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NK and CD4⁺ T Cell Cooperative Immune Responses Correlate with Control of Disease in a Macaque Simian Immunodeficiency Virus Infection Model

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Control of infectious disease may be accomplished by successful vaccination or by complex immunologic and genetic factors favoring Ag-specific multicellular immune responses. Using a rhesus macaque model, we evaluated Ag-specific T cell-dependent NK cell immune responses in SIV-infected macaques, designated “controlling” or “noncontrolling” based on long-term chronic viremia levels, to determine whether NK cell effector functions contribute to control of SIV infection. We observed that Gag stimulation of macaque PBMCs induced subset-specific NK cell responses in SIV-controlling but not SIV-noncontrolling animals, as well as that circulatory NK cell responses were dependent on Ag-specific IL-2 production by CD4⁺ central memory T cells. NK cell activation was blocked by anti–IL-2–neutralizing Ab and by CD4⁺ T cell depletion, which abrogated the Gag-specific responses. Among tissue-resident cells, splenic and circulatory NK cells displayed similar activation profiles, whereas liver and mucosal NK cells displayed a decreased activation profile, similar in SIV-controlling and -noncontrolling macaques. Lack of T cell-dependent NK cell function was rescued in SIV-noncontrolling macaques through drug-mediated control of viremia. Our results indicate that control of disease progression in SIV-controlling macaques is associated with cooperation between Ag-specific CD4⁺ T cells and NK cell effector function, which highlight the importance of such cell-to-cell cooperativity in adaptive immunity and suggest that this interaction should be further investigated in HIV vaccine development and other prophylactic vaccine approaches.

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Recent evidence suggests that innate immunity may play a crucial role in the control of HIV infection at all stages of disease. NK cell functions, such as production of IFN-γ and β-chemokines and direct killing of HIV-infected cells, have been hypothesized as potential correlates of protection in HIV-1 highly exposed seronegative subjects. The possibility that cooperation with the adaptive immune system may impact NK cells, providing a de facto potential for T cell-dependent effector responses, has important implications for HIV/SIV vaccine development and would provide yet another mechanism available for prophylactic and/or therapeutic protection. We studied rhesus macaques, the model of choice for evaluating SIV vaccines, to determine whether T cell-dependent NK cell immune responses contribute to control of SIV infection. We asked whether memory CD4⁺ T cells cooperate with NK cells and whether such an interaction affects SIV replication in controlling versus noncontrolling SIV-infected macaques. We found that subpopulation-specific circulatory and tissue NK cell responses were observed only in SIV-controlling animals. These responses were directly correlated with, and dependent on, Ag-specific IL-2 production by SIV-specific memory CD4⁺ T cells and inversely correlated with viral load. Our results suggest that NK and CD4⁺ T cell responses cooperate in the control of SIV replication and disease progression, providing another potential correlate of protective immunity.

Materials and Methods

Animals and cell collection

Assays used freshly isolated (n = 25) and frozen (n = 20) PBMCs from naive or SIVmac255-infected rhesus macaques (Macaca mulatta). Macaques were categorized as SIV controllers if they exhibited a chronic viral load of 10⁵ copies/ml plasma for ≥16 wk prior to sample collection. SIV noncontrolling animals had a chronic viral load of 10⁵ copies/ml plasma. Frozen cells were obtained from previously identified SIV controlling and
noncontrolling macaques (13, 14). Animals were housed at Bioqual (Gaithersburg, MD) or at Advanced BioScience Laboratories (Kensington, MD) and maintained according to institutional Animal Care and Use Committee guidelines. Blood samples were collected by venipuncture of anesthetized animals into EDTA-treated collection tubes. PBMCs were obtained by centrifugation on Ficoll-Paque PLUS gradients (GE Health-care, Piscataway, NJ). Cells were washed and resuspended in R-10 medium (RPMI 1640 containing 10% FBS, 2 mm glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and antibiotics). Frozen cells were thawed and rested for 8 h in R-10 medium before stimulation. For evaluation of tissue-specific T cell-dependent NK cell responses, 11 macaques (3 naive, 4 SIV controlling, and 4 SIV noncontrolling) were sacrificed 64–76 wk after initial SIV infection for collection of peripheral blood, spleen, liver, jejunum, and colon biopsies. Spleen and liver biopsies were minced, passed through a 40-μm cell strainer, and lysed to remove contaminating RBCs. Jejunum and colon biopsies were teased apart with 23G needles and then digested for two 30-min intervals on an orbital shaker in R-10 medium containing 1 mg/ml collagenase II (Sigma, St. Louis, MO), Liver, jejunum, and colon mononuclear cells were further enriched by centrifugation on Percoll gradients.

