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Antigen-specific NK cell memory in rhesus macaques

R Keith Reeves[1,2], Haiying Li[1,2], Stephanie Jost[3], Eryn Blass[1], Hualin Li[1], Jamie L Schafer[1], Valerie Varner[1], Cordelia Manickam[1], Leila Eslamizar[1], Marcus Altfeld[3,4], Ulrich H von Andrian[3,5] & Dan H Barouch[1,3]

Natural killer (NK) cells have traditionally been considered nonspecific components of innate immunity, but recent studies have shown features of antigen-specific memory in mouse NK cells. However, it has remained unclear whether this phenomenon also exists in primates. We found that splenic and hepatic NK cells from $SHIV_{SF162P3}$-infected and SIV_{mac251}-infected macaques specifically lysed Gag- and Env-pulsed dendritic cells in an NKG2-dependent fashion, in contrast to NK cells from uninfected macaques. Moreover, splenic and hepatic NK cells from Ad26-vaccinated macaques efficiently lysed antigen-matched but not antigen-mismatched targets 5 years after vaccination. These data demonstrate that robust, durable, antigen-specific NK cell memory can be induced in primates after both infection and vaccination, and this finding could be important for the development of vaccines against HIV-1 and other pathogens.

NK cells have traditionally been associated with nonspecific innate killing of virus-infected and neoplastic cells. However, increasing evidence suggests that NK cells also cooperate with adaptive humoral immune responses to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and modulate the responses of $CD4^+$ and $CD8^+$ T cells[1–6]. In the specific context of HIV-1 infection, NK cells have been reported to proliferate during primary infection[7] before the development of $CD8^+$ T cell responses. In addition, NK cells lyse HIV-1–infected cells through a variety of mechanisms such as ADCC[8], downmodulation of major histocompatibility complex (MHC) class I molecules[9] and upregulation of ligands of NKG2D, a stimulatory NK cell receptor[10]. NK cells can also inhibit CCR5-dependent entry of HIV-1 by secreting the β-chemokines CCL3, CCL4 and CCL5 (ref. 11). In rhesus macaques, NK cells have been shown to lyse simian immunodeficiency virus (SIV)-infected cells[12] and SIV-pulsed cells[13]. Other studies have shown that acute infection of rhesus macaques with SIV_{mac251} induces rapid NK cell activation and increased cytotoxicity[14], and longitudinal studies suggest that NK cells may be associated with the prevention of disease progression in SIV-infected macaques[15,16].

So far, antigen-specific NK cell memory has been described only in mice[17–23]. Mice lacking T and B cells develop immunologic memory for haptens and viral antigens that is mediated by a transferrable subset of liver-restricted NK cells[18,19,21,23]. Certain activating receptors on human and murine NK cells have also been shown to recognize proteins from several viruses and to modulate disease[24–27]. However, expression of those surface molecules on NK cells has not been associated with the acquisition of antigen-specific NK cell memory responses. Long-lived and transferrable memory responses against murine cytomegalovirus (MCMV) were shown to induce binding of the virus-encoded protein m157 to the activating receptor Ly49H on murine NK cells[28], although antigen specificity was not formally tested in that study. Antigen-specific NK cell memory has not been demonstrated previously in any primate species, but a large body of work has long suggested that the NK cell response might not be entirely nonspecific. Increased NK cell antiviral functions in HIV-1–exposed seronegative individuals have been associated with protection[29,30], and uninfected infants of HIV-1–positive mothers can mount potent NK cell responses that are associated with blocking transmission *in utero*[31], both of which indicate potential pre-sensitization to the virus. Similarly, killer immunoglobulin-like receptor (KIR)-MHC interactions have been shown to exert significant immunological pressure on HIV-1 replication, thereby driving virus evolution and escape[32]. During infection with human cytomegalovirus, $NKG2C^+CD57^+$ NK cells expand in a virus-specific manner[33], and, similarly, $CD27^+$ NK cells are associated with spontaneous control of the virus[34], but no previous study has definitively shown acquired antigen specificity of NK cells in humans or other primate species. To address this deficit, in this study we assessed potential NK cell memory in both SIV-infected and long-term vaccinated rhesus macaques.

RESULTS

Identification and purification of rhesus macaque NK cells

We identified bulk NK cells as $CD3^-CD14^-CD20^-HLA\text{-}DR^{lo}NKG2A^+$ mononuclear cells as described[15,35,36] (**Fig. 1a**) and confirmed their identity by demonstrating coexpression of the NK cell–related markers CD8α, NKp30 and NKp46 (**Fig. 1b**). Macaque NK cells also had the expected distribution of $CD56^+$ and $CD16^+$ subpopulations[15,35,36] (**Fig. 1c**). To evaluate the antigen specificity of NK cells, we developed a flow cytometry assay to measure the ability of purified NK cells to lyse autologous dendritic cells (DCs). We purified NK cells from bulk mononuclear cells using magnetic beads and then sorted either

[1]Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA. [2]Division of Immunology, New England Primate Research Center, Harvard Medical School, Southborough, Massachusetts, USA. [3]Ragon Institute of Massachusetts General Hospital, MIT, and Harvard, Cambridge, Massachusetts, USA. [4]Heinrich-Pette-Institut, Leibniz Institute for Experimental Virology, Hamburg, Germany. [5]Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA. Correspondence should be addressed to R.K.R. (rreeves@bidmc.harvard.edu) or D.H.B. (dbarouch@bidmc.harvard.edu).

