Natural killer cells in HIV controller patients express an activated effector phenotype and do not up-regulate NKp44 on IL-2 stimulation

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Control of HIV replication in elite controller (EC) and long-term nonprogressor (LTNP) patients has been associated with efficient CD8$^+$cytotoxic T-lymphocyte function. However, innate immunity may play a role in HIV control. We studied the expression of natural cytotoxicity receptors (NKp46, NKp30, and NKp44) and their induction over a short time frame (2–4 d) on activation of natural killer (NK) cells in 31 HIV controller patients (15 ECs, 16 LTNPs). In EC/LTNP, induction of NKp46 expression was normal but short (2 d), and NKp30 was induced to lower levels vs. healthy donors. Notably, in antiretroviral-treated aviremic progressor patients (TAPPs), no induction of NKp46 or NKp30 expression occurred. More importantly, EC/LTNP failed to induce expression of NKp44, a receptor efficiently induced in activated NK cells in TAPPs. The specific lack of NKp44 expression resulted in sharply decreased capability of killing target cells by NKp44, whereas TAPPs had conserved NKp44-mediated lysis. Importantly, conserved NK cell responses, accompanied by a selective defect in the NKp44-activating pathway, may result in lack of killing of uninfected CD4$^+$NKp44Ligand$^+$ cells when induced by HIVgp41 peptide-S3, representing a relevant mechanism of CD4$^+$ depletion. In addition, peripheral NK cells from EC/LTNP had increased NKG2D expression, significant HLA-DR up-regulation, and a mature (NKG2A−CD57$^+$killer cell Ig-like receptor$^+$CD85j$^+$) phenotype, with cytolytic function also against immature dendritic cells. Thus, NK cells in EC/LTNP can maintain substantially unchanged functional capabilities, whereas the lack of NKp44 induction may be related to CD4 maintenance, representing a hallmark of these patients.

A benign disease course with long-term nonprogressing disease (LTNP) up and beyond 20 y is observed in a minority (<1–2%) of HIV-1–infected patients who maintain high CD4$^+$ T-cell counts (>500 μL) with low-level viremia (<1,000 cp/mL) without progression to AIDS in the absence of antiretroviral treatment (ART). A subset of LTNPs is aviremic virus-controlling (<50–75 cp/mL) patients who are considered to represent a distinct clinical entity defined as elite controllers (ECs) because of their efficient and extensive spontaneous control of viral replication (1, 2). Understanding of the mechanisms that underlie the lack of disease progression in EC and LTNP patients has attracted relevant scientific focus over the years, with the ultimate goal to exploit this understanding for therapeutic or vaccination purposes.

Viral replication may be decreased in LTNP/EC because of virus mutations or host genetic background conferring reduced CD4$^+$ T-cell susceptibility. However, both an intact viral replication capacity and a conserved CD4$^+$ T-cell susceptibility to HIV infection in vitro have recently been proven in most HIV controller patients (3–5). Among cytotoxic effector cells, an acknowledged role in the control of viremia and disease has been attributed to CD8$^+$ cytotoxic T lymphocytes (CTLs), which in these patients, display an exceptionally high avidity and breadth against HIV epitopes (1, 2, 6, 7). Vigorous and effective CTL responses associated to HLA class I haplotype (e.g., B*57 and B*27 alleles) represent an example of genetic background positively affecting HIV control (1, 2, 6, 7). Also, HLA-C polymorphisms have been implicated in the control of HIV (8). Unique allele carriage is, however, not a feature uniquely characterizing LTNPs/ECs. HIV controllers may lack this genetic background, but they have CTL responses with high avidity and breadth against HIV_{gag}. Conversely, this immunogenetic background may be present in progressors who display poorer CTL response quality (5, 9–11). Also, HLA B*5701 LTNPs/ECs and HLA-matched progressors cannot be distinguished by the clonal composition of HIV-specific CD8$^+$ T cells (12).

The relevance of natural killer (NK) cell function in the setting of HIV controller status has been suggested by genetic studies showing the association between HLA-$Bw4_{80I}$ DNA carriage and specific killer cell Ig-like receptors (KIRs; i.e., KIR3DL1/S1) (13, 14). NK cell-associated control of HIV replication in vitro occurs with KIR3DS1$^+$ NK cells in a HLA-$Bw4_{80I}^+$ target cell genetic background (15); however, this result has not been subsequently reproduced in vivo in EC/LTNP cohorts (16). Various combinations of these mechanisms seem to be involved in the successful control of HIV replication in some LTNP and EC patients; however, none of them taken alone can fully explain this condition, and it has not been shown to identify all of these patients.

Involvement of the activating NK receptors in disease progression was suggested by the demonstration that HIV-1 infection was associated to profoundly decreased expression of natural cytotoxicity receptors (NCRs; i.e., NKp46, NKp30, and NKp44) (17). This decrease, in turn, leads to an impaired cross-talk between NK cells and dendritic cells (DCs), resulting in an altered DC editing (18). Moreover, rates of CD4$^+$ T-cell loss after ART interruption are inversely associated with NCR expression on NK cells before ART discontinuation (19).

