Asymptomatic CMV infections in long-term renal transplant recipients are associated with the loss of FcRγ from LIR-1$^+$ NK cells

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Abbreviations: NK cells, natural killer cells; CMV, cytomegalovirus; RTR, renal transplant 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 FcRγ, 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 $FcR\gamma^{-}LIR\text{-}1^{+}NKG2C^{-}$ 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 FcRγ but express LIR-1.

INTRODUCTION

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 $LIR\text{-}1^{+}$ NK cells, whereas $LIR\text{-}1^{-}$ 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

and functionally differentiated NK-cells. NKG2C^{hi}CD57^{hi} NK cells have increased effector function and can lyse CMV-infected macrophages in the presence of CMV-specific antibodies via an ADCC mechanism [12,15]. In the presence of CMV-reactive antibodies, FcRγ-deficient (FcRγ-) NK cells from CMV-seropositive healthy donors display higher ADCC responses against CMV-infected targets than conventional NK cells. These NK cells lack the FcRγ signaling adaptor molecule, one of two adaptor chains (the other being CD3ζ) known to associate with the transmembrane domain of CD16 [16]. Thus CMV alters NK cell surface receptors and intracellular signaling adaptor molecules.

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

RESULTS

CMV antibody levels are higher in RTRs than in healthy controls

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

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

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

CMV infection alters the phenotype of $CD3^-CD56^{dim}$ NK cells in RTRs

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

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

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

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

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

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

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

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

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

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

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

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

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

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

After anti-CD16 stimulation, expression of CD107a on $CD56^{dim}$, $CD57^+$, LIR-1^+ and $NKp46^+$ NK cells from CMV Ab^+ RTR ($p<0.05$) and CMV Ab^+DNA^+ RTR ($p<0.05$) was higher, compared to CMV Ab^- RTR (Figure 4C-H). $NKG2C^+CD56^{dim}$ NK cells had higher expression of CD107a in CMV Ab^+ RTR than CMV Ab^+ controls ($p=0.004$; Figure 4E). TNF-α production was higher on $CD57^+$, LIR-1^+ and $NKp46^+$ NK cells from CMV Ab^+ RTR ($p\leq0.05$) than CMV Ab^- RTR (data not shown). These differences were not evident among controls. Thus persistent CMV in RTR enhances NK-mediated ADCC responses.

Loss of FcRγ is a feature of LIR-1^+ NK cells from CMV-seropositive individuals

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

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

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

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

FcRγ$^-$LIR-1$^+$ NK cells from CMV$^+$ RTR display the highest ADCC responses

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

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

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

DISCUSSION

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

297 FcRγ^{-}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 FcγRIII (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

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

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

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

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

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

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

promyelocytic leukemia zinc finger) and in turn epigenetically resemble $CD8^+$ T cells [39]. The resultant cells are termed *adaptive NK cells*. The underlying mechanisms remain unclear. LIR-1 binds with high affinity to the CMV protein UL18 [9]. This interaction may trigger epigenetic changes so higher proportions of $FcR\gamma^-$ LIR-1^+NKG2C$^-$ NK cells are observed in CMV-positive people. Further studies are required to understand the stages of differentiation.

$FcR\gamma^-$ NK cells display increased ADCC responses in the presence of CMV-specific antibodies [16, 39]. Similarly, we observed higher CD107a and TNF-α expression in $FcR\gamma^-$ LIR-1^+NKG2C$^-$ and in $FcR\gamma^-$LIR-1^+NKG2C$^+$ NK cells (Figure 6). Increased polyfunctional responses in $FcR\gamma^-$LIR-1^+NKG2C$^-$ NK cells were also induced by K562 target cells (data not shown). Thus CMV driven $FcR\gamma^-$LIR-1^+NKG2C$^-$ NK cells have enhanced ADCC and NK cell cytotoxicity. However, whether $FcR\gamma^-$LIR-1^+NKG2C$^-$ NK cells can induce higher NK cell cytotoxicity in the presence of CMV-infected cells requires investigation.

