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LETTER TO THE EDITOR

CD4$^+$ AND CD4$^-$ CD1D-RESTRICTED NATURAL KILLER T CELLS IN PERINATALLY HIV-1 INFECTED CHILDREN RECEIVING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

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We conducted a cross-sectional study on 43 Italian perinatally human immunodeficiency virus-type 1 (HIV-1) infected children receiving highly active antiretroviral therapy (HAART) and 26 age-matched healthy controls to explore CD1d-restricted NKT subsets. CD4$^+$ CD1d-rectricted natural killer (NKT) cell depletion was evidenced in 26 HIV-1 infected children with active viral replication despite HAART. Conversely, no alteration was evidenced in 17 children with undetectable viral load, suggesting full recovery in both CD4$^+$ and CD4$^-$ CD1d-rectricted NKT cell subsets. The loss of CD4$^+$ NKT cells in unresponsive children may have clinical consequences, including autoimmune disorders or cancer development. Future therapeutic perspectives are suggested.

CD1d-restricted NKT cell is a recently characterized lineage, recognizing glycolipids presented by CD1d molecule on the surface of antigen-presenting cells. CD1d-restricted NKT cell exerts immunoregulatory functions between innate and adaptive immune systems, and favours early control of several pathogens, including human immunodeficiency virus-type 1 (HIV-1) (1). Conversely, CD1d-restricted NKT cells are a favoured target for HIV-1 infection and their depletion may contribute to impaired immune response to tumour cells and opportunistic pathogens (2).

The mechanisms responsible for the CD1d-restricted NKT depletion in HIV-1 infected adults have been previously described (3). Biological bases for functional disruption of surviving NKT cells, mainly mediated by the HIV-1 Nef protein, have also been reported (3). Interestingly, CD1d-restricted NKT cell recover under highly active antiretroviral therapy (HAART) seems to be only partial. After an initial reconstitution phase (largely caused by the CD4$^-$ NKT subset redistribution), CD1d-restricted NKT cells are newly produced, leading to the expansion of both their CD4$^+$ and the CD4$^-$ subsets (3). Nevertheless, the CD4$^+$ subset recovery remains somewhat depressed in adult patients even during successful HAART (3).

Immune reconstitution processes in children with perinatal HIV-infection are substantially different from those described in adults. The redistribution of lymphocytes sequestered in tissues is minimal, while the thymic activity sustains the generation of CD4$^+$ subpopulations and the expansion of naïve CD45RA$^+$ CD4$^+$ T-lymphocyte subset (4). Similarly, it is

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possible that the recovery of CD1d-restricted NKT cells under HAART is different between infected children and adults. In this study we investigated CD1d-restricted NKT cell subsets in perinatally HIV-1 infected children treated with HAART

MATERIALS AND METHODS

Definitions

HIV-1 infection was diagnosed by the persistence of HIV-1 antibodies after 18 months or, before 18 months, by detection of viral markers on at least 2 occasions (5). HAART was defined as a combined therapy with three o more antiretroviral drugs of at least two different classes (5).

Children

This cross-sectional study included HIV-1 perinatally-infected children referring to the Infectious Disease Unit, Anna Meyer Children University Hospital, Department of Sciences for Women and Children's Health, University of Florence, Florence, Italy. Blood was taken during routine examination after the parent's or tutor's informed consent had been obtained. Controls were healthy age-matched children undergoing minor elective surgery with no known immune diseases, who were HIV-1 seronegative and free from infections and medication for at least one month previous to the blood sample. The study received local Ethical Committee approval.

Viral load measurement

Plasma viral load was evaluated quantitatively before starting therapy and 12 weeks after. Amplicor HIV Monitor test (Roche Diagnostic Systems, Inc., Branchburg, NJ) was used. Results are expressed as Log_{10} HIV-1 RNA copies/mL.

