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Justin Harper, Emory University
Nicolas Huot, Institut Pasteur, Unité HIV, Inflammation et Persistance
Luca Micci, Emory University
Gregory Tharp, Emory University
Colin King, Emory University
Philippe Rascel, Institut Pasteur, Unité HIV, Inflammation et Persistance
Neeta Shenvi, Emory University
Hong Wang, Emory University
Cristin Galardi, University of North Carolina
Amit Upadhyay, Emory University

Only first 10 authors above; see publication for full author list.

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IL-21 and IFNα therapy rescues terminally differentiated NK cells and limits SIV reservoir in ART-treated macaques

Justin Harper1,11, Nicolas Huot2,11, Luca Micci1, Gregory Tharp3, Colin King1, Philippe Rascle2,4, Neeta Shenvi5, Hong Wang1, Cristin Galardi6,7, Amit A. Upadhyay3, Francois Villinger8, Jeffrey Lifson9, Guido Silvestri1,10, Kirk Easley5, Beatrice Jacquelin2, Steven Bosinger1,3,10, Michaela Müller-Trutwin2,11 & Mirko Paiardini1,10

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.
Natural killer (NK) cells are “licensed” with functional competence following education with self-major histocompatibility complex (MHC) class I molecules. In particular human leukocyte antigen (HLA)-E, the ligand for the inhibitory CD94/NKG2a receptor2–4, is positively regulated by HLA-A expression and inhibits NK cell-mediated lysis5,6. In a companion manuscript7, Huot et al. define NK cell differentiation states based on their education via NKG2a and expression of CD16 (FeCRII), an activating Fc receptor that mediates antibody-dependent cell-mediated cytotoxicity (ADCC),8, and demonstrate that nonpathogenic SIVagm infection in African green monkeys (AGMs) imprints the maturation of NK cells inducing terminally differentiated NKG2a(low)CD16(high) NK cells, which express high levels of interleukin (IL)−12 and IL-15. It has been previously demonstrated that NK cell functionality is responsive to immunotherapies with IL-21 and interferon alpha (IFNα). For example, ex vivo IL-21 treatment expanded CD16+ NK cells9,10, antagonized the IL-15-dependent expansion of resting NK cells9,11, and reverses hyporesponsiveness via the STAT1 and PI3K-AKT-FOXO1 pathway11. Likewise, ex vivo IFNα treatment upregulates IL-15-mediated NK cell cytotoxicity12, including CD107a degranulation and ADCC activity13,14, and downregulates IL-21R expression15; furthermore, in vivo IFNα-induced NK cell cytotoxicity correlates with reductions in HIV-DNA during antiretroviral therapy (ART)16. In SIVagm 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 NKG2α(low)CD16(high) 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 ART18, 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-naive, ART-only controls (i.e., controls). ART was withdrawn at d402 p.i. and, given attenuation in IFN signaling upon sustained therapy19, 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 RMs20,21. 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 ART20,22. 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. 2); 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 cohort23, we also confirmed that ISGs are induced by pathogenic SIVmac infection and are significantly reduced, but not fully normalized, by ART (Supplementary Fig. 4), as is observed in natural hosts24,25. 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+CD8+ T-cells (Fig. 1b, representative gating strategy in Supplementary Fig. 1c) nor did it enhance T-cell responses whether by T-bet expression, which regulates Th1 cytokine expression26, 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 SIVmac239/251 Env peptides. By measuring levels of surface CD107a expression in CD107a+ NK cells, 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; i.e., see Eq. 1). IL-21 administration led to a significant retention, 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 imprints 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 viral content, which remained significantly lower than controls also at d374 p.i. (Fig. 2g). Consistent with previous observations20, 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 expected27,28. 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 tissues29, it is plausible that the levels of T-cell activation and
Cytokine therapy promotes the maturation of NKG2a/a-lowCD16+ 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 frequency of total NK cells (CD45+CD8−CD3−NKG2a/c+); 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) with the caveat that the anti-NKG2a monoclonal antibody (mAb) (clone Z199) cannot distinguish between NKG2a and NKG2c in nonhuman pri- mates (NHPs). Cytokine therapy significantly favored the generation of the terminally differentiated NKG2a/a-lowCD16+ subset (Stage 3; Fig. 3f, g) with a concomitant loss of the intermediate NKG2a/a-highCD16+ subset as a proportion of NK cells (Stage 2; Fig. 3e, g). Specifically, at d374 p.i., stage 3 NK cells constitute 46.3 ± 7.52% of total NK cells in cytokine-treated RMs as compared to 7.59 ± 2.24% in controls. Notably, cytokine therapy did not impact the frequency of these NK cell subsets relative to CD45+ lymphocytes (Supple- mentary Fig. 9a–d). These data suggest that maturation was blocked in SIVmac infection, even under ART, in favor of intermediate NKG2a/a-highCD16+ NK cells, while cytokine therapy allowed NK cells to attain terminal differentiation.

