Dynamic changes in natural killer cell subset frequencies in the absence of cytomegalovirus infection

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Individuals lacking functional natural killer (NK) cells suffer severe, recurrent infections with cytomegalovirus (CMV), highlighting the critical role of NK cells in antiviral defense. Therefore, ongoing attempts to develop an efficacious vaccine to prevent CMV infection should potentially aim to elicit NK-cell antiviral responses as an accessory to conventional T- and B-cell based approaches. In this regard, CMV infection provokes marked phenotypic and functional differentiation of the NK-cell compartment, including development of adaptive NK cells that exhibit enhanced antiviral activity. We examined longitudinal blood samples collected from 40 CMV-seronegative adolescents to ascertain whether a CMV glycoprotein B (gB) vaccine can stimulate differentiation or expansion of CMV-associated subsets of NK cells. Study participants uniformly lacked the CMV-dependent NKG2C^{high} subset of NK cells, suggesting that an adjuvanted CMV gB vaccine is an inadequate stimulus for sustained expansion of these cells. In contrast, we observed unexpected dynamic fluctuations in the frequency of NK cells lacking FcRγ, EAT-2, and SYK, which were independent of vaccination or CMV infection. These results suggest that frequencies of some NK cell subsets may increase in response to unknown environmental or inflammatory cues, where greater understanding of the nature of such signals should permit vaccine-driven expansion of CMV-reactive NK cells.

Vaccine | HCMV | Innate lymphoid cell | NK cell | Memory | CD56 | CD57 | FcRγ | SYK | NKG2C | EAT-2

Introduction

Cytomegalovirus (CMV) is a significant global cause of morbidity and physical impairment, with manifestations of infection ranging from subclinical disease to death. Congenital infection and the infection of immunocompromised patients, including transplant recipients, represent the most severe consequences of CMV in the human population. Congenital CMV accounts for roughly 400 deaths and more than 5,000 developmentally impaired children each year in the United States [1]. Therefore, effective strategies to prevent or control infection are desperately needed.

Unfortunately, CMV has proven to be a challenging target for vaccine development. To date, most CMV vaccine efforts focus on elicitation of antibodies against viral glycoproteins or generation of antiviral T-cell responses. Administration of a MF59-adjuvanted CMV glycoprotein B (gB) vaccine to CMV-seronegative adolescent girls induced strong gB-specific antibody responses and afforded 43% protective efficacy [2]. The same vaccine conferred short-lived, 50% protection, against CMV infection in seronegative post-partum women [3] and reduced post-transplant viral load when given to patients

awaiting a kidney or liver transplant [4]. While promising, these results indicate that humoral responses against gB may be insufficient to effectively prevent CMV infection in many individuals. DNA vaccines aimed at eliciting CMV-reactive T cells have also afforded minimal protection in a transplant patient-based clinical trial [5]. These advances prompted development of new vaccines aimed at eliciting both humoral and cellular immunity [6], but it remains unclear whether other arms of the immune response must be engaged to effectively prevent CMV infection.

Natural killer (NK) cells are critical antiviral effectors that produce IFN-γ [7], lyse virus-infected cells [8], and regulate adaptive immune responses [9-14]. NK cells play an important role in control of CMV infection in both mice and humans [15, 16]. Since NK cells lack the somatically rearranged antigen-specific receptors characteristic of T and B cells, and because they were previously thought to be short-lived cells [17], vaccine triggering of NK cells has historically been considered of little value. However, recent data suggests that the innate immune system makes important contributions to vaccine-elicited protection against infection [18]. Specifically, long-lived populations of adaptive NK cells with antigen-specific features similar to those of memory T cells have emerged as potential new targets of vaccines aimed at preventing CMV infection [19-22].

Immunological memory in virus-specific NK cells is widely described in the context of murine CMV. In C57BL/6 mice, a mouse CMV gene product engages an activating NK cell receptor, Ly49H (*Klra8*), promoting clonal expansion and contraction of the Ly49H-expressing subset of NK cells [23-27]. Thereafter, a subset of memory Ly49H$^+$ NK cells with enhanced antiviral effector functions persists indefinitely [28]. Similar types of adaptive NK cells develop in response to hapten sensitization [29], vaccinia virus infection [30], and virus-like particle immunization of mice [31]. Likewise, simian immunodeficiency virus-reactive memory NK cells develop in rhesus macaques after virus infection or immunization [32]. Collectively, animal studies point to existence of long-lived, virus-dependent subpopulations of memory NK cells that are likely better antiviral effectors than their naïve counterparts.

Several types of memory NK cells have been characterized in humans. These include memory NK cells induced by cytokines [33], varicella zoster virus exposure [34], antibody-mediated stimulation [35], or CMV-derived peptides [36]. High frequencies of NK cells expressing the activating receptor NKG2C are frequently observed in CMV seropositive individuals [37]. These NKG2C^{high} cells undergo proliferative expansion during primary CMV infection in transplant patients [38] and in response to CMV-infected fibroblasts [39], IL-12-producing infected monocytes [40], and CMV UL40-derived peptides [36]. CMV-associated adaptive NK cells expressing NKG2C display altered DNA methylation patterns and reduced expression of signaling molecules, including FcRγ, spleen tyrosine kinase (SYK), and EWS/FLI1-associated transcript 2 (EAT-2) [41, 42]. CMV-associated NK cell expansions with reduced expression of FcRγ, SYK, and/or EAT-2 generally lack expression of the transcription factor PLZF and may also express activating KIR or lack DAP12-coupled receptors altogether. NK cells with reduced expression of FcRγ, SYK, or EAT-2 are also detected in CMV seronegative individuals, with 10% of individuals displaying significant expansions of this population. These phenotypic changes are linked to more potent antibody-dependent activation, expansion, and function of these adaptive NK cells relative to other NK-cell subsets.

