SIV-induced terminally differentiated adaptive NK cells in lymph nodes associated with enhanced MHC-E restricted activity

Nicolas Huot, Institut Pasteur, Unité HIV, France
Philippe Rascle, Institut Pasteur, Unité HIV, France
Caroline Petitdemange, Institut Pasteur, Unité HIV, France
Vanessa Contreras, Université Paris Sud-Inserm
Christina M Stuerzel, Ulm University
Eduard Baquero, Institut Pasteur, Unité HIV, France
Justin L Harper, Emory University
Caroline Passaes, Institut Pasteur, Unité HIV, France
Rachel Legendre, Institut Pasteur, Unité HIV, France
Hugo Varet, Institut Pasteur, Unité HIV, France

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

Journal Title: NATURE COMMUNICATIONS
Volume: Volume 12, Number 1
Publisher: NATURE PORTFOLIO | 2021-02-24, Pages 1282-1282
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1038/s41467-021-21402-1
Permanent URL: https://pid.emory.edu/ark:/25593/vsrx8

Final published version: http://dx.doi.org/10.1038/s41467-021-21402-1

Copyright information:
© The Author(s) 2021

This is an Open Access work distributed under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/rdf).

Accessed September 30, 2023 11:05 AM EDT
SIV-induced terminally differentiated adaptive NK cells in lymph nodes associated with enhanced MHC-E restricted activity

Nicolas Huot, Philippe Rascle, Caroline Petitdemeange, Vanessa Contreras, Christina M. Stürzel, Eduard Baquero, Justin L. Harper, Caroline Passaes, Rachel Legendre, Hugo Varet, Yoann Madec, Ulrike Sauermann, Christiane Stahl-Hennig, Jacob Nattermann, Asier Saez-Cirion, Roger Le Grand, R. Keith Reeves, Mirko Paiardini, Frank Kirchhoff, Beatrice Jacquelin & Michaela Müller-Trutwin

Natural killer (NK) cells play a critical understudied role during HIV infection in tissues. In a natural host of SIV, the African green monkey (AGM), NK cells mediate a strong control of SIVagm infection in secondary lymphoid tissues. We demonstrate that SIVagm infection induces the expansion of terminally differentiated NKG2a\textsuperscript{low} NK cells in secondary lymphoid organs displaying an adaptive transcriptional profile and increased MHC-E-restricted cytotoxicity in response to SIV Env peptides while expressing little IFN-γ. Such NK cell differentiation was lacking in SIVmac-infected macaques. Adaptive NK cells displayed no increased NKG2C expression. This study reveals a previously unknown profile of NK cell adaptation to a viral infection, thus accelerating strategies toward NK-cell directed therapies and viral control in tissues.
The impact of NK cells on viremia in HIV-1 infection has been clearly demonstrated through studies on HLA-I alleles. Burgeoning data show that the NK cell repertoire is not stable and that its diversity increases with age. The NK cell repertoire diversity reflects immune history and correlates with viral susceptibility. This raises the possibility that there are unexpected functional specialization and distinct adaptive capabilities among NK cell subpopulations in response to viral infections and immunizations, a mechanism which could be exploited to promote antiviral immunity. African non-human primates, such as African green monkeys (AGM), have been a natural host for simian immunodeficiency viruses (SIV) for probably over 1 million years. In contrast to people living with HIV (PLH), natural SIV hosts typically do not progress toward disease despite persistent high viremia. We have previously shown that AGMs efficiently control viral replication in SLT (both in the T zone and in B follicles), and that this viral control is mediated predominantly by NK cells. No such viral control is generally observed in lymph nodes (LN) of PLH and in the non-human primate model of HIV, i.e., SIVmac-infected macaques (MAC), where SLT constitute a major viral reservoir. The capacity to control viral replication in B follicles of AGM was associated with the presence of CXCR5 NK cells.

Here, we hypothesize that non-pathogenic (AGM) and pathogenic HIV-1/SIVmac infections differentially imprint NK cells and thereby contribute to the the distinct capacity of the NK cells to control SIV/HIV replication in SLT. In this work, we show that SIVagm infection induces the expansion of terminally differentiated NKG2a low NK cells in SLT displaying an adaptive transcriptional profile and increased MHC-E-restricted cytotoxicity in response to SIV Env peptides. THEMIS but not NKG2C expression was increased in the adaptive NK cells. Our longitudinal analysis also uncovers, that such NK cell terminal differentiation is blocked in SIVmac-infected macaques, in which NK cells showed a decrease in MHC-E-restricted cytotoxicity in response to SIV Env peptides and rather frequently expressed IFN-γ. This study reveals a distinct NK cell adaptation to a viral infection. It also improves our understanding of NK cell dysfunction in HIV infection and thereby opens avenues to improve NK-cell mediated viral control in HIV cure strategies.

Results

Blood NK cell diversity during SIV infection in the natural versus heterologous host. NK cell effector function is known to increase together with the diversity of the receptor repertoire on NK cells. We first estimated and compared phenotypic changes in blood NK cells following SIV infection between pathogenic and natural host models. The NK cell gating strategy and animals are describe in, respectively, Supplementary Figs. 1 and 2. Force-directed clustering analysis was performed based on expression data from nine markers of NK cells followed longitudinally (between day 0 and day 240 p.i.) in 6 MAC and 6 AGM. This generated NK cell cluster profiles that were different between AGM and MAC following SIV infection but consistent between monkeys from a same species (Fig. 1a, b and Supplementary Fig. 3a, b). Both in AGM and MAC, IFN-γ and perforin showed the strongest positive correlation to each other (Supplementary Fig 3c, d). However, only in AGM, markers studied longitudinally throughout infection were often inter-correlated, while less or not correlated in MAC (Supplementary Fig. 3c, d). For instance, in SIVagm infection, IFN-γ correlated with the expression of the activating receptors Nkp46, Nkp30, and NKG2D, but this was not the case during SIVmac infection. These results suggest a more coordinated action of NK cells during SIVagm than SIVmac infection. When we analyzed the association between viremia levels and the receptor repertoire, the only negative correlation observed was between viremia and CD16 in AGM (p = 0.006, p = −0.3; Supplementary Fig. 3c, d).

We next analyzed distinct NK cell subpopulations stratified by CD16 and NKG2a/c, two markers commonly used to define NK cell terminal maturation in humans (Supplementary Fig. 1). Follow up of these subsets during infection in blood did not reveal major differences within each species studied, but highlighted profound differences between AGM and MAC, such as an expansion of NKG2a high/CD16− NK cells in SIVmac infection (Fig. 1c, d). We then analyzed the functional activity of these blood NK cell subsets based on the ex vivo expression of the CD107a surrogate marker. The activity decreased in all subsets in response to SIV infection for both AGM and MAC (Supplementary Fig. 3e, f). However, the activity of NKG2a low/CD16++ NK cells returned rapidly to normal levels in AGM but not in MAC.

