Early antiretroviral therapy-treated perinatally HIV-infected seronegative children demonstrate distinct long-term persistence of HIV-specific T-cell and B-cell memory

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Objective: To investigate long-term persistence of HIV-specific lymphocyte immunity in perinatally HIV-infected children treated within the first year of life.

Design: Twenty perinatally HIV-infected children who received ART therapy within the first year of life (early treated) and with stable viral control (>5 years) were grouped according to their serological response to HIV.

Methods: Western blot analysis and ELISA defined 14 HIV-seropositive and six seronegative patients. Frequencies of gp140-specific T-cell and B-cell, and T-cell cytokine production were quantified by flow cytometry in both seronegatives and seropositives. Transcriptional signatures in purified gp140-specific B-cell subsets, in response to in-vitro stimulation with HIV peptides was evaluated by multiplex RT-PCR.

Results: Gp140-specific T cells and B cells persist at similar levels in both groups. A higher production of IL-21 in gp140-specific T cells was found in seropositives vs. seronegatives ($P = 0.003$). Gene expression in switched IgM–IgD– gp140-specific memory B cells after stimulation with HIV peptides *in vitro* demonstrated a differential expression of genes involved in signal transduction and activation after BCR/TLR triggering and B-cell activation. Genes relating to antibody production (PRDM1) and T–B cognate stimulation (CXCR4, IL21R) were differentially induced after in-vitro stimulation in seronegatives vs. seropositives suggesting a truncated process of B-cell maturation.

Conclusion: HIV-specific memory B and T cells persist in early treated regardless their serological status. Seronegatives and seropositives are distinguished by gp140-specific T-cell function and by distinct transcriptional signatures of gp140-specific B cells after in-vitro stimulation, presumably because of a different antigen exposure. Such qualitative insights may inform future immunotherapeutic interventions.

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Introduction

Strong evidence indicates that antiretroviral treatment (ART) should be initiated in HIV infected infants early in the first year of life in order to reduce morbidity and mortality [1]. Early control of HIV-1 replication within the first months of life was accompanied by preservation of CD4⁺ T-cell counts, absence of circulating activated T cells, and normal immune function [2]. However, these early treatment studies also identified a lack of circulating HIV-1-specific antibodies and T-cell responses as a hallmark of early suppression of HIV-1 replication [2,3]. Several research groups have also found that a consistent proportion of these patients do not display HIV-specific responses [4,5]. The percentage of HIV-infected children who are HIV seronegative has been shown to range between 36 and 46% of HIV-infected children treated within the first year of life [3,5,6]. This condition of seronegativity persists over time after long-term follow-up. A number of studies showed that the absence of HIV antibodies indicates a smaller size of HIV reservoir [4,5,7] making these seronegative children ideal candidates for immune intervention studies towards HIV remission [8,9].

On the other hand, however, it is not known whether the absence of HIV-specific antibodies could represent a limitation during periods of viral re-exposure because of poor ART adherence, or in the perspective of cure strategies.

In this controversial scenario, two main scientific questions arise: if HIV-specific cells persist despite HIV serostatus, and whether, if present, these cells act differentially according to the patient serostatus once they re-encounter the virus [10].

Herein, we explored these two questions in a group of seronegative patients in comparison to seropositive patients treated with ART within the first year of life.

Methods

Study participants

Study participants were enrolled at Bambino Gesù Children's Hospital between December 2016 and June 2017. Only patients who started ART within 1 year of life with durable viral control (at least 2 years) were considered eligible. Plasma samples were tested to define HIV-1 serostatus by fourth generation Chemiluminescent

Microparticle Immune Assay (CMIA) (Abbott Architect HIV Ag/Ab Combo Assay) and western blot analysis (WB) (Medical Systems, Italy). Western blot was considered positive when at least one antigen gave a positive result. Timing of blood draw and design of the study has been visualized in Supplementary Figure 1, <http://links.lww.com/QAD/B647>.

Ethics statement

Bambino Gesù Children's Hospital review board approved the study and written informed consent was obtained from all participants or their legal guardians.

