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Increased CTLA-4^{+} T-cells may contribute to impaired Th1 immune responses in patients with chronic obstructive pulmonary disease (COPD)

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List of abbreviations

AECOPD	Acute exacerbations of COPD
BFA	Brefeldin-A
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
GOLD	Global initiative for chronic obstructive lung disease
HC	Healthy controls
IFNγ	Interferon-gamma
IL	Interleukin
NTHI	Non-typeable *Haemophilus influenzae*
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
RPMI	Roswell Park Memorial Institute
sCOPD	Stable COPD
sTNFR1	Soluble tumour necrosis factor receptor-1

Th1	T-helper type 1
TNFα	Tumour necrosis factor-alpha
Treg	Regulatory T-cells
UWA	University of Western Australia

ABSTRACT

Impaired T-helper (Th)-1 function is implicated in the susceptibility of patients with chronic obstructive pulmonary disease (COPD) to respiratory infections, which are common causes of acute exacerbations of COPD (AECOPD). To understand the underlying mechanisms, we assessed regulatory T-cells (Treg) and the expression of an inhibitory T-cell receptor, cytotoxic T-lymphocyte-associated antigen (CTLA)-4. Cryopreserved peripheral blood mononuclear cells (PBMC) from patients with AECOPD (n=17), patients with stable COPD (sCOPD; n=24) and age-matched healthy non-smoking controls (n=26) were cultured for 24hr with brefeldin-A or monensin to detect *intracellular* or *surface* CTLA-4 (respectively) by flow cytometry. T-cells in PBMC from AECOPD (n=9), sCOPD (n=14) and controls (n=12) were stimulated with anti-CD3 with and without anti-CTLA-4 blocking antibodies and cytokines were quantified by ELISA. Frequencies of circulating T-cells expressing intracellular CTLA-4 were higher in sCOPD (p=0.01), whilst AECOPD patients had more T-cells expressing surface CTLA-4 than healthy controls (p=0.03). Increased frequencies of surface CTLA-4$^+$ CD4$^+$ T-cells and CTLA-4$^+$Treg paralleled increases in plasma sTNFR1 levels (r=0.32, p=0.01 and r=0.29, p=0.02 respectively) in all subjects. IFNγ responses to anti-CD3 stimulation were inversely proportional to frequencies of CD4$^+$ T-cells expressing intracellular CTLA-4 (r= -0.43, p=0.01). Moreover, CTLA-4 blockade increased the induction of IFNγ, TNFα and IL-6 in PBMC stimulated with anti-CD3. Overall, chronic inflammation may expand sub-populations of T-cells expressing CTLA-4 in COPD patients

and therefore impair T-cell function. CTLA-4 blockade may restore Th1 function in COPD patients and thus aid the clearance of bacterial pathogens responsible for AECOPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is one of the top five causes of morbidity and mortality worldwide. Smoking is the most common cause of COPD, but genetic factors and environmental pollutions are also implicated (1). The disease is characterized by progressive limitation of airflow, with abnormal inflammatory responses to inhaled particles or gases (2). The chronic inflammation of the airways involves infiltration and activation of neutrophils, macrophages and T-cells (3-5). Subsequent systemic inflammation contributes to co-morbidities (e.g. cardiovascular disease and cachexia) (6).

Most patients are relatively stable with treatment (sCOPD), but some experience acute exacerbations of COPD (AECOPD), which increase mortality and morbidity (7). AECOPD is defined as "an event in the natural course of the disease characterized by a change in the patient's baseline dyspnea, cough and/or sputum, and beyond normal day-to-day variations, that is acute in onset and may warrant a change in regular medication in a patient with underlying COPD'' (2). Respiratory bacterial infections account for up to 50% of AECOPD events. Non-typeable *Haemophilus influenzae* (NTHI), *Streptococcus pneumoniae and Moraxella catarrhalis* are the major bacterial pathogens isolated from patients with AECOPD (8). As NTHI oral vaccines do not reduce the frequency and severity of AECOPD (9), the capacity to mount a protective anti-bacterial immune response may be limited in patients with COPD.

