

## **NKG2D expression on HIV-specific CD8+ T cells is reduced in viremic HIV-1-infected patients but maintained in HIV controllers**

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## Brief Report

### NKG2D expression on HIV-specific CD8<sup>+</sup> T cells is reduced in viremic HIV-1-infected patients but maintained in HIV controllers

Running head: NKG2D expression on CD8<sup>+</sup>T cells in HIV infection

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The authors have no conflicts of interest to declare.

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## **Abstract**

NKG2D mediates an important co-stimulatory pathway in CD8<sup>+</sup> T cells. In HIV infection, we found that NKG2D expression on both total and HIV-specific CD8<sup>+</sup> T cells was significantly lower in viremic patients than in HIV controllers. Antiretroviral therapy partially restored NKG2D expression on HIV-specific CD8<sup>+</sup> T cells. We observed a negative correlation between the respective expression levels of CD38 and NKG2D on total CD8<sup>+</sup> and HIV-specific CD8<sup>+</sup> T cells. The maintenance of NKG2D expression on CD8<sup>+</sup> T cells in HIV controllers may contribute to better cell function.

Keywords: NKG2D, HIV-specific CD8<sup>+</sup> T lymphocytes, inflammation, HIV controllers

## Introduction

The CD8<sup>+</sup> T cell response has a major role in limiting HIV replication, as has been demonstrated in simian models (1), primary HIV infection (2) and HIV controllers (HICs, i.e. patients in whom HIV RNA is spontaneously undetectable) (3,4). The mechanisms underlying the strong antiviral potential and long-term persistence of HIV-specific CD8<sup>+</sup> T cells in HICs are not well understood. The NKG2D pathway could be involved in this phenomenon but remains largely unexplored in CD8<sup>+</sup> T cells in HIV-infected patients.

NKG2D is expressed on natural killer (NK) cells and has a major role in NK-mediated cell lysis (5,6). It is also present on the vast majority of CD8<sup>+</sup>  $\alpha\beta$ T cells (5,7). On CD8<sup>+</sup> T cells, NKG2D mediates a co-stimulatory pathway favoring proliferation, cytotoxicity and a Th1 antigen-specific cell response (7-11). Conversely, the protein is also involved in CD8<sup>+</sup> T cell immunoregulation and death. NKG2D ligands (NKG2DLs, such as the stress-inducible proteins MICA, MICB and ULBPs) are induced on activated T cells. The expression of NKG2DLs may lead to the elimination of activated CD8<sup>+</sup> T cells by NK cells, thus limiting their expansion (12,13). This could promote chronic viral infection, as has been recently reported (12). Moreover, the release of NKG2DLs downregulates NKG2D expression on CD8<sup>+</sup> T cells (14,15).

Although it was recently reported the NKG2D pathway may have an impact on CD8<sup>+</sup> T cells in HIV infection (11), the mechanism remains largely unexplored. Thus, we decided to use flow cytometry to study the expression of NKG2D and its ligands on T cells, with a focus on HIV-specific CD8<sup>+</sup> T cells in different groups of HIV-infected patients. We hypothesized that decreased expression of NKG2D and over-expression of NKG2DLs in viremic patients would lead to the death of HIV-specific T cells. In contrast, normal NKG2D expression in HICs would optimize cytotoxicity and cell proliferation.

## Patients and Methods

Four groups of patients were studied: primary HIV infection patients (PHI) (n=11) enrolled in the French ANRS PRIMO cohort (16), chronically HIV-1-infected patients with a viral load above 10,000 RNA copies/mL ("viremic patients") (n=10), patients with undetectable plasmatic RNA viral load after long-term (> 2 years) highly active anti-retroviral treatment (HAART) ("treated patients") (n=11) and, lastly, HIV controllers (HICs (n=15) enrolled in the French ANRS HIV Controllers cohort (17). The latter patients had been infected for more than 10 years and had never received HAART. Ninety percent of their plasma viral RNA assays were below 400 copies/mL.

In addition, 13 healthy donors (HDs) were also studied. The experimental procedures with human blood were approved by an independent institutional review board (*Ile de France VII*) and were performed according to European Union guidelines and the Declaration of Helsinki.

HIV-specific CD8<sup>+</sup> T cells were identified with soluble allophycocyanin (APC)-labeled peptide-HLA class 1 multimers (Proimmune, Oxford, United Kingdom) derived from HIV proteins and then stained with antibodies directed against NKG2D (coupled to phycoerythrin (PE) (R&D Systems Europe, Lille, France)), CD8 (coupled to peridin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)) and CD3 (coupled to APC-H7 (BD Biosciences, San Jose, CA)). CD38 staining was performed with an antibody coupled to fluorescein isothiocyanate (FITC) (BD Bioscience, San Jose, CA).

