CONCISE COMMUNICATION

The impact of active HIV-1 replication on the physiological age-related decline of immature-transitional B-cells in HIV-1 infected children

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Objective: To characterize the level of immature-transitional B-cells in blood during pediatric HIV-1 infection in relation to active or suppressed viremia. We also aimed at characterizing the level of expression of CXCR4, CXCR5 and CCR7 on immature-transitional B-cells, as these receptors are important mediators for homing of B-cells.

Design: Forty-eight HIV-1 vertically infected children (33 viral controllers and 15 viremic patients) and 33 age-matched healthy controls were enrolled in a cross-sectional study.

Methods: We measured the levels of peripheral immature-transitional B-cells in all groups in relation to switched memory B-cells by flow cytometry. In parallel we evaluated CXCR4, CXCR5 and CCR7 expression on immature-transitional B-cells and measured plasma levels of CXCL12, BAFF and interleukin-7 by ELISA.

Results: We observed a lack of physiological age-related decline of immature-transitional B-cells in viremic children in parallel to a decreased level of switched memory B-cells. Interestingly, immature-transitional B-cells from viremic children presented with high levels of CXCR4. On the contrary, the level of CXCL12, the natural ligand for CXCR4, was lowest in the HIV-1 infected group, as compared with controls.

Conclusion: Control of HIV-1 viremia through antiretroviral treatment appears to be crucial in decreasing the expansion and alteration of immature-transitional B-cells.

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Introduction

Immature-transitional B-cells represent a critical link between immature B-cells in the bone marrow and mature naïve B-cells, which in turn differentiate into switched memory B-cells, in the periphery [1]. In the spleen, immature-transitional B-cells are thought to directly differentiate into IgM memory B-cells [1]. The

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levels of both switched and IgM memory B-cells were recently reported to be altered during HIV-1 infection in children contributing to a defective memory B-cell function [2,3]. An expansion of immature-transitional B-cells has been reported in adults with advanced HIV-1 infection [4]. Whether immature-transitional B-cells in HIV-infected children display an altered frequency and/or phenotype has not been previously investigated. This study aims at characterizing immature-transitional B-cells in childhood in relation to active HIV-1 replication or suppressed viremia.

Methods

Study design

Forty-eight HIV-1 vertically infected children were enrolled in a cross-sectional study at the Children Hospital Bambino Gesù, Rome, Italy. Children undergoing HAART and maintaining undetectable levels of HIV-1 plasma viremia ($n = 33$) were defined as 'Viral controllers' whereas children with detectable viral load in plasma and experiencing virological failure despite HAART ($n = 15$) were defined as 'Viremic patients'. Thirty-three age-matched healthy controls were also included. Characteristics of all individuals enrolled are reported in Table 1. The study was approved by the ethics committee at the Children Hospital Bambino Gesù and written informed consent from parents was obtained.

Immature-transitional and switched memory B-cell phenotyping

Peripheral blood mononuclear cells (PBMCs) and plasma were purified from Ficoll-Hypaque EDTA-treated blood. PBMCs were stained with FITC-conjugated, PE-conjugated, PE-Cy5-conjugated and APC-conjugated antihuman monoclonal antibodies (Abs) binding to surface CD10, CD19, CD24, CD27, CD38, IgM, CXC chemokine receptor 4 and 5 (CXCR4 and CXCR5), CC chemokine receptor 7 (CCR7) and interleukin-7 receptor (R)-α (BD-Pharmingen, San Diego, California, USA), acquired using a FACScalibur instrument and analyzed by the CellQuest software (both from Becton Dickinson, San Jose, California, USA). In this study, immature-transitional B-cells have been identified as CD19+CD24^{high}CD38^{high} [1] and confirmed as CD19+CD38^{high}IgM^{high} [5] and as CD10+[4].

In addition, we stained the cells for surface CD27 in order to exclude the possible presence of circulating CD27+ CD10+ germinal center founder B-cells [6]. Also CD19+CD38+ plasmablasts were excluded according to their bigger size. Switched memory B-cells were defined as CD19+CD27+IgM-[7].

Measurement of plasma CXC chemokine ligand 12, B-cell activating factor and interleukin-7

Plasma CXC chemokine ligand 12 (CXCL12), B-cell activating factor (BAFF) and interleukin-7 titers were measured using the Human CXCL12/SDF-1α Duo Set, the Human BAFF/BLyS/TNFSF13B Quantikine ELISA Kit and the Quantikine HS Human interleukin-7 Immunoassay respectively (all from R&D Systems, Abingdon, UK) following the manufacturer's instructions.