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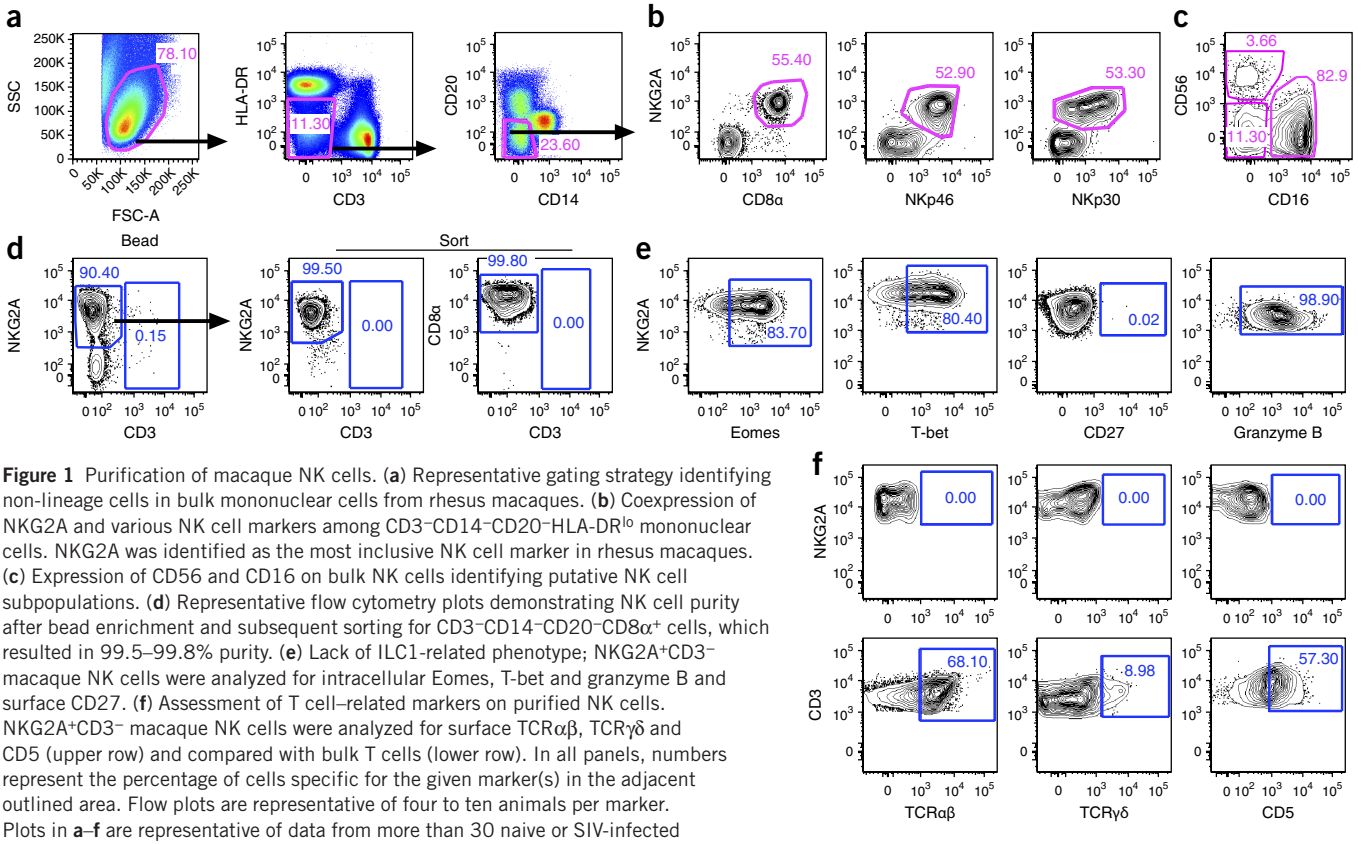

Figure 1 Purification of macaque NK cells. (**a**) Representative gating strategy identifying non-lineage cells in bulk mononuclear cells from rhesus macaques. (**b**) Coexpression of NKG2A and various NK cell markers among CD3$^-$CD14$^-$CD20$^-$HLA-DR^{lo} mononuclear cells. NKG2A was identified as the most inclusive NK cell marker in rhesus macaques. (**c**) Expression of CD56 and CD16 on bulk NK cells identifying putative NK cell subpopulations. (**d**) Representative flow cytometry plots demonstrating NK cell purity after bead enrichment and subsequent sorting for CD3$^-$CD14$^-$CD20$^-$CD8α$^+$ cells, which resulted in 99.5–99.8% purity. (**e**) Lack of ILC1-related phenotype; NKG2A$^+$CD3$^-$ macaque NK cells were analyzed for intracellular Eomes, T-bet and granzyme B and surface CD27. (**f**) Assessment of T cell–related markers on purified NK cells. NKG2A$^+$CD3$^-$ macaque NK cells were analyzed for surface TCRαβ, TCRγδ and CD5 (upper row) and compared with bulk T cells (lower row). In all panels, numbers represent the percentage of cells specific for the given marker(s) in the adjacent outlined area. Flow plots are representative of four to ten animals per marker. Plots in **a**–**f** are representative of data from more than 30 naive or SIV-infected rhesus macaques and were obtained in six independent experiments.