In order to evaluate the differentiation and functionality of the NK cells during SIV infection in a comprehensive way, we implemented a genome-wide transcriptional mapping. We sorted the four NK cell subpopulations stratified by CD16 and NKG2a/c expression from the blood of chronically infected monkeys. We identified 2586 and 1895 genes to be differentially regulated between the distinct NK cell subpopulations in MAC and AGM, respectively (Fig. 2a). We analyzed in more detail the gene expression profiles of NK cell receptors (Fig. 2b), transcription factors (Fig. 2c), and effector molecules (Fig. 2d). Genes upregulated in NKG2a low/CD16− NK cells included those associated with tissue homing (CXCR3), proliferation (MKI67), and transcription factors involved in cellular stemness and quiescence such as Tcf7 (TCF-1), indicating a low differentiation stage when compared to the other subsets. In contrast, NKG2a high/CD16− NK cells expressed elevated levels of genes associated with tissue egress and circulation (CX3CR1) and genes encoding transcription factors associated with effector function and terminal differentiation, including TBX21 (T-bet) and ZEB2. The other two NK cell subpopulations displayed gene expression profiles of intermediate differentiation. Thus, the combination of NKG2a/c and CD16 allowed the determination of distinct NK cell differentiation stages in non-human primates (NHP). Together these results uncovered profound differences in NK cell behavior following SIV infection between the two models that seem to be driven by differences in the terminal differentiation process.
infection (Fig. 2b and Supplementary Fig. 4a, b). In SIVagm infection, the two NKG2a/c\textsuperscript{low} subsets (NKG2a/c\textsuperscript{low}CD16\textsuperscript{+} and NKG2a/c\textsuperscript{low}CD16\textsuperscript{−}) increased in acute and chronic infection, respectively (Fig. 3b). These expansions were probably not a consequence of proliferation as the Ki-67 expression levels did not increase in the NKG2a/c\textsuperscript{low} cells (Fig. 3c). The NKG2a/c\textsuperscript{low} NK cells exhibited increased cytolytic activity (CD107a, Perforin) during primary and chronic SIVagm infection, but not in SIVmac infection, except for a transient response between days 2 and 14 p.i. (Fig. 3c and Supplementary Fig. 4e, f). NK cells from MAC showed ex vivo increases of IFN-γ expression in response to SIVmac infection, while in AGM, the expression level of IFN-γ decreased in LN NK cells (Fig. 3c). AGM rather displayed increased expression of Perforin during primary SIVagm infection in contrast to SIVmac infection (Fig. 3c and Supplementary Fig. 4a, b). A correlation matrix showed indeed a positive correlation of NKG2a/c with CD16, IFN-γ, and NKG2D for MAC (Supplementary Fig. 4c), while AGM showed a negative correlation between NKG2a/c and perforin (PRF) (Supplementary Fig. 4d). In addition, more correlations were noticed again for NK cells in SIVagm as compared to SIVmac infection, suggesting, as in blood, a better coordination of NK cell response in SIVagm than SIVmac infection. Altogether, LN NK cells displayed higher expressions of NKG2a/c with a more
pronounced cytokine (IFN-γ) profile in SIVmac infection, while a more cytotoxic profile (Perforin) correlating with low NKG2a/c expression was observed in SIVagm infection.

We analyzed if there was a relation between the observed NK cell subsets and viral load in LN. In MAC, only weak positive correlations were observed, i.e. between the NKG2a/chighCD16+ NK cells and both cell-associated viral (ca-v) RNA (viral load in LN ($p = 0.02$) and ca-vDNA ($p = 0.048$) (Supplementary Table S1). In AGM, a positive correlation was observed for NKG2a/clowCD16− NK cells ($p < 0.008$ for ca-vRNA and ca-vDNA), while a negative correlation was observed with the NKG2a/chighCD16− NK cells ($p < 0.03$ for ca-vRNA and ca-vDNA; Supplementary Table S1). No significant negative correlations were measured between NK cell markers and viral load in LN in MAC or AGM (Fig. 3d, e). The data suggest that viral replication is driving the expansion of NKG2a/chighCD16+ NK cells in SIVmac infection but of NKG2a/clowCD16− NK cells in SIVagm infection.

In order to perform an unbiased analysis of NK cell subsets in SLT, we performed a genome-wide transcriptional mapping of the LN NK cells. Four NK cell subsets were sorted from the LN of the same animals and same time points as described above for the blood. The four subsets corresponded to NKG2a/clowCD16−, NKG2a/chighCD16−, NKG2a/clowCD16+, and CXCR5+ NK cells. The latter were known to play a role on viral replication in SLT20. The NKG2a/chighCD16+ subset was not sorted due to the extremely low number of these cells in LN. Heatmaps were drawn for genes coding for NK cell markers (Supplementary Fig. 5a), transcription factors (Supplementary Fig. 5b), and receptors associated with trafficking and immune function (Supplementary Fig. 5c). The CXCR5+ NK cells clustered separately from the other NK cell subpopulations in LN, but consistently closely to the NKG2a/clowCD16− NK cells (Fig. 4a and Supplementary Fig. 5a–c).

The genome-wide analysis of all subsets in LN showed, in line with the observations made in blood (Fig. 2 and Supplementary
**Fig. 3 Dynamics of NK cell repertoire and activity in response to SIV infection in LN.**

**a** Pie charts and arcs showing the proportion of different combinations of markers on NKG2a+ NK cells isolated from LN during SIVmac (left panel) and SIVagm (right panel) infections, as analyzed using SPICE 5.3.

**b** Frequency of NK cell subpopulations out of total NK cells in LN. Colors are the same as in Fig. 1. Cells were sampled before and during SIVmac and SIVagm infection (n = 6 animals per species) at the time point indicated by the X axis. The full line indicates the median, and the interquartile range is indicated by the area between the dotted lines. For Groups comparisons two-sided Wilcoxon signed-rank test with Bonferroni correction were used (n = 6). P values ≤ 0.05 were considered statistically significant. Asterix indicate significant change when compared to the base line. Exact P values are provided on the graphs.

**c** Longitudinal profiles of intracellular levels of Perforin, interferon gamma, Ki-67, and surface level of CD107a on NK cell subsets ex vivo during SIVmac (left panel) and SIVagm (right panel) infections without any prior stimulation. The colors for each NK cell subpopulation are the same as in Fig. 1. Each symbol represents one monkey, the black line indicates the median and error bars the interquartile range. For group comparisons, two-sided Wilcoxon signed-rank test with Bonferroni correction was used (n = 6). P values ≤ 0.05 were considered statistically significant. Asterix indicate significant change when compared to the base line, graphically annotated as follows: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

**d, e** Pearson correlation matrix between viral load in LN (ca-vDNA and ca-vRNA) and expression level of functional markers showed in Fig. 3c for d MAC and e AGM. The orange and blue colors indicate higher and lower r value. The p value is indicated by the size of the circle. All p and r values are given in the Data Source file.
Fig. 4 Deep transcriptional differences between blood and pLN NK cells subsets during chronic SIVagm infection. a Heatmap showing the 5,489 genes differentially regulated between blood and LN NK cells in chronically SIVagm-infected AGM. The orange and blue colors indicate higher and lower levels of transcripts measured in blood and LN NK cell subsets from three chronically infected monkeys. Each row represents a variable gene among clusters, and each column represents the NK cell subset for an individual monkey. The NK cell subsets are indicated in distinct colors. b Clustering of samples using the first five principal components (PC) of the indicated subset with percentages of variance associated with each axis based on the mean expression of the genes with variable expression. Each dot represents one monkey and circle around the dot represents one NK subset.

Fig. 5a–c), that the LN NKG2a/clowCD16+ NK cells expressed high levels of markers known to be associated with NK cell terminal differentiation. Also, the NKG2a/chighCD16– NK cells showed again the less differentiated phenotype and expressed high levels of transcripts coding for NKG2D (KLRK1), Nkp46 (NCR1), and CD56 (NCAM-1) similar to the CD56high NK cell population observed in human SLT.15. Differential expression of NK cell markers at the transcription level were confirmed at the protein level by flow cytometry (Supplementary Fig. 5d). This analysis confirmed that CD16 combined with NKG2a/c allowed distinguishing NK cell differentiation stages in LN. Taken together, this demonstrates that SIVmac infection led to an accumulation of only the intermediate, stage of NK cell differentiation in LN, whereas in SIVagm infection, the fully differentiated, cytolytic NKG2a/clowCD16+ NK cells expanded.

LN-specific NK cell gene signature during SIVagm infection in the natural host. We next hypothesized that NK cells in LN, which are known to be able to eliminate SIVagm-infected cells in AGM, display LN-specific features distinct from NK cells in blood where the virus is not controlled. We therefore compared the genome-wide transcriptome profiles between blood and LN (Fig. 4a, b). Surprisingly, the CD16– and CD16+ NK cells from LN were more closely related to each other than to their respective subsets in the blood. As many as 5489 genes displayed expression differences between LN and blood NK cells (Fig. 4a). The four NK cell subpopulations in the LN segregated clearly into distinct clusters, while they were less separated from each other in the blood (Fig. 4a,b). Gene Ontology (GO) enrichment analysis indicated that in LN from SIVagm-infected animals, NK cells displayed an enrichment for pathways involved in viral responses, regulation of defense, IFN response, pattern recognition receptor signaling and inflammasome activation (NLRP3, NLRP1, and NLRC4) compared to blood NK cells (Supplementary Fig. 6a–c). In contrast, blood NK cells displayed an enrichment in biological process terms, such as regulation of stress-activated mitogen-activated protein kinase (MAPK) cascade and of histone modifications (Supplementary Fig. 6d–f). Thus, LN NK cells displayed a common gene signature distinct from blood during SIVagm infection in the natural host showing for instance stronger viral defense and inflammasome activation.

