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Graphical Abstract

Highlights

- NK cells with multiple protein deficiencies are present in HCMV-infected individuals
- SYK deficiency is associated with hyper-methylation of the gene promoter
- Memory-like NK cells have protein deficiencies in combination with FcRγ deficiency
- FcRγ-deficient NK cells expand preferentially in an antibody-dependent manner

In Brief

Long-lived “memory-like” NK cells have been identified in HCMV-infected individuals at variable frequencies, but little is known about how this NK cell pool is formed. Kim and colleagues show data that support epigenetic modifications and antibody-dependent expansion as mechanisms underlying the formation of this memory-like NK cell pool.

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Epigenetic Modification and Antibody-Dependent Expansion of Memory-like NK Cells in Human Cytomegalovirus-Infected Individuals

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SUMMARY

Long-lived “memory-like” NK cells have been identified in individuals infected by human cytomegalovirus (HCMV), but little is known about how the memory-like NK cell pool is formed. Here, we have shown that HCMV-infected individuals have several distinct subsets of memory-like NK cells that are often deficient for multiple transcription factors and signaling proteins, including tyrosine kinase SYK, for which the reduced expression was stable over time and correlated with epigenetic modification of the gene promoter. Deficient expression of these proteins was largely confined to the recently discovered FcRγ-deficient NK cells that display enhanced antibody-dependent functional activity. Importantly, FcRγ-deficient NK cells exhibited robust preferential expansion in response to virus-infected cells (both HCMV and influenza) in an antibody-dependent manner. These findings suggest that the memory-like NK cell pool is shaped and maintained by a mechanism that involves both epigenetic modification of gene expression and antibody-dependent expansion.

INTRODUCTION

NK cells constitute a critical component of innate immunity and serve as a first line of defense against malignancy and viral infections, particularly herpesvirus infections (Biron et al., 1989; Orange, 2002; Vivier et al., 2011). Many recent studies have revealed adaptive immune or “memory-like” properties of NK cells, including long-term persistence and enhanced functional responsiveness, after pathogen infection or exposure to other stimuli (Béziat et al., 2012; Cooper et al., 2009; Foley et al., 2012; Gumá et al., 2004; Lopez-Vergès et al., 2011; O’Leary et al., 2006; Paust et al., 2010; Petitdemange et al., 2011; Sun et al., 2009). Although some of these characteristics might be transient or reflect a pre-activation state, it is also possible that some NK cells have undergone stable changes that serve to maintain memory-like properties, analogous to changes that occur during the differentiation of memory T cells (Farber et al., 2014). However, little is known about such changes that might stably alter the transcriptional programs of memory-like NK cells.

In humans, elevated and variable frequencies of memory-like NK cells, characterized by the expression of the activation receptor NKG2C, have been observed in association with prior infection by human cytomegalovirus (HCMV) (Gumá et al., 2004, 2006b; Monsivais-Urenda et al., 2010; Muntasell et al., 2013; Noyola et al., 2012), a common herpesvirus that establishes life-long latent infection in the majority of human populations (Dowd et al., 2009). It has also been observed that NKG2C+ NK cells expand in number in transplant patients experiencing HCMV reactivation and persist long term, even after clearance of active infection (Della Chiesa et al., 2012; Foley et al., 2012; Lopez-Vergès et al., 2011). NKG2C might be a useful marker for identifying memory-like NK cells, but more recent studies have shown that HCMV-infected individuals also have expanded populations of NK cells that persist long term and express certain activation forms of killer-cell immunoglobulin-like receptors (KIRs), including KIR2DS2 and KIR2DS4, even in the absence of NKG2C (Béziat et al., 2013; Della Chiesa et al., 2014). Thus, the memory-like NK cell pool in HCMV-infected individuals is likely to include a variety of expanded NK cell subsets expressing different activation receptors. Yet, despite the association with HCMV infection, there has been no direct evidence that these receptors themselves are responsible for activation of NK cells in response to HCMV-infected target cells. In fact, NKG2C+ NK cells display poor functional responses toward HCMV-infected cells (Magri et al., 2011; Petersen et al., 2010; Zhang et al., 2013).