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Interestingly, in the AIDS-free HIV infection model of chimpanzees, peripheral NK cells have absent/low baseline expression of NKp30, which was, however, inducible on cytokine-mediated in vitro NK cell activation (20). In addition, activating NK cell receptor induction/modulation has been reported in vivo and in vitro during treatment of human HCV infection involving NKp30 (21) and DNAX-accessory molecule 1 (DNAM-1,CD226) (22), which are both involved in DC–NK cell cross-talk (23, 24). In addition, activating NK cell receptor ligands are lost in $CD4^+$ T cells of infected patients, with the exception of NKG2D-Ligands (e.g., MHC class I polypeptide-related sequence A/B,MICA/B) (25). Furthermore, HIV_{nef} and HIV_{vpu} have been shown to directly target NKG2D and DNAM-1 ligands (i.e., MICA/B and poliovirus receptor, PVR) (26, 27). These immune evasion mechanisms are in line with the idea that NK cells may exert a critical control of HIV-1 infection. In this context, an as yet uncharacterized NKp44-L is reported to be induced in uninfected $CD4^+$ T cells by an HIV_{gp41} peptide inducing innocent $CD4^+$ T-cell bystander lysis (28, 29). These observations, thus, raise the question of whether differences in NCR surface expression may help to explain the different disease course observed in HIV controllers—LTNPs, ECs, or both.

Here, we report a study addressing the activating NK cell receptors expression, their modulation, and the consequences on NK cell function in a cohort of HIV controller (LTNP and EC) patients. The data provide evidence that differences in inducibility/modulation of NCR may offer clues on how successful disease-free HIV-1 control may be achieved in these patients.

Results

Increased NKG2D and Reduced NKp46 Surface Expression in ECs and LTNPs. We first analyzed the expression of activating NK cell receptors on peripheral NK cells from EC and LTNP patients enrolled in the study compared with a group of healthy, uninfected donors (HDs) by flow cytometry.

No differences were observed in the proportion of activating receptor-expressing NK cells comparing EC and LTNP patients. Compared with HDs, however, reduced proportions of $NKp46^+$ $CD56^+$-expressing NK cells were detected in both ECs and LTNPs (Fig. 1*A* and Fig. S1). LTNP and EC patients had significant increases in NKG2D expression, whereas the proportions of NKp30- and DNAM-expressing NK cells were similar to HDs (Fig. 1*A*).

To assess whether differences in the proportion of cells expressing a given receptor also reflected changes in molecule densities, mean fluorescence intensity ratio (MFIr) was analyzed. NKp46 MFIr was decreased in both total $CD56^+$ and $CD56^{dim}CD16^+$ NK cells from LTNPs ($P = 0.016$ and $P = 0.0098$, respectively) and ECs ($P = 0.0326$ and $P = 0.035$, respectively) compared with HD (Figs. S1 and S2). NKG2D molecule densities on peripheral NK cells of LTNP patients were not increased compared with HDs, even if the proportion of $CD56^+NKG2D^+$ NK cells was increased (Fig. 1*A* and Fig. S2). On the contrary, EC patients had significant increases of NKG2D molecule density, which was evaluated by MFIr, compared with both HD and LTNP patients ($P = 0.009$ and $P = 0.04$, respectively) (Figs. S1 and S2).

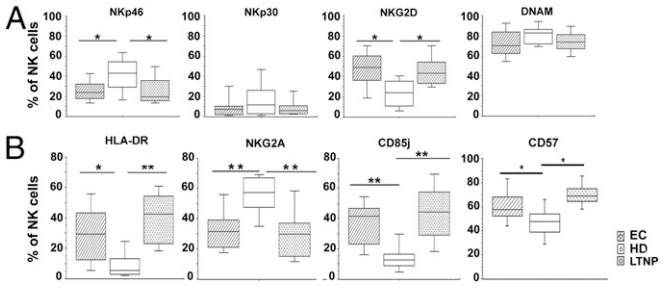

Fig. 1. Flow cytometric analysis of peripheral NK cells in HIV-1–positive patients, ECs/LTNPs, and HDs. In the box plots, bars represent range, lines represent median values, and boxes represent 75% and 25% percentiles. Data are representative of all patients and HDs. $*P < 0.05$, $**P < 0.001$, Mann–Whitney *u* test. (*A*) Activating receptor expression. Proportion of NK cell expressing each receptor. (*B*) NK cell expression of HLA-DR, NKG2A, CD85j, and CD57.

Expression of HLA-DR on $CD56^{dim}CD16^+$ NK Cells from LTNP and EC Patients. To assess whether the altered expression of activating NK receptors could be associated with NK cell activation in LTNPs/ECs, peripheral NK cells were analyzed by flow cytometry for the expression of HLA-DR.

Increased expression of HLA-DR was observed in $CD56^+$NK cells in both LTNPs (42.5 ± 16.84; median ± SD) and ECs (29.2 ± 18.26) compared with HDs (5.43 ± 8.38; $P = 0.0002$ and $P = 0.016$, respectively) (Fig. 1*B*). Notably, this increase was caused by increased expression of HLA-DR on $CD56^{dim}16^+$ NK cells (Fig. S3), whereas no differences were observed among $CD56^{bright}CD16^{+/-}$ NK cells of LTNPs or ECs compared with HD (47.65 ± 18.95, 35.2 ± 24.15, and 45 ± 28.28, respectively; P = not significant).

NK Cell Activation in HIV Controller (LTNP/EC) Patients Is Associated with an Increase of $NKG2A^-KIR^+$ $CD57^+CD56^{dim}$ Effector NK Cells. We next studied whether NK cell activation would result in a skewing of peripheral NK cell maturation, leading to mature effector cells characterized by strong cytolytic activity and rapid cytokine production (30–33).

