Critical Role of CD2 Co-stimulation in Adaptive Natural Killer Cell Responses Revealed in NKG2C-Deficient Humans

Graphical Abstract

Highlights
- NKG2C$^{-/-}$ donors have normal T cell immunity to cytomegalovirus
- NKG2C$^{-/-}$ donors have normal frequencies of adaptive NK cells
- CD2 is critical for antibody-triggered responses by adaptive NK cells
- CD2 synergizes with NKG2C in classical adaptive NK cells

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In Brief
Liu et al. demonstrate the emergence of redundant adaptive NK cell subsets in NKG2C$^{-/-}$ donors. Functional studies unraveled a critical role for CD2 in antibody-dependent responses by adaptive NK cells, paving the way for new strategies to harness their cytotoxic potential in cell therapy.

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Critical Role of CD2 Co-stimulation in Adaptive Natural Killer Cell Responses Revealed in NKG2C-Deficient Humans

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SUMMARY

Infection by human cytomegalovirus (HCMV) leads to NKG2C-driven expansion of adaptive natural killer (NK) cells, contributing to host defense. However, approximately 4% of all humans carry a homozygous deletion of the gene that encodes NKG2C (NKG2C<sup>-/-</sup>). Assessment of NK cell repertoires in 60 NKG2C<sup>-/-</sup> donors revealed a broad range of NK cell populations displaying characteristic footprints of adaptive NK cells, including a terminally differentiated phenotype, functional reprogramming, and epigenetic remodeling of the interferon (IFN)-γ promoter. We found that both NKG2C<sup>−</sup> and NKG2C<sup>+</sup> adaptive NK cells expressed high levels of CD2, which synergistically enhanced ERK and S6RP phosphorylation following CD16 ligation. Notably, CD2 co-stimulation was critical for the ability of adaptive NK cells to respond to antibody-coated target cells. These results reveal an unexpected redundancy in the human NK cell response to HCMV and suggest that CD2 provides “signal 2” in antibody-driven adaptive NK cell responses.

INTRODUCTION

Human cytomegalovirus (HCMV) is a persistent betaherpes virus with a worldwide prevalence ranging between 50% and 100% of the population depending on socioeconomic factors. Congenital HCMV infection is a leading cause of sensorineural hearing loss in children and a significant cause of neurodevelopmental delay (Manicklal et al., 2013). Immunocompromised patients with AIDS, severe combined immunodeficiency (SCID), or those having received immunosuppressive treatment in conjunction with hematopoietic stem cell transplantation (HSCT), frequently experience life-threatening HCMV infection. Adaptive immunity plays a crucial role in the control of HCMV (Crough and Khanna, 2009). HCMV-specific T cells have a terminally differentiated phenotype and can represent up to 40% of the total T cell memory pool (Sylwester et al., 2005; van Lier et al., 2003).

Natural killer (NK) cells are innate lymphocytes involved in numerous physiological processes including reproduction (Parham and Moffett, 2013) and immunity to infections (Jost and Altfeld, 2013). Recent advances in NK cell biology suggest that NK cells display adaptive features during CMV infection, contributing to viral control (Vivier et al., 2011). Unlike T and B cell immune responses, CMV-driv...
The absence of more severe HCMV infection in NKG2C−/− donors indicates the existence of redundant pathways for the control of the infection. To enable a comprehensive analysis of the immune system in NKG2C−/− donors, we screened 2,208 healthy blood donors and identified 81 NKG2C−/− donors, corresponding to a frequency of 3.7% in the Swedish population, in line with frequencies reported in other populations (Miyashita et al., 2004; Moraru et al., 2012b; Thomas et al., 2012). We then prospectively obtained buffy coats from 60 of these for downstream analyses. The demographics of the NKG2C−/− cohort and of the age-matched NKG2C+ controls (NKG2C+/− and NKG2C+/+) are summarized in Table S1.