Adaptive NKG2a/clowCD16+ NK cells in LN during SIVagm infection. We next addressed the question of the overall anti-viral activity profiles in the NK cells which expanded in LN upon acute (CXCR5+), NKG2a/clowCD16+ and/or chronic SIVagm infection (CXCR5+, NKG2a/clowCD16−) using the genome-wide expression data (Fig. 5). We compared these three NK cell subsets to the remaining subset (NKG2a/chighCD16−). The NKG2a/clowCD16+ NK cells were those with the highest number of upregulated genes (1072 genes) compared to NKG2a/chighCD16− NK cells followed by 432 upregulated genes in NKG2a/clowCD16− and 294 genes in CXCR5− NK cells. As many as 176 genes were commonly upregulated among the three expanded subsets, and one gene was downregulated, CCDC102B (Fig. 5a). Among the 176 upregulated genes, GO enrichment analysis uncovered pathways important for the lytic activity of NK cells, such as pathways associated with the biology of cytotoxic granules, including lytic vacuoles, secretion by cells, lysosomes and lytic vacuole membranes, cellular assembly and microtubule cytoskeleton (Fig. 5b, c). Among the genes related to cytokines were CXCL11, CXCL12, IL-27, IFNγR, IL15RA, and IL21R and inflammasome-related proteins included NLRP3 and IL1B (Fig. 5c). Many of the other upregulated genes are known to be associated with metabolism, such as GLUL (glutamine synthetase); H6PD (Glucose-6-phosphate-dehydrogenase), ENTPD1 (CD39), and ATP13A2. The NKG2a/clow NK cells also expressed high levels of Lamp1 (CD107a; p < 0.0000000005) and CCL5 (p = 0.0003; Fig. 5c). These data are consistent for CXCR5+ and NKG2a/clow NK cells, and in particular for NKG2a/clowCD16+ NK cells, displaying a strong degranulation activity in LN.
Strong cytotoxic potential and inflammasome activation are compatible with the generation of adaptive NK cells. We next analyzed the expression of markers which are so far described as the most specific to adaptive NK cells, meaning the modulation of transcription factors as well as the variable loss of some intracellular adapter signaling molecules. The NKG2a^{high}CD16− NK cells did not resemble adaptive NK cells, since they expressed high levels of LCK, CD247 (CD3z), ZAP70, SYK, Fyn, SH2D1B, TYROBP (DAP12), and HCST (DAP10). In contrast, the NKG2a^{low}CD16+ NK cells in LN fully displayed gene expression profiles characteristic of adaptive NK cells, such as low expression of LCK, ZAP70, FceRy, and SYK.
and high expression of GRAP2 (Fig. 5d). The NKG2a/clowCD16- and CXCR5+ NK cells also shared several adaptive NK cell-like features, including low expression of FceRIy and VAV1 (Fig. 5d). The levels of KLRC2 (NKG2C) were negligible when compared to KLRC1 (NKG2A) transcripts in all NK cell subsets. Altogether, these findings revealed that during SIVagm infection, LN NKG2a/clowCD16+ NK cells, and to a certain extent also NKG2a/clowCD16- and CXCR5+ NK cells, displayed a transcriptional profile of adaptive NK cells.

We then searched for genes that could be linked to the generation of adaptive NK cells and identified 15 genes that were strongly expressed in NKG2a/clowCD16+ NK cells (Fig. 5e). Among the highly expressed genes were those coding for ZNF831, JAKMIP2, PLBD1, UBASH3, S100A9, LAT (linker for activation of T cell), GRAP2 (GRB2-related adapter protein 2) and THEMIS (Thymocyte-Expressed Molecule Involved in Selection; Fig. 5d, e). THEMIS showed a positive correlation with GRAP2 ($p < 0.004, r = 0.74$) and a negative correlation with NKG2A ($p < 0.002, r = −0.83$) expression (Fig. 5f). Altogether, the data revealed the presence of adaptive NK cells during SIVagm infection expressing low levels of NKG2C and high levels of Themis.

High MHC-E expression on LN memory CD4+ T cells. SIVagm-infected AGMs were thus characterized by an expansion of NKG2Aclow in SLT. NKG2A is an inhibitory receptor that binds to a non-classical MHC type I, the MHC-E. Shaping of NK cell function via self-reactive inhibitory NK cell receptors, such as NKG2A, is a well-described process of education39-41. Decreased NKG2A expression is thus indicative of NK cell education. The education via NKG2A involves recognition of peptides presented by MHC-E. MHC-E generally binds self-peptides encoded in the leader sequence (LS) of classical MHC class I molecules, such as the VL9 nonamer peptide42. MHC-E can also bind nonamer peptides derived from the stress protein HSP60 as well as pathogen-derived peptides43-46. MHC-E is generally expressed at lower levels than other MHC-I molecules and binding of peptides stabilizes MHC-E expression at the cellular surface42. The expansion of NKG2Aclow NK cells in the LN of AGM might be indicative that they became educated to be less inhibited by peptides presented by MHC-E. We evaluated the levels of MHC-E expression on the viral target cells in LN. We found that before SIV infection, among all CD4+ T cells in LN, MHC-E+ cells were found most often among memory CD4+ T cells and Tfh cells, both in MAC and AGM (Fig. 6a, c and Supplementary Fig. 7a-c). After SIVmac infection in MAC, both the percentage of MHC-E+ memory CD4+ T cells as well as the expression intensity of MHC-E on CD4+ T cells increased and were particularly high for Tfh cells (Fig. 6b, d and Supplementary Fig. 7a, b, d, g, i). In contrast, in SIVagm infection, MHC-E expression was only transiently increased in acute phase and the percentage of MHC-E+ Tfh cells decreased (Fig. 6d and Supplementary Fig. 7). Co-staining of MHC-E expression with SIV RNA could be observed (Fig. 6e). AGM B cell follicles showed accumulation of NK cells close to the MHC-E+ cells (Fig. 6f and Supplementary Fig. 8). Therefore, MHC-E expression was most frequent among the preferential target cells for SIV and HIV infection, i.e. memory CD4+ T cells and in particular on Tfh cells.

MHC-E restricted SIV-specific NK cell activity increased after SIV infection in the natural host. We set up a functional assay to analyze if NK cells show a different ability of MHC-E restricted viral suppressive activity during pathogenic and non-pathogenic SIV infection. To this end, we searched for SIV-derived peptides that would have a high probability for binding to MHC-E and examined whether the SIV genome sequence encodes amino acid sequences similar to the canonical MHC-E binding motif. As described in more detail in the Methods section, we identified in the LS of ENV, nonamer peptides containing the canonical sequence motif of MHC-E binding peptides. These sequences were in addition close to the signal-peptidase cleavage site, therefore showing a potential for the identified peptides for being cleaved in vivo. The identified SIV ENV peptides stabilized HLA-E expression on MHC-I devoid K562 cells stably transduced with HLA-E*0101 (K562-E*0101) (Fig. 7a) indicating their ability for binding to HLA-E. To further analyze the efficacy of binding, competition-binding assays were performed (Fig. 7b). The SIV ENV peptides displaced peptides known to strongly bind to HLA-E (VL9 or HSP60) already at low concentrations (1 μM). The concentrations needed by SIV ENV peptides for displacement of VL9 and HSP60 were in a similar range as those needed by other viral peptides known to efficiently bind to HLA-E (i.e. HIV-1 GAG and HCV peptides47) and even lower than for the previously reported SIVmac GAG peptide48 (Supplementary Fig. 9a). These analyses demonstrate that peptides derived from SIV ENV efficiently bind to HLA-E.