NKG2DLs staining was performed using anti- MICA, -MICB, -ULBP1, -ULBP2 and -ULBP3 antibodies coupled to PE (MICA, MICB, ULBP2), FITC (ULBP1) and APC (ULBP3) (R&D Systems Europe).

The soluble MICA assay was kindly performed in Sophie Caillat-Zucman's lab.

Statistical analysis was performed using ANOVA tests.

## Results

NKG2D was strongly expressed on total CD8<sup>+</sup> T cells from HDs (as described in the literature (5)) and HICs (82±15% and 77±20%, respectively) (Figure 1a). In contrast, PHI patients showed significantly lower NKG2D expression on their CD8<sup>+</sup> T cells (41±22%; p<0.001 and p<0.01 when compared with HDs and HICs, respectively). Low expression was also observed on CD8<sup>+</sup> T cells from viremic patients and treated patients (54±18% and 52±16%, respectively; p<0.05 vs. HDs for both comparisons).

NKG2D expression was then studied on HIV-specific CD8<sup>+</sup> T cells in HIV-infected patients (Figure 1b). Importantly, expression was high in HICs (83±14%). In contrast, viremic patients and PHI patients, all with high viral loads, displayed significantly lower expression of NKG2D on their HIV-specific CD8<sup>+</sup> T cells, relative to HICs (59±16% for PHI patients and 53±18% for viremic patients; p<0.001 vs. HICs for both comparisons). Treated patients displayed significantly higher expression of NKG2D on their HIV-specific CD8<sup>+</sup> T cells (73±16%) than viremic patients did (p<0.05).

Comparisons of the mean fluorescence intensity for NKG2D in each group of patients yielded similar results (data not shown).

This defect in NKG2D expression may contribute to impaired function in HIV-specific CD8<sup>+</sup> T cells, since we observed a positive correlation between NKG2D expression on one hand and the percentages of both IL-2<sup>+</sup> and CFSE<sup>low</sup> CD8<sup>+</sup> T cells on the other (p=0.002, r=0.49, and p=0.031, r=0.38, respectively).

Our results suggest that HIV infection lowers the expression of NKG2D on total and HIV-specific CD8<sup>+</sup> T cells. We then investigated several possible mechanistic explanations for the much lower levels of NKG2D on CD8<sup>+</sup> T cells from viremic patients.

Firstly, reports in many settings have demonstrated that soluble forms of MHC Class I chain-related (MIC) molecules induce NKG2D down-modulation in NK and T cells (14,15). We therefore

tested for the presence of soluble MICA in the patients' plasma; however, low levels were found in all groups (data not shown).

Secondly, activated CD8<sup>+</sup> T cells may express NKG2DLs, which would lead to their killing by NK cells (12,13). We and others have reported that viremic subjects have higher activated CD8<sup>+</sup> T cell counts than HICs and HAART-treated patients (4,18). Thus, we hypothesized that there could be a negative correlation between NKG2D expression and CD38 expression and a positive correlation between NKG2DLs expression and CD38 expression in CD8<sup>+</sup> T cells from untreated patients. Indeed, we observed a negative correlation between the respective expression levels of CD38 and NKG2D on total CD8<sup>+</sup> T cells (Figure 2a,  $p=0.0045$ ,  $r=-0.35$ ) and HIV-specific CD8<sup>+</sup> T cells (Figure 2b  $p=0.0003$  and  $r=-0.46$ ). In contrast, we did not detect any significant *ex vivo* expression of MICA, MICB or ULBP1, 2, and 3 on CD8<sup>+</sup> T cells (data not shown) and therefore no correlation with activation of expression.

This finding suggests either that there is a defect in NKG2DLs expression on CD8<sup>+</sup> T cells in the setting of HIV infection or that NKG2DL-expressing activated CD8<sup>+</sup> T lymphocytes are rapidly killed by NK cells.

To test the first hypothesis, we investigated the expression of NKG2DLs on CD8<sup>+</sup> T cells after strong mitogenic activation (with a combination of anti-CD2 and anti-CD28 antibodies) in the different groups of patients. Each ligand tested was significantly and similarly induced *in vitro* on activated CD8<sup>+</sup> T cells from each group (data not shown). Next, we looked at whether the level of NKG2D expression on NK cells could account for the killing of NKG2DL<sup>+</sup> CD8<sup>+</sup> T cells. We found that HDs and viremic patients did not differ significantly in terms of NKG2D expression on their NK cells ( $74\pm 6\%$  and  $69\pm 7\%$ , respectively), as previously reported (19). Unexpectedly, NKG2D expression was significantly lower on NK cells from HICs, when compared with those from viremic patients or HDs ( $13\pm 3\%$ ,  $p<0.0001$  for both comparisons).

