to suppress T cells, and tumour-associated CD8⁺ and CD4⁺ T cells have reduced IFN-γ-mediated effector function. The regulatory environment in TDLNs and spleens established during healthy aging was increased in elderly tumour-bearing hosts, which could further disable anti-mesothelioma immune responses in the elderly. This may contribute to increased tumour emergence in the elderly, and impact on responses of elderly hosts chemotherapy and immunotherapy, which will be examined in the next two chapters.
Chapter 7  Examining the effects of aging, cancer and chemotherapy on murine dendritic cells and T cells

7.1  Introduction

The studies in Chapter 6 demonstrated that mesothelioma further skews lymphoid CD11c+ cells and T cells towards a suppressive/exhausted phenotype during aging. This may affect responses to chemotherapy in the elderly as some chemotherapeutic agents augment anti-tumour immunity (1180-1182, 1199). However, the effects of aging on immune responses during chemotherapy have not been studied in depth (31) as elderly patients are often excluded from clinical trials due to increased risk of adverse responses (1277-1280) and use of aged mice is difficult and expensive (1281).

Chemotherapeutic agents that have been/are being used to treat mesothelioma patients include gemcitabine, a synthetic pyrimidine nucleoside analogue that disrupts DNA synthesis (1248, 1249), and platinum-based compounds, such as cisplatin, that inhibit DNA replication and transcription by cross-linking DNA (1260-1263). Gemcitabine and cisplatin have been shown to exert immunomodulatory effects in cancer patients and in murine models using young mice (1182, 1199). For example, gemcitabine and cisplatin relieve immunosuppression by reducing MDSC numbers (1217, 1228, 1230-1233, 1271), and cisplatin reduces PD-L2 expression on tumour cells and DCs (1264). These agents can increase numbers of DCs (1198, 1271) and tumour-specific cytotoxic effector T cells (1271, 1811-1813), further promoting anti-tumour immunity. Studies using young mice with syngeneic mesothelioma (aged 2-3 months) have shown that gemcitabine: inhibits B cell proliferation and antibody production in response to tumour antigens, which may alleviate T cell suppression (1205); augments tumour-specific CD8+ and CD4+ T cell responses (1205); restores defects in antigen cross-presentation by tumour-infiltrating DCs; and increases generation of tumour-specific cytotoxic CD8+ T cells (1193, 1258). Furthermore, others using young mesothelioma-bearing mice have
shown that gemcitabine and cisplatin act synergistically with immunotherapies, such as agonist anti-CD40 antibody (1251) or CTLA-4 blockade (1246, 1484, 1814) to enhance tumour-infiltrating effector CD8+ and CD4+ T cells, leading to tumour regression. However, these studies did not consider the effects of age-related changes in immune function.

Previous studies in our laboratory using young mice (aged 1.5-2 months) with AE17 mesothelioma tumours have shown that CD8+ T cells are required for gemcitabine- and cisplatin-induced tumour regression, and these chemotherapies enhance generation of CD8+ T cells specific to subdominant tumour-derived antigens (1222). However, no studies have examined the effects of aging on DC and T cell function during treatment with chemotherapy in mesothelioma. Thus, the aims of the studies in this chapter were to examine the influence of aging, cancer and chemotherapy on CD11c+ cells, DC subsets and T cells in mesothelioma-bearing mice.
7.2 Results

7.2.1 Examining the effects of aging, cancer and chemotherapy on murine dendritic cells

7.2.1.1 Gemcitabine and cisplatin slow tumour growth, regardless of age

The first studies investigated if the effects of chemotherapy on tumour growth were influenced by aging. Young (2-5 months; n=9) and elderly (22-24 months; n=11-12) AE17 mesothelioma tumour-bearing C57BL/6J mice were treated with gemcitabine or cisplatin, using previously described doses (1193, 1222). To ensure measurable tumours were present for analysis and that the window in which the immune response was being activated was not missed, mice were sampled when they received one-third of the treatment schedule (i.e. two doses of gemcitabine or one dose of cisplatin). Gemcitabine induced tumour regression in young and elderly mice, relative to age-matched PBS controls (Figure 7.1A). At the experimental end point, young and elderly gemcitabine-treated mice demonstrated reduced tumour sizes (young \( p < 0.0001 \), elderly \( p = 0.0001 \); Figure 7.1B) and tumour weights (young \( p < 0.0001 \), elderly \( p = 0.001 \); Figure 7.1C), compared to their PBS controls; no age-related differences in tumour size and weight were seen.

Cisplatin slowed tumour growth rates in both age groups relative to age-matched PBS controls (Figure 7.1A). Tumour sizes (young \( p < 0.0001 \), elderly \( p = 0.0008 \); Figure 7.1B) and weights (young \( p < 0.0001 \), elderly \( p = 0.009 \); Figure 7.1C) were reduced, compared to PBS controls. There was a trend for increased tumour weight from elderly, compared to young, cisplatin-treated mice (\( p = 0.08 \); Figure 7.1C).

7.2.1.2 Chemotherapy reduces CD8α⁺CD11b⁻ cDCs in elderly TDLNs

The effects of aging, cancer and chemotherapy on DCs in young (2-5 months; n=6-9) and elderly (22-24 months; n=7-12) mesothelioma-bearing mice were examined. Correlations between cell proportions and tumour weights in chemotherapy-treated mice were also analysed to account for any influence of tumour size, with increasing tumour size considered a weak or non-response to therapy.
Figure 7.1 Gemcitabine and cisplatin slow tumour growth, regardless of age

Young and elderly mice were inoculated subcutaneously with $5 \times 10^5$ AE17 cells, and tumours left to develop before treatment with intraperitoneal (i) gemcitabine (120 $\mu$g/g/dose) or PBS diluent (100 $\mu$l/dose) for 2 doses, each 3 days apart, or (ii) cisplatin (6 $\mu$g/g/dose) or PBS diluent (100 $\mu$l/dose), for 1 dose. Gemcitabine treatment began 9-11 days after tumour cell inoculation, and cisplatin treatment occurred 12-14 days after tumour cell inoculation; the shaded bar in (A) represents treatment duration. Tumour size (in mm$^2$) was measured daily to determine tumour growth rates (A). At the experimental end point (2-3 days after the last dose), tumour sizes (B) and tumour weights (C) were measured. Data are shown as mean ± SEM, n = 9 young and n = 12 elderly gemcitabine-treated mice, n = 9 young and n = 11 elderly cisplatin-treated mice, n = 13-15 young and n = 12-16 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing chemotherapy-treated mice to age-matched PBS control mice.

TDLN CD11c$^+$ cell proportions in chemotherapy-treated mice were similar to those in age-matched PBS controls (Figure 7.2A). The significant increase in TDLN CD11c$^+$ cell proportions with increasing tumour size seen in young untreated tumour-bearing mice in Chapter 6 (Figure 6.2B) was maintained in young cisplatin (r =
0.8452, \( p = 0.006 \); Figure 7.2B), but not in young gemcitabine-treated mice (Figure 7.2C). In Chapter 6, elderly untreated tumour-bearing mice did not demonstrate a significant correlation between TDLN CD11c\(^+\) cell proportions and tumour size (Figure 6.2C); yet their gemcitabine- and cisplatin-treated counterparts demonstrated increasing TDLN CD11c\(^+\) cell proportions with increasing tumour size (likely non-responders), reaching significance for cisplatin (\( r = 0.7, p = 0.02 \); Figure 7.2D), and gemcitabine (\( r = 0.5245, p = 0.08 \); Figure 7.2E).

**Figure 7.2 Increased proportions of CD11c\(^+\) in TDLNs of elderly chemotherapy-treated mice with increasing tumour size**

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c\(^+\) cells and analysed by flow cytometry. Proportions of CD11c\(^+\) cells in TDLNs (A), and the correlation between proportions of TDLN CD11c\(^+\) cells and tumour weight in cisplatin-treated young (B) and elderly mice (D), and gemcitabine-treated young (C) and elderly mice (E) were measured. Data in (A) are shown as mean ± SEM, \( n = 9 \) young and \( n = 12 \) elderly gemcitabine-treated mice, \( n = 9 \) young and \( n = 11 \) elderly cisplatin-treated mice, \( n = 14 \) young and \( n = 16 \) elderly PBS control mice.
Gemcitabine reduced CD11b⁺CD8α⁺CD4⁻ cDC proportions in TDLNs in young and elderly mice, relative to their age-matched PBS controls (young p = 0.0003, elderly p < 0.0001; Figure 7.3A); nonetheless, there were still more CD11b⁺CD8α⁺CD4⁻ cDCs in TDLNs in elderly relative to young gemcitabine-treated mice (p = 0.008; Figure 7.3A). Gemcitabine also reduced CD8α⁺CD11b⁻ cDC proportions in elderly, but not young TDLNs, relative to their age-matched PBS controls (p = 0.003; Figure 7.3B), however the variable data seen in young mice blurred potential age-related differences. TDLN pDC proportions remained elevated in elderly, compared to young, gemcitabine-treated mice (p = 0.0007; Figure 7.3C). Interestingly, gemcitabine-treated elderly mice demonstrated reduced proportions of TDLN pDCs with increasing tumour size (weak responders; r = -0.8571, p = 0.01; Figure 7.4A); this was not observed in age-matched PBS control mice (Figure 7.4B), or young gemcitabine-treated mice (Figure 7.4C). Gemcitabine did not affect TDLN CD11b⁺CD8α⁺CD4⁺ cDC proportions (Figure 7.3D).

Unlike gemcitabine, cisplatin reduced elderly, but not young, CD11b⁺CD8α⁺CD4⁻ cDC TDLN proportions, relative to PBS controls (p = 0.003; Figure 7.3A), and did not affect CD8α⁺CD11b⁻ cDC proportions (Figure 7.3B). However, as tumour size increased, elderly cisplatin-treated mice demonstrated significantly decreasing TDLN CD8α⁺CD11b⁻ cDCs (r = -0.8469, p = 0.02; Figure 7.5A); this was not seen in PBS controls (Figure 7.5B), or in young cisplatin-treated mice (Figure 7.5C). Cisplatin also did not affect pDC (Figure 7.3C) or CD11b⁺CD8α⁺CD4⁺ cDC (Figure 7.3D) proportions in either age group, and the age-related difference in pDC proportions was maintained (p = 0.001; Figure 7.3C). No correlations were observed between the other TDLN DC subsets and tumour size in chemotherapy-treated mice. For the remainder of this chapter, correlation data is summarised in tables and only graphs with statistically significant correlation data shown.
Figure 7.3 Reduced proportions of CD11b⁺CD8α⁺CD4⁻ cDCs and CD8α⁺CD11b⁻ cDCs in TDLNs of chemotherapy-treated mice

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for DC subsets and analysed by flow cytometry. Proportions of CD11b⁺CD8α⁺CD4⁻ cDCs (A), CD8α⁺CD11b⁻ cDCs (B), pDCs (C), and CD11b⁺CD8α⁺CD4⁺ cDCs (D) are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
**Figure 7.4 Reduced proportions of TDLN pDCs with increasing tumour size in elderly gemcitabine-treated mice**

Young and elderly tumour-bearing mice were treated with gemcitabine or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for pDCs and analysed by flow cytometry. The correlation between proportions of TDLN pDCs and tumour weight in elderly (A) and young (C) gemcitabine-treated mice, and elderly PBS control mice (B) was measured, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 12 elderly PBS control mice.

**Figure 7.5 Reduced proportions of TDLN CD8α⁺CD11b⁻ cDCs with increasing tumour size in elderly cisplatin-treated mice**

Young and elderly tumour-bearing mice were treated with cisplatin or PBS diluent as per Figure 7.1. 2-3 days later, tumour-draining lymph nodes (TDLNs) were stained for CD8α⁺CD11b⁻ cDCs and analysed by flow cytometry. The correlation between proportions of TDLN CD8α⁺CD11b⁻ cDCs and tumour weight in elderly (A) and young (C) cisplatin-treated mice and elderly PBS control mice (B) was measured, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 12 elderly PBS control mice.

### 7.2.1.3 Chemotherapy increases CD80, TNF-α, GAL-9, PD-L1, IL-10 and TGF-β on CD11c⁺ cells in elderly TDLNs

The effects of gemcitabine and cisplatin on aging CD11c⁺ cell function was also examined, and where changes to the percentage of cells positive for a marker were similar to expression levels of that marker, only the percentage data is shown.
Regardless of age, gemcitabine increased expression levels of MHC class II (young $p = 0.03$, elderly $p = 0.003$; Figure 7.6A), CD86 (young $p = 0.048$, elderly $p = 0.01$; Figure 7.6B), IFN-γ (young $p = 0.035$, elderly $p = 0.03$; Figure 7.6C) and CD39 (young $p = 0.01$, elderly $p = 0.04$; Figure 7.6D) on TDLN CD11c+ cells, relative to age-matched PBS controls; although percentages of cells positive for these markers were not altered (Appendix F: Figures 1A-1D).

Figure 7.6 Gemcitabine increases MHC-II, CD86, IFN-γ and CD39 on CD11c+ cells in young and elderly TDLNs
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c+ cells, MHC class II, CD86, CD39 and intracellular IFN-γ, and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of MHC class II (A), CD86 (B), IFN-γ (C) and CD39 (D) on CD11c+ cells were measured. Data are shown as mean ± SEM, $n = 6$ young and $n = 8$ elderly gemcitabine-treated mice, $n = 6$ young and $n = 7$ elderly cisplatin-treated mice, $n = 11$ young and $n = 12$ elderly PBS control mice, * $p<0.05$, ** $p<0.005$ comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
Gemcitabine increased expression levels and percentages of IL-12+ (young p = 0.01, elderly p < 0.0001; Figure 7.7A) and CD73+ (young p = 0.02, elderly p = 0.001; Figure 7.7B) CD11c+ cells in young and elderly TDLNs, relative to age-matched PBS control mice.

Figure 7.7 Gemcitabine increases IL-12 and CD73 on CD11c+ cells in young and elderly TDLNs

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c+ cells, CD73 and intracellular IL-12, and analysed by flow cytometry. Percentages of CD11c+ cells positive for IL-12 (A) and CD73 (B) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.

Chemotherapy exerted several age-specific effects on TDLN CD11c+ cells. In elderly, but not young mice, gemcitabine and cisplatin induced up-regulation of percentages of IL-10+ TDLN CD11c+ cells (gemcitabine p = 0.01, cisplatin p = 0.04; Figure 7.8A) that were associated with increased IL-10 (data not shown) and GAL-9 expression levels (gemcitabine p = 0.025, cisplatin p = 0.03; Figure 7.8B), relative to controls. Elderly, but not young, gemcitabine-treated mice also up-regulated percentages and expression levels of TNF-α+ cells (p = 0.0005; Figure 7.8C), as well as CD80 expression (p = 0.016; Figure 7.8D), relative to controls. Additionally, elderly gemcitabine-treated mice demonstrated significantly higher expression levels and percentages of CD11c+ cells positive for IL-10 (p = 0.04; Figure 7.8A) and TNF-α (p = 0.0007; Figure 7.8C) compared to their younger counterparts. In contrast, cisplatin
induced increased expression levels and percentages of IL-12⁺ (p = 0.06; Figure 7.7A) and CD73⁺ (p = 0.04; Figure 7.7B) TDLN CD11c⁺ cells, in elderly, but not young, mice relative to controls.

![Graphs A, B, C, D showing data distribution for IL-10, GAL-9, TNF-α, and CD80 expression levels.]

**Figure 7.8** Chemotherapy increases IL-10, GAL-9, TNF-α and CD80 on CD11c⁺ cells in elderly TDLNs
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c⁺ cells, CD80, galectin-9 (GAL-9), and intracellular TNF-α and IL-10, and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for IL-10 (A) and TNF-α (C), and expression levels (measured as geometric mean fluorescence intensity; MFI) of GAL-9 (B) and CD80 (D) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.

Examination of the relationship between marker expression on TDLN CD11c⁺ cells and tumour size revealed further age-specific effects of chemotherapy. Young gemcitabine-treated mice responding to chemotherapy demonstrated significant
negative correlations between tumour size and percentages of CD40⁺, CD80⁺, CD86⁺ and CD39⁺ CD11c⁺ cells (data shown in Figures 7.9A-7.9D and Table 7.1) and CD86 expression levels (data not shown); no correlations were seen in young untreated mice or elderly gemcitabine-treated mice (Table 7.1).

Figure 7.9 Percentages of CD40⁺, CD80⁺, CD86⁺ and CD39⁺ CD11c⁺ cells decrease with tumour size in TDLNs of young gemcitabine-treated mice
Young tumour-bearing mice were treated with gemcitabine as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c⁺ cells, CD40, CD80, CD86 and CD39, and analysed by flow cytometry. The correlation between percentages of CD11c⁺ cells positive for CD40 (A), CD80 (B), CD86 (C) and CD39 (D) and tumour weight was measured, n = 6 young gemcitabine-treated mice.

Table 7.1 Correlation between markers on TDLN CD11c⁺ cells and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice Gemcitabine</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD40⁺ CD11c⁺ cells</td>
<td>r = 0.3576</td>
<td>r = -0.9429</td>
<td>r = 0.2797</td>
<td>r = 0.4286</td>
</tr>
<tr>
<td></td>
<td>p = 0.3</td>
<td>p = 0.02</td>
<td>p = 0.4</td>
<td>p = 0.3</td>
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<tr>
<td>% CD80⁺ CD11c⁺ cells</td>
<td>r = 0.4061</td>
<td>r = -0.9429</td>
<td>r = 0.1399</td>
<td>r = 0.3571</td>
</tr>
<tr>
<td></td>
<td>p = 0.2</td>
<td>p = 0.02</td>
<td>p = 0.7</td>
<td>p = 0.4</td>
</tr>
<tr>
<td>% CD86⁺ CD11c⁺ cells</td>
<td>r = -0.1033</td>
<td>r = -0.9429</td>
<td>r = -0.0455</td>
<td>r = 0.5714</td>
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<tr>
<td></td>
<td>p = 0.8</td>
<td>p = 0.02</td>
<td>p = 0.9</td>
<td>p = 0.2</td>
</tr>
<tr>
<td>% CD39⁺ CD11c⁺ cells</td>
<td>r = 0.2249</td>
<td>r = -0.9429</td>
<td>r = 0.3147</td>
<td>r = 0.3810</td>
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<tr>
<td></td>
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<td>p = 0.02</td>
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</table>
In contrast, elderly weak/non-responding cisplatin-treated mice showed significant increases in percentages of CD80⁺, CD86⁺, PD-L1⁺ and TGF-β⁺ CD11c⁺ cells (Figures 7.10A-7.10D and Table 7.2), as well as increases in expression levels of CD80, CD86, PD-L1 (data not shown) and IL-10 (Figure 7.10E and Table 7.2), with increasing tumour size; again, this was not observed in age-matched PBS control mice or young cisplatin-treated mice.

Figure 7.10 Increased CD80, CD86, PD-L1, TGF-β and IL-10 on TDLN CD11c⁺ cells from elderly cisplatin-treated mice as tumour size increases
Elderly tumour-bearing mice were treated with cisplatin as per Figure 7.1. 2-3 days later, tumour-draining lymph nodes (TDLNs) were stained for CD11c⁺ cells, CD80, CD86, PD-L1, and intracellular IL-10 and TGF-β, and analysed by flow cytometry. The correlation between tumour weight and percentages of CD11c⁺ cells positive for CD80 (A), CD86 (B), PD-L1 (C), and TGF-β (D), and expression levels of IL-10 (shown as geometric mean fluorescence intensity; MFI; E) was measured, n = 7 elderly cisplatin-treated mice.

Table 7.2 Correlation between markers on TDLN CD11c⁺ cells and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice Cisplatin</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD80⁺ CD11c⁺ cells</td>
<td>r = 0.4061</td>
<td>r = 0.4857</td>
<td>r = 0.1399</td>
<td>r = 0.7748</td>
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</tr>
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<td>% CD86⁺ CD11c⁺ cells</td>
<td>r = -0.1033</td>
<td>r = 0.2571</td>
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<td>% PD-L1⁺ CD11c⁺ cells</td>
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<td>p = 0.7</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>% TGF-β⁺ CD11c⁺ cells</td>
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<td>r = -0.2571</td>
<td>r = -0.0839</td>
<td>r = 0.8571</td>
</tr>
<tr>
<td></td>
<td>p = 0.7</td>
<td>p = 0.7</td>
<td>p = 0.8</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>IL-10 MFI of CD11c⁺ cells</td>
<td>r = -0.1152</td>
<td>r = 0.2571</td>
<td>r = 0.1189</td>
<td>r = 0.7857</td>
</tr>
<tr>
<td></td>
<td>p = 0.8</td>
<td>p = 0.7</td>
<td>p = 0.7</td>
<td>p = 0.048</td>
</tr>
</tbody>
</table>
Age-related differences in marker expression on TDLN CD11c+ cells observed in tumour-bearing untreated mice were maintained in chemotherapy-treated mice: i.e. increased IL-12 (Figure 7.7A), CD73 (Figure 7.7B), MHC class I (Appendix F: Figure 2A), CD80 (Appendix F: Figure 2B), and TGF-β (Appendix F: Figure 2C) on elderly CD11c+ cells, relative to their younger counterparts. No age-related differences or correlation between marker expression and tumour size were seen in the remaining markers examined (Appendix F: Figures 3A-3D and data not shown).

7.2.1.4 Chemotherapy increases pDCs and reduces CD11b+CD8α-CD4- cDCs in tumours

Cisplatin increased CD11c+ cell proportions in young, but not elderly, tumours relative to age-matched PBS controls (p = 0.03; Figure 7.11A) contributing to higher proportions of tumour-associated CD11c+ cells in young, compared to elderly, cisplatin-treated mice (p = 0.0005; Figure 7.11A). In contrast, gemcitabine did not induce any age-related differences in CD11c+ cell proportions in tumours (Figure 7.11A). Both chemotherapies led to: (i) increased tumour-associated pDC proportions in both age groups (gemcitabine: young p = 0.09 and elderly p < 0.0001; cisplatin: young p = 0.01 and elderly p < 0.0001; Figure 7.11B) leading to significantly elevated pDCs in elderly gemcitabine-treated tumours (p = 0.04; Figure 7.11B); and (ii) decreased CD11b+CD8α-CD4- cDCs (gemcitabine: young p = 0.005 and elderly p = 0.0001; cisplatin: young p = 0.01 and elderly p = 0.04; Figure 7.11C) relative to age-matched PBS controls, leading to significantly reduced CD11b+CD8α-CD4- cDCs in elderly gemcitabine-treated tumours (p = 0.008; Figure 7.11C). Both chemotherapies reduced CD8α+CD11b- cDCs in tumours from young, but not elderly, mice compared to PBS controls (p = 0.001 for gemcitabine, p = 0.04 for cisplatin; Figure 7.11D). Increasing tumour size correlated with increased CD11b+CD8α-CD4- cDCs in elderly gemcitabine-treated tumours, as well as increased pDCs and reduced CD8α+CD11b- cDCs in elderly cisplatin-treated tumours (Figures 7.12A-7.12C and Table 7.3); whilst no correlations were observed for these subsets in age-matched PBS-treated tumours or their younger counterparts (Table 7.3). CD11b+CD8α-CD4+ cDCs were not affected by gemcitabine or cisplatin (Appendix F:...
Figure 4), and the other DC subsets examined did not show any correlation with tumour size (data not shown).

Figure 7.11 Increased pDCs and reduced CD11b^CD8α^CD4^- cDCs in tumours of chemotherapy-treated mice
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11c^ cells and DC subsets and analysed by flow cytometry. Proportions of CD11c^ cells (A), pDCs (B), CD11b^CD8α^CD4^- cDCs (C), and CD8α^CD11b^- cDCs (D) within tumours are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 7-11 elderly cisplatin-treated mice, n = 11-14 young and n = 12-16 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
Figure 7.12 Increased CD11b⁺CD8α⁺CD4⁻ cDCs and pDCs, and reduced CD8α⁺CD11b⁻ cDCs in tumours of elderly chemotherapy-treated mice with increasing tumour size
Elderly tumour-bearing mice were treated with gemcitabine or cisplatin as per Figure 7.1. 2-3 days after the last dose, tumours were stained for DC subsets and analysed by flow cytometry. The correlation between tumour weight and proportions of CD11b⁺CD8α⁺CD4⁻ cDCs (A), pDCs (B) and CD8α⁺CD11b⁻ cDCs (C) was measured, n = 8 elderly gemcitabine-treated mice, and n = 7 elderly cisplatin-treated mice.