Flow cytometric analysis showed significantly decreased proportions of $NKG2A^+$ peripheral NK cells in both LTNPs and ECs (Fig. 1*B*). The decrease in the proportion of $NKG2A^+$ $CD56^+$ NK cells relied exclusively on the decrease in $NKG2A^+$ $CD56^{dim}16^+$ NK cells ($P = 0.0017$ and $P = 0.0068$, respectively) (Fig. S3*A*). Analysis of CD85j, which is usually expressed by peripheral $NKG2A^-CD56^{dim}16^+$ NK cells during maturation (31), confirmed an increase in the proportion of $CD85j^+$ cells on both total $CD56^+$ and $CD56^{dim}16^+$ NK cells in EC and LTNP patients ($P = 0.0014$ and $P = 0.0018$ vs. HDs, respectively) (Fig. 1*B*). Because CD57 and KIRs are considered to represent a hallmark of NK cell effector maturation among $CD56^{dim}16^+$ $NKG2A^-$ NK cells, we also studied these molecules in addition to CD85j. Indeed, increased CD57 expression was detected in LTNPs and ECs ($P < 0.05$) (Fig. 1*B* and Fig. S3).

We then studied the relationship between NK cell activation and NKG2A expression to identify a possible association between NK cell activation and their peripheral maturation. A negative correlation was observed between $HLA\text{-}DR^+CD56^+$ and $NKG2A^+CD56^+$ NK cells and between $CD85j^+$ and $NKG2A^+$NK cells (Spearman test; $P = 0.0144$ and $P = 0011$, respectively) (Fig. 2).

Thus, NK cell activation in ECs/LTNPs is associated with increased/accelerated peripheral transition, which is revealed by the loss of NKG2A and the expression of KIRs, CD57, and CD85j, suggesting a more mature phenotype.

Different Kinetics of NKp46 and NKp30 Induction on NK Cells from ECs and LTNPs Compared with HDs and Progressor Patients. The above findings of reduced triggering NK cell receptor expression (NKp46), which were paralleled by an increased NK cell maturation, could either represent an inherent characteristic of EC/LTNP patients (21, 22) or be secondary to HIV-1 infection (17). To address this issue, LTNP and EC NK cells were purified and activated in vitro in the presence of rhIL-2 in short-term cultures. Flow cytometric analysis of NCR expression was performed at baseline (before activation) and after 2 and 4 d of culture.

After 2 d, the proportion of $NKp46^+CD56^+$ cells increased to comparable levels in ECs, LTNPs, and HDs (Kruskall–Wallis test: not significant) (Fig. 3, *Upper*). In HDs after 4 d, the increase in NKp46 expression persisted, whereas in LTNPs and ECs, a significant reduction in the proportion of $NKp46^+CD56^+$ NK cells was observed with a return to (and in some cases,

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reduction to below) baseline values ($P = 0.012$ and $P = 0.0129$, respectively; u test) (Fig. 3). Analysis of NKp46 fold change over baseline confirmed the difference for LTNPs and ECs (Fig. 3, *Upper*). Similar changes were observed for NKp46 molecule density as well, which was assessed by MFIr (Fig. 3). To understand whether this difference could be attributed to HIV infection or intrinsic patient characteristics, purified NK cells from a group of aviremic ART-treated patients were evaluated in parallel. In these patients, NKp46 was not induced after 2 d of in vitro activation, and it steadily decreased after 4 d (Fig. 3).

A comparable increase in NKp30 expression and fold change over baseline was observed after 2 d in HDs, LTNPs, and ECs (P = not significant; Kruskall–Wallis test) (Fig. 3, *Lower*). After 4 d, however, an increase in NKp30 expression was significantly less pronounced in LTNPs and ECs for both the proportion of positive cells ($P = 0.0082$ and $P = 0.02$, respectively) and the fold change over baseline expression ($P = 0.01$ and $P = 0.033$, respectively). Interestingly, in the case of NKp30, molecule densities were conserved comparing ECs, LTNPs, and HDs after both 2 and 4 d (Fig. 3). ART-treated aviremic progressor patients showed significantly lower and almost noninducible NKp30 expression compared with HDs at 2 and 4 d of culture and EC/LTNP patients after 4 d. MFIr values in ART-treated progressors were consistently below values in HDs, ECs, and LTNPs. (Fig. 3).

Thus, NK cells from LTNP and EC patients maintain the ability to display increases of NCR expression (NKp30 and NKp46) on activation but show blunted responses with contained increase (NKp30) or rapid return to preinduction surface levels (NKp46); however, aviremic ART-treated patients have significant defects in both NKp30 and NKp46 surface expressions induction. Analysis of overall KIR expression on NK cells from ECs, LTNPs, and progressor patients showed no differences, whereas KIR expression was increased in all groups compared with HDs (Fig. S3*B*).

Markedly Impaired NKp44 Inducibility Characterizes HIV Controllers (LTNPs/ECs) from Progressor Patients. Previous data indicated that de novo expression of NKp44 is defective in HIV-viremic patients (17). Thus, we analyzed its expression in EC/LTNP NK cells on rhIL-2–mediated activation. Evaluation of the de novo expression of NKp44 on purified in vitro rhIL-2–activated NK cells revealed that EC and LTNP patients, compared with HDs, had markedly reduced NKp44 expression ($P < 0.05$) (Fig. 4). Indeed, NKp44 was virtually absent in NK cells of EC/LTNP. To understand whether this defect was related to latent/low-level HIV replication or rather, an inherently different regulation of its expression characterizing these subjects, we compared these subjects with HIV progressor patients. Aviremic ART-treated progressors showed inducible NKp44, with significant increases on in vitro short-term activation with rhIL-2 ($P < 0.05$) (Fig. 4). No significant differences in the ability to induce NKp44 surface expression on in vitro rhIL-2 activation were observed between ECs and LTNPs (Fig. 4).