Antiretroviral treatment

To study the impact of viral load on T cell-dependent NK cell activation, seven SIV noncontrolling macaques were treated for a period of 8 wk with a triple mixture of antiretroviral therapy (ART) containing didanosine (Videx; Bristol-Myers Squibb, Princeton, NJ), stavudine (Zerit; Bristol-Myers Squibb), and a 9-[2-(R)-[[bis(isopropoxycarbonyl)oxy]methyl]phosphonoyl] methoxy[propyl]adenine fumurate (Gilead Sciences, Foster City, CA). Videx was given i.v. as a single daily dose of 10 mg/kg. Zerit was administered twice a day orally at a 1.2-mg/kg dose, and 9-[2-(R)-[[bis(isopropoxycarbonyl)oxy]methyl]phosphonoyl] methoxy[propyl]adenine fumurate was given as a single daily s.c. dose of 20 mg/kg. To prevent damage to the pancreas, the Videx dose was decreased to 5 mg/kg after 3 wk of treatment.

Flow cytometry

Anti-human fluorochrome-conjugated mAbs known to cross-react with rhesus macaque Ags were used, including FITC anti-CD69 (FN50), V450 anti–IFN-γ (B27), PE-Cy7 anti-CD95 (DX2), PE-Cy7 anti-CD56 (B159), Alexa Fluor 700 anti-CD3 (SP34-2), allophycocyanin-Cy7 anti-IL-2 (MQ1-17H12), and allophycocyanin-Cy7 anti-CD16 (3G8) (all from BD Biosciences, San Jose, CA); Alexa Fluor 647 anti-CD107a (eBioH4A3), PE-Cy7 anti-CD28 (CD28.2), and eFluor 650NC anti-CD8 (RPA-T8) (all from eBioscience, San Diego, CA); QDot605 anti-CD4 (eBioH4A3), PE-Cy7 anti-CD28 (CD28.2), and eFluor 650NC anti-CD8a (RPA-T8) (all from eBioscience, San Diego, CA); QD605QD655 anti-CD4 (custom conjugation of clone L200; BD Biosciences) and QD605QD655 anti-CD8a (3B5) (BioLegend, San Diego, CA); PE anti-CD49d, PE anti–NK-KG2A (199) (Beckman Coulter, Fullerton, CA); allophycocyanin anti–α4β7 (rehsus recombinant) and QD605QD655 anti-CD4 (T4/19Thy5D7) (National Institutes of Health [NIH] Nonhuman Primate Reagent Resource); and Pacific Blue anti-CCR7 (TGR/CCR7) and PerCP/Cy5.5 anti–TNF-α (Mab11) (BioLegend, San Diego, CA). The yellow and aqua LIVE/DEAD viability dyes (Invitrogen) were used to exclude dead cells. After SIV peptide stimulation, PBMCs and T cells were stained with on-chip GAM antibodies and 2 μg/ml un conjugated anti-CD49d and anti-CD28 (BD Biosciences). BD GolgiPlug, BD GolgiStop, and Alex Fluor 647 anti-CD107a were added for the last 5 h of culture at the manufacturer’s recommended concentrations. Subsequently, cells were washed and stained with a panel of mAbs for detection of multiple parameters of T and NK cell activation. CD3+ T cells were divided into CD4+ and CD8+ populations and, for each, cells were subdivided into CD28–CD95+ central memory (CM) and CD28+CD95+ effector memory cells. The percentage of cytokine-secreting cells among each memory subset was determined. NK cells (CD3+CD8–NK2G2A+) were subdivided by their CD16 and CD56 expression patterns (Supplemental Fig. 1A). Next, the upregulation of IFN-γ, TNF-α, CD107a, and CD69 in each subpopulation was calculated (Supplemental Fig. 1B). For both T and NK cell-activation assays, nonstimulated and Staphylococcal enterotoxin B (SEB)-treated (5 μg/ml, Sigma) tubes were used as negative and positive controls, respectively. In some assays, 10 μg/ml of an anti–IL-2 mAb (BioLegend) was added to neutralize IL-2. Nonhuman primate CD3 and CD4 MicroBead Kits (Miltenyi Biotec, Auburn, CA) were used to deplete these cells from PBMCs. Recombinant macaque IL-15 (150 ng/ml) and IL-2–Fc (a fusion of macaque IL-2 and IgG2c, 400 ng/ml), both obtained from the NIH/National Center for Research Resources-funded Resource for Nonhuman Primate Immunoregulatory Reagents (Emory University, Atlanta, GA), were used for activation of enriched NK cells (PBMCs depleted of CD3+ T cells). To increase the number of animals assessed, 20 viably frozen PBMC samples (3 naive, 9 SIV controllers, and 8 noncontrollers) were evaluated for T and NK cell activation. Experimental results using fresh or frozen PBMCs yielded comparable data and, therefore, were combined for analysis (data not shown).