Infection of HCMV-seropositive individuals by certain other viruses, including hantavirus, HIV-1, and EBV, is associated with further elevation of NKG2C+ NK cell frequencies (Björkström et al., 2011; Brunetta et al., 2010; Mela and Goodier, 2007; Petitdemange et al., 2011; Saghaﬁan-Hedengren et al., 2013), illustrating the potential impact of other viral infections on the expansion of the memory-like NK cell pool in HCMV-infected individuals. Again, there is no direct evidence that NKG2C is responsible for activation of NK cells in response to these viral infections. Importantly, these memory-like NK cells differ from conventional NK cells in their turnover rates and functional responses to tumor cells and cytokines (Béziat et al., 2012,
ory-like NK cells that exist within either NKG2C+ or NKG2C−
NK cells. We have recently discovered a distinct subset of NK cells characterized by deficiency in expression of FcRγ (also known as FcεRγ) (Hwang et al., 2012b; Zhang et al., 2013), a signaling adaptor associated with the Fc receptor CD16 (Lanier, 2008). These FcRγ−deficient (FcRγ−) NK cells, termed “g” NK cells,” express normal amounts of CD3ζ, another signaling adaptor associated with CD16, and often display predominant expression of NKG2C or particular KIRs, reflecting clonal-like expansion (Hwang et al., 2012b; Zhang et al., 2013). Compared to conventional NK cells, FcRγ− NK cells express lower amounts of major tumor recognition receptors (Nkp30 and Nkp46) and show generally poor responsiveness to tumor targets (Hwang et al., 2012b). FcRγ− NK cells are stably maintained and constitute as much as 85% of the total NK cell population but persist at variable frequencies depending on the donor. Despite the association with HCMV infection, FcRγ− NK cells respond poorly to HCMV-infected cells, reminiscent of the poor response of NKG2C− NK cells (Magri et al., 2011; Petersen et al., 2010; Zhang et al., 2013). However, FcRγ− NK cells display greatly enhanced functional responsiveness to HCMV-infected cells in the presence of HCMV-specific antibody (Ab), superior to conventional NK cells (Zhang et al., 2013), indicating that the response of FcRγ− NK cells toward HCMV-infected cells is not through direct recognition, but through Ab-dependent recognition via CD16. Compatible with our finding, a more recent study showed that NKG2C− NK cells also display enhanced functional responses to HCMV-infected cells in an Ab-dependent manner (Wu et al., 2013). Importantly, the discovery of FcRγ− NK cells illustrates a specific protein deficiency associated with pathogen infection in the human body.

Because FcRγ deficiency can identify Ab-dependent memory-like NK cells that exist within either NKG2C+ or NKG2C− populations (Zhang et al., 2013), FcRγ deficiency itself provides a molecular signature for memory-like cells in HCMV-infected individuals. In this study, we sought to identify molecular changes among CD16 downstream signaling molecules, SYK expression, and other signaling protein deficiencies associated with HCMV infection, FcRγ− NK cells, in these donors expressed SYK, whereas in donor#221, nearly all FcRγ− NK cells expressed SYK, consistent with the gene profiling analysis of our cohort of 62 healthy donors revealed that SYK-deficient NK cells were present in many donors, constituting as much as 60% of the total NK cell pool (Figure 2A). However, we found no evidence of ZAP70-deficient NK cells in these donors. Given the association of FcRγ− NK cells with HCMV infection, we performed flow cytometric analysis after intracellular staining with anti-SYK mAb. We observed abundant expression of SYK in NK cells from many donors (e.g., donor #204) (Figure 1D). However, the majority of FcRγ− NK cells in donor #214 were completely deficient for the expression of SYK, whereas in donor #221, nearly all FcRγ− NK cells expressed SYK, consistent with the gene profiling analysis. These data confirm that there was a distinct difference in SYK transcript and protein amounts between FcRγ− NK and conventional NK cells in donor #214, and that FcRγ− NK cells can express variable amounts of SYK. In contrast, all NK cells, including SYK-deficient NK cells, in these donors expressed the other SYK family kinase ZAP70 at uniformly high abundance (Figure 1D).

RESULTS

Gene Expression Profiling of FcRγ− NK and Conventional NK Cells
As a starting point for exploring potential differences in gene expression between conventional NK and FcRγ− NK cells, and to screen for genes that might contribute to the enhanced CD16 responsiveness of FcRγ− NK cells, we performed gene expression profiling studies on sorted samples of NK cells. Because intracellular staining of FcRγ requires cell fixation, we used cell surface markers that had predominant expression on FcRγ− NK cells in individual donors: NKG2C for donor #214 and KIR2DL2 for donor #221 (Figure 1A). By using this strategy and two different markers to avoid generating skewed data associated with a specific marker, CD56+CD3− NK cells were sorted into enriched FcRγ− NK and conventional NK cell samples, which were used to generate gene-expression profiles. This comparison revealed 407 transcripts in common that were differentially expressed (>1.75-fold different; 218 lower and 189 higher in FcRγ− NK cells) between FcRγ− NK and conventional NK cells within the same donor (Table S1), including many transcripts encoding cell surface markers. Consistent with microarray data, we found that on the surface of FcRγ− NK cells, ITGA6, SIGLEC7, CD7, PECAM1, and TIM-3 were expressed at lower amounts, whereas FAS, CD2, and ILT2 were expressed at higher amounts in all donors (n = 7) examined (Figure 1B). These data illustrate distinct transcriptional differences that contribute to the phenotypic characteristics of FcRγ− NK cells compared to conventional NK cells.

SYK Expression Varies Widely in NK Cells
To gain insight into the mechanism of FcRγ− NK cell enhanced responsiveness to CD16 stimulation, we looked for differences in expression of genes encoding signaling molecules that function downstream of CD16 (Colucci et al., 1999; Lanier, 2008). The gene profiling data showed that the amount of transcript encoding the protein tyrosine kinase SYK was less than 5% in FcRγ− NK cells compared to conventional NK cells in the donor #214 (Figure 1C). However, there was minimal difference in mRNA amounts for SYK in donor #221 or for other signaling proteins, including ZAP70 and PLCγ2, in either donor. Thus, among CD16 downstream signaling molecules, SYK expression can be quite different between FcRγ− NK and conventional NK cells but might also vary greatly between individuals.