We analyzed the impact of homozygous NKG2C deletion on the differentiation profile and the anti-HCMV response of CD4 and CD8 T cells from NKG2C−/− donors as compared to NKG2C+ (NKG2C+/− or NKG2C+/+) donors (Figures 1, S1, and S2). We found that the NKG2C deletion resulted in a slight but statistically significant accumulation of terminally differentiated effector memory CD45RA+ cells in the CD8+ T cell compartment (24.1 ± 14.4 versus 32.3 ± 16.9, p = 0.014), whereas no significant changes were observed for any of the other CD8 T cell subsets studied (Figures S1A and S1B). Interestingly, the accumulation of mature CD8 T cells was particularly visible in young and middle-age individuals (17.8 ± 9.6 versus 32.07 ± 17.2, p = 0.001; Figures 1A–1C). However, CD8 T cell responses following stimulation with overlapping peptide pools derived from the HCMV proteins IE-1, IE-2, and -deficient individuals revealed an unexpected redundancy in the adaptive NK cell response and point to a critical role for CD2 in providing co-stimulation for NKG2C- and antibody-mediated triggering of adaptive NK cells.

**RESULTS**

**Minimal Imprint in T Cell Immunity in Individuals Carrying Homozygous Deletion of NKG2C**

The absence of more severe HCMV infection in NKG2C−/− donors indicates the existence of redundant pathways for the control of the infection. To enable a comprehensive analysis of the immune system in NKG2C−/− donors, we screened 2,208 healthy blood donors and identified 81 NKG2C−/− donors, corresponding to a frequency of 3.7% in the Swedish population, in line with frequencies reported in other populations (Miyashita et al., 2004; Moraru et al., 2012b; Thomas et al., 2012). We then prospectively obtained buffy coats from 60 of these for downstream analyses. The demographics of the NKG2C−/− cohort and of the age-matched NKG2C+ controls (NKG2C+/− and NKG2C+/+) are summarized in Table S1.

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Adaptive NK Cell Response to HCMV in NKG2C−/− Donors

We recently reported that, among HCMV+ individuals, some displayed an expansion of NKG2C-negative NK cells, all of which expressed activating KIRs (Béziat et al., 2013). Such adaptive NK cells were identified by their highly differentiated phenotype, manifested by reduced expression of CD7, CD161, and FcεR1γ and higher expression of CD57 and LILRB1 (Béziat et al., 2013, 2014; Zhang et al., 2013). To examine the possible existence of adaptive NK cells in NKG2C−/− donors, we analyzed multiparametric flow cytometry data by non-linear dimensionality reduction using t-distributed stochastic neighbor embedding (t-SNE) (Figure 2A) (Amir et al., 2013). The t-SNE algorithm clusters cells according to their expression of multiple parameters and visualizes high-dimensional data in two-dimensional representations, avoiding the bias introduced by manual gating of specific subsets. This analysis clearly revealed clusters of cells sharing the phenotypic hallmarks of adaptive NK cells in NKG2C−/− donors (Figures 2A and 2B). In fact, NKG2C was the only marker that distinguished adaptive NK cell clusters in NKG2C-sufficient and -deficient donors. Applying stringent phenotypic criteria to assign a given cell population as adaptive,
we quantified the frequency of NKG2C+ and NKG2C−/− donors with imprints of adaptive NK cell responses (Figures 2C and 2D). Donors considered as carrier of an adaptive NK cell subset were defined on the basis of significant expansion of cells, representing >10% of CD56dim NK cells, displaying at least four of the five phenotype characteristics (low CD7, low CD161, low FcγRIγ, high CD57, or high LILRB1). Among the 47 HCMV+/NKG2C+ individuals in the control cohort, 16 (34%) had a population of adaptive NK cells, in agreement with the 38% reported previously (Bézia et al., 2013). Surprisingly, we found that 11 (25%) of the 44 HCMV+/NKG2C−/− donors had a significant population (>10%) of adaptive NK cells, a proportion not significantly different from the NKG2C+ individuals (Fisher’s exact t test: p = 0.27) (Figure 2D). Notably, none of the 14 NKG2C+ or 16 NKG2C−/− HCMV seronegative donors harbored such expansions (data not shown), suggesting that, like NKG2C+ donors, the expansions observed in NKG2C−/− individuals were related to HCMV infection.