We then analyzed the MHC-E-dependent activity of NK cells toward target cells presenting the SIV ENV peptides via MHC-E.
We first studied NK cells from uninfected AGM and MAC. NK cells were co-cultured with K562-E*0101 cells pre-loaded or not with the peptides and their activity was measured by CD107a expression (Fig. 7c, d). As expected, the HLA-I-derived VL9 peptide inhibited NK cell activity, while the HSP60 peptide did not, validating our assay. When pulsing the cells with the SIVmac ENV peptide, NK cell activity from both MAC and AGM were inhibited. The SIVagm peptide displayed an intermediate inhibitory activity on NK cells. We confirmed these results with NK cells from another MAC species (i.e. Indian rhesus macaques; Fig. 7d). The peptides tested did not cover the potential variability of the virus during the infection. However, it should be noted that they were localized in a region which is among the most conserved in the SIV genome\textsuperscript{49–51}. Altogether, the data show that
the NK cells from uninfected animals showed a MHC-E restricted activity that varied dependent on the SIV ENV sequence and that they were more inhibited by the SIVmac ENV than the SIVagm ENV peptides tested.

As a proof of concept of the capacity of the ENV peptides to be processed in primary cells and to be recognized differentially by NK cells depending on their sequence when presented by simian MHC-E, we applied a functional assay using AGM primary CD4+ T cells as targets and full-length infectious SIV as a source for the peptides. Two infectious molecular mutant viruses deriving from the wild-type SIVagm.sab92018 backbone were generated. In this backbone, the ENV sequence coding for the nonamer peptide of the wild-type virus (IGIVVIVKL) was replaced either by the sequence coding for the SIVmac239/251 peptide (NQLLIAILL) or by the VL9 sequence. The mutant viruses replicated efficiently in primary CD4+ T cells from non-infected AGM (Fig. 7e). The in vitro infected primary CD4+ T cells were co-cultured with autologous NK cells. NK cells strongly suppressed replication of the wild-type SIVagm (Fig. 7e). They controlled the replication of the VL9 mutant less than the wild-type SIVagm, in line with a stronger inhibitory effect of the VL9 peptide. CD4+ T cells infected with the virus containing the sequence coding for the SIVmac ENV peptide escaped NK cell suppressive capacity. These results confirmed a distinct MHC-E restricted NK cell suppressive activity depending on the ENV sequence.

We then reasoned that if NK cells are educated through the NKG2α/MHC-E axis, then the MHC-E restricted viral suppressive capacity of the NK cells after SIVagm infection should be even higher than before SIV infection. We therefore compared the MHC-E dependent suppressive activity of NK cells before and after SIV infection. We exposed NK cells from blood and SLT to K562-E+0101 cells loaded or not with peptides. NK cells from LN of SIVagm-infected AGM, while NK cells from SIVmac-infected AGM, showed an increased MHC-E restricted NK cell activity in presence of all SIV ENV peptides tested (Fig. 8c, d). We also analyzed NK cells from spleen. As observed for LN, MHC-E restricted activity of NK cells from spleen showed trends for decreased activity toward cells presenting ENV peptides from SIVmac239/251, SIVagm.sab, SIVagm.tang, and SIVgsn in MAC after SIVmac infection, while this activity increased after SIVagm infection in the presence of SIVagm.sab92018, SIVagm.sab, and SIVmac239/251 peptides (Supplementary Fig. 9c). Altogether, our results revealed that the MHC-E restricted suppressive NK cell activity decreased in SIV-infected MAC in SLT, while it was enhanced in SIV-infected AGM.

**Discussion**

Previous studies mostly focused on circulating NK cells during HIV/SIV infection, and only a few studies on tissue NK cells have been reported. Here, we designed a study comparing NK cells associated with a strong viral control in SLT (SIVagm infection) to NK cells lacking the capacity of an efficient viral control (SIVmac infection). Our results show that SIV infection in the natural host induced terminally differentiated NKG2AlowCD16+ NK cells in LN displaying a strong degranulation activity and a transcriptional profile of adaptive NK cells. Consistently, the MHC-E restricted NK cell activity against target cells presenting SIV peptides was increased after SIVagm infection. In contrast, the process of terminal NK cell differentiation was blocked in LN during SIVmac infection of MAC in early infection, despite the generation of a highly diverse NK cell pool. Our results uncover that NK cells are educated to efficiently kill SIV-infected cells in SLT during non-pathogenic SIVagm infection while this education is not occurring in pathogenic SIV infection.

The notion that NK cell differentiation is the result of a continuous process that can adapt in response to a viral infection is relatively recent. By analyzing distinct NK cell subsets from AGM and MAC at the transcriptomic and protein level we demonstrate here that NKG2A combined with CD16 defines distinct stages of NK cell differentiation in monkeys. This provides a tool for preclinical studies in NHP for the investigation of other infections and for vaccine studies in general.

The NKG2αlowCD16+ NK cells clearly displayed the most differentiated profile and strong cytotoxic activity. We also observed cytotoxic activity for the NKG2αlowCD16− and CXCR5+ NK cells. CD16 can be quickly shed from the NK cell surface upon activation. Under these conditions, NKG2αlowCD16− and CXCR5+ NK cells might be a mixture of less differentiated NK cells together with terminal differentiated NKG2αlowCD16+ cells that exert the viral control. The NKG2αlowCD16−xCR5+ NK cells might be responsible for the viral control in B cell follicles and the NKG2α+CD16+CXCR5− NK cells in the T cell zone of lymphoid tissues. Future studies will need to address which of the activatory NK cell receptors are involved in the suppressive activity.
**Fig. 7 Analysis of MHC-E restricted NK cell activity.**

**a** Each histogram shows HLA-E expression after loading K562-E*0101 cells with the different peptides. Light gray curves indicate the mean fluorescent intensity (MFI) of HLA-E on K562 cells (control), the dark gray curves the MFI of HLA-E on K562-E*0101 cells without peptides, and the red curves the MFI of HLA-E on K562-E*0101 cells loaded with the given peptide. VL9 and HSP60 are control peptides well known to bind to MHC-E and deriving respectively from HLA-I and heat-shock protein. The SIVmac251 and SIVmac239 peptide sequences were identical. One representative experiment out of more than three is shown. On the right side are shown the localization of the peptide stained with Alexa 568 (red). Nuclei were stained with DAPI (blue).

**b** Competition assay for binding to HLA-E. K562-E*0101 cells were loaded with biotin-A2 VL9 (left) or HSP60 peptides (right). An increasing concentration of the indicated unlabeled competitor peptides were introduced into the culture. HLA-E-bound biotinylated peptide was measured at the cell surface. line represent the median of three independent experiments. Error bars represents the range.

**c** Analysis of HLA-E dependent activity of NK in presence of K562-E*0101 cells loaded or not with peptides. Activity was evaluated by CD107a cell surface expression. Dot plots show representative examples for one animal per species.

**d** HLA-E dependent activity of NK cells from blood of six uninfected cynomolgus MAC (orange squares), four uninfected rhesus MAC (red circles) and six uninfected AGM (blue triangles) in the absence or presence of peptides, as indicated. The black line indicates the median and error bars the interquartile range. Each symbols represents a unique monkey. For Groups comparisons two-sided Wilcoxon signed-rank test with Bonferroni correction were used (n = 5). P values of less or equal to 0.05 were considered statistically significant. Asterix indicate a statistically significant difference. The exact P value is provided.

**e** SIV suppressive capacity of autologous NK cells depending on the ENV nonamer sequence in the infectious virus. The panels show in vitro replication levels of SIVagm.sab92018 (gray), SIVagm.sab92018 mutant with the sequence coding for the VL9 peptide (purple) and the SIVagm.sab92018 mutant with the sequence coding for the SIVmac nonamer peptide (black) in primary CD4+ T cells from 8 uninfected AGM cultured alone or in presence of autologous NK cells (green). For each of the three viruses, left panels show longitudinal viral replication pattern (days 0, 7, 11 and 15 p.i.) and right panels viral replication at the end time-point of the culture (day 15 p.i.). The y-axis reports viral replication level as quantified by SIV p27. The black line indicates the median and error bars the interquartile range. Significant difference between the two conditions was determined using using two-tail Mann–Whitney per experiment. Asterix indicate a statistically significant difference. The exact P value is provided.
Also, while our study demonstrates a superior MHC-E restricted NK cell activity in the natural host, this might not be the unique mechanism of viral suppression, since terminally differentiated cells also up-regulate for example ADCC-mediated killing capacity\(^5\).

