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1 Asymptomatic CMV infections in long-term renal transplant
2 recipients are associated with the loss of FcR γ from LIR-1⁺ NK cells

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26 **Key words:** CMV, cytokines, cytotoxicity, natural killer cells, renal transplantation

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28 **Abbreviations:** NK cells, natural killer cells; CMV, cytomegalovirus; RTR, renal transplant
29 recipients.

ABSTRACT

Whilst it is established that CMV disease affects NK-cell profiles, the functional consequences of asymptomatic CMV replication are unclear. Here we characterise NK cells in clinically stable renal transplant recipients (RTR; n=48) >2 years after transplantation. RTRs and age-matched controls (n=32) were stratified by their CMV serostatus and the presence of measurable CMV DNA. CMV antibody or CMV DNA influenced expression of NKG2C, LIR-1, NKp30, NKp46 and FcR γ , a signaling adaptor molecule, on CD56^{dim} NK cells. Phenotypic changes ascribed to CMV were clearer in RTRs than in control subjects, and affected NK-cell function as assessed by TNF- α and CD107a expression. The most active NK cells were FcR γ ⁻LIR-1⁺NKG2C⁻ and displayed high antibody-dependent cell cytotoxicity (ADCC) responses in the presence of immobilised CMV glycoprotein B reactive antibody. However, perforin levels in supernatants from RTRs with active CMV replication were low. Overall we demonstrate that CMV can be reactivated in symptom-free renal transplant recipients, affecting the phenotypic and functional profiles of NK-cells. Continuous exposure to CMV may maintain and expand NK cells that lack FcR γ but express LIR-1.

INTRODUCTION

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Natural Killer (NK) cells are large granular lymphocytes involved in control of virus-infected and tumour cells. Their function is regulated by diverse families of surface receptors with no gene rearrangement as the cells differentiate. NK cells are divided into two groups based on cell surface density of CD56 (cytokine-producing CD56^{bright} and cytotoxic CD56^{dim} cells). CD56^{dim} NK cells are more mature, less proliferative and express inhibitory and activating receptors such as Killer Immunoglobulin-like receptors (KIR), C-type lectin-like receptors (e.g. CD94/NKG2C or NKG2A), leukocyte immunoglobulin receptors (e.g. LIR-1) and natural cytotoxicity receptors (NCRs) such as NKp30 and NKp46 [1, 2]. These receptors monitor changes in the expression of class I molecules on stressed cells to induce target cell lysis. Target cell lysis can also be initiated by CD16, a low-affinity Fc receptor on NK cells that initiates antibody dependent cellular cytotoxicity (ADCC) after it is cross-linked by an IgG antibody [2–4]

Cytomegalovirus (CMV) is a herpes virus that persists without symptoms in an immunocompetent host, but can cause serious complications in immunosuppressed individuals such as renal transplant recipients (RTRs) [5]. NK cells can control CMV infection even in the absence of an effective T cell response [6]. Moreover, CMV has the unique ability to imprint on the NK-cell receptor repertoire by altering expression of both inhibitory and activating receptors [7,8]. CMV also encodes proteins able to subvert NK-cell recognition of target cells. For example, LIR-1 binds with much higher affinity to CMV UL18 than to its natural ligand HLA-G [9]. CMV-infected cells expressing UL18 inhibited LIR-1⁺ NK cells, whereas LIR-1⁻ NK cells were activated in the presence of CMV UL18 [10]. Increased expression of LIR-1 on NK cells has been observed in CMV-seropositive individuals [7,11], but the function of these cells is unclear. CMV also encodes UL40 that up-regulates cell surface expression of HLA-E. NKG2C can recognize HLA-E carrying an appropriate peptide, but HLA-E binds with much higher affinity to NKG2A (an inhibitory receptor) to inhibit NK cell mediated lysis [9].

CMV promotes the accumulation of NKG2C⁺ NK cells in healthy individuals [7,12,13]. In recipients of solid organ and haematopoietic stem cell transplants, NKG2C⁺ NK cells expand following CMV reactivation and remain stable over time whilst there is no detectable CMV viremia [12,14]. Most CMV-induced NKG2C⁺ NK cells express CD57, a marker of mature

100 and functionally differentiated NK-cells. NKG2C^{hi}CD57^{hi} NK cells have increased effector
101 function and can lyse CMV-infected macrophages in the presence of CMV-specific
102 antibodies via an ADCC mechanism [12,15]. In the presence of CMV-reactive antibodies,
103 FcR γ -deficient (FcR γ -) NK cells from CMV-seropositive healthy donors display higher
104 ADCC responses against CMV-infected targets than conventional NK cells. These NK cells
105 lack the FcR γ signaling adaptor molecule, one of two adaptor chains (the other being CD3 ζ)
106 known to associate with the transmembrane domain of CD16 [16]. Thus CMV alters NK cell
107 surface receptors and intracellular signaling adaptor molecules.

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109 In the present study, we define the effects of CMV on NK-cell surface receptors, FcR γ and
110 NK cell function in RTR stable more than two years after transplantation with no symptoms
111 of active CMV infection. Using a sensitive PCR, we detect subclinical CMV reactivations in
112 RTR and assess their effects on the NK cell receptor repertoires and function.

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114

115 RESULTS

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117 **CMV antibody levels are higher in RTRs than in healthy controls**

118 RTRs with a median (range) age of 54 (27 – 71) years (15 females, 33 males) were recruited
119 8 (2-18) years after transplantation. All patients were free from clinical CMV disease or
120 reactivation within 6 months of blood collection and receiving no current anti-viral treatment.
121 All patients were stable on maintenance immunosuppressive therapy (Tacrolimus (n=28),
122 Sirolimus (n=12), Cyclosporin (n=8)]. The drug regimen was not associated with CMV status
123 (χ^2 , p=0.26). Age-matched healthy controls (13 females, 19 males) were studied in parallel.

124

125 To understand the effect of CMV on NK-cell receptor repertoires and function, patients and
126 controls were stratified by the presence of CMV antibody and CMV DNA in plasma.
127 Antibodies were detected using CMV lysate, CMV gB and CMV IE-1 preparations (Figure
128 1). Eight RTR (17%) were classified as CMV seronegative (CMV Ab⁻). The 40 CMV
129 seropositive RTR were further subdivided into 33 RTR (82%) without measurable CMV
130 DNA (CMV Ab⁺) and 7 RTR (18%) with measurable CMV DNA (CMV Ab⁺DNA⁺; 22–2717
131 CMV copies/ml). Of the 32 controls, 12 were CMV Ab⁻ (38%). A representative sample of
132 control plasmas were screened for CMV DNA and all were negative, as expected.

133 CMV Ab⁺ RTR had higher levels of IgG reactive with all three CMV antigens compared to
134 CMV Ab⁺ controls (p<0.05 to <0.0001). CMV Ab⁺DNA⁺ RTR had slightly higher levels of
135 antibody recognizing gB antigen than those without detectable viremia (p=0.03), but the
136 presence of CMV DNA did not alter humoral responses to CMV lysate or IE-1 antigen
137 (Figures 1A-C).