Adaptive NKG2C+ NK Cells Preferentially Express Self HLA-Specific KIR and Share Functional Attributes with NKG2C+ Adaptive NK Cells

To further characterize the adaptive NK cell population in NKG2C+ donors, we assessed their KIR repertoires. Strikingly, as in NKG2C+ donors (Bézia et al., 2012, 2013), adaptive NK cell populations in NKG2C−/− donors displayed profound deviations in their KIR repertoires (Figures 3A and S4) with preferential expression of self-KIR and low frequencies of NKG2A (Figures 3B and 3C). More importantly, they shared functional attributes of adaptive NK cells observed in NKG2C+ individuals. First, they had a poor ability to produce interferon (IFN)-γ after interleukin-12 (IL-12)/IL-18 stimulation (Figure 3D). Second, they displayed decreased degranulation capacity, measured via cell-surface expression of CD107a, upon direct interaction with K562 (p = 0.0046) and RAJI (p = 0.0005) cells and displayed enhanced IFN-γ (p < 0.0001) and tumor necrosis factor (TNF) (p = 0.0002) production against antibody-coated target cells compared to conventional NK cells (Figures 3E–3G). Third, in line with the enhanced ability to produce IFN-γ, compared to conventional NK cells, they displayed a clear epigenetic remodeling associated with demethylation of CpG motifs in the conserved noncoding sequence (CNS) 1 of the IFNG locus (Figure 3H), which was shown to be exclusively demethylated in NKG2C+ expressing expansions from HCMV+ individuals (Luetke-Eversloh et al., 2014).

Altogether, these data demonstrate that NKG2C−/− individuals develop HCMV-driven adaptive NK cell responses at similar frequencies and with similar epigenetic, phenotypic, and functional properties as NKG2C+ individuals.

Adaptive Response to HCMV Occurs Independently of Activating KIRs in NKG2C−/− Individuals

The identification of adaptive NK cells in donors lacking NKG2C raised the question of which potential activating receptors might contribute to the expansion of this subset. Among other genes, the NK gene complex on chromosome 12 encodes NKG2E, an activating receptor that also forms functional heterodimers with CD94 and recognizes HLA-E (Lanier et al., 1998; Lazetic et al., 1996). Since CD94 was at least weakly expressed on all NK cells in both NKG2C+ and NKG2C−/− donors (Figure 4A), we asked whether an alternative activating CD94/NKG2 heterodimer was functional on adaptive NK cells in the absence of NKG2C. To this end, we stimulated NK cells with 221.AEH cells or with P815 target cells coated with anti-CD94 (Figures 4B–4D). These experiments demonstrated that neither triggering of CD94 (P815 + anti-CD94) nor stimulation with the natural HLA-E ligand (221.AEH) induced functional responses in adaptive NK cells from NKG2C−/− donors when compared with NKG2C-expressing adaptive NK cells. These results exclude the involvement of NKG2E in the expansion of adaptive NK cell subsets in NKG2C−/− donors.