The reasons why NKG2\(^{a/c}\)low NK cells expanded in SIVagm infection in AGM but not in SIVmac infection in MAC are unclear. These cells did not show increased proliferation and it is unlikely that they result from increased trafficking to lymphoid tissues, since we reported that LN homing receptors decrease on NK cells from AGM in SIVagm infection similarly to SIVmac and HIV infection\(^6\). It is therefore more likely that this expansion of NKG2\(^{a/c}\)low NK cells is due to NK cell differentiation in situ\(^1\). A major difference between the two infections consists in the persistence of an inflammatory environment in SLT during SIVmac infection while inflammation is resolved in SIVagm infection\(^5\). Systemic inflammation has been shown to drive the expansion of IFN-\(\gamma\) producing NK cells in mice\(^5\). Similarly, it has been recently shown that inflammatory cytokines drive the expansion of CD94\(^\*\)CD56\(^{bright}\) NK cells with an increased capacity to produce IFN-\(\gamma\) in HIV infection that could themselves contribute to the inflammation\(^6\). These cells might correspond to the NKG2\(^{a/c}\)highCD16\(^{+}\) NK cells that were the predominant NK cell subset in LN of SIVmac-infected MAC and expressing IFN-\(\gamma\)\(^6\). Notably, the terminally differentiated NK cells in AGM LN expressed high levels of IL15 and IL21. Higher levels of IL-15 and IL-21 in the local environment during SIVagm infection as compared to SIVmac infection might allow NK cell terminal differentiation. Considering that AGMs are ancient hosts of SIV, it is possible that the long-term co-existence in AGM selected a specific tissue environment in response to SIV infection that allows proper education of NK cells. By driving NK cells toward a terminal differentiated, cytotoxic phenotype, this would allow the presence of NK cells with capacity to kill infected cells in LN, while avoiding the inflammation associated with NK-cell mediated IFN-\(\gamma\) production.

The distinct MHC-E expression profiles on CD4\(^+\) T cells in LN between SIVmac and SIVagm infection might be determined by host and/or viral factors\(^1\). The frequency of productively infected cells in chronic SIVagm infection in SLT is too low to explain the decreased levels of MHC-E solely by virus-mediated down-regulation of MHC-E or NK-cell mediated killing of MHC-E\(^+\) cells. A distinct inflammatory environment between SIVmac and SIVagm infections, in particular higher levels of IFN-\(\gamma\) in SIVmac than SIVagm infection\(^6\), could be one reason for the higher MHC-E expression in SIVmac infection.

It has been shown that genetic factors determining MHC-E expression levels strongly affect viral load in HIV-infected individuals\(^1\). This is in line with reports on the importance of MHC-E in SIV control. It has previously been reported that MHC-E-dependent NK cell suppressive capacity is dependent of the nature of the peptide loaded by MHC-E\(^1\) and that peptides derived from SIVmac can inhibit NK cells\(^1\). Only few MHC-E binding viral peptides have been described so far\(^5\). We define here a lentiviral region (ENV-LS), that can code for peptides with the capacity to bind to MHC-E. This region might thus serve as a critical target for NK cell directed
HIV vaccines, cure strategies, or other therapeutic modalities. Further analyses are needed to identify the sequences of peptides bound to MHC-E in vivo. Of note, the target cells and peptides used in our assays were the same before and after SIV infection, clearly demonstrating that the increased MHC-E restricted activity of NK cells in SIVagm infection must be due to changes at the level of the NK cells themselves.

Examples are known where NKG2A-dependent NK cell education is a determining factor for NK cell responses in infectious conditions, such as CMV infection in humans5,6,7. The IDMIT center complies with the Standards for Human Care and Use of the approved and accredited under statement number A15-035 by the ethics committee. The IDMIT center complies with the Standards for Human Care and Use of the approved by the Ethical Committee of Animal Experimentation (CETEA-DSV, Lyon, France) before handling. African green monkeys (Caribbean Chlorocebus sabaeus, AGM) and cynomolgus macaques (Macaca fascicularis, cynMAC) were infected intravenously with 250 median tissue culture infectious dose (TCID50) of SIVagm.sab92018 and 5000 or 1000 median animal infectious dose (AID50) of SIVmac251 respectively. The rhesus macaques (Macaca mulatta, rhMAC) were infected intravenously with 300 TCID50 SIVmac251. The viremia levels are shown in Supplementary Fig. 2. All cynMAC where viremic. Five rhMAC were used as unaffected controls. Six out of the 13 SIVmac-infected rhMAC were viremic and seven were SIV Controllers (SIC)79. If not specified otherwise in the text, the species of the macaques used was cynomolgus.

Tissue collections and processing. Whole venous blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient centrifugation. Biopsies of peripheral lymph nodes (pLN) were performed by excision. Other tissues were collected at autopsy. After careful removal of adhering connective and fat tissues, LN and spleen cells were dissociated using the gentleMACS® Dissociator technology (Miltenyi Biotec, Germany). The cell suspension was subsequently filtered through 100- and 40-μm cell strainers, and cells were washed with cold phosphate-buffered saline (PBS). Cells were either immediately stained for flow cytometry or cryopreserved in 90% foetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

Methods

Monkeys. Twelve African green monkeys (Caribbean Chlorocebus sabaeus, AGM), 9 cynomolgus macaques (Macaca fascicularis, cynMAC) and 18 Indian rhesus macaques (Macaca mulatta, rhMAC) were housed at the IDMIT Center (Fontenay-au-Rouge, France) and the rhMAC at the German Primate Center (DPZ). All experimental procedures were conducted in strict accordance with the international European guidelines 2010/63/EU on the protection of animals used for experimentation and other scientific purposes and with the French and French law (French decree 2013-118), and the German Animal Welfare Act and also with the recommendations of the Weatherall report. The IDMIT center complies with the Standards for Human Care and Use of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number A5826-86. Monitoring of the monkeys was under the supervision of the Animal facility in charge of the animal facilities. Animal experimental protocols were approved by the Ethical Committee of Animal Experimentation (CETEA-DSV, IDF, France; Notification 12-098 and A17-044). The pVISCONTI study was approved and accredited under statement number A15-035 by the ethics committee “Comité d’Ethique en Expérimentation Animale du CEA”, registered and authorized (243/2011/13F/SU/04) by the French Ministry of Research and Education. The study at DPZ was approved by the Lower Saxony State Office for Consumer Protection and Food Safety and performed with the project licences 33.19-42502-04-12/0830 and 33.19-42502-04-17/2500. The DPZ has the permission to breed and house non-human primate under license number 33/2001/17 granted by the local veterinary office and conforming with § 11 f. of the German Animal Welfare act.

The animals were healthy and seronegative for SIV, type D retrovirus, and simian T-cell lymphotropic virus type 1 at the time of infection and were housed in single cages within level 3 biosafety facilities after infection. At the inclusion in the study the average weight of the monkeys was between 3 and 6 kg. All monkeys were young adults with an average age of 3–5 years at inclusion. Both males and females were used (60% females and 40% males for each species). Because H6 haplotypes are notably associated with viral control in cynomolgus macaques, macaques with H6 haplotype were excluded from this study. The sample size varied between 3 and 9 monkeys per group (n = 6 in most experiments), chosen according to the tripartite harmonized International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline on Methodology (previously coded Q2B). Sample collection was performed in random order. The investigators were not blinded while the animal handlers were blinded to group allocation.

SIV infections. Briefly, Monkeys were sedated with Ketamine Chlorhydrate (Rhone-Mérieux, Lyons, France) before handling. African green monkeys (Car-ibbean Chlorocebus sabaeus, AGM) and cynomolgus macaques (Macaca fascicu-laris, cynMAC) were infected intravenously with 250 median tissue culture infectious dose (TCID50) of SIVagm.sab92018, and 5000 or 1000 median animal infectious dose (AID50) of SIVmac251, respectively. The rhesus macaques (Macaca mulatta, rhMAC) were infected intravenously with 300 TCID50 SIVmac251. The viremia levels are shown in Supplementary Fig. 2. All cynMAC where viremic. Five rhMAC were used as unaffected controls. Six out of the 13 SIVmac-infected rhMAC were viremic and seven were SIV Controllers (SIC)79. If not specified otherwise in the text, the species of the macaques used was cynomolgus.