138

139 **CMV infection alters the phenotype of CD3⁻CD56^{dim} NK cells in RTRs**

140 CMV may alter NK-cell receptor expression [7,17]. We compared the effect of persistent
141 (Ab⁺) and active (Ab⁺DNA⁺) CMV infection in RTR and healthy controls. The proportions of
142 CD3⁻CD56^{bright} NK cells were similar in all groups (data not shown; p=0.08-0.75), but
143 proportions of CD3⁻CD56^{dim} NK cells were lower in CMV Ab⁺DNA⁺ RTR (p=0.01) and
144 CMV Ab⁺ RTR (p=0.004) than CMV Ab⁻ RTR. Frequencies were similar in CMV Ab⁻ and
145 CMV Ab⁺ controls (Figure 2A). Accordingly, proportions of CD3⁻CD56^{dim} NK cells were
146 inversely related to levels of antibody reactive with CMV gB or lysate in RTR (r = -0.35, -
147 0.36; p=0.01, 0.01, resp). The relationship was marginal in controls (r = -0.23, -0.30; p=0.20,
148 0.10, resp.).

149

150 As proportions of CD56^{dim} NK cells were affected by CMV in RTRs, we restricted our
151 analysis to this subset. Consistent with earlier studies [7,8,14], persistent and/or active CMV
152 in RTRs affected expression of NKG2C, LIR-1, NKp30 and NKp46 (Figures 2B-E) on
153 CD56^{dim} NK cells (for gating strategy please refer to the supplementary figure). Expression
154 of NKG2C was higher in CMV Ab⁺ RTR (p<0.0001) and CMV Ab⁺DNA⁺ RTR (p=0.002)
155 than CMV Ab⁻ RTR. The median (range) frequency was marginally greater (p=0.09) in CMV
156 Ab⁺DNA⁺ RTR [20 (3 – 60) %] than CMV Ab⁺ RTR [5 (0.72 – 38) %]. Moreover, NKG2C
157 expression was higher in CMV Ab⁺ controls (p=0.0004) than CMV Ab⁻ controls (Figure 2B).
158 To investigate variability in NKG2C expression observed in CMV-positive RTRs, all
159 participants were genotyped for a deletion known to abrogate expression of NKG2C [18–20].
160 As expected, heterozygous carriers (+/-) displayed lower expression of NKG2C. Induction of
161 NKG2C expression by CMV was clearest in CMV-seropositive patients without the deletion
162 (+/+) (Figure 3).

163

164 When LIR-1 expression was assessed as median fluorescence intensity (MFI), levels were
165 higher in CMV Ab⁺DNA⁺ RTR than CMV Ab⁺ RTR (p=0.007) or CMV Ab⁻ RTR (p=0.005),

166 showing an effect of active CMV. CMV Ab⁺ RTR had higher LIR-1 expression than CMV
167 Ab⁺ controls (p=0.001), but CMV Ab⁺ and Ab⁻ controls were similar (Figure 2C).

168

169 Expression of NKp46 and NKp30 was lower in CMV Ab⁺DNA⁺ RTR than CMV Ab⁻ RTR
170 (p<0.05). CMV Ab⁺DNA⁺ RTR also had lower expression of NKp46 compared to CMV Ab⁺
171 RTR (p=0.03) showing an effect of active CMV. Moreover, compared to CMV Ab⁺ controls,
172 CMV Ab⁺ RTR had decreased expression of NKp46 (p=0.0007). Expression of NKp30 was
173 lower in CMV Ab⁺ controls (p=0.04) than CMV Ab⁻ controls, but NKp46 expression was not
174 affected by CMV in controls (Figure 2D-E).

175

176 Expression of NKG2A, perforin, CD57, KLRG1, CD16, NKG2D and CD62L was also
177 assessed. CMV Ab⁺ RTR had slightly less NKG2A⁺ cells compared to CMV Ab⁻ RTR
178 (p=0.05) (Figure 2F). Perforin expression was higher in CMV Ab⁻ RTR than CMV Ab⁻
179 controls (Figure 2G), whilst expression of KLRG1 (Figure 2I) was slightly lower, suggesting
180 an effect of transplantation or immunosuppressive drugs. Whereas CD57, CD16, NKG2D
181 and CD62L did not exhibit any differences between groups (Figures 2H, 2J, 2K, 2L).

182

183 We also assessed expression of KIR2DL1, KIR3DL1, KIR2DL2/DL3/DS2 and KIR2DS4 on
184 CD56^{dim} NK cells from individuals shown to carry the relevant genes after PCR amplification
185 of extracted DNA (PCR data not shown). Expression of KIR2DL1, KIR3DL1 and
186 KIR2DL2/2DL3/2DS2 were similar in RTR and controls, with and without CMV (Figures
187 2M, 2N, 2O). Most (32/48) RTR did not express KIR2DS4 as they carried the KIR2DS4*003
188 variant in which a 22bp deletion abrogates cell surface expression [21] or lacked the
189 KIR2DS4 gene.

190

191 **Perforin secretion after stimulation is diminished in RTRs with active CMV infection**

192 Natural cytotoxicity depends upon the secretion of perforin and granzymes following contact
193 with a potential target. Lower perforin secretion has been reported in NK cells from older
194 adults following stimulation with K562 cells [22], but the authors did not assess CMV
195 serostatus or reactivation. This is addressed here following stimulation of PBMC with anti-
196 CD16 or K562 cells.

197

198 Before in vitro stimulation, cells from CMV Ab⁺ RTR had lower expression of perforin than
199 CMV Ab⁻ RTR (p=0.01) (Figure 2G). After stimulation, perforin levels were similar in
200 cultures from CMV Ab⁻ RTR and CMV Ab⁺ RTR or CMV Ab⁻ controls, so transplantation
201 itself did not impair perforin secretion. However, perforin levels in the supernatant of NK
202 cells from CMV Ab⁺DNA⁺ RTR was lower compared to CMV Ab⁺ RTR (anti-CD16
203 p=0.004; K562 p=0.027) and CMV Ab⁻ RTR (anti-CD16 p=0.05) so active CMV replication
204 may suppress or exhaust perforin release. Intermittent CMV reactivation in CMV Ab⁺ RTR
205 may activate NK cells as perforin secretion was higher in cultures from CMV Ab⁺ RTR than
206 CMV Ab⁺ controls (K562 p=0.0009) (Figures 4A, 4B).

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208

209 **CMV enhances the induction of CD107a on NK cells stimulated with anti-CD16**

210 We assessed changes in NK cell function (expression of CD107a and TNF- α) following in
211 vitro stimulation with cytokines (IL-12 + IL-15 + IL-18), K562 cells or anti-CD16 (Figures
212 4C-4H). Expression of CD107a and TNF- α were measured on NK cells expressing NKG2C,
213 LIR-1, NKp30 and NKp46 as CMV affected expression of these markers (Figure 2). We
214 included CD57⁺ CD56^{dim} NK cells as CD57 is a marker of mature and functional NK cells.
215 Expression of TNF- α or CD107a on NKG2C⁺CD56^{dim} NK cells was not quantitated in CMV
216 Ab⁻ subjects, as there were too few events (Figure 4E).

217

218 Following cytokine stimulation, there were few differences between the five groups.
219 However, CD107a expression by CD57⁺ and LIR-1⁺CD56^{dim} NK cells was lower in CMV
220 Ab⁺DNA⁺ RTR than CMV Ab⁻ RTR (p=0.02) (Figures 4D and 4F) and CD107a expression
221 by NKG2C⁺ CD56^{dim} NK cells was higher in CMV Ab⁺ RTR than CMV Ab⁺ controls
222 (p=0.01) (Figure 4E).