Previous reports suggest that activating KIRs may compensate for the loss of NKG2C in donors with a homozygous NKG2C deletion (Bézia et al., 2013; Della Chiesa et al., 2014). Accordingly, we examined the relative contribution of NKG2C and activating KIRs to the adaptive NK cell pool in each donor (Figure 4E). In NKG2C+ donors, 60% of the expansions expressed only NKG2C, 27% co-expressed NKG2C and an activating KIR (KIR2DS1, KIR2DS2, KIR3DS1, or KIR2DS4), whereas 13% of the expansions expressed predominantly an activating KIR. Surprisingly, we found similar overall frequencies of activating KIR-positive expansions in NKG2C−/− individuals (36%) (Figure 4E). Moreover, the magnitude of the adaptive response, determined as the fraction of adaptive NK cells within the total NK cell subset, did not differ in donors with and without the NKG2C deletion and seemed to be independent of the activating receptor composition (Figure 4F). Although our phenotypic analysis did not include KIR2DS3 and KIR2DS5, the detection of three haplotype A/A donors among the 11 NKG2C−/− donors with an expansion allowed us to conclude that the expression of NKG2C and/or activating KIRs are not prerequisites for the emergence of adaptive NK cells.

CD2 and CD16 Synergistically Activate Adaptive NK Cells

The finding that adaptive NK cell responses can occur independently of NKG2C and activating KIRs prompted us to revisit the potential role of other activating receptors expressed by NK cells. We found a profound downregulation of Nkp46, stable expression or weak downregulation of 2B4, NTB-A, CRACC, and NKG2D and an increased expression of DNAM-1 and CD2 in adaptive NK cells of both NKG2C−/− and NKG2C+ donors (Figures 5A, 5B, and S5A). Next, we addressed whether any particular activating receptors could co-stimulate human adaptive NK cells in a fashion similar to that described for DNAM-1 in Ly49H-driven responses in the mouse (Nabekura et al., 2014). Since it was recently shown that antibody-mediated recognition of CMV-infected cells can drive the expansion of adaptive NK cells (Lee et al., 2015), we tested the ability of CD2 (Figures 5C and S5B), 2B4, and DNAM-1 (Figure S5C) to co-stimulate the CD16 pathway in adaptive NK cells from both NKG2C−/− and NKG2C+ donors. Agonistic stimulation of CD2 and CD16 using antibody-coated P815 cells revealed a striking synergistic interaction between CD2 and CD16 that was not observed for any other receptor combinations or in conventional NK cells. Thus, ligation of CD16 together with CD2 led to an
Figure 3. Adaptive NK Cells in NKG2C−/− Donors Share Most Attributes of NKG2C-Expressing Adaptive NK Cells

(A) NKG2A and KIR repertoire in conventional (left column) and adaptive (right column) NK cells of NKG2C−/− donors.

(B) Frequency of NKG2A+ NK cells (B) and educated cells (C) in adaptive and conventional NK cells of NKG2C+ and NKG2C−/− donors.

(D) Representative intracellular IFN-γ production by conventional and adaptive NK cells in NKG2C+ (n = 16) and NKG2C−/− (n = 11) donors after overnight stimulation with IL-12 and IL-18.

(E–G) Functional assay of conventional CD56dim NK cells as compared to adaptive NK cells from HCMV+NKG2C+ (n = 16) or HCMV+NKG2C−/− (n = 11) donors.

Cell-surface expression of CD107a (E) and intracellular expression of TNF (F) and IFN-γ. (G) were assessed after 6 hr of stimulation with K562 target cells or RAJI target cells in the presence of anti-CD20 (rituximab, 1 μg/ml).

(H) CpG methylation profile relative to transcriptional start site (TSS) of the IFN-γ promoter in adaptive NK cells compared to NKG2A+ and NKG2A+CD57− subsets. Four NKG2C−/− and six NKG2C+/− donors were analyzed. The error bars represent the SEM.
increase in IFN-γ and TNF-producing cells compared to CD16 stimulation alone (Figures 5C and S5B). Titrating the dose of CD16 revealed that CD2 engagement enhanced the maximum response compared to CD16 crosslinking alone without influencing the response threshold (Figure 5D).