We next analyzed the innate activity of NK cells ex vivo. In cytokine-treated RMs, we observed a transient induction of IFN-γ expression (Fig. 3h, representative gating strategy in Supplementary Fig. 1c), but an increase in CD107a degranulation, specifically within the NKG2a/a-lowCD16+ subset (Fig. 3i)30. Although other NK cell differentiation subsets displayed variable levels of ex vivo innate degranulation activity, cytokine therapy skewed the total ex vivo innate activity toward being dominated by stage 3 NK cells by d374 p.i. (Supplementary Fig. 9e–h). Indeed, at all measured experimental points (d77, d217, and d374), the average %CD107a+ stage 3 NK cells were more than seven-fold higher in cytokine-treated than control RMs. The formation and activity of NKG2a/a-lowCD16+ NK cells correlate with viral recrudescence following ATI. To better 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 ATI31,32, whereas in SIV-infected RMs, prior IL-21 monotherapy during ART is not beneficial. 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 (Supple- mentary 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 rIFNα 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 rIFNα 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<sup>low</sup>, 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<sup>high</sup>, 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 (Fig. 5e, f). In summary, our results indicate that cytokine treatment during ART is effective in promoting an immunological memory response after SIV infection, with comparable levels of cell-associated SIV-RNA and SIV-DNA (Fig. 5g, h).
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 the frequency and innate activity of terminally differentiated NK cells, both on-ART (d374 p.i.) and following ATI (d13), were associated with depletions targeting the CD8β+ T-cells; the frequency of HLA-E-restricted CD8β+ T-cells and NKG2a/c+ CD8β+ T-cells; and the Env-specific NK cell activity (as indicated at left) in all RMs (n = 13; days 35, 217, and 339/374 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 log2-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 infections33-35. The formation of a terminally differentiated NK cell subset (NKG2a/e_lowCD16+) with robust innate and adaptive antiviral activities, which was found in nonpathogenic infections in AGMs7, was blocked in pathogenic SIVmac infection in favor of intermediate differentiation (NKG2a/c_highCD16+) with heightened pro-inflammatory potential36. Notably, we have demonstrated in a pathogenic model of infection that IL-21 and IFNα treatment during ART were effective in removing this block and in promoting NK cell terminal differentiation without altering their follicular homing or inducing a de novo expansion. As terminally differentiated NK cells were correlated with reductions in lymphoid replication competent virus during ART and the delay in viral rebound after ATI, these data support a role for SIV-Env-specific, HLA-E-restricted NKG2a/c+CD16+ 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 for this study and housed at YNPRC (Supplementary Table 1). All animals were Mamu-A*011:*, B08* and B17*, whereas 4 RMs were Mamu-A*01:1*, R2R12, RN122, Rsp14, and RQb13. RMs were deemed pathogen-free and housed as previously described41. 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 plasma viral loads at day 35 p.i. and their Mamu-A*01 haplotype (Supplementary Table 1). At d35 p.i. RMs began a daily, s.c. triple for- mulation ART regimen consisting of tenofovir disoproxil fumarate (TDF; 5 mg/kg/d; Gilead Sciences), emtricitabine (FTC; 40 mg/kg/d; Gilead Sciences), and dolutegravir (DGTG; 2.5 mg/kg/d; ViVi HealthCare)19 that were obtained via a material transfer agreement (MTA). Nine RMs were administered two cycles of rhSIV 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. 500 μg/kg) 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 controls (i.e., ART (controls)) and two RMs (RbK11 and RNα12) were projected to be controllers based on pre-established criteria45; 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/c⁹⁶CD16⁺ 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, 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/c⁹⁶CD16⁺), d Stage 1 (blue, NKG2a/c⁹⁶CD16⁺), e Stage 2 (orange, NKG2a/c⁹⁶CD16⁺), and f Stage 3 (purple, NKG2a/c⁹⁶CD16⁺). g The mean frequency of each NK cell differentiation stage from above was also re-visualized 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/c⁹⁶CD16⁺) NK cells. a–i Data from individual RMs (staggered open circles) are overlaid against the mean (solid line) ± SEM (shaded region within the dashed lines): control (ART-only, black; 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’s 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 (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.