Statistical analyses

All statistical analyses were performed by using Sigma Stat for Windows software (SPSS Inc, Chicago, USA). Student t-test or Mann–Whitney Rank Sum test were applied to analyze the percentage of immature-transitional B-cells and switched memory B-cells, the mean fluorescence intensity (MFI) of CXCR4 and the levels of plasma CXCL12, BAFF and interleukin-7 among the groups. Multiple and linear regression analyses were performed to correlate age, CD4+ T-cell percentage, HIV-1 viral load and years with ongoing HIV-1 replication to the above parameters.

Results

Immature-transitional B-cells increase in viremic children

In healthy children, the level of immature-transitional B-cells is physiologically high and slowly decreases with age. We observed an age-related decline of immature-transitional B-cells, in both healthy controls (R = −0.5; $P = 0.02$) and viral controllers (R = −0.5; $P = 0.004$) but not in viremic patients (R = 0.4; $P = 0.2$) (Fig. 1a). As a consequence, viremic patients presented with higher levels of immature-transitional B-cells as compared with viral controllers (6.6% ± 3.1%/22 ± 16 cells/μl vs. 4.2% ± 2.6%/15 ± 11 cells/μl) ($P = 0.008$) (Fig. 1b). As the expansion of immature-transitional B cells has previously been related to a decrease in switched memory

Table 1. Characteristics of the individuals.

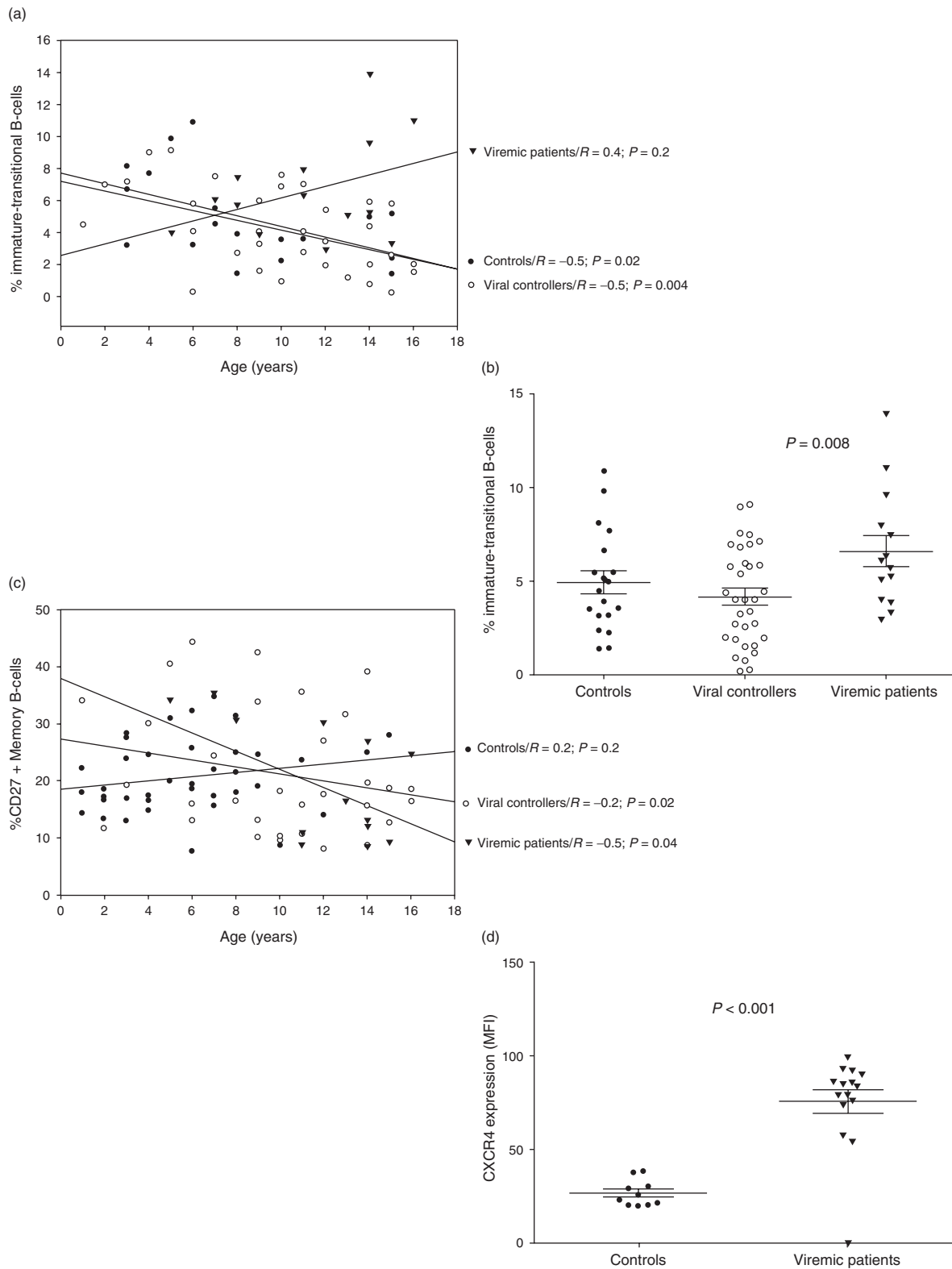| Group	Controls	Viral controllers	Viremic patients
Number of subjects	33	33	15
Age mean (±SD)	8.8 (±4.4)	10.0 (±4.2)	11.4 (±3.3)
HIV-1 viral load copies/ml mean (±SD)	Not determined	<50	19480 (±22186)
Years with active HIV-1 replication mean (±SD)	0 (±0)	6.2 (±4.2)	11.4 (±3.3)
CD4+ % and absolute number (cells/μl) mean (±SD)	Not determined	34.5 (±6.9)/937 (±584)	24.0 (±5.6)/515 (±217)
IL-7 (pg/ml) mean (±SD)	6.3 (±3.3)	3.1 (±5.3)	0.7 (±1.8)