B-cell and T-cell stimulation

Cryopreserved PBMCs were thawed and resuspended in R10 medium [RPMI 1640 and 10% fetal bovine serum (FBS) supplemented with 1% Pen/Strep and L-Glu. For B cell stimulation, 3×10^6 cells/ml were incubated for 16 h at 37 °C and 5% CO₂ with a mix of three different pools of ENV peptides (100 µg/ml, EURIPRED) and CD40L (250 ng/ml, ENZO Life Sciences, Pero, Italy). For T-cell stimulation, 5×10^6 PBMCs were rested over night at 37 °C, 5% CO₂ in R10 medium prior to 12 h incubation with 2 µg/ml gp140 (kindly provided by Dr Kalyanaraman, ABL, Maryland, USA), 1 µg/ml of staphylococcal enterotoxins B (SEB, List Biological Laboratories, Inc., Campbell, California, USA) or medium (negative control) with the addition of 1 µg/ml of anti-CD28 (BD Biosciences, San Jose, California, USA).

Flow cytometry, cell sorting, RNA extraction and reverse transcription

Following specific stimulation, PBMCs were stained for B-cell phenotype with the following monoclonal antibodies (mAbs, from BD Biosciences): CD3⁺, CD10⁺, CD16⁺ (BV510), CD19⁺ (APC-R700), CD21⁺ (APC), CD27⁺ (FITC), CD38⁺ (PE-Cy7), CD73⁺ (PErCP-Cy5.5), CD80⁺ (APC-H7) IgD (BV421), IgM (PE-CF594), IgG (BV605, HIV-1 envelope trimeric gp140 protein (gp140-R-Phycoerythrin (-R-PE)) and Live/Dead (BV510; BD Horizon, Franklin Lakes, New Jersey, USA). T-cell phenotype using the following mAbs: CD3⁺ (BUV496), CD4 (APC-Cy7), CD27⁺ (BV480), CXCR5 (Alexa647), IFNγ (PE-Cy7), IL-2 (BV711) and TNFα (FITC) from BD Biosciences, CD8 (PerCP) and CD40L (BV605) from BD BioLegend, IL-21 (PE, eBioscience), CD45RO (PE-TexasRed, Beckman Coulter, Irving, Texas, USA) and Live/Dead Fixable Blue Dead Stain Kit (ThermoFisher Scientific, Rome, Italy). The gp140 protein was obtained from European Research Infrastructures for

Poverty Related Disease (EURIPRED) and Lightning-Link R-PE Conjugation Kit (Innova Biosciences) was used to obtain a labelled gp140-R-PE. Cells were acquired on a BD LSRFortessa and analyzed by FlowJo v10.0.8r1 (Treestar, Inc., Ashland, Oregon, USA). Sorting was performed as shown in Supplementary Figure 2, <http://links.lww.com/QAD/B648> using FACSAriaII (BD Biosciences); sorted cells were collected in prelabelled tubes containing reverse transcription buffer for consequent PCR prior to Fluidigm [11].

Multiplexed reverse transcription-PCR (Fluidigm)

Amplified cDNAs were loaded onto 96.96 integrated fluidic circuit chip (Fluidigm; Biomark, South San Francisco, California, USA), according to manufacturer's instructions. Previously validated Taqman probes (Supplementary Table 1, <http://links.lww.com/QAD/B652>), polymerases and primers were mixed with samples as described earlier [11]. Cycle threshold (Ct) values were normalized as already described [12].

Statistical analysis

Unpaired *t*-test or Mann-Whitney U-test were performed to analyze normally and not-normally distributed FACS data; paired Wilcoxon test was used to compare different conditions among the same study participant. Boolean analysis and figures were produced using Simplified Presentation of Incredibly Complex Evaluations (SPICE, distributed by the National Institute of Allergy and Infectious Diseases, NIH (<http://exon.niaid.nih.gov/spice>)). Statistical tests were performed with Prism 6.0 (GraphPad, Prism). Gene expression data analysis and outlier identification were executed using Singular loaded on R. ANOVA was used to identify DEGs and DIGs. FC was determined using mean Expression threshold (Et)

values and volcano plots were generated in Prism 6.0. Statistical differences in gene expression between unstimulated and stimulated samples were determined by Wilcoxon matched paired tests. Pearson or Spearman correlation tests were used, respectively for normally or not-normally distributed data. Correlation matrix analysis was performed on ggplot2 package loaded on R.