Table 7.3 Correlation between tumour-associated DC subsets and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice Gemcitabine</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD11b⁺CD8α⁺CD4⁻ cDCs</td>
<td>r = 0.3697 p = 0.3</td>
<td>r = -0.0857 p = 0.9</td>
<td>r = 0.4615 p = 0.1</td>
<td>r = 0.8333 p = 0.02</td>
</tr>
<tr>
<td>% pDCs</td>
<td>r = -0.4182 p = 0.2</td>
<td>r = 0.3714 p = 0.5</td>
<td>r = -0.4273 p = 0.2</td>
<td>r = 0.9643 p = 0.003</td>
</tr>
<tr>
<td>% CD8α⁺CD11b⁻ cDCs</td>
<td>r = 0.0243 p = 0.9</td>
<td>r = -0.0883 p = 0.8</td>
<td>r = -0.2867 p = 0.4</td>
<td>r = -0.8929 p = 0.01</td>
</tr>
</tbody>
</table>

7.2.1.5 Chemotherapy increases MHC class I/II, CD86, IFN-γ, CD73, A2A and A2B receptors and TGF-β on tumour CD11c⁺ cells

Gemcitabine induced several changes in young and elderly tumour-associated CD11c⁺ cells that were not observed with cisplatin; these were: increased expression of MHC class I (young p = 0.02, elderly p = 0.0003; Figure 7.13A), CD73 (young p = 0.04, elderly p = 0.06; Figure 7.13B), A2A receptor (young p = 0.08, elderly p = 0.01; Figure 7.13C) and A2B receptor (young p = 0.03, elderly p = 0.006; Figure 7.13D), although percentages of cells positive for these markers did not change (Appendix F: Figures 5A-5D), as well as increased expression levels and percentages of TGF-β⁺ cells (young p = 0.001, elderly p < 0.0001; Figure 7.14A), relative to age-matched PBS controls.
Gemcitabine and cisplatin also induced some shared changes in CD11c+ cells, regardless of age: expression levels and percentages of CD11c+ cells positive for MHC class II (young p = 0.0003 and p = 0.02, elderly p = 0.0002 and p = 0.006; Figure 7.14B), CD86 (young p = 0.002 and p = 0.08, elderly p < 0.0001 and p = 0.002; Figure 7.14C) and IFN-γ (young p = 0.001 and p = 0.048, elderly p < 0.0001 and p = 0.0049; Figure 7.14D) increased relative to age-matched PBS controls.

![Figure 7.13 Gemcitabine increases MHC-I, CD73, A2AR and A2BR on tumour-associated CD11c+ cells](image)

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, MHC class I, CD73, A2A receptor (A2AR) and A2B receptor (A2BR), and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of MHC class I (A), CD73 (B), A2AR (C), and A2BR (D) on CD11c+ cells were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 7-10 elderly cisplatin-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice. * = p<0.05, *** = p<0.0005, comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
Figure 7.14 Chemotherapy increases MHC-II, CD86, IFN-γ and TGF-β on tumour-associated CD11c+ cells

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, MHC class II, CD86, and intracellular IFN-γ and TGF-β, and analysed by flow cytometry. Percentages of CD11c+ cells positive for TGF-β (A), MHC class II (B), CD86 (C), and IFN-γ (D) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 11-12 elderly PBS control mice. * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001, comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.

Age-specific effects of chemotherapy were observed as gemcitabine and cisplatin up-regulated expression levels and percentages of CD39+ (p = 0.0003 and p = 0.01; Figure 7.15A) and PD-L1+ (p = 0.0025 and p = 0.008; Figure 7.15B) tumour-associated CD11c+ cells in elderly mice only, relative to PBS controls. This removed the age-related differences in CD39 and PD-L1 expression seen in untreated
tumour-bearing mice (Figures 7.15A and 7.15B). Higher percentages of CD11c+ cells expressed increased levels of CD80 (p = 0.03 and p = 0.0047; Figure 7.15C) in elderly, compared to young, chemotherapy-treated tumours.

Figure 7.15 Chemotherapy increases CD80, CD39 and PD-L1 on elderly tumour-associated CD11c+ cells
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, CD80, CD39 and PD-L1, and analysed by flow cytometry. Percentages of CD11c+ cells positive for CD39 (A), PD-L1 (B), and CD80 (C) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 11-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.

Elderly weak/non-responding gemcitabine-treated mice demonstrated significant negative correlations between tumour size and MHC class I expression on CD11c+ cells and percentages of CD40+ CD11c+ cells (Figures 7.16A and 7.16B and Table
7.4); this was not seen in elderly controls or their younger counterparts (Table 7.4). In elderly cisplatin-treated mice, increasing tumour size correlated with reduced percentages of CD11c⁺ cells expressing IL-12 and CD39, as well as reduced levels of CD80 and the A2B receptor (Figures 7.16C-7.16F and Table 7.4); this was not seen in elderly controls or young cisplatin-treated mice (Table 7.4). Also, the reduction in CD86⁺ CD11c⁺ cells with increasing tumour size observed in elderly tumour-bearing untreated mice was maintained in elderly cisplatin-treated mice (Table 7.4). No changes were seen for the other markers examined (Appendix F: Figures 6A-6E and data not shown).

Figure 7.16 Reduced MHC-I, CD40, IL-12, CD39, CD80 and A2BR on CD11c⁺ cells in elderly chemotherapy-treated tumours with increasing tumour size
Elderly tumour-bearing mice were treated with gemcitabine or cisplatin as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11c⁺ cells, MHC class I, CD40, CD80, CD39, A2B receptor (A2BR), and intracellular IL-12, and analysed by flow cytometry. The correlation between tumour weight and expression levels (measured as geometric mean fluorescence intensity; MFI) of MHC class I (A) and percentages of CD11c⁺ cells positive for CD40 (B) in elderly gemcitabine-treated mice, and the correlation between tumour weight and percentages of CD11c⁺ cells positive for IL-12 (C) and CD39 (D), and MFI expression levels of CD80 (E) and A2BR (F) in elderly cisplatin-treated mice was measured, n = 8 elderly gemcitabine-treated mice, and n = 7 elderly cisplatin-treated mice.
### Table 7.4 Correlation between markers on tumour CD11c+ cells and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice Gemcitabine</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC-I MFI of CD11c+ cells</td>
<td>r = -0.2242, p = 0.5</td>
<td>r = -0.4286, p = 0.4</td>
<td>r = -0.1401, p = 0.7</td>
<td>r = -0.8095, p = 0.02</td>
</tr>
<tr>
<td>% CD40+ CD11c+ cells</td>
<td>r = -0.0909, p = 0.8</td>
<td>r = 0.3143, p = 0.6</td>
<td>r = 0.0454, p = 0.9</td>
<td>r = -0.7381, p = 0.046</td>
</tr>
</tbody>
</table>

% CD86+ CD11c+ cells
% IL-12+ CD11c+ cells
% CD39+ CD11c+ cells
CD80 MFI of CD11c+ cells
A2BR MFI of CD11c+ cells

In summary, regardless of age: (i) chemotherapy further reduces CD11b+CD8α+CD4+ cDCs in TDLNs and tumours whilst increasing tumour-associated pDCs, suggesting compromised Th2 activation and increased suppression within tumours; (ii) chemotherapy increases the ability of tumour-associated CD11c+ cells to activate effector CD4+ T cells via increased MHC class II, CD86 and IFN-γ; however, gemcitabine may simultaneously increase the suppressive activity of tumour-associated CD11c+ cells due to elevated CD73, A2A receptor, A2B receptor and TGF-β.

With aging: (i) pDC proportions remain higher in elderly TDLNs and tumours, suggesting increased regulatory activity with chemotherapy; (ii) whilst chemotherapy improves the T cell-activating potential of elderly TDLN CD11c+ cells due to increased CD80, TNF-α and IL-12, it also increases their suppressive activity via CD73, GAL-9 and IL-10, and (iii) chemotherapy increases the regulatory activity of elderly tumour-associated CD11c+ cells via increased CD39 and PD-L1. In young mice with small tumours responding to gemcitabine, TDLN CD11c+ cells are more activated due to higher CD40, CD80 and CD86. In elderly mice with larger tumours that are not responding to cisplatin: (i) reductions in TDLN and tumour-associated CD8α+CD11b+ cDCs with increasing tumour size suggests that generation of CD8+...
cytotoxic T cells is compromised; and (ii) TDLN CD11c+ cells are more suppressive due to increased PD-L1, IL-10 and TGF-β. In elderly weak/non-responders to gemcitabine and cisplatin, tumour-associated CD11c+ cells have reduced MHC class I, CD40, CD80, CD86 and/or IL-12 with increasing tumour size, suggesting lower T cell activation in tumours.

7.2.2 Examining the effects of aging, cancer and chemotherapy on murine T cells

7.2.2.1 Gemcitabine reduces Treg proportions in young TDLNs

The effects of aging, cancer and chemotherapy on T cells in TDLNs and tumours of young (2-5 months; n=6-9) and elderly (22-24 months; n=7-12) mesothelioma tumour-bearing mice treated with chemotherapy were also examined. Gemcitabine reduced TDLN Treg proportions in young mice, relative to age-matched PBS controls (p = 0.04; Figure 7.17), removing the age-related differential (Figure 7.17). In contrast, cisplatin did not affect TDLN Treg proportions (Figure 7.17). No chemotherapy-induced changes in TDLN CD8+ and CD4+ T cell proportions were seen, and the age-related differences remained (Appendix F: Figures 7A and 7B). No correlation was observed between proportions of TDLN T cell subsets and tumour size in chemotherapy-treated mice (data not shown).

Figure 7.17 Gemcitabine reduces Treg proportions in TDLNs of young mice
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4+CD25+FoxP3+ Tregs and analysed by flow cytometry. Proportions of Tregs are shown as mean ± SEM, n = 9 young and n = 12 elderly gemcitabine-treated mice, n = 9 young and n = 11 elderly cisplatin-treated mice, n = 14 young and n = 16 elderly PBS control mice, * = p<0.05, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
7.2.2.2 Chemotherapy increases PD-1, IL-10 and TGF-β on T cells in elderly TDLNs

To investigate the effects of chemotherapy on T cell function, T cells in TDLNs and tumours from young and elderly mice were stained for T cell activation/effector and regulatory markers as previously described.

Chemotherapy exerted age-specific effects on TDLN CD8+ T cells. Gemcitabine and cisplatin reduced the percentage of CD8+ T cells in elderly TDLNs expressing CD73, compared to age-matched controls (gemcitabine p = 0.07, cisplatin p = 0.04; Figure 7.18A), resulting in similar CD73 expression in elderly and young chemotherapy-treated mice (Figure 7.18A). Gemcitabine, but not cisplatin, further increased expression levels and percentages of elderly CD8+ T cells positive for PD-1 (p = 0.047; Figure 7.18B) and IL-10 (p = 0.01; Figure 7.18C), as well as expression levels of ICOS (p = 0.04; Figure 7.18D) and TGF-β (p = 0.01; Figure 7.18E), relative to controls. The age differentials in PD-1, IL-10, TGF-β and ICOS were maintained in TDLNs of chemotherapy-treated mice, whereby elderly CD8+ T cells had higher expression compared to their younger counterparts (Figures 7.18B-7.18E). When considering the effects of tumour size, elderly weak/non-responders to gemcitabine demonstrated reduced percentages of perforin+ CD8+ T cells and TGF-β expression levels in TDLNs as tumour size increased (Figures 7.19A and 7.19B and Table 7.5); this was not seen in PBS controls or young gemcitabine-treated mice (Table 7.5). TDLN CD8+ T cells from elderly cisplatin-treated mice had increased percentages of A2B receptor+ cells, but reduced percentages of LAG-3+ cells as tumour size increased (Figures 7.19C and 7.19D and Table 7.5), whilst this was not seen in PBS controls and young counterparts (Table 7.5). No further chemotherapy-induced changes were seen in the other markers examined on TDLN CD8+ T cells (Appendix F: Figures 8A-8D and 9A-9D).
Figure 7.18 Chemotherapy reduces CD73 and increases PD-1, ICOS, IL-10 and TGF-β on CD8+ T cells in elderly TDLNs

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8+ T cells, CD73, PD-1, ICOS, and intracellular IL-10 and TGF-β, and analysed by flow cytometry. Percentages of CD8+ T cells positive for CD73 (A), PD-1 (B) and IL-10 (C), and expression levels (shown as geometric mean fluorescence intensity; MFI) of ICOS (D) and TGF-β (E) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 6-7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
Elderly tumour-bearing mice were treated with gemcitabine or cisplatin as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8⁺ T cells, LAG-3, A2B receptor (A2BR), and intracellular perforin and TGF-β, and analysed by flow cytometry. The correlation between tumour weight and percentages of perforin⁺ CD8⁺ T cells (A) and TGF-β expression levels (shown as geometric mean fluorescence intensity; MFI) on CD8⁺ T cells (B) in elderly gemcitabine-treated mice, and A2BR⁺ (C) and LAG-3⁺ (D) CD8⁺ T cells in elderly cisplatin-treated mice were measured, n = 8 elderly gemcitabine-treated mice, n = 7 elderly cisplatin-treated mice.

Table 7.5 Correlation between markers on TDLN CD8⁺ T cells and tumour size

<table>
<thead>
<tr>
<th>Markers</th>
<th>Young mice PBS</th>
<th>Young mice Gemcitabine</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>% perforin⁺ CD8⁺ T cells</td>
<td>r = -0.0546, p = 0.9</td>
<td>r = -0.0290, p = 0.9</td>
<td>r = 0.1226, p = 0.7</td>
<td>r = -0.7619, p = 0.04</td>
</tr>
<tr>
<td>TGF-β MFI of CD8⁺ T cells</td>
<td>r = -0.5879, p = 0.1</td>
<td>r = 0.3714, p = 0.5</td>
<td>r = -0.0979, p = 0.8</td>
<td>r = -0.8571, p = 0.01</td>
</tr>
</tbody>
</table>

Again, gemcitabine, but not cisplatin, up-regulated expression levels and percentages of CD4⁺ T cells expressing IL-10 (p = 0.04; Figure 7.20A) and TGF-β (p = 0.04; Figure 7.20B) in elderly TDLNs, relative to their PBS controls, with the age-related differences in their expression maintained.
Figure 7.20 Gemcitabine increases IL-10 and TGF-β on CD4⁺ T cells in elderly TDLNs

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4⁺ T cells and intracellular IL-10 and TGF-β, and analysed by flow cytometry. Percentages of CD4⁺ T cells positive for IL-10 (A) and TGF-β (B) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.

A significant negative correlation was observed between tumour size and TGF-β expression levels on CD4⁺ T cells from TDLNs of elderly gemcitabine-treated mice (Figure 7.21A and Table 7.6). In contrast, elderly cisplatin-treated mice showed increasing percentages of IFN-γ⁺ cells and expression levels of CD25 and perforin on CD4⁺ T cells in TDLNs as tumour size increased (Figures 7.21B-7.21D and Table 7.6). These correlations were not observed in age-matched PBS control mice or chemotherapy-treated counterparts (Table 7.6). Chemotherapy did not alter the other markers examined on TDLN CD4⁺ T cells (Appendix F: Figures 10A-10F and 11A-11E).

### Table 7.6 Correlation between markers on TDLN CD4⁺ T cells and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice Gemcitabine</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Gemcitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TGF-β MFI of CD4⁺ T cells</strong></td>
<td>r = -0.5152 p = 0.1</td>
<td>r = 0.5429 p = 0.3</td>
<td>r = 0.0979 p = 0.8</td>
<td>r = -0.7857 p = 0.03</td>
</tr>
<tr>
<td><strong>% IFN-γ⁺ CD4⁺ T cells</strong></td>
<td>r = -0.3212 p = 0.4</td>
<td>r = 0.3769 p = 0.5</td>
<td>r = 0.0421 p = 0.9</td>
<td>r = 0.8571 p = 0.03</td>
</tr>
<tr>
<td><strong>CD25 MFI of CD4⁺ T cells</strong></td>
<td>r = 0.2473 p = 0.4</td>
<td>r = 0.45 p = 0.2</td>
<td>r = 0.1382 p = 0.6</td>
<td>r = 0.6182 p = 0.048</td>
</tr>
<tr>
<td><strong>Perforin MFI of CD4⁺ T cells</strong></td>
<td>r = -0.0667 p = 0.9</td>
<td>r = 0.0286 p &gt; 0.9999</td>
<td>r = 0.3077 p = 0.3</td>
<td>r = 0.8929 p = 0.01</td>
</tr>
</tbody>
</table>

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Elderly tumour-bearing mice were treated with gemcitabine or cisplatin as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4+ T cells, CD25, and intracellular IFN-γ, perforin and TGF-β, and analysed by flow cytometry. The correlation between tumour weight and TGF-β expression levels (shown as geometric mean fluorescence intensity; MFI) on CD4+ T cells in elderly gemcitabine-treated mice (A), and percentages of IFN-γ+ CD4+ T cells (B) and levels of CD25 (C) and perforin (D) on CD4+ T cells in elderly cisplatin-treated mice were measured, n = 8 elderly gemcitabine-treated mice, n = 7-11 elderly cisplatin-treated mice.

7.2.2.3 Chemotherapy reduces perforin, and increases the A2A and A2B receptors, PD-1, LAG-3 and/or TGF-β on elderly tumour CD8+ and CD4+ T cells

No chemotherapy-induced changes were observed in tumour-associated CD4+ T cell and Treg proportions, meaning age-related differences were maintained (p = 0.02 and p = 0.002 for CD4+ T cells; Appendix F: Figure 12A, p = 0.02 and p = 0.002 for Tregs; Appendix F: Figure 12B). No age- or chemotherapy-induced changes were seen in tumour-associated CD8+ T cell proportions (Appendix F: Figure 12C). The negative correlation observed between tumour size and tumour-associated CD8+ T cell proportions in young tumour-bearing untreated mice was maintained in young chemotherapy-treated mice (gemcitabine: r = -0.6167, p = 0.08, cisplatin: r
= -0.7333, p = 0.03). Furthermore, young cisplatin-treated mice demonstrated increasing proportions of tumour-associated Tregs as tumour size increased (Figure 7.22 and Table 7.7); this was not seen in PBS controls or elderly cisplatin-treated mice (Table 7.7).

![Image](image.png)

**Figure 7.22 Tumour Treg proportions increase with tumour size in young cisplatin-treated mice**

Young tumour-bearing mice were treated with cisplatin as per Figure 7.1. 2-3 days after the last dose, tumours were stained for Tregs, and analysed by flow cytometry. The correlation between tumour weight and percentages of Tregs in tumours in young cisplatin-treated mice was measured, n = 9 young cisplatin-treated mice.

**Table 7.7 Correlation between tumour Treg proportions and tumour size**

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice Cisplatin</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Tregs</td>
<td>r = -0.0551</td>
<td>r = 0.7833</td>
<td>r = -0.1740</td>
<td>r = 0.1909</td>
</tr>
<tr>
<td></td>
<td>p = 0.9</td>
<td>p = 0.02</td>
<td>p = 0.5</td>
<td>p = 0.6</td>
</tr>
</tbody>
</table>

Gemcitabine, but not cisplatin, induced common changes in young and elderly CD8+ T cells within tumours; i.e. increased expression levels and percentages of tumour-associated CD8+ T cells expressing CD25 (young p = 0.03, elderly p = 0.001; Figure 7.23A) and IFN-γ (young p = 0.0003, elderly p = 0.004; Figure 7.23B), relative to age-matched PBS controls, and the age differential in IFN-γ expression remained (p = 0.003; Figure 7.23B). Gemcitabine also induced small, but significant, increases in the percentage of tumour-associated CD8+ T cells expressing LAG-3 (young p = 0.01, elderly p = 0.02; Figure 7.23C), which was associated with increased expression levels of LAG-3 (data not shown) and ICOS (young p = 0.0047, elderly p = 0.01; Figure 7.23D), compared to age-matched PBS controls.
Figure 7.23 Gemcitabine increases CD25, IFN-γ, LAG-3 and ICOS on CD8+ T cells in tumours

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, CD25, LAG-3, ICOS, and intracellular IFN-γ, and analysed by flow cytometry. Percentages of CD8+ T cells positive for CD25 (A), IFN-γ (B) and LAG-3 (C), and expression levels (measured as geometric mean fluorescence intensity; MFI) of ICOS (D) were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 7-11 elderly cisplatin-treated mice, n = 6-9 young and n = 8-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.

Chemotherapy also exerted age-specific effects on tumour-associated CD8+ T cells.

Both gemcitabine and cisplatin reduced perforin expression levels on elderly, but not young, CD8+ T cells, relative to PBS controls (gemcitabine p = 0.047, cisplatin p = 0.04; Figure 7.24A). Additionally, gemcitabine increased expression levels and percentages of CD8+ T cells positive for the A2A receptor (p = 0.002; Figure 7.24B), PD-1 (p = 0.03; Figure 7.24C), and TGF-β (p = 0.01; Figure 7.24D) in elderly, but not
young, tumours compared to controls, nonetheless, elderly TGF-β expression remained below that seen in young mice (p = 0.03; Figure 7.24D). CD8+ T cells from young gemcitabine-treated tumours up-regulated CD73 (p = 0.01; Figure 7.24E), thus removing the age differential seen in untreated mice (Figure 7.24E).

Figure 7.24 Chemotherapy reduces perforin and increases A2AR, PD-1 and TGF-β on elderly tumour CD8+ T cells
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, A2A receptor (A2AR), PD-1, CD73, and intracellular perforin and TGF-β, and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of perforin (A), and percentages of CD8+ T cells positive for A2AR (B), PD-1 (C), TGF-β (D) and CD73 (E) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 6-7 elderly cisplatin-treated mice, n = 6-9 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
In contrast, cisplatin up-regulated the percentage of CD25+ CD8+ T cells in young tumours only (p = 0.04; Figure 7.23A), accounting for lower relative CD25+ CD8+ T cells in elderly mice (p = 0.01; Figure 7.23A). Cisplatin also increased percentages of IFN-γ+ CD8+ T cells in elderly tumours, relative to PBS controls (p = 0.03; Figure 7.23B), removing age-related differences in seen in untreated tumour-bearing mice (Figure 7.23B). When considering the effects of tumour size, expression levels and percentages of tumour-associated perforin+ and TGF-β+ CD8+ T cells decreased, whilst expression levels of the A2B receptor increased, with increasing tumour size in elderly weak/non-responding cisplatin-treated mice (Figures 7.25A-7.25C and Table 7.8); this was not seen in age-matched PBS controls or young cisplatin-treated mice (Table 7.8). Chemotherapy did not induce any changes in the other markers examined (Appendix F: Figures 13A-13D).

![Figure 7.25 Reduced perforin and TGF-β, and increased A2BR on CD8+ T cells in elderly cisplatin-treated tumours with increasing tumour size](image)

Elderly tumour-bearing mice were treated with cisplatin as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, A2B receptor (A2BR), and intracellular perforin and TGF-β, and analysed by flow cytometry. The correlation between tumour weight and percentages of perforin+ CD8+ T cells (A), TGF-β+ CD8+ T cells (B), and expression levels (shown as geometric mean fluorescence intensity; MFI) of A2BR on CD8+ T cells (C) in elderly cisplatin-treated tumours was measured, n = 7 elderly cisplatin-treated mice.

| Table 7.8 Correlation between markers on tumour CD8+ T cells and tumour size |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | Young mice PBS  | Young mice Cisplatin | Elderly mice PBS | Elderly mice Cisplatin |
| % perforin+ CD8+ T cells       | r = 0.0061      | r = -0.1429       | r = -0.1958      | r = -0.7857       |
|                                 | p = 0.99        | p = 0.8           | p = 0.5          | p = 0.048         |
| % TGF-β+ CD8+ T cells          | r = 0.0909      | r = -0.6          | r = -0.1399      | r = -0.9286       |
|                                 | p = 0.8         | p = 0.2           | p = 0.7          | p = 0.007         |
| A2BR MFI of CD8+ T cells       | r = 0.6         | r = -0.3714       | r = 0.6429       | r = 0.7857        |
|                                 | p = 0.4         | p = 0.5           | p = 0.1          | p = 0.048         |
Regardless of age, gemcitabine increased expression levels and percentages of tumour-associated CD4⁺ T cells expressing CD39 (young p = 0.0002, elderly p = 0.002; Figure 7.26A), A2A receptor (young p = 0.03, elderly p = 0.001; Figure 7.26B), PD-1 (young p = 0.006, elderly p < 0.0001; Figure 7.26C) and LAG-3 (young p = 0.0002, elderly p = 0.001; Figure 7.26D), as well as expression levels of ICOS (young p = 0.04, elderly p = 0.01; Figure 7.26E), relative to age-matched PBS controls.

Figure 7.26 Gemcitabine increases CD39, A2AR, PD-1, LAG-3 and ICOS on CD4⁺ T cells in tumours
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD4⁺ T cells, CD39, A2A receptor (A2AR), PD-1, LAG-3, and ICOS, and analysed by flow cytometry. Percentages of CD4⁺ T cells positive for CD39 (A), A2AR (B), PD-1 (C), and LAG-3 (D), and expression levels (measured as geometric mean fluorescence intensity; MFI) of ICOS (E) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 6-7 elderly cisplatin-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
Age-specific changes in tumour-associated CD4⁺ T cells were also observed with both chemotherapies reducing expression levels of perforin on elderly, but not young, CD4⁺ T cells, compared to age-matched PBS controls (gemcitabine p = 0.02, cisplatin p = 0.007; Figure 7.27A). Gemcitabine also increased expression levels and percentages of elderly CD4⁺ T cells expressing the A2B receptor (p = 0.0003; Figure 7.27B), and TGF-β (p = 0.007; Figure 7.27C), relative to PBS controls, removing the age differential seen in TGF-β expression in untreated mice (Figure 7.27C). However, CD4⁺ T cells from elderly gemcitabine-treated mice had increased expression levels and percentages of CD73⁺ cells (p = 0.03; Figure 7.27D) compared to their younger counterparts. Cisplatin increased percentages of PD-1⁺ (p = 0.02; Figure 7.26C) and LAG-3⁺ (p = 0.04; Figure 7.26D) CD4⁺ T cells, and reduced CD73⁺ CD4⁺ T cells (p = 0.02; Figure 7.27D) in elderly mice, compared to age-matched controls, again removing the age differential in CD73 expression seen in untreated mice (Figure 7.27D). Lower expression of CD25 (p = 0.02; Figure 7.27E) and ICOS (p = 0.002; Figure 7.26E) was seen on tumour-associated CD4⁺ T cells from elderly, compared to young, cisplatin-treated mice.

