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Th1 and Th17 proinflammatory profile characterizes invariant natural killer T cells in virologically suppressed HIV+ patients with low $CD4^+/CD8^+$ ratio

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Introduction: Scanty data exist on the phenotype and functionality of invariant natural killer T (iNKT) cells in HIV-infected (HIV+) patients.

Methods: By flow cytometry, we studied iNKT cells from 54 HIV+ patients who started combined antiretroviral therapy and had undetectable viral load for more than 1 year. Twenty-five maintained a $CD4^+/CD8^+$ ratio less than 0.4, whereas 29 reached a ratio more than 1.1; 32 age-matched and sex-matched patients were healthy controls (CTR).

Results: Patients with low ratio had lower percentage of $CD4^+$ iNKT cells compared with patients with high ratio and higher $CD8^+$ iNKT cell percentage; double-negative iNKT cells were lower in HIV+ patients compared with CTR. Patients with low ratio had higher percentage of $CD4^+$ and double-negative iNKT cells expressing CD38 and HLA-DR compared with patients with high ratio. $CD4^+$ iNKT cells expressing PD-1 were higher in patients with $CD4^+/CD8^+$ ratio less than 0.4, whereas double-negative iNKT cells expressing PD-1 were lower compared with patients with ratio more than 1.1. Patients with low ratio had higher $CD4^+$ iNKT cells producing IL-17, $CD8^+$ iNKT cells producing IFN-γ, TNF-α or IFN-γ and TNF-α, and double-negative iNKT cells producing IL-17 or IL-17 and IFN-γ compared with CTR. Activated $CD4^+$ (or $CD8^+$) T cells correlated with activated $CD4^+$ (or $CD8^+$) iNKT cells, as well as the percentages of $CD4^+$ (or $CD8^+$) T cells expressing PD-1 was correlated to that of $CD4^+$ (or $CD8^+$) iNKT cells expressing PD-1.

Conclusion: Low $CD4^+/CD8^+$ ratio despite effective combined antiretroviral therapy is associated with altered iNKT cell subsets, enhanced activation, and prominent Th1/Th17 proinflammatory profile.

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Keywords: antiretroviral therapy, $CD4^+/CD8^+$ ratio, flow cytometry, iNKT cells, polyfunctionality

Introduction

Despite full viral suppression by combined antiretroviral therapy (cART), some HIV-infected (HIV+) patients maintain high levels of circulating $CD8^+$ T cells and a persistently low $CD4^+/CD8^+$ ratio. The normalization of the $CD4^+/CD8^+$ ratio has a great clinical relevance, and it has been shown that patients

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with low ratio are characterized by altered T-cell subsets, high $CD8^+$ T-cell activation, and increased risk of non-AIDS-related morbidity and mortality [1,2]. The mechanisms at the basis of the failure of immune recovery in these patients are not well understood, but likely include those involved in the regulation of T-cell homeostasis. Among such mechanisms, those based upon invariant natural killer T (iNKT) cells have never been investigated in detail.

iNKT cells are innate-like lymphocytes that recognize glycolipid antigens presented by major histocompatibility complex class-I-related glycoprotein CD1d and exert important immunoregulatory functions [3–5]. These cells represent about 0.01–1.0% of peripheral blood mononuclear cells (PBMCs) and are characterized by the presence of a semi-invariant T-cell receptor (iTCR), formed by a Vα24Jα18 α-chain coupled with a Vβ11 β-chain [6]. Upon stimulation, this rare population rapidly produces proinflammatory and/or antiinflammatory cytokines able to orchestrate innate immunity through the recruitment and activation of different types of effector cells (NK cells and dendritic cells) as well as B and T cells [7–11]. iNKT cells can be divided into different subsets on the basis of the expression of $CD4^+$ and $CD8^+$; in turn, each subset is functionally distinct: $CD4^+$ subset mainly produces Th2 cytokines, whereas $CD8^+$ and $CD4^-CD8^-$ (double-negative) iNKT cells are characterized by the production of Th1 cytokine and a strong cytolytic activity [12].

The frequency and activity of iNKT cells have been studied in several acute and chronic infections, including HIV. In this case, it was shown that iNKT cells are highly susceptible to the infection and constitute a considerable viral reservoir [13]. Concerning their subpopulations, $CD4^+$ iNKT cells are rapidly depleted in the gut after primary infection and are further lost in the following phases [14], but are preserved in gut-associated lymphoid tissue (GALT). In the GALT, iNKT cells are characterized by production of Th2 cytokines [15]. Circulating iNKT cells are functionally impaired and express high levels of PD-1 [16], most of them produce IFN-γ [16], and a high expression of CD161 is associated with poor cytokine secretion [17]. To note, a successful cART induces a rapid recovery of NKT cells [18], but the reconstitution of the pool of $CD4^+$ iNKT cells remains delayed [19].

The phenotype and the polyfunctionality of iNKT cells and their subsets have never been investigated in details in patients with different $CD4^+/CD8^+$ ratio after successful cART. Thus, we have characterized iNKT cells in patients with low and normal $CD4^+/CD8^+$ ratio and evaluated their capacity to produce *in vitro* proinflammatory or anti-inflammatory cytokines upon stimulation.

Materials and methods

Patients

The study was approved by the Institutional Review Board of the Department of Surgery, Medicine, Dentistry and Morphological Sciences of the University of Modena and Reggio Emilia. We enrolled a total of 54 HIV+ patients with undetectable viral load (<50 copies/ml), and in particular 25 patients (24 men) who displayed a $CD4^+/CD8^+$ ratio less than 0.4 have been taking cART since 7.12 ± 5.50 years (group A). They were compared with 29 patients (20 men) with a $CD4^+/CD8^+$ ratio more than 1.1 who had been under cART since 7.47 ± 5.83 years (group B). As controls, we studied a total of 32 age-matched and sex-matched healthy donors (CTR). Patients' characteristics and treatments are reported in Table 1.

Polychromatic flow cytometry

To identify circulating iNKT cells, whose percentage in peripheral blood is typically less than 1%, and in HIV+ patients even less, it is mandatory to acquire a huge number of cells [20,21]. Thus, up to 50 ml of venous blood was collected from each patient into ethylenediaminetetraacetic acid tubes. PBMCs were isolated by Ficoll-Hypaque density gradient according to standard procedures and immediately processed for immunophenotypic analysis.

Freshly isolated PBMCs were stained with fluorochrome-labeled mAbs at previously defined optimal concentrations. The following mAbs were used to identify iNKT cells: anti-Vα24Jα18 TCR-PE (Becton Dickinson, San José, California, USA), -CD161-PC7 (Beckman Coulter, Brea, California, USA), -CD4-AF700, -CD8-APC-Cy7, -CD3-PE-Cy5, -HLA-DR-FITC, -CD38-BV605, and anti-programmed cell death protein-1 (PD-1, or CD279)-BV421 (from eBiolegend, San Diego, California, USA). PBMCs were incubated for 20 min at room temperature, washed with Stain Buffer (Becton Dickinson), and immediately analyzed. A minimum of 10 million cells were acquired on an acoustic focusing flow cytometer (Attune NxT; ThermoFisher, Eugene, Oregon, USA).