Statistical analysis

Results are shown as mean ± SEM. Data were analyzed using Prism (v5.03, GraphPad Software). A p value ≤ 0.05 was considered statistically significant for each test.

Results

Gag-specific IFN-γ production by lymphocytes of SIV-controlling macaques

SIVmac251-infected macaques were categorized as controlling or noncontrolling based on their chronic viral load levels (Fig. 1A). No difference was observed in the percentage of NK cells (CD3–CD8–NK2G2A+) in either group of SIV-infected macaques compared with naive animals (Fig. 1B). As expected, CD4+ T cells were significantly decreased in both groups of infected macaques, more so in noncontrollers, whereas CD8+ T cells were proportionally increased (as a percentage of CD3+ T cells) in both SIV-infected groups. Stimulation of PBMCs of SIV-infected animals with Gag or Env peptide pools for 24 h significantly upregulated Gag-specific IFN-γ–producing cells only in SIV-controlling macaques (Fig. 2A, 2C), although all groups responded equally to SEB. IFN-γ–producing cells included NK, CD4+, and CD8+ T cells (Fig. 2B, 2D). Among SIV controllers, the proportion of IFN-γ–producing cells was significantly higher in NK and CD8+ T cells only in response to Gag stimulation (Fig. 2D). Both the proportion (Fig. 2D) and the amount of IFN-γ produced per cell (mean fluorescence intensity; data not shown) were significantly higher in NK cells after Gag stimulation of PBMCs. Similarly, NK and CD8+ T cells from SIV controllers significantly increased the expression of CD107a (Fig. 2E) and TNF-α (p < 0.05, data not shown) in response to Gag stimulation. To rule out a lack of response in NK cells of noncontrollers as the result of an intrinsic SIV-associated cell dysfunction, PBMCs of SIV-controlling and -noncontrolling macaques were depleted of CD3+ T cells to generate enriched NK cells. On average, CD3+ T cell depletion resulted in a 2.5-fold enrichment of NK cells (from 4.5 ± 3.3 to 11.2 ± 6.6% of live lymphocytes). Neither the enriched NK cells of SIV-controlling or -noncontrolling macaques responded to direct Gag peptide stimulation, indicating a lack of direct recall-response capability and implicating T cells for SIV peptide recognition (Fig. 2F). Nevertheless, NK cells of both groups were equally capable of producing IFN-γ and upregulating CD69 and CD107a expression in response to IL-2 plus IL-15 stimulation.
Subset-specific NK cell activation in SIV-controlling macaques