Despite its inflammatory aetiology, COPD is considered as an immune-deficient state as the abundant activated T-cells in the airways of COPD patients do not eradicate bacterial infections. Indeed, T-helper (Th)-1 immune responses [e.g. production of interferon (IFN)γ and phagocytosis] are impaired in COPD patients (10-13). Exogenous IFNγ can enhance killing of NTHI by monocytes from patients with bronchiectasis (14), confirming the necessity for appropriate Th1 responses for clearance of bacterial infections. Here we address the regulators of T-cell responses in COPD patients and search for means to improve host production of IFNγ.

An important regulator of T-cell function is cytotoxic T-lymphocyte antigen-4 (CTLA-4), which blocks the CD28-mediated activation of T-cells (15). CTLA-4 is constitutively expressed by regulatory T-cells (Tregs) and activated effector T-cells. It inhibits the proliferation of T-cells and production of pro-inflammatory cytokines, and so prevents continuous T-cell activation (15, 16). Polymorphisms in the gene encoding CTLA-4 (e.g. rs231775 and rs5742909) and increased serum levels of the soluble form of CTLA-4 have been associated with dysregulated T-cell responses and increased susceptibility to COPD and chronic bronchitis (17-20). Furthermore, increased levels of soluble CTLA-4 in the serum of COPD patients paralleled decreased lung function and increased C-reactive protein (CRP) (21). Frequencies of circulating CTLA-4$^+$ T-cells and CTLA-4$^+$ Tregs were also higher in COPD patients than controls (22, 23). Kalathil *et al.* reported that CTLA-4 blockade *in vitro* increased the proliferation of CD4$^+$ and CD8$^+$ T-cells and production of IFNγ by peripheral blood mononuclear cells (PBMC) from three COPD patients (24).

Here in a larger patient cohort, we address the possibility that chronic inflammation in COPD patients may increase CTLA-4 expression or proportions of Tregs which constitutively express CTLA-4, thus limiting protective Th1-cell responses (e.g. IFNγ production). Little is known about the role of CTLA-4 in AECOPD in terms of levels of expression and anti-bacterial function. Furthermore, most studies have only assessed intracellular expression as surface expression is complicated by the rapid endocytosis of CTLA-4. Thus we have addressed the expression of intracellular and surface CTLA-4 using novel assays and hypothesized that the expression of CTLA-4 is elevated in AECOPD which reduces antibacterial responses such as IFNγ production.

METHODS

Study subjects and sample collection

Patients with AECOPD (n=17; 7 current smokers and 10 ex-smokers) were recruited on admission to the Emergency Department in Royal Perth Hospital in Western Australia. Patients with stable COPD (sCOPD; n=24, all ex-smokers) were recruited from a dedicated COPD clinic at Royal Perth Hospital. All AECOPD and sCOPD patients had a smoking history of >15 pack-years and ex-smokers were defined as those who had ceased smoking >1 year earlier. The diagnosis and severity of COPD was established by a respiratory physician according to the GOLD criteria (Stages 2-4) (25). All COPD patients had been treated with anticholinergic drugs, long-acting beta agonists and inhaled corticosteroids for >3 months prior to participating in the study. Co-morbidities included hypertension, osteoporosis and ischemic heart disease. No patients were receiving systemic corticosteroids or had diabetes, neuromuscular, allergic or rheumatological disease. Age-matched healthy non-smoking controls with no clinical evidence of COPD and not taking any antibiotics or anti-inflammatory medications were tested in parallel (HC; n=26). This study was approved by

the Royal Perth Hospital Human Research Ethics Committee (EC2012/23) and all participants gave informed consent.

Blood samples were collected in lithium heparin tubes, centrifuged at 1000g for 10 minutes and plasma was stored in aliquots at -80°C. PBMC were isolated by Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare, Uppsala, Sweden) and cryopreserved in 10% dimethyl sulfoxide/fetal calf serum (FCS; GIBCO by Invitrogen, Carlsbad, CA).