Adaptive NK cells have memory-like properties [16]. Expansion of NKG2C$^+$ NK cells was observed in CMV seropositive but not seronegative bone marrow transplant recipients given NKG2C$^+$ NK cells from a CMV-seropositive donor [40]. Here some CMV Ab^+DNA^+ RTR had high proportions of $FcR\gamma^-$LIR-1^+NKG2C$^+$ NK cells and raised strong ADCC responses but this was variable. Many patients with high ADCC responses may have active CMV replication so active CMV may be essential to expand the *adaptive NK cells* which may in turn contribute to the control of CMV. Longitudinal studies should address whether these cells are a stable feature of some RTR or are a response to protracted CMV replication.

Immunosuppressive drugs or other clinical variables such as age at transplant, CMV serostatus of donor and the recipient before transplantation may have altered the NK cell receptors in RTR however, due to small sample size and lack of clinical data the effect of these variables on NK cell receptor expression could not be determined. In spite of these limitations, our findings align with the other studies [16, 39] that demonstrate that the loss of FcRγ and increase in the expression of LIR-1 or NKG2C is associated with CMV seropositivity. Expansion of $FcR\gamma^-$LIR-1^+NKG2C$^+$ and $FcR\gamma^-$LIR-1^+NKG2C$^-$ NK cells should be confirmed in future in a larger cohort of CMV Ab^+DNA^+ RTR.

The changes to NK cell phenotypes associated here with CMV seropositivity (which carries a

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

MATERIALS AND METHODS

Patients and Controls

Renal transplant recipients were recruited from Royal Perth Hospital (RPH), Western Australia. All patients were clinically stable, free from clinical CMV disease or reactivation within 6 months of blood collection and receiving no current anti-viral treatment. All patients were on stable maintenance immunosuppressive therapy. Age-matched healthy controls were recruited from laboratory staff and colleagues. All participants provided written informed consent and the project was approved by the Human Research Ethics Committees of RPH, the University of Western Australia and Curtin University. PBMC were isolated from heparin-treated blood using Ficoll–Paque (GE Healthcare, Little Chalfont, UK) density gradient centrifugation and cryopreserved in liquid nitrogen. Plasma samples were aliquoted and stored at -80^0C.

Detection of CMV DNA and antibody

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

CMV-reactive IgG was quantitated by ELISA using a lysate of human foreskin fibroblasts (HFF) infected with AD169. Cells were harvested after 7 days, sonicated and stored at -80^0C. Uninfected HFF were prepared as a negative control. Parallel microtitre plates were coated with CMV glycoprotein prepared in hamster ovary cells (Chiron, CA) and CMV immediate-early 1 (IE-1) prepared in *E.coli* (Miltenyi Biotec, Germany) at 0.5µg/ml. Plates were coated overnight (4^0C), washed with PBS/0.05% Tween, blocked with 5%BSA/PBS and washed. Plasma samples pre-diluted in 2%BSA/PBS were run alongside control plasma from a CMV-seropositive healthy individual assigned a value of 100 arbitrary units (AU). Plates were washed after 2 hours and horse-radish peroxide conjugated anti-human IgG (Sigma-Aldrich, MO) diluted 1:4000 in 2%BSA/PBS was added. Tetramethylbenzidine substrate (Sigma-Aldrich) was added, colour development was stopped with 1M H_2SO_4 and plates were read at 450nm. CMV seropositivity was defined as >2 standard deviations above the mean antibody levels derived for a set of 11 samples which had been deemed seronegative by the ARCHITECT CMV IgG assay (Abbott Diagnostics, IL).