Results

Limited exposure to HIV replication after perinatal infection and early treatment initiation correlated with lower HIV antibody response

ART-treated HIV-perinatally infected children who started therapy within the first year of life ($N=20$) with durable viral control (see Table 1) were investigated for their HIV antibody response to determine their serostatus. Six participants (6/20, 30%) were seronegatives as defined by complete absence of antibodies against all antigens tested, whereas seropositives were defined as positive for at least one antigen in either ELISA or western blot analysis (10 HIV antigens tested) (Supplementary Figure 3A, <http://links.lww.com/QAD/B649>). Patients' characteristics are shown in Table 1. No differences in terms of age, time on suppressive ART, serum measurements of IgG, IgM and IgA, CD4⁺ frequency and count were found between seronegatives and seropositives (Table 1). Timing of ART initiation, time to virus suppression (time from ART start to HIV RNA <50 copies/ml), duration of plasma viremia (time from birth and HIV-RNA <50 copies/ml), and area under the curve of HIV RNA, as revealed by longitudinal collection of viral load analysis (Supplementary Figure 3B and C, <http://links.lww.com/QAD/B649> and Table 1) were lower in seronegative

Table 1. Patients' characteristics.

	Seronegative	Seropositive	<i>P</i> value
Participants, <i>n</i> (female)	6 (5)	14 (8)	n.s.
Age (years), mean (SEM)	9.16 (2.1)	8.06 (1.5)	n.s.
Time of ART start (weeks) mean (SEM)	7.33 (2.6)	22.21 (3.9)	0.014
Time on suppressive ART (years), mean (SEM)	8.43 (1.9)	6.78 (1.4)	n.s.
Time to viral suppression (week) Mean (SEM)	10.17 (2.4)	32.21 (7.3)	0.024
Duration of plasma viremia (week), mean (SEM)	21.62 (2.75)	62.31 (7.55)	0.0001
AUC HIV RNA, area (95% CI)	2.04·10 ⁶ (85 915–223 962)	1.06·10 ⁷ (11 370–60 077)	0.02
Peak viremia, mean (SEM)	5.1 (4.9)	5.5 (4.7)	n.s.
% CD4 ⁺ T cells, mean (SEM)	38.9 (4.1)	38.1 (1.9)	n.s.
CD4 ⁺ cell count nadir, mean (SEM)	845.5 (164.2)	725.2 (92.39)	n.s.
CDC clinical stage (N-A/B/C)	(6/0/0)	(11/1/2)	—
CDC immunological stage (1/2/3)	(4/2/0)	(7/5/2)	—
IGM (MG/DL), mean (SEM)	92.9 (12.6)	97.5 (18.6)	n.s.
IGG (MG/DL), mean (SEM)	980.3 (76.5)	869.8 (183.2)	n.s.
IGA (MG/DL), mean (SEM)	147.7 (19.3)	106.5 (7.4)	n.s.
ART therapy, <i>N</i> Patients (drugs)	2 (2 NRTIs + 1 PI) 4 (2 NRTIs + 1 NNRTI)	7 (2NRTIs + 1 PI) 4 (2 NRTIs + 1 NNRTI) 3 (2 NRTIs + 1 NNRTI+ 1 PI)	n.s.

ART, antiretroviral therapy; AUC, area under the curve; CDC, Center for Disease Control and Prevention; CI, confidence interval; n.s., not significant; ND, not determined; NNRTI, non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; SEM, standard error of the mean.

compared with seropositive study participants ($P=0.014$, $P=0.024$, $P=0.0001$ and 0.02) respectively; Table 1 and Supplementary Figure 3B, <http://links.lww.com/QAD/B649>). Asterisks in Supplementary Figure 3 B, <http://links.lww.com/QAD/B649>, indicate 3 seropositives who despite a similar exposition to seronegatives to the antigen, still maintain a positivity for HIV antigens. All patients have been treated according to PENTA guidelines [13] at the time of diagnosis irrespective of age.