Fig. 1. Multiple regression analyses on the immature-transitional B-cell percentages among the different groups in relation to age (a) and scatter plot analyses on the immature-transitional B-cell percentages among the different groups (b). Multiple regression analyses on the switched memory B-cells percentages in relation to age among the different groups (c). Scatter plot analyses on the CXCR4 MFI on immature-transitional B-cells of HIV-1 infected viremic children and healthy controls (d).

B-cells in diseases characterized by antigenic persistence such as systemic lupus erythematosus (SLE) [8], the level of switched memory B-cells was also analyzed. We found an age-related, significant decrease of switched memory B-cells in viremic patients (R = −0.5; P = 0.04) whereas no significant differences were observed for viral controllers (R = −0.2; P = 0.2) and healthy controls (R = 0.2; P = 0.2) (Fig. 1c). In the infected children, no significant correlation was found between the percentages or absolute numbers of immature-transitional B-cells and either the percentages or absolute numbers of CD4 T-cells or HIV-1 viral load (not shown).

Expanded immature-transitional B-cells from viremic patients present with high levels of CXCR4

Immature-transitional B-cells in viremic children might be retained in the periphery as a result of impaired homing capacity due to altered expression of chemokine receptors [9]. In order to test this hypothesis we measured the levels of CXCR4, CXCR5 and CCR7 in the expanded immature-transitional B-cell population in the blood of viremic children, as compared with healthy controls. Although the levels of CXCR5 and CCR7 did not show relevant differences between patients and controls (not shown), we observed an increase of CXCR4 in immature-transitional B-cells from viremic patients compared with controls (P < 0.001) (Fig. 1d). Interestingly, the level of CXCL12, the natural ligand for CXCR4, was lowest in the HIV-1 infected groups with 70% of the viremic children and 66% of the viral controllers having undetectable CXCL12 in plasma. In healthy controls, 35% of the children had undetectable plasma CXCL12. We also measured BAFF but there were no differences between the controls and HIV-1 infected children (not shown). No significant correlation was found between the levels of CXCR4 expression on immature-transitional B-cells of infected children and either the percentages or absolute numbers of CD4 T-cells or HIV-1 viral load (not shown).

The level of immature-transitional B-cells in the periphery does not increase in parallel with the levels of interleukin-7 in children

The expansion of immature-transitional B-cells in adults with advanced HIV-1 infection has been related to high levels of interleukin-7 [4]. Conversely, we observed decreased levels of interleukin-7 in HIV-1 infected children compared with healthy controls (P < 0.001) (Table 1). Interestingly, we observed a strong correlation between interleukin-7 levels and years of ongoing HIV-1 replication (R = −0.4; P < 0.001).

Discussion

Immature-transitional B-cells represent a critical link between immature B-cells in the bone marrow and mature B-cells in the periphery [1]. The level of immature-transitional B-cells is elevated in healthy children and slowly decreases with age. Immature-transitional B-cells have never been investigated in HIV-1 infected children but understanding the biology of this population in early life might have important implications to prevent impaired B-cell functions in both HIV-1 infected children and adults. We observed lack of physiological age-related decline of this population in viremic children, whereas the level of immature-transitional B-cells was comparable between children under successful antiretroviral therapy and healthy controls. This is in line with findings from HIV-1 infected adults showing a decrease of this expanded B-cell subpopulation following initiation of effective HAART [10].