Fig. 4 Cytokine therapy delays the rebound of plasma viremia following ATL. 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. e The log₁₀ SIV-RNA copies/ml are given as a longitudinal mean (solid line with closed circles) ± SEM (color-coded shaded region within the dashed lines), 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 described. 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 prepped. 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 U/ml 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 we have shown to be cross-reactive in RMs20,44,46,47 and are supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio), 100 U/ml 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.

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**Fig. 5 The formation and activity of NGK2α/c^CD16^ NK cells correlate with viral recrudescence following ATI.** a From PBMCs taken 24 h following the first (d6 post ATI) and the fifth PEG-IFNrx dose (d37 post ATI), the expression of interferon-stimulated genes was calculated as a cross-sectional log fold-change between cytokine-treated (n=8) and control (n=RMs, which is represented as a double-gradient heatmap. The size of each data point corresponds inversely to the log_{10}-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, NGK2α/c^CD16^), Stage 1 (blue, NGK2α/c^hghCD16^), Stage 2 (orange, NGK2α/c^hghCD16^), and Stage 3 (purple, NGK2α/c^hghCD16^). NK cells isolated from PBMCs at d13 post ATI were used to determine c the frequency of ex vivo innate activity (CD107a surface expression) or d the Env-specific activity against co-culture with K562 cells expressing HLA-E loaded with SIYVmac Env peptides. e Data from individual cytokine-treated (PEG-IFNrx with prior ART + IL-21 + rIfNrx, blue; n=8) and control (prior ART-only; black; n=5) RMs is 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. e 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.) 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_{10}-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 included in a 4°C for 1 h at room temperature in the dark. Data was acquired on a minimum of 50,000 live CD4+ T cells on an LSR II (BD Biosciences) using FACs Diva software (v8.0.1) and analyzed using FlowJo software (version 10.2.4; TreeStar).

Intracellular cytokine staining. Th17 and Th22 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 peripheral blood mononuclear cells derived from PBMCs were pulsed with PMA and ionomycin (24 h) in a 96-well plate for an enzyme-linked immunosorbent assay (ELISA) was used for measurement of TNF-α. DNA samples for which there were fewer than ten positive replicates for amplification were cross-reactive in RMs as validated by NHP Reagent Resource (MassBiologics): anti-human mAbs that were cross-reactive were validated using FlowJo software (version 10.4.2; TreeStar). The intracellular stain was incubated for 1 h at 37 °C under 5% CO2, 100 U/mL IL-2, and 10 ng/mL of IL-15, and then analyzed for cell surface CD107a expression via flow cytometry.

Evivo innate NK cell activity assay. NK cell degranulation activity was determined by expression of cell surface CD107a, as previously described. Cryopreserved PBMCs were labeled with anti-NK2Gr1/c-PE (clone Z199, cat. IM3291U; Beckman Coulter) conjugated with anti-PE Microbeads (Miltenyi Biotec) and magnetically isolated according to the manufacturer's instructions. NK2Gr1+ cells were cultured overnight at 37 °C with 5% CO2 in RPMI supplemented with 10% FBS, 100 U/mL IL-2, and 10 ng/mL of IL-15, and then analyzed for cell surface CD107a expression via flow cytometry.

The expression of HLA-E* and K562* cells was isolated to >95% purity by Protein-G sepharose afmed endotoxin free. Activity was verified using a Vero/EMCV bioassay. The frequency of NK cells expressing surface CD107a was measured by flow cytometry per each culture condition to assess the background (%CD107a+cells), the maximum (%CD107a+cells), and the peptide-specific degranulation activity (%CD107a+cells). From these measurements the Env-specific NK cell activity was calculated as previously described: 

\[
\text{log}_{10} \left( \frac{\%\text{CD107a}_{\text{ENV}} - \%\text{CD107a}_{\text{NK}}}{\%\text{CD107a}_{\text{NK}} - \%\text{CD107a}_{\text{NK}+\text{Env}}} \right) = \text{Env-specific NK cell activity (1)}
\]

Production and testing of rhesus rIL-21-IgFc. A fusion protein of rhesus IL-21-IgFc was produced as previously described by the Resource of Nonhuman Primate Immune Reagents at New Iberia Research Center and were provided via MTA (P20125,56,57). Using the Drosophila S2 system, a fusion protein was produced between rMamuIL-21 and a macaque IgG2 Fc, which was mutated (L235A and P331S) to block complement or Fc receptor binding. The IL-21 was isolated to >95% purity by Protein-G sepharose affinity chromatography, dialyzed against PBS, and tested for sterility and confirmed endotoxin free.

