Unlike HIV infection, which progresses to AIDS absent suppressive anti-retroviral therapy, nonpathogenic infections in natural hosts, such as African green monkeys, are characterized by a lack of gut microbial translocation and robust secondary lymphoid natural killer cell responses resulting in an absence of chronic inflammation and limited SIV dissemination in lymph node B-cell follicles. Here we report, using the pathogenic model of antiretroviral therapy-treated, SIV-infected rhesus macaques that sequential interleukin-21 and interferon alpha therapy generate terminally differentiated blood natural killer cells (NKG2a/CD16+) with potent human leukocyte antigen-E-restricted activity in response to SIV envelope peptides. This is in contrast to control macaques, where less differentiated, interferon gamma-producing natural killer cells predominate. The frequency and activity of terminally differentiated NKG2a/CD16+ natural killer cells correlates with a reduction of replication-competent SIV in lymph node during antiretroviral therapy and time to viral rebound following analytical treatment interruption. These data demonstrate that African green monkey-like natural killer cell differentiation profiles can be rescued in rhesus macaques to promote viral clearance in tissues.
Natur al killer (NK) cells are “licensed” with functional competence following education with self-major histo-compatibility complex (MHC) class I molecules. In particular human leukocyte antigen (HLA)-E, the ligand for the inhibitory CD94/NKG2a receptor, is positively regulated by HLA-A expression and inhibits NK cell-mediated lysis. In a companion manuscript, Huot et al. define NK cell differentiation states based on their education via NKG2a and expression of CD16 (FcγRIII), an activating Fc receptor that mediates antibody-dependent cell-mediated cytotoxicity (ADCC), and demonstrate that nonpathogenic SIVgag infection in African green monkeys (AGMs) imprints the maturation of NK cells inducing terminally differentiated NKG2aCD16+ NK cells, which express high levels of interleukin (IL)–12. It has been previously demonstrated that NK cell functionality is responsive to immunotherapies with IL-21 and interferon alpha (IFNa). For example, ex vivo IL-21 treatment expanded CD16+ NK cells, antagonized the IL-15-dependent expansion of resting NK cells, and reverses hyporesponsiveness via the STAT1 and PI3K-AKT-FOXO1 pathway [1]. Likewise, ex vivo IFNα therapy upregulates IL-15-mediated NK cell cytotoxicity, including CD107a degranulation and ADCC activity [13,14], and downregulates IL-21R expression [15], furthermore, in vivo IFNα-induced NK cell cytotoxicity correlates with reductions in HIV-DNA during antiretroviral therapy (ART) [16]. In SIVgag infection, systemic IL-15 was associated with NK cell proliferation in lymph node (LN), while systemic IFNα correlated with NK cell cytotoxicity in LN [17]. Given previous findings on a role of IL-21 and IFNα in regulating NK cell function, and that NKG2aCD16+ NK cells are generated while expressing high levels of IL-21R in nonpathogenic SIV infection, we sought to determine whether immunotherapy with IL-21 and IFNα rescues AGM-like profiles of NK cell maturation and activity in SIV-infected rhesus macaques (RMs).

Results

IL-21 and rIFNα immunotherapies are biologically active in SIV-infected, ART-treated RMs. Sixteen RMs were intravenously (i.v.) infected with SIVmac239 and at day (d) 35 post-infection (p.i.) initiated triple formulation ART, which was maintained for 13 months (Fig. 1a and Supplementary Table 1). Prior to ART initiation, the RMs RPK11 and RNA12 mimicked pre-established virologic and immunologic features of controllers; hence, both were not assigned to an experimental group, but excluded from analyses and followed as a part of a study aimed at characterizing post-treatment viral control. Among the remaining 14 RMs, 9 were administered rhesus IL-21-IgFc (IL-21) at d42 and d189 p.i. in two cycles of four doses given once per week followed by weekly rhesus IFNα-IgFc (rIFNα) starting at d323 (3 doses) and d383 p.i. (2 doses; i.e., ART + IL-21 + rIFNα, cytokine-treated). The cytokine-treated RM 172_10 was euthanized at d66 p.i. due to AIDS-defining conditions. Five RMs served as cytokine treatment-naïve, ART-only controls (i.e., controls). ART was withdrawn at d402 p.i. and, given attenuation in IFN signaling upon sustained therapy [19], cytokine treatment–experienced RMs were transitioned to human PEGylated-IFNα (PEG-IFNα; 7 doses, once every 6–8 days, subcutaneous (s.c.), 7 µg/kg; i.e., cytokine-treated) followed by necropsy in 6 months. IL-21 and PEG-IFNα sequential therapies were well tolerated without clinical complications as anticipated based on prior monotherapy administration in SIV–infected RMs [20,21]. Plasma viral loads amid ART revealed no treatment–related impact on the kinetics of viral suppression or rate of viral reactivation (Fig. 1b, c).