Immunophenotyping

Cryopreserved PBMC were thawed, washed and aliquots of 10^6 cells were stained for 15 minutes in the dark at room temperature (RT) with antibodies recognizing cell surface antigens: V500-anti-CD3 (UCHT1), V450-anti-CD56 (B159), APC-H7-anti-CD16 (3G8), APC-anti-CD57 (NK-1), PE-anti-NKp46 (9E2/NKp46), PE-anti-NKp30 (p30-15), PE-Cy7-anti-NKG2D (1D11; BD Biosciences, NJ), PerCPCy5.5-anti-CD85j (HP-F1; eBioscience, CA), Alexa Fluor 488 anti-NKG2C (134591), PE-anti-KIR2DL1 (143211; R&D systems, MN), PE-anti-KIR2DL2/L3/DS2 (GL183; Beckman Coulter, CA), PerCPCy5.5-anti-CD62L (DREG-56), PE-KIR3DL1 (DX9; Biolegend, CA), PerCPCy5.5-anti-KIR2DL1/S1 (HP-MA4; eBioscience). For intracellular markers, cells were incubated for 20 minutes at 4^0C with Cytofix/Cytoperm solution after surface staining and washed twice with cold 1X perm wash buffer (BD Cytofix/Cytoperm kit). Cells were then stained with 5µL PerCPCy5.5-anti-perforin (DG9; Biolegend) for 30 minutes in the dark at RT. After incubation, cells were washed twice with cold 1X perm wash buffer. At least 100,000 events were acquired in the lymphocyte gate on a FACSCanto II instrument (BD Biosciences). Data were analysed with FlowJo v10 software (Tree Star, OR).

Perforin ELISA

PBMCs were stimulated with anti-CD16 or K562 cells for 6 hours and supernatants were collected after 6 hours and stored at -80^0C. Perforin levels were measured using a commercial human Perforin ELISA kit (Abcam, Cambridge, UK) able to detect <40 pg/mL.

Functional Assays

Cryopreserved PBMC were thawed, washed and stimulated (0.5 x 10^6 cells/100 µL) with cytokines [IL-12 (10ng/mL; R & D systems) + IL-15 (10ng/mL; R & D systems) + IL-18 (10 ng/mL; MBL, Japan)] for ~ 16 hours at 37^0C and 5% CO_2. Remaining PBMC were rested overnight and next day these cells ($5x10^5$/100 µL) were stimulated for 6 hours at 37°C with RPMI 1640/10% FBS, 10µg anti-CD16 (BD Biosciences) bound to flat-bottomed 96-well plates or K562 cells at an effector-to-target ratio of 2:1. BV786-anti-CD107a (H4A3; BD Biosciences) was added to all wells. After 1 hour, brefeldin A and monensin (BD

Biosciences) were added and cells were incubated for a further 5 hours at 37°C. Cells were then stained with a live/dead dye; FVS700 (BD Biosciences) for 30 minutes, followed by BUV395-anti-CD3 (UCHT1), BV510-anti-NKp46 (9E2/NKp46), PE-CF594-anti-CD57 (NK-1), BV421-anti-NKp30 (p30-15; BD Biosciences), PE.Cy7-anti-CD56 (HCD56; Biolegend), APC-anti-NKG2C (134591; R&D systems) and PE-anti-LIR-1 (HP-F1; eBioscience) for 15 minutes in the dark at RT. Cells were then fixed for 20 minutes at 4^0C, permeabilized (BD Cytofix/Cytoperm™; BD Biosciences), stained for intracellular antigens using BV650-anti-TNF-α (MAb11; Biolegend) and FITC-anti-FceRIγ (Merck Millipore, Germany) for 30 minutes and was washed twice with cold 1X perm wash buffer. At least 100,000 events were acquired in the lymphocyte gate on a BD LSR II Fortessa Instrument (BD Biosciences). Data were analysed with FlowJo v10 software (Tree Star, OR).

ADCC Assay

96-well plates were coated with CMV gB antigen at 0.5µg/ml and incubated overnight at 4^0C. Wells were washed and blocked for 60 minutes at RT with 10% FBS/RPMI. 100µL aliquots of heat-inactivated (56°C, 1 hour) autologous plasma were added to the coated wells, incubated overnight at 4^0C. The optimal dilution (1:300) was selected in preliminary experiments that displayed highest expression of CD107a and production of TNF-α. Wells were washed and $5x10^5$ PBMCs were added with BV786-anti-CD107a (H4A3; BD Biosciences) for 1 hour followed by brefeldin A and monensin (BD Biosciences) for a further 5 hours at 37°C. Cells were stained as described under the functional assay.

Genotyping

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

Statistical analyses

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

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

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FIGURE LEGENDS

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

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

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

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

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

FIGURE 4. Analysis of perforin secretion and CD107a expression

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

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

Figure 5. Co-expression of $FcR\gamma^{-}$, $LIR\text{-}1^{+}$ and $NKG2C^{+}$ NK cells in RTRs and controls

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

Figure 6. ADCC by different subsets of $FcR\gamma^{-}$, $LIR\text{-}1^{+}$ and $NKG2C^{+}$ NK cells.