Elderly non-responder gemcitabine-treated mice demonstrated significant correlations between increasing tumour size and decreasing expression levels and percentages of tumour-associated PD-1⁺ CD4⁺ T cells, yet increasing CD25 expression on CD4⁺ T cells (Figures 7.28A and 7.28B and Table 7.9). In contrast, elderly non-responder cisplatin-treated mice displayed a significant correlation between increasing tumour size and increasing CD73⁺ CD4⁺ T cells (Figure 7.28C and Table 7.9). No chemotherapy-induced changes were seen in the other markers examined on tumour-associated CD4⁺ T cells (Appendix F: Figures 14A-14C and data not shown).
Figure 7.27 Chemotherapy increases A2BR and TGF-β, and reduces perforin, CD25 and CD73 on elderly CD4+ T cells in tumours

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD4+ T cells, CD25, CD73, A2B receptor (A2BR), and intracellular perforin and TGF-β, and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of perforin (A), and percentages of CD4+ T cells positive for A2BR (B), TGF-β (C), CD73 (D), and CD25 (E), and were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 7-11 elderly cisplatin-treated mice, n = 6-14 young and n = 7-16 elderly PBS control mice, * = p<0.05, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
Elderly tumour-bearing mice were treated with gemcitabine or cisplatin as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD4+ T cells, CD25, CD73, and PD-1, and analysed by flow cytometry. The correlation between tumour weight and percentages of PD-1+ CD4+ T cells (A) and levels of CD25 on CD4+ T cells (B) in elderly gemcitabine-treated mice, and percentages of CD73+ CD4+ T cells (C) in elderly cisplatin-treated mice were measured, n = 8-12 elderly gemcitabine-treated mice, n = 7 elderly cisplatin-treated mice.

<table>
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<th>Young mice PBS</th>
<th>Young mice Gemcitabine</th>
<th>Elderly mice PBS</th>
<th>Elderly mice Gemcitabine</th>
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<tr>
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<td>r = -0.2571</td>
<td>r = 0.1818</td>
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<td>CD25 MFI of CD4+ T cells</td>
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<td>r = 0.4667</td>
<td>r = 0.1176</td>
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<tr>
<td></td>
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<td>p = 0.2</td>
<td>p = 0.7</td>
<td>p = 0.049</td>
</tr>
<tr>
<td>% CD73+ CD4+ T cells</td>
<td>r = 0.4061</td>
<td>r = 0.2</td>
<td>r = 0.2028</td>
<td>r = 0.8571</td>
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<tr>
<td></td>
<td>p = 0.2</td>
<td>p = 0.7</td>
<td>p = 0.5</td>
<td>p = 0.02</td>
</tr>
</tbody>
</table>

In summary, irrespective of age: (i) chemotherapy may improve the effector function of tumour-associated CD8+ T cells by increasing CD25 and IFN-γ, however they also adopt an exhausted/regulatory phenotype due to up-regulation of CD39, ICOS and LAG-3; and (ii) chemotherapy increases the regulatory activity of tumour-associated CD4+ T cells due to increased CD39, A2A receptor, PD-1 and LAG-3.

With aging: (i) reduced proportions of CD8+ and CD4+ T cells were maintained in elderly TDLNs; (ii) the suppressive/exhausted phenotype displayed by elderly CD8+ and CD4+ T cells is exacerbated by chemotherapy, due to increased ICOS, PD-1, IL-10 and/or TGF-β; and (iii) chemotherapy may reduce the cytotoxic effector function of elderly tumour-associated CD8+ and CD4+ T cells due to decreased perforin and increase suppression/exhaustion via increased A2A and A2B receptors, PD-1, LAG-3.
and/or TGF-β. In elderly non-responders to gemcitabine, increasing tumour size reduces the effector function of elderly TDLN CD8+ T cells via reduced perforin, and increases their ability to respond to suppressive adenosine via the A2B receptor; however, the effector function of elderly TDLN CD4+ T cells may be improved due to increased CD25, IFN-γ and perforin. In elderly non-responders to cisplatin, increasing tumour size is associated with tumour-associated CD8+ T cells with reduced perforin-mediated effector function, and CD8+ and CD4+ T cells with increased regulatory activity due to increased A2B receptor or CD73 expression, respectively.
7.3 Discussion

The studies in this chapter examined whether aging influenced the effects of chemotherapy on DCs and T cells in TDLNs and tumours. Data from this study shows that one-third of the way through treatment with chemotherapy, young and elderly tumour-associated CD11c+ cells increased MHC class I and II, CD86 and IFN-γ, suggesting increased capacity to present antigens to, and activate, tumour-specific CD8+ and CD4+ T cells. In gemcitabine-treated mice, this was reflected by increased activation and effector function of tumour-associated CD8+ T cells, i.e. increased CD25 and IFN-γ. This is supported by a previous study in our laboratory showing that gemcitabine and cisplatin promote the generation of effector CD8+ T cells specific to subdominant tumour antigens (1222). Others have shown that gemcitabine improves tumour antigen cross-presentation by DCs leading to activation and proliferation of tumour antigen-specific effector CD4+ and CD8+ T cells (1193, 1258). Moreover, tumour-associated DCs from gemcitabine-treated mice were reported to increase antigen-presenting and co-stimulatory molecules that induce IFN-γ-producing CD8+ and CD4+ T cells (1217). Cisplatin has been shown to activate DCs that induce high levels of IFN-γ and IL-2 production by T cells (1264) and promote recruitment of tumour-specific, IL-2+ IFN-γ+ CD8+ T cells into tumours (1206). Chemotherapy-induced increases in APC and T cell function may contribute to mesothelioma tumour regression observed in this and other studies (1193, 1222, 1251).

There are several mechanisms by which chemotherapy may promote immune activation. A major mechanism is through immunogenic tumour cell death, which involves the release of tumour antigens and immune-activating molecules, such as calreticulin, HMGB-1 and ATP (948, 1182, 1183, 1185, 1815). Our laboratory has shown that gemcitabine and cisplatin are cytotoxic to AE17 mesothelioma cells (1222), and others have shown that both chemotherapeutic agents induce HMGB-1 and ATP release by tumour cells (1187, 1190, 1194, 1272, 1273). Chemotherapy-induced release of HMGB-1 and ATP by tumour cells promotes DC activation. HMGB-1 activates DCs by binding TLR-4 on DCs (951), whilst ATP binds P2RX7
purinergic receptors on DCs, facilitating activation of the NLRP3 inflammasome leading to IL-1β release which is required for activation and recruitment of IFN-γ-producing tumour-specific CD8+ T cells (947, 948, 1194, 1196). ATP also promotes recruitment and differentiation of activated, inflammatory DCs within chemotherapy-treated tumours (1197). The immunostimulatory effects of chemotherapy-induced release of ATP and HMGB-1 from tumour cells may account for the increased activation status of CD11c+ cells (characterised by increased MHC class I/II, CD86, IFN-γ and/or IL-12) from TDLNs and tumours seen in this study. Nonetheless, cisplatin induces early apoptosis and unlike other platinum-based chemotherapies, does not elicit all components of ICD (1187, 1190, 1191, 1272), which may account for the observation in this study that cisplatin was less efficient in mediating tumour regression than gemcitabine. As well as activating APCs by promoting ICD, chemotherapy may directly activate DCs (1201-1203) and gemcitabine exposure leads to increased expression of antigen-presenting and co-stimulatory molecules (1217) and improved T cell stimulatory ability (1220). Gemcitabine can activate the NFκB pathway (1256, 1257) which is involved in activation/maturation of APCs (658). Additionally, chemotherapy may facilitate antitumour immunity by relieving immune suppression. Gemcitabine has been reported to eliminate/reduce suppressive MDSCs (1217, 1228, 1230-1233) and Tregs (1223, 1224, 1259), whilst cisplatin reduces PD-L2 on DCs and tumour cells (1264). In this study, a reduction in TDLN Tregs was observed in young gemcitabine-treated mice, suggesting reduced suppression. Further studies are required to examine chemotherapy-induced changes in MDSCs and PD-L2 in the AE17 model.

Despite evidence of immune activation, results from this study suggest that in the later stages of chemotherapy and/or once chemotherapy ceases, an immunosuppressive environment may develop within tumours. In gemcitabine-treated tumours, CD11c+ cells, CD8+ and CD4+ T cells demonstrated simultaneous increases in activation and regulatory markers, implying transitioning to suppressor cells. In particular, up-regulation of CD73 by tumour-associated CD11c+ cells, and CD39 by tumour-associated CD8+ and CD4+ T cells suggests increased conversion of ATP (released from immunogenic tumour cell death) into suppressive adenosine
within tumours, thus confounding the immunostimulatory effects of chemotherapy (545, 1197, 1243, 1244). Additionally, tumour-associated CD11c+ cells and T cells may be more responsive to adenosine, due to up-regulation of A2A and A2B receptor expression in response to chemotherapy, leading to suppression of effector function and skewing towards regulatory function (545, 571, 1566, 1816, 1817). Administration of chemotherapy, followed by immunotherapies to block CD39, CD73 and adenosine receptors may be a beneficial strategy to alleviate adenosine-mediated suppression, improve T cell anti-tumour function and improve the efficacy of chemotherapy (545, 1243, 1320, 1815-1820). Other suppressive mechanisms also increased in tumours of chemotherapy-treated mice. Up-regulation of TGF-β by CD11c+ cells suggests increased potential to inhibit effector T cells and promote Treg development (1691), and increases in PD-1, ICOS and LAG-3 by CD8+ and/or CD4+ T cells in gemcitabine-treated tumours suggests development of exhaustion (1102, 1119). Immune regulation may also be increased via elevated proportions of tumour-associated pDCs with suppressive function within tumours. Overall, these results suggest that suppressive signals are increased within tumours and there may be a greater number of negative signals during cross-talk between tumour-associated CD11c+ cells and T cells, leading to T cell suppression. This may contribute to tumour outgrowth once chemotherapy ceases, which has been described in murine models of mesothelioma (1193, 1245-1247) and other cancers (1233). Thus, regardless of age, chemotherapy appears to exert dual effects on tumour-associated CD11c+ cells and T cells, by initially increasing their effector function, but later skewing them towards regulatory function. However, in this study, mice received one-third of the usual schedule of gemcitabine and cisplatin, and further studies are required to examine chemotherapy-induced changes after a full schedule of chemotherapy.

It is possible that tumour-associated CD11c+ cells in chemotherapy-treated mice migrate to TDLNs as evidenced by similarities to TDLN CD11c+ cells in gemcitabine-treated mice, with CD11c+ cells in both tissues expressing elevated MHC class II, CD86, IFN-γ and CD73. One study has shown that DCs from gemcitabine-treated mice retain their ability to migrate to TDLNs (1821), and another study has shown
that another chemotherapeutic agent (cyclophosphamide) improves CD8α+ cDC migration (1,199), supporting the idea that DCs can migrate from tumours to TDLNs in chemotherapy-treated mice. Further studies into the effects of gemcitabine and cisplatin on DC migration in the AE17 mesothelioma model are required. Again, CD11c+ cells within TDLNs of chemotherapy-treated mice have the potential to present antigens to, and activate T cells, particularly CD4+ T cells, due to increased MHC class II, CD86, IFN-γ and IL-12, but also have increased capacity to produce adenosine (via CD39 and CD73), and suppress T cells.

Results from this study suggest elderly mice respond to chemotherapy, and decreasing tumour burden, indicating responses to chemotherapy, was accompanied by increased cytotoxic CD8+ T cell activation. In elderly gemcitabine-treated mice, increased MHC class I and CD40 on tumour-associated CD11c+ cells with decreasing tumour burden suggests increased local tumour antigen presentation and co-stimulation of tumour-specific cytotoxic CD8+ T cells. In elderly cisplatin-treated mice, decreasing tumour burden was associated with: (i) increased CD8α+CD11b− cDCs which are highly efficient DC subsets at capturing and cross-presenting antigens from dead cells, such as tumour cells undergoing chemotherapy-induced ICD (127, 952, 1822), and are critically required for activation of tumour-specific CD8+ T cells (124, 125, 127), (ii) increased T cell-activating capacity of CD11c+ cells, due to increased CD80, CD86 and IL-12, and (iii) reduced tumour-associated pDCs. These changes suggest that in elderly mice responding to cisplatin, pDC-mediated suppression is reduced, and tumour antigen cross-presentation and activation of cytotoxic CD8+ T cells is improved; this is supported by the observation that CD8+ T cell perforin expression increased with decreasing tumour size. Furthermore, the suppressive function of elderly tumour-associated CD8+ and CD4+ T cells via the A2B receptor and CD73 appeared to be alleviated with decreasing tumour burden in elderly cisplatin-treated mice. These changes may contribute to the tumour regression observed in elderly chemotherapy-treated mice despite receiving one-third of the usual schedule. Further studies should assess the functional activity of elderly tumour-associated CD8+ T cells.
There was also evidence that elderly mice may not respond as efficiently to chemotherapy as their younger counterparts, and that additional suppressive mechanisms with age subvert responses to chemotherapy. In particular, there was a trend for increased tumour weights from elderly, compared to young, cisplatin-treated mice. This may be explained by the relationship between tumour burden and CD11c+ cell and T cell function. With increasing tumour burden, i.e. tumours less responsive to chemotherapy: (i) tumour-associated CD8α+CD11b+ cDC proportions reduced, whilst pDC proportions increased, (ii) the T cell-activating potential of CD11c+ cells reduced, and (iii) the perforin-mediated effector activity of CD8+ T cells reduced. This suggests that in larger tumours, CD8+ T cell activation is reduced, and as CD8+ T cells are required for cisplatin-mediated tumour regression (1222), this could reduce the efficacy of cisplatin in elderly mice. Additionally, age-related changes in APCs, such as reduced phagocytic capacity (68, 241, 614), antigen-presenting ability (234, 241, 244, 249) and reduced expression of danger-sensing molecules, such as TLR-4 (1823), may impair their ability to take up tumour antigens and respond to activation signals following chemotherapy-induced immunogenic tumour cell death, leading to reduced T cell activation. Furthermore, there were several age-related suppressive changes in tumours which could impair elderly immune responses during the later stages of chemotherapy and/or after chemotherapy ceases. Proportions of pDCs remained elevated in tumours of elderly gemcitabine-treated mice that could contribute to immune suppression. CD11c+ cells within elderly gemcitabine- and cisplatin-treated tumours displayed increased CD80 and PD-L1, suggesting increased potential to interact with T cells via the CD80/CTLA-4 and PD-L1/PD-1 pathways; the latter is likely to be increased due to elevated PD-1 on elderly tumour-associated CD8+ and CD4+ T cells, leading to T cell inhibition (883, 1824). Elevated CD39 on elderly CD11c+ cells suggests that conversion of ATP to adenosine may be further increased in elderly tumours. Chemotherapy may also enhance the responsiveness of elderly tumour-associated CD8+ and CD4+ T cells to adenosine via increased A2A and A2B receptors, leading to suppression (545, 547, 554, 920, 1825). This is supported by the observation that tumour-associated CD8+ and CD4+ T cells from elderly gemcitabine- and cisplatin-treated mice had decreased perforin and/or IFN-γ expression, suggesting reduced
anti-tumour cytotoxic activity (371, 405, 1826). Thus, the sequential dual effects of chemotherapy starting with enhanced immune stimulation, yet later promoting immune suppression, appear enhanced with aging and could reduce the effectiveness of chemotherapy in the elderly. Further studies examining comparisons between responders and non-responders to chemotherapy within each age group are required. Additionally, interventions such as depletion of immune cells, or blocking regulatory molecules during chemotherapy will help to determine which immune cells/pathways confer an age advantage or disadvantage to responses to chemotherapy.

The hypothesis that suppressive effects are enhanced during aging is further supported by changes in TDLNs of elderly chemotherapy-treated mice. Reduced tumour size in elderly cisplatin-treated mice was associated with higher CD8α+CD11b−cDC proportions and reduced PD-L1, IL-10 and TGF-β expression by TDLN CD11c+ cells, suggesting increased cytotoxic CD8+ T cell activation and alleviation of suppression in TDLNs of elderly mice with regressing tumours. Similarly, decreased tumour burden in elderly gemcitabine-treated mice was associated with increased perforin+ CD8+ T cells in TDLNs, suggesting increased cytotoxic activity which may contribute to the tumour regression seen in elderly mice in this study. However, these changes may be eventually outweighed by the observation that chemotherapy enhanced the regulatory environment already established in TDLNs of elderly untreated tumour-bearing mice. Gemcitabine and cisplatin increased regulatory CD73, GAL-9 and IL-10 on CD11c+ cells in elderly TDLNs, suggesting these cells are more likely to inhibit T cells through: (i) adenosine production (545, 547, 554, 920, 1825), and (ii) negative interactions via the GAL-9/TIM-3 pathway (913). Gemcitabine also increased CD80 and TNF-α on elderly CD11c+ cells, which can negatively regulate T cells via interactions with CTLA-4 (831) and induce T cell exhaustion (1810). Gemcitabine also further enhanced the age-related increases in PD-1, ICOS, IL-10 and TGF-β on elderly TDLN CD8+ and/or CD4+ T cells, suggesting increased suppressive function and/or exhaustion. Overall, this suggests that exacerbation of the regulatory environment during chemotherapy may compromise immune responses in elderly mesothelioma-bearing mice. These
observations suggest that chemotherapy should be combined with immunotherapies that block inhibitory molecules, such as CD73 (1097, 1243, 1321-1324), PD-1 (1827) and TGF-β (1828-1830), to relieve immunosuppression and improve chemotherapy efficacy in the elderly.

In summary, chemotherapy appears to initially promote activation of APCs and effector T cells, leading to tumour regression, yet may later promote suppression contributing to tumour outgrowth. With aging, chemotherapy further enhances immunosuppressive mechanisms in TDLNs and tumours, which may reduce chemotherapy efficacy in elderly mesothelioma-bearing mice. Age-related changes in DCs and T cells may also impact on immunotherapy, and this is examined in Chapter 8.
Chapter 8  
Examining the effects of aging on murine dendritic cells and T cells during IL-2/CD40 immunotherapy

8.1 Introduction

The studies in Chapter 6 demonstrated that mesothelioma exacerbates the regulatory status of lymphoid tissue CD11c⁺ cells and T cells that develops during healthy aging. The studies in Chapter 7 showed chemotherapy further promotes immune suppression in TDLNs and tumours of elderly mesothelioma-bearing mice. These changes in immune function may also impact on immunotherapeutic strategies to treat cancer in the elderly. However, the majority of studies to-date used young mouse models to evaluate immunotherapies, and this may not reflect the human setting, where most cancers emerge in the elderly (1166, 1167, 1281, 1282), furthermore, little is known about the effects of aging on responses to immunotherapy in mesothelioma. Of the few studies that have considered aging, several indicate that cancer immunotherapies are less effective in elderly hosts (608, 614, 1169, 1170, 1335-1343, 1345). Specific strategies providing appropriate stimulatory signals such as agonist anti-CD40 antibody alone or combined with IL-2, and combining cancer vaccines with agonist anti-OX40 or anti-4-1BB monoclonal antibodies (1169, 1345, 1416, 1419, 1420, 1831) may be required to overcome age- and tumour-induced dysfunction in elderly hosts (1166, 1167, 1282).

Previous studies from our laboratory, using young mice (1.5-2 months of age), have shown that intra-tumoural administration of IL-2 in combination with agonist anti-CD40 antibody induces permanent regression of large AE17 mesothelioma tumours mediated by CD8⁺ T cells, neutrophils (1351), B cells (1350) and pro-inflammatory M1 macrophages (1245). Cured mice remained tumour-free for the remainder of their natural lives and were protected from tumour re-challenge by CD8⁺ and CD4⁺ T cells and NK cells (1348, 1349). Studies from our laboratory have also shown that elderly macrophages activated with IL-2 and agonist anti-CD40 antibody restore the capacity of elderly CD8⁺ T cells to produce IFN-γ and perforin (1419, 1420).
However, the influence of aging on DC and T cell function during treatment with IL-2 and agonist anti-CD40 antibody in vivo had not been investigated. Therefore, the aims of the studies in this chapter were to examine changes in CD11c+ cells, DC subsets, and CD8+ and CD4+ T cells in TDLNs and tumours of young and elderly AE17 mesothelioma-bearing mice during treatment with the IL-2 and agonist anti-CD40 antibody combination.
8.2 Results

8.2.1 The effects of aging and cancer on dendritic cells during IL-2/CD40 immunotherapy

8.2.1.1 IL-2/CD40 is less efficient in slowing tumour growth in elderly mice

The first studies examined the effects of IL-2/agonist anti-CD40 antibody (IL-2/CD40) immunotherapy and aging on tumour growth. Young (2-5 months; n=7-10) and elderly (22-24 months; n=7-11) AE17 mesothelioma tumour-bearing mice were treated with IL-2/CD40, using previously described doses (1351). Mice received one-third of the treatment schedule (i.e. two doses of IL-2/CD40), to enable identification of tumours responding to treatment and still have enough tumour sample to collect for analysis, as young mice given the full regimen demonstrate complete resolution of tumours which therefore cannot be sampled (1348, 1349, 1351). Tumour growth rates and tumour sizes of IL-2/CD40-treated mice were compared to age-matched AE17 tumour-bearing mice treated with the PBS diluent. IL-2/CD40 slowed tumour growth rates in both age groups, although the effects were more pronounced in young mice (Figure 8.1A). At the experimental end point, young and elderly IL-2/CD40-treated mice showed reductions in tumour sizes (young p < 0.0001, elderly p < 0.0001; Figure 8.1B) and weights (young p < 0.0001, elderly p = 0.002; Figure 8.1C), relative to PBS controls. However, elderly IL-2/CD40-treated mice demonstrated significantly increased tumour sizes (p = 0.003; Figure 8.1B) and weights (p = 0.0004; Figure 8.1C), compared to their younger counterparts.

8.2.1.2 IL-2/CD40 increases TDLN CD11b⁺CD8α⁻CD4⁺ and CD11b⁺CD8α⁺CD4⁻ cDCs

CD11c⁺ cells and DC subsets in IL-2/CD40-treated mice were compared to age-matched tumour-bearing mice treated with the PBS diluent. Regardless of age, IL-2/CD40 increased proportions of CD11b⁺CD8α⁻CD4⁺ cDCs (young p = 0.008, elderly p = 0.047; Figure 8.2A) and CD11b⁺CD8α⁺CD4⁻ cDCs (young p < 0.0001, elderly p <
0.0001; Figure 8.2B), relative to controls, and the age differential seen in TDLN CD11b⁺CD8α⁻CD4⁻ cDCs in untreated tumour-bearing mice was lost (Figure 8.2B). No IL-2/CD40-induced differences were seen in proportions of TDLN CD11c⁺ cells (Appendix G: Figure 1A), CD8α⁺CD11b⁻ cDCs (Appendix G: Figure 1B) or pDCs (Appendix G: Figure 1C).

**Figure 8.1** IL-2/CD40 is less efficient in slowing tumour growth in elderly mice
Young and elderly mice were inoculated subcutaneously with 5 x 10⁵ AE17 cells, and tumours left to develop before treatment with intra-tumoural IL-2 (20 μg/dose) and agonist anti-CD40 antibody (40 μg/dose) or PBS diluent (100 μl/dose) for 2 doses, each 3 days apart. IL-2/CD40 treatment began 9-11 days after tumour cell inoculation; the shaded bar in (A) represents treatment duration. Tumour size (in mm²) was measured daily to determine tumour growth rates (A). At the experimental end point (2-3 days after the last dose), tumour sizes (B) and tumour weights (C) were measured. Data are shown as mean ± SEM, n = 10 young and n = 11 elderly IL-2/CD40-treated mice, n = 14-15 young and n = 16 elderly PBS control mice, ** = p<0.005, **** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
Figure 8.2 IL-2/CD40 increases TDLN CD11b^+CD8α^+CD4^+ cDCs and CD11b^+CD8α^+CD4^− cDCs
Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for DC subsets, and analysed by flow cytometry. Proportions of CD11b^+CD8α^+CD4^+ cDCs (A) and CD11b^+CD8α^+CD4^− cDCs (B) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 11 young and n = 12 elderly PBS control mice. * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.

8.2.1.3 IL-2/CD40 reduces IFN-γ, TNF-α, IL-12, CD39, CD73, A2A receptor, IL-10 and TGF-β on CD11c^+ cells in young and elderly TDLNs
The effect of IL-2/CD40 on aging CD11c^+ cell function was examined and where changes to the percentage of cells positive for a marker were similar to expression levels of that marker, only the percentage data is shown.