CD3$^-$CD14$^-$CD20$^-$NKG2A$^+$ or CD3$^-$CD14$^-$CD20$^-$CD8α$^+$ NK cells by flow cytometry. This resulted in 99.5–99.8% pure NK cells with no detectable (0.00%) contaminating T cells (**Fig. 1d**). NK cells showed high expression of intracellular transcription factors Eomes and T-bet and the serine protease granzyme B, but they did not express CD27 (**Fig. 1e**). Given the highly overlapping phenotypes, it is possible that purified NK cells could contain minor frequencies of phylogenetically related innate lymphoid cell group 1 (ILC1)[37], but as primate ILC1 cells generally do not express granzyme B, are CD27$^+$ and have low expression of Eomes[38], contamination is likely to be minimal. To confirm a lack of T cell contamination, we further analyzed purified NK cells by flow cytometry and found no detectable expression of the T cell markers CD5, TCRαβ and TCRγδ (**Fig. 1f**). Collectively, these data present a comprehensive definition of NK cells in rhesus macaques and validate our methodologies for highly efficient purification.

Antigen-specific NK cell responses in SHIV$_{SF162P3}$ infection

To evaluate the ability of purified NK cells to lyse cells in an antigen-specific manner, we cultured NK cells with autologous DCs pulsed with antigens and labeled with the red dye PKH26. Cultures also contained unpulsed autologous DCs labeled with the green dye CFSE as internal controls (**Fig. 2a**). Although many NK cell–killing assays call for cultures of only 6 h, time-course experiments showed that maximum killing was achieved with an 18-h incubation in our study (**Fig. 2b**); this might have been due in part to our use of frozen cells, differences among species or differences in the mechanism of 'innate' versus 'antigen-specific' killing by NK cells. Using this assay, we first tested the ability of purified splenic and hepatic NK cells from a cohort of eight macaques infected with simian-human immunodeficiency virus (SHIV) strain SF162P3 (SHIV$_{SF162P3}$) to lyse DCs pulsed with SIV$_{mac239}$ Gag or HIV-1 Env. Splenic NK cells killed DCs pulsed with Gag or Env (median specific lysis of 13% and 10%, respectively, at a 10:1 effector:target (E:T) ratio), but not cells pulsed with the irrelevant protein ovalbumin (Ova) (median specific lysis of 0.3% at a 10:1 E:T ratio) (**Fig. 2c**). Responses to the viral proteins were significantly different from those to Ova at both 10:1 (Gag, $P = 0.015$; Env, $P = 0.001$) and 5:1 (Gag, $P = 0.017$; Env, $P = 0.023$) E:T ratios. Hepatic NK cells showed a median specific lysis of 16–18% of Gag-pulsed DCs (**Fig. 2d**). As an additional positive control, we also demonstrated that bulk NK cells, regardless of their antigen experience, were functionally capable of nonspecific lysis of MHC-devoid K562 cells, standard NK cell targets (**Fig. 2e**). These data confirmed that highly purified tissue NK cells from SHIV-infected macaques could recognize and lyse autologous DCs in an antigen-specific manner.

Antigen-specific NK cell responses in SIV$_{mac251}$ infection

We next evaluated NK cells from a cohort of eight rhesus macaques chronically infected with SIV$_{mac251}$ and six naive uninfected macaques. Splenic NK cells from infected animals were highly reactive to Gag-pulsed DCs at a 10:1 ratio with a median specific lysis of 40%, compared with 0.1% in uninfected age-matched controls (**Fig. 3a**) ($P = 0.018$). In contrast, NK cells from SIV-infected animals were not reactive to unpulsed DCs (**Fig. 3b**). These data demonstrate robust anti-Gag NK cell responses in the spleen of SIV-infected animals but not in uninfected animals, confirming true antigen specificity. Only marginal Gag-specific NK cell responses were observed in peripheral blood, although these responses were still slightly stronger than those observed in uninfected animals ($P = 0.05$) (**Fig. 3c**). These findings suggest that antigen-specific NK cells either are located primarily