Flow cytometry

Lymphocyte numbers were calculated from heparinized peripheral blood samples using MultiTEST reagents in combination with MultiSET software (Becton Dickinson, San Jose, CA). The following monoclonal antibodies were used: FITC-labeled Vα24 and PE- and biotin-labeled Vβ11 (Oxford Immunotech, U.K.); PerCP-Cy5.5-labeled CD3, allophycocyanin-labeled CD4 (Becton Dickinson, San Jose, CA). NKT cells were defined by co-expression of CD3, Vα24, and Vβ11, because this combination was shown to be highly specific for α-galactosylceramide (α-GalCer)-reactive, CD1d-restricted NKT cells (6).

Statistical analysis

Age, viral load, T-lymphocyte and NKT subsets counts were expressed as median and interquartile range (IQR). Statistical comparison between the two groups was performed by the Mann-Whitney U test. Linear regression analysis, calculating the regression coefficient (r), was used to evaluate the relationship between CD1d-restricted NKT and $CD4^+$ T-lymphocyte counts or viral load. $P<0.05$ was considered as statistically significant. Statistical analyses were performed using SPSS software package (SPSS 13.0; Chicago, IL).

RESULTS

Forty-three perinatally HIV-1-infected children and 26 controls were included in the study. Of the HIV-1-infected patients, 26 children exhibited undetectable viral loads, while a sustained viral replication was evident in 17 children. The two groups of patients did not differ in terms of age and time on HAART (5.8 years [IQR:2.2-13.0] *vs* 4.7 years [IQR:1.2-13.2]; P=0.561).

Among children with undetectable viral load: 16 (61.5%) were receiving a protease inhibitor-based regimen (containing lopinavir/ritonavir [n=15] or atazanavir [n=1]), while 10 (38.5%) children were receiving an efavirenz-based regimen. In the group of children with sustained viral load, 8 (47.1%) children were receiving a protease inhibitor-based regimen (containing lopinavir ritovavir [n=5] or darunavir [n=2], or tenofovir [n=1]),while 9 children (52.9%) were receiving an efavirenz-based regimen.

$CD4^+$CD1d-restricted NKT percentages in HIV-1-infected children with or without virological failure, and healthy children are reported in Table I. No statistical difference was evidenced between children with virological success and healthy children (P=0.196), suggesting a complete immunological recovery. Differently, children with virological failure displayed values significantly lower than healthy children (P=0.002) and lower than HIV-infected children with undetectable viral load (P=0.046), indicating that a persistent depletion in the $CD4^+$ CD1d-restricted NKT subset was associated with virological failure. Results were confirmed considering absolute values (Table I). With regard to $CD4^-$CD1d-restricted NKT percentages, no significant difference among the groups was observed. Similar findings were evidenced considering absolute cell numbers (Table I).

Table I. *Clinical and laboratory findings of the study children.*

	Group 1	*Group 2*	*Group 3*	*P*--*	*P**	*P**
	HIV-1 infected children with viral load < 50 copies /mL (n=26)	**HIV-1 infected children with viral load > 50 copies /mL (n=17)**	**Healthy children (n=26)**	**group 1 *vs.* group 3**	**group 2 *vs.* group 3**	**group 1 *vs.* group 2**
Median and IQR Age (years)	14.52 (9.96-17.77)	12.51 (9.90-15.20)	13.81 (5.81-17.00)	0.168	0.142	0.254
Median and IQR Viral load (Log_{10} copies/mL)	<1.69	4.12 (3.50-4.42)	Not applicable	-	-	<0.0001
$CD4^+$ T-lymphocyte percentages (median and IQR)	29.0 (23-33)	24.5 (19-28)	41.0 (37-44)	<0.0001	<0.0001	<0.064
$CD4^+$ T-lymphocyte cells/μL (median and IQR)	811 (472-1054)	712 (581-876)	1241 (984-1690)	0.009	0.006	0.434
$CD4^+CD1d$-restricted NKT percentage (median and IQR)	0.03 (0.01-0.06)	0.01 (0.01-0.03)	0.03 (0.02-0.08)	0.196	0.002	0.046
$CD4^+CD1d$-restricted NKT cells/μL (median and IQR)	0.60 (0.27-1.26)	0.24 (0.00-0.65)	0.87 (0.54-1.91)	0.100	0.001	0.037
$CD4^-CD1d$-restricted NKT percentage (median and IQR)	0.05 (0.01-0.27)	0.03 (0.00-0.04)	0.03 (0.01-0.08)	0.131	0.153	0.069
$CD4^-CD1d$-restricted NKT cells/μL (median and IQR)	0.79 (0.23-1.63)	0.69 (0.25-3.67)	0.66 (0.51-1.50)	0.724	0.938	0.539

