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Forkhead box protein 3$^+$ regulatory T cells and Helios$^+$ subset in perinatally acquired HIV

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Summary

Forkhead box protein 3 (FoxP3)$^+$ regulatory T cells (T_{regs}) are important not only in regulating the development of autoimmune conditions, but also in chronic infectious diseases. Given their cardinal function in suppressing immune activation, research has focused upon whether they play a detrimental role in chronic infections, particularly HIV. While the role of T_{regs} in HIV has been investigated intensively, it remains an unresolved topic. However, it is generally accepted that T_{regs} are susceptible to HIV infection and are preferentially preserved over conventional CD4$^+$ T cells. It is unknown whether the peripheral-induced or the thymic-derived T_{regs} are more susceptible to HIV cytotoxicity. It has been recognized that T_{regs} can be segregated into two subsets based on Helios expression, with the vast majority being Helios$^+$. This study examines the impact of HIV infection on total T_{regs} and their Helios subsets in a perinatal-acquired HIV-infected paediatric population. The finding indicates a selective expansion or survival of T_{regs} in association with CD4 depletion and increased viraemia. The Helios$^+$ and Helios$^-$ subsets within T_{regs} appear to be equally affected. However, the Helios$^+$ T_{regs} seem to be more preserved in patients with low CD4$^+$ ≤ 25% and detectable plasma HIV RNA >20 copies/ml. In this group, the frequencies of T_{regs} are increased, but their numbers appear insufficient to restrain immune activation. In conclusion, our findings suggest that both Helios subsets of T_{regs} are susceptible to HIV infection and are preferentially preserved compared to conventional CD4$^+$ T cells.

Keywords: AIDS, regulatory T cells, T cells

Introduction

Since the demonstration that depletion of CD4$^+$CD25$^+$ T cells results in the development of organ-specific and systemic autoimmune disease, accumulating evidence increasingly supports the involvement of forkhead box protein 3 (FoxP3)$^+$ regulatory T cells (T_{regs}) in diverse immune disorders, cancers and infectious diseases [1–3]. T_{regs} are thought to be involved in modulating the responses of the immune system, thereby affecting the outcome of many chronic viral infections such as herpes simplex virus (HSV), hepatitis C virus (HCV) and retroviruses, including HIV [4]. In HIV, demonstration of a direct and predictable relationship of T_{regs} with the disease process in acute or chronic infection or subsequent immune dysregulation (as evidenced by increased activation markers) has been extremely challenging [5]. It remains unclear whether T_{regs} are beneficial to the host by regulating chronic immune inflammation or detrimental by suppressing anti-HIV immunity [6,7].

Studies in adults show that CD4$^+$CD25$^+$ T_{regs} and *in-vitro* FoxP3 transduced conventional CD4$^+$ T cells are susceptible to HIV infection [8,9]. In addition to CD4 and CD25, they can express the chemokine co-receptor CCR5, a required co-receptor for HIV entry into cells [8]. CXCR4 co-receptor is expressed, but at lower levels compared to CCR5. The use of replication-competent HIV demonstrates that HIV replicates efficiently in T_{regs} and is cytotoxic to the cells. While some studies report that T_{regs} may be preferentially infected and depleted [10], one study showed variable susceptibility of T_{regs} to HIV depending on trophism, virus strain and viral life-cycle timing [9]. However, the T_{regs} remained suppressive 24 h after infection *in vitro*.

T_{regs} may suppress immune activation, as demonstrated by enhanced *in-vitro* HIV-specific activity, cytokine

production and proliferative responses of T cells [11,12]. Therefore, T_{regs} may have a protective role in the pathogenesis of HIV by limiting the dysregulated immune activation seen in HIV that precedes the collapse of the immune system. In contrast, T_{regs} may suppress effective anti-viral responses to HIV infection by targeting HIV-specific effectors. These seemingly dichotomous and antagonistic roles of T_{regs} are difficult to delineate clearly [13,14]. On one hand, T_{regs} may facilitate the establishment of HIV by inhibiting HIV-specific immunity. On the other hand, T_{regs} may modulate the non-specific inflammation that is detrimental. Still others propose that the perturbation of T_{regs} in HIV is not the direct cause of immune activation noted in HIV infection and that the data do not show T_{regs} as playing a significant role in temporizing the immune response to HIV [15].

There are conflicting data in the literature regarding the role of T_{regs} in HIV infection and their subsequent interaction. Some studies in adults demonstrated the proportion (%) of T_{regs} (defined as $CD4^+CD25^+FoxP3^+$) to be lower in viraemic patients, with a concomitant increase in activation markers, human leucocyte antigen D-related (HLA-DR) and CD38 on CD8 [16]. Similarly, another study showed a gradual decrease of the absolute and proportion of T_{regs} (defined as $CD3^+CD4^+CD25^{hi}FoxP3^+$) during HIV disease progression, together with increased immune activation [17]. In one study of patients with acute primary HIV infection (median 13 days), the frequency of T_{regs} was found to be lower than in chronic patients and, over time, the frequency of T_{regs} decreased in untreated patients [18]. In addition, the elevated proportion of T_{regs} and low levels of immune activation, evidenced by reduced expression of the activation marker CD69 in a cohort of HIV-resistant sex workers exposed to HIV regularly who remained negative, was reported in another study [19]. Alternatively, studies showed that in HIV patients with low CD4 counts (<200), absolute T_{regs} (defined as $CD4^+FoxP3^+$) were lower but constituted a higher proportion of the CD4 population compared to HIV-positive patients with higher CD4 counts and healthy adults [20].