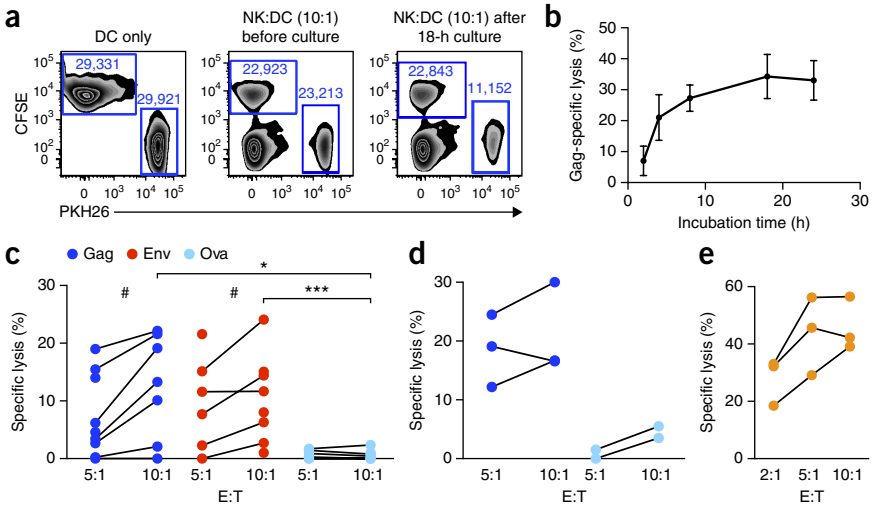

Figure 2 Antigen-specific lysis of autologous DCs by NK cells in chronically $SHIV_{SF162P3}$-infected macaques. (**a**) Flow cytometric visualization of NK cell–DC coculture. Plots are representative of more than 50 NK cell–DC coculture assays visualizing DCs only, cultures immediately after the addition of NK cells, and lysis after coculture. Collected numbers of events are indicated and were used to calculate lysis. (**b**) Results of a time-course experiment demonstrating maximum killing at 18 h of coincubation. Data shown are the mean ± s.e.m. of four independent experiments. (**c**) Specific lysis of Gag- and Env-pulsed DCs from $SHIV_{SF162P3}$-infected macaques by splenic NK cells at 5:1 and 10:1 E:T ratios. Statistical comparisons between parallel E:T ratios comparing antigen response (Gag or Env) with response to Ova: $^{*}P < 0.05$, $^{***}P < 0.001$, Mann-Whitney *U*-test. Statistical comparisons between 10:1 and 5:1 E:T ratios of the same animals: $^{\#}P < 0.05$, Wilcoxon matched-pairs test. (**d**) Specific lysis of Gag- and Env-pulsed DCs from $SHIV_{SF162P3}$-infected macaques by hepatic NK cells at 10:1 and 5:1 E:T ratios. (**e**) Specific lysis of PKH26-labeled K562 cells by splenic NK cells from $SHIV_{SF162P3}$-infected macaques. NK cell–resistant RAJI cells labeled with CFSE were included in all wells as internal controls.

in tissues or possibly reflect sequestration in sites of higher antigen concentration. In a confirmatory assay, we found that CD107a was also specifically upregulated on splenic NK cells cultured with Gag-pulsed DCs (**Fig. 3d**), and that CD107a expression correlated with NK cell lysis ($P = 0.02$; **Fig. 3e**). These data corroborate the antigen-specific NK cell killing assays and also suggest that CD107a might be a relevant surrogate marker for antigen-specific NK cell cytotoxicity. Taken together, these results demonstrate antigen-specific NK cell activity in both SHIV- and SIV-infected rhesus macaques. The greater NK cell killing in animals infected with SIV_{mac251} compared with that in $SHIV_{SF162P3}$-infected animals may reflect higher viral loads in those animals or virus-specific differences. Indeed, viral loads modestly correlated with both percentage specific lysis and CD107a expression (**Fig. 3f**), suggesting an antigen-driven expansion of SIV Gag-specific NK cells.

Ad26 vectors induce potent memory NK cell responses

We then evaluated whether NK cells are capable of long-term antigen-specific memory after vaccination in the absence of ongoing antigen stimulation. We administered two vaccinations to nine rhesus macaques. The vaccinations were 6 months apart, and each contained 10^9 viral particles of either replication-incompetent Ad26 vectors[39] expressing HIV-1 Env ($n = 4$) or a DNA-Ad26 prime-boost regimen expressing SIV_{mac239} Gag ($n = 5$). Vaccinated animals developed durable Env- and Gag-specific T cell responses to their respective vaccine antigens as expected (**Fig. 4a**,**b**) and were not boosted or challenged further. Five years after the final vaccination, we necropsied both