Three subpopulations of macaque circulatory NK cells exist based on CD16 and CD56-expression patterns (Supplemental Fig. 1A). CD16+CD56−dim cells (CD16+ NK cells) represent ∼85% of circulatory NK cells, have low cytokine-producing capacity, and are primarily considered cytotoxic effectors. CD16−CD56+ cells (CD56+ NK cells) represent ∼5% of circulatory NK cells and are primarily considered cytokine-producing cells. Double-negative (DN; CD16−CD56−) NK cells (∼10% of circulatory NK cells) do not have a human counterpart and appear to have both cytolytic and cytokine-producing potential (15). In agreement with previous results from our laboratory and those of other investigators, CD16+ NK cells were significantly reduced in SIV-noncontrolling macaques, which, in turn, led to a significant increase in the proportion of DN NK cells (Supplemental Fig. 2A) (16–18). Upon stimulation of PBMCs with Gag or Env peptides, IFN-γ production by CD4+ and CD8+ T cells (Supplemental Fig. 3C). A positive correlation was seen between both IFN-γ-producing (Fig. 4B, p = 0.0004) and TNF-α-producing (Fig. 4C, p = 0.0257) CD56+ NK cells and IL-2-producing CM CD4+ T cells, supporting the role of IL-2 in activating NK cells during T cell recall responses. IL-2 production by CM

NK cell activation correlates with IL-2 production by CD4+ CM T cells

Because enriched macaque NK cells did not respond to Gag peptide stimulation in the absence of T cells (Fig. 2F), we evaluated the T cell contribution to the NK cell responses. Ag-specific IL-2-producing T cells were evaluated in a 6-h stimulation assay, because T cells elicit a faster response to peptide stimulation than do NK cells. A significant upregulation of IL-2-producing cells among total CD4+ T cells of SIV-controlling macaques was observed in response to Gag, but not Env, stimulation (Supplemental Fig. 3A). CD8+ T cells did not produce any IL-2 in response to either Gag or Env stimulation (Supplemental Fig. 3B). Gag-specific IL-2–producing CD4+ T cells were confined to the CM (CD28+CD95+) compartment (Fig. 4A). No significant IL-2 production was observed in effector memory (CD28−CD95+) CD4+ T cells (Supplemental Fig. 3C). A positive correlation was seen between both IFN-γ-producing (Fig. 4B, p = 0.0004) and TNF-α-producing (Fig. 4C, p = 0.0257) CD56+ NK cells and IL-2–producing CM CD4+ T cells, supporting the role of IL-2 in activating NK cells during T cell recall responses.
CD4+ T cells also correlated positively with IFN-γ production by CD16+ (p = 0.0278) and DN (p = 0.0373) NK cells (data not shown).

Addition of an IL-2–neutralizing Ab abrogated IFN-γ production by CD16+ NK cells (Fig. 4D). Further, the same Ab also significantly (p < 0.05) reduced IFN-γ–producing CD56+ NK cells, although the highest Gag responders were only partially diminished (Fig. 4E). Depletion of either CD3+ or CD4+ T cells from macaque PBMCs reduced Gag-specific T cell-dependent NK cell responses by >90%, as assessed by IFN-γ production (Fig. 4F).

Immune responses by tissue-resident NK cells

We evaluated T cell-dependent NK cell responses in spleen, liver, jejunum, and colon NK cells. Initially, we examined tissue-homing potential and the proportional distribution of NK and T cells in the tissues of the naive and infected macaques. In SIV noncontrollers, expression of α4β7, the gut-homing marker, was significantly decreased in CD16+ NK cells and increased in DN NK cells compared with naive animals (p < 0.05, Supplemental Fig. 2B). Expression patterns of CCR7, the lymph node-homing marker, were similar in naive and SIV-infected animals. Despite the subset-specific alterations in α4β7 expression, NK cells were present at comparable proportions in all tissues assayed in both naive and SIV-infected macaques (Supplemental Fig. 4A).