## Discussion

Our results suggest that HIV replication lowers the expression of NKG2D on total and HIV-specific CD8<sup>+</sup> T cells. This co-stimulatory defect may contribute to impaired function in anti-HIV CD8<sup>+</sup> T cells. In contrast, NKG2D expression is maintained on total and HIV-specific CD8<sup>+</sup> T cells in HICs.

The level of immune activation may play a role in the regulation of NKG2D expression on CD8<sup>+</sup> T cells, since we observed a negative correlation between the respective expression levels of CD38 and NKG2D on total CD8<sup>+</sup> T cells and HIV-specific CD8<sup>+</sup> T cells. In the context of antiretroviral therapy, reduced activation might allow the reconstitution of a pool of HIV-specific NKG2D<sup>+</sup> CD8<sup>+</sup> T cells. Interestingly, normal expression of NKG2D on HIV-specific CD8<sup>+</sup> T cells in HICs may be favored by the lower level of immune activation seen in these patients, when compared with viremic individuals (18). Given that the MICA and MICB proteins are highly polymorphic (20), rare alleles associated with reduced cell membrane expression of these proteins could be over-represented in HICs (thus reducing CD8<sup>+</sup> T cell killing by NKG2D<sup>+</sup> NK cells). Interestingly, a genome-wide association study of the HIC cohort showed that most of the tag single nucleotide polymorphisms associated with low viral replication were located in the region ranging from HLA-Cw to MICB (21). Low *in vivo* induction of MICA or MICB on CD8<sup>+</sup> T cells by physiological stimuli could lead to the greater maintenance of HIV-specific NKG2D<sup>+</sup> CD8<sup>+</sup> T cell function in HICs. This point deserves further attention.

However, we also showed that there was neither an overall nor a selective impairment of the expression of the various NKG2DLs on CD8<sup>+</sup> T cells after *in vitro* cell activation. Our results thus suggest that NKG2DL<sup>+</sup> CD8<sup>+</sup> T cells are not detected *ex vivo* because they are killed *in vivo* by NKG2D<sup>+</sup> NK cells. After looking at NKG2D expression on NK cells in viremic patients and HICs, our results are in accordance with the literature: in viremic patients, NKG2D expression is maintained on NK cells (in contrast to other killer cell immunoglobulin-like receptors) (19). The



lower expression of NKG2D on NK cells in HICs has not been previously described and remains puzzling. Other peculiar phenotypic features in controllers have suggested the specific regulation of activating NK cell receptors in these patients (22). Low expression of NKG2D on NK cells might also facilitate the survival of HIV-specific CD8<sup>+</sup> T cells in HICs.

This extensive study is the first to have described NKG2D expression on total and HIV-specific CD8<sup>+</sup> T cells in different groups of HIV-infected patients. We suggest that the maintenance of NKG2D expression on CD8<sup>+</sup> T cell in HICs may help to optimize CD8<sup>+</sup> T cell function and the antiviral immune response in these patients, as recently suggested (11). Therapeutic strategies based on reduced immune activation should be also beneficial in the maintenance of this cell activation pathway.

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## Figure legend

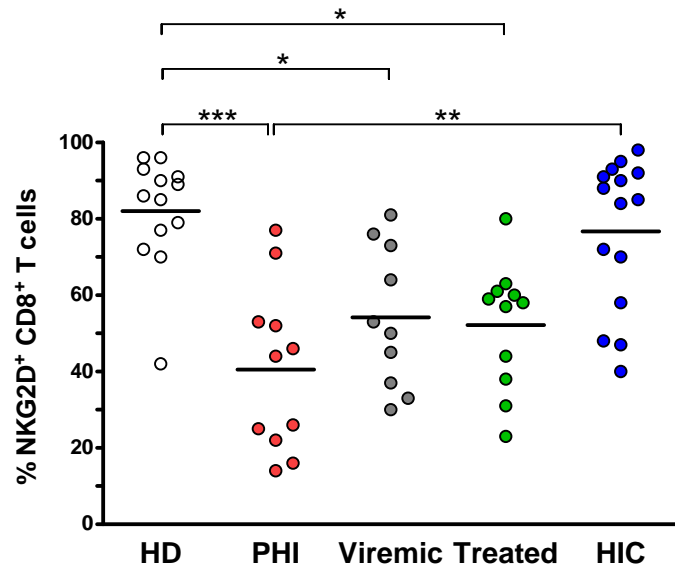
### Figure 1

1a: NKG2D expression on total CD8<sup>+</sup> T cells (\*\*= p<0.001, \*= p<0.01, \* = p<0.05 in an analysis of variance (ANOVA)). 1b: NKG2D expression on HIV-specific CD8<sup>+</sup> T cells from HIV-infected patients (\*\*= p<0.001, \*\* = p<0.01, \* = p<0.05 in an ANOVA). HD: healthy donor, HIC: HIV controller, PHI: primary HIV infection