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

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

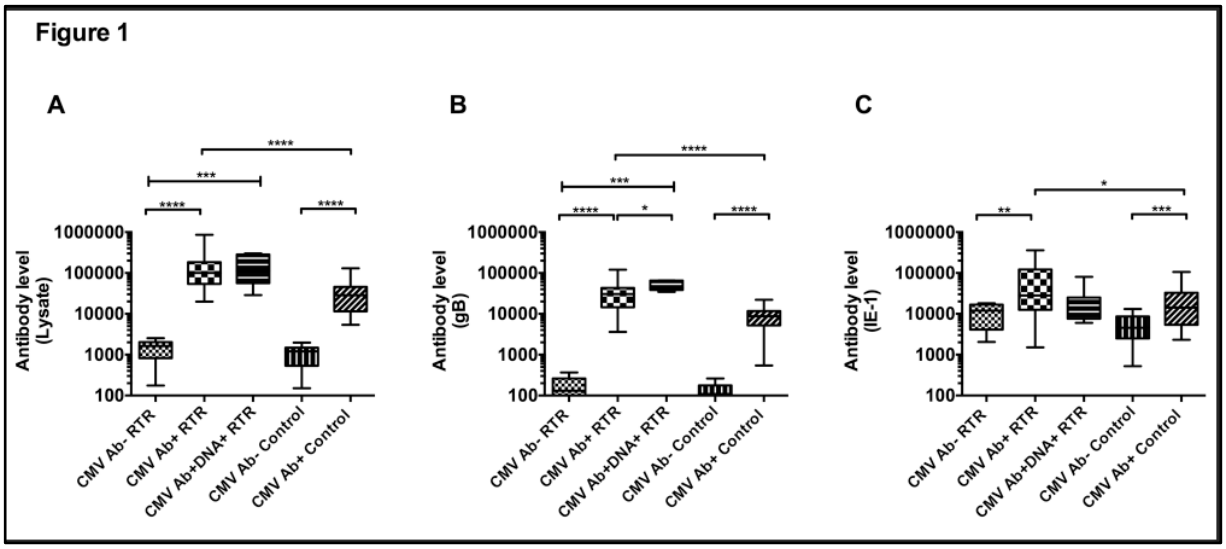


Figure 1
A
B
C
Antibody level (Lysate)
Antibody level (gB)
Antibody level (IE-1)
1000000
100000
10000
1000
100
CMV Ab- RTR
CMV Ab+ RTR
CMV Ab+DNA+ RTR
CMV Ab- Control
CMV Ab+ Control

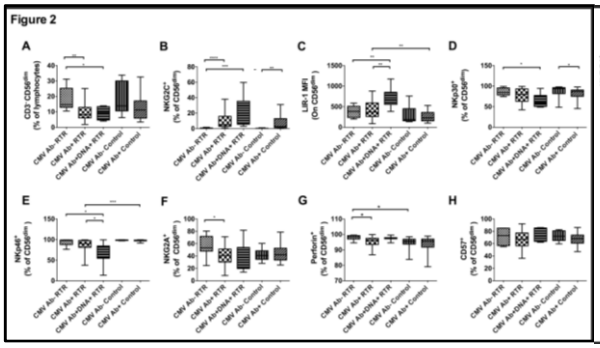
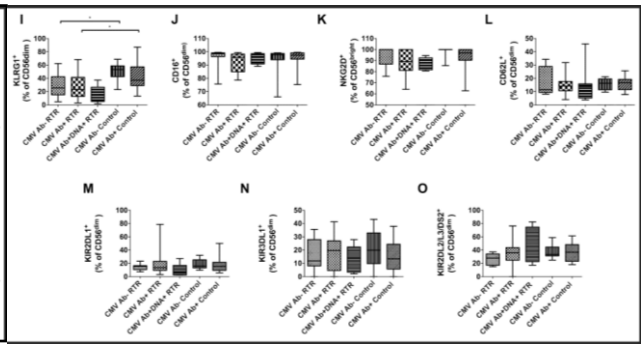