T-cell subsets

PBMC (1 x 10^6 cells/mL) were cultured at 37°C, 5% CO_2 for 24hr in polypropylene tubes at a 5° incline in 10% FCS/RPMI. BD GolgiPlug^{TM} (Brefeldin-A, BFA; 1 μg/mL) or BD GolgiStop^{TM} (monensin; 2μM) plus CTLA-4-APC antibodies (BD Biosciences, San Jose, CA) was added after 2hr. BFA prevents secretion of proteins including CTLA-4 and hence measures *intracellula*r CTLA-4. As *surface* expression of CTLA-4 is transient due to rapid endocytosis, PBMC were cultured in the presence of CTLA-4-APC antibodies plus monensin (which prevents acidification and subsequent degradation of endocytosed CTLA-4 antibody complexes) prior to staining for immunophenotypic markers. Cells were washed and stained with the BD Horizon^{TM} Fixable Viability Stain 450, followed by surface-staining with CD3-APC-H7, CD4-V500, CD8-PerCP-Cy5.5 and CD45RA-PE-Cy7 antibodies. Intracytoplasmic staining was done using the BD Pharmingen^{TM} Human Foxp3 buffer sets with Foxp3-PE and CTLA-4-APC antibodies (BD Biosciences). 200,000 events were acquired using a BD FACSCanto II cytometer and analyzed with FlowJo v5.7.2 software (Tree Star, Ashland, OR). The expression of *intracellular* or *surface* CTLA-4 was measured in Tregs (CD25$^+$Foxp3$^+$CD4$^+$CD3$^+$ lymphocytes), total CD4$^+$ T-cells (CD4$^+$CD3$^+$ lymphocytes) or total CD8$^+$ T-cells (CD8$^+$CD3$^+$ lymphocytes). Gating strategies are shown in Supplementary Figure 1.

CTLA-4 blocking assay

PBMC (2×10^5 cells/well at 1×10^6 cells/mL) from 9 AECOPD patients, 14 sCOPD patients and 12 controls were stimulated with 10ng/mL anti-CD3 (Mabtech, Nacka Strand, Sweden), with or without 10μg/mL anti-CTLA-4 antibody (clone BNI3; BD Biosciences) for 24hr in 96-well flat bottom plates. Culture supernatants were stored at -80°C. To measure T-cell production of IFNγ or TNFα, PBMC (1×10^6 cells/mL) from 6 subjects (AECOPD, n=2; sCOPD, n=2; controls, n=2) were cultured with 10ng/mL anti-CD3 for 24hr, with or without 10 μg/mL anti-CTLA-4 antibody. BFA was added 2hr after the start of culture. Cells were washed and stained with the BD Horizon^{TM} Fixable Viability Stain 450, followed by surface-staining with CD3-V500, CD4-PerCP, and CD8-APC-H7 antibodies (BD Biosciences). Intracytoplasmic staining was performed using the BD Pharmingen^{TM} Cytofix Cytoperm buffer sets, IFNγ-BV421 and TNFα-PE antibodies (BD Biosciences). 200,000 events were acquired using a BD FACSCanto II cytometer and analyzed with FlowJo v5.7.2 software (Tree Star).

Quantitation of soluble biomarkers

Plasma levels of CRP and soluble tumour necrosis factor receptor-1 (sTNFR1) were measured by ELISA (R&D Systems, Minneapolis, MN). Concentrations of IFNγ, IL-6, IL-10 and TNFα (BD Biosciences) were measured in culture supernatants by ELISA with the diluent (10% FCS/PBS) as a negative control. A sample with known cytokine concentrations (QC) was assayed on each plate to assess inter-plate variation (coefficient of variance was <10%).

Data Analysis

Non-parametric Mann-Whitney U-tests were used to compare data between groups. Wilcoxon matched-pairs signed rank tests were used to compare data within groups (e.g. with and without CTLA-4 blocking). Correlations were assessed using Spearman's rank correlation coefficients. All analyses were performed with GraphPad Prism 5.04 software (La Jolla, CA). Statistically significant differences (p<0.05) are indicated in the figures.

RESULTS

Systemic inflammation in patients with AECOPD is higher than in sCOPD patients.