To examine SYK protein expression, we performed flow cytometric analysis after intracellular staining with anti-SYK mAb. We observed abundant expression of SYK in NK cells from many donors (e.g., donor #204) (Figure 1D). However, the majority of FcRγ− NK cells in donor #214 were completely deficient for the expression of SYK, whereas in donor #221, nearly all FcRγ− NK cells expressed SYK, consistent with the gene profiling analysis. These data confirm that there was a distinct difference in SYK transcript and protein amounts between FcRγ− NK and conventional NK cells in donor #214, and that FcRγ− NK cells can express variable amounts of SYK. In contrast, all NK cells, including SYK-deficient NK cells, in these donors expressed the other SYK family kinase ZAP70 at uniformly high abundance (Figure 1D).

SYK-Deficient NK Cells Are Associated with HCMV Infection
Analysis of our cohort of 62 healthy donors revealed that SYK-deficient NK cells were present in many donors, constituting as much as 60% of the total NK cell pool (Figure 2A). However, we found no evidence of ZAP70-deficient NK cells in these donors. Given the association of FcRγ− NK cells with HCMV infection...
infection, we suspected that there might also be a correlation between SYK-deficient NK cells and HCMV infection. Testing of plasma samples for HCMV-specific Ab revealed that all donors with SYK-deficient NK cells (using 3% as the detection threshold), except for one, were seropositive for HCMV IgG (Figure 2B). The exceptional donor, in which SYK-deficient NK cells constituted 5% of total NK cells, had substantial memory T cells specific for HCMV pp65 antigen (Zhang et al., 2013), demonstrating that all donors with SYK-deficient NK cells were previously exposed to HCMV. In contrast, SYK-deficient NK cells were not associated with HSV-1 or HSV-2 infection. Further serological analysis indicated that SYK-deficient NK cells were detectable in HCMV-infected donors regardless of HSV-1 and HSV-2 infection \((p < 0.05)\) (Figure 2C). Thus, the presence of SYK-deficient NK cells is associated with prior infection by HCMV, but not by HSV-1 or HSV-2.

**SYK Deficiency Is Associated with Epigenetic Modifications**

To determine whether SYK-deficient NK cells are a transient or stable population, we performed longitudinal studies. Analysis of samples collected from several donors at different time points showed no noticeable decrease in the frequencies of SYK-deficient NK cells (Figure 3A), suggesting that the SYK deficiency is stably maintained. Given the importance of Syk in CD16 signaling in mouse NK cells (Colucci et al., 1999), we sought to examine whether SYK deficiency affects the functional responsiveness to CD16.
engagement in human NK cells. Despite SYK deficiency, cross-linking of CD16 led to production of interferon-γ (IFN-γ) by SYK-deficient NK cells at amounts significantly higher than those produced by SYK-expressing NK cells in peripheral blood mononuclear cells (PBMCs) (p < 0.01) (Figure 3B), indicating that SYK deficiency does not impair NK cell responsiveness to CD16 in human NK cells.

To examine whether SYK deficiency is maintained after cell division, NK cell clones were generated under limiting dilution conditions and analyzed for phenotype and functional attributes. The status of SYK expression (either present or absent) did not change over the course of several weeks of culturing (data not shown). Upon CD16 crosslinking, SYK-deficient NK cell clones displayed higher IFN-γ production compared to SYK-expressing NK cell clones (Figure 3C), consistent with observations of fresh NK cells. Thus, these data indicate that SYK-deficient NK cells are stably maintained even after several rounds of cell division and that their functional characteristics are inheritable by daughter cells.

As a potential mechanism underlying the SYK deficiency, we examined the methylation status of the SYK promoter region and found evidence of hyper-methylation within a CpG-rich region in which methylation has been correlated with reduced expression of SYK in human tumor cell lines (Goodman et al., 2003). In our studies, methylation was detected at several cytosines within the CpG island proximal to the SYK transcription initiation site in SYK-deficient NK cell clones but not SYK-expressing clones (Figures 3D and S1). In contrast, cytosines further upstream did not show such a difference. These data indicate that the SYK deficiency is associated with hyper-methylation of a specific region in the SYK promoter DNA sequences, suggesting that epigenetic modifications lead to silencing of SYK in memory-like NK cells.