The crucial function of NK cells in immune defense against CMV coupled with the discovery that distinct subsets of NK cells emerge after infection, collectively suggest that targeted induction of these subsets of NK cells during immunization may provide enhanced protection against CMV infection. The capacity of existing vaccines to elicit transient or sustained expansion of CMV-associated human NK

Table 1. Patient Demographics. Age, race, and ethnicity of female subjects who received placebo or gB/MF59 vaccine (n=20 per group).

	gB/MF59	**Placebo**	**Total**
# subjects	20	20	40
Age Category at Vaccination, n (%)			
12-15 years	14 (70)	11 (55)	25
15-17 years	6 (30)	9 (45)	15
Race, n (%)			
Black	7 (35)	7 (35)	14
Caucasian	12 (60)	11 (55)	23
Other	1 (5)	2 (10)	3
Ethnic Origin, n (%)			
Hispanic/Latino	1 (5)	0 (0)	1
Not	19 (95)	20 (100)	39

cells has not been reported. In this study, we interrogate longitudinal peripheral blood mononuclear cell (PBMC) samples collected from MF59-adjuvanted CMV glycoprotein B (gB) vaccine or placebo recipients who locally participated in clinical trial NCT00133497 [2]. Our study reveals vaccine-independent oscillation of FcRγ^{neg} NK cell frequencies, but not those of NKG2C^{high} NK cells, in human blood from CMV seronegative individuals. These finding provoke re-evaluation of the paradigm concerning NK-cell subset dynamics in humans.