To confirm biological activity, we sought to recapitulate observations that IL-21 attenuates residual T-cell immune activation and improves mucosal immunity during ART [20,22]. In cytokine-treated RMs, ART with IL-21 treatment was superior, as compared to ART-only controls, in rapidly and significantly reducing immune activation (HLA-DR+CD38+) in memory CD4+ T-cells from peripheral blood mononuclear cells (PBMCs; Fig. 1d with representative stains and gating strategy in Fig. 1e and Supplementary Fig. 1a, respectively). A similar early reduction following IL-21 treatment was found in rectal biopsy (RB) for the levels of immune activation in memory CD4+ T-cells (Supplementary Fig. 2a) and proliferation (Ki-67+) in CD8+ T-cells (Supplementary Fig. 2b); however, changes in RB did not sustain for long term and treatment did not substantially impact activation or proliferation in CD4+ and CD8+ T-cells from LN (Supplementary Fig. 2). In addition, IL-21 therapy significantly enhanced Th17/Th22 functionality based on the expression of IL-2 and TNF-α (Supplementary Fig. 3). The efficacy of rIFNα amid long-term ART was confirmed by the upregulation of IFN-stimulated genes (ISGs) in PBMCs at 2 h post-treatment relative to control RMs (Fig. 1f), which utilized six rIFNα–treated and two of five control RMs that were not described in this manuscript. In a different historical cohort [23], we also confirmed that ISGs are induced by pathogenic SIV infection, and are significantly reduced, but not fully normalized, by ART (Supplementary Fig. 4), as is observed in natural hosts [24,25]. Cytokine treatment reduced the frequency of HLA-E+CD4+ T-cells (Fig. 1g, representative gating strategy in Supplementary Fig. 1b); however, it did not impact the frequency of NKG2α/γ+CD8+ T-cells (Fig. 1h, representative gating strategy in Supplementary Fig. 1c) nor did it enhance T-cell responses whether by T-bet expression, which regulates Th1 cytokine expression [26], or by IFN-γ ELISPOT following stimulation with SIV-Gag and -envelope (Env) peptides (Supplementary Fig. 5; T-bet gating strategy given in Supplementary Fig. 1d). We then analyzed if the cytokine therapy enhanced NK cell activity by exposing them to MHC-I-deficient target cells with induced HLA-E loaded with SIVmac239 Env peptides. By measuring levels of surface CD107a expression, we calculated the SIV–Env–specific, HLA-E–restricted NK cell activity (raw data of CD107a expression by co-culture condition are given in Supplementary Fig. 6–c; representative stains shown in Supplementary Fig. 6–d; see Eq. 1). IL-21 administration led to a significant reduction, which was sustained following rIFNα administration, of the Env–specific NK cell activity independent of viremia (Fig. 1i), indicating that the designed immunotherapy impacted NK cell imprinting while CD8+ T-cell responses remained tepid.

