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NK cells of HIV-1-infected patients with poor CD4$^+$ T-cell reconstitution despite suppressive HAART show reduced IFN-γ production and high frequency of autoreactive CD56^{bright} cells

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ABSTRACT

HIV-1-infected patients failing to recover CD4$^+$ T-cell count despite HAART (immunological non-responders, NRs), are at increased risk of disease progression and death. To better understand the NR status, we performed a comprehensive assessment of NK cells in NR patients as compared to immunologic responders. NRs exhibited an accumulation of CD56^{bright} NK cells inversely correlated with CD4$^+$ counts. Both CD56^{bright} and CD56^{dim} NK cells of NRs displayed unimpaired degranulation ability, but poorly responded to cytokine stimulation in terms of NKp44 up-regulation and IFN-γ production that may explain the susceptibility of NRs to infections and tumors. Notably, CD56^{bright} NK cells from NRs showed higher cytotoxicity against autologous activated CD4$^+$ T cells. Moreover, NRs had reduced Treg cell counts that showed an inverse correlation with autoreactive CD56^{bright} cells. These data suggest that accumulation of CD56^{bright} NK cells, possibly linked to decreased homeostatic control by Tregs, contributes to poor immune reconstitution in NRs.

1. Introduction

In human immunodeficiency virus (HIV)-infected patients, long-term highly active antiretroviral therapy (HAART) suppresses viral replication below detection levels and leads to peripheral CD4$^+$ T-lymphocyte count reconstitution and a drastic reduction of AIDS-related morbidity and mortality rates. However, despite complete HIV-1 suppression, 15–30% of treated patients fail to recover CD4 counts, particularly those starting HAART late in the course of disease when severe CD4$^+$ T-cell depletion has occurred [1,2]. These critical patients, identified as immunological non responders (NR), display various immunologic alterations that are not present in patients with complete CD4 counts restoration, including highly differentiated T-cell maturation profile, heightened CD8$^+$ T-cell activation, and apoptotic/exhausted CD4$^+$ T-cell phenotype [3–8]. Importantly, NR patients are at high risk of serious clinical events (e.g. malignancies, cardiovascular events, severe infections) and death [9–13], thus a better understanding of the immune dysfunction and specific adjuvant therapies for the NR status are needed.

Poor immunologic reconstitution in NRs has been associated with a central defect in CD4$^+$ T-cell regeneration caused by reduced thymopoiesis and advanced thymus involution [14–16]. Conversely, various studies on NRs reported normal thymic output [17,18], and emphasized depletion of peripheral CD4$^+$ T cells via apoptotic death associated with high level of immune activation [5,7,17]. More recently, an additional mechanism sustaining CD4$^+$ T cell depletion in NR patients has been proposed, consisting in the up-relation of NKp44L, the ligand for an activating receptor of natural killer (NK) cells, on the surface of uninfected CD4$^+$ T cells that, as a consequence, become susceptible to NK-cell mediated killing [18]. This interesting model, however, has not yet been supported by NK cell functional studies in NR patients and, more in general, the role of NK cell function in incomplete CD4$^+$ T cell recovery has been basically disregarded. NK cells are a crucial component of the innate response against viruses and tumors endowed with

Abbreviations: FMO, fluorescence minus one control; HAART, highly active antiretroviral therapy; HD, healthy donor; HIV, 1 human immunodeficiency virus type 1; IFN-γ, interferon-γ; LPS, lipopolysaccharides; mAb, mouse monoclonal antibody; MFI, Mean fluorescence intensity; NK, natural killer cell; NRs, immunological non-responders; PBL, peripheral blood lymphocytes; PBMCs, peripheral blood mononuclear cells; Rs, immunological responders; Treg, regulatory T cell; SEM, standard error of the mean

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the capacity to directly recognize and kill infected and transformed cells, secrete anti-viral chemokines and cytokines, and amplify adaptive immune responses by interactions with various cell types [20]. Two functionally distinct subsets of NK cells can be defined based on the differential expression of CD56, CD16, and of many receptors regulating cell function and homing [21]. Most NK cells in peripheral blood belong to the highly cytotoxic $CD56^{dim}$ subset, while a small percentage (5–10%) represents $CD56^{bright}$ cells that display relatively lower cytotoxicity and yet release relevant amounts of cytokines upon stimulation. $CD56^{bright}$ are believed to be precursors of $CD56^{dim}$ cells based on in vitro and in vivo studies [22–24]. In addition, a minor $CD56^{neg}$ subset of anergic NK cells may accumulate with systemic inflammation [25]. Chronic HIV-1 infection leads to pathologic changes in the NK cell compartment, such as abnormal repertoire of inhibitory and activating receptors and defective functionality, characterized by $CD56^{dim}$ cell depletion and expansion of the $CD56^{neg}$ subset [26]. HAART-induced suppression of HIV-1 replication has been reported to normalize subset distribution and phenotype of NK cells, although there is no consensus on the degree of NK-cell function restoration [26]. In this study, we investigated the frequency, phenotype and various functions of NK-cell subsets in a cohort of NRs compared with full immunologic responders (R). Our results show that the function of NK cells is altered in NRs and identify the accumulation of a regulatory $CD56^{bright}$ NK cell population as a novel mechanism behind poor $CD4^+$ T cell recovery during suppressive HAART.