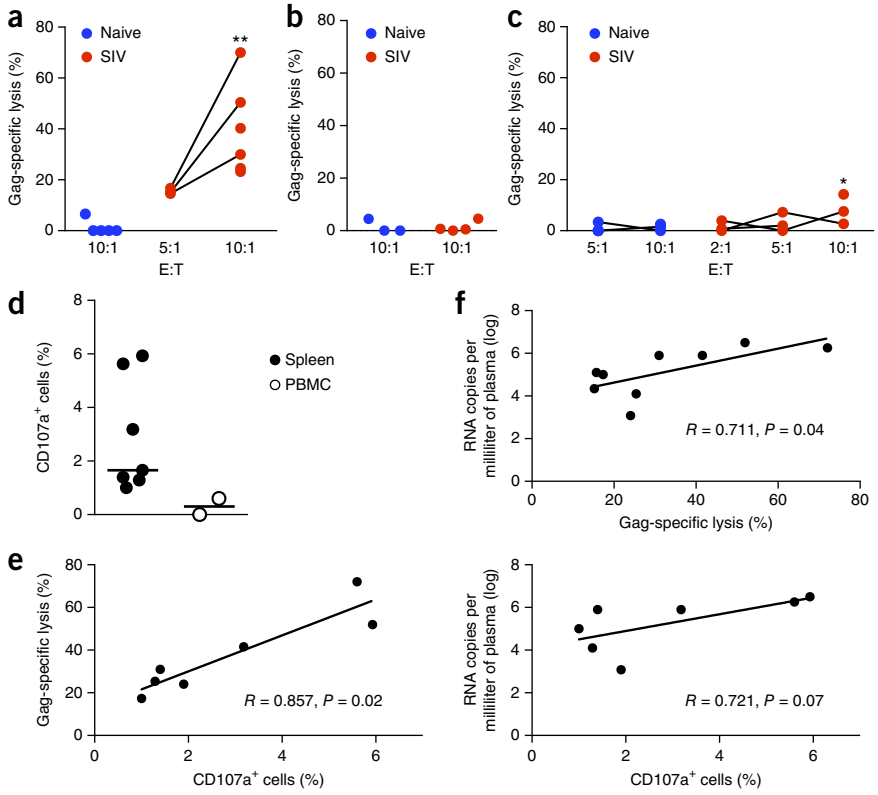

Figure 3 Antigen-specific NK cell responses in SIV_{mac251}-infected macaques. (**a**) Specific lysis of Gag-pulsed DCs from SIV-infected and SIV-uninfected rhesus macaques in NK cell–DC cocultures from spleen at 10:1 and 5:1 E:T ratios. (**b**) Specific lysis of mock-pulsed DCs in splenic NK cell–DC cocultures at 10:1 E:T ratios. (**c**) Specific lysis of Gag-pulsed DCs in NK cell–DC cocultures from peripheral blood at various E:T ratios. (**d**) CD107a expression in splenic and peripheral NK cells in response to Gag-pulsed DCs from SIV-infected rhesus macaques in NK cell–DC (10:1) cocultures. Horizontal bars indicate medians. (**e**) Relationship between data from **b** and **d** ($n = 7$). (**f**) Correlation of SIV-specific NK cell responses with plasma viral load (top, $n = 9$; bottom, $n = 7$). Cross-sectional statistical comparisons are between SIV-infected and SIV-uninfected samples at 10:1 E:T. $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U*-test. Spearman's test was used for all correlations; $P < 0.05$.

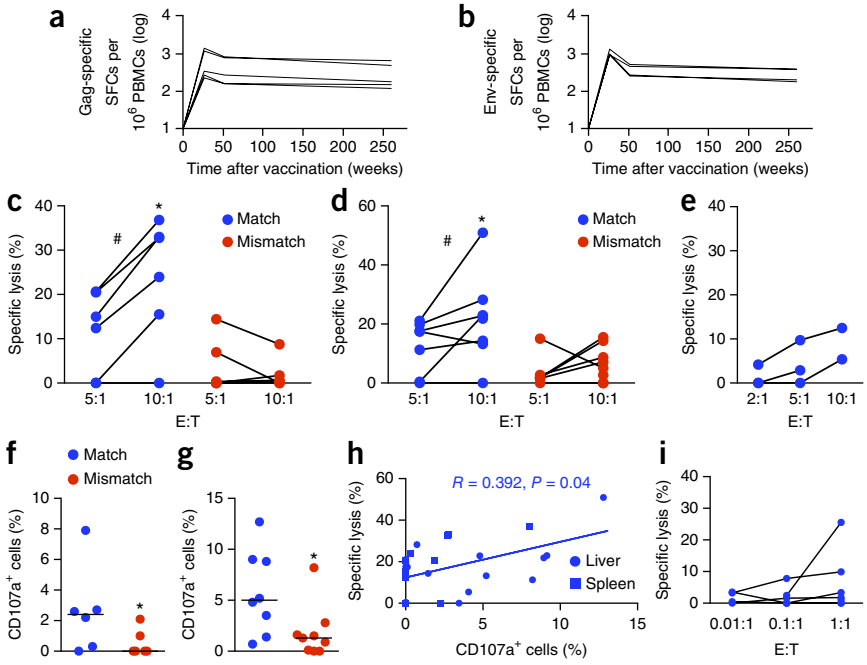

Figure 4 Antigen-specific NK cell memory in vaccinated macaques. (**a**,**b**) Gag-specific (**a**) and Env-specific (**b**) T cell responses in Ad26-vaccinated macaques measured longitudinally by interferon-γ ELISPOT as described[39]. SFC, spot-forming cell. (**c**,**d**) Specific lysis of antigen-pulsed DCs with 'matched' antigens (i.e., Gag-pulsed DCs in Gag-vaccinated animals and Env-pulsed DCs in Env-vaccinated animals) and 'mismatched' antigens (i.e., Gag-pulsed DCs in Env-vaccinated animals and Env-pulsed DCs in Gag-vaccinated animals) by purified splenic (**c**) and hepatic (**d**) NK cells at 10:1 and 5:1 E:T ratios. (**e**) Specific lysis of Gag-pulsed DCs by purified NK cells from PBMCs of Gag-vaccinated rhesus macaques. (**f**,**g**) CD107a expression in (**f**) splenic and (**g**) hepatic NK cells in response to antigen-pulsed DCs with matched antigens and mismatched antigens. Assays were performed at 10:1 NK cell:DC ratios and were background subtracted using unpulsed DC coculture controls. Horizontal bars indicate medians. (**h**) Correlation of Gag-specific lysis and CD107a upregulation in matched and mismatched animals ($n = 28$). (**i**) Lack of $CD8^+$ T cell response at low E:T ratios to exclude the possibility of contaminating $CD8^+$ T cell–mediated killing. $CD8^+$ T cells were bead purified from Ad26-Gag–vaccinated macaques as described in the Online Methods and cultured with Gag-pulsed DCs. Statistical comparisons between parallel E:T ratios comparing matched antigen with mismatched antigen, $^*P < 0.05$, Mann-Whitney *U*-test. Statistical comparisons between 10:1 and 5:1 E:T ratios of the same animals, $^{\#}P < 0.05$, Wilcoxon matched-pairs test. Spearman's test was used for all correlations; $P < 0.05$.