Cytokine therapy reduces replication competent virus in lymphoid tissue, which is uniquely correlated with Env–specific NK cell activity. As cytokine therapy enhanced Env–specific NK cell activity, we sought to determine the impact on viral persistence amid ongoing ART. Independent of ART–mediated viral suppression, cytokine therapy failed to reduce the content of total cell–associated SIV–RNA (Fig. 2a–c) or -DNA (Fig. 2d–f) as determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in bulk PBMCs or LN when compared to controls; yet, cell–associated SIV–DNA in RB was lower in cytokine–treated animals as compared to controls (p = 0.0576 at d374 p.i.; Fig. 2f). Moreover, in cytokine–treated RMs, IL-21 therapy (d217 p.i.) significantly decreased the frequency of LN CD4+ cells harboring replication competent virus relative to controls, as determined by quantitative viral outgrowth assay (QVOA) (Fig. 2g). In three out of four controls, viral suppression due to ongoing ART (d217 to d374 p.i.) resulted in a non–significant reduction of replication competent virus. In cytokine–treated RMs, subsequent rIFNα therapy did not further decline the replication competent virus content, which remained significantly lower than controls also at d374 p.i. (Fig. 2g). Consistent with previous observations [20], the IL-21 impact on replication competent virus was likely unrelated to SIV–specific T-cell responses.
(Supplementary Fig. 5d, e). To further understand the effect on the viral reservoir, measures of SIV reservoir content were correlated against measures of T-cell immune activation, T-cell proliferation, HLA-E+ CD4+ T-cells, NKG2a/c+ CD8+ T-cells, and Env-specific NK cell activity (Fig. 2h). The content of cell-associated SIV-RNA and -DNA, which were not impacted by therapy, positively correlated with T-cell activation and proliferation as expected\cite{27,28}. The frequency of LN CD4+ cells harboring replication competent virus was also positively correlated with T-cell activation and proliferation in blood, but not in LN. Given the enrichment of effector cells in blood as compared to lymphoid tissues\cite{29}, it is plausible that the levels of T-cell activation and
proliferation in blood may better represent therapy-induced immunologic and virologic changes, including in the content of replication competent virus, in LN and other, not accessed anatomical sites. Of note, the content of LN replication competent virus, but not cell-associated SIV-DNA in tissues, displayed a unique positive correlation with HLA-E+ CD4+ T-cell levels and a negative correlation with Env-specific NK cell activity; suggesting that enhanced NK cell functionality is an important mechanism for IL-21-mediated reduction of the replication competent viral reservoirs.

Cytokine therapy promotes the maturation of NKG2a/c\textsubscript{low}CD16\textsuperscript{+} NK cells with enhanced ex vivo innate activity, which correlates with the content of lymphoid replication competent virus. To better characterize the NK cell-mediated response during ART, blood NK cells were immunophenotyped for biomarkers of homing and differentiation. Cytokine therapy resulted in a non-significant increase in the population of total NK cells (CD45\textsuperscript{+}CD3\textsuperscript{-}NKG2a/c\textsuperscript{+}; Fig. 3a) and did not impact homing to the B-cell follicle as gauged by CXCR5 expression (Fig. 3b), the expression of activating receptors (i.e., NKp30, NKp80, and NKp46), or cellular activation (HLA-DR) (Supplementary Fig. 7, representative gating strategy in Supplementary Fig. 1c). NK cells were divided into distinct differentiation stages (stages 0–3) based on their expression of CD16 and NKG2a/c (Fig. 3c–f; representative gating strategy and plots in Supplementary Fig. 8)\textsuperscript{7} with the caveat that the anti-NKG2a monoclonal antibody (mAb) (clone Z199) cannot distinguish between NKG2a and NKG2c in nonhuman primates (NHPs). Cytokine therapy significantly favored the generation of the terminally differentiated NKG2a/c\textsubscript{low}CD16\textsuperscript{+} subset (Stage 3) (Fig. 3g, h; representative flow cytometry plots are given at critical time points (as indicated above) for a cytokine-treated (RQM14) and control RMs (RSp14; 16 of 260 single-replicate stains). The formation and activity of NKG2a/c\textsubscript{low}CD16\textsuperscript{+} NK cells correlate with viral recrudescence following ATI. To further analyze the functional relevance of NK cell differentiation in viral persistence, all RMs underwent ART analytical treatment interruption (ATI) with cytokine-treated RMs additionally receiving ongoing PEG-IFNα (Fig. 1a). Of note, PEG-IFNα therapy has previously been suggested as able to delay viral rebound when initiated prior to ATI\textsuperscript{31,32}, whereas in SIV-infected RMs, prior IL-21 monotherapy during ART is not\textsuperscript{20}. Based on longitudinal plasma viremia following ATI (Fig. 4a, b), cytokine-treated RMs exhibited a significant delay in rebound (>200 copies/mL) both by survival curve analysis (Fig. 4c) and by day of rebound (average 22.1 ± 4.27 days versus 10.6 ± 0.98 days; Fig. 4d). Thus, cytokine treatment modulated the kinetics of plasma rebound, as a slope (d13–d20 ATI) and mean analysis (d13 ATI, Fig. 4e). Cytokine therapy did not however impact the peak or set-point viremia relative to controls (Supplementary Fig. 10a, b) or the content of cell-associated SIV-DNA or -RNA in PBMCs (Supplementary Fig. 10c, d). Transitioning treatment-experienced RMs (i.e., cytokine-treated RMs with prior rIFNa during ART) to PEG-IFNα led to a reset in ISG expression as of 24 h following the first administration (d6 ATI); however, this effect was largely lost following the fifth dose by which nearly all RMs had rebounded (d37 ATI; Fig. 5a). As with rIFNa on-ART, PEG-IFNα therapy following ATI failed to improve SIV-specific T-
cell responses by SIV-Gag-stimulated IFN-γ ELISpot (Supplementary Fig. 10e). At d13 post ATI, the distribution of NK cell maturation subsets in PBMCs was similar to that observed during ART with higher levels of terminally differentiated (NKG2a/CD16+low, stage 3) NK cells with strong, innate degranulation activity (i.e., ex vivo CD107a surface expression) in the cytokine-treated animals in contrast to higher levels of the intermediate (NKG2a/CD16+high, stage 2) NK cells with weak innate degranulation activity in the controls (Fig. 5b, c). In contrast, at d13 post ATI, the Env-specific activity of bulk NK cells in cytokine-treated animals was no longer statistically significantly different from controls (Fig. 5d) and tended to converge with the levels observed in controls (ART-only).
chronic infection prior to ART initiation (d35 p.i.) (Fig. 1f). Furthermore, at d58 post ATI, by which all RMs had experienced virologic rebound and PEG-IFNα therapy was no longer effective (Figs. 4c and 5a), the frequency of terminally differentiated NK cells in cytokine-treated RMs converged with levels observed in controls (Supplementary Fig. 10f); indicating that during viremic conditions in cytokine-treated RMs converged with levels observed in controls mediated differentiation of SIV-Env-specific T-cells; and the Env-specific point corresponds inversely to the log10-transformed Spearman rank correlation coefficient (r) is represented as a double-gradient heatmap and the size of each data point corresponds inversely to the log10-transformed Spearman’s p value. The false discovery rates (FDR) were calculated using SAS and significant values (Q < 0.05) are represented by a black border.