Plasma viral load. The viral RNA copy numbers in plasma and the cell-associated viral DNA and RNA of the animals were quantified by real-time PCR assay5,8,77,78,79,80,81,82. The number of SIV RNA copies was measured by qPCR in duplicate. SIVagm and SIVmac251 products of 77 transcription from plasmids were used as standards to calculate SIV RNA copy numbers. 18S ribosomal RNA and CRCS DNA quantitation were used for normalization. Sample preparation, enzyme mix preparation and PCR setup were performed in three separate rooms to avoid PCR contamination. Negative controls were used to exclude sample contamination. The reactions were run in an ABI Prism 7500 with one cycle at 95°C (15 min) followed by 40 cycles at 95°C (15 s) and 55°C (1 min). The cut-off value was 6 viral copies or below per ml of plasma.

Viral RNA from rhesus macaques was extracted from 140 µl plasma using QIAamp viral RNA isolation mini kit (QiaGen). For qRT-PCR, 8.5 µl RNA solution was reverse transcribed and amplified using TaKaRa Prime-Script-One-Step-RT-PCR kit (TaKaRa Bio Europe). Reverse transcription was performed at 45°C for 5 min, amplification was started by an initial denaturation step at 95°C for 10 s, followed by 45 cycles of denaturation for 5 s at 95°C and annealing and elongation phase for 10 s at 72°C. For 30 s using the RotorGene Q apparatus (BD Biosciences). The cut-off value was 40 viral copies per ml plasma.

Construction of the infectious molecular clones of SIVagm. Two infectious recombinant molecular clones were derived from the SIVagm.sab92018 clone (SIVagm.Sab92018ivTF) which itself derived from a SIVagm virus that was isolated from a naturally infected AGM (92018) and has never been in vitro cultured before cloning5,78,79,80. The recombinant clones harbor the ENV LS sequence coding either for the nonamer peptide of SIVmac251/13 (QNLIGILAI; atcgactgtgcgc- catcgct) or VL9 (VMAPRITL; ggtaggtgcgcgccactgtcagtc). The mutants were generated by replacement of the wild-type ENV LS sequence of SIVagm.sab using site-directed mutagenesis (cat:0554; NEB New England Biolabs). The primers used to construct the different mutants are list in Supplementary Table 2.

Polychromatic flow cytometry. Flow cytometric analysis was performed on fresh or Frozen, PBMCs and pLN cells. Samples were stained utilizing standard procedure employing clones of anti-human monoclonal antibodies (mAbs) that we have shown to be cross-reactive in the NHP species used in the study. The antibody clones and references are listed in Supplementary Table 3. The anti-NKG2A antibody used recognizes both NKG2A and NKG2C on simian cells80. Gating strategy used for NK and CD4 T cells analysis are shown in Supplementary Fig. 1. Flow cytometry acquisitions were done on a LSRFortessa (BD Biosciences). Intracellular staining was performed using BD Cytofix/Cytoperm®. The data were further analyzed using FlowJo 10.4.2 software (FlowJo, LLC, Ashland, OR, USA). Multimparametric analyses were performed using SPICE (version 5.1). t-SNE was performed with the cytobank (Cytobank, Inc.), using 2000 iterations and a perplexity of 60.

Immunofluorescence staining. K562-E™101 cells were incubated 16 h with biotinylated peptides. Cells were then fixed with PFA 4% and staining was performed using DAPI and Streptavidin coupled with Alexa 568 for 1 h at 37°C followed by three washing steps. Cells were analyzed using a Nikon microscope.
Isolation of CD4+ T cells and NK cells and Cell sorting of NK cell sub-populations. CD4+ T cells and NK cells from fresh or frozen PBMC, pLN and spleen were isolated using positive selection with respect to the anti-human CD4 (CD4 Microbeads, human Miltenyi Biotec (USA)), or the anti-NKG2a/c monoclonal antibody-PE conjugated (clone Z199 Beckman Coulter (USA)) reveals by anti-PE microbeads (Miltenyi Biotec (USA), the staining and the positive selection of the cells was done according to the supplier’s instructions. Eight color panels were used to phenotype, surface stain and sort NK cells from blood and pLNs from chronically infected AGMs. Cells were thawed in 20% FBS-containing media supplemented with benzamide nuclease, and counts and viabilities were performed (Life Technologies). Cells were washed and stained with Aqua Live/Dead stain (Molecular Probes). Cells were washed and blocked using normal mouse IgG (CalTag). For the NKG2a/c (HLA-E) expression panel, pLMCs and pLNs were surface stained for CD3 (SP34.2, 1:10 dilution; BD), CD8 (B1W3S/80, 1:20 dilution; Miltenyi), CD16 (3G8, 1:20 dilution; Beckman Coulter, Inc.), NKG2a/c (Z199, 1:20 dilution; Beckman Coulter, Inc.), CD27 (2H7, 1:20 dilution 1/20; Biologend), and CD14 (M5E2, 1:25 dilution; BD). Post-staining, cells were washed and stained on a FACSARIA II (BD). Cells were directly collected in a lysis buffer that contained TCEP. The purity of the cells was >97%.

Cell culture. K562 (human HLA class I-negative erythroleukemia) (ATCC® CCL-243) as well as HLA-E transduced K562 cells were maintained in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. CD4+ T cells were cultured in RPMI 1640 with Glutamax (Life Technologies) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 U/ml of IL-2. NK cells were cultured in the same medium supplemented with 10 ng/ml of IL-2.

Infection of primary CD4+ T cells with SiVagm viruses. CD4+ T cells from SiV-negative AGMs rested overnight in the medium described above supplemented with IL-2 (20 ng/ml). They were stimulated with anti-CD4 and -CD3. After 2 days, 5 x 10^5 cells CD4+ T cells were exposed to virus (4 ng of p27 capsid antigen) for 1 h. Subsequently, cells were washed extensively to remove cell-free virus and maintained in IL-2 medium. Virus production in culture supernatants was monitored at regular intervals by SiV p27 antigen capture assay (Zetaprints) according to the manufacturer’s instructions.

Design of SiVagm and SiVmac ENV peptides with potential for efficient binding to HLA-E. We searched for SiV-derived peptides that would have a high probability for binding to MHC-E and examined whether the SiV genome sequence encodes amino acid sequences similar to the canonical MHC-E binding motif found in the leader sequence (LS) of classical MHC class I42 and some cellular proteins such as the stress protein HSP6043. Since retroviral ENV proteins contain a LS, we focused on that region and screened the ENV LS sequences from eight distinct SiV. We identified 28 peptides showing similarities with the MHC-E permissive motif (Supplementary method Fig. 2a). We used a prediction algorithm to examine these 28 ENV LS peptides as well as two control peptides (VL9 (VMAEPTYLL) derived from HLA-B*0701 and HSP60 (QMRPVSVRKL)) for their binding affinity to HLA-E*0101 and HLA-E*0103, the only two functional HLA-E alleles in humans (Supplementary method Fig. 2b and Supplementary method Table 3).58,59,61 We selected for each SiV the ENV peptide with a high probability for binding to MHC-E (Supplementary method Fig. 2c and Supplementary method Table 3). The peptides selected for SiVmac239 and SiVmac251 were identical to each other. The MHC-E peptide-binding groove is conserved among primates and it has been shown that human and MAC MHC-E are promiscuous and able to bind identical peptides47,48. Three-dimensional analyses indicated that AGM MHC-E was also conserved with respect to human and other non-human primate MHC-E (Supplementary method Fig. 2d).

Peptides and HLA-E stabilization assay. Synthetic peptides biotinylated or not were purchased from Proimmune (United Kingdom) and Proteogenix (France) and immobilized in DMSO at the concentration of 2 mg/ml. The peptides used were VL9 (VMAEPTYLL), HSP60 (QMRPVSVRKL), SiVmac239/251 (NQLIALLI), SiVagmAB0249 (IGGVIVTVKL), SiVagmAR2 (SGCWSLWYKL), SiVagmF191 (GLVGIVQVQL), and SiVGN (KMLVSVLKL). K562-E*0101 cells were incubated with these peptides (5–300 µM) at 37 °C for 15–20 h in serum-free AIM-V medium (GIBCO BRL) at a concentration of 1–3 x 10^6 cells/ml. Control cultures were kept at 37 °C for over 16 h without peptides. Cells were then harvested, washed in PBS, and cell-surface expression of HLA-E was determined by incubation with PE-conjugated anti-HLA-E antibody, washed twice with PBS and fixed in 100 µl of Cytofix (BD Biosciences). Cells were acquired using a LSR II (BD Biosciences), and FlowJo software (version 9.6.4, Tree Star, Ashland, OR) was used for all analyses. Results were expressed either directly as mean fluorescent intensity (MFI).