Next, we explored the potential contribution of CD2 to antibody-triggered responses by adaptive and conventional NK cells. To this end, we made use of the CD20+ RAJI B cell lymphoma line, which expresses CD58, the ligand of CD2. We monitored functional responses in the two subsets following incubation with anti-CD20 (rituximab)-coated RAJI cells. CD2 blockade led to a profound decrease of IFN-γ production as well as degranulation (Figure 5E). The costimulatory effect of CD2 was evident at low rituximab concentrations, suggesting that this pathway may boost the CD16 responses in the context of low levels of immunoglobulins. The blocking of CD2 also revealed that CD2 engagement enhanced the maximum response compared to CD16 crosslinking alone without influencing the response threshold (Figure 5D).

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In NKG2C+ donors, NKG2C has a unique ability, alongside CD16, to trigger functional responses in resting NK cells without the need for additional co-activation (Luetke-Eversloh et al., 2014). Therefore, we tested the potential synergy between CD2, CD16, and NKG2C in the NKG2C-expressing subset from HCMV− (conventional phenotype) and HCMV+ (adaptive phenotype) donors. Functional assays with agonistic monoclonal antibodies (mAbs) revealed synergies between NKG2C and CD2 in adaptive NK cells of HCMV+ individuals but not in conventional NK cells (Figure 5F). Blockade of CD2 interactions with its natural ligand in target cell assays with HLA-E-expressing 221 cells had a broader effect and diminished the response in both conventional and adaptive NK cells (Figure 5G), potentially attributable to an additional effect on target cell adhesion (Hahn and Bierer, 1993). Together these results reveal a unique role for CD2 in specifically boosting functional responses mediated through CD16 and NKG2C in adaptive NK cells.

**CD2 Co-stimulation Boosts the CD16 Signaling Cascade**

To dissect the mechanism underlying CD2 costimulation in adaptive NK cells, we monitored the phosphorylation kinetics of key signaling molecules downstream of CD16, including CD3ζ, ZAP70/Syk, SLP76, LAT, ERK1/2 (MAP kinase pathway), and S6RP (mTORC pathway), using phosphoflow cytometry (Figures 6A–6D) (Long et al., 2013). Unlike T cells (Kaizuka et al., 2009), no signaling events was induced in conventional NK cells when triggered with anti-CD2 alone (Figures 6B–6D). In contrast, CD16 crosslinking induced phosphorylation of all signaling molecules tested in both NK subsets. In line with the functional readouts, CD16-induced phosphorylation of SLP76, ERK1/2, and S6RP was stronger in adaptive compared to conventional NK cells (Figure 6D). In conventional NK cells, we noted a weak synergy between CD2 and CD16 that was significant for the phosphorylation of ERK1/2 after 1 min (mean ratio 1.6 versus 1.9, p = 0.0006) and S6RP after 10 min (mean ratio 6.6 versus 10.3, p < 0.0001). In adaptive NK cells, however, co-ligation of CD2 and CD16 led to significantly higher levels of SLP76, ERK1/2, and S6RP.
Figure 5. Synergistic Effect of CD2 and CD16 in Adaptive NK Cells

(A and B) Representative FACS (A) plot and summary graph (B) of CD2 expression in adaptive NK cells of NKG2C* and NKG2C−/− donors.

(B) Functional assay of conventional (white) CD66* NK cells as compared to adaptive (red) NK cells from NKG2C−/− (n = 10) donors. Cell-surface expression of CD107a and intracellular expression of TNF and IFN-γ were assessed after 6 hr of redirected stimulation with P815 using mouse anti-human antibodies, as indicated (50 μg/ml).

(D) Cell-surface expression of CD107a and intracellular expression of TNF and IFN-γ production in conventional (black lines) versus adaptive (red lines) NK cells of five NKG2C−/− donors after 6 hr of redirected stimulation with P815 cells and anti-CD16 alone (closed symbols) or anti-CD2 (5 μg/ml) together with anti-CD16 (open symbols) at indicated concentrations. Significant differences of adaptive NK cell responses after CD16 stimulation compared to CD16*CD2 stimulation are depicted.