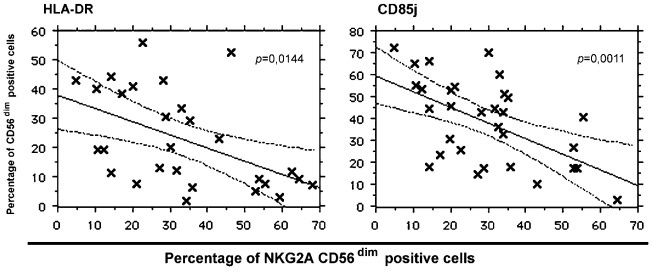

Fig. 2. Correlation analysis of NKG2A with HLA-DR and CD85j expression (%) on CD56 ^{dim}CD16^{+}–positive cells (Spearman test).

Induction in NKp44 expression directly correlated with baseline NKp46 and DNAM-1 expression ($P < 0.001$ in both cases, Spearman test) and indirectly correlated with CD4^{+} T-cell counts ($P = 0.0087$, Spearman test). CD4^{+} cell counts indirectly correlated with molecule density of NKp46, NKp30, and DNAM-1 on NK cells (Fig. S3*C*). No correlation was found between NK cell parameters and viral load.

Analysis of Cytolytic Activity in EC/LTNP Patients with Impaired NKp44 Expression. NK cell cytotoxic activity is dependent, among other factors, on relevant activating receptor triggering by target cells. In view of the peculiar modulation of different NCRs in EC and LTNP during in vitro activation and culture, we sought to confirm their functional correlate. Flow cytometric analysis of NK cell cytotoxicity was performed using PKH-26/TO-PRO-3 staining after coculture with NK cells.

Purified NK cell cultures, activated for 4 d in the presence of rhIL-2, were first evaluated for their activity against K562 target cells and immature DCs (iDCs) to explore the activity through NKp46 and NKG2D (predominantly involved in K562 lysis) and through NKp30 and DNAM-1 (predominantly involved in iDC lysis). With both targets, NK cells from ECs and LTNPs showed similar cytolytic activity compared with NK cells from HD (Fig. S4) (P = not significant; Kruskall–Wallis test).

To further explore how differential NKp44 expression on NK cells from HIV controller (EC/LTNP) and aviremic progressor patients (ART-treated) may influence NK cell function, we next studied lysis of FcγR^{+}P815 cells in the presence of NKp44-specific mAbs (redirected lysis assay). Parallel experiments were performed using anti-NKp46 mAbs at a time point (2 d) when its expression differed in aviremic progressors and ECs/LTNPs (Fig. 3, *Upper*). As shown in Fig. 5, the low expression of NKp44 on NK cells from ECs/LTNPs was associated to a highly reduced lysis of P815 cells. As shown by the proportion of TO-PRO-3/PKH-26–labeled target cells, lysis was two- or sixfold higher in progressor patients and HDs, respectively (Fig. 5, *Left* and Fig. S5, *Upper*). Using anti-NKp46 mAbs to redirect NK cell triggering in a parallel experiment, ECs/LTNPs had proportionally higher expression of NKp46 and threefold higher cytolytic activity compared with aviremic ART-treated progressors (Fig. 5, *Right* and Fig. S5, *Lower*). This result confirmed that the cytolytic potential of activated NK cells in ECs/LTNPs is conserved and that only NKp44- but not NKp46-mediated lysis is impaired because of lack of its expression on activation.

Discussion

NK cells play an important role in antiviral responses and are involved in maintenance of adaptive lifesaving defenses (34–37). NK cell involvement in continuous active control of viremia in LTNP or EC patients is, however, still partly disputed. Indeed, involvement of a protective genetic background for HLA:KIR interaction involves only some patients in any given cohort and only a fraction of NK cells in any given patient (9, 11, 13–15), and it may, rather, involve KIR-expressing HIV-specific CTLs (38, 39).

In the present study, we show that, in all patients analyzed belonging to the EC/LTNP category, there are relevant imbalances in NCR expression on cell activation. Although both NKp46 and NKp30 display a bunted inducibility, virtual lack of NKp44 expression was observed. This finding was accompanied by relevant HLA-DR expression and a phenotypic shift of peripheral blood CD56dim NK cells to NKG2A^{-}CD57^{+}CD85j^{+}. These data suggest a possible role of activating NK cell receptors and NK cell effector function in the EC/LTNP control of HIV replication.

The percentages of activated NK cells (30–40%), which are defined by HLA-DR expression, in all EC and LTNP patients are remarkable, and they are comparable with those percentages originally described in viremic patients (40) and those percentages recently observed after successful cART (41). Here, the concomitant finding that NK cell activation is associated

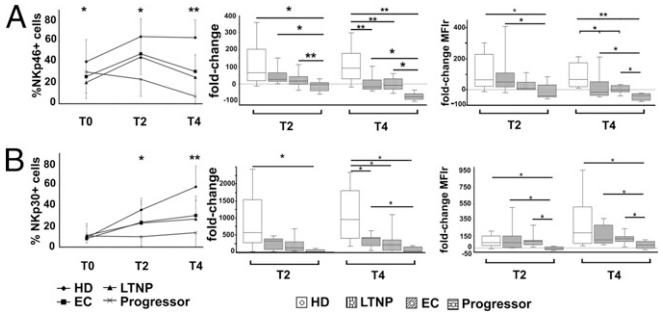

Fig. 3. Cytokine-induced in vitro modulation of NK cell receptors. Expression of (*Upper*) NKp46 and (*Lower*) NKp30. (*Left*) Line plots represent the mean expression of given receptors (± SD) at baseline (T0) and after 2 (T2) and 4 (T4) d (Kruskall–Wallis test). Box plots indicate fold change compared with respective baseline values in the proportion of (*Center*) NK cells expressing given receptors or (*Right*) NK cell molecule density (MFI; 30 experiments). $*P < 0.05$, $**P < 0.001$, Mann–Whitney *u* test.

with decreased NKG2A expression and increased presence of $NKG2A^{-}KIR^{+}CD57^{+}CD85j^{+}CD56^{dim}$ NK cells (30–32) suggests an accelerated transition to more functionally differentiated NK effectors that are associated with their activation in vivo. In view of the capability of NK cells to edit DCs and influence downstream adaptive immune responses, our data are in agreement with the reported continuous $CD8^{+}$ CTLs functional control of low-level HIV replication in all EC patients (42, 43), and they support the notion that maintenance of EC status requires combined NK cell–$CD8^{+}$ CTL-mediated inhibition of replicating HIV (11). In this regard, the increased proportion of activated, peripheral, tissue-bound $NKG2A^{-}KIR^{+}CD57^{+}CD85j^{+}CD56^{dim}CD16^{+}$ NK cells (30) could also contribute to chronic immune activation (44) and end organ damage (45), which has recently been reported in EC.