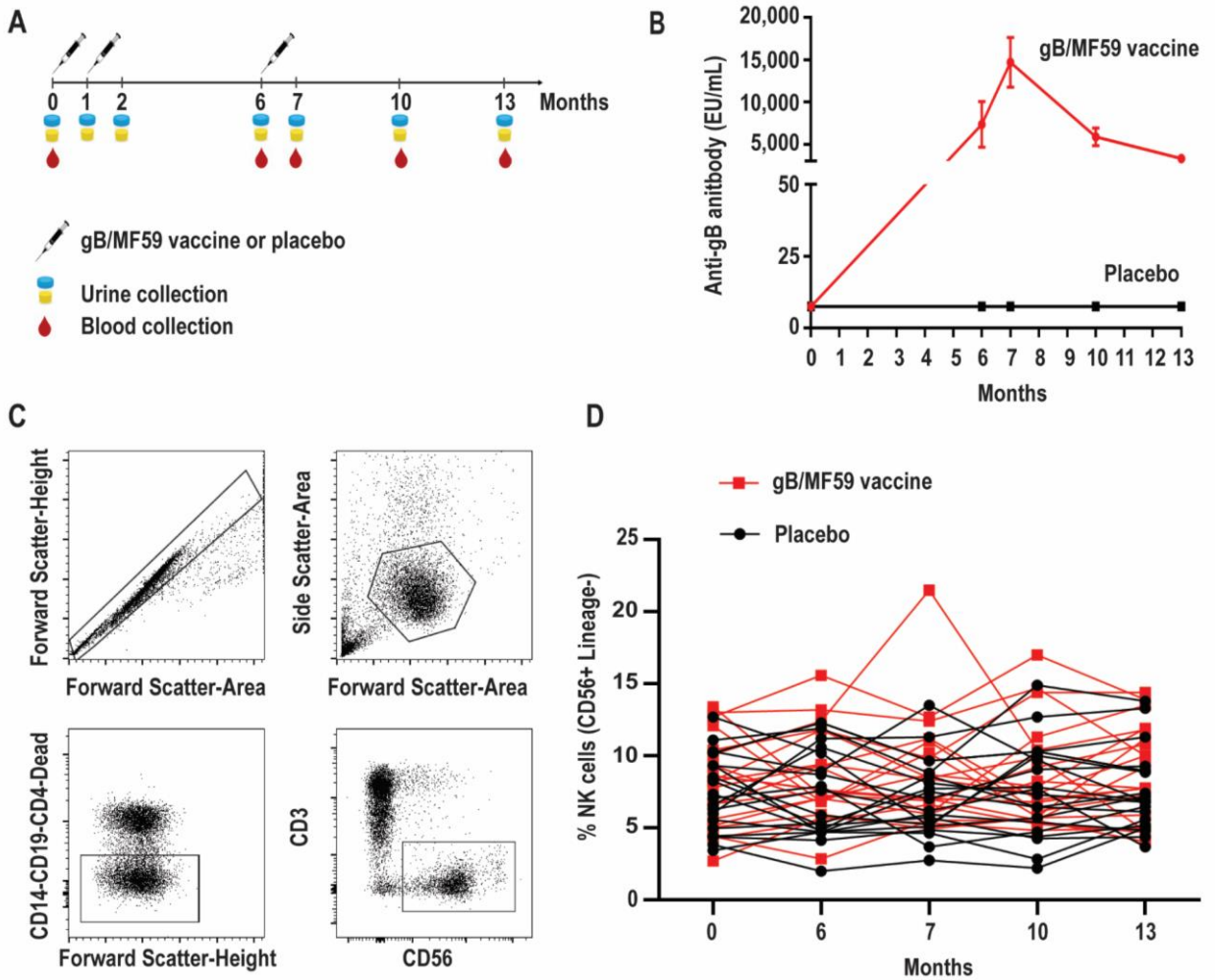

Figure 1. Longitudinal antibody and NK-cell responses of vaccine study participants. (A) Schematic representation of the timeline of vaccine trial depicting three administrations of gB/MF59 vaccine or placebo (sterile saline) and timing of urine and blood samples collection. (B) Sera anti-gB antibody titers for placebo (n=20) and vaccine (n=20) recipients analyzed in present study. (C) Representative flow cytometry gating of singlets, live lymphocytes, lineage-negative (CD19, CD14, CD4, CD3) CD56$^+$ NK cells based on forward scatter (height and area), side scatter (area), viability dye uptake, and surface marker expression. (D) Resulting frequencies of gated NK cells in individual vaccine and placebo recipients over sampling period.

Results

Vaccine trial cohort. To determine whether CMV vaccination strategies can trigger emergence of CMV-associated NK cell subsets, we examined a longitudinal series of PBMC from a subset (n=40, **Table 1**) of CMV vaccine trial participants (NCT00133497) for whom a full set of samples were available. Half of the study participants received three intramuscular injections of CMV gB in MF59 adjuvant while the placebo group was administered sterile saline in place of the vaccine (**Figure 1A**). Vaccine recipients exhibited a robust gB-specific antibody response (**Figure 1B**). None of the selected 40 study participants acquired CMV infection during the study period, as measured by PCR testing for CMV in urine and for seroconversion against non-vaccine CMV antigens [2].

Minimal variation in NK-cell frequencies over time. We first assessed the proportion of total NK cells (CD56$^+$ CD3$^-$ CD19$^-$ CD14$^-$ CD4$^-$) in PBMC. **Figure 1C** depicts the gating scheme used to identify NK cells in our samples. There was a broad range (2.0-21.5%) of NK-cell proportions across study

participants and across sampling time points (**Figure 1D**). The mean proportion of NK cells across all time points is similar in groups receiving placebo or vaccine (Placebo=7.4%, Vaccine=8.6%, p=0.16), while the changes in NK cell proportions over time between the placebo or vaccine group were not statistically significantly different (p=0.71).

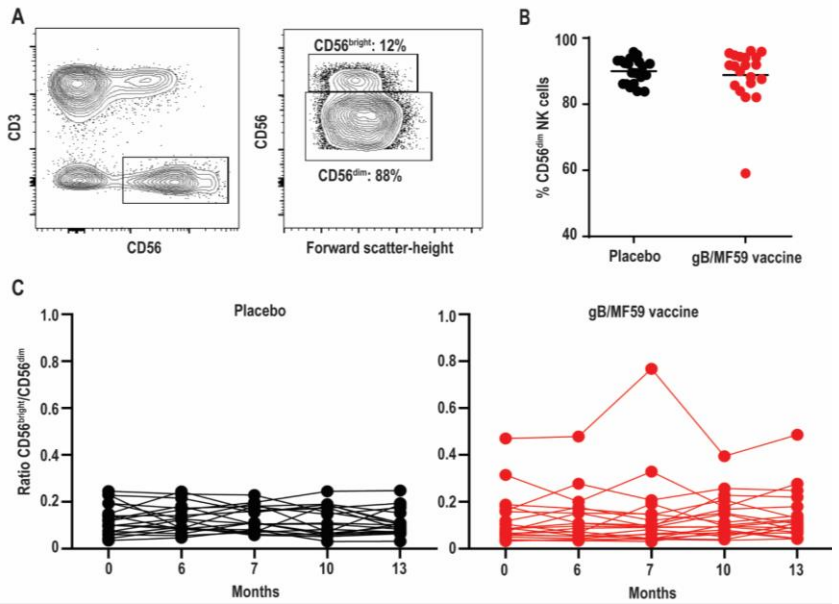

Figure 2. Stable proportions of CD56^{dim} and CD56^{bright} NK cells over time. (A) Representative gating of CD56^{bright} and CD56^{dim} events within the Lineage^{-} CD3^{-}CD56^{+} NK cell gate, and (B) percentage of CD56^{dim} NK cells in each study participant averaged across all time points. Bar represents mean among treatment group (n=20/group). (C) Ratio of CD56^{bright} to CD56^{dim} NK cells in each study participant across vaccine trial time points. Statistically significant changes in subset proportion over time evaluated using generalized linear mixed model as described in Methods, with results presented in **Table 2**.

NK cells can be stratified based on CD56 expression into CD56^{dim} and CD56^{bright} subsets (**Figure 2A**) that exhibit distinct phenotypic and functional characteristics [43]. The CD56^{dim} subset comprises a mean 88.8±1.14% (average of all time points) of circulating NK cells in study participants (**Figure 2B**), where the ratio between CD56^{bright} and CD56^{dim} cells in vaccine and placebo groups is relatively consistent over study time points (**Figure 2C**). Specifically, time did not modify the effect between the placebo and vaccine groups regarding CD56^{dim} cell counts (p=0.38). In addition, neither time (p=0.97) nor treatment group (mean vaccine: 6.50% (95% CI: 5.34%, 7.91%); mean placebo: 5.51% (95% CI: 4.51%, 6.74%), p=0.24) were independently associated with CD56^{dim} cell counts. For CD56^{bright} cell counts, time modified the effect of placebo and vaccine groups (p=0.01); wherein CD56^{bright} count increased by a factor of 1.25 (95% CI: 1.07, 1.46, p=0.01) in the vaccine group but the change in the placebo group was not statistically significant (**Table 2**).