Figure 2
A
B
C
D
E
F
G
H


I
J
K
L
M
N
O

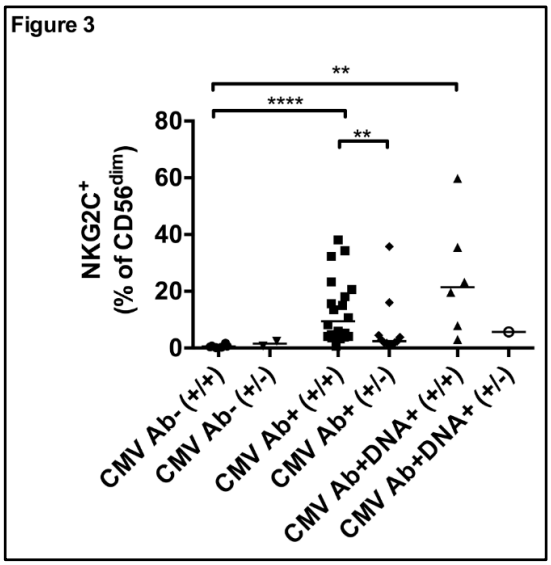


Figure 3
NKG2C+ (% of CD56dim)
80
60
40
20
0
CMV Ab- (+/+)
CMV Ab- (+/-)
CMV Ab+ (+/+)
CMV Ab+ (+/-)
CMV Ab+DNA+ (+/+)
CMV Ab+DNA+ (+/-)

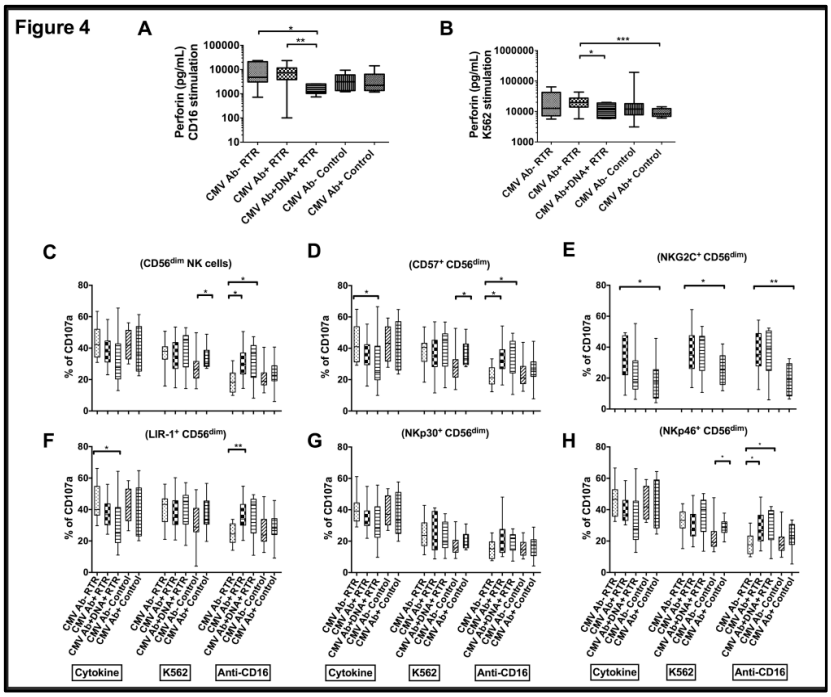


Figure 4
A
Perforin (pg/mL) CD16 stimulation
B
Perforin (pg/mL) K562 stimulation
CMV Ab- RTR
CMV Ab+ RTR
CMV Ab+DNA+ RTR
CMV Ab- Control
CMV Ab+ Control
C
(CD56dim NK cells)
D
(CD57+ CD56dim)
E
(NKG2C+ CD56dim)
F
(LIR-1+ CD56dim)
G
(NKp30+ CD56dim)
H
(NKp46+ CD56dim)
% of CD107a
Cytokine
K562
Anti-CD16