Discussion
Although historically underappreciated in curative approaches for chronic viral infections, these data demonstrate that HLA-E-restricted NK cell responses impact SIV control in vivo, as has been found in mouse models for other viral infections. The formation of a terminally differentiated NK cell subset (NKG2a/cloreCD16+) with robust innate and adaptive antiviral activities, which was found in nonpathogenic infections AGMs, was blocked in pathogenic SIVmac infection in favor of intermediate differentiation (NKG2a/bchighCD16+) NK cells and their activity during ART with the subsequent delay in viral rebound following ATI.

Methods
Study design. Sixteen female Indian-origin RMs (Macaca mulatta) were recruited to this study and housed at YNPRC (Supplementary Table 1). All animals were Mamu-B08+ and -B17+, whereas 4 RMs were Mamu-A01+; RH12, RNA12, Rsp14, and RB13. RMs were deemed pathogen-free and housed as previously described. RMs were i.v. infected with 300 TCID50 SIVmac251 (Fig. 1a), which was purchased from Koen Van Rompay at UC-Davis. RMs were stratified into in vivo therapy cohorts balancing for their set point viral plasma loads at day 35 p.i. and their Mamu-A01 haplotype (Supplementary Table 1). At d35 p.i. RMs began a daily, s.c. triple for maintenance ART regimen consisting of tenofovir disoproxil fumarate (TDF; 3 mg/kg/d; Gilead Sciences), emtricitabine (FTC; 40 mg/kg/d), Gilead Sciences), and dolutegravir (DTG; 2.5 mg/kg/d; ViVi Health) that were obtained via a material transfer agreement (MTA). Nine RMs were administered two cycles of rhesus IL-21-IgFc (IL-21; 4 doses, once per week, s.c. 100 µg/kg) starting at day 42 p.i. and again at day 189 p.i. All animals with prior IL-21 therapy were subsequently administered rhesus IFNα-IgFc (rIFNα; once per week, s.c. 50,000 units) starting at day 323 p.i. (3X) and day 383 p.i. (2X; i.e., ART = IL-21 + IFNα, cytokine-treated). Cytokine-treated RM 172.10 was euthanized at day 66 p.i. due to rapid progression to AIDS-defining endpoints related to weight loss. Five RMs were utilized as ART-only control (i.e., ART+/-) and two RMs (RPk11 and RNA12) were projected to be controllers based on pre-established criteria; hence they were excluded from analyses and not assigned to an experimental group but followed as a part of a study aimed at characterizing post-treatment control. Following ART ATI (day 402 p.i.), the eight remaining cytokine-treated RMs transitioned to human PEG-IFNα (7 doses, once every 6–8 days, s.c. 7 µg/kg) starting at day 5 post ATI. Animals were followed for 6 months following ATI and subjected to necropsy. The longitudinal characterization of ISGs via RNA-seq in PBMCs was performed in a historical cohort (n = 6;
Supplementary Fig. 4 in which RMs were infected i.v. with SIVmac251 and treated with an ART regimen consisting of s.c. 3TC and TDF daily; intramuscular brecanavir weekly; and intramuscular cabotegravir once every 3 weeks.