Study demographics are presented in Table 1. Relative to healthy controls, AECOPD and sCOPD patients exhibited increased systemic inflammation as defined by higher levels of plasma CRP (p<0.001 for both) and sTNFR1 (p=0.007 & p<0.001, respectively). Levels of CRP and sTNFR1 were also higher in AECOPD than sCOPD patients (p=0.02 & p=0.04, respectively; Table 1).

AECOPD patients exhibit higher frequencies of circulating Tregs and CTLA-4$^+$ T-cells

The frequency of Tregs (CD4$^+$CD25$^+$Foxp3$^+$) was higher in AECOPD than sCOPD patients (p=0.01) or healthy controls (p=0.001), but similar in sCOPD patients and controls (Figure 1A). Frequencies of total and memory (CD45RA^{neg}) CD4$^+$ and CD8$^+$ T-cells were similar in all groups (data not shown). The frequency of Tregs expressing *intracellular* CTLA-4 was higher in sCOPD patients than controls (p=0.01; Figure 1B) and correlated with plasma levels of sTNFR1 (r=0.26, p=0.04). The frequency of CD4$^+$ T-cells expressing *intracellular* CTLA-4 was marginally higher in sCOPD patients than controls (p=0.06; Figure 1C). Expression of

intracellular CTLA-4 was low in CD8^{+} T-cells (<5%) and did not differ between groups (Figure 1D).

Interestingly, the frequency of *surface* CTLA-4^{+} Tregs or CD4^{+} T-cells are significantly lower than the frequency of *intracellular* CTLA-4^{+} Tregs or CD4^{+} T-cells respectively (p<0.0001; Figure 1B *vs.* 1E and Figue 1C *vs.* 1F respectively). The median frequency of *surface* CTLA-4^{+} Tregs was higher in AECOPD patients than sCOPD patients (p=0.15) or controls (p=0.17), but the difference was not significant (Figure 1E). AECOPD patients had a higher frequency of CD4^{+} T-cells with *surface* CTLA-4^{+} than controls (p=0.03), but there was no increase in sCOPD patients (Figure 1F). Levels of sTNFR1 correlated with the proportion of *surface* CTLA-4^{+} Tregs (r=0.29, p=0.02) and *surface* CTLA-4^{+} CD4^{+} T-cells (r=0.32, p=0.01). Few CD8^{+} T-cells expressed *surface* CTLA-4 (<1%). The proportions of Tregs, CD4^{+} T-cells or CD8^{+} T-cells expressing *intracellular* or *surface* CTLA-4 did not differ between AECOPD who were current smokers *vs.* those who were ex-smokers (p=0.67-1.00).

Levels of pro-inflammatory cytokines induced by anti-CD3 were increased by CTLA-4 blockade in all subjects

Sufficient PBMC were available from 9 AECOPD patients, 14 sCOPD patients and 12 healthy controls. PBMC were cultured with anti-CD3 to induce a pan T-cell response, with or without added anti-CTLA-4 antibody. Levels of IFNγ, TNFα, IL-6 and IL-10 in supernatants of PBMC cultured with anti-CD3 only (no CTLA-4 blockade) were similar in the three groups (Figure 2A-D). Overall the production of IFNγ correlated inversely with the proportions of Tregs (r=-0.46, p=0.004) and *intracellular* CTLA-4^{+} CD4^{+} T-cells (r=-0.52, p<0.001).

CTLA-4 blockade increased the anti-CD3-induced IFNγ, TNFα and IL-6 responses of PBMC from from healthy controls, sCOPD and AECOPD patients (Figure 2A-C), whilst IL-10 levels were marginally reduced (p=0.10-0.32; Figure 2D). In a pilot study of 2 AECOPD patients, 2 sCOPD patients and 2 healthy controls analysed as a group, CTLA-4 blockade increased the proportions of IFNγ^{+} or TNFα^{+} CD4^{+} and CD8^{+} T-cells (Figure 2E). Hence CTLA-4 blockade may promote production of pro-inflammatory cytokines whilst inhibiting the production of IL-10.