In-vitro stimulation

For functional assays, freshly isolated PBMCs were stimulated for 4 h at 37 °C in 5% CO_2 atmosphere with phorbol myristate acetate (PMA; final concentration 200 ng/ml, Sigma Aldrich, St. Louis, Missouri, USA) and ionomycin (1 μg/ml, Sigma Aldrich) in complete culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and 1% each of L-glutamine, sodium pyruvate, nonessential amino acids, antibiotics, 0.1 mol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 55 μmol/l β-mercaptoethanol). For each sample, up to 15 million cells were left unstimulated as negative control and up to 15 million cells were stimulated. All samples were

incubated with a protein transport inhibitor containing brefeldin A (Golgi Plug, Becton Dickinson). Cells were stained with Live Dead AQUA (ThermoFisher) and surface mAbs anti-Vα24Jα18 TCR-PE (Becton Dickinson), -CD3-PE-Cy5, -CD4-AF700, and -CD8-APC-Cy7 (Biolegend). Cells were washed with Stain Buffer (Becton Dickinson), fixed and permeabilized with the Cytofix/Cytoperm buffer set (Becton Dickinson) for cytokine detection [22]. Finally, cells were stained with mAbs anti-IL-17A-BV421, anti-TNF-α-BV605, IFN-γ-FITC, and IL-4-APC according to standard procedures. Up to 15 million cells per sample were acquired by an acoustic focusing flow cytometer (Attune NxT).

Flow cytometry

Data were acquired in list mode using Attune NxT 2.1 software and analyzed by FlowJo 9.9 (Tree Star Inc., Ashland, Oregon, USA) [23]. Samples were compensated by software after acquisition. Single staining and Fluorescence Minus One controls were performed for all mAbs of the panel to set proper compensation and define positive signals. 'Simplified Presentation of Incredibly Complex Evaluations' (SPICE, kindly provided by Dr Mario Roederer, NIH, Bethesda, Maryland, USA) software 5.3 was used to graphically depict polychromatic flow cytometry data [24,25]. For the functional analysis of iNKT cells, a threshold of 0.05% was set on the basis of the distribution of negative values generated after background subtraction [26,27].

Statistical analysis

Quantitative variables were compared with nonparametric Mann–Whitney *U* test. The correlations between clinical parameters and molecular data were performed by Spearman correlation test and linear regression analysis. *P* values less than 0.05 were considered statistically significant. Permutation test was used to compare SPICE pies. The results were confirmed with rank sum test. The Bonferroni correction was applied for comparison between CTR vs less than 0.4 group or CTR vs more than 1.1 group. Data are represented as mean ± SEM. Statistical analyses were performed using Prism 6.0 (Graphpad Software Inc., La Jolla, California, USA).

Results

Identification of invariant natural killer T cells among freshly isolated peripheral blood mononuclear cells

The gating strategy used to identify iNKT cells with their subsets is shown in Fig. 1a. Freshly isolated lymphocytes were identified by their physical parameters [forward side

Table 1. Clinical characteristics of the patients, and type of therapy.

	CTR	Group A CD4+/CD8+ ratio <0.4	Group B CD4+/CD8+ ratio >1.1	*P* (A vs B)
Patients *N* (men)	32 (17)	25 (24)	29 (20)	NS
CD4+/CD8+ ratio[a]	1.65 ± 0.74	<0.4	>1.1	<0.001
Age (years)[a]	47.28 ± 8.05	51.08 ± 11.15	47.14 ± 10.11	NS
Time of HAART (years)[a]	–	7.12 ± 5.50	7.47 ± 5.83	NS
CD4+ cell count (cells/μl)[a]	773 ± 300	479 ± 179	913 ± 355	<0.0001
CD8+ cell count (cell/μl)[a]	479 ± 197	1155 ± 463	616 ± 279	<0.0001
Viral load <50 copies/ml [N (%)]		25 (100)	29 (100)	NS
HAART regimen (N)				
3TC/ATV/RAL		0	1	
3TC/ATV/RTV		1	0	
3TC/DRV/RTV		4	2	
3TC/DTG		0	2	
3TC/LPV/RTV		0	1	
ABC/3TC/ATV/RTV		1	1	
ABC/3TC/DRV/RTV		1	2	
ABC/3TC/DTG		1	1	
ABC/3TC/EFV		1	2	
ABC/3TC/NEV		0	2	
ABC/ZDV/3TC/LPV/RTV		1	0	
ATV/RTV/MVC		1	0	
ZDV/DRV/RTV/MVC		1	0	
DRV/RTV		0	3	
DRV/RTV/RAL		2	0	
TDF/FTC/ATV/RTV		2	1	
TDF/FTC/DRV/RTV		1	0	
TDF/FTC/EFV		5	7	
TDF/FTC/ELV/COBI		0	1	
TDF/FTC/RPV		3	3	

[a]Mean ± SD. ABC, abacavir; ATV, atazanavir; COBI, cobicistat; DRV, darunavir; DTG, dolutegravir; EFV, efavirenz; ELV, elvitegravir; FTC, emtricitabine; LPV, lopinavir; MVC, maraviroc; NEV, nevirapine; RAL, raltegravir; RPV, rilpivirine; RTV, ritonavir; TDF, tenofovir disoproxil fumarate; ZDV, zidovudine.