Previous reports showed that tissue-resident NK cells in rhesus macaques are mostly CD16+; however, a fourth subpopulation, characterized by the surface expression of both CD16 and CD56, also exists (15). Given the CD16/CD56 expression differences that exist between circulatory and tissue-resident NK cells, we expressed NK immune responses as a percentage of total NK cells in lymphocytes isolated from the various tissues to compare up-regulation of activation markers in NK cells across different compartments. Similarly to circulatory NK cells, splenic NK cells of SIV-controlling macaques significantly upregulated the expression of IFN-γ (Fig. 5A), CD107a (Fig. 5B), TNF-α (Fig. 5C), and CD69 (Fig. 5D) in response to Gag stimulation. Interestingly, in the spleen of SIV-controlling macaques, the proportion of IFN-γ and TNF-α–producing NK cells also increased in response to Env stimulation. Furthermore, in contrast to circulatory NK cells,
FIGURE 4. Gag-specific IL-2 production by CM T cells is essential for NK cell effector responses. Fresh and frozen PBMCs were stimulated for 6 h in the presence of SIV Gag or Env peptides or SEB. (A) Production of IL-2 by CD28+CD95+ CM CD4+ T cells. Correlation between IL-2 production by CM CD4+ T cells (at 6 h) and CD56+ NK cell IFN-γ (B) and TNF-α (C) responses at 24 h (all Gag-specific by subtraction of nonstimulated controls). Reduction of T cell-dependent NK cell responses in CD16+ (D) and CD56+ (E) NK cells by the addition of an IL-2-neutralizing Ab. Suppression of IFN-γ (F) and CD107a (G) upregulation in total NK cells by depletion of CD3+ or CD4+ T cells. Data are pooled from 36 macaques (8 naive, 13 SIV controllers, and 15 SIV noncontrollers) (A–C), from 12 SIV controllers (D, E), and from 3 SIV controllers for (F, G). Spearman’s correlation analysis was used to determine statistical significance in (B) and (C). *p < 0.05, **p < 0.001, two-way ANOVA (D, E).

splenic NK cells from noncontrolling macaques upregulated the expression of CD107a and CD69 in response to Gag (Fig. 5B, 5D). Hepatic NK cell responses were almost undetectable for all animals assayed, with the exception of SIV-noncontrolling animals, which exhibited a low (albeit significant) upregulation of IFN-γ–producing cells in response to Gag (Fig. 5A, Liver). We observed an upregulation of IFN-γ–producing mucosal NK cells in response to Gag in SIV-controlling macaques (Fig. 5A, Mucosal).

FIGURE 5. Activation of tissue-resident NK cells is observed mostly in SIV-controlling macaques. Lymphocyte single-cell suspensions were purified from spleen, liver, and mucosal tissues (jejunum and/or colonic biopsies) and stimulated for 24 h in the presence of SIV Gag or Env peptides. Upregulation of IFN-γ (A), CD107a (B), TNF-α (C), and CD69 (D) in response to Gag and Env peptides was measured within total NK cells (CD3+ CD56+ NKG2A+ live lymphocytes) for each tissue. Data are pooled from 11 macaques (3 naive, 4 SIV controllers, and 4 SIV noncontrollers). For each parameter, activation of NK cells was determined by subtracting nonstimulated control values. *p < 0.05 (Gag), *p < 0.05 (Env) versus naive group, Mann–Whitney t test. C, SIV controlling; N, naive; NC, SIV noncontrolling.

Impact of viral load on CD4+-dependent NK cell responses

During chronic SIV/HIV infection, high viral loads are often associated with phenotypic and functional abnormalities of immune cell subpopulations including T cells (19), B cells (20), and NK cells (17, 21). In this study, SIV-noncontrolling macaques displayed impaired T cell Ag-specific recall responses and CD4+-dependent NK cell responses. Given that both Ag-specific T cell production of IL-2 (p = 0.0019, data not shown) and T cell-dependent NK cell activation levels are inversely correlated with plasma viremia (p = 0.0036, Fig. 6A), we investigated whether decreasing viral loads through ART would improve the responsiveness of NK and T cells. Seven SIV-noncontrolling macaques were treated with ART for 8 wk, which significantly reduced plasma viremia in all macaques (Fig. 6B). Four of seven animals displayed undetectable viral loads. However, only nonsignificant trends toward increased proportions of CD4+ T cells and decreased proportions of CD8+ T cells resulted (Fig. 6C). Nevertheless, ART had a positive impact on the capacity of NK cells to respond in T cell-dependent assays, because the proportion of CD69+ and IFN-γ+ Gag-specific NK cells increased significantly after 8 wk of therapy compared with pre-ART levels (Fig. 6D). Although T cell-dependent NK cell responses were partially rescued during ART, no significant increase in the production of IL-2 by either total or CM CD4+ T cells in response to Gag stimulation was observed (Fig. 6E). Despite this, the marginal Gag-specific increases in IL-2 production observed in CM CD4+ T cells after 8 wk of ART correlated significantly with the increased production of IFN-γ (p = 0.0004, Fig. 6F) and expression of CD69 (p = 0.0456, data not shown) by NK cells at the same time point.