Interestingly, the expansion of immature-transitional B-cells in viremic children did not stringently follow the levels of HIV-1 viral load or the CD4 T-cell counts. This observation possibly reflects the fact that the expansion of immature-transitional B-cells may be an indirect consequence of immune-activation rather than a direct effect of HIV-1 viremia. However, the mechanism through which HAART impacts on the different B-cell subpopulations remains the focus of intense research [11]. HAART invariably leads to two interlinked events, namely, control of HIV-1 plasma viremia and reduced production of inflammatory cytokines, which together lead to control of immune-activation. Whether clonal expansion and sustained high frequency of immature-transitional B-cells in different settings reflect a common driving factor upon antigenic persistence (such as in SLE, malaria and chronic hepatitis C) is not clear [8]. However, the presence of high levels of immature-transitional B cells in these diseases is frequently associated with depleted switched memory B-cells as reported in this present work on pediatric HIV-1 infection. In addition, immature-transitional B-cells have been shown to persist for several years, at the expense of switched memory B-cells, in a subset of patients with SLE who have been treated with an anti-CD20 antibody (rituximab) [8].

We also found high levels of CXCR4 expression in the expanded population of immature-transitional B-cells in the blood of viremic children. Altered expression of CXCR5 in B-cells during HIV-1 infection was previously shown in adults [12]. The expression of CXCR4 is developmentally regulated; in peripheral blood, the majority of mature naïve and memory B-cells express CXCR4 and remain positive for this receptor following activation and differentiation into plasma cells [13]. Thus, it is likely that expanded immature-transitional B-cells lay between IgG-CXCR4^{low} immature B-cells and IgG+CXCR4^{high} mature B-cells.

The natural ligand of CXCR4 is CXCL12, which normally orchestrates migration of CXCR4+ B-cells in

different tissue compartments [9]. In order to assess whether the increased presence of immature-transitional B-cells, which we found to be CXCR4+, could be due to altered levels of CXCL12 in blood, we measured the plasma levels of this chemokine in the different groups. The majority of HIV-1 infected patients had CXCL12 plasma levels below detectable values compared with healthy controls. As CXCL12 downregulates the cell surface expression of the receptor CXCR4 [14], one possibility is that the low level of CXCL12 in viremic children may partially contribute to the high expression of CXCR4 on immature-transitional B-cells in this group. CXCL12 is produced mainly from stromal cells in lymphoid tissue and bone marrow, and it is therefore interesting that CXCL12 mRNA levels in tissue decreased upon SIV infection [15]. Thus, it is also conceivable that HIV-1 infection may alter the production of CXCL12 in lymphoid tissue of children leading to low levels of CXCL12 in plasma as reported by others and us [16]. All in all, it is, therefore, likely that the CXCR4 expression in immature-transitional B-cells from viremic children mainly represents a marker of differentiation [13], as stated earlier, and that although HAART might be able to quickly normalize the levels of immature-transitional B-cells [10], restoring the levels of plasma CXCL12 might follow a slower kinetic.

Another factor that may have a role for the expansion of immature-transitional B-cells in viremic children is BAFF; BAFF is an HIV-1-inducible (particularly Env-inducible) factor that plays a key role in the survival and differentiation of peripheral B-cells, including immature-transitional B-cells [17]. However, we measured the levels of BAFF and we could not find any significant difference between the groups suggesting that BAFF may not have a role for the expansion of immature-transitional B-cells observed in viremic children.

The expansion of immature-transitional B-cells has been related to high levels of interleukin-7 in adults with advanced HIV-1 infection [4]. Conversely, we observed a different scenario in vertically HIV-1 infected children for whom decreased levels of interleukin-7 were found in both the viremic group and viral controllers compared with healthy children. Decreased levels of interleukin-7 are unlikely due to a direct consumption of interleukin-7 by immature-transitional B-cells in the periphery as we found that the interleukin-7Rα (CD127) is not expressed on B-cells (results not shown). Recent data reported interleukin-7 to be more critical for B-cell development in adults than in children suggesting that other mechanisms may determine the decline of interleukin-7 observed in HIV-infected children [18]. For instance, the presence of a functional thymus leading to a fast T-cell turnover in children might determine a different interleukin-7 consumption [19]. Therefore, the low levels of interleukin-7 that we observed, might be due to a greater consumption of interleukin-7 by T-cells newly produced by the thymus in response to active viral replication. In line with this, we observed a strong correlation between interleukin-7 levels and years of ongoing HIV-1 replication. Accordingly, recent data suggest low levels of serum interleukin-7 as a predictable marker of virological failure in children [20].

In summary, we report that ongoing HIV-1 replication has an impact on the physiological age-related decline of immature-transitional B-cells in parallel with a decline of switched memory B-cells in viremic children. Immature-transitional B-cells from viremic children also presented with high levels of CXCR4 expression suggesting that this B-cell subset also has an altered differentiation stage. Control of HIV-1 viremia through antiretroviral treatment appears to be crucial in preventing or decreasing the expansion of these altered immature-transitional B-cells and should be achieved prior to vaccination [3] in order to maximize the generation of specific switched memory B-cells.

Acknowledgement

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