The presently observed changes in the expression of activating NK receptors, with increased NKG2D and reduced NKp46, could hardly be explained within the background of NK cell activation in uninfected subjects. NK cell activation leads to increases in NKp46, NKp30, and NKG2D with de novo in vitro NKp44 expression in HDs (17). NKG2D expression was unchanged in viremic patient NK cells, even in the presence of NK cell activation (46). Under in vitro experimental conditions, NKG2D plays a more relevant role in the control of autologous infected $CD4^{+}$ cells in vitro than NKp46 and NKp30 (46), and therefore, its increased expression in ECs/LTNPs could, indeed, be relevant for the control of HIV replication. Accordingly, NK cell cytotoxic function in ECs/LTNPs was comparable with function in HDs in a K562 and iDC killing assay. Thus, both receptor expression pattern and cytotoxic function confirm the overall maintained functionality of NK cells in EC/LTNP patients.

Reduced NKp46 expression may represent either an acquired characteristic after virus infection (17) or a distinct regulatory pattern in some patients, which was recently evidenced in HCV infection (22, 37). To address this issue, we tested the hypothesis that EC/LTNP patients, while maintaining NK cell cytotoxic activity, may display a different regulation of NCR expression on activation in vitro. Indeed, we observed that NKp46, which is decreased in ECs/LTNPs, increases in surface density, reaching the levels observed in HDs, with subsequent earlier return to baseline levels. This pattern is compatible with a conserved but time-limited NK cell response (20). Interestingly, ART-treated patients, different from ECs/LTNPs, have a significant defect in NKp46 up-regulation. This NK cell regulation is also reflected by a blunted but not significantly impaired NKp30 expression on in vitro activation in ECs/LTNPs compared with HDs. Because these patterns were observed in all EC/LTNP patients, although they were not found in HDs or ART-treated aviremic HIV-infected patients, we conclude that this regulation is likely to represent an inherent difference of these subjects rather than be related to HIV infection. Because NK cell function depends on a balance of activating and inhibitory stimuli, the present finding of similarly increased KIR expression in ECs/LTNPs and aviremic ART-treated patients further supports the functional relevance of these differences in activating receptor expression induction.

In the same context, the observation that NKp44 is poorly/not inducible in vitro only in EC/LTNP patients but not ART-treated progressors is particularly remarkable and may have additional relevant implications. It has been shown that an as yet unknown NKp44 ligand is induced on uninfected $CD4^{+}$ T cells by a shedded HIV_{gp41} peptide(peptide S3) (28, 29), thus inducing their susceptibility to NK-mediated killing. Antibodies specific for this peptide protected viremic patients from $CD4^{+}$ T-cell loss (47). The present demonstration that all EC/LTNP patients fail to induce NKp44 on short-term NK activation suggests that they may be efficiently protected from innocent bystander killing of uninfected $CD4^{+}$ T cells, even after NKp44 ligand expression mediated by HIV_{gp41} peptides.

Although at a first glance, failure to express NKp44 may catch relevant attention, this finding should not convey the view that ECs/LTNPs are protected because of a defective NK cell response. Rather, they are likely to be protected by conserved NK cell responses, including cytolytic activity, lysis of iDC (with maintenance of appropriate DC editing and downstream adaptive responses), and conserved inducibility of major NCRs (NKp46 and NKp30). In this context, the putative reduction of NKp44-dependent innocent bystander CD4+ lysis may provide an additional mechanism for conserved DC maturation/antigen presentation without central memory $CD4^{+}$ T-cell clearance (48). Together with preserved NK cell function, these events would explain the control of viremia in these patients.

Based on these observations, HIV control and nonprogressive disease would rely on a pattern of NKp46, NKp30, and NKp44 induction/modulation. In combination, this pattern may effectively control virus replication: on one hand, it avoids killing innocent bystander $CD4^{+}$ T cells, and on the other hand, it ensures maintenance of efficient downstream adaptive responses with broad and polyfunctional $CD8^{+}$ CTL responses.

The present study was not designed to gather an insight into transcriptional regulation of NCRs. This shortcoming needs to be addressed in a more focused study. It is, indeed, unclear to what extent NCR expression/regulation is affected by low-level viral replication in ECs/LTNPs or whether it is an intrinsic characteristic of these subjects. In addition, it is only indirectly assumed that the described NCR regulation is instrumental in determining the benign disease course. Additional work is needed to identify HDs with this peculiar NCR regulation to rule out that NCR induction/regulation is affected by HIV.