Very few studies have investigated T_{regs} in HIV-infected paediatric patients [21,22]. In one such study, the frequency of T_{regs} correlated positively with viraemia but negatively with CD4 cells, suggestive of T_{reg} expansion with CD4 decline [21]. T_{regs} declined at a slower rate than other CD4 cells. There is a selective expansion of T_{regs} associated with viraemia and CD4 depletion. In another study of T_{regs} in HIV-exposed but uninfected neonates, unexposed neonates and infected neonates, high levels of T_{regs} and low levels of $CD4^+$ and $CD8^+$ T cell activation were documented in the exposed uninfected neonatal cord blood [22]. Unlike in the unexposed neonates, exposed neonates had an HIV-1-specific T cell response. The depletion of $CD4^+CD25^+CD127^-$ T_{regs} augmented this HIV-specific response, suggesting that T_{regs} may contribute to protection from vertical transmission by suppressing T cell activation, which is important for virus uptake into cells.

It is recognized that T_{regs} exist as two subsets, thymic-derived (tT_{regs}) and peripheral-induced (pT_{regs}), with many similarities and differences [23]. The challenge has been the difficulty in distinguishing these two subsets *in vivo* in order to understand their roles in different diseases. The transcription factor Helios is suggested as a potential biomarker expressed preferentially in tT_{regs} [24]. However, subsequent studies have challenged this claim, resulting in a controversial and unresolved topic [25]. In HIV, there is no study that investigates whether the $Helios^+$ and $Helios^-$ T_{reg} subsets are affected differentially during the different stages of infection. Given the profound level of CD4 depletion, regeneration and activation depending on the HIV infectious status of the patients, we investigated the impact of CD4 depletion and HIV viraemia on T_{regs} and their $Helios^+$ subset in our perinatally acquired HIV-infected paediatric cohort. We found that a selective expansion or survival of T_{regs} associated with CD4 depletion and increased viraemia. Interestingly, both $Helios^+$ and $Helios^-$ subsets within T_{regs} appeared to be equally affected. However, when segregating our patients into three groups based on CD4% and HIV RNA, the poor status group with low $CD4^+ \leq 25\%$ and detectable plasma HIV RNA >20 copies/ml had increased frequencies of T_{regs} and preservation of $Helios^+$ T_{regs}. Nevertheless, this group also had the highest immune activation based on increased $CD8^+$ T cells and their up-regulation of CD38 and HLA-DR expression. While there was a selective preservation of T_{regs} and the $Helios^+$ subset, it remains unclear whether they played a positive or negative role in paediatric HIV disease and progression.

Materials and methods

Patients

All perinatally HIV-infected patients attending the Pediatric HIV Clinic of UTHealth (Houston, Texas, USA) and providing informed consent were eligible for inclusion into this prospective cross-sectional study. Sixty patients meeting these criteria provided blood samples during the study interval; this group represented 87% of the clinic's perinatally infected patients. No perinatal transmissions were from breastfeeding. The fraction of *intra-utero* and intrapartum transmissions was not available. The study was approved by the Institutional Review Board of UTHealth. Clinical and related laboratory data were from medical records. The percentages and numbers for CD3, CD4, CD8, CD38 and HLA-DR were obtained commercially through Labcorp.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from ethylenediamine tetraacetic acid (EDTA)

anti-coagulated fresh whole blood by Ficoll gradient centrifugation. A total of 50 000 cells were surface-stained with anti-CD4 (RPA-T4; Biolegend, San Diego, CA, USA) and intracellularly stained with anti-Helios (22F6; BioLegend) and anti-FoxP3 (259D; BioLegend) using the FoxP3/transcription factor staining buffer set, as per the manufacturer's protocol (eBioscience, San Diego, CA, USA). The fluorescence activated cell sorter (FACS)Calibur (BD Biosciences, San Jose, CA, USA) was used for data acquisition. Data analysis employed FlowJo version 7·6 (Tree Star, Inc., Ashland, OR, USA).

Analysis

Because previous studies [6,21] have demonstrated that T_{regs} may associate with clinical measures of HIV disease, patients were classified into cohorts based on clinical laboratory indicators of HIV disease status, where good status was defined as $CD4^+ \geq 25\%$ (CD4↑) and undetectable plasma HIV RNA <20 copies/ml [viral load (VL)↓]. Of the four resulting cohorts (CD4↑VL↓, CD4↓VL↑, CD4↑VL↑, CD4↓VL↓), the cohort with CD4↓VL↓ was excluded because it comprised only one patient. The remaining cohorts were referenced as HIV clinical status good (CD4↑VL↓), intermediate (CD4↑VL↑) or poor (CD4↓VL↑).

Clinical laboratory findings were reviewed to characterize patients' status near the time of T_{reg} measurements (± 7 days). Indicators of the severity of HIV disease during the course of infection included Centers for Disease Control (CDC) immunological and clinical status (which provided an estimate of a patient's most severe immune suppression and worst clinical status), nadir CD4% and nadir number of $CD4^+$ cells/μl. Anti-retroviral drug treatment (ART) status at the time of T_{reg} measurement was classified as (1) combined ART (cART), which most often comprises dual-nucleoside/nucleotide reverse transcriptase inhibitors with either a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor; (2) ART, for those who did not meet the inclusions for cART; or (3) no ART regimen. Patient history of exposure to ART comprised classifications cART, ART and no ART during the course of infection. An individual patient may be classified into one or more than one of these groupings. Cumulative anti-retroviral drug major resistance mutations (mRM) was the sum of a cumulative list of unique mRM found in each patient's records from the first RM test to the day of the T_{reg} blood sample that are clinically significant, and resulted in resistance to the ART in use. mRM were those defined in the Stanford database (http://hivdb.stanford.edu/; date accessed 15 July 2013).

Measured outcomes (T_{regs} and $Helios^+$ subset) and clinical results were compared between the three HIV disease status cohorts by Kruskal–Wallis test with pairwise comparisons. Main-effects generalized linear models with either identity or log link was used to test the association of T_{reg} outcomes (both as percentage and cell number for each outcome) with the three clinical cohorts while controlling for age, gender, ethnicity, CDC clinical categories and CDC immunological categories. Residuals for all models were checked for normality by Shapiro–Wilks test to ensure the appropriate fit of the model to the outcome measure.