Collectively, our data illustrate that control of SIV disease progression in macaques is associated with increased T cell-dependent NK cell effector function in blood, as well as in spleen and mucosal tissues. The circulatory NK cell responses correlated directly with IL-2 production by Ag-specific CM CD4+ T cells. Further, although viral load negatively affects NK cell function, T cell-dependent NK cell responses in SIV-noncontrolling macaques can be at least partially rescued by short-term ART. Overall, our data support the notion that T cell-dependent NK cell recall responses are potential immune correlates of protection and should be monitored both in vaccine-development and therapeutic studies.
FIGURE 6. Therapeutic control of viremia in SIV-noncontrolling macaques improves T cell-dependent NK cell effector responses. Seven SIV-infected noncontrolling macaques were treated with ART for 8 wk. Peripheral blood was obtained before and after ART. (A) Correlation between viral load and the percentage of CD56+ NK cells producing IFN-γ in response to 24-h stimulation (using same animals as in Fig. 4B and 4C). Effect of ART on plasma viral loads (B) and on the proportion of NK and T (CD4+ and CD8+) cells in peripheral blood (C). (D) T cell-dependent Gag-specific NK cell effector responses measured after 24 h of stimulation. Responses without stimulation have been subtracted. (E) Gag-specific T cell responses measured after 6 h of stimulation. (F) Correlation between Gag-specific production of IL-2 by CM (CD28+CD95+) CD4+ T cells and production of IFN-γ by NK cells post-ART. Data are combined from seven macaques. Dashed line in B represents the limit of detection (50 copies/ml) for the viral load assay. Spearman’s correlation analysis was used to determine statistical significance in (A) and (F). *p < 0.05, **p < 0.01, Mann–Whitney t test.

Discussion
Recent studies examining vaccine-induced immunity in humans highlighted the often-overlooked importance during Ag-specific immune responses of NK cells that mediate cytotoxic and cytokine-producing effector functions (7–9). Several NK cell functional responses have been correlated with either the prevention from acquisition or the immunological control of SIV and HIV infection (10, 11, 22–24). Despite the fact that nonhuman primate-based vaccination models serve as surrogates for HIV/SIV vaccine development (12), few preclinical studies have evaluated the role played by NK cells during vaccine-elicited immune responses. In this study, we examined, in detail, the IL-2–dependent activation of circulatory and tissue-resident NK cells and its association with Ag-specific CD4+ T cell responses. We demonstrated that T cell-dependent NK cell activation is primarily dependent on IL-2 produced by Ag-specific memory CD4+ T cells. To the best of our knowledge, this is the first report to characterize SIV-specific T cell-dependent NK cell responses in circulatory, as well as tissue-resident, NK cells. Notably, this study identifies NK cells as key players in adaptive immune responses. Although a greater proportion of NK cells compared with CD4+ or CD8+ T cells produced IFN-γ in response to Gag stimulation of PBMCs (Fig. 2B, 2D), the greater absolute number of CD3+ CD8+ lymphocytes makes the CD3+CD8+IFN-γ T cell subset most abundant. Therefore, although NK cells provide an early, rapid response, it must be noted that overall control of SIV infection in macaques has been associated with strong and Ag-specific CD4+ and CD8+ T cell responses (25, 26). CD8 cells have long been known to contribute to the control of SIV viremia (27). More recently, CD4+ T cells were shown to be associated with the postpeak decline of viremia in SIV-infected macaques (28). Furthermore, their direct cytolytic function was linked to clearance of SIV-infected macrophages (29) and used as a predictor of disease outcome in HIV-infected individuals (30). In addition to cellular immune control, multiple genetic correlates of protection have been identified. For example, certain TRIM5α polymorphisms are associated with viral susceptibility/resistance, and particular MHC class I molecules are correlated with control of HIV and SIV (31, 32). Similar associations with some MHC class II molecules in both nonhuman primates (33, 34) and humans (35) were reported, although not all HLA class II associations have been reproducible (36). Thus, the NK response described in this article is one component of a complex immune approach that combats HIV/SIV infection and disease progression.