Peptide competition assay. Peptide-binding assays were performed according to methods described in the literature (36). In brief, K562-E*0101 cells were incubated overnight (18 h) at 37 °C and 5% CO2 with synthetic peptides (300 µM) at 37 °C for 15–20 h in serum-free AIM-V medium supplemented with benzonase nuclease, and counts and viabilities were assessed by flow cytometry. Cells were acquired using a LSR II (BD Biosciences), and FlowJo software (version 9.6.4, Tree Star, Ashland, OR) was used for all analyses. Results were expressed either directly as mean fluorescent intensity (MFI).

NK cell cytotoxic in vitro assay. NK cell activity was determined through expression of cell surface CD107a and CD107b. NK cells were cultured overnight in the presence of the MHC-class I-devoid target cell line K562 at an effector:target ratio of 1:5 for a total of 6–8 h in the presence of anti-human CD107a. Monensin (BD GolgiStop, BD Biosciences, Franklin Lakes, NJ, USA) was added 1 h after setup of the co-culture, followed by an additional 5 h of incubation at 37 °C in a humidified atmosphere with 5% (v/v) CO₂. Cells were then washed with PBS and used for viability and expression of CD3, CD14, CD16, CD28, NKG2a/c, CD16, and CD107a.

MHC-E-dependent NK cell viral suppressor assays. K562-E*0101 cells were incubated with 50 µM of a given peptide at 26 °C for 15–20 h. NK cells were co-cultured with 2 x 10^5 K562-E*0101 target cells pulsed or not with peptides at a NK cell/target cell ratio of 5:1. Target cells were in parallel cultured in the absence of NK cells. Anti-CD107a antibody was added at the start of the assay, and GolgiStop and GolgiPlug (both BD Biosciences) were added 1 h after the start of the stimulation. After 6 h of culture, cell suspensions were stained for viability and cytotoxic activity was analyzed through measurement of CD107a expression by flow cytometry. Percent activity was calculated as described above.

T cells (5 x 10^6 cells) activated for 2 days as described above were co-cultured with autologous NK cells (ratio 1:1) and from the time of virus exposure on.

RNA extraction, library preparation, and sequencing. RNA was isolated from the sorted NK cells using the RNeasy® Mini Kit (205113, Qiagen). RNA integrity was verified with the Agilent Bioanalyzer. DNase-treated RNA was treated with lithium diiodoacetamide stranded RNAse H Kit (illuminati, San Diego, CA), according to manufacturer’s instructions. An initial poly(A) RNA isolation step (included in the Illumina protocol) is performed on
10 ng of total RNA to keep only the polyadenylated RNA fraction and remove the ribosomal RNA. A step of fragmentation is then performed on the enriched fraction under high temperature. The fragmented RNA samples were randomly primed for reverse transcription followed by second-strand synthesis to create double-stranded cDNA fragments. No end repair step was necessary. An adenine was added to the 3′-end and specific Illumina adapters were ligated. Ligation products were subjected to PCR amplification. The obtained cDNA libraries were controlled by Bioanalyzer DNA1000 Chips (Agilent, # 5067-1504) and quantified by spectrophotometry (Quant-it™ High-Sensitivity DNA Assay Kit, #Q33120, Invitrogen). Sequencing was performed on the Illumina HiSeq2500 platform to generate single-end 100 bp reads bearing strand specificity.

**Homology modeling of HMC-E and model analysis.** Since there is no X-ray structure available for HMC-E from non-human primates, structures for rhCM1, rhCM2, monkey, baboon, and chimpanzee proteins were modeled with the software MODELLER using the crystal structure of HLA-E*01:03 (PDB code 3BZF) as template. HMC-E amino acid sequence conserved were analyzed with the Consurf server. Illustrations were rendered with PyMol.

**Bioinformatic analysis of the genome-wide sequence data.** Bioinformatic analyses were performed using the RNA-seq pipeline from Seqcan. Reads were cleaned and adapter sequences, and low-quality sequences were removed using cutadapt version 1.11. Only sequences >250 nucleotides (nt) in length were considered for further analysis. STAR version 2.5.0a, with default parameters, was used for alignment on the reference genome (Clorocercus sabaeus, from Ensembl release 90). Genes were counted using featureCounts version 1.6.8-p3 from Subread package (parameters: -t gene, -g 1). Data were analyzed using R version 3.4.3 and the Bioconductor package DESeq2 version 1.18.14. Normalization and dispersion estimation were performed with DESeq2, using the default parameters, and statistical tests for differential expression were performed by applying the independent filtering algorithm. A general linear model, including the monkey identifier as a blocking factor, was used to test for the differential expression between the biological conditions. For each pairwise comparison, raw p values were adjusted for multiple testing according to the Benjamini and Hochberg (BH) procedure. Genes with an adjusted p value < 0.05 were considered differentially expressed.

Analysis of pathways associated with differentially expressed genes were performed using ClueGo. Both groups of genes (up- and downregulated, p value < 0.05) were used as dual input for GO and pathway annotation networks of the expressed genes and proteins pathway enrichment analysis. Each list was used to query the Kyoto Encyclopedia of Genes and Genomes (KEGG), GO-biological function database and Wiki pathways. ClueGo parameters were set as follows: Go Term Fusion selected; only display pathways with p values ≤ 0.05; GO tree interval, all levels; GO term minimum genes, 3; threshold of 4% of genes per pathway; and a kappa score of 0.42. GO terms are presented as nodes and clustered together based on the similarity of genes present in each term or pathway. The most significant term was chosen as a representative of the group (Benjamini–Hochberg correction).

**Statistics.** A Wilcoxon matched-pairs signed-rank test was used, with subsequent Bonferroni correction to account for multiple testing, to evaluate whether there was a statistically significant difference in the level of one given marker at a given time point post-infection when compared to the baseline level (day 0: Figs. 1c, d and 3b, c; Supplementary Figures 1a, b, c, e, f, 4a, b, e, f, 5d, and 7a–d, f–i). Baseline level in blood consisted in the median of 3–6 pre-infection values per animal. In pLN3s, the baseline value consisted in the median of 1–2 pre-infection values per animal.

Cytotoxic activity of NK cells in various conditions was compared to the condition without peptide using the Wilcoxon matched-pairs signed-rank test within each species (AGM, rMAC, and rHMC, 7d). The same way, pre- and post-infection cytotoxic activity was compared using a Wilcoxon matched-pairs signed-rank test in cynMac and in AGM (the same animal providing both pre- and post-infection samples; Fig. 8a–d and Supplementary Figure 9b, c). For rhMAC, a Kruskall-Wallis was used as animals were independent and as we further distinguished SIV controllers from viremic animals (Supplementary Figure 9b).

Differences in viral replication level as quantified by SIV p27 according to the culture condition was determined with a Wilcoxon matched-pairs signed-rank test (Fig. 7e). For the analyses with the mutated SIV clones, we also considered a model where target cells were assumed to be at a constant level in the absence of NK cells and to decline exponentially in the presence of NK cells due to the changes in NK:target ratio.

In order to illustrate specific gene sets in the genome-wide RNAseq analysis, we draw heatmaps based on variance-stabilizing transformed counts (Fig. 2a, b, c, d; Figs. 3a and 4d, e; Supplementary Figures 5a–c and 6b, c, e; see also chapter above).

Correlation analyses were performed using Spearman’s coefficient (Figs. 3d, e and 4a; Supplementary Figures 3c, d and 4c, d). These statistical analyses were performed with Prism (GraphPad, La Jolla, CA) and SigmaStat software (Systat).

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

**Data availability**

Source data are provided with this paper. Deep sequencing results have been deposited in the Gene Expression Omnibus database; the accession number is GSE140609. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or are available from the authors upon request. Source data are provided with this paper.