Gp140-specific B and T cells persist in both seronegative and seropositive early treated group after long-term viral suppression and higher IL21 production distinguishes HIV-specific T cells of seropositives

To assess whether HIV serostatus was associated with differences in B-cell or T-cell phenotype, we investigated surface maturation markers of B and T cell by flow cytometry. Seropositive and seronegative study participants demonstrated similar B-cell compartment measured as frequency of IgD, CD27, and CD21 as well as IgG and IgM among live CD10⁺CD19⁺ B cells (Supplementary Figure 2a–d, <http://links.lww.com/QAD/B648>, as well as similar mature T-cell subsets and peripheral T-follicular helper cells (pTfh; live CD4⁺CD27⁺CD45RO⁺CXCR5⁺) (Supplementary Figure 4a–c, <http://links.lww.com/QAD/B650>, <http://links.lww.com/QAD/B651>).

We further investigated the frequency of gp140-specific B and T cells by flow cytometry. The presence of gp140-specific T cells was quantified as CD40L⁺ T cells following stimulation with gp140 and SEB [14] (representative gates in Fig. 1a). Seronegative and seropositive study participants were similar with respect to frequency of gp140-specific T cells (left panel, Fig. 1a). Characterization of the distribution of memory CD40L⁺ maturational T-cell subsets by expression of CD27, CD45RO (representative gate in Fig. 1b) and CXCR5 demonstrated that seronegative and seropositive study participants have similar gp140-specific T-cell distribution (Fig. 1c) and peripheral T-follicular helper cells (pTfh) (Fig. 1d). Both groups demonstrated a higher frequency of gp140-specific T cells within central memory T cells (CD27⁺CD45RO⁺, CM) compared with effector memory (CD27[−]CD45RO⁺, EM) in both seronegative and seropositive individuals ($P=0.041$ and $P=0.004$, respectively; Fig. 1c). To further explore gp140-specific T-cell function, cytokine production was investigated by intracellular staining after gp140 protein stimulation. Whereas no differences were found for IFN γ , IL-2 and TNF α CD40L⁺CD4⁺T cells revealed a higher expression of IL-21 in seropositives compared to seronegatives ($P=0.003$; Fig. 2a). We further evaluated the ability of gp140-specific T cells to produce cytokines simultaneously using Boolean analysis. Results confirmed the higher production of IL-21 by gp140-specific T cells

Fig. 1. Gp140-specific T cells. Left side panel of (a) shows a representative gate used to identify gp140-specific T-cell subsets according to the expression of CD40L after in-vitro stimulation. Right panel of (a) depicts frequencies of gp140-specific T-cell-gated on live CD3⁺CD4⁺ T cells. (b) Representative gate of gp140-specific T-cell distribution in CM, EM and EMRA. (c) Contingency plot representing the median values of gp140-specific T cells within T-cell subset. (d) Scatter dot plot shows gp140-specific peripheral T-follicular cells. Mann–Whitney test was used for all comparisons. SEB, Staphylococcal Enterotoxin B.

Fig. 2. Cytokine production in gp140-specific T cells. (a) Scatter dot plot representing intracellular staining measurement for IFN- γ , IL-2, TNF α , IL-21 production after in-vitro stimulation in gp140-specific CD4 $^{+}$ T cells. (b) SPICE program was used for Boolean analysis looking at the production of IFN- γ , IL-2, IL-21 and TNF α in gp140-specific CD4 $^{+}$ T cells in seronegatives and SP. The bar graph represents the median frequency of all the Boolean subsets. (c) Each color in the pie charts corresponds to a specific combination of markers indicated at the bottom of the bar graph in (b), whereas every arc indicates the presence of that specific cytokine. (d) Permutation test was performed through SPICE program. Mann–Whitney test was used for comparisons ($*P \leq 0.05$).