**Study approval.** All animal experimentation was conducted following guidelines set forth by the Animal Welfare Act and by the NIH’s Guide for the Care and Use of Laboratory Animals, 8th edition. All procedures were performed in accordance with institutional regulations and were approved by Emory University’s Institutional Animal Care and Use Committee (permit 3000434). Animal care facilities are accredited by the US Department of Agriculture and the Association for Assessment and Accreditation of Laboratory Animal Care International. Proper steps were taken to minimize animal suffering and all procedures were conducted under anesthesia with follow-up pain management as needed.
Fig. 3 Cytokine therapy promotes the maturation of NKG2a/cloCD16− NK cells with enhanced ex vivo innate activity, which correlates with the content of lymphoid replication competent virus. a The frequency of NK cells (CD45+CD20−CD3−NKG2a/c+) of PBMC CD45+ lymphocytes was longitudinally measured by flow cytometry, b as was their CXCR5 expression. The frequency of each differentiation stage of NK cells was determined based on the following definitions: c Stage 0 (red, NKG2a/cloCD16−), d Stage 1 (blue, NKG2a/cloCD16+), e Stage 2 (orange, NKG2a/chiCD16+), and f Stage 3 (purple, NKG2a/chiCD16−). g The mean frequency of each NK cell differentiation stage from above was also revisualized as a color-coded (as annotated below), parts-of-whole stacked bar plot for the cytokine-treated (n = 8; at left) and control RMs (n = 5; at right) over time (indicated at left). Flow cytometry was used to quantify the ex vivo innate frequency of h IFN-γ+ (intracellular) NK cells and i CD107a+ (surface) stage 3 (NKG2a/chiCD16+) NK cells. Data from individual RMs (tagged open circles) are overlaid against the mean ± SEM (shaded region within the dashed lines): control (ART-only, black; n = 5) and cytokine-treated (ART + IL-21 + IFNα, blue; n = 8). Treatment phases are indicated with the following background shading: IL-21 (orange), rIFNα (red), and ART (gray). Data were analyzed with a two-sided (95% CI), two-way ANOVA with Bonferroni correction with cross-sectional comparisons relative to controls. The frequencies of each differentiation stage of NK cells in PBMCs (as indicated below) were correlated against levels of cell-associated and replication competent SIV content in tissue, and the ex vivo innate and Env-specific NK cell activities in PBMCs (as indicated at left) in all RMs (n = 13; days 35, 77, 217, and 374 p.i. as matched data are available). Per each correlation the two-tailed (95% CI) Spearman’s rank correlation coefficient (ρ) is represented as a double-gradient heatmap and the size of each data point corresponds inversely to the log10-transformed Spearman’s p value. The false discovery rates (FDR) were calculated using SAS and significant values (Q<0.05) are represented by a black border.

SIV-RNA

Fig. 4 Cytokine therapy delays the rebound of plasma viremia following ATI. Following ART analytical treatment interruption (ATI), the plasma SIV-RNA copies/mL were measured by qRT-PCR in each a cytokine-treated (PEG-IFNα with prior ART + IL-21 + rIFNα, blue; n = 8 RMs) and b control RMs (prior ART-only, black; n = 5 RMs). The horizontal dashed line (200 copies/mL) represents the threshold for virologic rebound and PEG-IFNα treatments are indicated by the purple arrows above. These kinetics of plasma viremia following ATI were then re-visualized as follows: c The delay in rebound of plasma viremia was represented as a treatment-stratified survival curve, which was analyzed with a Log-rank Mantel-Cox test. d The delay in viral rebound, in days, per each RM was represented as a color and shape-coded symbol overlaid against the mean ± SEM (red), which was analyzed with a two-sided (95% CI) Mann–Whitney U test. The delay in viral rebound, in days, per each RM was represented as a color and shape-coded symbol overlaid against the mean ± SEM (red), which was analyzed with a two-sided (95% CI), two-way ANOVA with Bonferroni’s correction for multiple comparisons across treatments (d13 p = 0.0001), and a mixed-effects linear model was used to analyze the slope between d13 and d20 post ATI (as indicated by the bracket; p = 0.08).