223

224 Following K562 stimulation, CMV seropositivity or DNA in RTR did not alter NK cell
225 function, but NKG2C⁺CD56^{dim} NK cells had higher expression of CD107a in CMV Ab⁺ RTR
226 than CMV Ab⁺ controls (p=0.03; Figure 4E). Compared to CMV Ab⁻ controls, CMV Ab⁺
227 controls had higher expression of CD107a on CD56^{dim} (p=0.03; Figure 4C), CD57⁺CD56^{dim}
228 (p=0.03; Figure 4D) and NKp46⁺CD56^{dim} NK cells (p=0.03; Figure 4H). No significant
229 differences were observed with TNF- α responses (data not shown; p=0.07-0.99).

230

231 After anti-CD16 stimulation, expression of CD107a on CD56^{dim}, CD57⁺, LIR-1⁺ and NKp46⁺
232 NK cells from CMV Ab⁺ RTR (p<0.05) and CMV Ab⁺DNA⁺ RTR (p<0.05) was higher,
233 compared to CMV Ab⁻ RTR (Figure 4C-H). NKG2C⁺CD56^{dim} NK cells had higher
234 expression of CD107a in CMV Ab⁺ RTR than CMV Ab⁺ controls (p=0.004; Figure 4E).
235 TNF- α production was higher on CD57⁺, LIR-1⁺ and NKp46⁺ NK cells from CMV Ab⁺ RTR
236 (p \leq 0.05) than CMV Ab⁻ RTR (data not shown). These differences were not evident among
237 controls. Thus persistent CMV in RTR enhances NK-mediated ADCC responses.

238

239 **Loss of FcR γ is a feature of LIR-1⁺ NK cells from CMV-seropositive individuals**

240 FcR γ -deficient cells are reported to respond robustly to anti-CD16 stimulation and CMV
241 seropositivity increases the percentage of FcR γ ⁻ NK cells in healthy controls [16].
242 Accordingly, CMV Ab⁺ controls and CMV Ab⁺ RTR had higher proportions of FcR γ ⁻ NK
243 cells than CMV Ab⁻ controls (p<0.0001) and CMV Ab⁻ RTR (p<0.0001), respectively. CMV
244 Ab⁺DNA⁺ RTR had slightly higher proportions of FcR γ ⁻ NK cells than CMV Ab⁺ RTR
245 (p=0.08) and significantly higher than CMV Ab⁻ RTR (p<0.0001). Interestingly, CMV Ab⁺
246 RTR had higher proportions of FcR γ ⁻ NK cells than CMV Ab⁺ controls (p=0.04; Figure 5A).

247

248 We then assessed expression of LIR-1 and NKG2C on FcR γ ⁻ NK cells (Figure 5B). CMV
249 Ab⁺ RTR, CMV Ab⁺DNA⁺ RTR and CMV Ab⁺ controls had more FcR γ ⁻LIR-1⁺NKG2C⁺ NK
250 cells than CMV Ab⁻ RTR (p<0.0001) or CMV Ab⁻ controls (p=0.0007) (data not shown).
251 Interestingly, more CD56^{dim} NK cells had the phenotype FcR γ ⁻LIR-1⁺NKG2C⁻ than FcR γ ⁻
252 LIR-1⁻NKG2C⁺ or FcR γ ⁻LIR-1⁺NKG2C⁺. This was seen in CMV Ab⁺ RTR (p<0.05), CMV
253 Ab⁺DNA⁺ RTR (p<0.01) and CMV Ab⁺ controls (p<0.01) (Figure 5C). Expansion of
254 NKG2C⁻FcR γ ⁻ NK cells has been observed in NKG2C heterozygous carriers [23]. Here
255 expansion of FcR γ ⁻LIR-1⁺NKG2C⁻ NK cells was found irrespective of the NKG2C
256 genotype. When proportions of FcR γ ⁻LIR-1⁺NKG2C⁻ NK cells were assessed according to
257 NKG2C genotype, approximately 67% of CMV Ab⁺DNA⁺ RTR (6/9), 64% of CMV Ab⁺
258 RTR (9/14) and 61% of CMV Ab⁺ control (11/18) were homozygous for the NKG2C gene
259 whereas 33% of CMV Ab⁺DNA⁺ RTR (3/9), 36% of CMV Ab⁺ RTR (5/14) and 39% of
260 CMV Ab⁺ control (7/19) were heterozygotes (NKG2C^{+/-}). Proportions of FcR γ ⁻LIR-
261 1⁺NKG2C⁻ NK cells were then compared between NKG2C homozygous and heterozygous
262 carriers. Among CMV Ab⁺ RTR, higher proportions of these cells (p=0.01) were observed in
263 NKG2C homozygous carriers [9.5 (0.7 – 38)] than NKG2C heterozygous carriers [2.4 (0.9 –

264 36)]. Among controls, the median (range) in NKG2C homozygous carriers was higher [3.8
265 (1.1 – 31)] than NKG2C heterozygous carriers [1.7(0.2 – 28)], with no significant difference
266 between the two.

267

268 In comparison to CMV-positive RTR, higher expression of NKG2C on FcR γ ⁻LIR-1⁺ NK
269 cells was not observed in CMV Ab⁺ controls suggesting intermittent CMV reactivation may
270 be required to up-regulate and/or maintain NKG2C on FcR γ ⁻LIR-1⁺ NK cells

271

272 **FcR γ ⁻LIR-1⁺ NK cells from CMV⁺ RTR display the highest ADCC responses**

273 FcR γ ⁻ NK cells show increased production of IFN- γ and expression of CD107a following
274 stimulation designed to elicit ADCC [16]. Here we induced ADCC (induction of CD107a and
275 TNF- α) by cross-linking receptors on NK cell from RTR and healthy controls with CMV gB-
276 specific antibody bound to immobilised antigen. Purified anti-CD16 was used as a positive
277 control. Plasma from CMV-seronegative controls did not activate NK cells in PBMC
278 preparations or purified cultured NK cells (data not shown).

279 FcR γ ⁻LIR-1⁺NKG2C⁻ NK cells from CMV-positive RTR and controls had higher ADCC
280 responses than FcR γ ⁻LIR-1⁻NKG2C⁺ (p<0.05) NK cells when cross-linked with anti-CMV
281 gB antibody (Figure 6A) or with anti-CD16 (Figure 6B). FcR γ ⁻LIR-1⁺NKG2C⁻ also had
282 higher responses than FcR γ ⁻LIR-1⁻NKG2C⁺ following K562 stimulation (data not shown;
283 p=0.003 to 0.008). Thus FcR γ ⁻LIR-1⁺NKG2C⁻ NK cells exhibit higher ADCC and NK cell
284 cytotoxicity.

285

286 ADCC responses of cells defined by FcR γ ⁻, LIR-1 and NKG2C were similar in CMV Ab⁺
287 RTR and CMV Ab⁺DNA⁺ RTR after cross-linking with patient plasma or anti-CD16 (Figures
288 6A-B) but were much greater than the responses seen in CMV Ab⁺ controls (right hand axes).