In our study, Ag-specific T cell responses were observed mostly in SIV-controlling macaques, suggesting an increased level of protective immunity. However, the higher viral loads in the SIV-noncontrolling group may have had an overall negative effect on Ag-specific immune function. It is important to mention that although SIV-noncontrolling animals had decreased numbers and Ag-specific function of CD4+ T cells, their response to SEB as positive control was comparable to that of SIV-controlling and naive animals (Figs. 2C, 4A). Moreover, enriched NK cell cultures from SIV-controlling and -noncontrolling macaques were equally responsive to IL-2 plus IL-15 stimulation (Fig. 2F), although a more physiological assay (e.g., killing of MHC-devoid target cells) might allow for identification of more subtle functional differences between NK cells of SIV-controlling and -noncontrolling animals. Taken together, these results suggest that reduced T cell-dependent NK cell effector responses observed in SIV-noncontrolling macaques are not due to a generalized impaired capacity of CM CD4+ T cells to produce IL-2 or to a direct viral load-associated NK cell dysfunction; instead they are a result of the decrease in number of SIV-specific memory CD4+ T cells, which prevents effective cross-talk with NK cells. Similarly, the lack of peptide responsiveness observed in SIV-noncontrolling animals strongly suggests that Ag-specific CD4+ T cell help is required for optimal activation of NK cells, as well as for CD8+ T cell priming during Ag-recall assays (37).

Previous studies evaluated NK cells during chronic SIV infection in a variety of nonhuman primates (15, 38, 39), primarily assessing NK cell function based on responses to MHC-devoid target cells (e.g., K562 and 721.221) or to PMA/ionomycin stimulation. Therefore, the possible role of Ag-specific T cells and cross-talk with other cell types was overlooked. Further, detailed analyses of Ag-specific T cell-dependent functional responses within each circulatory NK cell subpopulation have not been described. Although we observed that T cell-dependent NK cell responses were subset specific, the capacity or threshold of each NK cell subset to interact and be activated by CD4+ T cells remains to be
determined. Interestingly, we observed that T cell-dependent NK cell effector functions were also Ag specific, as shown by limited responses to Env stimulation both in circulatory, as well as tissue-resident, NK cells. The importance of immune cell cooperation, specifically in the context of NK cell activation, was reported previously (6–9); however, our evaluation of such responses in SIV-infected macaques displaying different courses of disease progression has extended these observations, highlighting the specificity and relevance of these responses to disease control. In this regard, preliminary results from our laboratory suggest that the killing capacity of naive NK cells against MHC-devoid 721.221 target cells is enhanced (>15%) by IL-2 pretreatment (D.A. Vargas-Inchaustegui, unpublished observations). If such nonspecific enhancement in killing were also observed during SIV-specific T cell-dependent NK cell activation, it would have important implications for SIV/HIV immunopathology, prophylaxis, and vaccine design. Given that our experiments were conducted with chronically infected animals, detailed analysis of T cell-dependent NK cell effector function during vaccination protocols, as well as during the early acute phase of SIV infection, are underway. Similarly, we wish to evaluate whether increased frequencies of regulatory T cells or markers of immune exhaustion (e.g., PD-1 expression) can be correlated to the lack of SIV-specific responsiveness observed in our SIV-noncontrolling macaques. These future studies will elucidate the potential role of these collaborative responses in the prevention and/or early control of SIV infection.