Tissue collection and processing. Collections of peripheral blood (PB), RB punches, and LN biopsies were conducted longitudinally and upon necropsy (Fig. 1a) as previously described44. EDTA PB was used for complete blood counts, and plasma was separated by centrifugation within 1 h of phlebotomy. PBMCs were isolated from PB by density gradient centrifugation (Ficoll-Paque Premium, GE Healthcare). RB punches were obtained by inserting an anoscope a short distance into the rectum and 20 punches were collected using a biopsy forceps. To obtain gut-derived lymphocytes, RB punches were digested with 1 mg/ml collagenase for 2 h at 37 °C with agitation, and then filtered with a 100-μm strainer to remove residual tissue fragments. For LN biopsies, the skin over the axillary or inguinal region was clipped and surgically trepped. An incision was then made in the skin and the LN was exposed by blunt dissection and excised over clamps. LNs
were segmented using a sterile scalpel; macerated over RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio); 100 μL penicillin, and 100 μg/mL streptomycin; and filtered through a 100-μm strainer to isolate mononuclear cells. Tissue segments of LN and 2–4 RB punches were flash frozen in dry ice for SIV-DNA analysis, whereas processed mononuclear cells were cryo-preserved in 10% dimethyl sulfoxide (DMSO) in FBS.

**Flow cytometric analysis.** Fourteen-parameter flow cytometric analysis was performed on fresh PBMCs and mononuclear cells derived from LN biopsies and RB punches. Samples were stained utilizing standard procedures employing clones of anti-human mAbs that have been shown to be cross-reactive in RMs (Table S4) and are validated in databases maintained by NHP Reagent Resource (MassBiosource: https://www.nhpresource.org/ReactionivityDatabase). The following antibodies were utilized for the longitudinal staining panel per T-cell and B-cell characterization: anti-CD21-PE (clone B-ly4, 10 μL, cat. 555442), anti-CD28-PE-CF594 (clone CD28D2, 5 μL, cat. 562296), anti-CD95-PE-Cy5 (clone DX2, 10 μL, cat. 559773), anti-CCR7-PE-Cy7 (clone 3D12, 7.5 μL, cat. 557648), anti-CD45RA-APC (clone 54H9, 10 μL, cat. 561210), anti-Ki-67-A1700 (clone B36, 5 μL, cat. 561277), and anti-CD3-APC-Cy7 (clone SP34-2, 5 μL, cat. 557757) all from BD Biosciences; anti-CD3+ or per 4 RB punches were analyzed with a two-sided (95% CI) Mann-Whitney U test.