Descriptive data are reported as median with 25th and 75th percentiles and minimum and maximum values for continuous variables and counts (percentage) for categorical variables. Comparisons between groups were by non-parametric tests, as the data deviated from a normal distribution as assessed by Q–Q plots and Shapiro–Wilks test. The Mann–Whitney *U*-test was used for comparisons between two groups and the Kruskal–Wallis test was used for comparing more than two independent groups. Box-and-whiskers plots represented 10th and 90th percentiles; points outside whiskers were outliers. Correlations were calculated by Spearman's rho test. Data management and analysis employed Microsoft Access (Microsoft Inc., Redmond, WA, USA) and SPSS version 19 (Statistical Package for Social Sciences, Chicago, IL, USA), respectively. As the majority of outcomes were co-linear, multivariate analyses were limited.

Results

Patient population and selected characteristics

Sixty patients were included in the study. The population attended the UTHealth clinic for a median of 14·4 years and at the time of T_{reg} determinations were comprised mainly of male patients (48·3% female) on cART (83·3%), who were black (70%), teenagers (median, 14·4 years old), with good immunological status (32% CD4) and with low HIV viraemia (65 HIV RNA copies/ml). The population has had more severe HIV disease in the past with median 18% CD4 nadir (Table 1).

A median of 9% $CD4^+$ cells were T_{regs} based on FoxP3 expression and 69·5% of the T_{regs} were $Helios^+$. Notable abnormal clinical values at the time of T_{reg} measurements included increased $CD8^+$ cells and increased activated $CD8^+$ cells based on their expression of HLA-DR. The majority has mRM to ART drugs (82·4%) and were on cART at some point in their disease course (91·7%).

To further resolve the relationship between HIV disease status and T_{reg} findings, outcomes were parsed into cohorts comprising good (CD4↑VL↓), intermediate (CD4↑VL↑) and poor (CD4↓VL↑) disease status (Table 1), based on their immunological and virological status at the time of collection of the blood sample for T_{reg} measurements. There were significant differences in age and race/ethnicity distribution among the HIV disease status groups, with older patients being significantly more frequent in the poor disease status group (median age 18·7, 13·5 and 13·9 for the poor, intermediate and good groups, respectively,

Table 1. Patient characteristics and outcomes.

Measure	All patients	HIV disease status group†		
		Poor (CD4↓VL↑)	Intermediate (CD4↑ VL↑)	Good (CD4↑ VL↓)
Patients, *n*	60	9	26	24
Age, years	14·4 [8·8–16·7; *(0·8–21·3)*]	18·7 [15·0–19·7; *(10·6–20·5)*]	13·5 [9·1–16·5; *(0·8–21·3)*]*	13·9 [6·3–15·8; *(1·8–18·5)*]**
Female, %	48·3	55·6	46·2	50·0
Race/ethnicity, %				
Black	70·0	77·8	80·8	54·2
Hispanic (not black)	21·7	22·2	7·7	37·5
White	8·3	0	11·5	8·3
T_{reg}				
% of $CD4^+$	9·0 [7·0–11·0; *(4·0–35·0)*]	11·0 [8·5–18·5; *(7·0–29·0)*]	9·0 [7·1–13·0; *(4·2–35·0)*]	8·0 [5·9–9·4; *(4·0–15·0)*]**
Number/μl	63·0 [47·4–95·7; *(13·3–304·2)*]	33·7 [29·5–50·9; *(13·3–57·7)*]	72·6 [54·3–113·8; *(32·5–304·2)*]*	68·6 [50·3–113·3; *(35·3–194·9)*]**
% of $CD3^+$	3·5 [2·9–4·9; *(1·5–9·9)*]	2·7 [2·0–3·3; *(1·6–6·3)*]	4·3 [3·1–5·0; *(1·5–9·9)*]	3·6 [3·1–5·0; *(2·3–6·4)*]
% of lymphocytes	2·7 [2·1–3·6; *(1·3–8·7)*]	2·0 [1·6–2·6; *(1·3–5·8)*]	3·1 [2·4–4·1; *(1·3–8·7)*]*	2·6 [2·2–3·6; *(1·7–5·0)*]
% $Helios^+$	69·5 [61·0–77·8; *(37·0–93·0)*]	72·0 [57·0–80·0; *(42·0–86·0)*]	69·0 [60·8–78·0; *(44·0–93·0)*]	68·5 [61·3–78·5; *(37·0–90·0)*]
Number/μl $Helios^+$	41·8 [29·5–73·1; *(9·6–231·2)*]	18·9 [14·7–34·8; *(9·6–44·4)*]	55·9 [30·0–85·1; *(19·5–231·1)*]*	44·4 [31·6–76·4; *(21·9–102·3)*]**
At T_{reg} determination				
$CD4^+$ % of lymphocytes	32·0 [**26·4**–39·8; *(8·9–67·0)*]	**19·1** [**16·2–20·5; *(8·9–22·5)***]	31·5 [**27·7**–40·1; *(25·0–67·0)*]*	37·9 [31·3–41·0; *(26·0–50·1)*]**
Number/μl	793·5 [522·0–1068·0; *(160·0–3380·0)*]	**269·0** [**181·0–355·5; *(160·0–450·0)***]	778·0 [534·3–1134·0; *(364·0–3380·0)*]*	913·0 [744·0–1206·5; *(415·0–3120·0)*]**
$CD8^+$ % of lymphocytes	**41·0** [31·9–**50·6; *(16·0–70·0)***]	**61·1** [**45·7–67·3; *(37·0–70·0)***]	**47·8** [**38·0–51·7; *(16·0–62·3)***]	33·0 [24·3–**39·8; *(18·0–52·0)***]**,***
Number/μl	**934·0** [672·5–**1181·5; *(287·0–3210·0)***]	711·0 [610·5–**980·5; *(465·0–1228·0)***]	**1039·5** [858·0–**1248·0; *(516·0–2280·0)***]	830·0 [662·5–**1118·3; *(287·0–3210·0)***]
$CD38^+$ % of lymphocytes	14·0 [10·0–**22·0; *(5·0–55·0)***]	**26·0** [17·0–**48·5; *(15·0–55·0)***]	18·0 [12·0–**24·3; *(8·0–47·0)***]	10·0 [7·3–12·0; *(5·0–20·0)*]**,***
Number/μl	348·0 [231·0–**547·5; *(105·0–1276·0)***]	**441·0** [287·0–**555·0; *(240·0–611·0)***]	405·0 [327·5–**640·5; *(168·0–1276·0)***]	258·5 [143·3–**390·0; *(105·0–870·0)***]***
$HLA\text{-}DR^+$ % of lymphocytes	**12·5** [**9·0–22·3; *(2·0–48·0)***]	**24·0** [**21·5–32·0; *(18·0–48·0)***]	**17·0** [**10·0–23·0; *(2·0–43·0)***]*	**9·5** [**6·3–11·8; *(3·0–25·0)***]**,***
Number/μl	**302·0** [**238·5–420·0; *(87·0–891·0)***]	**384·0** [**334·5–426·0; *(288·0–480·0)***]	**366·0** [**278·0–516·0; *(105·0–891·0)***]	**265·5** [**156·0–323·5; *(87·0–750·0)***]**,***
HIV RNA (log_{10} copies/ml)	**1·8** [1·3–3·2; *(1·3–4·9)*]	**4·1** [3·6–4·3; *(1·5–4·9)*]	**2·0** [1·9–3·6; *(1·5–4·9)*]	**<1·3** [1·3–1·3; *(1·3–1·3)*]**,***
ART none %	11·7	33·3	15·4	0
ART not cART	5·0	0	11·5	0
cART	83·3	66·7	73·1	100
Any mRM to ART, % *n* = 51 (9 UNK)§	82·4	88·9	83·3	82·3
Count of mRM	2·0 [1·0–5·0; *(0–13·0)*]	4·0 [1·0–4·0; *(0–8·0)*]	1·5 [1·0–5·0; *(0–11·0)*]	3·0 [1·0–6·0; *(0–13·0)*]
NRTI %	66·7	66·7	62·5	76·5
NNRTI	47·1	66·7	50·0	35·3
PImRM	47·1	66·7	45·8	41·2
During course of HIV infection				
$CD4^+$ % nadir	**18·0** [**10·3–23·0; *(0·0–58·0)***]	**9·0** [**7·5–17·0; *(7·0–24·0)***]	**20·0** [**15·5–26·3; *(1·0–58·0)***]	**20·0** [**7·5–25·3; *(0·0–55·0)***]
Number/μl	371·0 [**170·5**–665·0; *(1·0–2772·0)*]	**165·0** [**129·5–317·0; *(99·0–460·0)***]	393·5 [**205·0**–651·0; *(11·0–2772·0)*]	495·5 [**186·0**–799·3; *(1·0–2499·0)*]
CDC clinical status B or C, %	48·3	66·7	34·6	58·3
CDC immunological status 2 or 3, %	55·0	77·8	50·0	50·0
History of no ART	3·3	11·1	3·8	0
Mono ART *n* = 59 (one UNK in good group)‡	23·7	44·4	19·2	21·7
Dual ART *n* = 59 (one UNK in good group)‡	37·3	66·7	38·5	26·1
cART *n* = 60	91·7	88·9	84·6	100