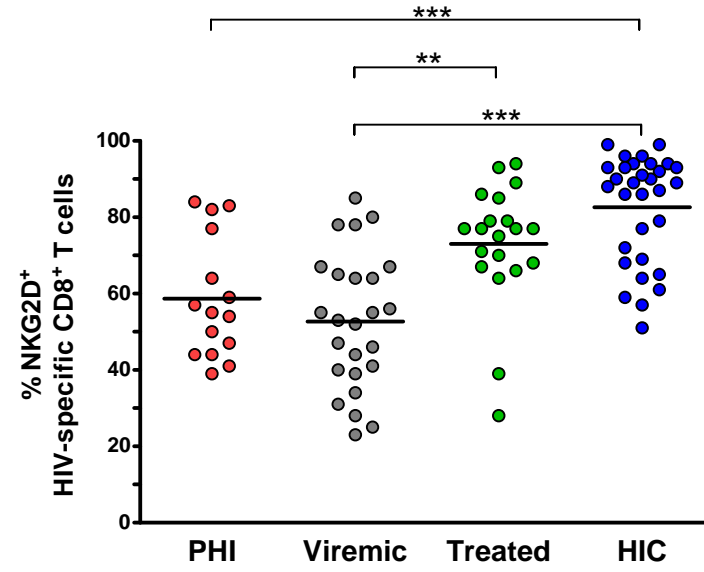
### Figure 2

2a: Negative correlation between the percentages of NKG2D<sup>+</sup> CD8<sup>+</sup> T cells and activated CD38<sup>+</sup> CD8<sup>+</sup> T cells. 2b: Negative correlation between the percentages of HIV-specific NKG2D<sup>+</sup> CD8<sup>+</sup> T cells and HIV-specific activated CD38<sup>+</sup> CD8<sup>+</sup> T cells. HIC: HIV controller

Figure 1

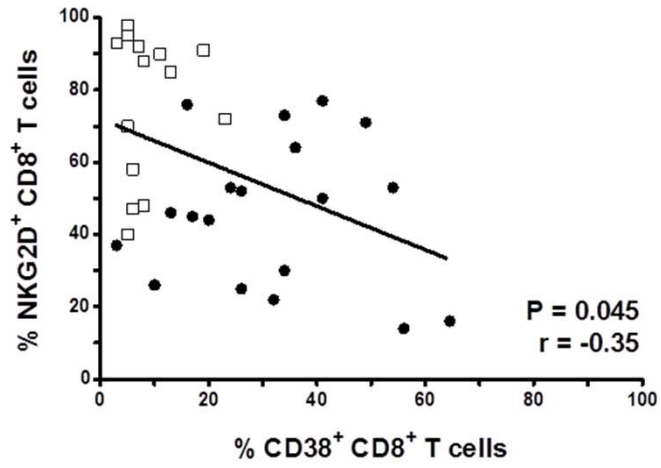


1a

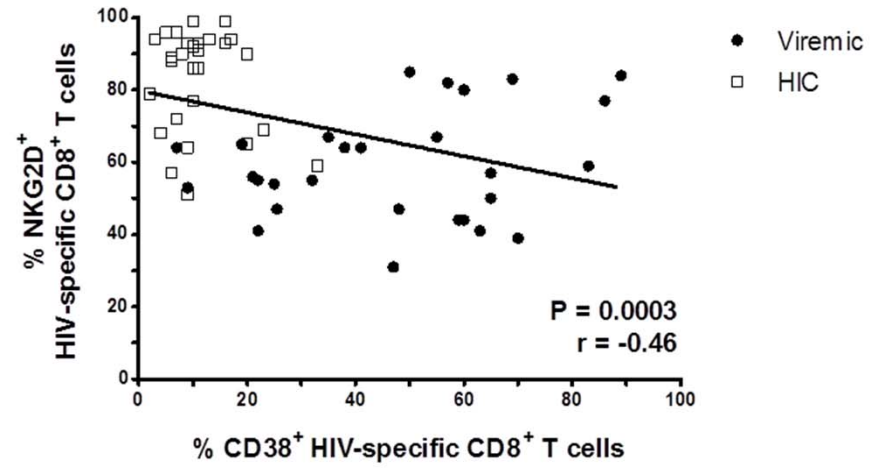


1b

**Figure 2**



**2a**



**2b**