**Fig. 5 The formation and activity of NKG2a<sup>+</sup>CD16<sup>+</sup> NK cells correlate with viral recrudescence following ATI.** (a) From PBMCs taken 24 h following the first (d6 post ATI) and the fifth PEG-IFNα dose (d37 post ATI), the expression of interferon-stimulated genes was calculated as a cross-sectional log<sub>10</sub>-fold change between cytokine-treated (n = 8) and control (n = 5) RMs, which is represented as a double-gradient heatmap. The size of each data point corresponds inversely to the log<sub>10</sub>-transformed nominal p value with significant (p < 0.05) adjusted p values indicated by a black border. Using DESeq2, data were analyzed with a two-sided (95% CI) Wald test using the Benjamini-Hochberg method for multiple comparisons. (b) In PBMCs at d13 post ATI, the distribution of the differentiation subsets was measured by flow cytometry as a frequency of NK cells: Stage 0 (red, NKG2a/CD16<sup>+</sup>), Stage 1 (blue, NKG2a/CD16<sup>+</sup>), Stage 2 (orange, NKG2a/CD16<sup>−</sup>), and Stage 3 (purple, NKG2a/CD16<sup>−</sup>). NK cells isolated from PBMCs at d13 post ATI were utilized to determine e the frequency of ex vivo innate activity (CD107α surface expression) or d the Env-specific activity upon co-culture with K562 cells expressing HLA-E loaded with SIVmac Env peptides. (c) Data from individual cytokine-treated (PEG-IFNα with prior ART + IL-21 + rIFNα, blue; n = 8) and control (prior ART-only; black; n = 5) RMs (open symbols) are overlaid against the mean ± SEM (in red) and were analyzed with b, c two-sided (95% CI), two-way ANOVA with Bonferroni’s correction for cross-sectional comparisons relative to controls or d with a two-sided (95% CI) Mann-Whitney U test. (d) The delay in the rebound of plasma viremia was correlated against measures of SIV content, NK cell differentiation, and NK cell activity (as indicated at left; n = 13) from the final on-ART measurement (d339–374 p.i.) or during rebound following ATI (d6–13 post ATI). Per each correlation the two-tailed (95% CI) Spearman’s rank correlation coefficient (rho) is represented as a double-gradient heatmap and the size of each data point corresponds inversely to the log<sub>10</sub>-transformed Spearman’s p value. The false discovery rates (FDR) were calculated using SAS and significant values (Q < 0.05) are represented by a black border. Correlations for which data did not exist during that experimental phase are indicated as “NA.”
mononuclear cells upon thawing. Cells were fixed and permeabilized with a Cytofix/ Cytoperm kit (BD Biosciences) and intracellular staining was incubated a 4 °C for 15 min and then washed with Perm/Wash buffer (BD Biosciences). Cells were stained for 4 h at 37 °C in RPMI 1640 medium supplemented with 10% FBS containing PMA. FACS analysis was performed using FlowJo software (version 10.4.2; TreeStar).

Intracellular cytokine staining. Th1 and Th2 cells were defined as the frequency IL-17 and IL-22 producing CD4+ T cells upon ex vivo stimulation with PMA and ionomycin. Freshly isolated mononuclear cells derived from PBMCs were incubated for 4 h at 37 °C in RPMI 1640 medium supplemented with 10% FBS containing PMA (80 ng/mL; cat. P8139, Millipore Sigma), calcium ionophore A23187 (500 ng/mL; cat. C9275, Millipore Sigma), brefeldin A (10 μg/mL; cat. 420601, BioLegend), and GolgiStop with monomycin (7 × 10−4 dilution; cat. 554724, BD Bioscapes). Samples were stained utilizing standard procedures employing clones of anti-human mAbs that were cross-reactive in RMs as validated by NHP Reagent Resource (MassBiologics): anti-CD8-PE-CF594 (clone RPA-T8, clone 562282), anti-CD95-PE-Cy5 (clone DX2, 10 μL; cat. 559773), anti-IFN-γ-PE-Cy7 (clone B27, 5 μL; cat. 555743), anti-TNFα-AL700 (clone Mab11, 1 μL; cat. 557996), and anti-CD3-CD7-AF488 (clone SP34-2, 5 μL; cat. 557757) all from BD Biosciences; anti-CD4-BV421 (clone OKT4, 4 μL; cat. 317343) and anti-IL-2-BV650 (clone MQ5-17H12, 1 μL; cat. 500332) both from BioLegend; and anti-IL-17-FTC (clone eBio164DEC17, 5 μL; cat. 53-7179-42), anti-IL-22-APC (clone IL22OP, 5 μL; cat. 17-7222-82), and Live/Dead Fixable Aqua (AmCyan, 2 μL of 1:20 PBS dilution, cat. L34957) from Thermof Fisher Scientific (Supplementary Table 2), conventional neutral bases are incorporated to avoid biasing in measuring off-target viral sequences. A Poisson method was employed to calculate values for which not all reactions were positive, which was otherwise calculated by interpolating individual cycle threshold values on a standard curve. Primers and probe sequences are given in Supplementary Table 2.

Quantification of cell-associated SIV-DNA and -RNA. Analyses were performed on cryo-preserved PBMCs, snap frozen LN biopsies, and snap frozen RB punches. Cell-associated SIV-DNA and -RNA were extracted with an AllPrep DNA/RNA Mini Kit (Qiagen), and were quantified via hybrid real-time/digital qPCR and RT-qPCR assays for SIV-DNA and -RNA, respectively, in ten replicates with single-copy clinical sensitivity as previously described. Viral copy numbers were normalized based on genomic CCR5. A Poisson method was employed to calculate viral copy loads (copy clinical sensitivity as previously described). Viral copy numbers were normalized based on genomic CCR5. A Poisson method was employed to calculate viral copy loads (copy clinical sensitivity as previously described).