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290

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DISCUSSION

292

293 We have shown that active and latent CMV infections alter NK cell profiles in RTR who
294 have been clinically stable for more than two years after transplantation. Specifically; CMV
295 influenced expression of NKG2C, LIR-1, NKp30, NKp46 and FcR γ . NK cell function was
296 assessed by induction of TNF- α and CD107a. Most functional NK cells had the phenotype

297 FcR γ -LIR-1⁺NKG2C⁻ and displayed high ADCC responses in the presence of anti-CMV gB
298 antibody.

299

300 To understand the effect of CMV, RTR and controls were stratified based on their CMV
301 serostatus and/or the presence of CMV DNA. A higher frequency of CMV reactivation in
302 RTR may plausibly explain their higher levels of CMV-reactive antibody. Interestingly,
303 CMV Ab⁺DNA⁺ RTR had particularly higher levels of antibody reactive with gB antigen.
304 CMV gB is present on the viral envelope or exists on the surface of an infected cell and is
305 crucial for entry into the cell [24,25]. In sera from CMV-seropositive individuals, up to 70%
306 of neutralizing antibodies can be gB-specific [26,27]. Thus CMV Ab⁺DNA⁺ RTR may have
307 higher gB neutralization antibody titres to control CMV replication. However, in an *in-vitro*
308 study, little role for gB was found in viral neutralisation [28] so anti-gB antibody in CMV
309 Ab⁺DNA⁺ RTR may be involved in activation of the complement pathway or cross-linking of
310 Fc γ RIII (CD16) receptor on NK cells. Alternatively, high levels may simply reflect higher
311 antigen burdens. Unlike gB antibody, IE-1 antibody levels were not higher in CMV
312 Ab⁺DNA⁺ RTR compared to other groups. This may arise because CMV-seronegative RTR
313 and controls had antibody able to bind bacterial contaminants of the IE-1 preparation.

314

315 The suggestion that CMV infection may imprint on the NK cell receptor repertoire is not
316 new [7, 29]. Similar to other studies [12-14], we show that CMV induced expression of
317 NKG2C on CD56^{dim} NK cells in RTR and controls. However, no greater expansion was
318 observed in CMV Ab⁺DNA⁺ RTR than CMV Ab⁺ RTR. This differs from an earlier study of
319 solid organ transplant patients [12], but the time of sample collection relative to a burst of
320 CMV replication may be critical. Also few RTR had detectable CMV DNA, limiting the
321 number of patients in this group. However, the patient with the highest levels of CMV DNA
322 had abundant NKG2C⁺ NK cells (data not shown).

323

324 CMV infection can also increase the expression of LIR-1 on NK cells from CMV-
325 seropositive healthy controls [7]. LIR-1 (CD85j/ILT2) is an inhibitory NK cell receptor that
326 binds to classical (HLA-A, B and C) and non-classical MHC (HLA-G) class-I molecules
327 [30]. We found the expression of LIR-1 on CD56^{dim} NK cells was high in CMV Ab⁺DNA⁺
328 and CMV Ab⁺ RTR. In a cohort of lung transplant patients, CMV disease was associated with
329 increased expression of LIR-1 that occurred even before viral DNA became detectable [31].
330 Thus, high LIR-1 expression in our cohort may reflect intermittent CMV reactivation and

331 mark patients at higher risk for progression to clinical disease. LIR-1 is known for its affinity
332 to UL18, a CMV evasive protein [9,32], so up-regulation of UL18 during intermittent CMV
333 reactivation may induce LIR-1 expression. CMV immune-evasion proteins can also suppress
334 the expansion of cytotoxic CD56^{dim} NK cells, so persistent and active CMV infections were
335 associated with lower proportions of CD56^{dim} NK cells in our cohort.

336

337 CMV infection increases degranulation and IFN- γ production by mature NK cells [15]. NK
338 cell cytotoxicity is mediated through the release of perforin and granzymes [33]. No previous
339 studies have assessed how CMV affects perforin secretion after NK cell activation. Here
340 perforin was assessed in culture supernatants following anti-CD16 and K562 stimulation.
341 RTR with CMV DNA generated lower levels of perforin in culture, suggesting active CMV
342 replication may impair perforin release. This needs further investigation by assessing perforin
343 levels at the immunological synapse and binding of perforin to the target cell membrane [22].
344 As the CMV viral load increases in healthy individuals above 70 years of age [34], it is
345 interesting that NK cells from older adults also displayed low perforin levels following K562
346 stimulation [22]. Whilst Hazeldine *et al* [22] did not assess CMV DNA, CMV reactivation in
347 elderly donors may impair polarization of lytic granules to the immunological synapse.
348 Intermittent CMV reactivation and effective control of the virus in CMV Ab⁺ RTR may also
349 explain why their levels of intracellular perforin were higher than CMV Ab⁺ controls or CMV
350 Ab⁺DNA⁺ RTR.

351

352 We also assessed expression of CD107a (also known as lysosome-associated membrane
353 protein; LAMP-1) and TNF- α after stimulating NK cells with cytokines (IL-12 + IL-15 + IL-
354 18), K562 target cells or anti-CD16. CD57 is a marker of T cell replicative senescence
355 whereas on NK cells it is regarded as a marker of maturation. CD57⁺ NK cells proliferate less
356 and produce less IFN- γ in response to cytokines but have higher cytotoxic capability and the
357 ability to produce abundant cytokines when activated by a potential target cell [35]. Unlike
358 other studies [12,36], we found no increase in CD57 expression with CMV. After cytokine
359 stimulation, expression of CD107a on CD57⁺ CD56^{dim} cells was lower in CMV Ab⁺DNA⁺
360 RTR. This may reflect lower expression of IL-12 and IL-18 receptors on CD57⁺ NK cells
361 [35,37].

362

363 Expression of CD107a on NK cells stimulated with anti-CD16 was high in RTR with active
364 or persistent CMV. CD56^{dim}, CD57⁺, LIR-1⁺ and NKp46⁺ NK cells exhibited higher CD107a
365 expression in CMV Ab⁺DNA⁺ RTR. This is a paradoxical finding in view of the low levels of
366 perforin in supernatants. CD107a (LAMP-1) binds adaptor-protein 1 (AP-1) sorting complex,
367 which is essential for perforin trafficking to lytic granules. Decreased levels of LAMP-1 or
368 defects in AP-1 can impair perforin secretion [38]. Increased expression of CD107a and
369 decreased perforin in the supernatants from CMV Ab⁺DNA⁺ RTR implies a defect in AP-1.
370 This warrants further investigation.

371

372 Another plausible explanation for higher expression of CD107a after anti-CD16 stimulation
373 can be a loss of FcR γ . CMV seropositivity correlated with loss of FcR γ , a signaling adaptor
374 molecule that associates with the transmembrane domain of CD16. This loss increases NK
375 cell-mediated ADCC [16]. The decrease in FcR γ expression seen in RTR could explain their
376 higher expression of CD107a after anti-CD16 stimulation. In haematopoietic cell transplant
377 recipients, CMV reactivation caused significant loss of FcR γ expression at 6 months and 1-
378 year post-transplant [39]. Accordingly, CMV Ab⁺DNA⁺ RTR had slightly more FcR γ - NK
379 cells (p=0.08) than CMV Ab⁺ RTR. An increase in FcR γ ⁻ NK cells in CMV Ab⁺ RTR
380 compared with CMV Ab⁺ healthy controls may simply reflect intermittent episodes of CMV
381 reactivation, driven by immunosuppressive drugs, inflammation or donor and recipient CMV
382 serostatus at the time of transplantation.