Absence of CMV-dependent NKG2C^{high} NK-cell subset. High frequencies of NKG2C-expressing NK cells have been almost exclusively observed in CMV seropositive individuals [37]. This subset expands after CMV reactivation in organ or tissue transplant recipients [38], and reflects activation of this subset by CMV UL40-derived peptides coupled with pro-inflammatory cytokines [36]. Due to the confirmed CMV negative status of vaccine trial participants throughout the duration of the vaccine study and the absence of UL40 antigens in the vaccine formulation, we hypothesized that NKG2C^{high} NK cell frequencies would be low at all time points. Using a positive control PBMC sample known to contain NKG2C^{high} NK cells [42], we confirmed that our staining protocol can effectively detect this subset (**Figure 3A**). As expected, NKG2C^{high} NK cells were largely undetectable in all vaccine trial participants at baseline and the average absolute change in frequency from baseline proportions of this subset hardly varied across time in both placebo (0.046-0.409% range of mean absolute change from baseline visit) and vaccine (-0.830-0.956% range of mean absolute change from baseline visit) recipients (**Figure 3B**). Analysis of additional samples from vaccine trial participants (n=3) at time points after natural acquisition of CMV infection (confirmed by PCR & seroconversion to non-vaccine CMV antigens) remained negative for NKG2C^{high} NK cells over the study timeframe (data not shown).

CMV- and vaccine-independent dynamic changes in NK-cell subset frequencies. Expanded subsets of NK cells that lose expression of FcRγ, EAT-2, and/or SYK are expanded in approximately half of CMV seropositive individuals but can also be observed in seronegative donors, albeit less commonly (≤10% of individuals) and at much lower frequencies [41, 42]. As these subsets can expand upon Fc receptor engagement by antibody [35, 41], we hypothesized that robust antibody responses against vaccine

antigens may trigger accumulation of these subsets after vaccine prime and boost administration. We could detect NK cells within the CD56^{dim} subset that exhibited loss of FcRγ expression (**Figure 4A**). These FcRγ^{neg} NK cells concomitantly lacked EAT-2 and SYK expression in most study participants relative to FcRγ^{+} NK cell counterparts (**Figure 4A**).

Table 2. Change in NK cell CD56^{bright} and CD56^{dim} subsets over time. Mean (95% confidence interval) percentage of CD56^{dim} and CD56^{bright} cells by time and treatment group.

	Placebo Mean (95% CI)	Vaccine Mean (95% CI)
CD56^{Dim} (interaction p-value = 0.38)		
0 m	5.95% (4.73%, 7.50%)	6.20% (4.95%, 7.76%)
6 m	5.41% (4.29%, 6.81%)	6.34% (5.07%, 7.94%)
7 m	5.15% (4.09%, 6.49%)	6.99% (5.58%, 8.75%)
10 m	5.41% (4.30%, 6.82%)	6.45% (5.15%, 8.07%)
13 m	5.67% (4.49%, 7.16%)	6.53% (5.21%, 8.20%)
CD56^{Bright} (interaction p-value = 0.01)		
0 m	0.62% (0.50%, 0.77%)	0.58% (0.47%, 0.72%)
6 m	0.61% (0.49%, 0.76%)	0.60% (0.48%, 0.74%)
7 m	0.60% (0.49%, 0.75%)	0.69% (0.56%, 0.86%)
10 m	0.55% (0.44%, 0.68%)	0.70% (0.57%, 0.87%)
13 m	0.59% (0.47%, 0.73%)	0.72% (0.58%, 0.90%)

Interestingly, we detected a progressive increase in the frequency of FcRγ^{neg} NK cells following prime and boost immunization in a subset of vaccine recipients (**Figure 4B**). However, a similar pattern was observed in some placebo recipients. Moreover, the majority of individuals given vaccine (n=11) or placebo (n=10) exhibited transient expansions and contractions in the frequency of FcRγ^{neg} NK cells over time (**Figure 4C**). A smaller number of individuals in both groups demonstrated FcRγ^{neg} NK cells at baseline that disappeared over time, or lacked this subset of cells entirely (**Figure 4C**). High-dimensional analysis with t-SNE confirmed FcRγ^{neg} NK cells largely cluster as one subset, the frequency of which changes over time within the selected study participant (**Figure 4D**).

Distinct CD57 expression on FcRγ^{neg} NK cells in absence of CMV. While the proportions of FcRγ^{neg} NK cells vary among individuals and at different time points, the percentage of NK cells expressing other receptors associated with CMV infection, including CD57 (range 10-54%) or NKG2A (range 20-84%), exhibited little variation across time (**Figure 5A**). In fact, no statistically significant differences in CD57 (p=0.96) or NKG2A (p=0.75) expression were observed over time between both placebo and vaccine groups. The temporally stable but heterogeneous expression of CD57 and NKG2A among individuals in the present study is consistent with prior observations [44]. As CMV infection is associated with increased expression of CD57 and down-regulation of NKG2A [45], most notably among FcRγ^{neg} [35] and NKG2C^{high} [46] NK cells, the expression of these receptors was examined on the NK-cell subsets in vaccine trial participants (**Figure 5B**). FcRγ^{neg} NK cells detected in CMV-negative individuals in this study segregated as NKG2A^{low} relative to FcRγ^{+} cells (**Figure 5B**), consistent with previous studies [35, 42]. However, FcRγ^{neg} NK cells were not enriched for expression of the (**Figure 5B**). In fact, that totality