In both age groups, IL-2/CD40 exerted positive and negative effects on activation/maturation markers on TDLN CD11c^+ cells: i.e. increased expression levels and percentages of CD80^+ (young p < 0.0001, elderly p < 0.0001; Figure 8.3A) and CD86^+ CD11c^+ cells (young p < 0.0001, elderly p = 0.007; Figure 8.3B), yet reduced IFN-γ (young p = 0.02, elderly p = 0.056; Figure 8.3C), TNF-α (young p = 0.008, elderly p = 0.04; Figure 8.3D) and IL-12 (young p < 0.0001, elderly p < 0.0001; Figure 8.3E), relative to age-matched PBS controls. Elderly IL-2/CD40-treated mice showed a small, but significant, decrease in expression of CD80 on CD11c^+ cells, compared to their younger counterparts (p = 0.02; Figure 8.3A), whilst CD86, IFN-γ, TNF-α and IL-12 expression levels were similar with age (Figures 8.3B-8.3E).
Figure 8.3 IL-2/CD40 increases CD80 and CD86, and reduces IFN-γ, TNF-α and IL-12 on TDLN CD11c⁺ cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c⁺ cells, CD80, CD86, and intracellular IFN-γ, TNF-α and IL-12, and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for CD80 (A), CD86 (B), IFN-γ (C), TNF-α (D) and IL-12 (E) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.

Importantly, IL-2/CD40 reduced expression levels and percentages of cells positive for a number of regulatory markers on young and elderly TDLN CD11c⁺ cells including: CD39 (young p = 0.08, elderly p = 0.035; Figure 8.4A), CD73 (young p < 0.0001, elderly p = 0.02; Figure 8.4B), IL-10 (young p = 0.0004, elderly p = 0.001; Figure 8.4C), TGF-β (young p < 0.0001, elderly p < 0.0001; Figure 8.4D), and the A2A
receptor (young $p = 0.003$, elderly $p = 0.056$; Figure 8.4E) relative to age-matched untreated tumour-bearing mice. Note that CD73 ($p = 0.03$; Figure 8.4B) and TGF-β ($p = 0.002$; Figure 8.4D) remained higher on elderly CD11c$^+$ cells in IL-2/CD40-treated mice.

Figure 8.4 IL-2/CD40 reduces CD39, CD73, A2AR, IL-10 and TGF-β on TDLN CD11c$^+$ cells
Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c$^+$ cells, CD39, CD73, A2A receptor (A2AR), and intracellular IL-10 and TGF-β, and analysed by flow cytometry. Percentages of CD11c$^+$ cells positive for CD39 (A), CD73 (B), IL-10 (C), and TGF-β (D), and expression levels (shown as geometric mean fluorescence intensity; MFI) of A2AR (E) were measured. Data are shown as mean ± SEM, $n = 7$-10 young and $n = 7$-11 elderly IL-2/CD40-treated mice, $n = 9$-11 young and $n = 11$-12 elderly PBS control mice, * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$, **** = $p<0.0001$ comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
Some age-specific effects were also seen. Young, but not elderly, IL-2/CD40-treated mice down-regulated MHC class I\(^+\) \((p = 0.01; \text{Figure 8.5A})\) and MHC class II\(^+\) cells \((p < 0.0001; \text{Figure 8.5B})\), relative to age-matched PBS controls, meaning an increased age-related differential for these markers \((p = 0.006; \text{Figure 8.5A}, p = 0.007; \text{Figure 8.5B})\). In contrast, CD40 on TDLN CD11c\(^+\) cells decreased \((p = 0.0006; \text{Figure 8.5C})\) in elderly, compared to young, IL-2/CD40-treated mice. IL-2/CD40 did not alter the other markers examined (Appendix G: Figures 2A-2D).

![Figure 8.5 IL-2/CD40 reduces MHC class I and II on CD11c\(^+\) cells in young TDLNs](image)

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c\(^+\) cells, MHC class I, MHC class II and CD40, and analysed by flow cytometry. Percentages of CD11c\(^+\) cells positive for MHC class I (A), MHC class II (B), and CD40 (C) were measured. Data are shown as mean ± SEM, \(n = 7\) young and \(n = 7\) elderly IL-2/CD40-treated mice, \(n = 11\) young and \(n = 12\) elderly PBS control mice, \(* = p<0.05, ** = p<0.005, **** = p<0.0001\) comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
8.2.1.4 Increased CD86, TGF-β and A2B receptor, but reduced CD40 on CD11c+ cells in elderly IL-2/CD40-treated tumours

IL-2/CD40 did not alter proportions of young or elderly tumour-associated CD11c+ cells and DC subsets, relative to untreated mice (Appendix G: Figures 3A-3E). However, elderly IL-2/CD40-treated mice demonstrated a small, but significant, increase in CD8α+CD11b+ cDCs in tumours, compared to their younger counterparts (p = 0.01; Appendix G: Figure 3B).

The only common effect of IL-2/CD40 on tumour-associated CD11c+ cells seen in both age groups was up-regulation of expression levels and percentages of IFN-γ+ cells (young p = 0.001, elderly p = 0.0004; Figure 8.6A), compared to age-matched PBS controls.

![Figure 8.6 IL-2/CD40 increases CD86, IFN-γ and TGF-β on CD11c+ cells in elderly tumours](image)

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, CD86 and intracellular IFN-γ and TGF-β, and analysed by flow cytometry. Percentages of CD11c+ cells positive for IFN-γ (A), CD86 (B) and TGF-β (C) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 11 young and n = 11-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
IL-2/CD40 induced several age-specific changes in activation and regulatory markers on tumour-associated CD11c+ cells. In elderly, but not young, IL-2/CD40-treated tumours expression levels and percentages of CD86+ (p = 0.01; Figure 8.6B) and TGF-β+ CD11c+ cells (p = 0.02; Figure 8.6C) increased compared to age-matched PBS controls. The age differential in TGF-β expression seen in untreated mice was removed in IL-2/CD40-treated mice (Figure 8.6C).

In contrast, IL-2/CD40 induced reduced expression levels and percentages of MHC class I+ (p = 0.04; Figure 8.7A) and CD80+ cells (p = 0.001; Figure 8.7B) in young, but not elderly, tumour-associated CD11c+ cells. Concurrently, IL-2/CD40 reduced expression of several regulatory markers on young CD11c+ cells only including: CD39 (p = 0.02; Figure 8.7C), A2A receptor (p = 0.004; Figure 8.7D), A2B receptor (p = 0.02; Figure 8.8A), and PD-L1 (p = 0.03; Figure 8.8B), yet expression of CD73 increased (p = 0.005; Figure 8.8C), relative to PBS controls. Note that in IL-2/CD40-treated tumours, the age-related differences in CD39 and PD-L1 seen in untreated tumours were removed (Figures 8.7C and 8.8B), whilst elderly CD11c+ cells demonstrated reduced CD40 expression levels (p = 0.04; Figure 8.8D) and increased A2B receptor+ cells (p = 0.007; Figure 8.8A) relative to young CD11c+ cells. The other markers examined on tumour-associated CD11c+ cells did not show any IL-2/CD40-induced differences (Appendix G: Figures 4A-4E).

### 8.2.1.5 Reduced CD40, CD80, CD86, IFN-γ, TNF-α and IL-12 on tumour CD11c+ cells from elderly non-responders to IL-2/CD40

To take into consideration the influence of tumour size, correlations between tumour weight and (i) DC subset proportions, and (ii) expression of activation and regulatory markers on CD11c+ cells were examined in TDLNs and tumours of young (n=7) and elderly (n=7) IL-2/CD40-treated mice. Elderly IL-2/CD40-treated mice separated into two groups: (i) those with larger tumours (> 200 mg; n=3), which are likely to be weak or non-responders to therapy, and (ii) those with smaller tumours (< 200 mg; n=4), which are likely to be responding to therapy. No clear distinction between responders and non-responders could be made in young mice. Therefore, only elderly responders versus weak/non-responders were considered.
Figure 8.7 IL-2/CD40 reduces MHC-I, CD80, A2AR and CD39 on CD11c+ cells in young tumours

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, MHC class I, CD80, CD39 and A2A receptor (A2AR), and analysed by flow cytometry. Percentages of CD11c+ cells positive for MHC class I (A), CD80 (B), CD39 (C) and A2AR (D) were measured. Data are shown as mean ± SEM, n = 7-10 young and n = 7-11 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
Figure 8.8 IL-2/CD40 reduces A2BR and PD-L1, and increases CD73 on CD11c+ cells in young tumours

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, CD40, CD73, PD-L1, and A2B receptor (A2BR), and analysed by flow cytometry. Percentages of CD11c+ cells positive for A2BR (A), PD-L1 (B) and CD73 (C), and expression levels (shown as geometric mean fluorescence intensity; MFI) of CD40 (D) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.

In TDLNs of elderly IL-2/CD40-treated mice, there was a significant correlation between increasing tumour size and decreasing pDC proportions (Figure 8.9A and Table 8.1), which was not seen in age-matched PBS controls or young IL-2/CD40-treated mice (Table 8.1).
Figure 8.9 Increased CD80, CD86 and IL-10, and reduced CD39 on elderly TDLN CD11c+ cells as tumour size increases

Elderly tumour-bearing mice were treated with IL-2/CD40 as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c+ cells, pDCs, CD80, CD86, CD39, and intracellular IL-10, and analysed by flow cytometry. The correlation between tumour weight and percentages of pDCs (A), expression levels (shown as geometric mean fluorescence intensity; MFI) of CD80 (B) and CD86 (C), and percentages of CD11c+ cells positive for IL-10 (D) and CD39 (E) were measured, n = 7 elderly IL-2/CD40-treated mice.

Table 8.1 Correlation between TDLN pDCs and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice IL-2/CD40</th>
<th>Elderly mice PBS</th>
<th>Elderly mice IL-2/CD40</th>
</tr>
</thead>
<tbody>
<tr>
<td>% pDCs</td>
<td>r = 0.1879</td>
<td>r = 0.7714</td>
<td>r = 0.2028</td>
<td>r = -0.8571</td>
</tr>
<tr>
<td></td>
<td>p = 0.6</td>
<td>p = 0.1</td>
<td>p = 0.5</td>
<td>p = 0.02</td>
</tr>
</tbody>
</table>

Elderly weak/non-responding IL-2/CD40-treated mice demonstrated increased CD80 and CD86 (Figures 8.9B and 8.9C and Table 8.2) on TDLN CD11c+ cells as tumour size increased, accompanied by increased IL-10+ CD11c+ cells, yet decreasing CD39+ CD11c+ cells (Figures 8.9D and 8.9E and Table 8.2). No correlations between tumour size and CD80, CD86, IL-10 and CD39 were seen in age-matched PBS controls or young IL-2/CD40-treated mice (Table 8.2).
Table 8.2 Correlation between markers on TDLN CD11c+ cells and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice IL-2/CD40</th>
<th>Elderly mice PBS</th>
<th>Elderly mice IL-2/CD40</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80 MFI of CD11c+ cells</td>
<td>r = 0.0546 p = 0.9</td>
<td>r = 0.1429 p = 0.8</td>
<td>r = 0.0350 p = 0.9</td>
<td>r = 0.9286 p = 0.007</td>
</tr>
<tr>
<td>CD86 MFI of CD11c+ cells</td>
<td>r = -0.0909 p = 0.8</td>
<td>r = 0.0714 p = 0.9</td>
<td>r = -0.0350 p = 0.9</td>
<td>r = 0.8214 p = 0.03</td>
</tr>
<tr>
<td>% CD39+ CD11c+ cells</td>
<td>r = 0.2249 p = 0.5</td>
<td>r = -0.4286 p = 0.4</td>
<td>r = 0.3147 p = 0.3</td>
<td>r = -0.8929 p = 0.01</td>
</tr>
<tr>
<td>% IL-10+ CD11c+ cells</td>
<td>r = -0.2242 p = 0.5</td>
<td>r = -0.6071 p = 0.2</td>
<td>r = 0.3152 p = 0.3</td>
<td>r = 0.8571 p = 0.02</td>
</tr>
</tbody>
</table>

Examination of tumours showed that intra-tumoural CD11b+CD8α+CD4+ cDCs increased as tumour burden increased in elderly mice (Figure 8.10A); this was not seen in elderly PBS controls or young IL-2/CD40-treated mice (Table 8.3).

Table 8.3 Correlation between tumour CD11b-CD8α-CD4+ cDCs and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice IL-2/CD40</th>
<th>Elderly mice PBS</th>
<th>Elderly mice IL-2/CD40</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD11b-CD8α-CD4+ cDCs</td>
<td>r = 0.2485 p = 0.5</td>
<td>r = -0.7143 p = 0.09</td>
<td>r = -0.1049 p = 0.7</td>
<td>r = 0.9643 p = 0.003</td>
</tr>
</tbody>
</table>

Furthermore, elderly mice responding poorly to IL-2/CD40 demonstrated reductions in expression of several co-stimulatory molecules and pro-inflammatory cytokines on intra-tumoural CD11c+ cells as tumour size increased, specifically: CD40, CD80, CD86, IFN-γ, TNF-α and IL-12 (Figures 8.10B-G and Table 8.4). Simultaneously, CD73 levels decreased on elderly tumour-associated CD11c+ cells with increasing tumour burden (Figure 8.10H and Table 8.4). TNF-α increased with increasing tumour size in young IL-2/CD40-treated mice (Table 8.4); no other correlations with tumour size were observed (Table 8.4).

Table 8.4 Correlation between markers on tumour CD11c+ cells and tumour size

<table>
<thead>
<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice IL-2/CD40</th>
<th>Elderly mice PBS</th>
<th>Elderly mice IL-2/CD40</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD40+ CD11c+ cells</td>
<td>r = -0.0909 p = 0.8</td>
<td>r = 0.2143 p = 0.7</td>
<td>r = 0.0455 p = 0.9</td>
<td>r = -0.9286 p = 0.007</td>
</tr>
<tr>
<td>% CD86+ CD11c+ cells</td>
<td>r = -0.2588 p = 0.5</td>
<td>r = -0.5357 p = 0.2</td>
<td>r = -0.6996 p = 0.02</td>
<td>r = -0.9643 p = 0.003</td>
</tr>
<tr>
<td>% IFN-γ+ CD11c+ cells</td>
<td>r = -0.4424 p = 0.2</td>
<td>r = 0.5714 p = 0.2</td>
<td>r = -0.4308 p = 0.2</td>
<td>r = -0.8469 p = 0.02</td>
</tr>
<tr>
<td>% TNF-α+ CD11c+ cells</td>
<td>r = -0.1000 p = 0.95</td>
<td>r = 0.8214 p = 0.03</td>
<td>r = -0.6786 p = 0.1</td>
<td>r = -0.9643 p = 0.003</td>
</tr>
<tr>
<td></td>
<td>Young mice PBS</td>
<td>Young mice IL-2/CD40</td>
<td>Elderly mice PBS</td>
<td>Elderly mice IL-2/CD40</td>
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<td>------------------</td>
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</tr>
<tr>
<td>% IL-12⁺ CD11c⁺ cells</td>
<td>r = 0.0424</td>
<td>r = 0.4286</td>
<td>r = -0.0547</td>
<td>r = -0.8214</td>
</tr>
<tr>
<td></td>
<td>p = 0.9</td>
<td>p = 0.4</td>
<td>p = 0.9</td>
<td>p = 0.03</td>
</tr>
<tr>
<td>CD80 MFI of CD11c⁺ cells</td>
<td>r = 0.1273</td>
<td>r = -0.6429</td>
<td>r = 0.2091</td>
<td>r = -0.7857</td>
</tr>
<tr>
<td></td>
<td>p = 0.7</td>
<td>p = 0.1</td>
<td>p = 0.5</td>
<td>p = 0.048</td>
</tr>
<tr>
<td>CD73 MFI of CD11c⁺ cells</td>
<td>r = -0.1273</td>
<td>r = 0.6786</td>
<td>r = -0.5909</td>
<td>r = -0.8571</td>
</tr>
<tr>
<td></td>
<td>p = 0.7</td>
<td>p = 0.1</td>
<td>p = 0.06</td>
<td>p = 0.02</td>
</tr>
</tbody>
</table>

Figure 8.10 Reduced CD40, CD80, CD86, IFN-γ, TNF-α, IL-12 and CD73 on elderly tumour CD11c⁺ cells as tumour size increases

Elderly tumour-bearing mice were treated with IL-2/CD40 as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD11c⁺ cells, CD11b⁺CD8α⁺CD4⁻ cDCs, CD40, CD80, CD86, CD73, and intracellular IFN-γ, TNF-α and IL-12, and analysed by flow cytometry. The correlation between tumour weight and percentages of CD11b⁺CD8α⁺CD4⁻ cDCs (A), percentages of CD11c⁺ cells positive for CD40 (B), CD86 (D), IFN-γ (E), TNF-α (F), and IL-12 (G), and expression levels (shown as geometric mean fluorescence intensity; MFI) of CD80 (C) and CD73 (H) were measured, n = 7 elderly IL-2/CD40-treated mice.
In summary, in both age groups: (i) CD11b+CD8α+CD4+ and CD11b+CD8α+CD4− cDC proportions increased in TDLNs of IL-2/CD40-treated mice, suggesting improved stimulation of Th1 and Th2 responses, (ii) IL-2/CD40 increased the co-stimulatory capacity of TDLN CD11c+ cells due to increased CD80 and CD86, but their T cell-activating capacity may be diminished via reduced IFN-γ, TNF-α and IL-12, (iii) IL-2/CD40 reduced the suppressive potential of TDLN CD11c+ cells due to reduced CD39, CD73, A2AR, IL-10 and TGF-β, and (iv) IL-2/CD40 promoted pro-inflammatory activity in tumour-associated CD11c+ cells through increased IFN-γ.

With aging: (i) despite IL-2/CD40 treatment, age-related differences in elderly TDLN CD11c+ cells were maintained, i.e. elderly CD11c+ cells had increased antigen-presenting capacity via increased MHC class I and II, however, their co-stimulatory capacity via CD40 and CD80 was reduced, and their potential to exert suppressive activity through CD73 and TGF-β increased, (ii) IL-2/CD40 increased the co-stimulatory capacity of elderly intra-tumoural CD11c+ cells due to increased CD86, but simultaneously increased suppressive activity through TGF-β, (iii) in contrast, CD11c+ cells from young IL-2/CD40-treated tumours had reduced antigen-presenting capacity via MHC class I, reduced co-stimulatory capacity via CD80, and diminished regulatory potential through reduced CD39, A2A and A2B receptors, and PD-L1. Furthermore, in elderly weak/non-responders to IL-2/CD40: (i) TDLN CD11c+ cells have increased co-stimulatory capacity due to increased CD80 and CD86, which may yield Tregs by concomitant increases in IL-10 with increasing tumour burden, and (ii) the ability of tumour-associated CD11c+ cells to co-stimulate and activate effector T cells may be compromised on account of declining expression of CD40, CD80, CD86, IFN-γ, TNF-α and IL-12 as tumour size increases.

8.2.2 The effects of aging and cancer on T cells during IL-2/CD40 immunotherapy

8.2.2.1 IL-2/CD40 reduces CD4+ T cell proportions in young and elderly TDLNs

The effects of aging, cancer and IL-2/CD40 on CD8+ and CD4+ T cells and Tregs in TDLNs and tumours of young (2-5 months; n=7-10) and elderly (22-24 months; n=7-
11) mesothelioma-bearing mice treated with IL-2/CD40 were also examined. IL-2/CD40 reduced TDLN CD4+ T cell proportions in young and elderly mice, relative to age-matched PBS controls (young p < 0.0001, elderly p = 0.005; Figure 8.11A). However, only young IL-2/CD40-treated mice showed a reduction in TDLN CD8+ T cell proportions relative to their PBS controls (p < 0.0001; Figure 8.11B). IL-2/CD40 did not affect Tregs (Figure 8.11C), and age-related reductions in CD4+ (p < 0.0001; Figure 8.11A), CD8+ T cell (p = 0.02; Figure 8.11B) and Treg (p < 0.0001; Figure 8.11C) proportions were maintained in TDLNs of elderly, compared to young, mice.

![Diagram](image)

**Figure 8.11 IL-2/CD40 reduces proportions of CD4+ T cells in young and elderly TDLNs**

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8+ T cells, CD4+ T cells and CD4+CD25+FoxP3+ Tregs, and analysed by flow cytometry. Proportions of CD4+ T cells (A), CD8+ T cells (B) and Tregs (C) are shown as mean ± SEM, n = 10 young and n = 11 elderly IL-2/CD40-treated mice, n = 14 young and n = 16 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
IL-2/CD40 increases PD-1, ICOS and LAG-3, but reduces CD73 and TGF-β on TDLN CD8+ T cells

To investigate the effects of IL-2/CD40 on T cell function, expression of activation and regulatory markers on CD8+ and CD4+ T cells was examined.

IL-2/CD40 exerted positive and negative effects on regulatory marker expression on TDLN CD8+ T cells in both age groups including increased expression levels and percentages of cells expressing PD-1 (young p = 0.003, elderly p < 0.0001; Figure 8.12A), ICOS (young p < 0.0001, elderly p = 0.0003; Figure 8.12B) and LAG-3 (young p = 0.001, elderly p = 0.002; Figure 8.12C), compared to age-matched PBS controls. Nonetheless, age-related differences in PD-1, ICOS and LAG-3 were maintained, whereby elderly CD8+ T cells had higher expression of these markers (Figures 8.12A-8.12C). In contrast, IL-2/CD40 induced reduced expression levels and percentages of CD73+ (young p = 0.004, elderly p = 0.0001; Figure 8.12D) and TGF-β+ CD8+ T cells (young p < 0.0001, elderly p = 0.0008; Figure 8.12E) in young and elderly TDLNs, relative to age-matched PBS controls. The reduction in CD73 was more striking in elderly mice, resulting in a trend for lower CD73 expression on CD8+ T cells in elderly, compared to young, IL-2/CD40-treated mice (p = 0.051; Figure 8.12D). However, IL-2/CD40-treated young mice demonstrated a more striking reduction in latent TGF-β, which led to an increase in the age differential (p = 0.0006; Figure 8.12E).

IL-2/CD40 also exerted age-specific effects on TDLN CD8+ T cells. Young, but not elderly, IL-2/CD40-treated mice up-regulated expression levels and percentages of TDLN CD8+ T cells expressing perforin (p < 0.0001; Figure 8.13A), relative to their PBS controls resulting in higher perforin expression on CD8+ T cells from young, compared to elderly, IL-2/CD40-treated mice (p = 0.002; Figure 8.13A). Young IL-2/CD40-treated mice also down-regulated expression levels of the A2A receptor, relative to their PBS controls (p = 0.003; Figure 8.13B), resulting in higher A2A receptor expression in elderly, relative to young, IL-2/CD40-treated mice (p = 0.0006; Figure 8.13B). No other age-associated IL-2/CD40-induced changes in TDLN CD8+ T cell marker expression were seen (Appendix G: Figures 5A-5F).
Figure 8.12 IL-2/CD40 increases PD-1, ICOS and LAG-3, and reduces CD73 and TGF-β on TDLN CD8+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8+ T cells, PD-1, ICOS, LAG-3, CD73, and intracellular TGF-β, and analysed by flow cytometry. Percentages of CD8+ T cells positive for PD-1 (A), ICOS (B), LAG-3 (C), CD73 (D) and TGF-β (E) were measured. Data are shown as mean ± SEM, n = 7 young and n = 6-7 elderly IL-2/CD40-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
Figure 8.13 IL-2/CD40 increases perforin and reduces A2AR on young TDLN CD8+ T cells
Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8+ T cells, A2A receptor (A2AR), and intracellular perforin, and analysed by flow cytometry. Percentages of CD8+ T cells positive for perforin (A) and expression levels (shown as geometric mean fluorescence intensity; MFI) of A2AR (B) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 5-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.

8.2.2.3 IL-2/CD40 increases CTLA-4, and ICOS, and reduces A2AR and TGF-β on CD4+ T cells in young and elderly TDLNs

IL-2/CD40 also exerted shared effects on several regulatory markers on TDLN CD4+ T cells from both age groups. Expression levels and percentages of TDLN CD4+ T cells expressing CTLA-4 (young p = 0.002, elderly p = 0.03; Figure 8.14A) and ICOS (young p < 0.0001, elderly p = 0.0008; Figure 8.14B) increased in IL-2/CD40-treated mice, relative to age-matched PBS controls. In contrast, IL-2/CD40 reduced percentages of latent TGF-β+ CD4+ T cells (young p < 0.0001, elderly p = 0.01; Figure 8.14C), which was associated with reduced levels of the A2A receptor (young p = 0.003, elderly p = 0.002; Figure 8.14D) in both age groups, compared to age-matched PBS controls. Despite these changes, expression of CTLA-4 (p = 0.051), ICOS (p = 0.001), TGF-β (p = 0.0006), and the A2A receptor (p = 0.001; Figures 8.14A-8.14D) remained higher on elderly TDLN CD4+ T cells, compared to young IL-2/CD40-treated mice.
Figure 8.14 IL-2/CD40 increases CTLA-4 and ICOS, and reduces TGF-β and A2AR on TDLN CD4+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4+ T cells, CTLA-4, ICOS, A2A receptor (A2AR), and intracellular TGF-β, and analysed by flow cytometry. Percentages of CD4+ T cells positive for CTLA-4 (A), ICOS (B), and TGF-β (C), and expression levels (measured as geometric mean fluorescence intensity; MFI) of A2AR (D) were measured. Data are shown as mean ± SEM, n = 7 young and n = 6-7 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.