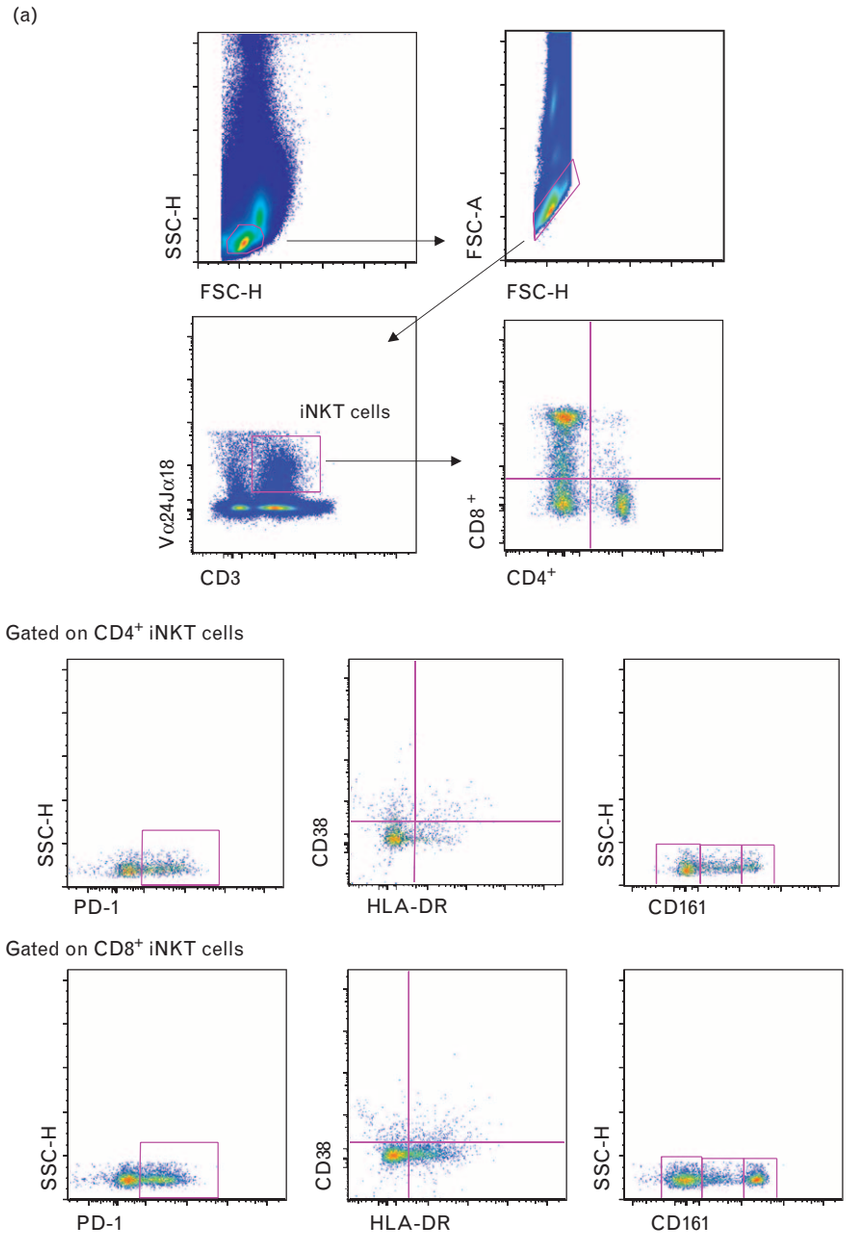

Fig. 1. Phenotype of invariant natural killer T cells in healthy patients and HIV+ patients with ratio less than 0.4 (group A) and more than 1.1 (group B). (a) Gating strategy used to identify circulating invariant natural killer T cells. Lymphocytes were identified on the basis of physical parameters (forward side scatter-H and side scatter (SSC)-H, then forward side scatter-H and forward side scatter-A), debris and doubles were removed and invariant natural killer T lymphocytes were identified on the basis of the expression of CD3 and Vα24Jα18. Then, $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ (double-negative) invariant natural killer T cells were identified. In these three subpopulations, the expression of PD-1, CD161, and activation markers CD38 and HLA-DR was analyzed. In controls ($N = 32$), group A ($N = 25$) and group B ($N = 29$): (b) percentage of circulating invariant natural killer T cells, (c) absolute number of circulating invariant natural killer T cells/ml blood, (d) percentage of circulating $CD4^+$ invariant natural killer T cells, (e) percentage of circulating $CD8^+$ invariant natural killer T cells, (f) percentage of circulating $CD4^-CD8^-$ invariant natural killer T cells, (g) percentage of $CD4^+$ invariant natural killer T cells expressing CD161, (h) percentage of $CD8^+$ invariant natural killer T cells expressing CD161, and (i) percentage of double-negative invariant natural killer T cells expressing CD161. In group A ($N = 13$) and group B ($N = 15$): (l) percentage of circulating activated $CD4^+$ invariant natural killer T cells, (m) percentage of circulating activated $CD8^+$ invariant natural killer T cells, (n) percentage of circulating activated double-negative invariant natural killer T cells, (o) percentage of circulating $CD4^+$ invariant natural killer T cells expressing PD-1, (p) percentage of circulating $CD8^+$ invariant natural killer T cells expressing PD-1, and (q) percentage of circulating double-negative invariant natural killer T cells expressing PD-1. Quantitative variables were compared with nonparametric Mann–Whitney U test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Data are represented as the mean ± SEM.

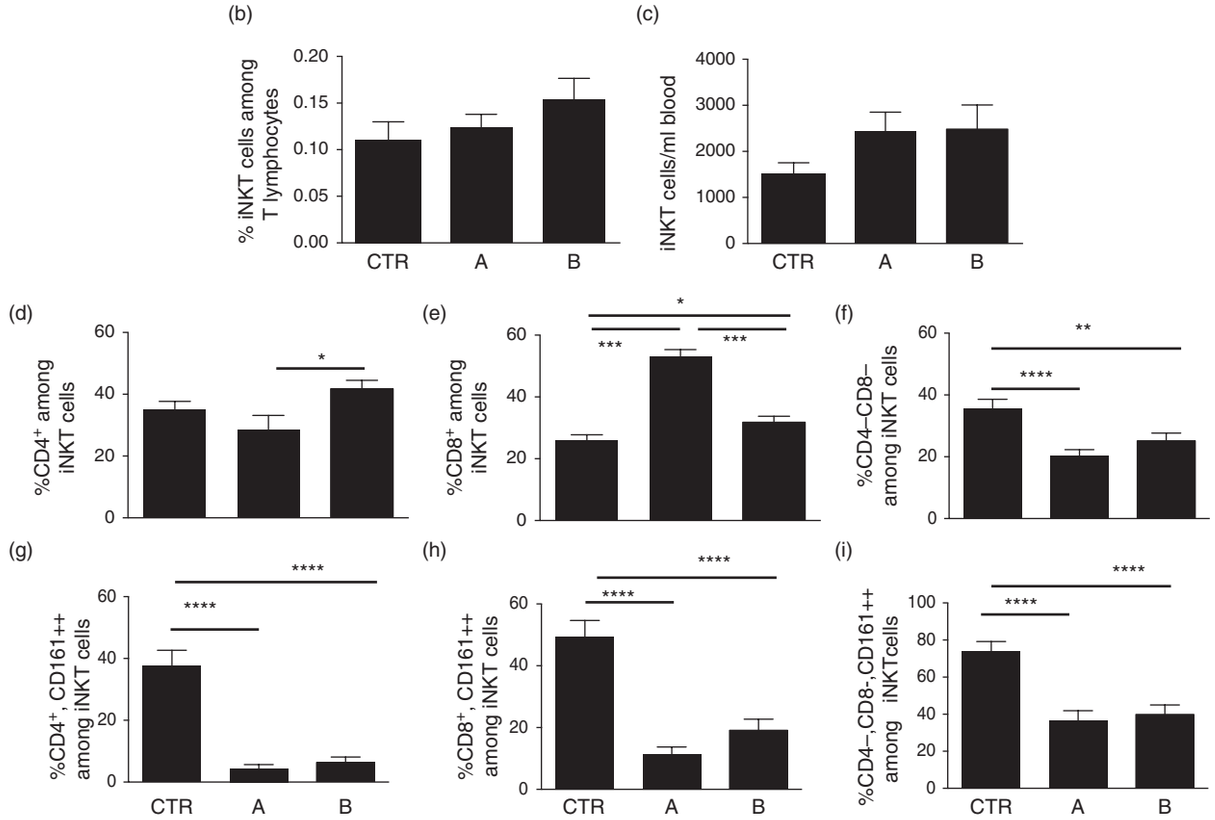

Fig. 1. (*Continued*).