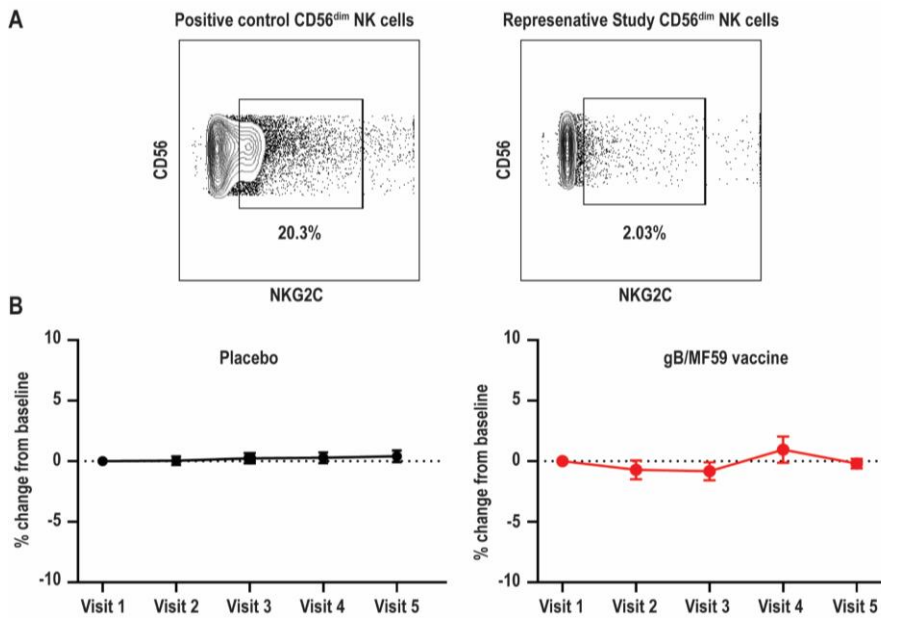

Figure 3. Absence of NKG2C^{high} NK cells in vaccine trial cohort. (A) Flow cytometry gating of NKG2C^{high} events among gated Lineage^{-}CD56^{dim} NK cells in positive control sample and negligible staining for NKG2C on NK cells in a representative vaccine study participant. (B) For each study participant, the absolute change in proportion of NKG2C^{high} NK cells over time relative to measurement at baseline is calculated and presented as average (± standard error of the mean) of treatment group (n=20/group).