Age-specific effects of IL-2/CD40 on TDLN CD4+ T cells were also seen. Elderly, but not young, IL-2/CD40-treated mice demonstrated reductions in expression levels and percentages of CD4+ T cells expressing CD73 (p = 0.03; Figure 8.15A), relative to age-matched PBS controls. CD4+ T cells from young, but not elderly, IL-2/CD40-treated mice up-regulated expression levels and percentages of cells positive for CD39 (p = 0.03), LAG-3 (p = 0.0001), PD-1 (p = 0.001) and CD25 (p < 0.0001; Figures 8.15B-8.15E), compared to their PBS controls. However, these changes did not alter
the age differentials in CD73, CD39 and LAG-3 seen in untreated mice, as expression of these markers remained higher on CD4+ T cells from elderly IL-2/CD40-treated mice, compared to young controls (Figures 8.15A-8.15C). In contrast, age differentials in PD-1 and CD25 expression seen in untreated mice were lost in IL-2/CD40-treated mice (Figures 8.15D and 8.15E). No further IL-2/CD40-induced differences were seen on CD4+ T cells (Appendix G: Figures 6A-6D).

Figure 8.15 IL-2/CD40 increases CD39, LAG-3, PD-1 and CD25 on young TDLN CD4+ T cells
Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4+ T cells, CD25, CD39, CD73, LAG-3, and PD-1, and analysed by flow cytometry. Percentages of CD4+ T cells positive for CD73 (A), CD39 (B), LAG-3 (C), PD-1 (D), and CD25 (E) were measured. Data are shown as mean ± SEM, n = 7-10 young and n = 7-11 elderly IL-2/CD40-treated mice, n = 11-14 young and n = 12-16 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
8.2.2.4 IL-2/CD40 reduces IFN-γ, perforin, CD73 and IL-10 on elderly tumour CD8+ T cells

Examination of tumour-associated T cells showed that IL-2/CD40 reduced proportions of young, but not elderly, CD4+ T cells (p = 0.0004; Figure 8.16A) and Tregs (p < 0.0001; Figure 8.16B), relative to age-matched PBS controls, removing the age differential in CD4+ T cells (Figure 8.16A), and elevating Treg proportions in elderly, compared to young, IL-2/CD40-treated tumours (p = 0.002; Figure 8.16B). No age-related or IL-2/CD40-induced differences were seen in proportions of tumour-associated CD8+ T cells (Figure 8.16C).

Regardless of age, IL-2/CD40 up-regulated expression levels and percentages of cells expressing CD25 on tumour-associated CD8+ T cells (young p < 0.0001, elderly p = 0.0501; Figure 8.17A) relative to PBS controls. This increase was more striking in young compared to elderly IL-2/CD40-treated tumours (p < 0.0001; Figure 8.17A).
Several age-specific effects of IL-2/CD40 were also observed. In elderly IL-2/CD40-treated tumours percentages of IFN-γ+ CD8+ T cells (p = 0.005; Figure 8.17B), and expression levels of perforin (p = 0.005; Figure 8.17C) reduced compared to age-matched PBS controls. This was accompanied by reductions in expression levels and percentages of tumour-associated CD8+ T cells expressing CD73 (p = 0.004; Figure 8.17D) and IL-10 (p = 0.047; Figure 8.17E) in elderly, but not young, IL-2/CD40-treated mice, relative to PBS controls. The former resulted in removal of the age-related differential in CD73 expression in IL-2/CD40-treated tumours (Figure 8.17D).

**Figure 8.17** IL-2/CD40 reduces IFN-γ, perforin, CD73 and IL-10 on elderly tumour CD8+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, CD25, CD73, and intracellular IFN-γ, perforin and IL-10, and analysed by flow cytometry. Percentages of CD8+ T cells positive for CD25 (A), IFN-γ (B), CD73 (D) and IL-10 (E), and expression levels (shown as geometric mean fluorescence intensity; MFI) of perforin (C) were measured. Data are shown as mean ± SEM, n = 7-10 young and n = 7-11 elderly IL-2/CD40-treated mice, n = 11-14 young and n = 12-16 elderly PBS control mice, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
IL-2/CD40 promoted a more regulatory phenotype in young tumour-associated CD8⁺ T cells, on account of increases in expression levels and percentages of cells positive for several regulatory markers, relative to PBS controls, specifically: CD39 (p = 0.02), A2A receptor (p = 0.046), A2B receptor (p = 0.002), ICOS (p = 0.002), LAG-3 (p < 0.0001), and PD-1 (p = 0.02; Figures 8.18A-8.18F). As a result, expression of CD39 (p = 0.04), A2A receptor (p = 0.0006), A2B receptor (p = 0.0006), ICOS (p = 0.002) and LAG-3 (p = 0.02; Figures 8.18A-8.18E) was higher on tumour-associated CD8⁺ T cells from young, compared to elderly, IL-2/CD40-treated mice. The remaining markers examined on tumour-associated CD8⁺ T cells did not show any IL-2/CD40-induced changes (Appendix G: Figures 7A and 7B).

IL-2/CD40 induced reduced expression levels and percentages of tumour-associated CD4⁺ T cells expressing CD25 (young p = 0.04, elderly p = 0.057; Figure 8.19A) and IFN-γ (young p = 0.04, elderly p = 0.003; Figure 8.19B), in both age groups, compared to age-matched PBS controls. This was associated with reductions in expression levels and percentages of cells expressing the regulatory markers CD73 (young p = 0.058, elderly p = 0.005; Figure 8.19C) and CTLA-4 (young p = 0.0004, elderly p = 0.07; Figure 8.19D), in young and elderly IL-2/CD40-treated tumours, relative to PBS controls. The reduction in CTLA-4 was more striking for young mice, resulting in higher expression of CTLA-4 on CD4⁺ T cells from elderly, compared to young, IL-2/CD40-treated mice (p = 0.03; Figure 8.19D).

Young, but not elderly, IL-2/CD40-treated mice demonstrated additional reductions in regulatory markers, as percentages of ICOS⁺ (p = 0.02; Figure 8.19E) and IL-10⁺ CD4⁺ T cells (p = 0.0008; Figure 8.19F) in tumours decreased, compared to their PBS controls, leading to a removal of the age differential in ICOS seen in untreated mice (Figure 8.19E). The remaining markers examined on tumour-associated CD4⁺ T cells were not affected by IL-2/CD40 (Appendix G: Figures 8A-8D and 9A-9C).
Figure 8.18 IL-2/CD40 increases CD39, A2AR, A2BR, ICOS, LAG-3 and PD-1 on young tumour CD8+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, CD39, A2A receptor (A2AR), A2B receptor (A2BR), ICOS, LAG-3 and PD-1, and analysed by flow cytometry. Percentages of CD8+ T cells positive for CD39 (A), A2AR (B), A2BR (C), ICOS (D), LAG-3 (E) and PD-1 (F) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
Figure 8.19 IL-2/CD40 reduces CD25, IFN-γ, CD73, CTLA-4, ICOS and IL-10 on tumour CD4+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD4+ T cells, CD25, CD73, CTLA-4, ICOS, and intracellular IFN-γ and IL-10, and analysed by flow cytometry. Percentages of CD4+ T cells positive for CD25 (A), IFN-γ (B), CD73 (C), CTLA-4 (D), ICOS (E) and IL-10 (F) were measured. Data are shown as mean ± SEM, n = 7-10 young and n = 7-11 elderly IL-2/CD40-treated mice, n = 11-14 young and n = 12-16 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing (i) young to elderly mice, or (ii) IL-2/CD40-treated mice to age-matched PBS control mice.
8.2.2.5 Reduced perforin and increased CD39, CD73, A2BR, CTLA-4, ICOS, LAG-3 and IL-10 on tumour CD8\(^+\) T cells in elderly non-responders to IL-2/CD40

The functional phenotypes of CD8\(^+\) and CD4\(^+\) T cells were also examined by analysing the correlation between T cell marker expression and tumour size, with increasing tumour size considered a likely weak or non-response to therapy. TDLN CD8\(^+\) T cells from elderly IL-2/CD40-treated mice demonstrated increased IFN-\(\gamma\) expression, yet percentages of CTLA-4\(^+\) cells also increased (Figures 8.20A and 8.20B and Table 8.5) with increasing tumour size. However, expression of the A2A receptor and IL-10 (Figures 8.20C and 8.20D and Table 8.5) on TDLN CD8\(^+\) T cells decreased as tumour size increased. These correlations were not observed in age-matched PBS controls or young IL-2/CD40-treated mice (Table 8.5).

| Table 8.5 Correlation between markers on TDLN CD8\(^+\) T cells and tumour size |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                 | Young mice PBS                  | Young mice IL-2/CD40            | Elderly mice PBS                | Elderly mice IL-2/CD40          |
| IFN-\(\gamma\) MFI of CD8\(^+\) T cells | \(r = -0.5515\)  p = 0.1        | \(r = 0.0360\)  p = 0.95        | \(r = -0.1576\)  p = 0.6        | \(r = 0.7857\)  p = 0.048       |
| % CTLA-4\(^+\) CD8\(^+\) T cells     | \(r = -0.2242\)  p = 0.5        | \(r = -0.2857\)  p = 0.6        | \(r = 0.1329\)  p = 0.7         | \(r = 0.9429\)  p = 0.02        |
| A2AR MFI of CD8\(^+\) T cells      | \(r = -0.6\)  p = 0.4           | \(r = -0.3214\)  p = 0.5        | \(r = -0.3214\)  p = 0.5        | \(r = -0.8929\)  p = 0.01       |
| IL-10 MFI of CD8\(^+\) T cells     | \(r = -0.8\)  p = 0.1           | \(r = 0.5714\)  p = 0.2         | \(r = -0.2857\)  p = 0.6        | \(r = -0.8214\)  p = 0.03       |

Examination of TDLN CD4\(^+\) T cells showed that percentages of ICOS\(^+\) CD4\(^+\) T cells decreased (Figure 8.20E and Table 8.6), but expression levels of CD39 increased (Figure 8.20F and Table 8.6) with increasing tumour burden in elderly IL-2/CD40-treated mice; this was not seen in age-matched PBS controls or young IL-2/CD40-treated mice (Table 8.6).

| Table 8.6 Correlation between markers on TDLN CD4\(^+\) T cells and tumour size |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|                                 | Young mice PBS                  | Young mice IL-2/CD40            | Elderly mice PBS                | Elderly mice IL-2/CD40          |
| % ICOS\(^+\) CD4\(^+\) T cells  | \(r = -0.3697\)  p = 0.3        | \(r = 0.2143\)  p = 0.7        | \(r = -0.0420\)  p = 0.9        | \(r = -0.7857\)  p = 0.048       |
| CD39 MFI of CD4\(^+\) T cells   | \(r = -0.3576\)  p = 0.3        | \(r = 0.2883\)  p = 0.5        | \(r = -0.0070\)  p = 0.99       | \(r = 0.9429\)  p = 0.02         |
In elderly tumour-bearing mice, there was a significant negative correlation between expression levels and percentages of perforin⁺ CD8⁺ T cells and tumour burden in elderly weak/non-responders to IL-2/CD40 (Figure 8.21A and Table 8.7). This was associated with increased expression of several regulatory markers on intra-tumoural CD8⁺ T cells with increasing tumour burden i.e.: A2B receptor, ICOS, LAG-3, IL-10, CD39, CD73 and CTLA-4 (Figures 8.21B-8.21H and Table 8.7); this was not seen in elderly PBS control tumours or young IL-2/CD40-treated tumours (Table 8.7). No correlations were seen between markers on tumour-associated CD4⁺ T cells and tumour size.
Figure 8.21 Reduced perforin, and increased regulatory markers on CD8+ T cells in elderly IL-2/CD40-treated tumours with increasing tumour size

Elderly tumour-bearing mice were treated with IL-2/CD40 as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, A2B receptor (A2BR), ICOS, LAG-3, CD39, CD73, CTLA-4, and intracellular perforin and IL-10, and analysed by flow cytometry. The correlation between tumour weight and percentages of CD8+ T cells positive for perforin (A), A2BR (B), ICOS (C), LAG-3 (D), and IL-10 (E), and expression levels (shown as geometric mean fluorescence intensity; MFI) of CD39 (F), CD73 (G) and CTLA-4 (H) on CD8+ T cells were measured, n = 7 elderly IL-2/CD40-treated mice.

<p>| Table 8.7 Correlation between markers on tumour CD8+ T cells and tumour size |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                               | Young mice PBS | Young mice IL-2/CD40 | Elderly mice PBS | Elderly mice IL-2/CD40 |
| % Perforin+ CD8+ T cells | r = 0.0061 | r = 0.75 | r = -0.1958 | r = -0.7857 |
| p = 0.99 | p = 0.07 | p = 0.5 | p = 0.048 | |
| % A2BR+ CD8+ T cells | r = 0.6 | r = 0.5 | r = 0.5357 | r = 0.9643 |
| p = 0.35 | p = 0.3 | p = 0.2 | p = 0.003 | |
| % ICOS+ CD8+ T cells | r = 0.1394 | r = 0.75 | r = -0.0629 | r = 0.8571 |
| p = 0.7 | p = 0.07 | p = 0.9 | p = 0.02 | |</p>
<table>
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<tr>
<th></th>
<th>Young mice PBS</th>
<th>Young mice IL-2/CD40</th>
<th>Elderly mice PBS</th>
<th>Elderly mice IL-2/CD40</th>
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<tr>
<td>% LAG-3* CD8* T cells</td>
<td>r = -0.0423, p = 0.9</td>
<td>r = 0.6429, p = 0.1</td>
<td>r = -0.3706, p = 0.2</td>
<td>r = 0.9286, p = 0.007</td>
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<tr>
<td>% IL-10* CD8* T cells</td>
<td>r = 0.4182, p = 0.2</td>
<td>r = -0.4643, p = 0.3</td>
<td>r = 0.2028, p = 0.5</td>
<td>r = 0.8214, p = 0.03</td>
</tr>
<tr>
<td>CD39 MFI of CD8* T cells</td>
<td>r = 0.2242, p = 0.5</td>
<td>r = -0.7857, p = 0.048</td>
<td>r = 0.2448, p = 0.4</td>
<td>r = 0.9286, p = 0.007</td>
</tr>
<tr>
<td>CD73 MFI of CD8* T cells</td>
<td>r = 0.5879, p = 0.08</td>
<td>r = 0.6071, p = 0.2</td>
<td>r = 0.2587, p = 0.4</td>
<td>r = 0.7857, p = 0.048</td>
</tr>
<tr>
<td>CTLA-4 MFI of CD8* T cells</td>
<td>r = 0.3333, p = 0.3</td>
<td>r = -0.3571, p = 0.4</td>
<td>r = 0.1329, p = 0.7</td>
<td>r = 0.8571, p = 0.02</td>
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In summary, regardless of age: (i) IL-2/CD40 reduces TDLN CD4⁺ T cell proportions, (ii) TDLN CD8⁺ and CD4⁺ T cells of IL-2/CD40-treated mice may be exhausted as evidenced by increased PD-1, ICOS, LAG-3 and/or CTLA-4, and have reduced CD73, A2A receptor and/or latent TGF-β-mediated suppressive activity, (iii) IL-2/CD40 may increase activation of tumour-associated CD8⁺ T cells due to increased CD25, and (iv) tumour-associated CD4⁺ T cells in IL-2/CD40-treated mice may have decreased IFN-γ-mediated effector function, reduced activation and/or reduced skewing towards Tregs due to decreased CD25, and lowered suppressive activity via CD73 and CTLA-4.

With aging: (i) the regulatory environment established in elderly TDLNs may be enhanced in elderly IL-2/CD40-treated mice, due to further increases in expression of CTLA-4, PD-1, ICOS, LAG-3, A2A receptor and/or TGF-β on elderly CD8⁺ and CD4⁺ T cells, and (ii) in IL-2/CD40-treated tumours, elderly CD8⁺ T cells may have reduced perforin-mediated effector function and but also reduced suppressive activity via CD73 and IL-10, whilst young CD8⁺ T cells may be more suppressive/exhausted due to increased CD39, A2A and A2B receptors, PD-1, ICOS and LAG-3. Furthermore, in elderly weak or non-responders to IL-2/CD40, tumour-associated CD8⁺ T cells may have reduced cytotoxic activity via perforin, but increased suppressive function due to elevated CD39, CD73, A2B receptor, CTLA-4, ICOS, LAG-3 and IL-10 with increasing tumour burden.
8.3 Discussion

The studies in this chapter examined the influence of aging and IL-2/CD40 immunotherapy on DCs and T cells in young and elderly mesothelioma-bearing mice. In both age groups, IL-2/CD40 increased activation, and reduced suppression within tumours. Elevations in IFN-γ expression by CD11c+ cells and CD25 expression by CD8+ T cells in tumours of both age groups suggests these cells are more activated with increased effector function that contributes to the slowing of tumour growth observed in young and elderly IL-2/CD40-treated mice. This is supported by our studies showing that CD8+ T cells are important anti-tumour effector cells during IL-2/CD40 treatment (1349, 1351), and other studies showing that DCs, CD8+ T cells and IFN-γ are required for IL-2/CD40-mediated tumour regression (1359, 1405-1407). Further studies are required to examine the cytotoxic effector function of CD8+ T cells in elderly mice. In contrast, young and elderly tumour-associated CD4+ T cells displayed reduced CD25 and IFN-γ effector function, suggesting that they may not contribute to IL-2/CD40-mediated tumour regression. This is supported by our previous studies (1349, 1351) and others (1359, 1405) demonstrating that CD4+ T cells are not critically required for IL-2/CD40-mediated effects on tumours. However, a reduction in CD25 expression on CD4+ T cells could be considered skewing away from a Treg-like phenotype, and this is supported by concomitant decreases in CD73 and CTLA-4 on CD4+ T cells in tumours, as both of these molecules are expressed by Tregs (544, 1618, 1621, 1832). Furthermore, others have shown that IL-2/CD40 reduced Tregs in murine tumours (1405, 1408). Thus, IL-2/CD40 may alleviate suppression in tumours via reductions in Tregs, which will contribute to tumour regression (1405, 1408). Other immune cell types may contribute to the IL-2/CD40-induced slowing of tumour growth observed in both age groups. Granulocytes (1351, 1364, 1365), macrophages (1245, 1360-1363, 1382-1386, 1833, 1834), B cells (1350), and NK cells (1379, 1405) can be activated in response to IL-2 and/or anti-CD40 antibody and exert anti-tumour activity. Additionally, IL-2/CD40 may relieve immune suppression within tumours via reductions in numbers and arginase-mediated inhibitory activity of MDSCs (1405, 1408). Thus, regardless of age, increased CD11c+ cell and CD8+ T cell activation, and
reduced inhibitory/Treg activity of CD4+ T cells in tumours may contribute to tumour regression.

It is possible that CD11c+ cells from IL-2/CD40-treated tumours migrate to TDLNs. Following CD40 ligation, DCs up-regulate molecules involved in migration, such as CCR7 (683, 1835), and have increased capacity to migrate to DLNs (683, 1836-1839). Data from this study suggests that in both age groups, CD11b+CD8αCD4+ cDCs and CD11b+CD8αCD4– cDCs may be migrating, as their proportions increased in TDLNs of IL-2/CD40-treated mice; further studies are required to assess this. Increased CD11b+CD8αCD4+ and CD11b+CD8αCD4– cDC proportions in TDLNs suggest increased activation of Th1 and Th2 responses (132, 142), which may be undermined by a concomitant decrease in TDLN CD4+ T cell proportions if they are helper cells but not if they are Tregs. Elderly non-responders showed a decrease in the Treg marker ICOS, but a simultaneous increase in the Treg marker CD39 on TDLN CD4+ T cells with increasing tumour size, making it difficult to determine how Tregs were changing with tumour burden; further studies are required to investigate which CD4+ T cell subsets are declining in TDLNs during IL-2/CD40 therapy.

TDLN CD11c+ cells displayed reduced suppressive potential, on account of reduced CD39, CD73, A2A receptor, IL-10 and TGF-β, suggesting that the TDLN environment of IL-2/CD40-treated mice is more permissive to T cell activation. However, CD11c+ cells only displayed a partially activated phenotype, characterised by increased CD80 and CD86, but reduced IFN-γ, TNF-α and IL-12, suggesting reduced T cell-activating capacity. A possible explanation is that the functional profile of TDLN CD11c+ cells reflects the analysis time point which was 2-3 days after IL-2/CD40 administration. It is possible that by this time point APCs and T cells have undergone activation to effector cells, mediated an anti-tumour immune response, and are in the attenuation phase of the response (9, 517, 707, 1732, 1840). Examination of young and elderly TDLN CD8+ and CD4+ T cells, and young tumour-associated CD8+ T cells supports this idea, as these cells displayed characteristics of exhaustion, specifically, up-regulated CD39, A2A and A2B receptors, PD-1, ICOS, LAG-3 and/or CTLA-4 expression (1101, 1105, 1740, 1741). Furthermore, in this study, mice
received one-third of the usual IL-2/CD40 schedule. In mice receiving a full IL-2/CD40 schedule the subsequent doses are likely to provide continued activation of the anti-tumour immune response, leading to complete tumour regression (1348, 1349, 1351). Further studies are required to examine CD11c+ cells/DCs and T cells after a complete schedule of IL-2/CD40.

IL-2/CD40 was less effective in slowing mesothelioma tumour growth in elderly, compared to young mice; thus, elderly responders versus weak/non-responders to IL-2/CD40 were examined to determine the potential mechanisms underlying this. In elderly weak/non-responding mice (i.e. those with larger tumour burdens), the T cell-activating capacity of tumour-associated CD11c+ cells appeared compromised, due to reduced expression of co-stimulatory molecules (CD40, CD80 and CD86) and pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-12). CD8+ T cell effector function also appeared compromised within elderly weak/non-responding tumours, due to reduced perforin. As our previous studies have shown that perforin-associated effector function is important for IL-2/CD40-mediated tumour regression (1351), this may be a contributing factor to the poor response of elderly mice to IL-2/CD40; further studies are required to assess the cytotoxic function of these cells. Additionally, CD8+ T cells in elderly weak/non-responding tumours expressed increased suppressive activity via elevated CD39, CD73, A2B receptor; i.e. increased adenosine (543, 565, 841, 1566), and elevated IL-10 (1841, 1842), as well as increased potential to respond to inhibitory signals via elevated CTLA-4, ICOS, and LAG-3 (801, 1843-1845). As CD8+ T cells are important effector cells during IL-2/CD40 therapy (1349, 1351, 1359, 1405-1407), their skewing towards suppressive activity is likely to contribute to the poor responses seen in some elderly mice. Furthermore, immune responses in TDLNs of elderly weak/non-responders were also skewed towards suppression. Elderly TDLN CD11c+ cells displayed a semi-mature, tolerogenic phenotype, characterised by increased CD80, CD86 and IL-10 expression; these cells are reported to promote T cell tolerance and generation of Tregs (513, 516, 775). Additionally, elevated CTLA-4 on TDLN CD8+ T cells of elderly weak/non-responders suggests that negative CD80/CTLA-4 interactions between CD11c+ cells and CD8+ T cells are increased, leading to T cell inhibition (831). Thus,
the simultaneously reduced immune-activating potential, and increased suppressive potential within tumours and TDLNs may explain the poor response of some elderly mesothelioma-bearing mice to IL-2/CD40.

When considering overall changes in elderly IL-2/CD40-treated tumours, CD11c+ cells and T cells have reduced anti-tumour activity. Specifically, elderly CD11c+ cells displayed reduced CD40 expression suggesting a reduced ability to activate T cells (1193, 1371, 1373-1376). This was associated with increased A2B receptor and latent TGF-β implying an increased capacity of CD11c+ cells to respond to adenosine (568-571, 1502), to suppress effector T cells and promote Tregs (162, 502, 512, 1691, 1720); the latter is supported by the observation that Tregs were elevated in elderly IL-2/CD40-treated tumours. Also, elderly tumour-associated CD8+ T cells have reduced effector function due to decreased perforin, which reduces the efficacy of IL-2/CD40-mediated tumour regression (1351). The effects of aging and IL-2/CD40 on other immune cells in tumours requires further study. For example, our previous studies suggest that IL-2/CD40-activated tumour-associated macrophages may play an important role in stimulating IFN-γ production by effector CD8+ T cells which may contribute to the slowing of tumour growth (1420). Thus, the anti-tumour function of elderly CD11c+ cells and CD8+ T cells appears compromised during IL-2/CD40 treatment contributing to reduced efficacy.

The reduced efficacy of IL-2/CD40 in elderly, compared to young, mesothelioma-bearing mice could also be attributed to an enhanced suppressive environment in elderly TDLNs. Increased MHC class I and II on TDLN CD11c+ cells in elderly IL-2/CD40-treated mice was accompanied by reduced CD40 and CD80 co-stimulatory signals and increased CD73 and TGF-β negative signals. Moreover, elderly TDLN CD8+ and CD4+ T cells may be more suppressive/exhausted and more responsive to negative signals from APCs, as age-related increases in CTLA-4, PD-1, ICOS, LAG-3, A2A receptor and TGF-β were further enhanced during IL-2/CD40 treatment. Further evidence for reduced effector T cells comes from the observation that elderly TDLN CD8+ T cells failed to up-regulate perforin in response to IL-2/CD40, unlike their younger counterparts. However, IL-2/CD40 did alleviate a few aspects of suppression within elderly TDLNs, such as removing the age differential in pDCs.
and CD11b⁺CD8α⁻CD4⁻ cDCs, and reducing CD73 expression on elderly CD4⁺ T cells. Thus, exacerbation of the regulatory environment within TDLNs is likely to contribute to reduced responses to IL-2/CD40 in elderly mice.