383

384 In CMV-positive people, the FcR γ ⁻ phenotype aligns with expression of NKG2C and
385 decreased expression of NKp30 [36], which was confirmed here in both the RTR and
386 controls. As in other studies, only a subset of FcR γ ⁻ NK cells expressed NKG2C. We noted
387 similar frequencies of LIR-1⁺ and FcR γ ⁻ NK cells in a patient with the highest levels of CMV
388 DNA (data not shown) and observed increased expression of LIR-1 and NKG2C on FcR γ ⁻
389 NK cells. Interestingly, FcR γ ⁻LIR-1⁺NKG2C⁻ NK cells were observed in CMV-positive RTR
390 and controls. Expansion of FcR γ ⁻NKG2C⁻ NK cells is observed in NKG2C heterozygous
391 people [23]. However in our study, expansion of FcR γ ⁻LIR-1⁺NKG2C⁻ NK cells was
392 independent of the NKG2C genotype so it may reflect CMV reactivation. It is unclear why
393 NK cells downregulate FcR γ in relation to CMV infection. Following CMV infection, NK
394 cells undergo profound epigenetic changes resulting in the downregulation of several proteins
395 including signaling adaptor proteins (eg. FcR γ) and transcription factors (eg. PLZF –

396 promyelocytic leukemia zinc finger) and in turn epigenetically resemble CD8⁺ T cells [39].
397 The resultant cells are termed *adaptive NK cells*. The underlying mechanisms remain unclear.
398 LIR-1 binds with high affinity to the CMV protein UL18 [9]. This interaction may trigger
399 epigenetic changes so higher proportions of FcRγ⁻ LIR-1⁺ NKG2C⁻ NK cells are observed in
400 CMV-positive people. Further studies are required to understand the stages of differentiation.
401
402 FcRγ⁻ NK cells display increased ADCC responses in the presence of CMV-specific
403 antibodies [16, 39]. Similarly, we observed higher CD107a and TNF-α expression in FcRγ⁻
404 LIR-1⁺ NKG2C⁻ and in FcRγ⁻ LIR-1⁺ NKG2C⁺ NK cells (Figure 6). Increased polyfunctional
405 responses in FcRγ⁻ LIR-1⁺ NKG2C⁻ NK cells were also induced by K562 target cells (data not
406 shown). Thus CMV driven FcRγ⁻ LIR-1⁺ NKG2C⁻ NK cells have enhanced ADCC and NK
407 cell cytotoxicity. However, whether FcRγ⁻ LIR-1⁺ NKG2C⁻ NK cells can induce higher NK
408 cell cytotoxicity in the presence of CMV-infected cells requires investigation.
409
410 *Adaptive NK cells* have memory-like properties [16]. Expansion of NKG2C⁺ NK cells was
411 observed in CMV seropositive but not seronegative bone marrow transplant recipients given
412 NKG2C⁺ NK cells from a CMV-seropositive donor [40]. Here some CMV Ab⁺ DNA⁺ RTR
413 had high proportions of FcRγ⁻ LIR-1⁺ NKG2C⁺ NK cells and raised strong ADCC responses
414 but this was variable. Many patients with high ADCC responses may have active CMV
415 replication so active CMV may be essential to expand the *adaptive NK cells* which may in
416 turn contribute to the control of CMV. Longitudinal studies should address whether these
417 cells are a stable feature of some RTR or are a response to protracted CMV replication.
418
419 Immunosuppressive drugs or other clinical variables such as age at transplant, CMV
420 serostatus of donor and the recipient before transplantation may have altered the NK cell
421 receptors in RTR however, due to small sample size and lack of clinical data the effect of
422 these variables on NK cell receptor expression could not be determined. In spite of these
423 limitations, our findings align with the other studies [16, 39] that demonstrate that the loss of
424 FcRγ and increase in the expression of LIR-1 or NKG2C is associated with CMV
425 seropositivity. Expansion of FcRγ⁻ LIR-1⁺ NKG2C⁺ and FcRγ⁻ LIR-1⁺ NKG2C⁻ NK cells
426 should be confirmed in future in a larger cohort of CMV Ab⁺ DNA⁺ RTR.
427
428 The changes to NK cell phenotypes associated here with CMV seropositivity (which carries a

429 likelihood of intermittent reactivation) are likely to be clinically important because CMV
430 may promote vascular disease directly via infection of the endothelium [41] or indirectly by
431 stimulating immune responses and inflammation [42]. In response to CMV infection, the
432 recruitment of NK or T cells for its control may induce endothelial injury and promote
433 atherogenesis. CMV-stimulated production of IFN- γ and TNF- α by CD4 T cells can induce
434 the chemokine fractalkine, which can activate NK cells to cause endothelial damage [43].
435 Moreover, NK cells can destroy endothelial cells following activation by xenoantigen-
436 specific antibodies [44]. *Adaptive NK cells* induced by CMV may play a role in the
437 pathogenesis of vascular disease as these cells are resistant to apoptosis during inflammatory
438 conditions, have high proliferation rates upon engagement of an activating receptor and are
439 highly responsive after cross-linking with CMV-specific antibodies [39]. Therefore, in an
440 attempt to control CMV infection, enhanced activation may drive NK cells to damage
441 endothelial cells and cause vascular disease. High CMV antibody levels in healthy
442 individuals are linked with ischemic heart disease and expansion of NKG2C⁺ NK cells is
443 associated with carotid atherosclerotic plaques in CMV seropositive patients [45,46]. Thus
444 expression of LIR-1 and/or loss of FcR γ could be used as a biomarker to assess the risk of
445 developing vascular disease. In summary, we demonstrate that CMV infection affects NK
446 cell receptor expression and expands FcR γ -LIR-1⁺ NK cells by reactivating intermittently in
447 clinically stable RTR.

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MATERIALS AND METHODS

451 **Patients and Controls**

452 Renal transplant recipients were recruited from Royal Perth Hospital (RPH), Western
453 Australia. All patients were clinically stable, free from clinical CMV disease or reactivation
454 within 6 months of blood collection and receiving no current anti-viral treatment. All patients
455 were on stable maintenance immunosuppressive therapy. Age-matched healthy controls were
456 recruited from laboratory staff and colleagues. All participants provided written informed
457 consent and the project was approved by the Human Research Ethics Committees of RPH,
458 the University of Western Australia and Curtin University. PBMC were isolated from
459 heparin-treated blood using Ficoll–Paque (GE Healthcare, Little Chalfont, UK) density
460 gradient centrifugation and cryopreserved in liquid nitrogen. Plasma samples were aliquoted
461 and stored at -80°C .

462

463 **Detection of CMV DNA and antibody**

464 Plasma from RTR was screened for CMV DNA in the Department of Microbiology (RPH)
465 using commercial kits (Abbott Diagnostics, IL) able to quantitate >20 copies/ml. This value
466 was used as a cut-off.

467 CMV-reactive IgG was quantitated by ELISA using a lysate of human foreskin fibroblasts
468 (HFF) infected with AD169. Cells were harvested after 7 days, sonicated and stored at -80°C .
469 Uninfected HFF were prepared as a negative control. Parallel microtitre plates were coated
470 with CMV glycoprotein prepared in hamster ovary cells (Chiron, CA) and CMV immediate-
471 early 1 (IE-1) prepared in *E.coli* (Miltenyi Biotec, Germany) at $0.5\mu\text{g/ml}$. Plates were coated
472 overnight (4°C), washed with PBS/0.05% Tween, blocked with 5% BSA/PBS and washed.
473 Plasma samples pre-diluted in 2% BSA/PBS were run alongside control plasma from a CMV-
474 seropositive healthy individual assigned a value of 100 arbitrary units (AU). Plates were
475 washed after 2 hours and horse-radish peroxide conjugated anti-human IgG (Sigma-Aldrich,
476 MO) diluted 1:4000 in 2% BSA/PBS was added. Tetramethylbenzidine substrate (Sigma-
477 Aldrich) was added, colour development was stopped with $1\text{M H}_2\text{SO}_4$ and plates were read at
478 450nm . CMV seropositivity was defined as >2 standard deviations above the mean antibody
479 levels derived for a set of 11 samples which had been deemed seronegative by the
480 ARCHITECT CMV IgG assay (Abbott Diagnostics, IL).