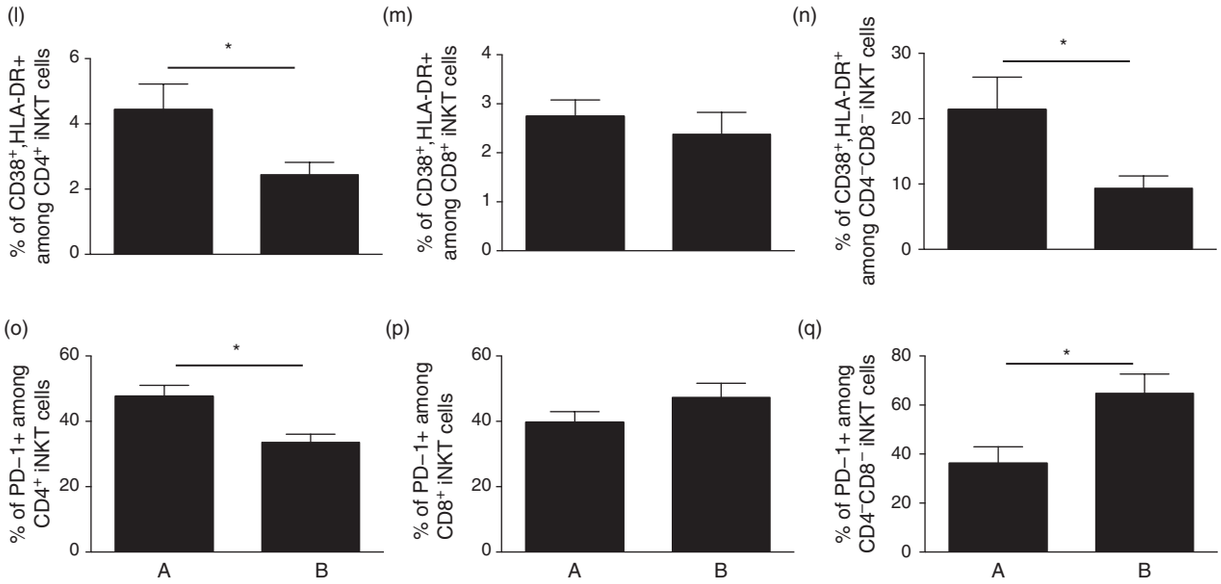

Fig. 1. (*Continued*).

scatter (FSC)-H vs SSC-H], doublets were removed from the analysis considering FSC)-H and FSC-Area, and then iNKT cells were selected on the basis of the expression of CD3 and Vα24Jα18 (using the 6B11 clone, which specifically binds the invariant CDR3 region of the iTCR). Among iNKT cells, we were able to identify $CD4^+$ iNKT cells, $CD8^+$ iNKT cells, and double-negative iNKT cells. Finally, PD-1 expression, activation status (by analyzing CD38 and HLA-DR), and CD161 expression were evaluated on different subpopulations of iNKT cells.

Changes in invariant natural killer T cell subpopulations in patients with low or high $CD4^+/CD8^+$ ratio

Considering iNKT cells as percentage of total T lymphocytes, both groups of HIV+ patients and healthy patients displayed a similar percentage and absolute number of iNKT cells (Fig. 1b and c, P = NS). The percentage of total iNKT cells expressing CD161, not shown in the figure, was lower in both groups of HIV+ patients (group A: 9.43 ± 2.87 and group B: 13.10 ± 3.84) than in CTR (56.90 ± 5.28, $P < 0.001$ in both cases).

We analyzed different subpopulations of iNKT cells, and we observed a significant decrease in $CD4^+$ iNKT cells in group A compared with group B (Fig. 1d, $P < 0.05$). On the contrary, the percentage of $CD8^+$ iNKT cells was higher in group A when compared with CTR (Fig. 1e, $P < 0.001$) and with group B (Fig. 1e, $P < 0.001$). The percentage of double-negative iNKT cells was lower in both groups of HIV+ patients if compared with healthy patients (Fig. 1f, group A vs CTR: $P < 0.0001$ and group B vs CTR: $P < 0.01$). Finally, the percentages of all subpopulations (i.e., $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ subsets) of iNKT cells expressing CD161 were significantly lower in both groups of HIV+ patients when compared with CTR (Fig. 1g–i, $P < 0.0001$ in all cases).

$CD4^+$ invariant natural killer T cells in group A displayed enhanced activation and exhaustion

We could analyze the activation status and the expression of PD-1 in different iNKT cell subpopulations in 13 patients of group A and 15 of group B. Group A displayed higher percentage of activated ($CD38^+$ and HLA-DR+) $CD4^+$ iNKT cells if compared with group B (Fig. 1l, $P < 0.05$). Patients with ratio less than 0.4 and more than 1.1 displayed similar percentage of activated $CD8^+$ iNKT cells (Fig. 1m, P = NS), whereas the percentage of activated $CD4^-CD8^-$ iNKT was higher in patients with low ratio (Fig. 1n, $P < 0.05$). The percentage of $CD4^+$ iNKT cells expressing PD-1 was higher in patients with ratio less than 0.4 (Fig. 1o, $P < 0.05$). Similar percentage was found among $CD8^+$ iNKT cell subset PD-1+ (Fig. 1p, P = NS) of different groups of patients. On the contrary to the $CD4^+$ iNKT cell subset, the percentage of $CD4^-CD8^-$ iNKT cells expressing PD-1 was higher in patients with ratio more than 1.1 compared with patients with ratio less than 0.4 (Fig. 1q, $P < 0.05$).