Data are counts, percentages (range where appropriate in italic type) or medians (25th and 75th quartiles); *,**,***$P \leq 0{\cdot}05$ (* poor *versus* intermediate; ** poor *versus* good; *** intermediate *versus* good). Clinical and clinical laboratory results outside normal range or indicative of poor HIV clinical status are shown in bold type. Exceptions in sample size: †*n* = 59 for HIV disease status groups because (CD4↓ VL↓) grouping comprising one individual was excluded from analysis; ‡One patient did not have history available; §Nine patients did not have history available. ART = anti-retroviral drug treatment; cART = combined ART; UNK = unknown; CDC = Centers for Disease Control; NRTI = nucleoside reverse transcriptase inhibitors; NNRTI = non-NRTI; PImRM = protease inhibitor major resistance mutations; mRM = major resistance mutations; T_{reg} = regulatory T cell; HLA-DR = human leukocyte antigen-D related; VL = viral load.

$P \leq 0{\cdot}05$) and white patients being significantly less likely to be in the good clinical status group compared to the intermediate clinical status group (8·3 *versus* 11·5%, $P \leq 0{\cdot}05$). Not surprisingly, there were more patients in poorer CDC immunological status in the poor disease status group than in the good disease status group (77·8 *versus* 50%, $P \leq 0{\cdot}05$), but this disparity was not seen for the CDC clinical status classification. There were more patients in the poor disease status group who were never on ART compared to the good disease status group (33·3 *versus* 0%, $P \leq 0{\cdot}05$) (Table 1).

As expected, VL was correlated negatively with percentage and absolute CD4 counts ($r = -0{\cdot}514$, $r = -0{\cdot}420$, $P < 0{\cdot}001$), consistent with depletion of $CD4^+$ T cells with viraemia. There was significant positive correlation between VL and percentages of $CD8^+$ T cells as well as their expression of CD38 and HLA-DR ($r = 0{\cdot}613$, $r = 0{\cdot}657$, $r = 0{\cdot}646$, $P < 0{\cdot}001$), indicating immune activation with increasing viraemia.

Preservation of T_{regs} and their $Helios^+$ subset in paediatric HIV

Figure 1 shows the gating strategy of two representative patients. Forward- and side-scatters were used to gate on lymphocytes (panel not shown) from which $CD4^+$ cells were subsequently identified (Fig. 1a). The percentage of $FoxP3^+$ T_{regs} was calculated within $CD4^+$ (Fig. 1b). Figure 1c shows the correlation of FoxP3 and Helios within $CD4^+$. The $Helios^+$ T_{reg} subset was determined based on the percentage of Helios within $FoxP3^+$ T_{regs} (Fig. 1d).