**SYK Deficiency Is Predominantly Confined to the FcRγ- NK Cell Subset**

Considering the association of SYK-deficient, FcRγ- NK, and NKG2C+ NK cells with HCMV infection (Guma et al., 2004; Zhang et al., 2013), we co-stained cells to further examine their relationship. This analysis showed that nearly all SYK-deficient NK cells were also deficient for FcRγ expression in most donors (Figures 4A and S2A), indicating that the majority of SYK-deficient NK cells are present within the FcRγ- NK cell subset. The SYK-deficient NK cells constituted the major population of FcRγ- NK cells in some donors, but FcRγ- NK cells expressed SYK frequently in other donors. Although a low frequency of SYK-deficient NK cells that expressed FcRγ were detectable in several donors, these cells generally had lower amounts of FcRγ than the conventional NK cells (Figures 4A and S2A). Thus, the NK cell population could be divided into three major subsets—conventional NK cells, FcRγ- NK cells that are SYK deficient, and FcRγ- NK cells that are SYK expressing—and these subsets were present at variable frequencies between different donors. With respect to NKG2C expression, SYK-deficient NK cells were often present within the NKG2C+ population (Figure S2B). However, in many donors, substantial proportions of SYK-deficient NK cells did not express NKG2C and, in two donors among our cohort, all SYK-deficient NK cells completely lacked expression of NKG2C (Figures S2B and S2C), indicating that there was not a strong relationship between SYK deficiency and NKG2C expression. Nonetheless, our data demonstrate that the SYK deficiency is largely confined to FcRγ- NK cells, but not NKG2C+ NK cells, revealing a close relationship between SYK deficiency and FcRγ deficiency.

**SYK Deficiency and Lack of NKG2C Expression Does Not Impair CD16 Responsiveness**

We next sought to examine whether SYK deficiency affects the functional responsiveness to CD16 engagement in FcRγ- NK cells. Crosslinking of CD16 led to production of IFN-γ by SYK-deficient FcRγ- NK cells at amounts significantly higher than those produced by SYK-expressing FcRγ- NK cells (p < 0.05) as well as conventional NK cells (p < 0.01), although there was substantial variation between donors (Figure 4B). However, there was no significant difference between these subsets in the degranulation response, as measured by cell surface expression of CD107a (Figure 4C).

We also examined the responses of these NK cell subsets to viral infection and observed that direct stimulation of NK cells...
with HCMV-infected target cells did not induce substantial IFN-γ production in any subset (Figure 4D, top). Considering the robust responses of SYK-deficient NK cells to Ab-mediated CD16 crosslinking, we examined whether the presence of naturally occurring Abs against HCMV would impact the NK cell responses to HCMV-infected cells. Addition of autologous plasma containing anti-HCMV IgG led to a dramatic production of IFN-γ by SYK-deficient FcRγ- NK cells at amounts significantly higher (p < 0.01) than those produced by conventional NK cells (Figure 4D, bottom). These data indicate that SYK-deficient FcRγ- NK cells respond poorly to virus-infected targets cells directly, but instead, respond strongly to infected target cells in the presence of virus-specific Abs. However, the difference between SYK-deficient FcRγ- NK cells and SYK-expressing FcRγ- NK cells was not significant in this target-cell-based system (Figure 4D). Importantly, the production of IFN-γ by SYK-deficient FcRγ- NK cells was higher than by conventional

Figure 3. Stability of SYK-Deficient Phenotype Associated with DNA Hyper-methylation
(A) Frequency of SYK-deficient NK cells collected at the initial time point and indicated months later from eight healthy donors.
(B) Dot plot shows IFN-γ production by indicated subsets of NK cells from a representative donor among 15 individuals after stimulation with immobilized anti-CD16 for 7 hr. Numbers represent the percentage of FcRγ- NK and conventional NK cells. Dot graphs show the percentage of SYK-expressing NK cells and SYK-deficient NK cells that produced IFN-γ. Circles connected by a line designate the same donor sample (n = 15); *p < 0.01.
(C) NK cell clones were generated via limiting dilution of sorted NK cells, then tested for functionality after CD16 stimulation. Dot plots show IFN-γ production by two representative clones from five independent experiments. Numbers represent the percentage of cells that produced IFN-γ. Dot graph shows the percentage of cells that produced IFN-γ after stimulation of SYK-expressing (open circle) and SYK-deficient (closed circle) clones (n = 20 each). Each dot represents an individual clone and error bars indicate the mean ± SEM percentage of IFN-γ-producing cells for each group. **p < 0.01.
(D) Schematic diagram of SYK including the promoter-associated CpG island and translation start codon. Arrow represents the transcription initiation site. Expanded region details the location of 20 specific CpG dinucleotides as potential methylation sites. Bar graphs below show the percentage of methylation detected at each individual site in SYK-expressing and SYK-deficient NK cell clones. Data shown are from one donor; similar patterns were observed from two additional donors.

See also Figure S1.

NK cells in either system, indicating that SYK itself does not play a positive role in CD16-induced cytokine production in human NK cells and that SYK deficiency can contribute in part to the enhanced CD16 responsiveness of the FcRγ- NK cell population. Moreover, when NK cells were categorized with respect to the presence or absence of NKG2C expression, FcRγ- NK cells also displayed superior responsiveness compared to conventional NK cell subsets regardless of NKG2C expression (Figure S3).