Production of cytokines by invariant natural killer T cells after in-vitro stimulation

We investigated the ability of iNKT cells to produce simultaneously different cytokines. The gating strategy is shown in Fig. 2a. Lymphocytes were selected according to physical parameters (FSC-H and SSC-H). Doublets and fluorochrome aggregates were removed by electronic gates (as in the plots 'FSC-A vs FSC-H' and 'Vα24Jα18 vs TIME'), and dead cells were identified on the basis of the SSC-H and negativity for Live/Dead. Among this population, iNKT cells were identified as $CD3^+$, Vα24Jα18+ cells. Then, the expression of $CD4^+$ and $CD8^+$ was analyzed among iNKT lymphocytes. In each subset of $CD4^+$, $CD8^+$, or $CD4^-CD8^-$ (double-negative) iNKT cells, the intracellular content of TNF-α, IFN-γ, IL-4, and IL-17 was simultaneously detected. We identified both the 'total' response, that is, the sum of all cells positive for at least one marker (this provides the overall 'frequency' of responding cells) and the 'qualitative' response, which describes the contribution of each functional pattern to the total specific response.

$CD4^+$ invariant natural killer T cells from HIV+ patients with ratio less than 0.4 display a skewed Th17 profile, whereas $CD8^+$ invariant natural killer T cells mainly produce TNF-α and IFN-γ

In Fig. 2b, the overall polyfunctional response of different subsets of iNKT cells in different groups of patients and in controls is represented as pie chart. Permutation test performed on 10 000 events revealed that a highly significant difference in the overall production of cytokines was detectable in $CD4^-CD8^-$ − iNKT cells. Changes among $CD4^+$ iNKT cells were at the limit of statistical significance, whereas $CD8^+$ iNKT cells behaved in a similar manner among the groups.

A more detailed analysis revealed that $CD4^+$ iNKT cells of group A were characterized by a higher production of IL-17 when compared with group B (Fig. 2c, $P < 0.025$) or healthy controls (Fig. 2c, $P < 0.001$). Concerning $CD8^+$ iNKT cells, we found that group A had higher percentage of cells producing IFN-γ (Fig. 2d, $P < 0.025$) or TNF-α (Fig. 2e, $P < 0.025$) compared with CTR. Furthermore, these patients also showed more $CD8^+$ iNKT cells able to simultaneously produce TNF-α and IFN-γ in comparison with CTR (Fig. 2f, $P < 0.025$). Finally, group A was characterized by higher percentages of double-negative iNKT cells that produced IL-17 (Fig. 2g, $P < 0.025$) and IL-17 and IFN-γ (Fig. 2h, $P < 0.025$) when compared with CTR. Regarding Th2 cytokine production, we did not find any production of IL-4 from different subsets of iNKT cells.

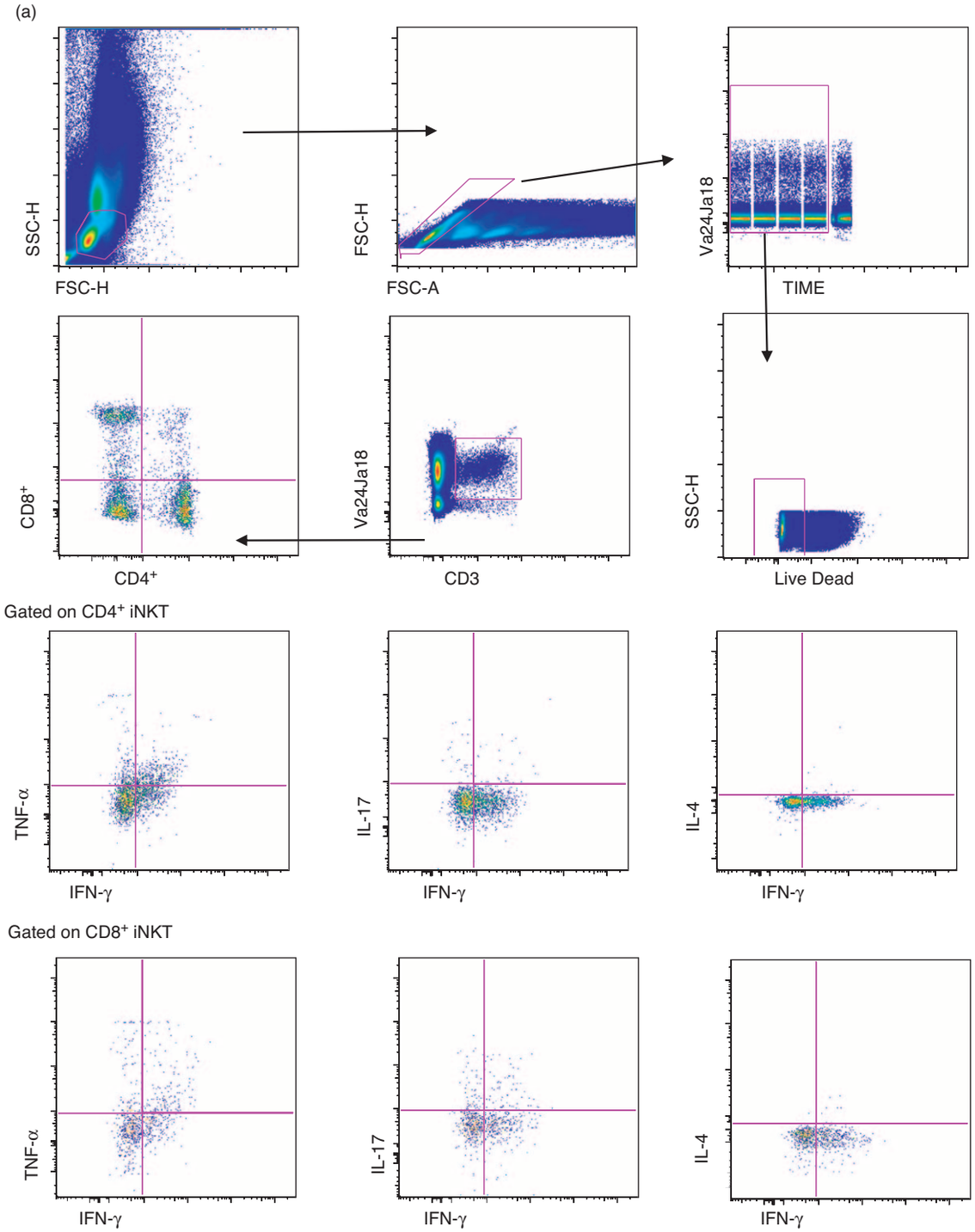

Fig. 2. Cytokine production and polyfunctionality of invariant natural killer T cells from controls and HIV+ patients with CD4+/CD8+ ratio less than 0.4 (group A) and more than 1.1 (group B). (a) Gating strategy used to identify different cytokines produced by invariant natural killer T cells. Several electronic, consecutive gates were set to exclude doublets, dead cells, and aggregates on the basis of physical parameters and fluorescent markers (forward side scatter-H vs SSC-H, forward side scatter-H vs forward side scatter-A, Vα24Jα18 vs Time, and SSC-H vs Live Dead). invariant natural killer T cells were recognized on the basis of the expression of CD3 and Vα24Jα18. Among this population, according to CD4+ and CD8+ expression, different subsets were identified (CD4+, CD8+, and CD4−CD8−). Among these invariant natural killer T subpopulations, the production of IL-4, IL-17, TNF-α, and IFN-γ was quantified after 4 h stimulation with phorbol myristate acetate/ionomycin. Nine-color flow cytometry was