*Note: IQR: interquartile range; * by Mann-Whitney test*

$CD4^+$, but not $CD4^-$, CD1d-restricted NKT absolute numbers inversely correlated with viral load in perinatally HIV-1 infected children receiving HAART (P=0.047; r=-0.285). $CD4^+$CD1d-restricted NKT cell absolute numbers directly correlated with $CD4^+$ T-lymphocyte count (P=0.05; r =0.308).

DISCUSSION

Our findings suggest that CD1d-rectricted NKT depletion occurs in HIV-1-infected children with active viral replication, similar to what already has been described for adults (6-9). In our study depletion affected mainly the $CD4^+$ CD1d-rectricted NKT cell subset. Conversely, no alteration occurred in children with undetectable viral load, suggesting full recovery in both $CD4^+$ and $CD4^-$ subsets of these cells. It may be speculated that, during a successful therapy, thymic function in children is adequate to generate a normal proportion of $CD4^+$ NKT subset. The finding

that CD4+CD1d-restricted NKT cell absolute numbers correlated with CD4+ T-lymphocyte count further corroborates this hypothesis.

Usuga et al. conducted a similar cross-sectional study on 23 Colombian HIV-infected children (10). Differently from our findings, HAART was not associated with full recovery in CD1d-restricted NKT subsets, either in children with or without undetectable viral load, and no prevalent depletion in CD4+ NKT cells was observed. However, CD1d-restricted NKT depletion was more profound in children with sustained viral replication (10). The authors speculate that irreversible damage occurring before HAART initiation may have prevented the full recovery in CD1d-restricted NKT cells. Differences between Usuga's study and our findings may be difficult to interpret. Differences in HAART regimens and/or HAART duration might have influenced the results. On the other hand, it must be considered that both studies included limited datasets and were cross-sectional. Moreover, no functional study was performed.

Consistently with our results, Sandberg and colleagues found that NKT cell depletion in HIV-1 infected individuals was limited to the CD4+ NKT cells (8). This finding is biologically plausible, since CD4+NKT cells are particularly vulnerable to HIV-1 infection, as indicated by their high expression of the HIV-1 co-receptor CCR5 with respect to CD4-NKT cells (8). R5-tropic HIV-1 is the predominant form of transmitted HIV-1 and this fact may account for the rapid depletion of NKT cells observed early in the course of HIV-1 infection (11).

The imbalance between the CD4+ and CD4- NKT cell subsets may have relevant consequences. The two subsets express differential homing receptors with CD4+NKT cells expressing the CD62L receptor for homing to lymph nodes and CD4+NKT cells expressing CD11a receptor for infiltration into tissues (8). Thus, the NKT cells that survey the secondary lymphoid organs are lost in individuals with high viral load, disrupting the interaction between the NKT-cell compartment and dendritic cells which is important for the immunoregulatory function of NKT cells (8). Moreover, CD4+NKT cell depletion leads to a shift in the pattern of NKT produced cytokines since CD4+ and CD4-NKT cells produce different cytokine profiles (i.e. CD4+NKT cells produce much more IL-4 then CD4-NKT cells) (12). The consequent immune disregulation may cause autoimmune disorders and impaired cancer surveillance (2). α-GalCer or other NKT activating glycolipids might have a therapeutic role in HIV-1 infected individuals, activating or expanding the CD4+NKT cell compartment, as suggested by data obtained in the mouse model (13-14).

In conclusion, our data suggest a preferential CD4+CD1d-rectricted NKT depletion in HIV-1 infected children with active viral replication despite HAART, and its restoration in HIV-1 infected children with undetectable viral load. Larger, longitudinal investigations are needed to better explore this issue.

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