FcRγ- NK Cells Are Deficient for Several Proteins Expressed in Conventional NK Cells
Based on the gene profiling data and evidence of epigenetic modification in FcRγ- NK cells, we suspected that there might be additional differences in factors influencing the transcriptional programs or signaling between FcRγ- NK and conventional NK cells. To explore the possibility, we examined protein expression of several other genes that showed differential mRNA amounts between FcRγ- NK and conventional NK cells.
Figure 4. Association and Functional Impact of SYK Deficiency with FcRγ Deficiency in NK Cells

(A) Flow cytometric analysis of FcRγ versus SYK expression in CD56+CD3−CD19−CD14− NK cells from four representative donors.

(B) Dot plots show IFN-γ production by indicated subsets of NK cells from a representative donor among ten individuals after CD16 stimulation. Numbers represent the percentage of NK cells within the designated quadrants. Dot graphs show the percentage of SYK-expressing conventional NK cells (FcRγ+SYK+; I), SYK-expressing FcRγ− NK cells (FcRγ−SYK+; II), or SYK-deficient FcRγ− NK cells (FcRγ− SYK−; III) that produced IFN-γ from several donors. Circles connected by a line designate the same donor sample (n = 10).

(C) Cell surface expression of CD107a was determined after stimulation as in (B). Numbers represent the percentage of NK cells within the designated quadrants. Dot graph shows the percentage of SYK-expressing conventional NK cells (I), SYK-expressing FcRγ− NK cells (II), or SYK-deficient FcRγ− NK cells (III) that displayed CD107a (n = 10).

(D) PBMCs were cultured for 3 days with mock- or HCMV-infected MRC-5 cells, with the last 6 hr in the presence or absence of autologous plasma as indicated. Dot plots show IFN-γ production by NK cells from one representative donor among nine individuals, and dot graph shows the percentage of NK cells that produced IFN-γ in SYK-expressing conventional NK cells (I), SYK-expressing FcRγ− NK cells (II), or SYK-deficient FcRγ− NK cells (III) from several donors (n = 9) ± autologous plasma. ns, not significant; *p < 0.05 and **p < 0.01.
These included the transcription factors PLZF (also known as ZBTB16) and IKZF2 (also known as HELIOS) and signaling molecules DAB2 and EAT-2 (Mathew et al., 2012; Pérez-Quintero et al., 2014; Shapira et al., 2014; Thornton et al., 2010).

The majority of conventional NK cells expressed PLZF (Figure 5A). In contrast, essentially all FcRγ− NK cells were deficient in PLZF expression. We noted that there were PLZF-deficient NK cells that expressed FcRγ in some donors, but these cells tended to express FcRγ at intermediate amounts. Similar to the pattern of PLZF expression, the signaling molecules DAB2 and EAT-2 were expressed by the majority of conventional NK cells (Figures 5B and 5C). However, FcRγ− NK cells generally displayed deficient expression of DAB2 and variable deficiency patterns of EAT-2. Furthermore, the majority of conventional NK cells expressed IKZF2 (Figure 5D). The pattern of IKZF2 expression in NK cells was more heterogeneous and complex, with evidence of multiple subsets in the FcRγ− NK cells, including a fraction of FcRγ− NK cells that expressed this transcription factor at amounts even higher than conventional NK cells. Thus, our study reveals the presence of several distinct subsets that are deficient for multiple transcription factors and signaling molecules. Importantly, these deficiencies are largely confined to the FcRγ− NK cell population, indicating a relationship between FcRγ deficiency and PLZF, DAB2, EAT-2, and IKZF2 deficiencies, resembling the relationship with SYK deficiency. Considering the heterogeneous expression of these proteins, we performed gene expression profiling on enriched NK cell samples sorted from two additional donors, which revealed many consistent differences between the populations of enriched FcRγ− NK cells and conventional NK cells (Table S2). Importantly, direct analysis of FcRγ− NK and conventional NK cells from multiple donors at the single-cell level revealed protein expression differences that were often more dramatic than observed differences in mRNA at the population level.

Ab-Dependent Expansion of FcRγ− NK Cells Occurs in Response to HCMV-Infected Cells

Hyper-methylation of the SYK promoter in SYK-deficient NK cells suggested that other protein deficiencies, including FcRγ deficiency in FcRγ− NK cells, were due to changes in DNA methylation. Because changes in methylation patterns on these target genes occur presumably in a stochastic manner, our observation that SYK-deficient NK cells, as well as other protein-deficient cells, were largely confined to the FcRγ− NK cell population suggested a potential effect of FcRγ deficiency, possibly through enhanced CD16 responsiveness, on the formation of memory-like NK cell pool.

Figure 5. Multiple Protein Deficiencies Correlate with FcRγ Deficiency
(A–C) PBMCs were co-stained for FcRγ and transcription factor PLZF (A), signaling molecules DAB2 (B), and EAT-2 (C). Shown are dot plots depicting CD56+CD3−CD19−CD14− NK cells from a representative HCMV-seropositive donor among at least seven HCMV-seropositive individuals. Dot graphs show mean fluorescence intensity (MFI) of indicated proteins in CD3−CD56+ T cells (T), conventional NK cells (NK), and FcRγ− NK cells from HCMV-seronegative and -seropositive donors. MFIs were each normalized by subtraction of the MFI from control staining. Symbols connected by lines are from the same donor sample.