Received: 27 August 2020; Accepted: 27 January 2021;
Published online: 24 February 2021

**References**

1. Martin, M. P. & Carrington, M. Immunogenetics of HIV disease. *Immunol. Rev.* **254**, 245–264 (2013).
2. Alter, G. & Altfeld, M. NK cells in HIV-1 infection: Evidence for their role in the control of HIV-1 infection. *J. Intern. Med.* **265**, 29–42 (2009).
3. Goulder, P. J. R. & Watkins, D. I. Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat. Rev. Immunol. **8**, 619–630 (2008).
4. Freud, A. G., Mundy-Bosse, B. L., Yu, J. & Caliguiri, M. A. The broad spectrum of human natural killer cell diversity. *Immunol. Today* **47**, 820–833 (2017).
5. Goodridge, J. P., Onfelt, B. & Malmberg, K.-J. Human NK cells: a broad toolbox for viral infection. *Immunol. Rev.* **267**, 197–213 (2015).
6. Blush, C. A. Natural killer cell diversity in viral infection: why and how much? *Pathog. Immunol.* **1**, 165–192 (2016).
7. Strauss-Albee, D. M. et al. Human NK cell repertoire diversity reflects immune experience and correlates with viral susceptibility. *Sci. Transl. Med.* **7**, 297ra115 (2015).
8. Palgen, J.-L. et al. NK cell immune responses differ after prime and boost vaccination. *J. Leukoc. Biol.* **105**, 1055–1073 (2019).
9. Reeves, R. K. et al. Antigen-specific NK cell memory in thymus macaques. *Nat. Immunol.* **16**, 927–932 (2015).
10. Paust, S., Semen, B. & von Andrian, U. H. Adaptive immune responses mediated by natural killer cells. *Immunol. Rev.* **235**, 286–296 (2010).
11. Peng, H. & Tian, Z. Natural killer cell memory: progress and implications. *Front. Immunol.* **8**, 1143 (2017).
12. Lucar, O., Reeves, R. K. & Jost, S. A natural impact: NK cells at the intersection of cancer and HIV disease. *Front. Immunol.* **10**, 1850 (2019).
13. Luteijn, R. et al. Early viral replication in lymph nodes provides HIV with a means by which to escape NK-cell-mediated control. *Eur. J. Immunol.* **41**, 2729–2740 (2011).
14. Schafer, J. L., Li, H., Evans, T. L., Estes, J. D. & Reeves, R. K. Accumulation of cytotoxic CD16+ NK cells in simian immunodeficiency-virus-infected lymph nodes associated with in situ differentiation and functional anergy. *J. Virol.* **89**, 6887–6894 (2015).
15. Freud, A. G. et al. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity* **22**, 295–304 (2005).
16. Svardal, H. et al. Ancient hybridization and strong adaptation to viruses across African vervet monkey populations. *Nat. Genet.* **49**, 1705–1713 (2017).
17. Muller, M. C. et al. Simian immunodeficiency viruses from central and western Africa: evidence for a new species-specific lentivirus in talanta monkeys. *J. Virol.* **87**, 1227–1235 (1993).
18. Raehlt, K., Pandreza, I. & Apetrei, C. The well-tempered SIV infection: pathogenesis of SIV infection in natural hosts in the wild, with emphasis on virus transmission and early events post-infection that may contribute to protection from disease progression. *Infect. Genet. Evol.* **46**, 308–323 (2016).
19. Sodora, D. L. et al. Toward an AIDS vaccine: lessons from natural simian immunodeficiency virus infections of African nonhuman primate hosts. *Nat. Med.* **15**, 861–865 (2009).
20. Huot, N. et al. Natural killer cells migrate into and control simian immunodeficiency virus replication in lymph node follicles in African green monkeys. *Nat. Med.* **23**, 1277–1286 (2017).
21. Hong, J. J., Chang, K.-T. & Villinger, F. The dynamics of T and B cells in lymph node during chronic HIV infection: Tfh and HIV, unhappy dance partners? *Front. Immunol.* **7**, 522 (2016).
22. Wong, J. K. & Yukl, S. A. Tissue reservoirs of HIV. *Curr. Opin. HIV AIDS* **11**, 362–370 (2016).
23. Strauss-Albee, D. M., Horowitz, A., Parham, P. & Blish, C. A. Coordinated regulation of NK receptor expression in the maturing human immune system. *J. Immunol.* **193**, 4871–4879 (2014).
24. Horowitz, A. et al. Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. *Sci. Transl. Med.* **5**, 208ra145 (2013).
25. Víctor, A. R. et al. Epigenetic and post-transcriptional regulation of CD16 expression during human natural killer cell development. J. Immunol. 200, 2677–2686 (2018).

26. Sivori, S. et al. TLR/NCR/KIR: which one to use and when? Front. Immunol. 5, 105 (2014).

27. Jeevan-Raj, B. et al. The transcription factor Tcf7 contributes to normal NK cell development and function by limiting the expression of granzymes. Cell. Rep. 20, 613–626 (2017).

28. Goh, W. & Huntington, N. D. Regulation of murine natural killer cell development. Front. Immunol. 8, 130 (2017).

29. Bézat, V., Descours, B., Parizot, C., Debâ, P. & Viéillard, V. NK cell terminal differentiation: correlated stepwise decrease of NGK2A and acquisition of KIRs. PLoS ONE 5, e16866 (2010).

30. Luette-Everslohy, N., Mullig, M. & Romagnani, S. Signatures of human NK cell development and terminal differentiation. Front. Immunol. 4, 499 (2013).

31. Freund, A. G., Yu, J. & Caligiuri, M. A. Human natural killer cell development in secondary lymphoid tissues. Semin. Immunol. 26, 132–134 (2017).

32. O’Sullivan, T. E., Johnson, L. R., Kang, H. H. & Sun, J. C. BNP3- and BNP3L-mediated misfolding promotes the generation of natural killer cell memory. Immunity 43, 331–334 (2015).

33. Evavold, C. L. & Kagan, J. C. How In...
Acknowledgements

We are grateful for the excellent help from the veterinarians and staff at the IDMIT Center (Benoit Delache, Jean-Marie Helies, Raphaël Ho Tsong Fang and Julie Morn). We thank M. Mietsch and M. Daskalaki for comprehensive veterinary support, and S. Hesse, J. Hampe, and N. Leuchte for excellent technical assistance. We also thank all the people from the Illumina Hisseq 2500 platform at the Institut Pasteur and especially Caroline Proux and Jean-Yves Coppee. The Biomics Platform (C2RT, Institut Pasteur) is supported by France Génomique (ANR-10-INBS-09-09) and IBISA NH was supported by the VRI Labex, the Fondation Jacqueline Beytouit and Institut Pasteur, and FR was recipient of a PhD fellowship from the University Paris Diderot, Sorbonne Paris Cité. C.P. was supported by a MSDAvenir Postdoctoral Fellowship Grant. FK was supported by the DFG (CRC 1279 and SPP 1937, SFB TR57), the Hector Foundation, and the DZIF (TTU-HIV). We gratefully acknowledge the support to IDMIT from the French government: Investments for the Future program for infrastructures (PIA) through the ANR-11-INBS-0008 grant as well as from the PIA grant ANR-10-EQPX-02-01 to the FlowCyTech facility at IDMIT. We equally acknowledge the Investments for Future grant ANR-10-INSB-04 to support the UtechS Photonic Bioimaging (Imagopole) and C2RT facilities at Institut Pasteur.

Author contributions

N.H. and M.M.T. designed the study; N.H. designed the experiments; N.H., B.J., and U.S. quantified the viral loads; C.S. and F.K. constructed the infectious SIVagm clones; N.H. and E.B. performed the prediction analyses; C.S.H. and C.P. provided samples; N.H., R.L., and H.V. performed the bioinformatic analyses; N.H., P.R., and Y.M. performed the statistical analyses; N.H., P.R., J.H., R.L.G., R.K.R., M.P., A.S.C., B.J., and M.M.T. analyzed the data; V.C., R.L.G., and B.J. coordinated the animal studies; M.M.T. obtained the funding; N.H. and M.M.T. wrote the manuscript and all co-authors reviewed it.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-21402-1.

Correspondence and requests for materials should be addressed to M.M-T.

Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.