In conclusion, NCR expression and regulation on NK cells of all ECs/LTNPs represent a distinctive characteristic not seen in

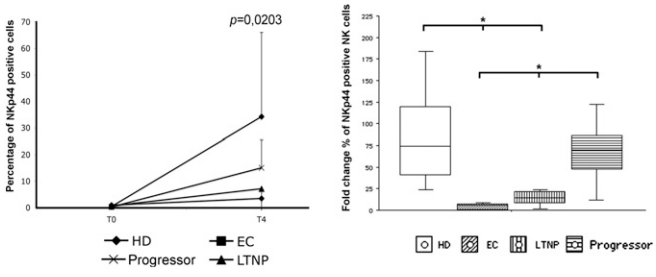

Fig. 4. IL-2–induced expression of NKp44 by purified NK cells in vitro. (*Left*) Line plot represents the mean percentage of NK cells (± SD) expressing NKp44 before and after 4 d (T4) of in vitro culture with rhIL-2 (Kruskall–Wallis test). (*Right*) Box plot indicates fold change in the proportion of activated NK cells expressing NKp44 ($*P < 0.05$, Mann-Whitney *u* test; 20 experiments).

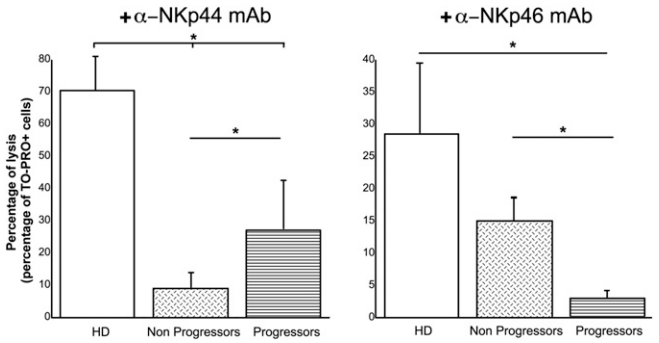

Fig. 5. Functional activity of purified NK cells after in vitro activation in the presence of rhIL-2 (200 U/mL). PKH-26/TO-PRO3 flow cytometric cytotoxicity assay. Bars represent variation of percentage of lysis (percentage of TO-PRO3$^+$/PKH$^+$ cells). Data are representative of 21 experiments. Mann–Whitney *u* test was used for comparison. Bars indicate percentage of purified NK cell-mediated FcγR$^+$P815 cell lysis in the presence of (*Left*) NKp44-specific mAbs and (*Right*) NKp46-specific mAbs after (*Left*) 4 or (*Right*) 2 d of culture in HD, nonprogressor (EC and LTNP), and progressor patients (mean + SD). Data are representative of 21 experiments. *$P < 0.05$, Mann-Whitney *u* test.

HDs or aviremic HIV progressors on ART. Its association with NK cell activation and increased proportions of mature NK cells is coherent with presently acknowledged mechanisms for HIV control in ECs/LTNPs with conserved DC-NK cell cross-talk and downstream CD8$^+$ CTL HIV-specific responses.

Materials and Methods

Patients. Patients were being followed up within a program for surveillance and treatment of HIV infection. EC patients had positive HIV-1 serology, were ART-naïve, had always undetectable HIV-RNA (<50–200 cp/mL according to the evolution of HIV-RNA assays over time), and had CD4$^+$ counts ≥450 cells/μL at all visits for ≥7 y of follow-up, with no clinical evidence of disease progression. LTNPs met the same definitions as ECs, with the exception of HIV-RNA being detectable during the years of observation and <1,000 cp/mL over the last 6 mo before study. Exclusion criteria were current or previous ART, age < 18 y, pregnancy, cancer, and treatment for HCV infection during the previous 6 mo. A group of HIV-infected patients on ART with HIV-RNA < 50 cp/mL for at least 12 mo and CD4$^+$ > 350/μL was considered as aviremic progressor controls (progressors).

The study was authorized by the local ethical committee. All patients already scheduled for a routine visit between October of 2011 and March of 2012 who provided informed consent were included. Nine Infectious Diseases Units caring for HIV patients enrolled 31 patients (15 ECs and 16 LTNPs) (Table S1).

HDs ($n = 10$) were recruited locally among blood bank donors.

Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation (Ficoll–Hypaque) and cryopreserved until processed. Analysis of genetic carriage (HLA and KIR) was performed on PBMCs and failed to show significant gene carriage enrichments (Table S2).

Abs. The following panel of mouse anti-human mAbs was used: PC5 and PC7 conjugated anti-CD56 (Immunotech-Coulter); FITC- and APC-conjugated anti-CD3 (BioLegend); APC-conjugated anti-CD19 and anti-CD14 (BioLegend); PE- and FITC-conjugated anti-CD16 (BD PharMingen); anti-NKp46 (BAB281 and IgG1), anti-NKp30 (7A6 and IgG1), anti-NKp44 (ZIN231 and IgG1), anti-NKG2D (BAT221 and IgG1), anti–DNAM-1 (F22 and IgG1), anti–LIR-1 (CD85j, F278, and IgG1), anti-KIR2DL2/S2 (CD158b1/CD158j), anti-KIR3DL1 (CD158e1), anti-KIR2DL1/S1 (CD158b/CD158ch; GL183, Z27, and 11pb6, respectively; and IgG1), and anti-NKG2A (Z199 and IgG2b). They were all produced in the laboratory (A. Moretta). Anti–HLA-DR (D1.12 and IgG2a) was provided by R. S. Accolla (University of Insubria, Varese, Italy). Anti-CD4 was from BD PharMingen (IgG1). FITC-conjugated (Southern Biotechnology) and PE-conjugated goat anti-mouse antiisotype Abs (BD PharMingen) were used for controls.

Immunofluorescence Analysis. Cells were analyzed by two-, three-, and four-color flow cytometry. Briefly, cells were incubated with primary mAbs followed by PE- or FITC-conjugated antiisotype-specific goat anti-mouse secondary reagents. Direct staining was performed by fluorochrome-conjugated mAbs as a third step. For cytofluorimetric analysis, cells were gated using forward and side light scatter parameters (FACSCantoII; BD), and 10,000 events were always acquired on CD3/14/19$^-$CD56$^+$ gating. MFI was used to represent molecule density on cell surface. To reduce interassay variability, the $MFI_{sample}/MFI_{control}$ was used (MFI_{ratio}) to compare sample/groups. Data were analyzed using FlowJo (Tree Star, Inc).