(E) Cell-surface expression of CD107a and intracellular expression of TNF and IFN-γ production in conventional (black lines) versus adaptive (red lines) NK cells of five NKG2C−/− donors after 6 hr of stimulation with RAJI cells coated with the indicated concentration of anti-CD20 (rituximab) in presence (close symbols) or absence (open symbols) of CD2 blocking. Significant differences of adaptive (red stars) and conventional (black stars) NK cell responses after rituximab stimulation with or without CD2 blocking are depicted.

(C–E) Adaptive subsets in NKG2C−/− donors were defined by the FcεR1−CD57+ phenotype.
phosphorylation of all signaling molecules. This synergistic induction of signaling in adaptive NK cells was particularly pronounced for ERK1/2 after 5 min (mean ratio 2.9 versus 3.9, \( p = 0.017 \)) and S6RP after 10 min (mean ratio 25.5 versus 39.8, \( p = 0.0068 \)). Taken together, these results demonstrate that the costimulation of CD16 by CD2 is mechanistically linked to the synergistic induction of the MAP kinase and mTORC pathways.

**DISCUSSION**

During the last decade, it has become clear that NK cells possess the ability to calibrate their functional potential and respond to pathogenic challenges in a fashion that is commonly attributable to cells within the adaptive immune system. However, the natural drivers behind these responses remain largely unknown. In the mouse, the activating receptor Ly49H has probably evolved to counteract the decoy major histocompatibility complex (MHC) class I molecule in MCMV, m157, which allows the virus to escape NK cells expressing the inhibitory receptor Ly49C (Forbes et al., 2014; Pyzik et al., 2014). However, Ly49H is not found in all mouse strains suggesting that it is not required for survival in natura, despite the protection it confers against MCMV in certain laboratory strains (Arase et al., 2002). In the human, the activating NKG2C receptor is expressed by a majority of adaptive NK cells responding to HCMV. However, individuals lacking this receptor are perfectly healthy and appear fully capable of controlling HCMV infection. Thus, the immunological control of both mouse and human CMV must involve other activating NK cell receptors, compensatory immune responses by other lymphocyte subsets or a combination thereof. The comparison of NK cell repertoires in two large cohorts of healthy donors either lacking or expressing the NKG2C gene allowed us to address these possibilities in the human.

Here, adaptive NK cell responses in NKG2C−/− individuals were limited to CMV-seropositive donors and occurred at similar frequencies as the previously described adaptive NK cell expansions in NKG2C+ donors. Although these expansions shared phenotypic, epigenetic, and functional attributes with NKG2C+ expressing expansions, most of the identified NK cell populations lacked all known drivers of adaptive NK cell responses, including activating KIRs (Béziat et al., 2013; Della Chiesa et al., 2014). The question raised, then, is which other activating signals may be involved in driving adaptive NK cell responses in NKG2C−/− donors. To address this, we first excluded the involvement of CD94/NKG2E by confirming that CD94 triggering including activating KIRs (Béziat et al., 2013; Della Chiesa et al., 2014). The question raised, then, is which other activating NK cell receptors, compensatory immune responses by other lymphocyte subsets or a combination thereof. The comparison of NK cell repertoires in two large cohorts of healthy donors either lacking or expressing the NKG2C gene allowed us to address these possibilities in the human.

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Adaptive NK cells displayed enhanced phosphorylation of ERK and S6RP upon CD16 and CD2 co-activation. ERK is a major hub of signal transduction in the MAP-kinase pathway.
whereas S6RP is downstream of the mTORC1 complex; both are late signaling events involved in numerous central processes in lymphocytes, including proliferation, differentiation, and cytokine production (Dong et al., 2002; Pollizzi and Powell, 2014). Recently, mTORC pathway activity was shown to be essential for mouse NK cell development (Marçais et al., 2014). Our results suggest that this pathway should also be scrutinized for its potential role in the development of adaptive NK cell responses.