To assess whether T_{regs} and their $Helios^+$ subset were more preserved relative to conventional $CD4^+$ T cells, we correlated their frequencies and absolute numbers with CD4 and viraemia. As $CD4^+$ cells declined in frequencies and numbers, there was an increase in the frequencies of T_{regs} ($r = -0{\cdot}439$ and $r = -0{\cdot}486$, $P < 0{\cdot}001$, respectively), while the $Helios^+$ subset remained unchanged (Fig. 2a,b). The absolute numbers of T_{regs} and $Helios^+$ correlated significantly and positively with absolute CD4 counts ($r = 0{\cdot}727$ and $r = 0{\cdot}603$, $P < 0{\cdot}001$, respectively) (Fig. 2c). There was also a positive correlation for the frequencies ($r = 0{\cdot}291$, $P = 0{\cdot}024$) and numbers ($r = 0{\cdot}924$, $P < 0{\cdot}001$) of T_{regs} *versus* their $Helios^+$ subsets (Fig. 2d). The direct positive correlation of the $Helios^+$ subset with T_{regs} suggested that this $Helios^+$ subset may be more preserved from thymic reconstitution or peripheral expansion as the frequencies of T_{regs} increase.

Consistent with a selective expansion or preservation in T_{regs}, the frequencies of T_{regs} ($r = 0{\cdot}382$, $P = 0{\cdot}003$) and $Helios^+$ subset ($r = 0{\cdot}101$, $P = 0{\cdot}442$) were not affected negatively by increased viraemia in contrast to $CD4^+$ cells ($r = -0{\cdot}382$, $P = 0{\cdot}003$) (Fig. 3). Similarly, absolute numbers of T_{regs} ($r = -0{\cdot}286$, $P = 0{\cdot}027$) and $Helios^+$ ($r = -0{\cdot}221$, $P = 0{\cdot}09$) were less affected by viraemia compared to $CD4^+$ cells ($r = -0{\cdot}420$, $P < 0{\cdot}001$). These results suggested that T_{regs} and their $Helios^+$ subset had relatively increased survival and/or expansion during viraemia and CD4 depletion.

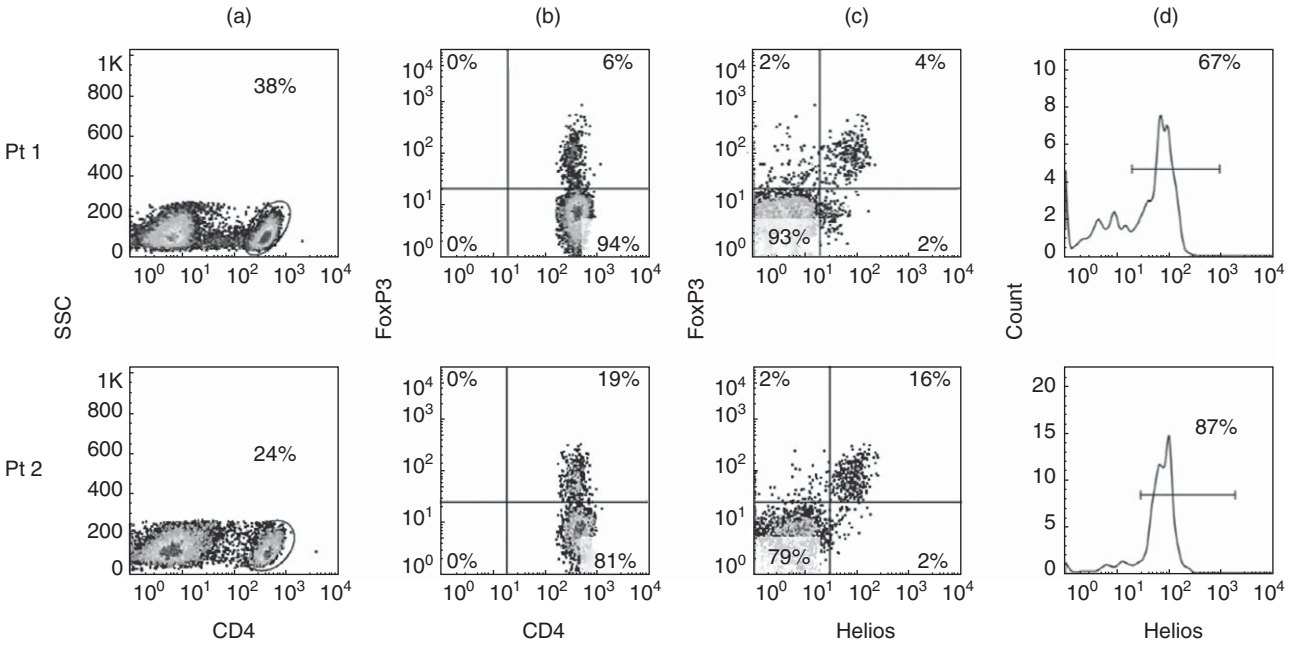

Fig. 1. Gating strategy for regulatory T cells (T_{regs}) and Helios subsets. Representative fluorescence activated cell sorter (FACS) plots of two patients showing (a) the gating for CD4 within lymphocyte gate; (b) forkhead box protein 3 (FoxP3) expression within CD4; (c) correlation of FoxP3 and Helios within CD4; and (d) percentage of Helios within $CD4^+FoxP3^+$ T_{regs}.