NK Cell-Activating Receptor Induction Assay. NK cells were isolated from PBMCs using biotin-conjugated antibody mixture (NK Cell Isolation Kit II; Miltenyi Biotec) as described (49) and either analyzed immediately after purification or after 2 and 4 d of in vitro culture with rhIL-2 (200 U/mL; Proleukin; Chiron Corp.). NK cell purity was ≥95%.

Cells were harvested and analyzed after 2 or 4 d by flow cytometry for the expression of NKp46, NKp30, and NKp44. Receptor expression changes were determined comparing MFI_r (or percentage of positive cells) with fresh NK cells MFI_r (or percentage of positive cells): $(MFI_{ratioTx} – MFI_{ratio\ baseline})/MFI_{ratio\ Tx} \times 100$.

Cytotoxicity Assay. Activated NK cell cytolytic activity was determined using PKH-26/TO-PRO3 (Sigma-Aldrich and Molecular Probes, respectively) assay as previously described (50). PKH-26–labeled target cells (2×10^4) were incubated with effector cells at different E:T ratios (1:1). K562, iDC, and FcγR$^+$ P815 mouse mastocytoma cell lines were used as target cells. Immature DCs were generated from peripheral blood monocytes as previously described (51). Cultures were incubated for 4 h at 37 °C in 5% (vol/vol) CO_2 in complete medium in the presence or absence of anti-NKp46 and anti-NKp44 mAbs and then placed on ice until flow cytometric analysis. Spontaneous and maximal target cell deaths (SD) were determined by PKH-26 labeling of cells cultured alone or permeabilized with BD Citofix/Citoperm reagent (BD Pharmigen). To identify dead cells, 5 μL 10 μM stock solution TO-PRO3 was added to each tube immediately before analysis. Cells were analyzed by FACSCanto II (BD), and 10,000 events were collected. Specific lysis was calculated by the formula (Sample– spontaneous dye/total dye –spontaneous dye) × 100.

Statistical Analysis. The Kruskall–Wallis and Mann–Whitney *u* tests for unpaired datasets were used for comparisons. Spearman test was used for correlation analysis. Tests were two-sided. Analysis was performed using StatView 4.2 program (Abacus Concepts).

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1. O'Connell KA, Bailey JR, Blankson JN (2009) Elucidating the elite: Mechanisms of control in HIV-1 infection. *Trends Pharmacol Sci* 30(12):631–637.
2. Poropatich K, Sullivan DJ, Jr. (2011) Human immunodeficiency virus type 1 long-term non-progressors: The viral, genetic and immunological basis for disease non-progression. *J Gen Virol* 92(Pt 2):247–268.
3. Lamine A, et al. (2007) Replication-competent HIV strains infect HIV controllers despite undetectable viremia (ANRS EP36 study). *AIDS* 21(8):1043–1045.
4. Blankson JN, et al. (2007) Isolation and characterization of replication-competent human immunodeficiency virus type 1 from a subset of elite suppressors. *J Virol* 81(5): 2508–2518.
5. Miura T, et al. (2008) Genetic characterization of human immunodeficiency virus type 1 in elite controllers: Lack of gross genetic defects or common amino acid changes. *J Virol* 82(17):8422–8430.
6. Geldmacher C, et al. (2007) CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients. *J Virol* 81(5): 2440–2448.
7. Migueles SA, et al. (2000) HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci USA* 97(6):2709–2714.