Figure 6. CD2 Co-activates the CD16-Induced Signaling Pathway in Adaptive NK Cells

(A) Barcoding and gating strategy used to identify adaptive and conventional NK cells in the phosphoflow cytometry analysis. 
(B–D) Phosphorylation of the indicated phospho-epitopes in adaptive or conventional NK cells after CD2 and/or CD16 crosslinking. (B) Representative histogram plots at the indicated stimulation time point. (C and D) Mean fluorescence intensity (MFI) fold change of the indicated phospho-epitopes in conventional subset (solid line) of four HCMV+ (C) and the conventional (solid line) or adaptive (dotted line) subsets of four HCMV+ (D) individuals. Among the four HCMV+ donors, three were NKG2C+ and one was NKG2C−.

Error bars represent the SEM.
The signaling cascade integrates different signals and functions as a signal amplifier. Therefore, an initially relatively small difference can get larger further down in the cascade. This fit with the gradually increasing differences observed in the present study: p-CD3z/p-ZAP70 < p-SLP76 < p-Erk. Indeed, we observed a tendency for increased phosphorylation of all proximal readouts following CD2 and CD16 costimulation of adaptive NK cells. Thus, although a major difference of phosphorylation can be excluded for these early signaling events, more subtle variations might remain unseen due to a lack of sensitivity.

Given the synergy observed between NKG2C and CD2, it is plausible that CD2 can also cooperate with other DAP12-coupled receptors such as activating KIRs, although this possibility was not specifically examined here. It will be of interest to explore whether CD2 costimulation of CD16-driven responses influence the functional reprogramming of the cell during expansion in a fashion similar to that noted in the context of T cell exhaustion in autoimmunity and infection (McKinney et al., 2015).

The expansion of adaptive NK cells in the absence of activating KIRs and NKG2C suggests that a combination of other NK receptors and or external factors contribute to elicit such responses. In this context, antibody-mediated recognition of viral antigens as a driver of adaptive NK cell responses is particularly appealing (Lee et al., 2015). Since CD2 is expressed at high levels on all adaptive NK cell subsets and significantly boost the response of NK cells to CD16 ligation, it is conceivable that recognition of CD58 on HCMV-infected cells play a role in shaping adaptive NK cell responses. HCMV has tropism for epithelial cells, endothelial cells, myeloid cells, and fibroblasts, all of which express CD58 (Revello and Gerna, 2010). CMV infection also causes loss of HLA class I (Tandon and Mocarski, 2012). Thus, CMV-infected cells fulfill all criteria for efficiently stimulating self-KIR+ adaptive NK cells via CD2 and CD16. Outstanding remaining questions are to define the cellular interactions that trigger the onset of adaptive NK cell responses as well as the cellular entitles that maintain stable repositories for many years during latency. Our results point to the necessity of looking beyond NKG2C and consider the possibility that broadly expressed receptors, such as CD2, provide essential co-stimulatory signals to NK cells, corresponding to signal 2 in the generation of adaptive T cell responses (Smith-Garvin et al., 2009).

Another major purpose of the current study was to examine whether the loss of NKG2C-driven responses had any influence on T cell-mediated immunity to HCMV. NKG2C−/− donors displayed similar frequencies of CMV-specific T cells as the NKG2C+ donors, suggesting that lack of NKG2C had no major impact on the T cell response to the virus. However, we observed an accumulation of effector memory CD45RA+ CD8 T cells earlier in life in HCMV+NKG2C−/− individuals, potentially indicating a stronger CD8 T cell response in the early phase of HCMV infection in the absence of NKG2C-driven adaptive NK cell immunity. This notion is supported by an extensive study of a rural population of Gambia, a country with almost universal HCMV seroprevalence; i.e., almost 100% of the population was HCMV+ by 6 years of age (Goodier et al., 2014). Although specific T cell immunity was not analyzed, immunoglobulin G (IgG) titers against HCMV were elevated in young individuals lacking the NKG2C gene. These results suggest that, despite a high level of redundancy within the NK cell compartment itself, the lack of NKG2C might also be partly compensated for by enhanced T and B cell responses, particularly during the early phases of HCMV infection. Possibly, an effective adaptive NK cell immunity helps to control the burden of HCMV infection before the emergence of efficient T and B cell immunity. Although adaptive NK cells displayed reduced degranulation responses, their enhanced ability to release cytokines in response to antibody-coated targets might help to fulfill this role and contribute to maintaining the virus silent during latency. The plasticity of adaptive NK cell responses in the absence of activating KIRs and NKG2C points to the importance of such responses within the innate immune system.