Fig. 3. Gp140-specific B cell distribution in total CD19⁺ cells and among maturational subsets. Left side panel in (a) depicts representative gates of gp140-specific staining in B cells with negative controls. Scatter dot plot on the right side of panel (a) represents the percentage of gp140-specific cells among total CD19⁺ B cells. (b) On the left side, demonstrative gate used to identify the surface expression of CD21⁺ and CD27⁺ on CD19⁺ B cells. On the right of panel (b), the scatter dot plot shows the frequencies of gp140-specific B cells among CD27⁺ and CD21⁺ subsets. Statistical analyses between the subsets were determined by Mann–Whitney test. (c and d) Median values of gp140-specific B-cell distribution among CD27⁺ and CD21⁺ subsets were used to build contingency plots showed in the pie charts. Unpaired *t*-test or Mann–Whitney U-test were used to compare normally or not-normally distributed data, respectively. AM, activated memory; FMO, fluorescence minus one; HC, healthy control; HIV+, HIV-positive patient; IM, intermediate memory; REM, resting memory; TLM, tissue-like memory. Asterisk (*) indicates statistical differences among REM cells and the other B cell populations.

from seropositive compared with seronegative study participants, and further revealed that most of these cells were IL-21 single producers (Fig. 2b and c). Results of the permutation test confirm the difference present between the two populations (Fig. 2d).

Gp140-specific B cells were detected using a previously validated fluorochrome-conjugate trimeric gp140 protein probe (left panel Fig. 3a). Regardless of serostatus, both early treated groups presented comparable levels of gp140-specific B cells in total live CD19⁺ B cells (right

panel Fig. 3a). In order to exclude low-affinity B-cell receptor (BCR) binding as previously shown [15–18], we further evaluated the gp140-specific IgD-B-cell subset among the maturational subsets and found no differences between seronegatives and seropositives (Fig. 3b). Gp140-specific B cell distribution were enriched within the resting memory (CD27⁺CD21⁺, REM) subset compared with other subsets in both seronegatives [*P*=0.006 vs. immature memory and *P*=0.015 vs. Tissue Like Memory (TLM)] and seropositive patients (*P*=0.005 vs. immature memory; *P*=0.0004 vs. TLM;

Fig. 3c and d). These observations support our previously published study suggesting that early ART preserves an intact immune B-cell response in HIV-infected children [19].

Early transcriptional signatures after HIV peptide stimulation distinguishes seropositives from seronegative early treated group

After an overnight *in-vitro* stimulation with HIV gp140 peptides and CD40L, we assessed transcriptional profiles and changes in early programming [20] in switched IgM–IgD– or in IgM+IgD– gp140-specific B cells. With such sorting strategy, we focused our analysis on high specificity gp140+ B cells avoiding the inclusion of low-affinity IgD+ gp140-binding cells. We sort-purified fixed numbers (100) of gp140-specific B cells by flow cytometry and analyzed transcript levels in 96 previously selected and validated genes through the multiplex RT-PCR (Fluidigm; Biomark) (Supplementary Table 1, <http://links.lww.com/QAD/B652>). Basal levels of differentially expressed genes (DEGs) in switched IgM–IgD– B cells revealed similar profiles between seronegatives and seropositives with the exception of the *SLAMF1* gene, encoding signaling lymphocytic activation molecule 1 (CD150), which was upregulated in seropositives vs. seronegatives (Fig. 4a). However, in gp140 peptide-stimulated samples, 13 DEGs were significantly upregulated in seropositives compared with seronegatives, including those involved in: signal transduction and activation after BCR/TLR triggering including MYD88, STAT3, LIGHT, MTOR, MAPK3, and SYK (Fig. 4a); (b) B-cell activation and proliferation after T-cell cognate stimuli, such as CD22⁺ and CD86⁺, and (c) antibody production and class switch recombination (CSR) via active control of AICDA–, that is, BATF.

DEGs were also investigated in the IgM+IgD– gp140-specific B-cell subset. In the unstimulated samples, seropositives showed upregulation of two DEGs, IL10 known to play a key role in the inhibitory B-cell activity and ABCB1, an ATP-dependent efflux pump, found as a discriminating factor of naive B cells [21]. Similar to what was found in stimulated IgM–IgD– gp140-specific B cells, the IgM+IgD– gp140-specific cells also presented a robust functional signature in seropositives compared with seronegatives. Indeed, 5 DEGs were found upregulated in seropositives compared with seronegatives in stimulated samples (Fig. 4a). PLCγ, mainly involved in B-cell proliferation after BCR triggering, activation markers BATF and CD38⁺, as well as CAMK4 and MZB1 downstream activators of antibody secretion through the control of Ca⁺⁺ stores were upregulated in seropositives compared with seronegatives.