The age-related differences in responses to IL-2/CD40 could be attributed to changes in the ability of elderly CD11c⁺ cells/DCs and T cells to respond to IL-2 and/or CD40 stimulation. Elderly T cells may be responsive to IL-2 stimulation, as no age-related differences in expression of the IL-2 receptor α chain, CD25, were observed and elderly CD4⁺ T cells respond to IL-2 in vitro (1846, 1847), and IL-2 promotes T cell anti-tumour immunity in aged mice (1174). DCs also express CD25 (1355, 1558, 1848, 1849), and IL-2 promotes the ability of DCs to prime naive T cells (1357, 1358), and augments CD40 expression on DCs, making them responsive to agonist anti-CD40 antibody stimulation (1359). However, others have shown that CD25 expression on DCs is reduced with aging (670), therefore elderly DCs may be less responsive to IL-2, which could affect responses to CD40 stimulation (1359). This study suggests that elderly CD11c⁺ cells in TDLNs and tumours will be less responsive to CD40 stimulation, on account of reduced CD40 expression. Whilst CD40 expression on specific DC subsets in IL-2/CD40-treated mice was not investigated, the studies in Chapter 5 showed that CD8α⁺CD11b⁻ and CD11b⁺CD8α⁺ CD4⁻ cDCs in elderly healthy LNs had reduced CD40 expression; if this was maintained in elderly IL-2/CD40-treated mice, these subsets would have reduced responses to CD40 stimulation, and reduced CD40-mediated activation of CD8α⁺CD11b⁻ cDCs may compromise generation of elderly anti-tumour CD8⁺ T cell responses (124, 125, 127). Reduced responsiveness of elderly CD11c⁺ cells/DCs to IL-2/CD40 could contribute to the reduced efficacy of this therapy in elderly mesothelioma-bearing mice. Further studies are required to examine CD25 and CD40 expression on DC subsets in elderly IL-2/CD40-treated mice.

In summary, regardless of age, IL-2/CD40 increased activation/effector function of CD11c⁺ cells and CD8⁺ T cells, whilst reducing the suppressive potential of CD4⁺ T cells within tumours, which may slow tumour growth. The poor responses to IL-2/CD40 observed in some elderly mice may be attributed to: (i) reduced T cell-activating potential of CD11c⁺ cells, (ii) skewing of CD8⁺ T cells towards
suppression/exhaustion in tumours, and (iii) increased likelihood of negative interactions between APCs and CD8\(^+\) T cells in TDLNs with increasing tumour burden. Furthermore, CD11c\(^+\) cells in elderly tumours develop increased suppressive function, whilst CD8\(^+\) T cell perforin-mediated effector function is reduced, and the regulatory environment in elderly TDLNs is enhanced, contributing to reduced efficacy of IL-2/CD40 in elderly mesothelioma-bearing mice.
Chapter 9  Final discussion

This thesis examined changes in human and murine DC and T cell functional status during healthy aging, and whether this was modulated by cancer (mesothelioma). This study also investigated whether anti-cancer therapies (gemcitabine and cisplatin chemotherapy and IL-2/CD40 immunotherapy) improved or worsened elderly murine DC and T cell function.

This study identified several mechanisms common for young and elderly hosts by which mesothelioma disables DC/APC function. The first is compromising cross-presenting DCs, demonstrated by reduced human mDC2 proportions following in vitro exposure to mesothelioma factors, and reduced CD8α⁺CD11b⁻ cDC proportions in murine TDLNs. Cross-presenting DCs are critically required for activation of tumour-specific cytotoxic CD8⁺ T cells (124, 125, 127, 145, 955, 956), and these data suggest this is compromised in mesothelioma-bearing hosts. Mesothelioma also promotes suppressive DC subsets, indicated by the predominance of pDCs within tumours, and increased pDC proportions within TDLNs and spleens of tumour-bearing mice. Plasmacytoid DCs can be key mediators of tumour-induced immunosuppression due to their ability to promote Tregs (203, 751, 754, 1079, 1080), as well as inhibit effector T cells via PD-L1 (762), IDO (753, 758, 1798) and granzyme B (763). Mesothelioma may further disable DCs/APCs by promoting semi-mature DCs/APCs. Exposure of human MoDCs to tumour cells in vitro (in an attempt to represent the effect of tumour cells on monocytes as they differentiate into DCs within tumours) revealed semi-mature DCs/APCs that up-regulated some co-stimulatory molecules and pro-inflammatory cytokines/chemokines (CD40, intracellular IL-12, and secreted TNF-α, MCP-1, IL-6, IL-8 and IL-18), and down-regulated other antigen-presenting molecules (CD1a) and pro-inflammatory cytokines (secreted IFN-γ, IL-12p70, IL-23 and IL-33). Similarly, CD11c⁺ cells within murine TDLNs displayed hallmarks of a semi-mature phenotype, characterised by increased expression of some antigen-presenting and co-stimulatory molecules (MHC class II and CD80), yet decreased expression of others (CD40, TNF-α and IL-12). As mentioned, limitations in flow cytometer capability made it difficult to
analyse DC subsets in the murine studies, and CD11c\(^+\) cells were analysed instead. In this study, CD11c was considered a marker of APCs, as it is expressed by DCs (3, 4), monocytes/macrophages (78-81), and B cells (82, 83). Semi-mature DCs/APCs cannot provide a full complement of co-stimulatory signals to T cells, preventing effector T cell activation, thus impairing anti-tumour immunity (516, 954, 1002, 1071, 1072). Pilot studies examining the interaction between tumour-exposed human MoDCs and T cells in vitro showed that young and elderly tumour-exposed MoDCs induced a suppressive/exhausted phenotype in young and elderly CD8\(^+\) and CD4\(^+\) T cells, characterised by up-regulation of several inhibitory markers on T cells, in particular TIM-3, PD-1, CTLA-4, ICOS, CD39 and TGF-\(\beta\) (Figures 9.1 and 9.2). This suggests that tumour-exposed DCs drive dysfunctional/exhausted T cells. However, further studies are required, including: (i) analysis of T cell function following interaction with DCs at earlier time points, and (ii) in-depth analysis of T cell function, for example, assessing CD8\(^+\) T cell cytotoxic function. Additional studies should include increased numbers of human volunteers and mice. Nonetheless, regardless of age, mesothelioma appears to impair the ability of DCs/APCs to stimulate anti-tumour T cell responses, which may contribute to tumour progression.

Promotion of semi-mature DCs/CD11c\(^+\) cells by mesothelioma in both age groups suggests that strategies to fully activate DC/APC function may be beneficial. This study examined the response of tumour-exposed DCs/CD11c\(^+\) cells to LPS/IFN-\(\gamma\), chemotherapy and IL-2/CD40 immunotherapy. Stimulation of tumour-exposed human MoDCs with LPS/IFN-\(\gamma\) only partially restored expression of co-stimulatory molecules (CD40, CD80 and CD86), and simultaneously increased expression of inhibitory molecules (CD39, CD73, PD-L1 and IL-10), suggesting these MoDCs are likely to inhibit T cells. This was supported by the observation that tumour-exposed, LPS/IFN-\(\gamma\)-activated MoDCs induced a suppressive/exhausted phenotype in young and elderly T cells. It is possible that tumour-exposed MoDCs cannot respond to LPS/IFN-\(\gamma\) due to tumour-induced downregulation of TLR-4 (1850). Alternatively, there may be a global impairment in the ability of tumour-exposed DCs to respond to immune stimulation, as tumour-derived factors, such as VEGF, IL-6, IL-10 and
TGF-β prevent DC maturation in response to LPS, TNF-α and CD40 ligation (1003, 1004, 1024, 1028-1030).

**Figure 9.1 Young and elderly tumour-exposed MoDCs induce a regulatory status in young CD8+ and CD4+ T cells**

Young T cells were stimulated by young and elderly tumour-exposed MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. In each heat map, percentages of cells positive for each marker were compared between parent (top values) and daughter (bottom values) CD8+ and CD4+ T cells stimulated by young (A, B) and elderly (C, D) tumour-exposed MoDCs. Data are shown as individual values, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs.
Figure 9.2 Young and elderly tumour-exposed MoDCs induce a regulatory status in elderly CD8+ and CD4+ T cells

Elderly T cells were stimulated by young and elderly tumour-exposed MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. In each heat map, percentages of cells positive for each marker were compared between parent (top values) and daughter (bottom values) CD8+ and CD4+ T cells stimulated by young (A, B) and elderly (C, D) tumour-exposed MoDCs. Data are shown as individual values, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs.

Tumour-exposed CD11c+ cells may also have diminished responsiveness to IL-2/CD40. Stimulation of DCs via CD40 induces potent DC maturation (809, 810), however, TDLN CD11c+ cells from IL-2/CD40-treated mice demonstrated reduced
IFN-γ, TNF-α and IL-12 levels, suggesting their ability to respond to stimulation via CD40 may be impaired, possibly due to the reduced CD40 expression on TDLN CD11c⁺ cells. A previous study from our laboratory showed that MoDCs from mesothelioma patients had reduced CD40 expression and only weakly responded to CD40L stimulation (322), supporting the theory that mesothelioma reduces the ability of DCs/APCs to respond to CD40 stimulation. In this study, an important effect of IL-2/CD40 on CD11c⁺ cells was reduced suppressive capacity, indicated by reductions in CD39, CD73, A2A receptor, IL-10 and TGF-β expression. However, tumour-bearing mice received one-third of the usual IL-2/CD40 schedule to enable examination of tumours before they resolved completely (in young mice at least), and further examination of TDLN DCs/CD11c⁺ cells after a full schedule of IL-2/CD40 is required.

The chemotherapies used this study, gemcitabine and cisplatin, can activate DCs by inducing release of immunostimulatory molecules (ATP and HMGB-1) from tumour cells (1187, 1190, 1194, 1272, 1273). The murine studies showed that whilst CD11c⁺ cells from TDLNs and tumours of chemotherapy-treated mice up-regulated antigen-presenting and co-stimulatory molecules, and pro-inflammatory cytokines (MHC class II, CD86, IFN-γ and/or IL-12), they simultaneously increased expression of inhibitory markers (CD39, CD73, A2A and A2B receptors and/or TGF-β), implying regulatory DCs. Regulatory APCs are more likely to inhibit T cells, and induce suppressive/exhausted T cells. This is supported by data showing that CD8⁺ T cells from gemcitabine-treated mice simultaneously displayed characteristics of activation/effector function (increased CD25 and IFN-γ) and exhaustion/suppressive function (increased CD39, LAG-3 and ICOS). This is likely to diminish anti-tumour immune responses following chemotherapy, which may contribute to the tumour outgrowth seen once chemotherapy ceases (1193, 1233, 1245-1247). These data suggest that combining chemotherapy with checkpoint blockade, in particular, targeting enzymes and receptors of the adenosine pathway, LAG-3 and TGF-β, may be beneficial. Again, further examination of DC and T cell function following a full schedule of chemotherapy is required. Taken together, these results suggest that mesothelioma-exposed DCs/APCs cannot respond to activation signals, and further
investigation of optimal strategies to augment DC function in mesothelioma is needed.

Age-specific changes further impair anti-mesothelioma immune responses in elderly hosts. Specifically, elderly human blood mDC1s, mDC2s and pDCs exposed to mesothelioma factors in vitro demonstrated greater reductions in antigen-presenting and co-stimulatory molecules and pro-inflammatory cytokines (MHC class I, CD40, CD80, TNF-α, IL-6 and/or IL-12), suggesting reduced capacity to activate tumour-specific effector T cells, relative to young hosts. In addition, tumour-associated CD11c+ cells from elderly mice displayed a more pronounced semi-mature status compared to their younger counterparts, due to reduced expression of antigen-presenting and co-stimulatory molecules (MHC class I and II, CD80, and CD86), and increased IL-10 expression. This suggests that the ability of elderly CD11c+ cells to present tumour antigens and provide appropriate co-stimulatory signals to CD8+ and CD4+ T cells is compromised, and that these CD11c+ cells are more likely to induce Tregs via IL-10 (465-468, 477), thereby preventing effective anti-tumour immune responses. This is supported by the observation that elderly tumour-associated CD8+ and CD4+ T cells had lower levels of IFN-γ implying weakened anti-tumour effector activity (1672, 1851, 1852). In-depth T cell functional studies, for example, using a tumour model expressing a known antigen (such as ovalbumin), and CD8+ transgenic T cells specific for that antigen (OT-I cells; 1853) could confirm that tumour antigen-specific CD8+ T cell cytotoxic function is impaired with aging.

An important finding of this study was that during aging (healthy aging, cancer, and treatment with chemo- or immunotherapy), elderly DCs/APCs and T cells were inevitably more skewed towards a suppressive/regulatory status. Whilst elderly human blood DCs and MoDCs, and elderly murine LN/TDLN and splenic CD11c+ cells retained or, in some cases, increased expression of antigen-presenting and co-stimulatory molecules and pro-inflammatory cytokines, they simultaneously increased expression of inhibitory molecules and/or anti-inflammatory cytokines. This was paralleled by a striking age-related increase in expression of the majority of regulatory markers examined on CD8+ and CD4+ T cells in elderly murine
LNs/TDLNs and spleens (Figures 9.3-9.6), suggesting elderly T cells may be more suppressive and/or exhausted (799, 1101, 1102, 1104, 1105).

### A Healthy mice: LN CD8⁺ T cells

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### B Tumour-bearing mice: TDLN CD8⁺ T cells

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**Figure 9.3 Increased regulatory markers on elderly LN/TDLN CD8⁺ T cells**

Tumour-draining lymph nodes (TDLNs) from young and elderly AE17 tumour-bearing mice, and lymph nodes (LNs) from young and elderly healthy mice were stained for CD8⁺ T cells, activation markers (CD25 and intracellular IFN-γ and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), CTLA-4, PD-1, ICOS, LAG-3, and intracellular IL-10 and TGF-β), and analysed by flow cytometry. In each heat map, percentages of CD8⁺ T cells positive for each marker were compared between young mice (top values) and elderly mice (bottom values), for healthy mice (A) and tumour-bearing mice (B). Data are shown as individual values, n = 6-14 young and n = 7-16 elderly tumour-bearing mice, n = 6-13 young and n = 5-14 elderly healthy mice.
Figure 9.4 Increased regulatory markers on TDLN CD8+ T cells in elderly chemothermal and immunotherapy-treated mice

Tumour-draining lymph nodes (TDLNs) from young and elderly AE17 tumour-bearing mice treated with gemcitabine, cisplatin or IL-2/CD40 were stained for CD8+ T cells, activation markers (CD25 and intracellular IFN-γ and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), CTLA-4, PD-1, ICOS, LAG-3, and intracellular IL-10 and TGF-β), and analysed by flow cytometry. In each heat map, percentages of CD8+ T cells positive for each marker were compared between young mice (top values) and elderly mice (bottom values), for gemcitabine-treated mice (A), cisplatin-treated mice (B), and IL-2/CD40-treated mice (C). Data are shown as individual values, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 6-11 elderly cisplatin-treated mice, n = 7-10 young and 6-11 elderly IL-2/CD40-treated mice.
**Figure 9.5 Increased regulatory markers on elderly LN/TDLN and splenic CD4^+^ T cells**

Tumour-draining lymph nodes (TDLNs) and spleens from young and elderly AE17 tumour-bearing mice, and lymph nodes (LNs) from young and elderly healthy mice were stained for CD4^+^ T cells, activation markers (CD25 and intracellular IFN-γ and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), CTLA-4, PD-1, ICOS, LAG-3, and intracellular IL-10 and TGF-β), and analysed by flow cytometry. In each heat map, percentages of CD4^+^ T cells positive for each marker were compared between young mice (top values) and elderly mice (bottom values), for healthy LNs (A), TDLNs (B), and spleens from tumour-bearing mice (C). Data are shown as individual values, n = 6-14 young and n = 7-16 elderly tumour-bearing mice, n = 6-21 young and n = 5-22 elderly healthy mice.
Figure 9.6 Increased regulatory markers on TDLN CD4+ T cells in elderly chemo- and immunotherapy-treated mice

Tumour-draining lymph nodes (TDLNs) from young and elderly AE17 tumour-bearing mice treated with gemcitabine, cisplatin or IL-2/CD40 were stained for CD4+ T cells, activation markers (CD25 and intracellular IFN-γ and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), CTLA-4, PD-1, ICOS, LAG-3, and intracellular IL-10 and TGF-β), and analysed by flow cytometry. In each heat map, percentages of CD4+ T cells positive for each marker were compared between young mice (top values) and elderly mice (bottom values), for gemcitabine-treated mice (A), cisplatin-treated mice (B), and IL-2/CD40-treated mice (C). Data are shown as individual values, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 6-11 elderly cisplatin-treated mice, n = 7-10 young and 6-11 elderly IL-2/CD40-treated mice.
These age-related changes may impact on the outcome of DC/T cell cross-talk, which depends on the summation of positive and negative signals (7, 707, 708, 713, 1854). The observations from this study suggest that: (i) elderly DCs/APCs have an increased potential to provide negative signals to T cells, and (ii) elderly T cells have an increased ability to respond to negative signals, meaning there is likely to be a greater summation of negative signals during DC/T cell interactions, leading to suppression of effector T cells and/or promotion of Tregs (707, 712). Inhibitory DC/T cell cross-talk is a key mechanism for attenuating T cell responses once they are no longer required (9, 710, 1854), and is important for maintaining immune tolerance under healthy conditions and preventing autoimmunity (7, 727). However, induction of a regulatory environment in elderly LNs/TDLNs, and the skewing of DC/T cell cross-talk towards an inhibitory outcome during aging may impair effector T cell responses against pathogens and tumours, and contribute to increased susceptibility to infections, reduced vaccination responses, and tumour progression in the elderly (31, 42-45).

The increased regulatory status of elderly DCs/APCs and T cells may also affect responses to anti-cancer therapies. In this study, chemotherapy appeared to exacerbate the age-related increase in expression of inhibitory markers, specifically, CD39, PD-L1 and TGF-β on elderly CD11c+ cells, and PD-1, LAG-3, ICOS, A2A and A2B receptors and TGF-β on elderly CD8+ and CD4+ T cells. This may further increase the likelihood of negative APC/T cell interactions, thus undermining chemotherapy-induced anti-tumour immunity, and leading to reduced efficacy of chemotherapy in the elderly, as observed for cisplatin in this study. Additionally, this study observed that IL-2/CD40 immunotherapy was less effective at reducing tumour burden in elderly, compared to young mice. This may be because IL-2/CD40 exacerbated the age-related increase in: (i) CD73 and TGF-β on TDLN CD11c+ cells; and (ii) CTLA-4, PD-1, ICOS, LAG-3, A2A receptor and TGF-β on TDLN CD8+ and/or CD4+ T cells, thereby further increasing the regulatory environment in elderly TDLNs. These results suggest that in order to improve the efficacy of chemotherapy and IL-2/CD40 immunotherapy in elderly mesothelioma-bearing hosts, these therapies should be combined with checkpoint blockade of multiple inhibitory molecules (CD39, CD73,
A2A and A2B receptors, PD-L1, PD-1, LAG-3, ICOS and TGF-β).

Combining chemotherapy and immunotherapy with checkpoint blockade may be possible in the clinic, based on the limited clinical evidence to-date showing that checkpoint blockade can be effective in elderly patients. A few recent studies evaluating anti-CTLA-4 and anti-PD-1 blockade in melanoma patients have included elderly patients (aged 65 years and above), and report that elderly patients show similar survival benefits and ability to tolerate treatments, compared to younger patients (1333, 1855, 1856). Although these studies did not perform in-depth analysis of immune cells in elderly patients treated with checkpoint blockade, the promising results seen provide support for the use of checkpoint blockade strategies in elderly patients, which could then potentially be combined with other therapies, as suggested by the results of this study.

Whilst the results from this study suggest that CD11c+ cells and T cells become more suppressive during aging, further functional studies are required to confirm this. For example, elderly LN/TDLN CD11c+ cells could be isolated, and co-cultured with T cells, followed by analysis of T cells to determine whether they are functional by measuring cytotoxic activity and production of effector molecules and cytokines (such as perforin, granzymes, IFN-γ and IL-2). In the murine studies, functional analyses, and analysis of inhibitory marker expression needs to be extended to DC subsets. To-date, one study has shown that splenic pDCs from middle-aged (12 months old) mice suppressed the ability of splenic cDCs to activate pathogen-specific IFN-γ+CD8+ T cells in a PD-L1-dependent manner (233); thus, studies to determine whether elderly DCs from hosts with cancer exert suppression in a similar manner should be performed.

Similarly, further investigation of elderly T cell function is required. The results from this study suggest that elderly CD8+ and CD4+ T cells may be exhausted, based on increased expression of multiple inhibitory molecules. Exhausted T cells are functionally impaired, including defects in proliferative capacity, cytotoxic activity, secretion of IL-2, IFN-γ and TNF-α, and impaired differentiation into memory T cells (1103, 1105, 1857-1860); the capacity of elderly LN/TDLN CD8+ and CD4+ T cells to
perform these functions should be tested. In addition, increased expression of multiple inhibitory/checkpoint molecules on elderly CD8⁺ and CD4⁺ T cells (such as CTLA-4, LAG-3, CD39, CD73, A2A and A2B receptors, IL-10 and TGF-β) suggests increased Treg activity in the elderly (450, 454, 491), and further studies are required to examine elderly Tregs. Functional assays to assess the ability of elderly CD8⁺ and CD4⁺ Tregs to suppress effector T cell proliferation and function should be performed, as there is evidence that Treg suppressive activity is increased during healthy aging (590, 592, 593, 598), and during cancer, as reducing Treg numbers restores T cell anti-tumour immunity in elderly mice (602). Additionally, studies assessing adenosine production by elderly CD11c⁺ cells and T cells should be performed, as this is likely to be increased due to age-related increases in CD39 and CD73 expression. One study demonstrated that healthy elderly human T cells have an increased capacity to produce adenosine (920). Thus, this needs to be studied in the context of aging and cancer as adenosine production is a major mechanism of immunosuppression within tumours (1094, 1095, 1566, 1751, 1752, 1861), and confounds immune responses following chemotherapy (545, 1243, 1244, 1816, 1817, 1819), as well as contributing to chemotherapy resistance (1243). Lastly, blockade of the inhibitory molecules that were up-regulated in this study should be performed to see if this restores elderly CD11c⁺ cell and T cell function, as others have shown that PD-L1/PD-1 blockade restores the proliferative capacity (788) and IFN-γ-mediated effector activity (245) of elderly T cells.

Whilst the increased inhibitory status of elderly DCs and T cells has implications for DC/T cell cross-talk, it may also affect their interactions with other cells. The regulatory molecules examined in this study are expressed on a wide range of immune and non-immune cells, allowing interactions with DCs and T cells expressing the appropriate receptor or ligand. For example, elevated PD-1 on elderly T cells increases the likelihood of negative interactions with: (i) PD-L1⁺ tissue cells, which maintains tolerance and prevents autoimmunity in the healthy steady state (883, 1862-1865), or (ii) PD-L1⁺ tumour cells, which terminates anti-tumour effector T cells (889, 900, 1126, 1866, 1867). This study observed age-related increases in several inhibitory molecules on T cells implying increased potential for
inhibitory interactions between T cells and other immune and non-immune cells in elderly hosts. Furthermore, expression of inhibitory receptors/ligands may also be altered on tissue and tumour cells during aging, which could further affect local T cell responses. Thus, age-related changes in DCs/APCs and T cells could contribute to a global increase in inhibitory interactions.

There are several possible factors and mechanisms that could contribute to the age-related changes in DCs/APCs and T cells seen in this study. These include changes in the aging environment, such as increases in circulating pro- and anti-inflammatory cytokines (32-34, 38, 1724, 1727), and age-associated changes in other circulating factors that modulate DCs/APCs and T cells, such as hormones and lipids (1868-1871). In particular, lipids may be key modulators of DCs during aging and cancer, as our previous studies have shown that: (i) mesothelioma factors promote lipid acquisition by young human MoDCs in vitro, which is associated with reduced antigen processing and a semi-mature tolerogenic status characterised by reduced CD1a and increased CD86 and IL-10 (228); (ii) the lipid content of tumour-associated CD11c+ cells in young mesothelioma-bearing mice increases with tumour burden, and is associated with reduced tumour antigen-specific T cell proliferation in TDLNs (228); and (iii) the lipid content of murine CD8α+ cDCs and pDCs in LNs and livers increases during healthy aging (240). Age-related changes occurring at the cellular and molecular levels may also play a role. Altered gene expression profiles in elderly DCs and T cells affect genes involved in key DC functions, such as anti-viral activity, migration, T cell priming and tolerance (662), as well as genes involved in cellular processes such as oxidative stress responses, apoptosis and signalling pathways, which could affect the function of elderly DCs and T cells (662, 1872). Signalling pathways are also altered in elderly DCs and T cells, leading to reduced DC and T cell activation, for example, pathways associated with DC activation/maturation (such as the IFN, NFκB, PI3 kinase and STAT pathways) have been shown to be impaired in elderly DCs (67, 68, 250-252, 661). Elderly T cells display defects in pathways associated with the TCR, CD28 and IL-2 receptor (717-721). In addition, age-related changes in DNA methylation patterns (1873, 1874), and metabolic pathways, such as those involved in oxidative stress responses (252, 631, 1875-1877), and
mitochondrial activity (241) affect immune cell (including DC and T cell) function. Additional studies are required to examine possible mechanisms underlying the changes in DCs and T cells seen in this study, in particular changes in gene expression profiles, for example, to determine if the exhausted phenotypic profile of elderly T cells is reflected at the transcriptional level.