In this study, NK cell activation was dependent on Ag-specific IL-2 production by CD4+ CM T cells (Fig. 4A), similar to the results of Horowitz et al. (7), who reported that, in humans vaccinated against rabies virus, NK cell effector responses were primarily dependent on IL-2 produced by memory CD4+ T cells, although IL-12 and IL-18 produced by myeloid accessory cells were also necessary for NK cell activation. In our study, IL-2 appeared to be the major activation molecule for two reasons: Ag-specific responses were measured using peptide pools for short stimulation periods, making TCR-positive cells the most likely candidates to respond in such short time periods, and IL-2 neutralization, as well as CD4+ T cell depletion, almost completely abrogated NK cell recall responses (Fig. 5D–G). Although our results suggest that 95% of effective NK cell activation is dependent on CM CD4+ T cell-derived IL-2, we cannot exclude a potential contribution of other NK cell activatory cytokines, such as IL-12, IL-15, or IL-18. Similarly, given that depletion of CD8+ T cells was not technically possible (macaque NK cells express CD8α), further experiments are needed to formally conclude that CD8+ T cells are not necessary for the NK cell effector functions described in this study. Finally, whether specific epitopes in Gag and/or Env are associated with CD4+ CM T cell development and IL-2 production, thus controlling T cell-dependent NK effector responses, requires further analysis, because peptide pools representing the entire Gag and Env proteins were used in this study for stimulation.

Although we detected an improvement in NK cell function after 8 wk of ART, we did not observe significantly increased T cell function (i.e. Gag-specific IL-2 production). Furthermore, only two of four markers of NK cell activation measured, CD69 and IFN-γ, were significantly increased after 8 wk of ART and only in the animals with the greatest recovery in IL-2 production (Fig. 6F). Collectively, our results suggest that earlier treatment initiation and/or longer treatment periods may be required for full recovery of both T and NK cell function. In this regard, it was reported that the kinetics of T and NK cell immune reconstitution during ART are very different (40) and that complete functional recovery of these cells is directly dependent on the level of viral suppression achieved (41).

Although the phenotype of rhesus macaque tissue-resident NK cells was described previously (15), the functional characteristics of these cells during SIV infection have not been reported. Surprisingly, immune responses by tissue-resident NK cells were location and Ag specific. We observed similar activation profiles in splenic and circulatory NK cells, but such responses were only partially present in mucosal NK cells and were nonexistent in hepatic NK cells of SIV-controlling macaques (Fig. 5). We hypothesize that, during SIV infection, tissue-resident NK cells may be functionally restricted because of their anatomic location, or alternatively, cells with which they react may regulate their activity. In support of this hypothesis, it was shown that intrahepatic levels of IL-10 maintain murine NKG2A+Ly49− liver NK cells in a functionally hyporesponsive state, as evidenced by their lack of IFN-γ production in response to IL-12/IL-18 stimulation (42). Furthermore, dampening of hepatic and mucosal NK cell activation can be controlled through the 2B4 inhibitory pathway, either by differential glycosylation of 2B4 (43) or by interaction with its ligand CD48 during NK/dendritic cell cross-talk (44). In recent years, a new subset of innate lymphocytes that express some NK cell markers and produce the cytokine IL-22 (NKp44+ NK cells) has been identified in different species (45, 46). Interestingly, IL-22 plays a crucial role in modulating tissue responses during inflammation (47), and IL-22-producing NKp44+ NK cells were found to be depleted during chronic SIV infection (46). Therefore, investigation of NKp44+ NK cell function using CD4-dependent Ag-specific and/or Ab-mediated functional assays could further improve our understanding of the role played by different types of tissue-resident NK cells in the control of SIV/HIV infection.

In summary, this study uncovered a novel effector function of NK cells during an SIV-specific adaptive immune response that is dependent on IL-2 produced by Ag-specific CD4+ CM T cells. The NK cell immune responses described are associated with strong and durable control of viremia. Therefore, T cell-dependent NK cell activation should be considered in vaccine strategies for HIV and other infectious diseases as a potential indicator of protective immunity.

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Disclosures
The authors have no financial conflicts of interest.

References
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