2. Materials and methods

2.1. Study participants

Twenty-eight HIV-1-infected patients receiving suppressive HAART were enrolled: 12 NRs with poor $CD4^+$ T-cell recovery (< 350 $CD4^+$ T cells/μl), and 16 Rs with immunological restoration (> 500 $CD4^+$ T cells/μl). All subjects were of Caucasian origin and without ongoing co-infections or other diseases and their characteristics are summarized in Table 1. For pilot experiments, we included 13 Caucasian healthy donors (HD) with a similar age (38 median years, 31–48.5 years interquartile range) and a lower proportion of males (1/12) if compared to patients. Ethical committee approval and written informed consent from all participants were obtained, in accordance with the Declaration of Helsinki.

2.2. PBMCs isolation and flow cytometry

Peripheral blood mononuclear cells (PBMCs) and plasma were obtained from EDTA-treated blood samples by Ficoll gradient technique and cryopreserved until used. PBMCs were thawed (80% minimum cell viability), then 0.5×10^6 cells were stained with mouse monoclonal antibodies (mAbs) in PBS containing 0.5% BSA and 0.1% NaN_3 for 20 min at 4 °C. For detection of intracellular IFN-γ, cells were treated with Cytofix/Cytoperm kit from BD Pharmingen (Franklin Lakes, NJ), then incubated with specific mAb for 30 min at room temperature. Stained cells were washed, fixed in 1% paraformaldehyde and acquired on a FACSCanto II (Becton Dickinson Italia Spa, Milan, Italy). Positive cell gating was set using fluorescence minus one control (FMO). Mean fluorescence intensity (MFI) was subtracted of the value obtained with isotype control antibody. Data analyses were performed using FlowJo software (TreeStar, Ashland, OR).

2.3. Enzyme-Linked immunosorbent assay (ELISA)

To quantify soluble CD14 (sCD14) and lipopolysaccharides (LPS) in plasma samples we used the Human sCD14 Quantikine (R & D Systems, Minneapolis, MN) and Human LPS (Cusabio Biotech, Hubei, P. R. China) ELISA Kit, respectively, following manufacturer's instructions.

Table 1
Characteristics of participants.

	R	NR	*P* values[a]
Number of individuals	16	12	
Male/Female (n)	13/4	9/3	1.000
Age [median years (IQR)]	41 (31–51)	45 (37–50)	0.220
Duration of HAART [median months (IQR)]	40 (23–111)	36 (24–49)	0.561
Type of HAART at time of analysis (n)			
NRTI + PI	9	7	
NRTI + NNRTI	7	1	
NRTI + II	0	1	
PI + II	0	3	
Plasma HIV RNA at time of analysis (median copies/ml)	< 20	< 20	
$CD4^+$ T cell count [median cells/μl (IQR)]			
At time of analysis	680 (552–903)	277 (200–328)	< 0.0001
Before HAART	315 (200–580)	94 (24–156)	0.004
$CD8^+$ T cell count [median cells/μl (IQR)]	638 (519–892)	646 (533–1091)	0.869
CD4/CD8 ratio [median (IQR)]	1.05 (0.93–1.22)	0.35 (0.24–0.59)	< 0.0001
Plasma sCD14 [median ng/ml (IQR)]	1692 (1471–2015)	1726 (1421–2393)	0.900
Plasma LPS [median pg/ml (IQR)]	272 (210–414)	286 (211–1007)	0.625

IQR, interquartile range; n, number of individuals; NRTI, nucleoside and nucleotide reverse transcriptase inhibitors; PI, protease inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitors; II, integrase inhibitor.

[a] Calculated by unpaired *t* test or Mann Whitney test; for categorical variables, X^2-test was used.

2.4. Antibodies

For flow cytometric analysis, the following mAbs against human antigens were used: NKG2D(CD314)/PE (1D11), CD3/FITC or APC (UCHT1), CCR7(CD197)/PE-Cy7 (3D12), CD27/PE (O323), CD38/PE-Cy7 (HB7), CD127/PE-Cy7 (RDR5), CD57/PE-Cy7 (TB01), CD16/APC-eFluor780 (CB16), PD-1(CD279)/PE-Cy7 (J105), FOXP3/Alexa488 (259D), CD127/PE-Cy7 (RDR5), CD45RA/APC-eFluor780 (HI100) from eBioscience (San Diego, CA); CD107a/FITC (H4A3), DNAM-1(CD226)/FITC (11A8), NKp30/PE (P30-15), NKp44/PE (P44-8), anti-NKp46/PE-Cy7 (9E2) from BioLegend (San Diego, CA); CD4/PerCp (L200), CD45RA/APC-H7 (HI100), CD25/FITC or PE (M-A251), CD69/PE (FN50), Annexin V/FITC, CD4/APC (RPA-T4), IFN-γ/FITC (B27) from BD Pharmingen; CD56/PerCp (MEM-188), CD62L/APC (LT-TD180) from Thermo Scientific (Waltham, MA); CD25/APC (MEM-181) from EuroBioSciences (Friesoythe, Germany). For isotype control staining, conjugated mouse IgG obtained from BD Pharmingen were used.

2.5. NKp44 up-regulation by IL-2

PBMCs were cultured for 5 days at 0.5×10^6 cells/ml in complete medium (RPMI 1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin; all from EuroClone, Pero, Italy) supplemented or not with 200 IU/ml of IL-2 (Peprotech, Rocky Hill, NJ), then immunolabeled for flow cytometry analysis of cell-surface receptors on gated $CD56^{bright}$ and $CD56^{dim}$ NK cells. Albeit 5-days culture with IL-2 up-regulated CD56 expression on both $CD56^{bright}$ and $CD56^{dim}$ as well as CD16 expression $CD56^{dim}$ cells, NK-cell subsets could be clearly distinguished in the established gates (data not shown).

2.6. NK cell degranulation assays

PBMCs were cultured overnight in complete medium and then