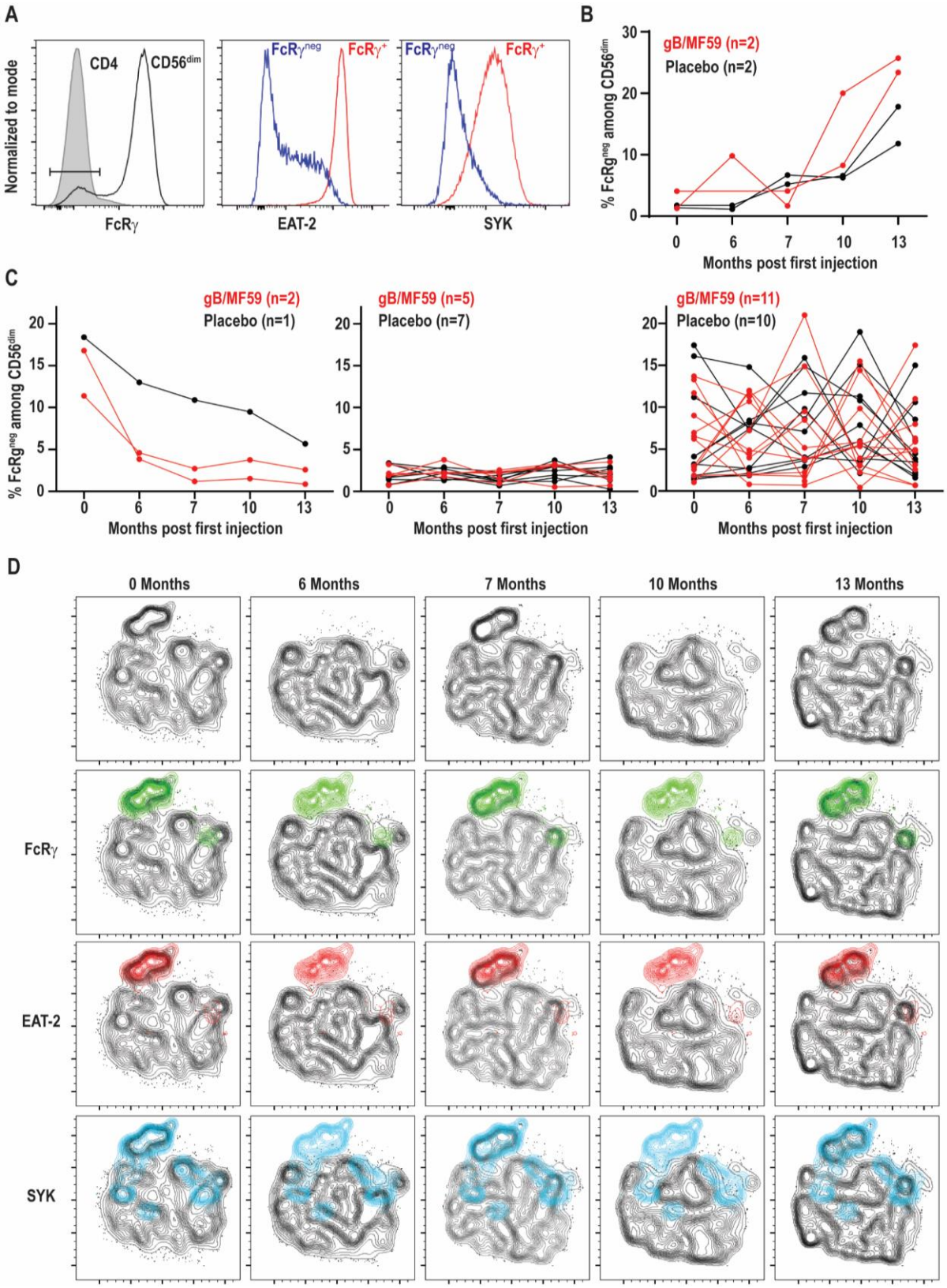

Figure 4. Dynamic vaccine-independent changes in NK-cell subset representation within repertoire over time. (A) Representative gating of FcRγ^{neg} CD56^{dim} NK cells (open histogram) relative to CD3^{+}CD4^{+} T cells (shaded histogram) in the same sample. EAT-2 and SYK expression on gated FcRγ^{neg} (blue histogram) and FcRγ^{+} (red histogram) subsets of CD56^{dim} NK cells. (B) Proportions of FcRγ^{neg} CD56^{dim} NK cells over time in a subset of gB-MF59 (red) or placebo (black) recipients revealing expansion of these cells within the repertoire. (C) Proportions of FcRγ^{neg} CD56^{dim} NK cells over time in remaining study participants grouped based on pattern of subset contraction (left), absence of subset (middle), or ebb-and-flow changes in repertoire. (D) Location of each adaptive NK-cell subset in t-SNE distribution of a single study participant CD56^{dim} NK cell repertoire over time course is highlighted in green (FcRγ^{neg}), red (EAT-2^{neg}), and blue (SYK^{neg}).

of FcRγ^{neg} NK cells observed across time points and individuals in this study expressed less CD57 than their FcRγ^{+} counterparts (**Figure 5C**). Thus, FcRγ^{neg} NK cells are more prevalent in the NK-cell repertoire in this longitudinally examined study cohort and frequently exhibit dynamic changes in frequency over time as well as distinct CD57 expression patterns relative to FcRγ^{neg} NK cells in CMV infected individuals.

Discussion

Past cross-sectional analyses suggest that adaptive subsets of NK cells are rarely present in the absence of CMV infection, whereas the frequencies of these adaptive NK cells are markedly increased in the majority of CMV seropositive individuals [41, 42, 46-48]. The present longitudinal analysis of NK cells in healthy CMV-negative individuals affirms the lack of NKG2C-expressing NK cells in CMV-naïve persons [42], yet challenges the paradigm that the FcRγ^{neg} NK cell subset is infrequent or absent in CMV seronegative individuals. In contrast to the current prototype, the majority of the healthy, demonstrably CMV-negative adolescent women profiled in this clinical study exhibit measurable frequencies of FcRγ^{neg} NK cells during at least one study time point, with dynamic changes in the frequency of these cells among circulating NK cells over time. Changes in frequencies of these subsets did not correlate with vaccine administration or vaccine-antigen-specific antibody titers, suggesting that undefined environmental factors promote oscillations in the representation of these subsets among total circulating NK cells.

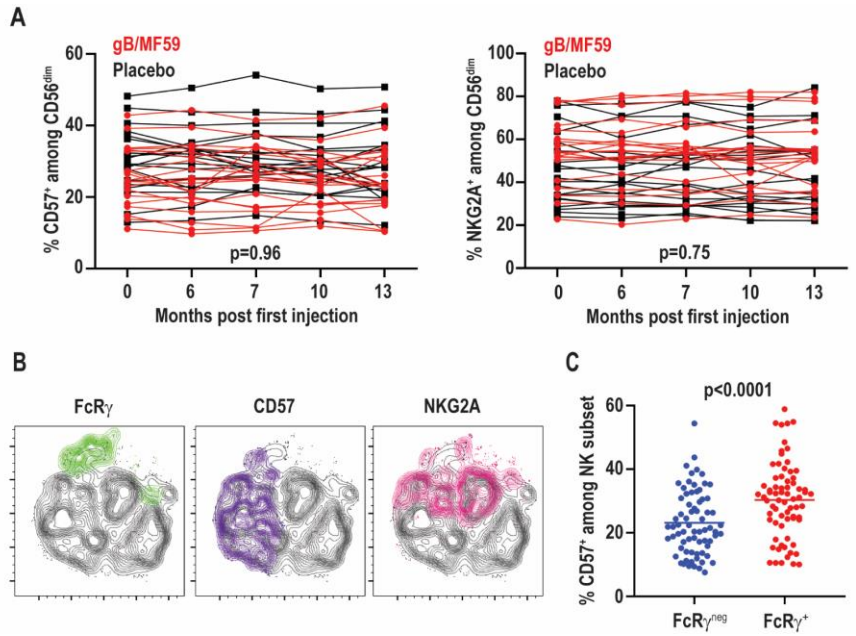

Figure 5. Figure 5. **No change in CD57 and NKG2A over time**. (A) Proportions of CD57^{+} and NKG2A^{+} CD3^{-}CD56^{dim} NK cells in individual gB/MF59 vaccine (red) and placebo (black) recipients over time. Mixed effects two way ANOVA with restricted maximum likelihood was used to compare mean differences over time between both placebo and vaccine groups for each marker. (B) Representative location of FcRγ^{neg} (green), CD57^{+} (purple), and NKG2A^{+} (pink) cells at single time point in t-SNE distribution of a single study participant CD56^{dim} NK cell repertoire. (C) Expression of CD57 on FcRγ^{neg} and FcRγ^{+} CD56^{dim} NK cells across all time points and study participants where the FcRγ^{neg} subset was detectable (≥ 5% of CD56^{dim} NK cells). Statistically significance differences between repeated measures determined by ANOVA.