In order to avoid interindividual gene expression variability, gene expression differences were also

calculated according to paired differences between stimulated and unstimulated samples and defined as Differentially Induced Genes (DIGs) as previously shown [12]. Within the switched IgM–IgD– gp140-specific B cells, seropositives demonstrated upregulation of IKBKG (NEMO), an NFκB activator, and IL2RA. A downregulation of the homing chemokine coreceptor CXCR4 was found only in seronegatives group in both IgM– and IgM+IgD– gp140-specific B cells (Fig. 4b). Consistent with this finding, CXCR4 was recently found to be downregulated in B cells of HIV-infected patients with lower serologic responses upon HBV vaccine [22]. Also, the IgM+IgD– gp140-specific counterpart showed similar results, indeed PRDM1 (BLIMP1) was positively induced only in seropositives, suggesting reduced plasma cell differentiation in IgM+ gp140-specific B cells in seronegative patients. Moreover, only seropositives had upregulation of genes involved in immune activation and lymphocyte proliferation pathways, such as CAMK4, CAV1, IL-21R. PDCD1 (PD-1), a regulator of B-cell activation [23], and SLAMF1 a regulator of antibody production [24] were downregulated only in seropositive patients (Fig. 4b).

HIV antibody response and higher activation pattern in gp140-specific B-cell gene signature is associated with HIV-specific T-cell function (IL-21)

To explore the most important variables defining humoral, and cellular responses upon HIV in early treated group, we performed a pairwise correlation matrix between gp140-specific T cells, gene expression of gp140-specific B cells and clinical data (Fig. 4c). A strong positive correlation was found between magnitude of antibody response, age at ART initiation and gene expression within the switched IgM–IgD– gp140-specific B cells with 10 and 9 positively correlated genes, respectively (Fig. 4c).

Analysis further revealed a positive correlation between IL-21-producing gp140-specific T cells and gene expression within the stimulated switched IgM–IgD– gp140-specific B-cell subsets. Indeed, 12 genes, including CCR7, CD22, CD86, CXCR5, MTOR and TLR7 positively correlated with IL-21-producing gp140-specific T cells. A positive correlation was also present between the magnitude of HIV antibody and PRDM1 gene expression after *in-vitro* stimulation further suggesting the greater ability of gp140-specific B cells of seropositives to undergo plasma cell differentiation after *in-vitro* stimulation. The frequency of gp140-specific B cells was negatively correlated with *NKRF* gene expression within the switched IgM–IgD– gp140-specific B cells further suggesting that *NKRF*, a negative regulator of NFκB, even in the context of *in vitro*-provided gp140 peptides, may impact on gp140-specific B cells over time.

Fig. 4. Gene expression analysis in gp140-specific B-cell subsets. (a) Volcano plot representing DEGs between seropositive and seronegative, with (red dots) or without (blue dots) in-vitro stimulation in sorted IgM- gp140-specific (triangle dots) and IgM+gp140-specific B cells (square dots). Only DEGs with a *P* value less than 0.05 are shown in the volcano plot. (b) Column heatmaps demonstrate differentially induced genes (DIGs) between stimulated and unstimulated samples in IgM- gp140-specific B cells (left side panel) and IgM+gp140-specific B cells (right side panel). Values depicted in this panel were calculated by the difference in the averages in Et between the two experimental conditions for both groups and displayed in the heatmap. Only genes resulted significantly induced with a *P* value less than 0.05 at paired analysis are showed in the heatmap. Paired Wilcoxon test for not-normally distributed data and paired *t*-test for normally distributed data were used for comparisons. (c) A correlation matrix is depicted with color heatmap showing *r* values derived from Spearman and Pearson correlation analysis, respectively used for normally and not-normally distributed data. A color was assigned only to *r* values resulting from significant correlations between data with a *P* value less than 0.05. An *r* value of 0 (white) was assigned to *P* value greater than 0.05. DEGs, differentially expressed genes.