481

482 **Immunophenotyping**

483 Cryopreserved PBMC were thawed, washed and aliquots of 10^6 cells were stained for 15
484 minutes in the dark at room temperature (RT) with antibodies recognizing cell surface
485 antigens: V500-anti-CD3 (UCHT1), V450-anti-CD56 (B159), APC-H7-anti-CD16 (3G8),
486 APC-anti-CD57 (NK-1), PE-anti-NKp46 (9E2/NKp46), PE-anti-NKp30 (p30-15), PE-Cy7-
487 anti-NKG2D (1D11; BD Biosciences, NJ), PerCPCy5.5-anti-CD85j (HP-F1; eBioscience,
488 CA), Alexa Fluor 488 anti-NKG2C (134591), PE-anti-KIR2DL1 (143211; R&D systems,
489 MN), PE-anti-KIR2DL2/L3/DS2 (GL183; Beckman Coulter, CA), PerCPCy5.5-anti-CD62L
490 (DREG-56), PE-KIR3DL1 (DX9; Biolegend, CA), PerCPCy5.5-anti-KIR2DL1/S1 (HP-
491 MA4; eBioscience). For intracellular markers, cells were incubated for 20 minutes at 4°C
492 with Cytofix/Cytoperm solution after surface staining and washed twice with cold 1X perm
493 wash buffer (BD Cytofix/Cytoperm kit). Cells were then stained with $5\mu\text{L}$ PerCPCy5.5-anti-
494 perforin (DG9; Biolegend) for 30 minutes in the dark at RT. After incubation, cells were
495 washed twice with cold 1X perm wash buffer. At least 100,000 events were acquired in the
496 lymphocyte gate on a FACSCanto II instrument (BD Biosciences). Data were analysed with
497 FlowJo v10 software (Tree Star, OR).

498

499 **Perforin ELISA**

500 PBMCs were stimulated with anti-CD16 or K562 cells for 6 hours and supernatants were
501 collected after 6 hours and stored at -80°C . Perforin levels were measured using a commercial
502 human Perforin ELISA kit (Abcam, Cambridge, UK) able to detect $<40\text{ pg/mL}$.

503

504 **Functional Assays**

505 Cryopreserved PBMC were thawed, washed and stimulated (0.5×10^6 cells/ $100\mu\text{L}$) with
506 cytokines [IL-12 (10ng/mL; R & D systems) + IL-15 (10ng/mL; R & D systems) + IL-18 (10
507 ng/mL; MBL, Japan)] for ~ 16 hours at 37°C and 5% CO_2 . Remaining PBMC were rested
508 overnight and next day these cells ($5 \times 10^5/100\mu\text{L}$) were stimulated for 6 hours at 37°C with
509 RPMI 1640/10% FBS, $10\mu\text{g}$ anti-CD16 (BD Biosciences) bound to flat-bottomed 96-well
510 plates or K562 cells at an effector-to-target ratio of 2:1. BV786-anti-CD107a (H4A3; BD
511 Biosciences) was added to all wells. After 1 hour, brefeldin A and monensin (BD

512 Biosciences) were added and cells were incubated for a further 5 hours at 37°C. Cells were
513 then stained with a live/dead dye; FVS700 (BD Biosciences) for 30 minutes, followed by
514 BUV395-anti-CD3 (UCHT1), BV510-anti-NKp46 (9E2/NKp46), PE-CF594-anti-CD57
515 (NK-1), BV421-anti-NKp30 (p30-15; BD Biosciences), PE.Cy7-anti-CD56 (HCD56;
516 Biolegend), APC-anti-NKG2C (134591; R&D systems) and PE-anti-LIR-1 (HP-F1;
517 eBioscience) for 15 minutes in the dark at RT. Cells were then fixed for 20 minutes at 4⁰C,
518 permeabilized (BD Cytotfix/Cytoperm™; BD Biosciences), stained for intracellular antigens
519 using BV650-anti-TNF-α (MAb11; Biolegend) and FITC-anti-FcεRIγ (Merck Millipore,
520 Germany) for 30 minutes and was washed twice with cold 1X perm wash buffer. At least
521 100,000 events were acquired in the lymphocyte gate on a BD LSR II Fortessa Instrument
522 (BD Biosciences). Data were analysed with FlowJo v10 software (Tree Star, OR).

523

524 **ADCC Assay**

525 96-well plates were coated with CMV gB antigen at 0.5µg/ml and incubated overnight at
526 4⁰C. Wells were washed and blocked for 60 minutes at RT with 10% FBS/RPMI. 100µL
527 aliquots of heat-inactivated (56°C, 1 hour) autologous plasma were added to the coated wells,
528 incubated overnight at 4⁰C. The optimal dilution (1:300) was selected in preliminary
529 experiments that displayed highest expression of CD107a and production of TNF-α. Wells
530 were washed and 5x10⁵ PBMCs were added with BV786-anti-CD107a (H4A3; BD
531 Biosciences) for 1 hour followed by brefeldin A and monensin (BD Biosciences) for a further
532 5 hours at 37°C. Cells were stained as described under the functional assay.

533

534 **Genotyping**

535 A deletion mutation abrogating expression of NKG2C was assessed by a nested PCR-based
536 method with two primer pair sequences [18] to distinguish homozygosity and heterozygosity.

537

538 **Statistical analyses**

539 Statistical analyses were performed using Prism 5 (GraphPad Software, CA). Nonparametric
540 tests were conducted to determine statistical significances. Mann–Whitney tests were used to
541 compare results between groups. Wilcoxon matched pairs tests were used to compare results

542 within a patient or control group. Correlation coefficients were calculated with Spearman's
543 tests. All p values ≤ 0.05 were considered to be statistically significant.

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545

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555

556 *Conflict of Interest:* The authors declare no financial or commercial conflict of interest.

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705 **FIGURE LEGENDS**

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707 **FIGURE 1. Antibody responses to CMV antigens.** (A-C) Plasma from healthy controls
708 and RTRs was collected to assess CMV-reactive IgG levels. IgG levels were assessed against
709 (A) CMV lysate antigen, (B) gB antigen and (C) IE antigen using ELISA in CMV Ab⁻ RTR
710 (n=8), CMV Ab⁺ RTR (n=33), CMV Ab⁺DNA⁺ RTR (n=7), CMV Ab⁻ control (n=12) and
711 CMV Ab⁺ controls (n=20). Data are shown as means derived from three-fold serial dilutions
712 of duplicate wells and are representative of one independent experiment. Groups were
713 compared using Mann-Whitney tests. The boxes represent the 25th and 75th percentiles and
714 the central line represents the median. The end of the whiskers represents the minimum and
715 maximum. Statistical significance is indicated as *p≤0.05, **p<0.01, ***p< 0.001, ****p<
716 0.0001.