In summary, this study has shown that, regardless of age: (i) mesothelioma reduces cross-presenting human mDC2s and murine TDLN CD8α−CD11b+ cDCs, which may impair the generation of tumour-specific cytotoxic CD8+ T cell responses, and increases pDCs in murine TDLNs and spleens which increases suppression of anti-mesothelioma immune responses, and (ii) mesothelioma further compromises DC/APC function by inducing semi-mature DCs/CD11c+ cells which are likely to promote T cell tolerance. Taken together, this may reduce anti-mesothelioma immune responses and contribute to tumour progression. Additionally, mesothelioma-exposed young and elderly DCs/CD11c+ cells demonstrated a diminished ability to respond to stimulation with LPS/IFN-γ and IL-2/CD40, and developed a regulatory status following chemotherapy, which may affect the efficacy of these therapies.

With aging, the mesothelioma-induced semi-mature status of DCs/CD11c+ cells was exacerbated in the elderly, which likely further compromises anti-mesothelioma effector T cell responses. Importantly, in all contexts of aging examined (healthy aging, cancer and treatment with chemo- or immunotherapy), elderly DCs/CD11c+ cells and T cells were skewed towards a suppressive/regulatory status, with LNs and spleens becoming the predominant sites of regulation in elderly mice. This suggests a greater summation of negative signals during APC/T cell interactions, leading to effector T cell suppression and/or generation of Tregs. An age-associated increase in immunosuppressive activity and T cell exhaustion may result in reduced anti-pathogen and anti-tumour immune responses, contributing to increased susceptibility to infections, increased incidence of cancer and tumour progression in the elderly. This may also reduce the efficacy of anti-cancer therapies, such as IL-2/CD40 immunotherapy and chemotherapy in elderly tumour-bearing hosts, as both therapies exacerbated the suppressive status of elderly TDLN CD11c+ cells and
T cells. This suggests that combining chemotherapy and IL-2/CD40 with checkpoint blockade targeting multiple regulatory molecules (particularly those for which chemotherapy and IL-2/CD40 exacerbated age-related increases, i.e. CD39, CD73, A2A and A2B receptors, PD-L1, PD-1, LAG-3, ICOS and TGF-β) is required in order to improve the efficacy of anti-cancer therapies in elderly hosts with mesothelioma.
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Chapter 12: Immune System

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Appendix A    Volunteer questionnaire

Volunteer Questionnaire

1. Age        _________years

2. Gender     Male    Female

3. Date of Birth  ____/____/____

4. Is there any history in your biological or blood relatives of:

☐ Heart problems  ☐ Rheumatoid arthritis
☐ High blood fats  ☐ Diabetes
☐ High blood pressure  ☐ Other autoimmune diseases (e.g. Lupus, MS)
☐ Stroke
☐ Easy bleeding  ☐ Cancer of  ____________
☐ Allergy
☐ Other  ____________
☐ Asthma

5. Have you ever been diagnosed with any of the following conditions?

☐ Heart problems  ☐ Rheumatoid arthritis
☐ High blood fats  ☐ Diabetes
☐ High blood pressure  ☐ Other autoimmune diseases (e.g. Lupus, MS)
☐ Stroke
☐ Easy bleeding  ☐ Cancer of  ____________
☐ Allergy
☐ Other  ____________
☐ Asthma

6. Statement of present health (please tick the box)

Excellent    Good    Fair    Poor

Please Explain

________________________________________________________________________________
7. Are you currently taking any steroid medicines?

☐ Cortisone  ☐ Dexamethasone

☐ Corticosteroids  ☐ Decadron

☐ Cortisone acetate  ☐ Hydrocortisone

☐ Prednisone  ☐ Solumedrol

☐ Prednisolone  ☐ Other ______________________

8. Are you currently taking any anticoagulant (blood thinning) medicines?

☐ Warfarin  ☐ Fondaparinux

☐ Heparin  ☐ Aspirin

☐ Cloxiar  ☐ Other ______________________

☐ Fragmin

9. Are you currently taking any medicines for lowering blood fats?

☐ Atorvastatin (Lipitor)  ☐ Gemfibrozil

☐ Simvastatin (Zocor)  ☐ Fenofibrate

☐ Rosuvastatin (Crestor)  ☐ Other ______________________

☐ Pravastatin (Pravachol)

10. Have you been prescribed anti-inflammatory medicines?

☐ Ibuprofen (Nurofen)  ☐ Other ____________

☐ Celecoxib

11. Please list all prescription medication currently being taken

_________________________________________________________________

_________________________________________________________________

12. Please list all non-prescription medication currently being taken

_________________________________________________________________

_________________________________________________________________
13. Do you currently smoke tobacco (cigarette, cigar or pipe)?

☐ Yes, on most or all days  ☐ Only occasionally  ☐ No

14. Have you been knowingly exposed to asbestos? E.g. Brake pads, asbestos fencing, shipbuilding industry, etc.

☐ Yes  ☐ No
Appendix B  Supplementary data for Chapter 3

Appendix Table B.1 Known medical conditions, medications, smoking status and asbestos exposure status of study volunteers

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Young Elderly

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**Smoking status**

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**Known asbestos exposure**

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</tr>
<tr>
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<td>18 (90%)</td>
<td>12 (60%)</td>
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**Appendix Figure B.1** Young and elderly immature MoDCs induce young CD4+ T cells with similar phenotypes

Young CD4+ T cells were stimulated by young and elderly immature MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD4+ T cells were compared for samples stimulated by young versus elderly immature MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001, comparing parent to daughter T cells.
Appendix Figure B.2 Young and elderly immature MoDCs induce elderly CD8+ T cells with similar phenotypes

Elderly CD8+ T cells were stimulated by young and elderly immature MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) markers from parent to daughter CD8+ T cells were compared for samples stimulated by young versus elderly immature MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, comparing parent to daughter T cells.
Appendix Figure B.3 Young and elderly immature MoDCs induce elderly CD4^+ T cells with similar phenotypes

Elderly CD4^+ T cells were stimulated by young and elderly immature MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4^+ T cells positive for activation (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) markers from parent to daughter CD4^+ T cells were compared for samples stimulated by young versus elderly immature MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, **** = p<0.0001, comparing parent to daughter T cells.
Young and elderly PBMCs were stimulated with LPS/IFN-γ for 24 hours, then activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and intracellular IL-10 were analysed on mDC1s via flow cytometry. Percentages of mDC1s positive for activation markers (A) and IL-10 (B), and expression levels (measured as geometric mean fluorescence intensity; MFI) of IL-10 (C) were measured. Each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005, comparing LPS/IFN-γ-mDC1s to unstimulated mDC1s from the same volunteer.
Appendix Figure B.5 No age-related differences in LPS/IFN-γ-mDC2 activation and regulatory markers

Young and elderly PBMCs were stimulated with LPS/IFN-γ for 24 hours, then stained for CD141+ mDC2s, activation markers (CD40, CD80, CD86, and intracellular TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2AR, A2BR, PD-L1, GAL-9, and intracellular IL-10 and TGF-β) for flow cytometric analysis. Percentages of mDC2s positive for activation (A) and regulatory markers (B) were measured. Each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers. * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing LPS/IFN-γ-mDC2s to unstimulated mDC2s from the same volunteer.
Appendix Figure B.6 No age-related differences in LPS/IFN-γ-pDC activation and regulatory markers

Young and elderly PBMCs were stimulated with LPS/IFN-γ for 24 hours, then stained for CD123+CD303+ pDCs, activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2AR, A2BR, GAL-9, and intracellular IL-10 and TGF-β) for flow cytometric analysis. Percentages of pDCs positive for activation (A) and regulatory markers (B) were measured. Each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing LPS/IFN-γ-pDCs to unstimulated pDCs from the same volunteer.
Appendix Figure B.7 Young and elderly PBMCs up-regulate pro-inflammatory cytokines after LPS/IFN-γ

Cytokine concentrations in young and elderly LPS/IFN-γ-stimulated PBMC culture supernatants were measured using a cytokine bead array. Concentrations of IFN-α, IFN-γ, TNF-α, MCP-1, IL-1β and IL-6 (A), and IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33 (B) were measured. Each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing LPS/IFN-γ-PBMCs to unstimulated PBMCs from the same volunteer.
Appendix Figure B.8 Young and elderly LPS/IFN-γ-MoDCs have similar regulatory marker expression

Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, then stimulated with LPS/IFN-γ for a further 2 days, then stained for CD11c⁺CD14⁻ MoDCs and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1 and galectin-9 (GAL-9), and intracellular IL-10 and TGF-β), and analysed by flow cytometry. Percentages of CD11c⁺CD14⁻ MoDCs positive for regulatory markers were measured; each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 7-10 young volunteers, n = 7-10 elderly volunteers. * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing LPS/IFN-γ-MoDCs to unstimulated MoDCs from the same volunteer.

Appendix Figure B.9 No age-related differences in cytokine secretion by LPS/IFN-γ-MoDCs

Concentrations of cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-10, IL-12p70, IL-17A, IL-18, and IL-33) were measured in culture supernatants from LPS/IFN-γ-stimulated young and elderly MoDCs via cytokine bead array. Each line represents an individual volunteer, and compares their LPS/IFN-γ-stimulated sample to their unstimulated control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, ** = p<0.005 comparing LPS/IFN-γ-MoDCs to unstimulated MoDCs from the same volunteer.
Appendix Figure B.10 Young and elderly LPS/IFN-γ-MoDCs induce young CD8+ T cells with similar phenotypes

Young CD8+ T cells were stimulated by young and elderly LPS/IFN-γ-MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD8+ T cells were compared for samples stimulated by young versus elderly LPS/IFN-γ-MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001, comparing parent to daughter T cells.
Appendix Figure B.11 Young and elderly LPS/IFN-γ-MoDCs induce young CD4⁺ T cells with similar phenotypes

Young CD4⁺ T cells were stimulated by young and elderly LPS/IFN-γ-MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4⁺ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD4⁺ T cells were compared for samples stimulated by young versus elderly LPS/IFN-γ-MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005, comparing parent to daughter T cells.
Appendix Figure B.12 Young and elderly LPS/IFN-γ-MoDCs stimulate similar levels of elderly T cell proliferation

Young and elderly LPS/IFN-γ-MoDCs were co-cultured with allogeneic, CFSE-labelled elderly T cells at a ratio of 1 DC: 5 T cells, for 5 days. Samples were stained for CD8+ T cells, CD4+ T cells, and CD4+CD25+CD127low Tregs and analysed by flow cytometry as per Figures 3.11A-3.11F. The percentage of T cell proliferation was calculated based on loss of staining intensity of the parent peak. Percentages of elderly CD8+ T cell, CD4+ T cell and Treg cell proliferation stimulated by LPS/IFN-γ-MoDCs were compared to those stimulated by age-matched unstimulated MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005 comparing LPS/IFN-γ-MoDCs to age-matched unstimulated MoDCs.
Appendix Figure B.13 Young and elderly LPS/IFN-γ-MoDCs induce elderly CD8+ T cells with similar phenotypes

Elderly CD8+ T cells were stimulated by young and elderly LPS/IFN-γ-MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD8+ T cells were compared for samples stimulated by young versus elderly LPS/IFN-γ-MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005, comparing parent to daughter T cells.
Appendix Figure B.14 Young and elderly LPS/IFN-γ-MoDCs induce elderly CD4⁺ T cells with similar phenotypes

Elderly CD4⁺ T cells were stimulated by young and elderly LPS/IFN-γ-MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4⁺ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD4⁺ T cells were compared for samples stimulated by young versus elderly LPS/IFN-γ-MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer's MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers' MoDCs, n = 3-10 elderly volunteers' MoDCs, * = p<0.05, ** = p<0.005, **** = p<0.0001, comparing parent to daughter T cells.
Appendix C  Supplementary data for Chapter 4

Appendix Figure C.1 No age-related differences in activation and regulatory markers on tumour-exposed mDC1s

Young and elderly PBMCs were exposed to conditioned media from the human JU77 mesothelioma cell line (JU77 tumour-conditioned media; TCM) for 24 hours, then stained for CD1c+ mDC1s, activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1, galectin-9 (GAL-9), and intracellular IL-10 and TGF-β) for flow cytometric analysis. Percentages of mDC1s positive for activation markers (A) and regulatory markers (B) were measured. Each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed to non-tumour-exposed mDC1s from the same volunteer.
Appendix Figure C.2 Young, but not elderly, tumour-exposed mDC2s up-regulate PD-L1

Young and elderly PBMCs were exposed to conditioned media from the human JU77 mesothelioma cell line (JU77 tumour-conditioned media; TCM) for 24 hours, then stained for CD141+ mDC2s, activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1, galectin-9 (GAL-9), and intracellular IL-10 and TGF-β) for flow cytometric analysis. Percentages of mDC2s positive for activation markers (A) and regulatory markers (B) were measured. Each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed to non-tumour-exposed mDC2s from the same volunteer.
Appendix Figure C.3 Young, but not elderly, tumour-exposed pDCs have increased CD73 and PD-L1

Young and elderly PBMCs were exposed to conditioned media from the human JU77 mesothelioma cell line (JU77 tumour-conditioned media; TCM) for 24 hours, then stained for CD123+CD303+ pDCs, activation markers (MHC class I, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1, galectin-9 (GAL-9), and intracellular IL-10 and TGF-β) for flow cytometric analysis. Percentages of pDCs positive for activation markers (A) and regulatory markers (B) were measured. Each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 13 young volunteers, n = 11 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing tumour-exposed to non-tumour-exposed pDCs from the same volunteer.
Appendix Figure C.4 JU77 tumour cells secrete TNF-α, MCP-1, IL-6 and IL-8
Concentrations of pro-inflammatory cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-6, IL-12p70, IL-17A, IL-18, IL-23 and IL-33), chemokines (monocyte chemoattractant protein-1 (MCP-1) and IL-8) and the anti-inflammatory cytokine IL-10 were measured in culture supernatants from human JU77 mesothelioma cells using a cytokine bead array. Data are shown as mean ± SEM, n = 2 JU77 cell culture supernatant samples, tested in separate experiments.
Appendix Figure C.5 Young and elderly tumour-exposed PBMCs have similar cytokine secretion

Young and elderly PBMCs were exposed to conditioned media from the human JU77 mesothelioma cell line (JU77 tumour-conditioned media; TCM) for 24 hours, and concentrations of IFN-α, IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1), IL-1β and IL-6 (A), and IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33 (B) were measured in culture supernatants from PBMC/JU77 TCM co-cultures using a cytokine bead array. Concentrations of cytokines measured in PBMC/JU77 TCM co-cultures were adjusted by subtracting the average concentration of each cytokine measured in JU77 TCM only. Each line in (A) and (B) represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05 comparing tumour-exposed to non-tumour-exposed PBMCs from the same volunteer.
Appendix Figure C.6 Young and elderly tumour-exposed MoDCs have similar expression of activation and regulatory markers

Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and during this period, MoDCs were exposed to human JU77 mesothelioma cells. On day 7, samples were stained for CD11c⁺CD14⁻ MoDCs, activation markers (MHC class I, CD1a, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1, galectin-9 (GAL-9), and intracellular IL-10 and TGF-β), and analysed using flow cytometry. Percentages of MoDCs positive for activation markers (A) and regulatory markers (B) were measured. Each line represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 7-12 young volunteers, n = 7-11 elderly volunteers, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing tumour-exposed to non-tumour-exposed MoDCs from the same volunteer.
Appendix Figure C.7 Young and elderly tumour-exposed MoDCs have similar cytokine secretion

Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and during this period, MoDCs were exposed to human JU77 mesothelioma cells. Concentrations of IFN-α, IFN-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1), IL-1β and IL-6 (A), and IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33 (B) were measured in culture supernatants from MoDC/JU77 cells co-cultures using a cytokine bead array. Concentrations of cytokines measured in MoDC/JU77 cells co-cultures were adjusted by subtracting the average concentration of each cytokine measured in JU77 TCM only. Each line in (A) and (B) represents an individual volunteer, and compares their tumour-exposed sample to their non-tumour-exposed control. Data are shown as mean ± SEM, n = 14 young volunteers, n = 12 elderly volunteers, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing tumour-exposed to non-tumour-exposed MoDCs from the same volunteer.
Appendix Figure C.8 Young and elderly tumour-exposed MoDCs stimulate similar levels of elderly T cell proliferation

Young and elderly MoDCs exposed to JU77 tumour cells were co-cultured with allogeneic, CFSE-labelled elderly T cells at a ratio of 1 DC: 5 T cells, for 5 days. Samples were stained for CD8+ T cells, CD4+ T cells, and CD4+CD25+CD127low Tregs and analysed by flow cytometry as per Figures 3.11A-3.11F. The percentage of T cell proliferation was calculated based on loss of staining intensity of the parent peak. Percentages of elderly CD8+ T cell, CD4+ T cell and Treg cell proliferation stimulated by tumour-exposed MoDCs were compared to those stimulated by age-matched non-tumour-exposed MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs.
Appendix Figure C.9 Young and elderly tumour-exposed MoDCs induce elderly CD8+ T cells with a regulatory phenotype

Elderly CD8+ T cells were stimulated by young and elderly tumour-exposed MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD8+ T cells were compared for samples stimulated by young versus elderly tumour-exposed MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing parent to daughter T cells.
Appendix Figure C.10 Young and elderly tumour-exposed MoDCs induce young CD4+ T cells with a regulatory phenotype

Young CD4+ T cells were stimulated by young and elderly tumour-exposed MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD4+ T cells were compared for samples stimulated by young versus elderly tumour-exposed MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing parent to daughter T cells.
Appendix Figure C.11 Young and elderly tumour-exposed MoDCs induce elderly CD4⁺ T cells with a regulatory phenotype

Elderly CD4⁺ T cells were stimulated by young and elderly tumour-exposed MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4⁺ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD4⁺ T cells were compared for samples stimulated by young versus elderly tumour-exposed MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 5-12 young volunteers’ MoDCs, n = 4-11 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing parent to daughter T cells.
Appendix Figure C.12 No age-related differences in activation and regulatory markers on tumour-exposed-LPS/IFN-γ mDC1s

Young and elderly PBMCs were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ for 24 hours, stained for CD1c+ mDC1s, activation markers (MHC class I, CD40, CD86, and intracellular TNF-α and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1, galectin-9 (GAL-9), and intracellular TGF-β), and analysed by flow cytometry. Percentages of mDC1s positive for activation markers (A), CD39, CD73, A2A receptor and A2B receptor (B), and PD-L1, GAL-9, and TGF-β (C) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer.
Appendix Figure C.13 No age-related differences in activation and regulatory markers on tumour-exposed-LPS/IFN-γ mDC2s

Young and elderly PBMCs were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ for 24 hours, stained for CD141+ mDC2s, activation markers (MHC class I, CD86, and intracellular IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), galectin-9 (GAL-9), and intracellular IL-10 and TGF-β), and analysed by flow cytometry. Percentages of mDC2s positive for activation markers (A), CD39, CD73, A2A receptor and A2B receptor (B), and GAL-9, IL-10 and TGF-β (C) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer.
Appendix Figure C.14 No age-related differences in activation and regulatory markers on tumour-exposed-LPS/IFN-γ pDCs

Young and elderly PBMCs were exposed to JU77 tumour-conditioned media (TCM) and stimulated with LPS/IFN-γ for 24 hours, stained for CD123+CD303+ pDCs, activation markers (MHC class I, and intracellular IFN-γ, TNF-α, IL-6 and IL-12), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), galectin-9 (GAL-9), and intracellular IL-10 and TGF-β), and analysed by flow cytometry. Percentages of pDCs positive for activation markers (A), CD39, CD73, A2A receptor and A2B receptor (B), and GAL-9, IL-10 and TGF-β (C) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer.
Appendix Figure C.15 CD1a, TNF-α and TGF-β decrease on young and elderly tumour-exposed-LPS/IFN-γ MoDCs
Young and elderly monocytes were differentiated into immature MoDCs using GM-CSF and IL-4 for 7 days, and during this period, MoDCs were exposed to human JU77 mesothelioma cells. On day 7, tumour-exposed MoDCs were stimulated with LPS/IFN-γ for a further 2 days. On day 9, samples were stained for CD11c+CD14- MoDCs, activation markers (CD1a and intracellular TNF-α), and regulatory markers (A2A receptor (A2AR), A2B receptor (A2BR), galectin-9 (GAL-9) and intracellular TGF-β), and analysed using flow cytometry. Percentages of MoDCs positive for activation markers (A), and regulatory markers (B) were compared, whereby each set of three points joined by a line represents an individual volunteer and compares their tumour-exposed-LPS/IFN-γ sample (middle point) to their tumour-exposed control (left point) and LPS/IFN-γ control (right point). Data are shown as mean ± SEM, n = 10 young volunteers, n = 10 elderly volunteers, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing tumour-exposed-LPS/IFN-γ samples to (i) tumour-exposed controls or (ii) LPS/IFN-γ controls within the same volunteer.
Appendix Figure C.16 Young and elderly tumour-exposed-LPS/IFN-γ MoDCs induce young CD8+ T cells with a regulatory phenotype

Young CD8+ T cells were stimulated by young and elderly tumour-exposed-LPS/IFN-γ MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD8+ T cells were compared for samples stimulated by young versus elderly tumour-exposed-LPS/IFN-γ MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing parent to daughter T cells.
Appendix Figure C.17 Young and elderly tumour-exposed-LPS/IFN-γ MoDCs induce young CD4+ T cells with a regulatory phenotype

Young CD4+ T cells were stimulated by young and elderly tumour-exposed-LPS/IFN-γ MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD4+ T cells were compared for samples stimulated by young versus elderly tumour-exposed-LPS/IFN-γ MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing parent to daughter T cells.
Appendix Figure C.18 Young and elderly tumour-exposed-LPS/IFN-γ MoDCs induce elderly CD8+ T cells with a regulatory phenotype

Elderly CD8+ T cells were stimulated by young and elderly tumour-exposed-LPS/IFN-γ MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD8+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD8+ T cells were compared for samples stimulated by young versus elderly tumour-exposed-LPS/IFN-γ MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing parent to daughter T cells.
Appendix Figure C.19 Young and elderly tumour-exposed-LPS/IFN-γ MoDCs induce elderly CD4+ T cells with a regulatory phenotype

Elderly CD4+ T cells were stimulated by young and elderly tumour-exposed-LPS/IFN-γ MoDCs in an MLR assay, as described in Figure 3.11. Percentages of parent and daughter CD4+ T cells positive for activation markers (CD25 and intracellular IFN-γ, IL-12 and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), CTLA-4, ICOS, TIM-3, PD-1, and intracellular IL-10 and TGF-β) were measured using flow cytometry. Changes in activation (A) and regulatory (B) marker expression from parent to daughter CD4+ T cells were compared for samples stimulated by young versus elderly tumour-exposed-LPS/IFN-γ MoDCs, where each line represents the change in marker expression from parent to daughter T cells induced by one volunteer’s MoDCs. Data are shown as mean ± SEM, n = 3-10 young volunteers’ MoDCs, n = 3-10 elderly volunteers’ MoDCs, * = p<0.05, **** = p<0.0001 comparing parent to daughter T cells.
Appendix D  Supplementary data for Chapter 5

Appendix Figure D.1 Absolute numbers of murine splenic and blood CD11c+ cells increase with age
Spleens and blood from young and elderly mice were stained for CD11c+ cells and analysed by flow cytometry. Total cell counts for spleens and blood were also performed to allow calculation of absolute numbers of CD11c+ cells per spleen (A) and per ml of blood (B). Data are shown as mean ± SEM, n = 9-12 young and n = 8-11 elderly mice, * = p<0.05 comparing young to elderly mice.