Discussion

The generation of an antigen-specific memory cell pool is a dynamic process that evolves from naïve cells to a memory cell pool able to rapidly react upon antigenic re-stimulation. In perinatally HIV-infected children treated with ART early after birth with sustained long-term viral suppression, this process is influenced by both the limited duration of antigen exposure after birth, and the distinct neonatal immune system [8,25]. HIV-specific responses in such early treated children appears to be low or absent [4,6], and this may be the result of a premature interruption of memory development. In line with previous reports [7,26,27], in our cohort, 30% of perinatally HIV-infected children treated within the first year of life resulted in seronegative status, and as expected, this sub-group was characterized by an earlier timing of ART start, lower antigen exposure and lower HIV-DNA levels compared with seropositives [7]. However, despite the rapid control of the plasma viremia compared with the other seropositive participants, three study participants were still positive for HIV-specific antibodies. Thus, it is not clear whether additional intrinsic characteristics other than viral exposure, may impact on HIV serostatus. It is also important to mention that, albeit not statistically significant, all but one of the seronegative patients are female patients. According to this data, and a recently published work [28] where 10 out of 11 posttreatment controllers were girls, we believe that sex may play a role in the ability to control HIV in early treated perinatally HIV-infected children. This aspect should be further investigated in larger studies. Indeed, one of the main limitation of the present study is the paucity of HIV Ab-negative patients analyzed. However, HIV seronegative status in children growing with HIV is a rare event where early treatment initiation (<3 months of age) followed by a continuous viral suppression together with still unknown host-related factors are necessary. In pediatric patients, such conditions, are critically limited by both adherence and compliance issues experienced in specific age ranges [29–31].

Regardless serological status, in the present study, we described that HIV-specific B and T cells persist at similar levels in both seronegative and seropositive groups of early treated children. Gp140-specific B cells were further investigated for their B-cell phenotype characteristics and showed that in both seronegatives and seropositives, gp140-specific IgD⁺ B cells were found to be enriched in REM B cells (gp140-specific REM: 35.46 and 43.36% in seropositives and seronegatives, respectively) (Fig. 3) rather than activated subsets (AM and TLM B cells) [19,32]. As recently reported, this was not the case in ART-naïve HIV-infected adults during the acute phase of the infection where gp140-specific B-cell responses were dominated by AM and TLM subsets rather than REM [15]. Overall, these results are consistent with our previous reports, demonstrating that early treatment in

perinatally HIV-infected children is able to preserve the memory B-cell compartment preventing B-cell exhaustion, commonly found in chronically infected patients and late treated patients [19,32].

To further evaluate whether qualitative differences within the gp140-specific B-cell subsets may underline the discordant ability of these patients to maintain HIV antibody over time, the transcriptional characteristics of switched IgM–IgD⁺ gp140-specific B cells and unswitched IgM+IgD⁺ gp140-specific B cells were further evaluated in two different ex-vivo conditions in seronegatives compared with seropositives. Regardless serostatus, no major differences emerged in terms of DEGs between the two groups in unstimulated conditions. However, switched IgM–IgD⁺ gp140-specific B cells, presented a different pattern of activation after an overnight stimulation with HIV peptides and CD40L. Indeed 13 genes mainly involved in intracellular signal transduction (*MYD88*, *STAT3*, *LIGHT*, *MTOR*), B-cell activation (*CD86*, *CD38*, *BATF* and *SYK*) as well as in CSR (*BATF*) were upregulated in seropositives compared with seronegatives overall showing a different gp140-specific activation signature in these B cells. These data were further analyzed by paired analysis to exclude inter-individual gene expression variability. We showed that CXCR4, a homing receptor, both in switched IgM–IgD⁺ and IgM+IgD⁺ gp140-specific B cells, is negatively induced after in-vitro stimulation only in seronegative patients. These results are in line with recent data showing defective CXCR4 expression in HIV-infected patients demonstrating suboptimal responses upon HBV vaccination [22]. Other vaccine-induced Ab responses were evaluated in order to exclude intrinsic defect of Ab production in seronegatives. The analysis of pneumococcus Ab showed seroprotective titers for the majority of early treated with higher levels in seronegatives compared with seropositives (data not shown). These data suggest, as previously shown [19,33], that early treated patients and especially seronegatives present a preserved Ab response and B-cell function compared with late-treated ones. In line with this, lack of HIV-specific Ab in seronegatives, may be because of a suppression of viral replication during a period of relative immunological immaturity rather than a B-cell intrinsic dysfunction [34]. A correlation matrix further revealed that the expression of PRDM1 in switched IgM–IgD⁺ gp140-specific B cells, necessary for the formation of mature Ab-secreting plasma cells [35,36], was positively associated to the magnitude of HIV Ab.