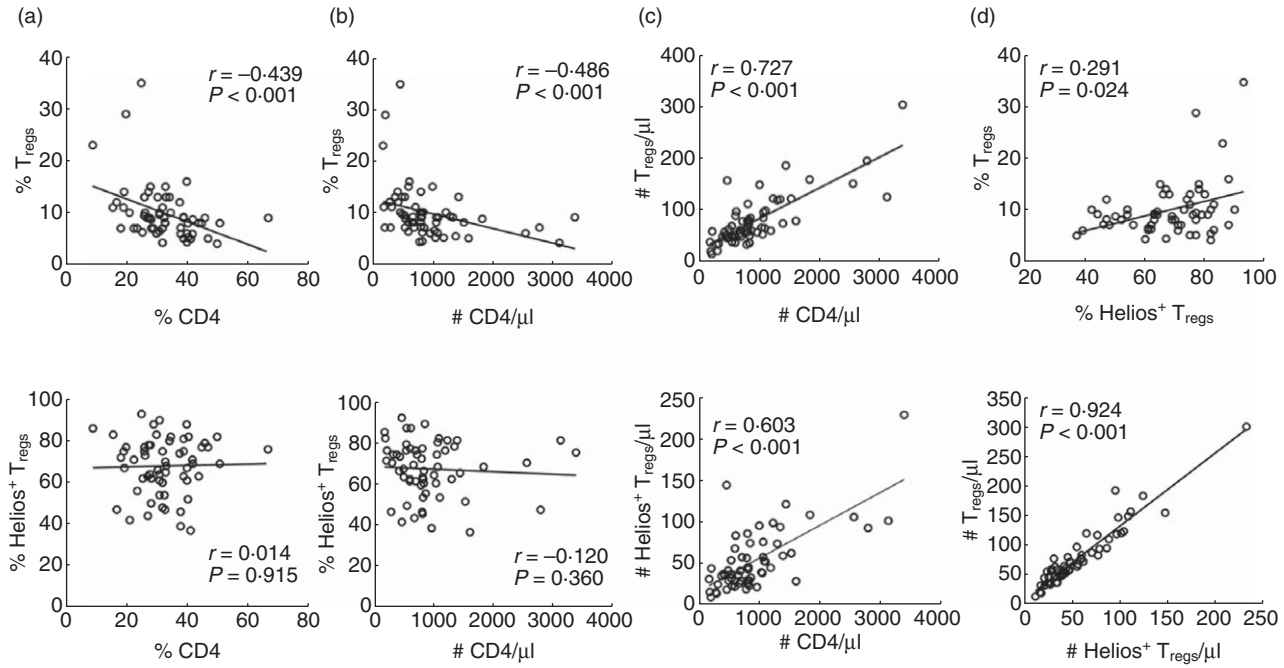

Fig. 2. Preservation of regulatory T cells (T_{regs}) and their Helios$^+$ subset in paediatric HIV. Correlation of the frequencies of T_{regs} and their Helios$^+$ subset with (a) the frequencies and (b) numbers of CD4. (c) Numbers of T_{regs} and their Helios$^+$ subset *versus* CD4 numbers. (d) Frequencies and numbers of T_{regs} *versus* Helios$^+$ subset; $n = 60$.

Effect of age on T_{regs} and their Helios$^+$ subset

While the frequencies and numbers of CD4 declined with age, there was no significant change for the percentage of T_{regs} and the Helios$^+$ subset ($r = 0{\cdot}168$, $P = 0{\cdot}202$ and $r = -0{\cdot}066$, $P = 0{\cdot}617$) (Fig. 4). However, the absolute numbers of T_{regs} and the Helios$^+$ subset showed a significant negative correlation to age ($r = -0{\cdot}610$, $r = -0{\cdot}571$, $P < 0{\cdot}001$, respectively), indicating a decline in their numbers with increasing age, even in those groups with good and intermediate clinical status (data not shown).

T_{regs} were increased during immune activation

The proportion of T_{regs} was correlated significantly and positively with total CD8$^+$ ($r = 0{\cdot}452$, $P < 0{\cdot}001$) and CD8$^+$ subsets of immune activation markers, CD38 ($r = 0{\cdot}462$, $P < 0{\cdot}001$) and HLA-DR ($r = 0{\cdot}459$, $P < 0{\cdot}001$), suggesting the preservation of T_{regs} in the face of increasing immune activation and destruction with progressive disease (Fig. 5a–c). This relationship was also kept for absolute numbers of T_{regs} *versus* CD8$^+$ and *versus* CD38$^+$ ($r = 0{\cdot}495$, $r = 0{\cdot}472$. $P < 0{\cdot}001$), but not HLA-DR$^+$ ($r = 0{\cdot}037$, $P = 0{\cdot}798$). It is

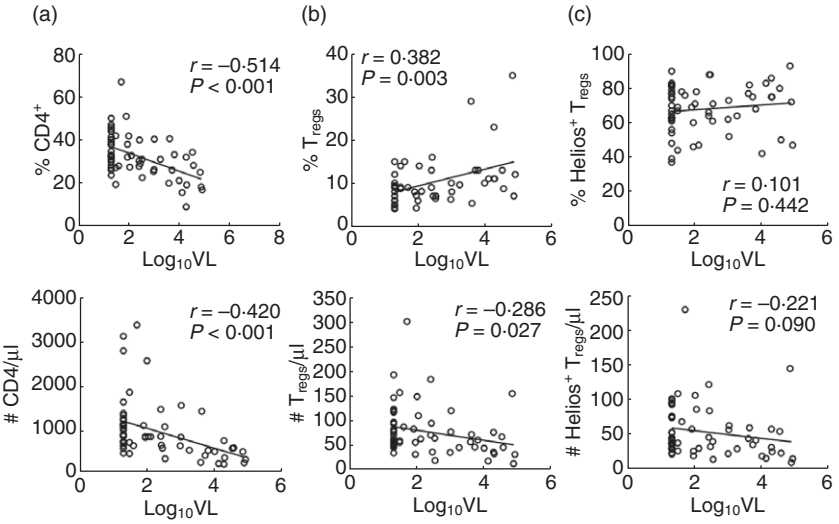

Fig. 3. Preservation of regulatory T cells (T_{regs}) and Helios$^+$ subset during viraemia. Correlation of percentages and numbers of (a) total CD4, (b) T_{regs} and (c) Helios$^+$ subset with viral load; $n = 60$.