Activation and exhaustion run in parallel in cells of the innate and adaptive immunity

We finally analyzed the correlation between activation markers, PD-1 expression, and cytokine production among $CD4^+$ or $CD8^+$ T cells and $CD4^+$ or $CD8^+$ iNKT cells. Our data revealed a strong correlation between activated $CD4^+$ T cells and activated $CD4^+$ iNKT cells (Fig. 3a, $R^2 = 0.42$, $P = 0.0002$), as well as between activated $CD8^+$ T cells and activated $CD8^+$ iNKT cells (Fig. 3b, $R^2 = 0.56$, $P < 0.0001$). The percentage of $CD4^+$ T cells expressing PD-1 was positively correlated with $CD4^+$ iNKT cells expressing PD-1 (Fig. 3c, $R^2 = 0.60$, $P < 0.0001$). We also noted a similar, striking correlation regarding $CD8^+$ cells (Fig. 3d, $R^2 = 0.63$, $P < 0.0001$).

Moreover, we compared the production of cytokines by $CD4^+$ T cells or $CD8^+$ T cells with the production by $CD4^+$ or $CD8^+$ iNKT cells. Our data revealed that there was a positive correlation between the percentage of $CD4^+$ T lymphocytes producing TNF-α and that of $CD4^+$ iNKT cells producing TNF-α (Fig. 3e, $R^2 = 0.63$, $P < 0.0001$). Similarly, a correlation existed between the percentage of $CD8^+$ T cells producing IFN-γ and that of $CD8^+$ iNKT cells producing IFN-γ (Fig. 3f, $R^2 = 0.74$, $P < 0.0001$).

Thus, these findings suggested that high and similar levels of activation are simultaneously present in different subsets of innate-like lymphocytes and in those that belong to adaptive immunity.

Discussion

The changes that occur in the immune system during HIV infection are under investigation since the beginning of the epidemics. Only recently, however, the development of novel technologies has allowed a deeper analysis of compartments that were almost unknown until a few years ago. One of these compartments regards cells the classification of which lies between innate and acquired immunity, that is, iNKT cells (that are also defined 'type I NKT cells'). Regarding HIV infection, few studies have been performed on this peculiar T-cell family, that is formed by different subsets, including that expressing $CD4^+$ (but not $CD8^+$) molecule, along with others characterized by the presence of $CD8^+$ only, the coexpression of $CD4^+$ and $CD8^+$, or the lack of both coreceptors. This is the first study that, using a novel and sophisticated cytometric approach, investigates the number, phenotype, and polyfunctionality of iNKT cells and their subtypes in HIV+ patients on cART who had been able to restore or not to a normal $CD4^+/CD8^+$ ratio after successful treatment.

Patients with different $CD4^+/CD8^+$ ratio after cART displayed similar percentages of iNKT cells. Previous studies reported that NKT cell frequency was significantly reduced among HIV+ individuals [28–30], with a specific depletion of $CD4^+$ iNKT cells compared with the $CD4^-$ subset [29]. In particular, this loss occurred within the first year of infection, with a continuous decline in the following 5 years. We could deeply investigate also the $CD4^-$ subset of iNKT cells and could evaluate the percentages of $CD8^+$ and $CD8^-$ iNKT cells as well, finding that the percentages of $CD4^+$ and $CD8^+$ iNKT cells mirrored those of T cells and that there was a marked reduction in double-negative iNKT cells in both HIV+ patients with high or low $CD4^+/CD8^+$ ratio.

We found that the expression of CD161 among iNKT cells was reduced in both HIV+ patient groups if compared with healthy patients. The expression of CD161 identifies more activated and mature subsets able to produce different cytokines such as IFN-γ, TNF-α, and IL-17 [31]. Data regarding the expression of CD161 among iNKT cells from HIV+ patients on cART are discordant. It has been reported that iNKT cells from HIV+ patients with bone and cardiovascular impairment expressed high levels of CD161 and predominantly secreted TNF-α [32]. However, in such study, the number of iNKT cells under investigation was too low to draw any statistically significant conclusion.

We found that HIV+ patients with $CD4^+/CD8^+$ ratio less than 0.4 were characterized by high levels of activated

used, acquiring a minimum of 10 million events per sample. (b) Polyfunctional response of different subsets of invariant natural killer T cells, as represented by 'Simplified Presentation of Incredibly Complex Evaluations' software. Arcs represent the total production of each cytokine, pie slices the polyfunctional capacity of cells. For the functional analysis of invariant natural killer T cells, a threshold of 0.05% was set on the basis of the distribution of negative values generated after background subtraction. Statistical analysis was performed by permutation test. (c) Total IL-17 production by $CD4^+$ invariant natural killer T cells from CTR, groups A and B, (d) total IFN-γ production by $CD8^+$ invariant natural killer T cells, (e) total TNF-α production by $CD8^+$ invariant natural killer T cells, (f) IFN-γ and TNF-α simultaneous production by $CD8^+$ invariant natural killer T cells, (g) total IL-17 production by double-negative invariant natural killer T cells, and (h) IFN-γ and IL-17 simultaneous production by double-negative invariant natural killer T cells. (b)–(h) Are related to 26 controls, 25 patients in group A, and 27 patients in group B. Quantitative variables were compared with nonparametric Mann–Whitney U test. $^*P < 0.05$ and $^{***}P < 0.001$. Data are represented as the mean ± SEM.