(D) PBMCs were co-stained for FcRγ and transcription factor IKZF2. Shown are dot plots depicting CD56+CD3−CD19−CD14− NK cells from one HCMV-seronegative and three representative HCMV-seropositive donors as indicated. All data are representative of at least two independent experiments.
As an attempt to address this possibility, we co-cultured PBMCs containing FcRγ− NK cells with HCMV-infected or mock-infected cells for 11–13 days in the presence or absence of autologous plasma containing anti-HCMV IgG. Measurement of the relative frequencies of FcRγ− NK cells showed that there was no increase in the frequency of FcRγ− NK cells in the presence of HCMV-infected cells compared to mock-infected cells (Figure 6A). In fact, the relative frequencies of FcRγ− NK cells generally decreased after culturing with HCMV-infected cells when compared to their frequencies before culture.

Figure 6. Ab-Dependent Expansion of FcRγ− NK Cell Population in Response to HCMV-Infected Target Cells
(A) PBMCs were cultured in the presence of mock- or HCMV-infected target cells with or without autologous plasma as indicated. Dot plots show NK cells from one representative donor among 14 individuals before and after 11–13 days culture in indicated conditions. Numbers represent the percentage of FcRγ− NK cells. Dot graph depicts the change in frequencies of conventional (open circle) and FcRγ− (closed circle) NK cells compared to initial frequencies for individual donors after culturing for 11–13 days as indicated (n = 14).
(B) Bar graph depicts absolute numbers of conventional (open circle) and FcRγ− (closed circle) NK cells from one representative among 14 individuals after culture in indicated conditions as described in (A). Dot graph shows the fold change in the absolute number of NK cells based on the absolute number of NK cells obtained from the control condition (PBMCs cultured with mock-infected cells without plasma) for each individual donor (n = 14).
(C) NK cells were sorted from PBMCs and cultured as described in (A). Dot plots show NK cells from one representative donor among nine individuals cultured for 11 days as indicated. Purified IgG (Ab) was also tested. Numbers represent percentages of FcRγ− NK cells.
(D) Dot graph depicts the fold change in the absolute number of NK cells based on the absolute number of NK cells obtained from the control condition (PBMCs cultured with mock-infected cells plus plasma) for each individual donor (n = 9, **p < 0.01). For each dot graph, error bars represent mean ± SEM.
Moreover, the absolute numbers of FcRγ− NK cells obtained after co-culture with HCMV-infected cells were not higher than those obtained from the culture with mock-infected cells for the majority of the donors examined, although some increases in the number of FcRγ− NK cells in a few donors after culture with HCMV-infected cells were observed (Figure 6B). However, the addition of autologous plasma to the HCMV culture led to significant increases in both relative frequencies (p < 0.01) and absolute numbers (p < 0.01) of FcRγ− NK cells, compared to those resulting from co-culturing with either mock-infected, HCMV-infected cells, or mock-infected cells plus plasma (Figures 6A and 6B). Importantly, under this condition, FcRγ− NK cells also underwent significant preferential expansion over conventional NK cells, which was apparently due to active proliferation of FcRγ− NK cells in a later phase (>6 days) of the culture period (Figure S4). Moreover, functional analysis of the expanded FcRγ− NK cells indicated that these cells maintained the enhanced ability to produce IFN-γ in response to CD16 crosslinking (Figure S5). Taken together, these data demonstrate that FcRγ− NK cells can expand robustly upon encounter of HCMV-infected cells in the presence, but not in the absence, of seropositive plasma, and that expanded FcRγ− NK cells maintain enhanced CD16 responsiveness.

To determine whether the plasma-dependent FcRγ− NK cell expansion requires other leukocytes, NK cells were sorted from PBMCs and co-cultured with mock- or HCMV-infected cells in the presence or absence of autologous plasma. Similar to the data obtained with PBMC cultures, inclusion of seropositive plasma with HCMV-infected target cells yielded substantial increase in the frequencies and numbers of FcRγ− NK cells compared to either the condition containing mock-infected cells with plasma or the condition containing HCMV-infected cells without plasma (Figures 6C and 6D and data not shown). Moreover, when compared to conventional NK cells, FcRγ− NK cells displayed significant preferential expansion under this condition (p < 0.01). Finally, the addition of purified IgG to the HCMV culture also led to preferential and dramatic expansion of FcRγ− NK cells at amounts comparable to those obtained with plasma, indicating that the plasma effect was primarily mediated by Abs. Taken together, these data demonstrate that FcRγ− NK cells can undergo dramatic and preferential expansion in response to HCMV-infected cells in the presence of Ab, regardless of other immune cells. Importantly, these data support the idea that, whereas HCMV infection drives the stochastic epigenetic changes leading to FcRγ deficiency, as well as other deficiencies, FcRγ deficiency itself might be an important part of a mechanism to select and promote FcRγ− NK cell expansion in the presence of virus-specific Ab during HCMV reactivation.