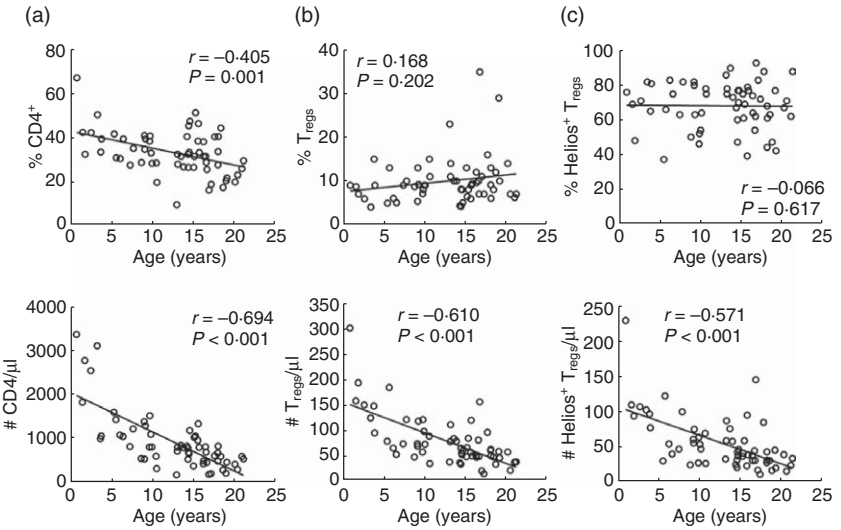

Fig. 4. Percentages of regulatory T cells (T_{regs}) and Helios$^+$ subset are unaffected by age. The frequencies and numbers of (a) total CD4, (b) T_{regs} and (c) Helios$^+$ subset with age; $n = 60$.

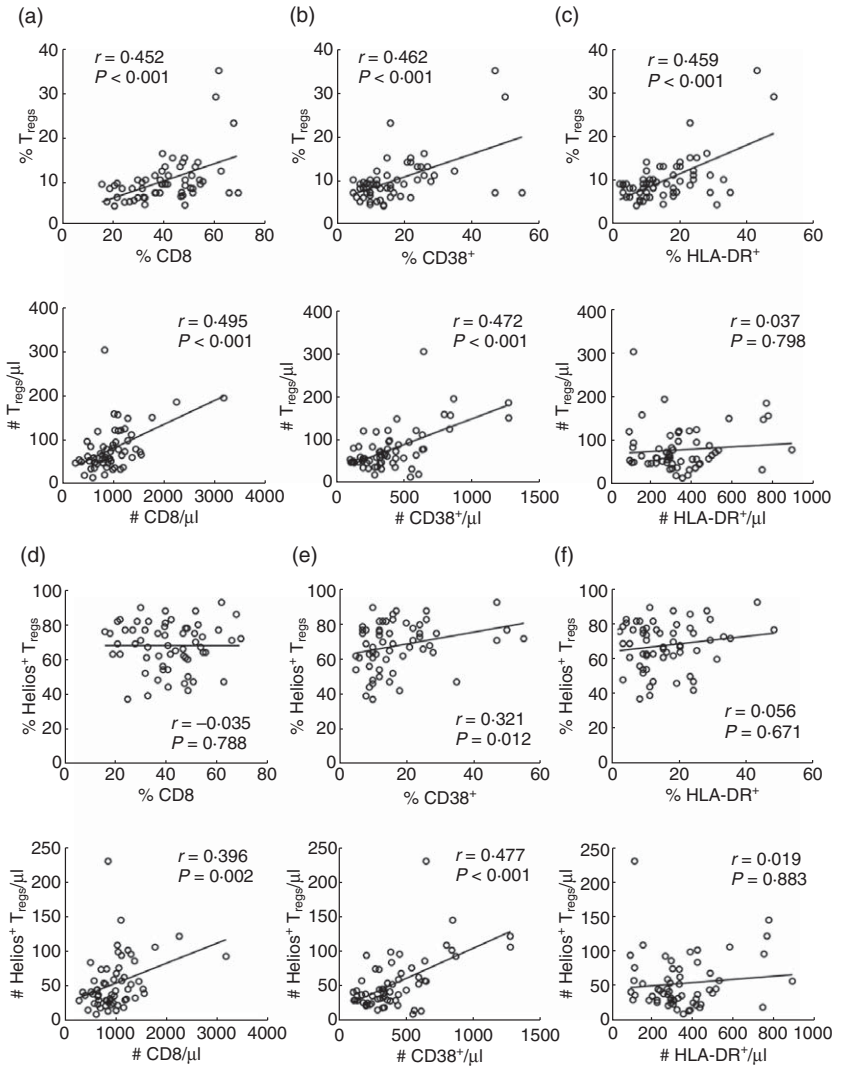

Fig. 5. Increasing regulatory T cells (T_{regs}) with immune activation. The frequencies and numbers of T_{regs} (a–c) and Helios$^+$ subset (d–f) *versus* total CD8, CD8$^+$CD38$^+$ and CD8$^+$ human leucocyte antigen D-related (HLA-DR)$^+$; $n = 60$.

unclear whether the T_{regs} were responding or reacting to the immune activation and whether, in their absence, the immune response would be more fulminant.

While the proportion of Helios$^+$ T_{regs} was correlated significantly and positively with CD38$^+$ ($r = 0{\cdot}321$, $P = 0{\cdot}012$), there was no correlation with proportions of total CD8$^+$ or HLA-DR$^+$ (Fig. 5d–f). Absolute numbers of Helios$^+$ T_{regs} were correlated significantly and positively with CD8 ($r = 0{\cdot}396$, $P = 0{\cdot}002$) and CD38 counts ($r = 0{\cdot}477$, $P < 0{\cdot}001$).