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719 **FIGURE 2. NK-cell phenotypes in association to CMV antibody and/or CMV DNA or**
720 **transplantation.** Multiparametric flow cytometry analysis were performed using
721 cryopreserved PBMCs from CMV Ab⁻ RTR (n=8), CMV Ab⁺ RTR (n=33), CMV Ab⁺DNA⁺
722 RTR (n=7), CMV Ab⁻ control (n=12) and CMV Ab⁺ controls (n=20) to assess the expression
723 of NK cell receptors. One independent experiment is shown here. Two subsets of NK cells
724 were identified using anti-CD3 and anti-CD56: CD3⁻CD56^{bright} and CD3⁻CD56^{dim}. Analyses
725 were restricted to CD3⁻CD56^{dim} NK cells. (A) Proportions of CD3⁻CD56^{dim} NK cells. (B)
726 Expression of NKG2C on CD56^{dim} NK cells. (C) MFI of LIR-1 on CD56^{dim} NK cells. (D)
727 Expression of NKp30 on CD56^{dim} NK cells. (E) Expression of NKp46 on CD56^{dim} NK cells.

728 (F) Expression of NKG2A on CD56^{dim} NK cells. (G) Expression of perforin on CD56^{dim} NK
729 cells. (H) Expression of CD57 on CD56^{dim} NK cells. (I) Expression of KLRG1 on CD56^{dim}
730 NK cells. (J) Expression of CD16 on CD56^{dim} NK cells. (K) Expression of NKG2D on
731 CD56^{dim} NK cells. (L) Expression of CD62L n CD56^{dim} NK cells. (M) Expression of
732 KIR2DL1 on CD56^{dim} NK cells. (N) Expression of KIR3DL1 and (O) Expression of
733 KIR2DL2/DL3/DS2 on CD56^{dim} NK cells. Groups were compared using Mann-Whitney
734 tests. The boxes represent the 25th and 75th percentiles and the central line in represents the
735 median. The end of the whiskers represents the minimum and maximum. Statistical
736 significance is indicated as * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

737

738

739 **FIGURE 3. Expression of NKG2C in association to CMV antibody and/or CMV DNA**
740 **and NKG2C genotype.**

741 A nested PCR was performed to detect a deletion mutation that abrogates expression of
742 NKG2C. Based on the NKG2C genotype, CMV antibody levels and DNA levels, RTRs and
743 controls [CMV Ab⁻ RTR (n=8), CMV Ab⁺ RTR (n=33), CMV Ab⁺DNA⁺ RTR (n=7), CMV
744 Ab⁻ control (n=12) and CMV Ab⁺ controls (n=20)] were further stratified to assess the
745 expression of NKG2C. Induction of NKG2C expression on NK cells by the presence of CMV
746 antibody was clearest in RTRs with homozygous presence of NKG2C (+/+). RTR with
747 heterozygous (+/-) genotype had decreased expression of NKG2C than homozygotes (+/+).
748 There were no individuals with the genotype (-/-). Each symbol represents an individual
749 donor. One independent experiment is represented here. Groups were compared using Mann-
750 Whitney tests. Medians are represented by lines and statistical significance is indicated as
751 ** $p < 0.01$, **** $p < 0.0001$.

752

753

754 **FIGURE 4. Analysis of perforin secretion and CD107a expression**

755 (A, B) Cryopreserved PBMCs from CMV Ab⁻ RTR (n=6), CMV Ab⁺ RTR (n=17), CMV
756 Ab⁺DNA⁺ RTR (n=7), CMV Ab⁻ control (n=7) and CMV Ab⁺ controls (n=9) were stimulated
757 with either (A) anti-CD16 or (B) K562 cells for 6 hours to assess perforin levels (pg/ml) in
758 the culture supernatants by ELISA. Data are means of duplicate wells in one independent
759 experiment. (C-H) PBMCs from CMV Ab⁻ RTR (n=10), CMV Ab⁺ RTR (n=15), CMV
760 Ab⁺DNA⁺ RTR (n=9), CMV Ab⁻ control (n=10) and CMV Ab⁺ controls (n=10) were
761 stimulated with either cytokines (IL-12 + IL-15 + IL-18), K562 target cells or anti-CD16 for

762 6 hours and expression of CD107a was assessed using multiparametric flow cytometry on (C)
763 CD56^{dim} NK cells, (D) CD57⁺CD56^{dim} NK cells, (E) NKG2C⁺CD56^{dim} NK cells, (F) LIR-
764 1⁺CD56^{dim} NK cells, (G) NKp30⁺CD56^{dim} NK cells and (H) NKp46⁺CD56^{dim} NK cells. One
765 independent experiment is represented here. Groups were compared using Mann-Whitney
766 tests. The boxes represent the 25th and 75th percentiles and the central line represents the
767 median. The end of the whiskers represents the minimum and maximum. Statistical
768 significance is indicated as *p ≤ 0.05, **p<0.01, ***p< 0.001.

769

770

771 **Figure 5. Co-expression of FcRγ⁻, LIR-1⁺ and NKG2C⁺ NK cells in RTRs and controls**

772 (A, B) Multiparametric flow cytometry analyses were performed on cryopreserved PBMCs
773 from CMV Ab⁻ RTR (n=10), CMV Ab⁺ RTR (n=14), CMV Ab⁺DNA⁺ RTR (n=9), CMV
774 Ab⁻ control (n=10) and CMV Ab⁺ controls (n=10) to determine the (A) percentage of
775 CD56^{dim} NK cells with the FcRγ⁻ phenotype. (B) Gating strategy of co-expression of LIR-
776 1⁺NKG2C⁺ on FcRγ⁻ CD56^{dim} NK cells is shown. (C) Boolean gating was performed to
777 compare different subsets of FcRγ⁻ NK cells expressing LIR-1 and NKG2C. Each symbol
778 represents an individual donor. One independent experiment is represented here. Groups were
779 compared using Mann-Whitney tests. Medians are represented by lines and statistical
780 significance is indicated as *p ≤ 0.05, **p<0.01, ****p< 0.0001.

781

782

783 **Figure 6. ADCC by different subsets of FcRγ⁻, LIR-1⁺ and NKG2C⁺ NK cells.**

784 (A, B) Cryopreserved PBMCs from CMV Ab⁻ RTR (n=10), CMV Ab⁺ RTR (n=14), CMV
785 Ab⁺DNA⁺ RTR (n=9), CMV Ab⁻ control (n=10) and CMV Ab⁺ controls (n=10) were
786 stimulated for 6 hours by autologous plasma bound to immobilised CMV gB antigen or
787 purified anti-CD16 to elicit an ADCC response (assessed by expression of CD107a and TNF-
788 α). Boolean gating was performed to assess different subsets of CD56^{dim} NK cells expressing
789 FcRγ, LIR-1, NKG2C, CD107a and TNF-α after cross-linking NK cells with either (A)
790 autologous plasma or (B) purified anti-CD16. For both figures, CMV Ab⁺ controls are plotted
791 on the right and RTR on the left Y-axis. Patient and control groups were compared using
792 Mann-Whitney tests. NK subsets were compared within patient/control groups using
793 Wilcoxon matched pairs tests. One independent experiment is represented here. The boxes

794 represents minimum to maximum, the line in the box represents the median and statistical
795 significance is indicated as * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 1