DISCUSSION

Acute exacerbations in COPD patients are commonly associated with respiratory infections (8), reflecting impaired Th1 responses to bacteria (10-13). For example; *in vitro* lymphoproliferative responses to NTHI protein P6 were lower in AECOPD patients whose sputum was culture positive for NTHI than in COPD patients with no exacerbations attributed to NTHI (12). However, the underlying mechanisms have not been elucidated. Here, we provide novel data to support a role for CTLA-4 and Tregs in limiting protective Th1 responses in patients with AECOPD. Expression of surface (not intracellular) CTLA-4 in patients with AECOPD was increased and associated with impaired IFNγ responses. Furthermore, the blockade of CTLA-4 improved production of Th1 cytokines.

Compared to controls, the frequencies of circulating CD4^{+} T-cells and Tregs expressing intracellular CTLA-4 were higher in sCOPD patients, whilst AECOPD patients exhibited increased frequencies of circulating Tregs and surface CTLA-4^{+} CD4^{+} T-cells, with no significant increase in surface CTLA-4^{+} Tregs. Our data extends previous studies which have only shown increased circulating Tregs in AECOPD patients (26). Importantly, we have linked the increased proportions of CD4^{+} T-cells and Tregs expressing surface CTLA-4 with

increased levels of plasma sTNFR1. We suggest that chronic inflammation experienced by all COPD patients may expand populations of *intracellular* CTLA-4$^+$ CD4$^+$ T-cells and CTLA-4$^+$ Tregs. Episodes of AECOPD may then further activate these cells, promoting the cycling of CTLA-4 between intracellular compartments and the cell surface, as shown previously in PHA-activated PBMC (27). This is the first demonstration of the critical need to distinguish surface from intracellular CTLA-4 in AECOPD and sCOPD patients.

Splice variants of the CTLA-4 gene may result in the expression of the transmembrane isoform (Tm-CTLA-4) or soluble isoform which lack the transmembrane domain (sCTLA-4). The expression of both isoforms can be induced by activated CD4$^+$ T-cells and both are immunosuppressive. Hence, increased cycling of CTLA-4 (e.g. during an AECOPD episode) could reflect increases in both transmembrane and soluble CTLA-4 because the commercially available clone of anti-CTLA-4 antibody (BNI3) used in our study targets both CTLA-4 isoforms. Future study should investigate the role of sCTLA-4 *vs.* Tm-CTLA-4 in AECOPD by utilizing an anti-CTLA-4 antibody clone that specifically target the sCTLA-4 isoform (e.g. those generated by Esposito L *et al*. 2014) (28).

The production of IFNγ was associated inversely with the proportions of circulating Tregs and intracellular CTLA-4$^+$ CD4$^+$ T-cells. Furthermore, the production of IFNγ, TNFα and IL-6 by PBMC and proportions of IFNγ- or TNFα-producing CD4$^+$ and CD8$^+$ T-cells from all subjects (including AECOPD patients) were increased by CTLA-4 blockade. Increased production of IFNγ and TNFα after CTLA-4 blockade may reflect the increased proportions of IFNγ- or TNFα-producing CD4$^+$ and CD8$^+$ T-cells found after CTLA-4 blockade, even though the expression of CTLA-4 is much lower in CD8$^+$ T-cells compared to CD4$^+$ T-cells. As the regulation of T-cell responses by CTLA-4 may be in an intrinsic or extrinsic manner (16), CTLA-4 blockade may enhance the activation of CTLA-4$^+$ T-cells and neighbouring T-

cells which may have low or no expression of CTLA-4 (e.g. CD8^{+} T-cells). Furthermore, improvement in CD4^{+} T-cell responses upon CTLA-4 blockade may also improve general Th1 responses (e.g. increased production in IFNγ) which may induce the activation of other Th1 cells such as CD8^{+} T-cells. Hence, increased proportions of CTLA-4-expressing CD4^{+} T-cells or Tregs in patients with COPD may impair Th1 responses needed for clearance of infections (29, 30).