**Ab-Dependent Expansion of FcRγ− NK Cells Occurs in Response to Influenza Virus-Infected Cells**

Considering the Ab-dependent expansion capability of FcRγ− NK cells in response to HCMV-infected cells, we sought to test FcRγ− NK cells in the setting of another common virus, influenza (flu). Via flow cytometry, we found that plasma from many healthy donors contained Ab that could bind to flu-infected, but not mock-infected, target cells (data not shown). Through experiments analogous to the HCMV co-culture system, we observed that the frequencies of FcRγ− NK cells increased in the presence of both flu-infected cells and plasma, but not in the absence of plasma, when compared to their frequencies before culture (Figure 7A). Importantly, the relative increase in absolute number of FcRγ− NK cells was significantly greater than that of conventional NK cells in the presence of both flu-infected cells and plasma (p < 0.01), compared to the control condition containing flu-infected cells without plasma (Figures 7A and 7B). Thus, similar to in vitro HCMV infection conditions, FcRγ− NK cells displayed robust and preferential expansion over conventional NK cells in response to flu-infected cells in an Ab-dependent manner.

**DISCUSSION**

Multiple lines of evidence support the concept that NK cells in humans and mice can exhibit several memory-like properties. These properties might be acquired through certain molecular
changes to maintain the memory-like state. Here, as a model for memory-like NK cells in humans, we have presented a stably maintained molecular signature of FcRγ− NK cells, which includes not only FcRγ deficiency but also differences in expression of multiple signaling molecules (SYK, DAB2, and EAT-2) and transcription factors (PLZF and IKZF2). For this study, the differences we observed were clear reductions from normal amounts and therefore are referred to as “deficiencies” for clarity. Unlike conventional NK cells that express these proteins, the majority of FcRγ− NK cells are deficient for PLZF and DAB2, while substantial, yet variable, fractions of FcRγ− NK cells are deficient for SYK, EAT-2, and IKZF2. Multi-protein-deficient FcRγ− NK cells were found almost exclusively in the HCMV-infected individuals of our cohort. Considering the prevalence of multi-protein-deficient memory-like NK cells.

Our study has also shown that SYK deficiency is associated with promoter DNA hyper-methylation, suggesting that epigenetic modifications are responsible for SYK deficiency. Consistent with our data, association between DNA hypermethylation and FcRγ and EAT-2 deficiencies has been reported (Y. Bryce-Bryson, 2014, Amer. Assoc. of Immunologists, conference). Thus, epigenetic modification might be a common mechanism for altered expression of these signaling proteins and transcription factors. Because epigenetic modifications occur presumably in a stochastic manner, our expansion data suggest that a subset of NK cells with particular epigenetic changes, specifically FcRγ deficiency, might be further selected and expanded preferentially in an Ab-dependent manner, especially during reactivation of HCMV or perhaps even during secondary infections by certain other viruses. It is possible that this process could continue with additional epigenetic changes during subsequent rounds of HCMV reactivation and Ab-dependent FcRγ− NK cell expansion, resulting in multiple protein deficiencies within the FcRγ− NK cell population. Taken together, we propose a model whereby HCMV infection directs stochastic epigenetic modifications and subsequent Ab-dependent expansion further selects and shapes the pool of memory-like NK cells.

Given that the majority of NKG2C+ NK cells belong to the FcRγ− NK cell population, the Ab-dependent expansion also provides a potential explanation for why HCMV-infected individuals with co-infections of other viruses had elevated frequencies of NKG2C+ NK cells (Björkström et al., 2011; Brunetta et al., 2010; Mela and Goodier, 2007; Petidemange et al., 2011; Safghafian-Hedengren et al., 2013). These viral infections might induce HCMV reactivation, which can in turn promote the expansion of NKG2C+ NK cells directly or in collaboration with HCMV-specific Ab. Other chronic or repeat viral infections might also have an impact on the size of the memory-like NK cell pool by promoting Ab-dependent expansion. Still, we expect that the degree of FcRγ− NK cell expansion might depend on the nature of the infection, because certain infections that are localized or that do not support sustained surface expression of antigens for Ab interaction might not be sufficient for substantial or detectable FcRγ− NK cell expansion. Furthermore, we speculate that during primary infection, precursor cells of FcRγ− NK cells might respond directly to HCMV-infected target cells by utilizing multiple activation receptors, including NKG2C, NKP46, DNAM1, and activating KIRs (Béziat et al., 2013; Gumá et al., 2006a; Long et al., 2013; Magri et al., 2011) and perhaps CD16 itself (Grier et al., 2012; Mandelboim et al., 1999), leading to expansion and differentiation into FcRγ− NK cells through epigenetic mechanisms that involve DNA methylation. It is also possible that other epigenetic mechanisms, such as chromatin remodeling or changes in transcription factor expression such as PLZF (Mathew et al., 2012), contribute to the formation of the memory-like NK cell pool. We are only just beginning to observe and understand dramatic and stable epigenetic alterations of the innate immune system after infection by a human pathogen.