The low frequencies of NKG2C-expressing NK cells across all time points in the forty vaccine trial participants is consistent with absence of CMV infection of these individuals and the purported link between CMV gene products and expansion of this subset of NK cells [49-51]. Within the limitations of our sampling scheme, these results also support the hypothesis that CMV gB and MF59 are insufficient to stimulate differentiation or accumulation of NKG2C^{high} NK cells. Since CMV UL40-derived peptides presented by HLA-E are critically required for HCMV-driven NKG2C expansion [36], incorporation of UL40 into next generation vaccines may more effectively elicit NKG2C^{high} memory NK cell expansion.

In contrast to both the tight link between CMV and NKG2C^{high} NK cells and the reported rarity of FcRγ^{neg} NK cells in CMV-seronegative individuals, the present longitudinal data suggest that the latter NK cell subset may be commonly present in some NK-cell repertoires and can exhibit dynamic changes in frequency. The majority (28 out of 40) CMV seronegative individual in our study exhibited populations of FcRγ^{neg} NK cells greater than 10% during at least one of the five time points analyzed over a year-long study period. These data contrast a previous cross-sectional studies which found expansions of NK cells lacking FcRγ, EAT-2, and/or SYK in 6/69 adults [42]. The fraction of study participants scoring positive for FcRγ^{neg} NK cell subsets at any given time point in our study ranged from 30% to 45%, suggesting that

additional factors may distinguish the two study populations. Moreover, the FcRγ^{neg} NK cells measured in this study appear to differ from those observed in CMV-infected individuals with regards to expression of the maturation marker CD57 [35, 42, 46, 52]. As CD57 expression on NK cells is putatively linked to increased cytolytic potential, decreased sensitivity to inflammatory cytokines, and reduced proliferative potential [53], this phenotypic disparity of FcRγ^{neg} populations of NK cells in the absence of CMV may reflect important functional distinctions as well.

A major distinction of the present study population is the restriction to analysis of adolescent females. The influence of puberty-associated hormones and other pediatric variables on adaptive NK cell subsets is unknown. Therefore, it is possible that the present longitudinal study reveals dynamics of NK cell subsets that are unique to adolescents, or even adolescent females, that are not shared by adult CMV seronegative populations. Of note, NK cells express the alpha and beta estrogen receptors (ERα and ERβ) and exhibit function alterations in response to estrogen [54, 55]. Moreover, while KIR, CD57, and NKG2A expression on NK cells remains stable across menstruation cycles [44, 56], the stability of the FcRγ^{neg} NK cell subset in this setting is less clear. Therefore, increased prevalence of CMV-associated NK cells or dynamic variation in the frequencies of the cells may reflect hormonal changes or environmental influences that are unique to or more common in adolescent females.

Besides these differences in age and gender of our study population, the participants in the CMV vaccine trial also exhibited a greater degree of racial diversity than was represented in previous cross-sectional studies [42]. Specifically, 35% of our vaccine trial participants were Black (i.e. African American). Although race assuredly impacts the NK-cell repertoire in the context of highly polymorphic receptors, including killer-cell immunoglobulin-like receptors (KIR), the effects of race on CMV-reactive NK cells and receptors associated with these subsets are less well defined. Intriguingly, 100% (15 of 15) of Black study participants demonstrated detectable FcRγ^{neg} NK cells at one or more time points of study, whereas only 58% (14 of 24) of Caucasian study participants exhibited FcRγ^{neg} NK cells in their repertoire. Thus, gender, race, genetics, and local environmental factors may all contribute to the distinct observations of adaptive NK cell frequencies in our study.

A key unanswered question concerns the nature of the stimuli provoking longitudinal changes in frequency of NK cell subsets. A recent study of barcoded hematopoietic cells in rhesus macaques noted significant fluctuations in the clonal composition of NK cells over time [57]. Our study stringently controlled for CMV exposure via urine and blood analyses [2]. Moreover, the results do not support a relationship between CMV gB vaccination or gB-specific antibody titers and altered frequencies of NK cell subsets. Nonetheless, other subclinical acute infections, vaccinations (e.g. seasonal influenza vaccine), inflammatory events, environmental exposures (i.e. allergens), or shifts in microbiota composition could alter the composition of the NK-cell repertoire. We speculate that these environmental stimuli or associated immune responses (i.e. antibody elaboration) provoke the expansion, differentiation, or release of FcRγ^{neg} NK cells into the circulation. The elevated frequency of these NK cell subsets in CMV-positive individuals may reflect an altered tempo or magnitude of these natural oscillations, or a greater regularity of the instigating stimulus. Alternatively, as the frequencies of these subsets appear to be more stable in CMV-seropositive individuals [38], aspects of the inflammatory environment during chronic CMV infection may more efficiently maintain these populations. Given that the MF59 adjuvant used in this CMV vaccine is designed for optimal stimulation of T and B-cell responses, future studies aimed at ascertaining the nature of inflammatory cues promoting adaptive NK cells will yield key insights into the types of adjuvants that may be applied to intentional promote sustained expansion of these NK cell subsets in next generation vaccines.