Appendix Figure D.2 Gating strategy for murine DC subsets using CD11c, CD4, CD8α, B220 and GR-1
Spleens, lymph nodes (LN), bone marrow (BM) and blood were collected from young and elderly mice and stained for markers of DC subsets (CD11c, B220, GR-1, CD8α and CD4) and analysed by flow cytometry. Within viable cells (A) and single cells (B) gates, CD11c+ cells were identified (C). Within the CD11c+ cells gate, pDCs were gated as B220+GR-1+ cells (D). Within the pDC exclusion gate (i.e. cells that are not B220 and GR-1 double positive; D), CD8α+CD4- cDCs, CD4+CD8α- cDCs and CD8α CD4- cDCs were gated (E).
Appendix Figure D.3 Absolute numbers of splenic CD11b^+CD8α^-CD4^- cDCs increase with age

Spleens and blood from young and elderly mice were stained for B220^+GR-1^- pDCs, CD8α^+CD11b^- cDCs, CD11b^-CD8α^-CD4^- cDCs, and CD11b^+CD8α^-CD4^- cDCs, and analysed by flow cytometry. Total cell counts for spleens and blood were also performed to allow calculation of absolute numbers of DC subsets per spleen (A) and per ml of blood (B). Data are shown as mean ± SEM, n = 9-12 young and n = 8-11 elderly mice, * = p<0.05 comparing young to elderly mice.
Appendix Figure D.4 No age-related differences in activation and regulatory markers on LN DC subsets

Lymph nodes (LNs) were stained for B220^GR-1^ pDCs, CD8α^CD11b^ cDCs, CD11b^CD8α^ CD4^ cDCs, the activation markers MHC class I, CD40, CD80 and CD86, and the regulatory marker PD-L1, and analysed by flow cytometry. Percentages of pDCs (A), CD8α^CD11b^ cDCs (B), and CD11b^CD8α^CD4^ cDCs (C) positive for each marker are shown as mean ± SEM, n = 8 young and n = 7 elderly mice.
Appendix Figure D.5 Elderly splenic CD11c+ cells have reduced A2A receptor expression

Young and elderly spleens were stained for CD11c+ cells, activation markers (MHC class I, MHC class II, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α, and IL-12), and regulatory markers (CD39, A2A receptor (A2AR), galectin-9 (GAL-9), and intracellular IL-10 and TGF-β), and analysed using flow cytometry. Percentages of CD11c+ cells positive for activation markers (A), and regulatory markers (B) are shown as mean ± SEM, n = 3-17 young and n = 3-21 elderly mice, * = p<0.05 comparing young to elderly mice.
Appendix Figure D.6 Elderly blood CD11c+ cells have increased CD73

Young and elderly blood samples were stained for CD11c+ cells, activation markers (MHC class I, MHC class II, CD40, CD80, CD86, and intracellular IFN-γ, TNF-α and IL-12), and regulatory markers (CD39, CD73, PD-L1, galectin-9 (GAL-9), and intracellular IL-10 and TGF-β), and analysed using flow cytometry. Percentages of CD11c+ cells positive for CD73 (A), activation markers (B), and other regulatory markers (C) were measured. Data are shown as mean ± SEM, n = 3-9 young and n = 3-7 elderly mice, ** = p<0.005 comparing young to elderly mice.
Appendix Figure D.7 Elderly splenic pDCs have reduced PD-L1
Young and elderly spleens were stained for B220^GR-1^ pDCs, CD8α^CD11b^- cDCs, MHC class I, CD40, CD86 and PD-L1 for flow cytometric analysis. Percentages of pDCs positive for MHC class I, CD40 and PD-L1 (A) and percentages of CD8α^CD11b^- cDCs positive for MHC class I, CD86 and PD-L1 (B) are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, **** = p<0.0001 comparing young to elderly mice.
Appendix Figure D.8 Elderly splenic CD11b+CD8αCD4− cDCs have increased activation markers and PD-L1

Young and elderly spleens were stained for CD11b+CD8αCD4− cDCs, activation markers (MHC class I, CD40, CD80 and CD86), and the regulatory marker PD-L1 for flow cytometric analysis. Percentages of cells positive for activation markers (A) and PD-L1 (C), and geometric mean fluorescence intensity (MFI) expression levels of activation markers (B) and PD-L1 (D) were analysed. Data are shown as mean ± SEM, n = 8 young and n = 7 elderly mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.
Appendix Figure D.9 No age-related changes in activation and regulatory markers on LN CD8+ T cells
Young and elderly lymph nodes (LNs) were stained for CD8+ T cells, activation markers (CD25, and intracellular IFN-γ and perforin), and regulatory markers (CD39, A2A receptor (A2AR), CTLA-4 and intracellular TGF-β), and analysed using flow cytometry. Percentages of CD8+ T cells positive for activation (A) and regulatory markers (B) are shown as mean ± SEM, n = 6-11 young and n = 5-12 elderly mice.

Appendix Figure D.10 No differences in activation and regulatory markers on LN CD4+ T cells with age
Young and elderly lymph nodes (LNs) were stained for CD4+ T cells, activation markers (intracellular IFN-γ and perforin), and regulatory markers (CD39, A2A receptor (A2AR) and intracellular TGF-β), and analysed using flow cytometry. Percentages of CD4+ T cells positive for activation (A) and regulatory markers (B) are shown as mean ± SEM, n = 6-8 young and n = 5-9 elderly mice.
Appendix Figure D.11 No differences in splenic CD8^+ T cell activation and regulatory markers with age
Young and elderly spleens were stained for CD8^+ T cells, activation markers (intracellular IFN-γ and perforin), and regulatory markers (CD39, PD-1, ICOS and LAG-3) for flow cytometric analysis. Percentages of CD8^+ T cells positive for activation (A) and regulatory markers (B) were measured. Data are shown as mean ± SEM, n = 5-7 young and n = 6-8 elderly mice.

Appendix Figure D.12 No differences in splenic CD4^+ T cell activation and regulatory markers with age
Young and elderly spleens were stained for CD4^+ T cells, activation markers (CD25, and intracellular IFN-γ and perforin), and regulatory markers (CD39, PD-1 and intracellular IL-10) for flow cytometric analysis. Percentages of CD4^+ T cells positive for activation (A) and regulatory markers (B) were measured. Data are shown as mean ± SEM, n = 5-13 young and n = 6-14 elderly mice.
Appendix E  Supplementary data for Chapter 6

Appendix Figure E.1 No age- or tumour-induced differences in MHC class II, CD40, CD80 and CD86 on TDLN CD11c⁺ cells

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c⁺ cells, MHC class II, CD40, CD80 and CD86, and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of MHC class II (A) and CD80 (B), and percentages of CD11c⁺ cells positive for CD40 (C) and CD86 (D) were measured. Data are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 10-18 young and n = 11-18 elderly healthy mice.
Appendix Figure E.2 Reduced TNF-α and IL-12 expression on CD11c+ cells in TDLNs
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c+ cells and intracellular TNF-α and IL-12, and analysed by flow cytometry. Expression levels (measured as geometric mean fluorescence intensity; MFI) of TNF-α (A) and IL-12 (B) were measured. Data are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 10-14 young and n = 11-15 elderly healthy mice, * = p<0.05, **** = p<0.0001 comparing tumour-bearing mice to age-matched healthy mice.

Appendix Figure E.3 Increased MHC-I, CD73 and TGF-β on CD11c+ cells in elderly TDLNs
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c+ cells, MHC class I, CD73, and intracellular TGF-β, and analysed by flow cytometry. Percentages of CD11c+ cells positive for MHC class I (A), CD73 (B) and TGF-β (C) were measured. Data are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 10-18 young and n = 11-18 elderly healthy mice, * = p<0.05, ** = p<0.005 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.
Appendix Figure E.4 No age-related differences in regulatory markers on TDLN CD11c⁺ cells

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice, and lymph nodes (LNs) from age-matched healthy mice were stained for CD11c⁺ cells and regulatory markers (CD39, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1, galectin-9 (GAL-9), and intracellular IL-10), and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for CD39, A2AR and A2BR (A), and PD-L1, GAL-9 and IL-10 (B) were measured. Data are shown as mean ± SEM, n = 6-11 young and n = 7-12 elderly tumour-bearing mice, n = 9-18 young and n = 8-18 elderly healthy mice, ** = p<0.005 comparing young to elderly mice.
Appendix Figure E.5 No age-related differences in activation and regulatory markers on splenic CD11c^+ cells from tumour-bearing mice

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Spleens from tumour-bearing mice and age-matched healthy mice were stained for CD11c^+ cells, activation markers (MHC class I, CD40 and intracellular IFN-γ, TNF-α and IL-12), and regulatory markers (CD39, A2B receptor (A2BR), galectin-9 (GAL-9), and intracellular IL-10), and analysed by flow cytometry. Percentages of CD11c^+ cells positive for activation markers (A and B), and regulatory markers (C) were measured. Data are shown as mean ± SEM, n = 3-8 young and n = 4-8 elderly tumour-bearing mice, n = 3-15 young and n = 3-15 elderly healthy mice, **** = p<0.0001 comparing young to elderly mice.
Appendix Figure E.6 Reduced CD86 and TGF-β and increased CD73 and PD-L1 on splenic CD11c+ cells from elderly tumour-bearing mice

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Spleens from tumour-bearing mice and age-matched healthy mice were stained for CD11c+ cells, CD86, CD73, PD-L1 and intracellular TGF-β, and analysed by flow cytometry. Percentages of CD11c+ cells positive for CD86 (A), TGF-β (B), CD73 (C) and PD-L1 (D) were measured. Data are shown as mean ± SEM, n = 8 young and n = 8 elderly tumour-bearing mice, n = 15 young and n = 15 elderly healthy mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Appendix Figure E.7 No age-related differences in activation and regulatory markers on tumour CD11c+ cells

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD11c+ cells, activation markers (CD40, CD86, and intracellular IFN-γ and IL-12), and regulatory markers (CD73, A2A receptor (A2AR), A2B receptor (A2BR) and galectin-9 (GAL-9)), and analysed by flow cytometry. Percentages of CD11c+ cells positive for activation markers (A), and regulatory markers (B) were measured. Data are shown as mean ± SEM, n = 6-11 young and n = 7-12 elderly tumour-bearing mice.

Appendix Figure E.8 Reduced proportions of CD8+ T cells and Tregs in elderly TDLNs

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice and lymph nodes (LNs) from age-matched healthy mice were stained for CD8+ T cells and CD4+CD25+FoxP3+ Tregs, and analysed by flow cytometry. Proportions of CD8+ T cells (A) and Tregs (B) are shown as mean ± SEM, n = 14 young and n = 16 elderly tumour-bearing mice, n = 13-20 young and n = 14-21 elderly healthy mice, * = p<0.05, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.
Appendix Figure E.9 Increased regulatory marker expression on elderly TDLN CD8$^+$ T cells

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice and lymph nodes (LNs) from age-matched healthy mice were stained for CD8$^+$ T cells, activation markers (intracellular IFN-γ and perforin), and regulatory markers (CD39, CD73, CTLA-4, PD-1, A2A receptor (A2AR), A2B receptor (A2BR), and intracellular IL-10), and analysed by flow cytometry. Percentages of CD8$^+$ T cells positive for CD39, CD73, CTLA-4 and PD-1 (A), activation markers (B), and A2AR, A2BR and IL-10 (C) were measured. Data are shown as mean ± SEM, n = 6-11 young and n = 7-12 elderly tumour-bearing mice, n = 6-8 young and n = 5-9 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.
Appendix Figure E.10 Elderly TDLN CD4+ T cells have increased regulatory markers

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice and lymph nodes (LNs) from age-matched healthy mice were stained for CD4+ T cells, and regulatory markers (CD39, CD73, A2B receptor (A2BR), CTLA-4, PD-1, ICOS, LAG-3, and intracellular IL-10), and analysed by flow cytometry. Percentages of CD4+ T cells positive for CD39, CD73, A2BR and CTLA-4 (A), and PD-1, ICOS, LAG-3 and IL-10 (B) were measured. Data are shown as mean ± SEM, n = 6-14 young and n = 7-16 elderly tumour-bearing mice, n = 6-11 young and n = 5-12 elderly healthy mice, * = p<0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) tumour-bearing mice to age-matched healthy mice.
Appendix Figure E.11 No age-related differences in activation and regulatory markers on CD4+ T cells in TDLNs
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumour-draining lymph nodes (TDLNs) from tumour-bearing mice and lymph nodes (LNs) from age-matched healthy mice were stained for CD4+ T cells, activation markers (intracellular IFN-γ and perforin), and regulatory markers (A2A receptor (A2AR), and intracellular TGF-β), and analysed by flow cytometry. Percentages of CD4+ T cells positive for activation markers (A), and regulatory markers (B) were measured. Data are shown as mean ± SEM, n = 6-11 young and n = 7-12 elderly tumour-bearing mice, n = 6-8 young and n = 5-9 elderly healthy mice.

Appendix Figure E.12 Reduced proportions of CD8+ T cells in elderly spleens
Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Spleens from tumour-bearing mice and age-matched healthy mice were stained for CD8+ T cells and CD4+CD25+FoxP3+ Tregs, and analysed by flow cytometry. Proportions of CD8+ T cells (A) and Tregs (B) are shown as mean ± SEM, n = 11 young and n = 12 elderly tumour-bearing mice, n = 10-18 young and n = 11-19 elderly healthy mice, * = p<0.05, **** = p<0.0001 comparing young to elderly mice.
Appendix Figure E.13 No age-related differences in activation and regulatory markers on CD8⁺ T cells in tumours

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD8⁺ T cells, activation markers (CD25 and intracellular perforin), and regulatory markers (CD39, A2A receptor (A2AR), A2B receptor (A2BR), CTLA-4, PD-1, ICOS, LAG-3, and intracellular IL-10), and analysed by flow cytometry. Percentages of CD8⁺ T cells positive for activation markers (A), and regulatory markers (B) were measured. Data are shown as mean ± SEM, n = 6-14 young and n = 7-16 elderly tumour-bearing mice.
Appendix Figure E.14 No age-related differences in activation and regulatory markers on CD4+ T cells in tumours

Young and elderly mice were inoculated with AE17 tumours as per Figure 6.2. Tumours were stained for CD4+ T cells, activation markers (CD25, and intracellular IFN-γ and perforin), and regulatory markers (CD39, CD73, A2A receptor (A2AR), A2B receptor (A2BR), CTLA-4, PD-1, LAG-3, and intracellular IL-10), and analysed by flow cytometry. Percentages of CD4+ T cells positive for activation markers (A), and regulatory markers (B) were measured. Data are shown as mean ± SEM, n = 6-14 young and n = 7-16 elderly tumour-bearing mice.
Appendix F  Supplementary data for Chapter 7

**Appendix Figure F.1** Chemotherapy does not alter percentages of MHC class II⁺, CD86⁺, IFN-γ⁺ and CD39⁺ CD11c⁺ cells in TDLNs

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c⁺ cells, MHC class II, CD86, CD39 and intracellular IFN-γ, and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for MHC class II (A), CD86 (B), IFN-γ (C) and CD39 (D) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice * = p<0.05 comparing young to elderly mice.
Appendix Figure F.2 Increased MHC class I, CD80 and TGF-β on elderly TDLN CD11c+ cells

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c+ cells, MHC class I, CD80, and intracellular TGF-β, and analysed by flow cytometry. Percentages of CD11c+ cells positive for MHC class I (A), CD80 (B), and TGF-β (C) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice. * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Appendix Figure F.3 No age-related differences in CD40 or regulatory markers on CD11c+ cells in TDLNs of chemotherapy-treated mice
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days later, tumour-draining lymph nodes (TDLNs) were stained for CD11c+ cells, CD40, and regulatory markers (A2A receptor (A2AR), A2B receptor (A2BR) and PD-L1), and analysed by flow cytometry. Percentages of CD11c+ cells positive for A2AR (A), A2BR (B), PD-L1 (C) and CD40 (D) were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 7-11 elderly cisplatin-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice.

Appendix Figure F.4 Chemotherapy does not alter proportions of CD11b+CD8αCD4+ cDCs in tumours
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11b+CD8αCD4+ cDCs and analysed by flow cytometry. Proportions of CD11b+CD8αCD4+ cDCs within tumours are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05 comparing young to elderly mice.
Appendix Figure F.5 No age-related differences in MHC-I, CD73, A2AR and A2BR on tumour CD11c+ cells
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, MHC class I, CD73, A2A receptor (A2AR) and A2B receptor (A2BR), and analysed by flow cytometry. Percentages of CD11c+ cells positive for MHC class I (A), CD73 (B), A2AR (C) and A2BR (D) were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6 young and n = 6-7 elderly cisplatin-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice.
Appendix Figure F.6 No chemotherapy-induced changes in activation and regulatory markers on CD11c+ cells in tumours

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD11c+ cells, CD40, galectin-9 (GAL-9), and intracellular TNF-α, IL-12 and IL-10, and analysed by flow cytometry. Percentages of CD11c+ cells positive for CD40 (A), TNF-α (B), IL-12 (C), GAL-9 (D) and IL-10 (E) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 11-12 elderly PBS control mice, * = p<0.05 comparing young to elderly mice.
Appendix Figure F.7 Reduced proportions of CD8⁺ and CD4⁺ T cells elderly TDLNs
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8⁺ and CD4⁺ T cells and analysed by flow cytometry. Proportions of CD8⁺ T cells (A) and CD4⁺ T cells (B) are shown as mean ± SEM, n = 9 young and n = 12 elderly gemcitabine-treated mice, n = 9 young and n = 11 elderly cisplatin-treated mice, n = 14 young and n = 16 elderly PBS control mice * = p<0.05, *** = p<0.0005, **** = p<0.0001 comparing young to elderly mice.

Appendix Figure F.8 Increased IFN-γ, perforin, CD39 and LAG-3 on elderly TDLN CD8⁺ T cells
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days later, TDLNs were stained for CD8⁺ T cells, CD39, LAG-3, and intracellular IFN-γ and perforin, and analysed by flow cytometry. Percentages of CD8⁺ T cells positive for IFN-γ (A), perforin (B), CD39 (C) and LAG-3 (D) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Appendix Figure F.9 No age-related differences in activation and regulatory markers on CD8+ T cells in TDLNs of chemotherapy-treated mice

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8+ T cells, CD25, A2A receptor (A2AR), A2B receptor (A2BR) and CTLA-4, and analysed by flow cytometry. Percentages of CD8+ T cells positive for CD25 (A), A2AR (B), A2BR (C) and CTLA-4 (D) were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 6-11 elderly cisplatin-treated mice, n = 6-14 young and n = 7-15 elderly PBS control mice.
Appendix Figure F.10 Increased CD25, CD39, CD73 and CTLA-4 on CD4+ T cells in elderly TDLNs

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4+ T cells, CD25, CD39, CD73, CTLA-4, and intracellular IFN-γ and perforin, and analysed by flow cytometry. Percentages of CD4+ T cells positive for CD25 (A), IFN-γ (B), perforin (C), CD39 (D), CD73 (E), and CTLA-4 (F) were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 6-11 elderly cisplatin-treated mice, n = 11-14 young and n = 12-16 elderly PBS control mice, * = p<0.05, ** = p<0.005, **** = p<0.0001 comparing (i) young to elderly mice, or (ii) chemotherapy-treated mice to age-matched PBS control mice.
Appendix Figure F.11 Increased A2BR, PD-1, ICOS and LAG-3 on CD4$^+$ T cells in elderly TDLNs

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4$^+$ T cells, A2A receptor (A2AR), A2B receptor (A2BR), ICOS, PD-1, and LAG-3, and analysed by flow cytometry. Percentages of CD4$^+$ T cells positive for A2AR (A), A2BR (B), ICOS (C), PD-1 (D) and LAG-3 (E) were measured. Data are shown as mean ± SEM, n = 6-9 young and n = 8-12 elderly gemcitabine-treated mice, n = 6-9 young and n = 6-11 elderly cisplatin-treated mice, n = 6-14 young and n = 7-16 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing young to elderly mice.
Appendix Figure F.12 Reduced proportions of CD4+ T cells and Tregs in elderly tumours
Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, CD4+ T cells and CD4+CD25+FoxP3+ Tregs and analysed by flow cytometry. Proportions of CD4+ T cells (A), Tregs (B) and CD8+ T cells (C) are shown as mean ± SEM, n = 9 young and n = 12 elderly gemcitabine-treated mice, n = 9 young and n = 11 elderly cisplatin-treated mice, n = 14 young and n = 16 elderly PBS control mice, * = p<0.05, ** = p<0.005, *** = p<0.0005 comparing young to elderly mice.
Appendix Figure F.13 No age-related differences in regulatory markers on CD8+ T cells in chemotherapy-treated tumours

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD8+ T cells, CD39, A2B receptor (A2BR), CTLA-4, and intracellular IL-10, and analysed by flow cytometry. Percentages of CD8+ T cells positive for CD39 (A), A2BR (B), CTLA-4 (C) and IL-10 (D) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 6-12 elderly cisplatin-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice.
Appendix Figure F.14 No age-related differences in IFN-γ, CTLA-4 and IL-10 on CD4+ T cells in chemotherapy-treated tumours

Young and elderly tumour-bearing mice were treated with gemcitabine, cisplatin or PBS diluent as per Figure 7.1. 2-3 days after the last dose, tumours were stained for CD4+ T cells, CTLA-4 and intracellular IFN-γ and IL-10, and analysed by flow cytometry. Percentages of CD4+ T cells positive for IFN-γ (A), CTLA-4 (B) and IL-10 (C) were measured. Data are shown as mean ± SEM, n = 6 young and n = 8 elderly gemcitabine-treated mice, n = 6 young and n = 7 elderly cisplatin-treated mice, n = 11 young and n = 12 elderly PBS control mice.
Appendix G  Supplementary data for Chapter 8

**Appendix Figure G.1** IL-2/CD40 does not affect proportions of CD11c⁺ cells, CD8α⁺CD11b⁻ cDCs and pDCs in young and elderly TDLNs

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD11c⁺ cells and DC subsets, and analysed by flow cytometry. Proportions of CD11c⁺ cells (A), CD8α⁺CD11b⁻ cDCs (B) and pDCs (C) are shown as mean ± SEM, n = 7-10 young and n = 7-11 elderly IL-2/CD40-treated mice, n = 11-14 young and n = 12-16 elderly PBS control mice, *** = p<0.0005 comparing young to elderly mice.
Appendix Figure G.2 No age-related differences in regulatory markers on CD11c⁺ cells in TDLNs of IL-2/CD40-treated mice
Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days later, tumour-draining lymph nodes (TDLNs) were stained for CD11c⁺ cells, A2A receptor (A2AR), A2B receptor (A2BR), PD-L1 and galectin-9 (GAL-9), and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for A2AR (A), A2BR (B), PD-L1 (C) and GAL-9 (D) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice.
Appendix Figure G.3 Increased CD8α⁺CD11b⁻ cDCs in elderly IL-2/CD40-treated tumours

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD11c⁺ cells and DC subsets, and analysed by flow cytometry. Proportions of CD11c⁺ cells (A), CD8α⁺CD11b⁻ cDCs (B), CD11b⁺CD8α⁺CD4⁺ cDCs (C), CD11b⁺CD8α⁻CD4⁻ cDCs (D), and pDCs (E) are shown as mean ± SEM, n = 7-10 young and n = 7-11 elderly IL-2/CD40-treated mice, n = 11-14 young and n = 12-16 elderly PBS control mice, * = p<0.05 comparing young to elderly mice.
Appendix Figure G.4 IL-2/CD40 does not alter activation and regulatory markers on tumour CD11c⁺ cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD11c⁺ cells, activation markers (MHC class II, and intracellular TNF-α and IL-12), and regulatory markers (galactin-9 (GAL-9) and intracellular IL-10), and analysed by flow cytometry. Percentages of CD11c⁺ cells positive for MHC class II (A), TNF-α (B), IL-12 (C), GAL-9 (D) and IL-10 (E) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Appendix Figure G.5 No IL-2/CD40-induced changes in activation and regulatory markers on TDLN CD8+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD8+ T cells, activation markers (CD25 and intracellular IFN-γ), and regulatory markers (CD39, A2B receptor (A2BR), CTLA-4, and intracellular IL-10), and analysed by flow cytometry. Percentages of CD8+ T cells positive for CD25 (A), IFN-γ (B), CD39 (C), A2BR (D), CTLA-4 (E) and IL-10 (F) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Appendix Figure G.6 No IL-2/CD40-induced changes in activation and regulatory markers on TDLN CD4+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumour-draining lymph nodes (TDLNs) were stained for CD4+ T cells, activation markers (intracellular IFN-γ and perforin), and regulatory markers (A2B receptor (A2BR) and intracellular IL-10), and analysed by flow cytometry. Percentages of CD4+ T cells positive for IFN-γ (A), perforin (B), A2BR (C), and IL-10 (D) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 6-11 young and n = 7-12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
Appendix Figure G.7 No IL-2/CD40-induced differences in CTLA-4 and TGF-β on tumour CD8\(^+\) T cells
Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD8\(^+\) T cells, CTLA-4, and intracellular TGF-β, and analysed by flow cytometry. Percentages of CD8\(^+\) T cells positive for CTLA-4 (A) and TGF-β (B) were measured. Data are shown as mean ± SEM, \(n = 7\) young and \(n = 11\) elderly IL-2/CD40-treated mice, \(n = 12\) elderly PBS control mice, \(* = p<0.05, ** = p<0.005\) comparing young to elderly mice.

Appendix Figure G.8 No IL-2/CD40-induced differences in perforin, CD39, A2AR and A2BR on tumour CD4\(^+\) T cells
Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD4\(^+\) T cells, CD39, A2A receptor (A2AR), A2B receptor (A2BR), and intracellular perforin, and analysed by flow cytometry. Percentages of CD4\(^+\) T cells positive for perforin (A), CD39 (B), A2AR (C), and A2BR (D) were measured. Data are shown as mean ± SEM, \(n = 7\) young and \(n = 7\) elderly IL-2/CD40-treated mice, \(n = 12\) elderly PBS control mice, \(* = p<0.05\) comparing young to elderly mice.
Appendix Figure G.9 No IL-2/CD40-induced differences in PD-1, LAG-3 and TGF-β on tumour CD4+ T cells

Young and elderly tumour-bearing mice were treated with IL-2/CD40 or PBS diluent as per Figure 8.1. 2-3 days after the last dose, tumours were stained for CD4+ T cells, PD-1, LAG-3, and intracellular TGF-β, and analysed by flow cytometry. Percentages of CD4+ T cells positive for PD-1 (A), LAG-3 (B), and TGF-β (C) were measured. Data are shown as mean ± SEM, n = 7 young and n = 7 elderly IL-2/CD40-treated mice, n = 11 young and n = 12 elderly PBS control mice, * = p<0.05, ** = p<0.005 comparing young to elderly mice.
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