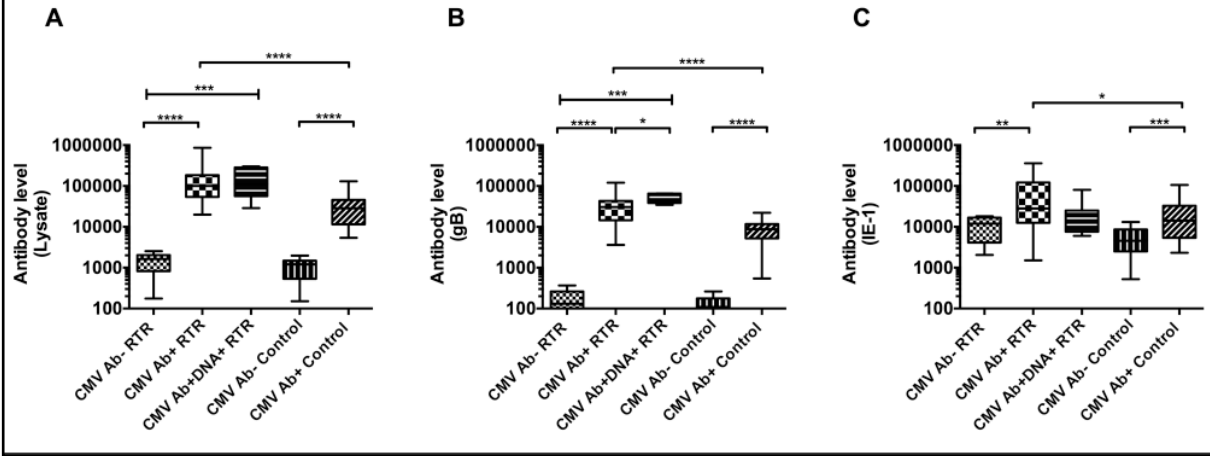


Figure 2

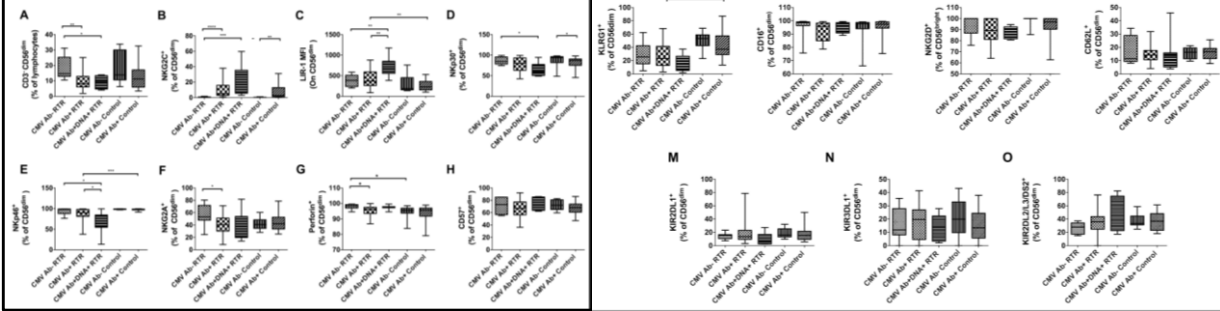


Figure 3

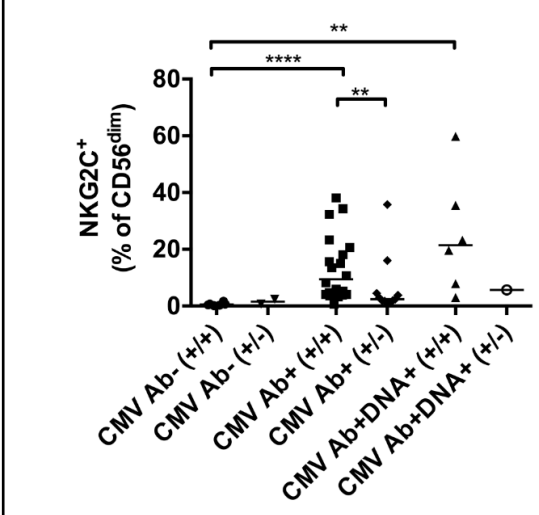


Figure 4

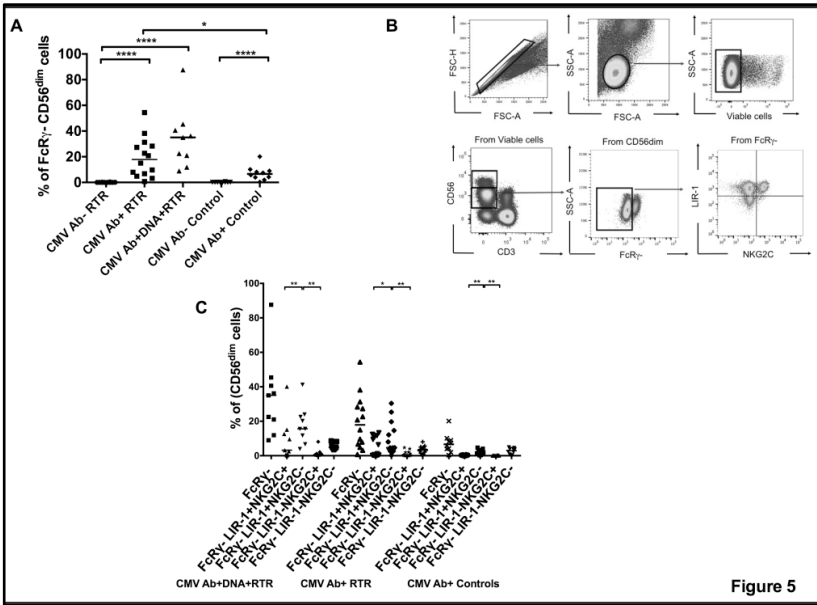
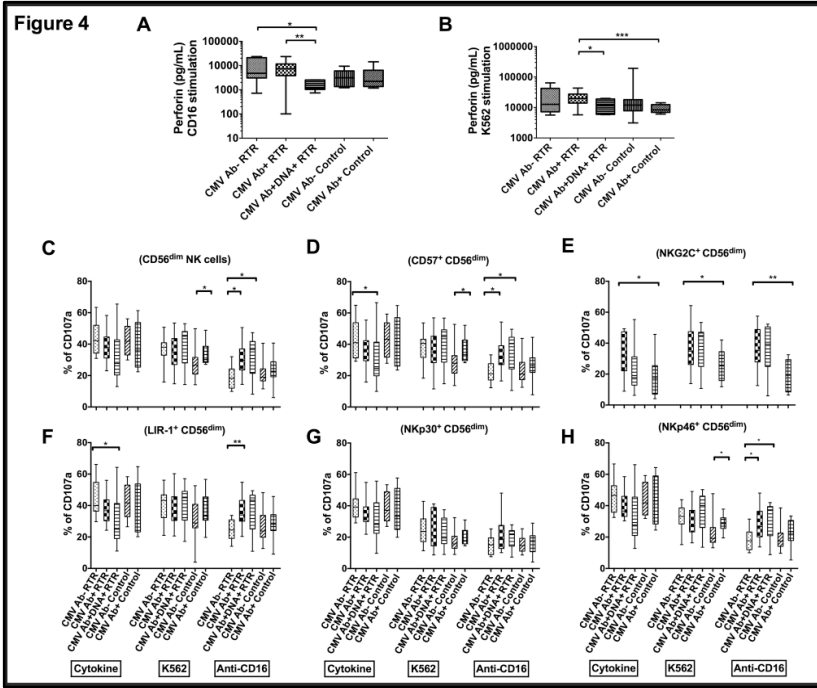


Figure 5