**EXPERIMENTAL PROCEDURES**

**Human Participants and Cells**

This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee in Stockholm, Sweden. 2,208 random healthy blood donors were screened for NKG2C expression by flow cytometry. Donors lacking NKG2C expression were confirmed by PCR using the protocol described by Moraru et al. verifying homozygous deletion of NKG2C gene (Moraru et al., 2012a). 60 controls expressing NKG2C and 60 donors lacking the NKG2C gene were identified and enrolled in the study. For all donors, peripheral blood mononuclear cells (PBMCs) were cryopreserved for later use. Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN).

**KIR and KIR-Ligand Typing and HCMV Serology**

KIR ligands were determined using the KIR HLA ligand kit (Olerup SSP; QIAGEN) for detection of the HLA-Bw4, HLA-C1, and HLA-C2 motifs. KIR genotyping was performed by using quantitative KIR automated typing (qKAT) (Jiang et al., 2012). HCMV serology was determined using an ELISA-based assay on plasma obtained during sample preparation. Purified nuclear CMV antigen (AD 169) was used, and the cut-off level for seropositivity was an absorbance of ≥0.2 at a dilution of 1/100.

**Flow Cytometry**

A list of fluorochrome-conjugated reagents used for stainings can be found in the Supplemental Experimental Procedures. Details protocols of flow cytometry staining, Stochastic neighbor embedding (SNE) analysis, functional flow cytometry assays, including T and NK cell functional assays and phospho-flow cytometry experiments, are provided in the Supplemental Experimental Procedures. KIR repertoire analyses were performed according to the strategy previously described (Béziat et al., 2014).

**DNA Methylation Analysis**

DNA methylation was analyzed as previously described (Luetke-Eversloh et al., 2014). The methylation levels of six CpG residues within the IFNG CNS1 region were analyzed via bisulfite conversion and pyrosequencing by Varionostic. Donors were selected based on the size of the three target sub-sets to ensure sufficient numbers of cells for methylation analysis after sorting.

**Statistics**

For multiple group comparisons, one-way ANOVA, two-way ANOVA, or Kruskal Wallis nonparametric tests were applied. For single comparisons of independent groups, the Student’s t test or the Mann-Whitney test was performed. For single comparisons of matched groups, the paired Student’s t test or the Wilcoxon matched pairs test was performed depending on the sample size and distribution. For comparisons of qualitative variable, the Fisher’s exact t test was performed. In the relevant figures, n.s. indicates not significant; ***p < 0.001; **p < 0.01; and *p < 0.05. Analyses were performed using GraphPad software.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.005.

AUTHOR CONTRIBUTIONS

L.L.L. performed experiments, analyzed data, and wrote the manuscript. J.L. performed and analyzed phospho-flow experiments. E.M.A. performed viSNE analysis. M.E., E.S., J.P.G., and V.Y.S.O. performed experiments. Q.H. performed epigenetic analysis. J.A.T. and J.J. performed KIR genetic analysis. S.L. provided crucial support for sample collection. M.S. performed HLA typing. K.T., H.G.L., C.R., and J.T. analyzed data and contributed to the writing of the manuscript. K.J.M. coordinated research efforts, supervised research work and data analysis, and wrote the manuscript. V.B. coordinated research efforts, performed experiments, supervised research work and data analysis, and wrote the manuscript.

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