groups of animals and assessed potential antigen-specific memory NK cell responses in peripheral blood, liver and spleen. As an added control, we evaluated antigen-specific responses to both the vaccine antigen (matched) and the non-vaccine antigen (mismatched—i.e., evaluation of anti-Env responses in Gag-vaccinated animals). At both 10:1 and 5:1 E:T ratios, splenic and hepatic NK cells lysed antigen-matched targets efficiently but exhibited minimal to no reactivity against antigen-mismatched targets (**Fig. 4c**,**d**) ($P = 0.02$ for hepatic NK cells; $P = 0.04$ for splenic NK cells). In particular, at the 10:1 E:T ratio, splenic NK cells from vaccinated animals showed a median of 27% specific lysis of antigen-matched targets but only 2% specific lysis of antigen-mismatched targets. Consistent with the previous experiment (**Fig. 3**), responses in peripheral blood were marginal (**Fig. 4e**). We also observed CD107a responses to the matched but not the mismatched antigen (**Fig. 4f**,**g**), and these data correlated with the killing assays ($P = 0.04$; **Fig. 4h**). The observation of antigen-specific NK cell responses 5 years after vaccination demonstrated the durability of these memory NK cell responses. Memory NK cell responses were comparable in liver and spleen, which contrasts with some evaluations of murine memory NK cell responses showing that memory NK cell activity is restricted to the liver[19,21,23].

Sorting of NK cells eliminated all contaminating $CD8^+$ T cells (**Fig. 1d**). To further exclude the remote possibility that contaminating T cells might still have contributed to the observed specific lysis, we carried out parallel cell-killing experiments with $CD8^+$ T cells from the vaccinated animals at 1:1, 0.1:1 and 0.01:1 E:T ratios. These low ratios were chosen to enable simulation of T cell contamination, rather than evaluation of $CD8^+$ T cell–mediated lysis, and little to no killing was observed in these experiments, even at the 1:1 ratio (**Fig. 4i**). In re-sort analyses, we observed 0.00% T cell contamination (**Fig. 1d**), and even if we consider the flow cytometer's potential theoretical error rate of 1 in 10,000 cells, the 1:1 E:T ratio represents a value 5 logs greater than the maximal possible levels of T cell contamination in any of the NK cell killing assays. These considerations collectively eliminate the possibility that the observed antigen-specific NK cell killing could have been due to contaminating $CD8^+$ T cells.

NK cell memory responses are NKG2 dependent

To determine the relative contributions of various NK cell subsets to the observed memory NK cell responses, we repeated the experiments described in **Figure 2** in the presence and in the absence of blocking antibodies to various NK cell receptors. Blocking NKp46 had no observable effect on NK cell–mediated killing, but blocking NKG2A

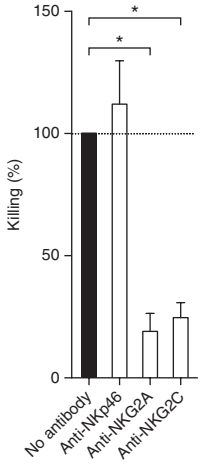

Figure 5 Antigen-specific NK cell killing is dependent on NKG2A and NKG2C. Specific lysis of antigen-pulsed DCs from animals referenced in **Figure 2**. Antibodies were added before coculture, and cell killing is shown as a fraction of that in control wells with no blocking (no antibody; normalized to 100%). The figure summarizes data from five independent experiments (mean and s.e.m.). $^*P < 0.05$, Wilcoxon matched-pairs test.

and NKG2C markedly reduced NK cell killing of Gag-pulsed DCs by 80% and 75%, respectively ($P < 0.05$; **Fig. 5**). These data suggest that molecules of the NKG2 family may have a role in target-cell recognition or as coreceptors, analogous to what has been shown for NKG2D in murine memory NK cell responses to haptens[21].

DISCUSSION

In these studies we observed clear evidence of antigen-specific memory NK cell responses in a primate species. Specifically, we observed anti-Gag and anti-Env NK cell responses in both SIV_{mac251}-infected and $SHIV_{SF162P3}$-infected rhesus macaques that demonstrated NK cell memory generated against active replication of lentiviruses. Furthermore, we found that antigen-specific NK cells were inducible after Ad26 vaccination and that the responses were intact 5 years after vaccination without challenge.