Production and testing of rhesus IFNα-IgFc. Using the Drosophila S2 system, a fusion protein was produced, as previously described, by a recombinant RM IFNα2 and a macaque IgG2 Fc by the Resource of Nonhuman Primate Immune Reagents at New Iberia Research Center and was provided via MTA. The IFNα was mutated at two positions (L235A and P331S) to block complement or Fc receptor binding. IFNα-IgFc was isolated to >95% purity by Protein-G sepharose affinity chromatography, dialyzed against PBS, lyophilized, and tested for sterility and confirmed endotoxin free. Activity was verified as 264 ± 10 μg/mg as determined by Vero/EMCV bioassay.

Peptigene alfa-2A. Pharmaceutical-grade human PEG-IFNa was purchased at cost (PEGASYS®, Roche), which was previously shown to be well tolerated in HIV-infected macaques.

RNA sequencing. RNA was extracted from PBMCs stored at −80 °C in BLT buffer with 1% 2-mercaptoethanol using RNaseasy Mini kits (QIAGEN, CA) with DNase digest and QIAcube automation stations. Extracted RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc. Wilmington, DE) and the quality was assessed by Bioanalyzer analysis (Agilent Technologies, Santa Clara, CA). Ten nanogram of total RNA was used as input for cDNA amplification using 5′ template-switch PCR with the Clontech SMART-Seq v4 Ultra Low Input RNA kit. Amplified cDNA was fragmented and amplified with dual indexed bar codes using Illumina NexeraXT DNA Library Prep kits. The amplified libraries from both sets were validated by capillary electrophoresis on the Agilent 4200 TapeStation. The libraries were normalized, pooled, and sequenced on the Illumina HiSeq 3000 system employing a single-end 101 cycles run at average read depths of 30×106 reads per sample. Reads were mapped to the MacaM version 7 assembly of the Indian rhesus macaque genomic reference by RhesusGenome using STAR (version 2.5.2b) with default alignment parameters. RNA sequencing reads 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 QVC), also 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's and/or mixed-effects models, in the event of absent data points, were performed with 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 p values were adjusted for multiple comparisons using the stepdown Bonferroni, Hochberg, and false discovery rate (FDR) 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 Permutation test (10^6 iterations) in SPICE version 6.0. Rates of increase in log2 SIV RNA copies per ml of plasma post AT1 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 slope and mean linear increase were estimated and compared between treatment conditions within the framework of the mixed-effects linear model. DESeq2 version 1.22.1R package was used to produce normalized read counts and compute the differential expression estimation using the Wald test. Multiple-test correction was performed with the Benjamini–Hochberg method and a FDR <0.05 was used to indicate statistical significance.

**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 GSE13443 with subseries GSE13440, GSE13441, and GSE13443. The MacaM version 7 assembly of the Indian rhesus macaque genomic reference is publicly available at https://www.unmc.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, and Supplementary Fig. 4, are available from the corresponding author on reasonable request. Source Data are provided with this paper.

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Author contributions
J.H. contributed to conceptualization, methodology, formal analysis, investigation, writing (original draft, review, and editing), and visualization. N.H. performed the phenotyping and ex vivo functional assays on NK cells and contributed to conceptualization, methodology, investigation, data analysis, and writing (review and editing). L.M. contributed to conceptualization, methodology, and investigation. G.T., A.A. U., and S.B. performed RNA-seq analyses. C.K. and H.W. performed longitudinal processing and flow cytometry of tissues. P. R. contributed to methodology and investigation. C.G. performed the ELLI-Spot experiments. N.S. and K.E. performed statistical analyses of the correlations and viral rebound kinetics. F.V. provided investigational compounds. J.L. measured virus content in plaque assays. A.A. and G.S. contributed to conceptualization. B.J. contributed to methodology. M.M.-T. contributed to conceptualization, methodology, writing (review and editing), supervision, and funding acquisition. M.P. contributed to conceptualization, methodology, resources, writing (original draft, review, and editing), supervision, and funding acquisition.

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The authors declare no competing interests.

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