8. Fellay JSK, et al. (2007) A whole-genome association study of major determinants for host control of HIV-1. *Science* 317(5840):944–947.
9. Emu B, et al. (2008) HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control. *J Virol* 82(11):5398–5407.
10. Mothe B, et al. (2012) CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. *PLoS ONE* 7(1):e29717.
11. Tomescu C, et al. (2012) Impact of protective killer inhibitory receptor/human leukocyte antigen genotypes on natural killer cell and T-cell function in HIV-1-infected controllers. *AIDS* 26(15):1869–1878.
12. Mendoza D, et al. (2012) HLA B*5701-positive long-term nonprogressors/elite controllers are not distinguished from progressors by the clonal composition of HIV-specific CD8+ T cells. *J Virol* 86(7):4014–4018.
13. Martin MP, et al. (2007) Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. *Nat Genet* 39(6):733–740.
14. Martin MP, et al. (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* 31(4):429–434.
15. Alter G, et al. (2007) Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* 204(12):3027–3036.
16. O'Connell KA, Han Y, Williams TM, Siliciano RF, Blankson JN (2009) Role of natural killer cells in a cohort of elite suppressors: Low frequency of the protective KIR3DS1 allele and limited inhibition of human immunodeficiency virus type 1 replication in vitro. *J Virol* 83(10):5028–5034.
17. De Maria A, et al. (2003) The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur J Immunol* 33(9):2410–2418.
18. Mavilio D, et al. (2006) Characterization of the defective interaction between a subset of natural killer cells and dendritic cells in HIV-1 infection. *J Exp Med* 203(10): 2339–2350.
19. Bozzano F, et al. (2011) NK-cell phenotype at interruption underlies widely divergent duration of CD4+-guided antiretroviral treatment interruption. *Int Immunol* 23(2): 109–118.
20. Rutjens E, et al. (2007) Differential NKp30 inducibility in chimpanzee NK cells and conserved NK cell phenotype and function in long-term HIV-1-infected animals. *J Immunol* 178(3):1702–1712.
21. Bozzano F, et al. (2011) Activating NK cell receptor expression/function (NKp30, NKp46, DNAM-1) during chronic viraemic HCV infection is associated with the outcome of combined treatment. *Eur J Immunol* 41(10):2905–2914.
22. Stegmann KA, et al. (2012) Interferon α-stimulated natural killer cells from patients with acute hepatitis C virus (HCV) infection recognize HCV-infected and uninfected hepatoma cells via DNAX accessory molecule-1. *J Infect Dis* 205(9):1351–1362.
23. Vitale M, et al. (2005) NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor. *Blood* 106(2):566–571.
24. Pende D, et al. (2006) Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: Relevance for natural killer-dendritic cell interaction. *Blood* 107(5):2030–2036.
25. Ward J, et al. (2007) HIV modulates the expression of ligands important in triggering natural killer cell cytotoxic responses on infected primary T-cell blasts. *Blood* 110(4): 1207–1214.
26. Cerboni C, et al. (2007) Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity. *J Gen Virol* 88(Pt 1):242–250.
27. Matusali G, Potestà M, Santoni A, Cerboni C, Doria M (2012) The human immunodeficiency virus type 1 Nef and Vpu proteins downregulate the natural killer cell-activating ligand PVR. *J Virol* 86(8):4496–4504.
28. Fausther-Bovendo H, Vieillard V, Sagan S, Bismuth G, Debré P (2010) HIV gp41 engages gC1qR on CD4+ T cells to induce the expression of an NK ligand through the PIP3/H2O2 pathway. *PLoS Pathog* 6(7):e1000975.
29. Vieillard V, Strominger JL, Debré P (2005) NK cytotoxicity against CD4+ T cells during HIV-1 infection: A gp41 peptide induces the expression of an NKp44 ligand. *Proc Natl Acad Sci USA* 102(31):10981–10986.
30. Moretta L (2010) Dissecting CD56dim human NK cells. *Blood* 116(19):3689–3691.
31. Lopez-Vergès S, et al. (2010) CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* 116(19):3865–3874.
32. Björkström NK, et al. (2010) Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* 116(19):3853–3864.
33. De Maria A, Bozzano F, Cantoni C, Moretta L (2011) Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. *Proc Natl Acad Sci USA* 108(2):728–732.
34. Vankayalapati R, et al. (2004) NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen. *J Immunol* 172(1):130–137.
35. Warfield KL, et al. (2004) Role of natural killer cells in innate protection against lethal ebola virus infection. *J Exp Med* 200(2):169–179.
36. Moretta A (2002) Natural killer cells and dendritic cells: Rendezvous in abused tissues. *Nat Rev Immunol* 2(12):957–964.
37. Marras F, Bozzano F, De Maria A (2011) Involvement of activating NK cell receptors and their modulation in pathogen immunity. *J Biomed Biotechnol* 2011(2011): 152430.
38. De Maria A, et al. (1997) Expression of HLA class I-specific inhibitory natural killer cell receptors in HIV-specific cytolytic T lymphocytes: Impairment of specific cytolytic functions. *Proc Natl Acad Sci USA* 94(19):10285–10288.
39. Costa P, et al. (2001) Differential disappearance of inhibitory natural killer cell receptors during HAART and possible impairment of HIV-1-specific CD8 cytotoxic T lymphocytes. *AIDS* 15(8):965–974.
40. Fogli M, et al. (2004) Significant NK cell activation associated with decreased cytolytic function in peripheral blood of HIV-1-infected patients. *Eur J Immunol* 34(8): 2313–2321.
41. Lichtfuss GF, et al. (2012) Virologically suppressed HIV patients show activation of NK cells and persistent innate immune activation. *J Immunol* 189(3):1491–1499.
42. Björkström NK, et al. (2010) Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* 116(19):3853–3864.
43. Miura T, et al. (2009) HLA-associated viral mutations are common in human immunodeficiency virus type 1 elite controllers. *J Virol* 83(7):3407–3412.
44. Miura T, et al. (2009) HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. *J Virol* 83(6): 2743–2755.
45. Sajadi MM, Pulijala R, Redfield RR, Talwani R (2012) Chronic immune activation and decreased CD4 cell counts associated with hepatitis C infection in HIV-1 natural viral suppressors. *AIDS* 26(15):1879–1884.
46. Pereyra F, et al. (2012) Increased coronary atherosclerosis and immune activation in HIV-1 elite controllers. *AIDS* 26(18):2409–2412.
47. Fogli M, et al. (2008) Lysis of endogenously infected CD4+ T cell blasts by rIL-2 activated autologous natural killer cells from HIV-infected viremic individuals. *PLoS Pathog* 4(7): e1000101.
48. Curriu M, et al. (2012) Viremic HIV infected individuals with high CD4 T cells and functional envelope proteins show anti-gp41 antibodies with unique specificity and function. *PLoS One* 7(2):e30330.
49. Vieillard V, Dereuddre-Bosquet N, Mangeot-Méderlé I, Le Grand R, Debré P (2012) An HIVgp41 vaccine protects CD4 central memory T cells in SHIV-infected macaques. *Vaccine* 30(48):6883–6891.
50. De Maria A, et al. (2007) Increased natural cytotoxicity receptor expression and relevant IL-10 production in NK cells from chronically infected viremic HCV patients. *Eur J Immunol* 37(2):445–455.
51. Lee-MacAry AE, et al. (2001) Development of a novel flow cytometric cell-mediated cytotoxicity assay using the fluorophores PKH-26 and TO-PRO-3 iodide. *J Immunol Methods* 252(1-2):83–92.
52. Ferlazzo G, et al. (2003) The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur J Immunol* 33(2):306–313.