Peptide-specific NK cell activity assay. K562 cells devoid of MHC-1-stably (ATCC) expressing HLA-A*0101 (K562-E011; Applied Biological Materials Inc.) cells were incubated with 50 μM of SIVmac239 Env peptide (NLQLIAIL at 26 °C for 15–20 h). Isolated NK cells were cultured for 6 h in the presence of an anti-CD107a-PE-Cy5 mAb (clone HA43, 5 μL; cat. 555802; BD Bioscapes), either alone (NK), or co-cultured at a 1:5 ratio with 2 × 105 K562-E011 cells that were either unpulsed (NK+K562-E) or loaded with SIVmac239/251 Env peptide (NK+K563+E-ENV). GolgiStop and GolgiPlug (BD Biosciences) was added 1 h following culture initiation. The frequency of NK cells expressing surface CD107a was measured by flow cytometry. The frequency of cells producing replication competent virus was determined from infectious units per million.

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Reagent Program managed by ATCC: ATCC (version 2.5.2b) with default alignment parameters (https://www.unmc.edu/immunology/IFN). The library was sequenced on the Illumina HiSeq 2500 system employing a single-end 101 cycles at average read depths of 30× to 40× reads per sample. Reads were mapped to the MacaM7 assembly of the Indian rhesus macaque genome reference (with RhesusGenome using STAR (version 2.5.2b) with default alignment parameters) (https://www.ummcl.edu/research/software/index.htm). After alignment, raw read counts per transcript was done internally with STAR using the HTSeq-count algorithm.

Statistics and reproducibility. Statistical tests were two-sided and p values ≤ 0.05 (95% confidence interval, CI) were considered statistically significant for each of the
specific statistical comparisons. All experiments were performed as a single technical replicate unless otherwise noted in the Methods (i.e., qRT-PCR, IFN-γ ELSpot, and QVCA/RT assays were repeated as independent experiments). Data were tested for Gaussian distribution using the D’Agostino-Pearson omnibus normality test. Data showing continuous outcomes are represented as mean ± SEM. Two-way ANOVA tests and/or mixed-effects models, in the event of absent data points, were performed for Bonferroni’s correction for multiple comparisons. Correlations were performed two-sided with a non-parametric Spearman correlation and were fitted with a simple linear regression. Comparisons of survival curves were conducted with a Log-rank (Mantel–Cox) test. All of the above analyses were conducted using GraphPad Prism version 8.1.2. Using SAS, Spearman’s ρ values were adjusted for multiple comparisons using the stepdown Bonferroni60, Hochberg61, and false discovery rate (FDR)62 methods. Correlation and RNA-seq data were visualized using ggplot2 (version 3.3.2) in RStudio (version 1.4.1103) with custom code. The distribution of cytokine co-expression (i.e., Boolean logical gates of IL-17 and IL-22 expression within CD4+ T-cells) was analyzed with a Permutest treatment (104 iterations) in SPICE version 60. Rates of increase in log10 SIV RNA copies per mL of plasma post ART were obtained using a mixed-effects linear model specifying that data follow a linear regression over time, with a random intercept for each animal. The mean slope and mean linear increase were estimated and compared between treatment conditions within the framework of the mixed-effects linear model63. DESeq2 version 1.22.1R package64 was used to produce estimated and compared between treatment conditions within the framework of the mixed-effects linear model65. The mean slope and mean linear increase were estimated and compared between treatment conditions within the framework of the mixed-effects linear model66.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Materials provided via MTA were supplied without restrictions on use. Source data are provided for the performed correlations, as are the statistical readouts for the correlations and RNA-seq analyses. RNA-seq data related to Figs. 1F and 5a, and Supplementary Fig. 4 are publicly available in GenBank (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) under GEO accession GSE163443 with subseries GSE163440, GSE163441, and GSE163443. The MacaM version 7 assembly of the Indian rhesus macaque genomic reference is publicly available at https://www.umc.edu/rhesusgenechip/index. htm. Source Data are provided with this paper.

**Code availability**

Other data that support the findings of this work, including custom ggplot2 (version 3.3.2) code for data visualization (RStudio version 1.4.1103) of Figs. 1F, 2h, 3j, 5a, e, and Supplementary Fig. 4, are available from the corresponding author on reasonable request. Source Data are provided with this paper.

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