Prior to these studies, antigen-specific NK cell memory had been described only in mice, first in studies showing that transferrable hapten-specific contact hypersensitivity (CHS) in mice lacking T and B cells was dependent on a subpopulation of liver NK cells[21]. This NK cell–mediated CHS was subsequently confirmed in other studies[17–19,22], and NK cell memory has now been demonstrated against vesicular stomatitis virus, HIV-1 and influenza vaccine antigens in mice and has been found to contribute to efficient protection against vaccinia and influenza challenges[19,23]. Pre-sensitized memory-like NK cells have also been described for MCMV and can expand upon virus exposure and to protect upon adoptive transfer[28].

Our data expand on these prior studies by showing antigen-specific NK cell memory in primates after both infection and vaccination. As SIV_{mac251} and $SHIV_{SF162P3}$ are not natural pathogens in macaques, it seems unlikely that memory responses to these viruses occur through typical NK cell–receptor interactions, such as those mediated by Ly49H-m157 in mice infected with MCMV[28]. The most specific associations with the control of HIV-1 by NK cells have been mediated by KIR; for example, it has been epidemiologically demonstrated that expression of KIR3DS1 on NK cells, in combination with its ligand HLA-Bw4-80I, is associated with slower disease progression[40], and there is *in vitro* evidence that $KIR3DS1^+$ NK cells can inhibit HIV-1 replication *in vitro*[41]. However, KIR-mediated effects require interaction with cognate ligands that are often disparately expressed in individuals. Perhaps a more likely pathway is antigen-specific imprinting and recall by a subset of NK memory cells that remain incompletely defined in primates, similar to observations in mice[21]. Our data also suggest that this process may be dependent on NKG2 molecules. Although the full mechanism is unclear, our data indicate that both inhibitory NKG2A and activating NKG2C are required, but the unrelated NKp46 is not. NKG2A is known to bind MHC-I, and absence of this inhibitory interaction results in cell killing, as demonstrated by lysis of MHC-devoid K562 cells. Our data show that NKG2 molecules may also be necessary for antigen-specific killing, which perhaps suggests a distinction specific to memory NK cells. NKG2C expression on human NK cells is associated with subset expansion in response to HCMV but not to other herpesviruses[33]. NKG2C also delineates a subpopulation of 'memory-like' NK cells that use antibody binding for specificity and display enhanced ADCC but become hyporesponsive to cytokine stimulation[42–44]. These alterations in NK cell function reflect widespread epigenetic modifications with changes in DNA methylation patterns resembling those of cytotoxic T cells[43,44]. NK cells and NKG2 expression have also been linked to elite control of viremia and reduced acquisition of HIV-1 (refs. 32,45,46).

The full role of NK cells in the modulation of HIV or SIV disease and in prevention of disease acquisition also remains uncertain. NK cells are known to lyse both HIV-infected and SIV-infected cells, but it has been difficult to determine the extent to which this occurs *in vivo*[7,11,14,41]. Increased NK cell function and sensitization in HIV-1–exposed seronegative individuals suggests that these cells could contribute, at least in part, to transmission blocking[29,30], although whether antigen specificity is generated in this context is unknown. Contradictory evidence suggests that NK cells may have little to no role in limiting lentivirus transmission or eliminating infected cells[36,47], but those studies did not address antigen specificity. Although significant empirical evidence exists, it has been difficult to define a specific role for NK cells in HIV or SIV disease, partly due to the fact that strategies for the full and accurate depletion of NK cells in SIV-infected macaque models are currently lacking[15,48]. SIV-specific NK cell activity positively correlated with plasma viral load, which indicates that the presence of antigen can probably influence at least the magnitude of antigen-specific NK cell responses. The longevity of memory NK cell responses after Ad26 vaccination in the absence of virus replication also suggests that ongoing antigenic stimulation is not necessarily required to maintain long-term responses. Future studies will be necessary to determine the kinetics and full anatomic distribution of antigen-specific NK cells after lentivirus infection and vaccination, as well as the relative contributions to disease modulation and the prevention of virus acquisition.