Overall, our observations suggest that despite similar frequency of gp140-specific B cells, qualitative differences may drive discordant ability of early treated patients to maintain antibody vs. HIV over time. The differential pattern showed by these cell subsets may be the result of a shorter antigen exposure in patients achieving a very early and sustained viral suppression in turn reducing cycles

within the germinal centers and provide a truncated maturation process of gp140-specific B and T cells [34].

We further evaluated the T-cell compartment in order to dissect whether T-cell specific differences in both antigen-specific and general subsets may influence the T-cell dependent ability of early treated to maintain HIV antibody over time. Whereas no differences were found in terms of total gp140-specific T cell and memory subsets distribution, IL-21 production after *in-vitro* stimulation was higher in seropositives compared with seronegatives suggesting that a longer viral exposure in seropositives allowed a more complete maturation process of these cells. These results were further confirmed by the correlation matrix showing strong correlation of IL-21 production and early transcriptional signatures of switched gp140-specific B cells after stimulation. IL-21 plays a critical role in B-cell fate decisions and differentiation and influences the expression of several functionally important B-cell surface molecules [37]. We here show for the first time in the context of pediatric HIV-infected children treated early after birth and under viral control, that lower production of IL-21 from gp140-specific T cells and concomitant lower gene expression after HIV peptide stimulation *in vitro* distinguish early treated seronegative children who fail to preserve HIV humoral immunity. Whereas these data particularly highlight the T-cell-dependent antibody response, additional experiments are needed in order to dissect the T-cell independent function of gp140-specific memory B cells in early treated patients.

Collectively, our data suggest that in seronegatives children undergoing eART, B cells and T cells are characterized by a reduced training process of maturation because of shorter antigen exposure, which provide qualitative differences both in terms of cytokine production and gene expression when the HIV antigen is re-encountered. In turn, this reduced training may hamper the ability of B cells to induce plasma cell differentiation upon re-stimulation and defective T/B cognate stimuli (e.g. IL-21) in the T-cell counterpart. It is still not clear, however, whether additional host-related characteristics, not solely driven by antigen exposure and the timing of ART initiation may impact the formation of a mature HIV-specific B cells able to induce and maintain HIV antibody in early treated individuals. Future studies with comprehensive multi-OMIC systems biology approaches may further define such characteristics thereby informing personalized new disease-modifying interventions.

Our observations suggest that a truncated memory process [38] could occur because of early control of HIV replication after early treatment initiation, visualized in Supplementary Figure 1, <http://links.lww.com/QAD/B647>. This finding is in line with other models of early and rapidly suppressed infection [39] with limited germinal center (GC) reactions that may be dominated

by IgM+ memory B cells rather than IgG [38]. In the present study, although no difference was found in frequencies of IgM+IgD- gp140-specific B cells between seronegatives and seropositives, positive induction of PRDM1 (Blimp1), IL21R, CAV1, and CAMK4 only in seropositives supports the ability of this B-cell subset to undergo a plasma cell differentiation upon re-stimulation. In addition, seronegative children, who failed to positively induce such genes, showed a negative induction of CXCR4. Although the role and function of IgM+ memory B cells is still extensively debated [40,41], recent studies revealed greater transcriptional analogies with classic IgG+ rather than naive B cells. Indeed, IgM+ memory subset demonstrated evidence of SHM and ability to provide rapid humoral response upon antigen re-challenge in a murine malaria model [39,42]. Additional studies are needed in order to fully characterize such responses and to define possible novel targets of immune interventions aiming at inducing long-term effective HIV immune responses.

In conclusion, although gp140-specific T-cell and B-cell immunity persists in perinatally HIV-infected children treated early after birth with discordant HIV antibody profile after several years of HIV viral control, responses to antigen re-stimulation differ markedly with respect to early transcriptional signatures and function. Albeit limited by the small sample size, we believe that our observation may further inform future studies aiming at viral remission through immunization strategies targeting specific molecular pathways in early treated children with discordant HIV serologic profiles.

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Conflicts of interest

There are no conflicts of interest.

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