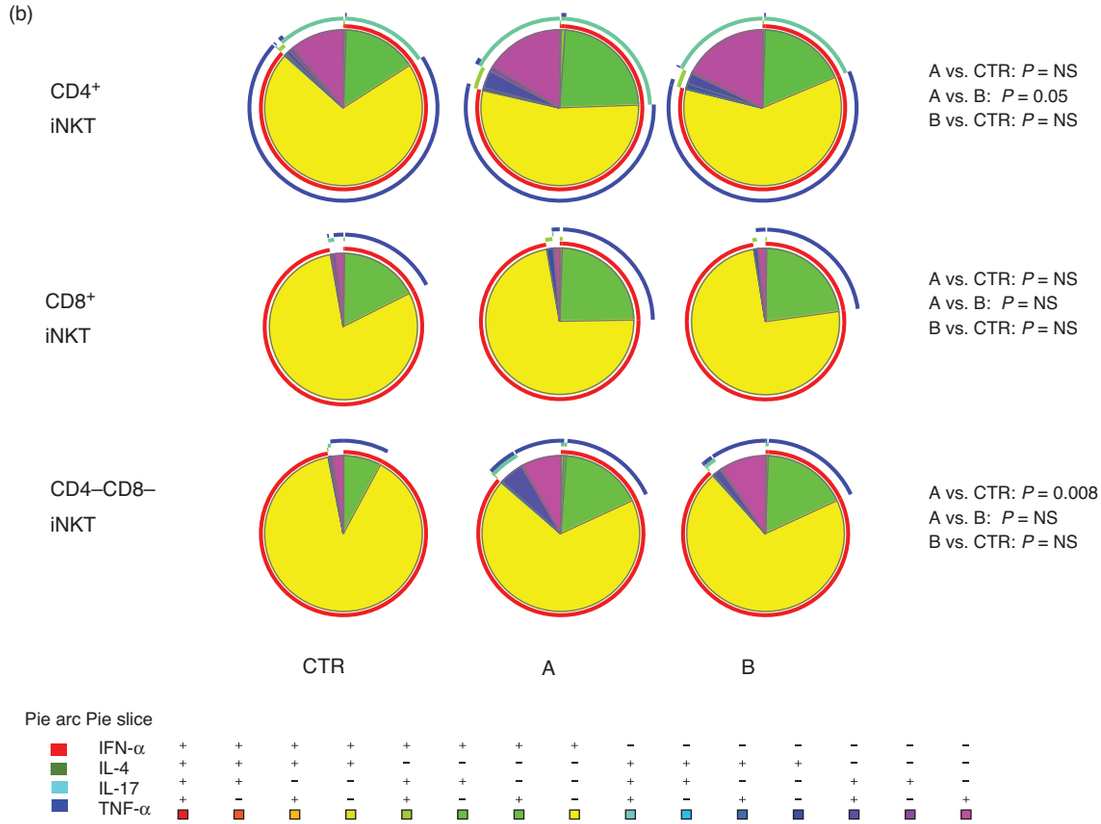

Fig. 2. (*Continued*).

CD4+ iNKT cells and high levels of CD4+ iNKT cells expressing PD-1 and that these percentages correlated with those of activated CD4+ T cells and CD4+ T cells expressing PD-1. Increased expression of exhaustion marker PD-1 in iNKT cells was reported among HIV+ individuals in one study, but PD-1 levels were not significantly correlated with IFN-γ production nor with proliferative capacity; blocking PD-1 did not restore iNKT cell function [15]. These could suggest that these cells acquire an irreversibly exhausted phenotype. However, it has to be considered that the expression and functional impact of other inhibitory receptors (such as the lymphocyte-activation gene 3) on iNKT cell subsets during HIV infection remain controversial [33]. Recently, it has been observed that PD-1+ NK cells display impaired cytotoxic activity and cytokine production, and that cytomegalovirus infection is correlated to the amount of NK cells expressing PD-1 [34]. Further analyses are needed to ascertain if such phenomenon also involves the iNKT subset.

We demonstrate here that, in patients with low CD4+/CD8+ ratio, the persistent level of activation among iNKT cells, the amount of IL-17-producing CD4+ iNKT cells and of IFN-γ-producing CD8+ iNKT cells could reflect what happens in the whole T-cell compartment. Activated iNKT cells produce multiple cytokines that can influence the outcome of the infection in favor of the host, although their potent activation may contribute to the onset of an uncontrolled cytokine storm, like that observed during sepsis [35]. It could be hypothesized that one of the crucial aspects of iNKT cell response to pathogens is the capacity to produce cytokines in a very rapid but short manner, which is typically followed by an extended time period of unresponsiveness to reactivation. This refractory period may represent a means to avoid chronic activation, thus protecting the host against some of the negative effects of iNKT cell activation, but potentially putting the host at risk for secondary infections [35]. The biphasic response of iNKT cells is thus of crucial importance for understanding the role of these cells in immune responses against infections [36]. Moreover, it has been shown that a sphingolipid produced by the human commensal *Bacteroides fragilis* can bind CD1d and modulate iNKT cells, indicating that the gut microbiome influences their post-thymic maturation. Thus, it is likely that iNKT cells in the GALT could play an important role in limiting microbial translocation and chronic pathologic immune activation [15]. Further studies are needed to clarify these aspects.

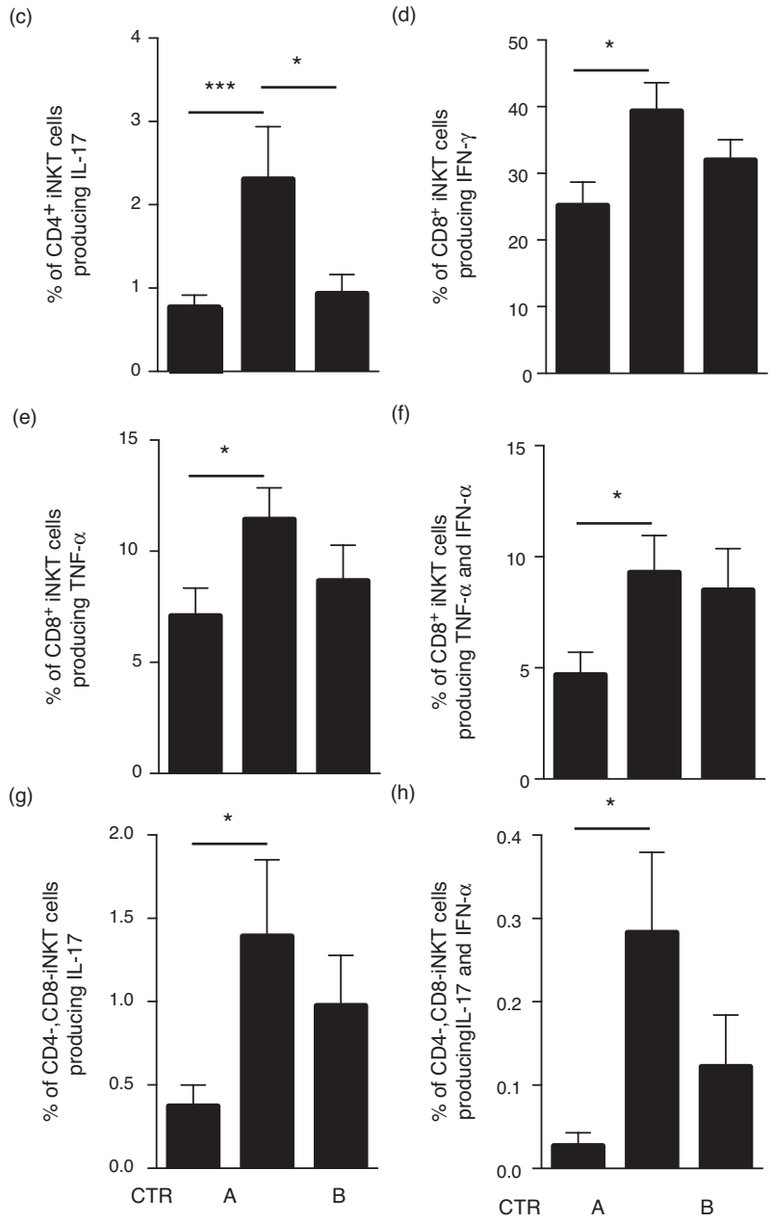

Fig. 2. (*Continued*).