Overall, our findings show antigen-specific NK cell responses in primates after both infection and vaccination. Although antigen-specific NK cells are likely modulated by ongoing virus replication in infected animals, the finding that Ad26 vaccination induced true memory NK cells that were maintained for 5 years after vaccination is particularly notable. The longevity, functionality and specificity of these responses suggest their potential utility in the development of vaccines against both HIV-1 and other pathogens. Further research to evaluate the protective efficacy of these responses in primates and to define the detailed molecular mechanisms of target-cell engagement is warranted. Regardless, our new data and the extensive work previously done in mice suggest that NK cell memory could be conserved among mammalian species and underscore the incomplete understanding and heterogeneity of innate versus adaptive immune responses.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.K.R., M.A., U.H.v.A. and D.H.B. designed the studies. Haiying Li, S.J., E.B., Hualin Li, J.L.S., V.V., C.M. and L.E. conducted the assays. R.K.R. and D.H.B. wrote the paper with the assistance of all other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals and SIV infections. Thirty-one adult Indian rhesus macaques were analyzed in this study: six naive, eight chronically infected with $SHIV_{SF162P3}$ (median duration of infection, 802 d), eight chronically infected with SIV_{mac251} (median duration of infection, 371 d), and nine vaccinated i.m. twice with 10^9 Ad26 vectors expressing HIV-1 Env inserts[39] or DNA prime-boost vaccines with Ad26 vectors expressing SIV_{mac239} Gag. Both male and female macaques were used. All animals were free of simian retrovirus type D and simian T-lymphotrophic virus type 1 and were housed at the New England Primate Research Center (Southborough, MA). All animal studies were performed in accordance with the American Association for Accreditation of Laboratory Animal Care standards and in compliance with protocols approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Tissue collection and processing. Macaques were euthanized and spleen and liver were collected and mechanically disrupted. EDTA-treated venous blood was also collected at various time points. Mononuclear cells were isolated from blood and single-cell spleen suspensions by density-gradient centrifugation over lymphocyte separation media (MP Biomedicals, Solon, OH). Hepatic mononuclear cells were isolated from the interface of a 30–60% discontinuous Percoll gradient. A hypotonic ammonium chloride solution was used to lyse contaminating red blood cells. Cells were either immediately cultured or cryopreserved in 90% FBS, 10% DMSO and stored in liquid nitrogen vapor.

Antigen-specific NK cell killing assay. Hepatic and splenic NK cells were isolated from bulk mononuclear cells with custom negative magnetic bead selection protocols that routinely yielded >90% $NKG2A^+CD3^-$ NK cell purity with <1% T cell contamination as described[49]. For some experiments, NK cells were further purified by positive sorting for $CD3^-CD14^-CD20^-CD8\alpha^+$ cells on a FACS Aria (Purity mode; 2,500 events per second), which resulted in 99.5–99.8% NK cell purity. Input numbers of between 2.0×10^7 and 1.0×10^8 mononuclear cells, depending on the experiment, yielded between 5.0×10^5 and 5.0×10^6 sort-purified NK cells. Dead cells were excluded by Aqua dye, and only live cells were sorted with >95% viability. Autologous DCs were similarly purified by bead-based separation with antibodies to macaque CD3 (clone SP34-2, BD Biosciences), CD20 (clone L27, BD Biosciences), CD8α (clone SK1, BD Biosciences), and NKG2A (clone Z199, Beckman-Coulter). DC purification resulted in >90% $HLA\text{-}DR^+NKG2A^-CD3^-$ cells, with 0% contamination by $CD3^+$ or $NKG2A^+$ cells. DCs were then pulsed with intact Gag or Env antigens (2 μg/ml) and labeled with the red dye PKH26 (Sigma-Aldrich) for identification in our flow cytometric assay. Non-pulsed DCs serving as intrawell controls were labeled with the green dye CFSE (Sigma-Aldrich). Purified NK cells were cultured with DCs at multiple effector-to-target (E:T) ratios (equal mixture of pulsed target DCs and non-pulsed control DCs) for 18 h, and the specific lysis of DCs was calculated as follows: (% sample lysis with NK effectors – % basal lysis without NK effectors)/(100 – % basal lysis without NK effectors) (**Fig. 2a**). In alternative experiments, anti-CD107a was added for the duration of culture, and CD107a expression was measured after 18 h as described previously[35]. In blocking experiments, 2 μg/ml of anti-NKG2A (clone Z199, Beckman-Coulter), anti-NKG2C (clone REA110, Bioss), or anti-NKp46 (clone BAB281, Beckman-Coulter) was added 1 h before coculture.

$CD8^+$ T cell isolation. Macaque $CD8^+$ T cells were isolated from blood with Miltenyi nonhuman primate $CD8^+$ T cell isolation kits according to the manufacturer's suggested protocol (Miltenyi Biotec). Purity was verified by flow cytometry, which showed that samples contained >95% $CD8^+CD3^+$ T cells.

Nonspecific NK cell killing assay. As an additional positive control and to ensure that our purified NK cells were reactive to known NK cell stimuli, we modified our killing assay to use MHC-devoid K562 cells as susceptible NK cell targets, labeling them with PKH26. NK cell–resistant RAJI cells served as intrawell controls and were labeled with the green dye CFSE. Purified NK cells were cultured with these cell lines at various E:T ratios for 18 h, and the specific lysis of K562 cells was calculated as follows: (% sample lysis with NK effectors – % basal lysis without NK effectors)/(100 – % basal lysis without NK effectors).

Flow cytometry. Isolated mononuclear cells or Q-Prep (Beckman-Coulter) on whole blood was used for surface staining. Caltag Fix & Perm (Invitrogen) was used for all intracellular staining. Antibodies used are shown in **Supplementary Table 1**. Flow cytometry acquisitions were done on an LSR II (BD Biosciences, La Jolla, CA), and FlowJo software (version 9.6.4, Tree Star, Ashland, OR) was used for all analyses.

Statistical analyses. All statistical and graphic analyses were done with GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA). Nonparametric Mann-Whitney *U*-tests were used where indicated, and a *P* value of <0.05 (by two-tailed test) was considered statistically significant.

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