As part of this study, analysis of mice that were infected with mouse cytomegalovirus (MCMV) yielded no evidence for the presence of FcRγ- or Syk-deficient NK cells within the MCMV-specific Ly49H+ memory NK cell pool (data not shown), revealing a difference between human and mouse memory NK cells. Nonetheless, the development of animal models as well as in vitro infection models will be useful for addressing key issues, such as the specific role of HCMV infection in the differentiation of these memory-like NK cells and functional impact of specific protein deficiencies. In addition to decreased gene expression, there are many factors that are more abundantly expressed in FcRγ− NK cells. Further exploration of our gene profiling data will probably reveal more differences between memory-like FcRγ− NK and conventional NK cells. Finally, the superior Ab-dependent expansion and functional capabilities of FcRγ− NK cells suggest that these memory-like NK cells might function to control HCMV reactivation from latency. Through these capabilities, FcRγ− NK cells might also aid in the control of multiple chronic or recurrent viral infections, especially other herpesviruses, thereby providing important protection for the host through an intriguing symbiotic relationship.

EXPERIMENTAL PROCEDURES

Human Subjects and Blood Samples
PBMCs were obtained from healthy volunteer donors with informed consent or from de-identified leukocyte reduction filters (American Red Cross), as approved by the Michigan State University Biomedical and Health Institutional Review Board.

Antibodies and Reagents
Sero logical status of donor plasma was determined with virus-specific ELISA kits (MP Biomedicala) according to the manufacturer’s instructions. The list of antibodies used in this study is included in the Supplemental Experimental Procedures.

Flow Cytometric Analysis of NK Cells and T Cells
For analysis of cell surface markers, FcRγ, SYK, IFN-γ, and CD107a expression, PBMCs or cultured cells were stained as previously described (Hwang et al., 2012c). For the analysis of DAB2 and EAT-2 intracellular molecules, cells were stained with anti-DAB2 or anti-EAT-2 Ab followed by fluorochrome-conjugated secondary Ab. For analysis of IKZF2 and PLZF, cells were fixed and permeabilized with Transcription Factor Buffer Set (BD). LIVE Cell Stain Kit (Invitrogen) was used to exclude dead cells from all analyses of NK cells.

Functional and Expansion Assays of NK Cells
NK cell functional assays with immobilized anti-CD16 mAb or HCMV-infected target cells were performed as previously described (Zhang et al., 2013). For
expansion assay, MRC-5 lung fibroblast cells were cultured in 96-well plates and either mock-infected or infected with HCMV (Towne strain, MOI = 1) (Zhang et al., 2013) or influenza virus (PR8 strain, 500 hemagglutination units per well) (Hwang et al., 2012a) for 2 hr, then washed with PBS to remove unabsorbed virus before addition of PBMCs or sorted NK cells, and cultured for 11–13 days in the presence of recombinant human IL-2 (10 U/ml). Plasma or purified IgG was added 2 days after plating of cells and half of culture media was removed and replenished with media containing IL-2 and plasma or IgG every 3–4 days.

**Microarray for Gene Profiling Analysis**

NK2G2C+ and NK2G2C− NK (CD56−CD3−CD19−CD14+) cells were sorted from the PBMCs of donor 214, KIR2DL2+ and KIR2DL2− NK cells were sorted from PBMCs of donor 221. Purified RNA from these cell preparations was analyzed by 4x44K Agilent human arrays (design ID 026652) in the RTSF at Michigan State University.

**NK Cell Cloning**

PBMCs were enriched for CD56+CD3−CD19− cells using NK Cell Isolation Kit (Miltenyi Biotech). Isolated NK cells were distributed into 96-well plates by limiting dilution in NK cell cloning medium (RPMI1640 supplemented with Miltenyi Biotec). Isolated NK cells were distributed into 96-well plates by

**DNA Extraction and Methylation Analysis**

DNA was extracted from SYK+ and SYK− NK clones with DNeasy Blood & Tissue Kit (QIAGEN) and bisulfite treated with the EZ DNA Bisulfite Kit (ZYMO). Nested PCR was performed on treated DNA to amplify the promoter region of SYK with primers and conditions previously described (Goodman et al., 2003).

**Statistics**

The Wilcoxon matched-pairs signed-rank test was used for comparison of the frequency change and fold change during NK cell expansion and functional assays. The chi-square test was used for comparison of ELISA data. Differences were considered significant when p < 0.05 (GraphPad Prism).

**ACCESSION NUMBERS**

The NCBI GEO accession number for the microarray data reported in this paper is GSE66124.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.02.013.

**AUTHOR CONTRIBUTIONS**

J.L. and J.M.S. performed experiments, interpreted data, and prepared the paper; T.Z. and I.H. conceived the study, performed experiments, and interpreted data; A.K. performed experiments and prepared the paper; L.N. and M.K. performed experiments; Y.K. and L.L.L. designed, performed experiments, and interpreted data; and S.K. conceived the study, interpreted results, and prepared the paper.

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**REFERENCES**


Guerri, J.T., Forbes, L.R., Monaco-Shawver, L., Oshinsky, J., Atkinson, T.P., Grier, J.T., C00, 1471–1479.


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