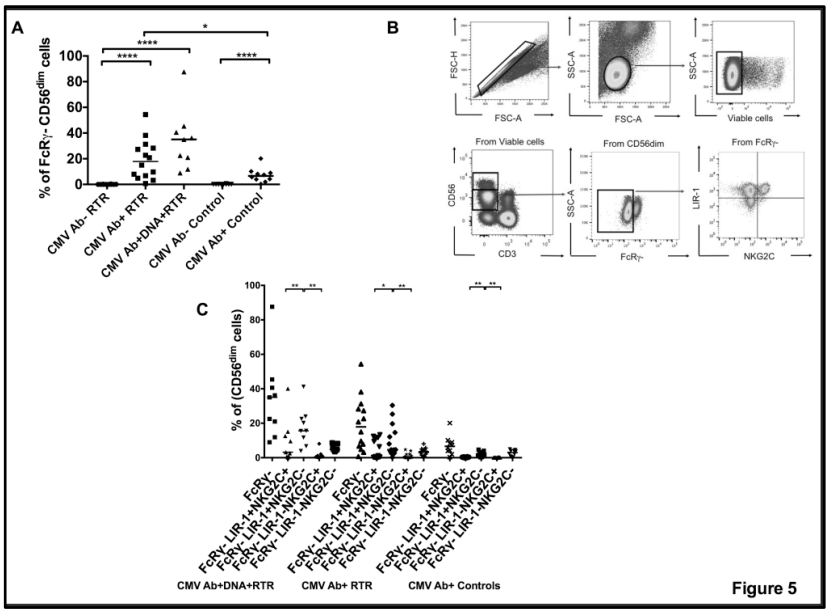

A
% of FcRγ- CD56dim cells
CMV Ab- RTR
CMV Ab+ RTR
CMV Ab+DNA+RTR
CMV Ab- Control
CMV Ab+ Control
B
FSC-H
FSC-A
SSC-A
Viable cells
From Viable cells
From CD56dim
From FcRγ-
CD56
CD3
FcRγ-
LIR-1
NKG2C
C
% of (CD56dim cells)
FcRγ-
FcRγ- LIR-1+NKG2C+
FcRγ- LIR-1+NKG2C-
FcRγ- LIR-1-NKG2C+
FcRγ- LIR-1-NKG2C-
CMV Ab+DNA+RTR
CMV Ab+ RTR
CMV Ab+ Controls

Figure 5

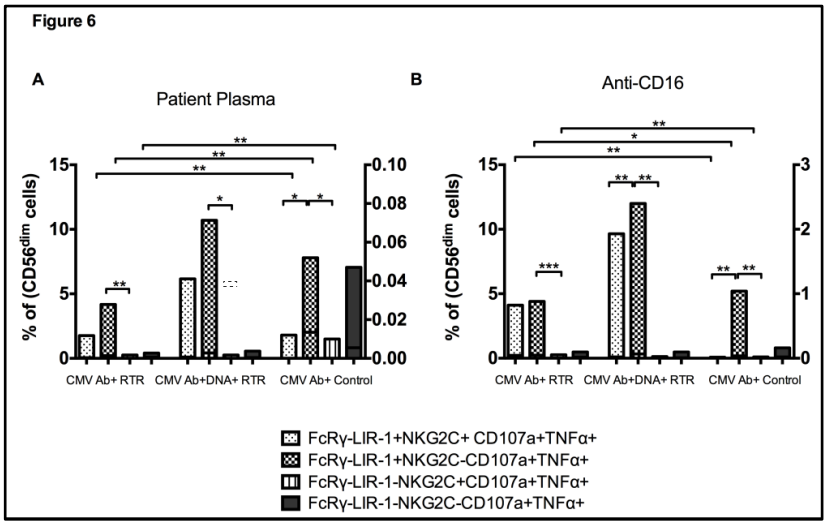


Figure 6
A
Patient Plasma
B
Anti-CD16
% of (CD56dim cells)
CMV Ab+ RTR
CMV Ab+DNA+ RTR
CMV Ab+ Control
FcRγ-LIR-1+NKG2C+ CD107a+TNFα+
FcRγ-LIR-1+NKG2C-CD107a+TNFα+
FcRγ-LIR-1-NKG2C+CD107a+TNFα+
FcRγ-LIR-1-NKG2C-CD107a+TNFα+