Even among individuals with minimal iNKT cell depletion during HIV infection, the iNKT subset displays functional impairment. Both $CD4^+$ and $CD4^-$ iNKTs exhibit reduced proliferation and IFN-γ, TNF-α, and IL-4 secretion in response to different stimuli [15,28,37], with variable restoration among cART recipients. Here we show that iNKT cells from patients with low $CD4^+/CD8^+$ ratio display a pronounced Th1 and Th17 proinflammatory profile.

Finally, $CD4^+/CD8^+$ ratio has become a marker of interest for immune dysfunction among HIV-infected patients. Recently, it was well evidenced that a low $CD4^+/CD8^+$ ratio is a risk factor for clinical progression and that $CD4^+/CD8^+$ ratio could become an easy and relatively inexpensive marker for clinicians to predict the risk of serious non-AIDS-defining events and death independently of $CD4^+$ restoration [2]. We found that such ratio can be associated with an impairment of iNKT cells, and we can thus hypothesize that their Th1 and Th17 profile, mirror of potent production of proinflammatory molecules, could contribute to the inappropriate and persistent immune system activation and to the persistent inflammation typical of HIV infection [38].

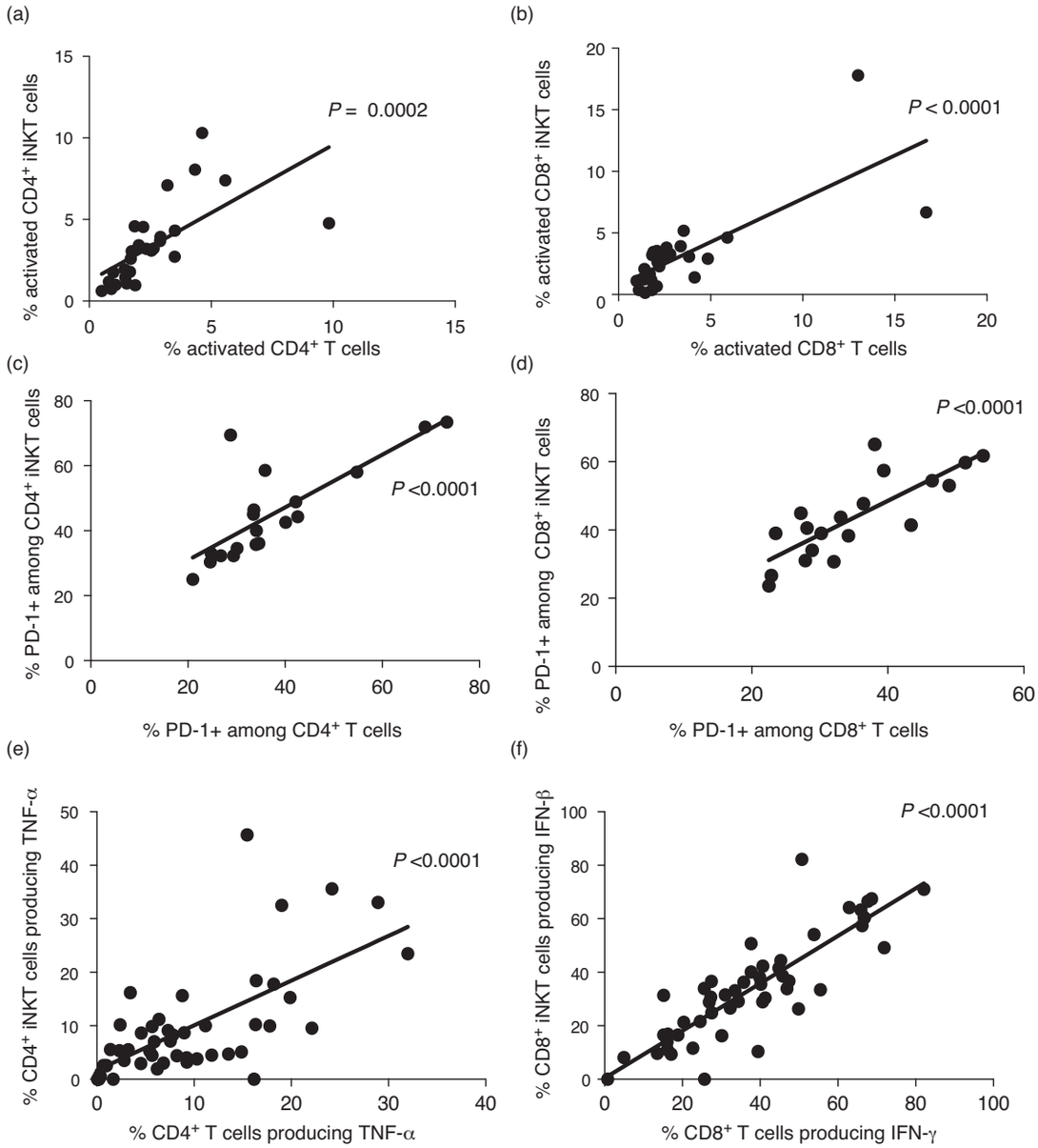

Fig. 3. In HIV+ patients, activation and exhaustion markers are expressed in the same manner by innate-like cells and in cells belonging to adaptive immunity. (a) Correlation between activated $CD4^+$ T cells and activated $CD4^+$ invariant natural killer T cells ($R^2 = 0.42$, $P = 0.0002$, $N = 28$), (b) correlation between activated $CD8^+$ T cells and activated $CD8^+$ invariant natural killer T cells ($R^2 = 0.56$, $P < 0.0001$, $N = 28$), (c) correlation between $CD4^+$ T cells expressing PD-1 and $CD4^+$ invariant natural killer T cells expressing PD-1 ($R^2 = 0.60$, $P < 0.0001$, $N = 19$), (d) correlation between $CD8^+$ T cells expressing PD-1 and $CD8^+$ invariant natural killer T cells expressing PD-1 ($R^2 = 0.63$, $P < 0.0001$, $N = 19$), (e) correlation between percentage of $CD4^+$ T cells producing TNF-α and $CD4^+$ invariant natural killer T cells producing TNF-α ($R^2 = 0.63$, $P < 0.0001$, $N = 52$), and (f) correlation between percentage of $CD8^+$ T cells producing IFN-γ and the percentage of $CD8^+$ invariant natural killer T cells producing IFN-γ ($R^2 = 0.74$, $P < 0.0001$, $N = 52$). Spearman correlation test and linear regression analysis were used.

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Conflicts of interest

There are no conflicts of interest.

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