Our results, to our knowledge, represent the first longitudinal study of CMV-associated NK-cell subsets in healthy CMV seronegative individuals. Here, we had the unique ability to gain insight into the intra-individual variation in the frequency of NK-cell subsets following gB/MF59 vaccination. We show that the lack of change in NKG2C expression was consistent with absence of CMV infection, confirming the stringent association of this virus with NKG2C^{high} NK cells. However, we also present evidence suggesting that presence of FcRγ^{neg}, EAT-2^{neg}, and SYK^{neg} NK cells in the repertoire may be more temporally dynamic and CMV-independent than previously thought. Future work examining age and gender related

differences as well as longitudinal analyses of post-transplant patients may give further insight into the variegated expression of CMV-associated NK-cell subsets.

Materials and Methods

CMV vaccine trial: This study was approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board and conducted by the Cincinnati Vaccine and Treatment Evaluation Unit (VTEU) as part of CMV vaccine trial NCT00133497. Study participants were 12- to 17-year-old healthy adolescent females confirmed CMV seronegative at the start of the study. Only samples from the Cincinnati site of this clinical trial were available for the purposes of the present study. Furthermore, only those subjects with available samples spanning trial duration were used for experimental analyses. As a result, a total of 40 participants were randomized into two groups (n=20/group) receiving either 3 doses of CMV gB subunit vaccine in MF59 adjuvant (20 µg gB and 10.75 mg MF59, Sanofi Pasteur) or sterile saline (Sodium chloride 0.9%) placebo by intramuscular injection in the deltoid on days 0, 30, and 180 of protocol [2]. Urine, saliva and blood were collected throughout time course to assess CMV infection by PCR and seroconversion to non-vaccine CMV antigens, respectively. The 40 subjects evaluated longitudinally in the present study remained CMV negative throughout sampling period. Three additional vaccine trial participants who were part of the placebo group and became positive for CMV infection during longitudinal sampling period were used to examine NK-cell subset frequencies at time points subsequent to natural acquisition of CMV infection. Peripheral blood mononuclear cells (PBMC) were collected and cryopreserved at screening and various times (days 0, 1, 30, 60, 180 and 210) of trial [2].

NK-cell analyses: PBMC were concomitantly stained and assessed by flow cytometry during a single experimental run (or block). A volunteer blood donor with a high percentage of NKG2C^{high} NK cells extraneous to vaccine trial was selected as a positive control for NKG2C staining and included in each block of vaccine trial participant samples to benchmark stain validity and reproducibility. Expression of FcRγ, SYK, and EAT-2 are benchmarked against CD4 T cells in the same sample, where the latter cells do not express these proteins [42]. Phenotypic analyses of PBMCs were performed using fluorochrome-conjugated antibodies. Cells were stained for surface markers using CD3 (OKT3, Biolegend), CD19 (HIB19, BD Biosciences), CD4 (RPA-T4, BD Biosciences), CD14 (M5E2, BD Biosciences), CD56 (N901, Beckman Coulter), NKG2C (REA205, Miltenyi Biotech), NKG2A (Z199, Beckman Coulter), CD57 (HCD57, Biolegend) and a fixable live-dead stain (Pacific Green, Invitrogen) in FACS buffer (HBSS supplemented with 5% fetal bovine serum and 2 µm EDTA). Following surface staining, cells were fixed in 2% paraformaldehyde (Fisher Scientific) and permeabilized with 0.04% Triton X-100 (Sigma Aldrich). Intracellular staining in FACS buffer with 2% bovine serum albumin was then performed to identify FcRγ (polyclonal rabbit, Millipore), EAT-2 (polyclonal rabbit, ProteinTech Group), SYK (4D10.1, eBioscience) markers. Intracellular EAT-2 staining was followed by secondary staining with polyclonal anti-rabbit IgG (Invitrogen). Flow cytometric data were obtained using an LSR Fortessa instrument (BD Biosciences) and analyzed via FlowJo_v10 software (Treestar).

T-distributed stochastic neighbor embedding (t-SNE) analyses: The tSNE algorithm of FlowJo_v10 was used to visualize dimensionality of NK cell subsets over time. For each donor, the data at individual time point was down sampled (gated on CD56^{dim} NK cells) and then concatenated to create three dimensionally reduced t-SNE plots. Populations expressing or lacking various proteins were overlaid on t-SNE plots to identify subset clusters.

Statistical analyses: Differences between placebo and vaccine recipients were compared using mixed effects two way ANOVA with restricted maximum likelihood. Changes over time (0, 6, 7, 10, and 13 months) and treatment group (placebo and vaccine) in the proportion of CD56^{bright} and CD56^{dim} cells were evaluated using generalized linear mixed models with a Poisson distribution, log link function, and an offset of the logarithm of the total NK cell count specified. A random intercept and a random slope and an interaction term between time and treatment group was included in the model. Graphs were generated using GraphPad Prism and statistical tests were performed in Prism and SAS 9.4 (SAS Institute Inc., Cary NC).

Author contribution

IG: conception and design of study, execution of experiments, acquisition of data, analysis and interpretation of data, drafting of the manuscript. HS: analysis and interpretation of data, critical revision of the manuscript. HJS and LA: statistical analyses and critical revision of the manuscript. HW: execution of experiments. YB: analysis and interpretation of results, critical revision of the manuscript. DB: conception and design of study, sample collection and regulatory approvals, critical revision of manuscript. SW: conception and design of study, analysis and interpretation of data, drafting and critical revision of the manuscript, obtained funding, study supervision.

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