Our data support the use of CTLA-4 blockade to restore Th1 responses (e.g. IFNγ production) in patients with COPD, which in turn should aid clearance of bacterial and/or viral pathogens thus reducing the risk of AECOPD. The blockade of "immune checkpoints" such as CTLA-4 has been used to inhibit Treg function and promote effector T-cell responses (31), showing promising clinical results in anti-tumor treatment (32). Similarly in mice infected with *Nippostrongylus brasiliensis*, *Listeria monocytogenes* or *Mycobacterium bovis*, CTLA-4 blockade enhanced the production of IFNγ and TNFα (33-35).

Although we had no access to cells from the lung, the observed increase in proportions of Tregs matches that seen in bronchoalveolar lavage and lymphoid follicles from COPD patients (23, 36). As responses by PBMC may not reflect respiratory mucosal responses to NTHI, studies of CTLA-4 expression by T-cells from the lung and challenge with bacteria implicated in AECOPD episodes (e.g. NTHI) are warranted to establish CTLA-4 blockade as a viable theraupetic strategy to improve the production of Th1 cytokines.

Author disclosures:

None of the authors has any potential financial conflict of interest related to this manuscript.

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Supplementary Figure 1 – Gating strategies used to identify CTLA-4$^+$ T-cell subsets. (A) Singlets were defined by forward scatter-area (FCS-A) and FCS-Height. (B) Dead cells were then excluded and (C) lymphocytes were gated based on side scatter (SSC)-A and FSC-A. (D) CD4$^+$ and (E) CD8$^+$ T-cells were identified by co-expression of CD3 with CD4 or CD8 respectively. (F) Co-expression of CTLA-4 and Foxp3 was assessed in CD4$^+$ T-cells.

Figure 1 – Proportion of Tregs and CTLA-4 expressing T-cells were increased in COPD patients. Circulating frequency of (A) Tregs (as % of CD4$^+$ T-cells), (B) intracellular CTLA-4$^+$ Tregs, (C) intracellular CTLA-4$^+$ CD4$^+$ T-cells, (D) surface CTLA-4$^+$ Tregs, (E), surface CTLA-4$^+$ CD4$^+$ T-cells, and (F) surface CTLA-4$^+$ CD8$^+$ T-cells. *$**p<0.01$; *$p<0.05$, Mann-Whitney U-test. The graphs show the interquartile range (box), median (middle line) and range (whiskers) for each group.*

Figure 2 – CTLA-4 blockade increased the production of IFNγ, TNFα and IL-6 by PBMC from AECOPD patients, sCOPD patients and controls. Levels of (A) IFNγ, (B) TNFα, (C) IL-6 and (D) IL-10 in cell culture supernatants of anti-CD3 stimulated PBMC with *(right)* or without *(left)* the addition of anti-CTLA-4 blocking antibodies. (E) Frequencies of IFNγ- and TNFα-producing CD4$^+$ and CD8$^+$ T-cells with CTLA-4 blockade *(right)* or without CTLA-4 blockade *(left)* from 6 subjects (2 AECOPD [▼], 2 sCOPD [▲], 2 controls [●]). *$***p<0.001$; $**p<0.01$; *$p<0.05$, Wilcoxon matched-pairs signed rank tests.*

Table 1 – Plasma biomarkers of systemic inflammation are increased in COPD patients

	Healthy controls	Stable COPD	AECOPD
N	26	24	17
Sex (Male:Female)[a]	14:12	13:11	9:8
Age in years[b]	75 [53-90]	71 [60-89]	78 [53-92]
Plasma CRP (mg/L)	0.66 [0.15-6.8]	8.4 [0.15-73][c]	33 [2.2-157][c, e]
Plasma sTNFR1 (ng/mL)	0.52 [0.35-1.0]	1.3 [0.79-2.48][d]	1.89 [1.13-6.41][c, e]

Data are presented as median [range]. C-reactive protein (CRP), soluble tumor necrosis factor receptor-1 (sTNFR1).

[a] Similar in all groups (Fisher's test, p>0.05)

[b] Similar in all groups (Mann-Whitney t-tests, p>0.05)

[c] Higher than controls, p<0.001

[d] Higher than controls, p<0.01

[e] Higher than sCOPD, p<0.05