Selective preservation of T_{regs} and their Helios$^+$ subset in the poor HIV category group

We next segregated our HIV cohort into three categories based on their CD4 and VL to examine the T_{regs} and their Helios$^+$ subset. As expected, patients in the defined poor clinical status group had significantly increased percentages of CD8$^+$ and CD8$^+$CD38$^+$ and both percentages and numbers of CD8$^+$HLA-DR$^+$ compared to patients in the good clinical status group (Table 1). These results support what is observed with immune dysregulation in poorly controlled or progressive HIV [26,27]. When examining the ratio of T_{regs} and Helios$^+$ subsets to their immune activation, the poor group had the lowest ratios to CD8, CD38 and HLA-DR, suggesting possible compromised immune regulation from the T_{regs} (Supporting information, Fig. S1).

T_{regs} in the poor clinical status group had significantly higher percentages than did the good group, but their numbers were lower (Fig. 6a). Non-parametric tests were used to compare groups in order to avoid the over-influence of outliers seen in the skewed data. The percentages of the Helios$^+$ subset were similar among the three groups, although the absolute numbers were lowest in the poor group (Fig. 6b). In support of a preferential survival or expansion of T_{regs} and their Helios$^+$ subset with increasing viraemia and CD4 depletion, there were higher T_{regs} and Helios$^+$ subset : CD4 ratios in the poor group (Fig. 6c,d).

Discussion

The outcome of this prospective cross-sectional study in a paediatric perinatal HIV-infected population revealed that while T_{regs} are susceptible to HIV-induced destruction based on reduced cell numbers, they have a higher preservation relative to CD4 decline associated with HIV viraemia. This selective conservation maintained a similar proportion of Helios$^+$ and Helios$^-$ subsets within T_{regs}. However, within the poor group that had CD4 lymphopenia and high VL, there is evidence of greater preservation of the Helios$^+$ T_{regs} within CD4$^+$ T cells. The mechanisms responsible for changes in T_{reg} numbers and precentages are not fully known, including the contribution of VL. The increased T_{regs} could be due to one or more of these possibilities: better survival, greater expansion, thymic reconstitution and/or *de-novo* generation. It is possible that the Helios$^+$ T_{regs} were more susceptible to HIV-induced death, but their loss was offset by thymic reconstitution. Alternatively, this increased T_{reg} proportion might have been contributed by generated pT_{regs} that had up-regulated FoxP3, with some expressing Helios [28,29]. It would be interesting to investigate whether this observation is similar or different in the adult HIV$^+$ population, where thymic function is less preserved. The small paediatric blood volume and the lack of a reliable cell

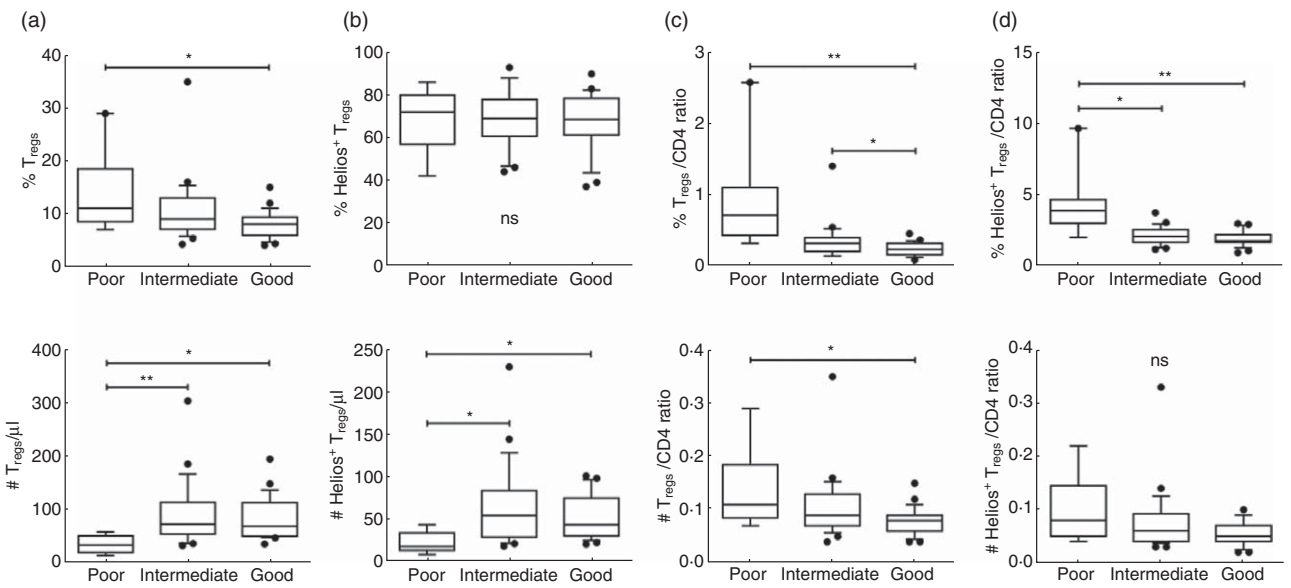

Fig. 6. Preservation of regulatory T cells (T_{regs}) and Helios$^+$ subset during CD4 lymphopenia and viraemia. The frequencies and numbers of (a) T_{regs} and (b) Helios$^+$ subset; and (c) T_{reg}/CD4 and (d) Helios$^+$ T_{reg}/CD4 ratios within the poor, intermediate and good HIV status groups. *$P < 0{\cdot}05$; **$P < 0{\cdot}001$.

surface marker that would allow for selective isolation of Helios$^+$ and Helios$^-$ subsets of T_{regs} limit the ability to examine their functions and T_{reg}-specific demethylation region (TSDR) that has been noted to be specific to bona fide, stable T_{regs} [30]. Moreover, it would be interesting to examine which Helios subsets might be more susceptible to HIV infection. Future longitudinal studies are needed to investigate T_{reg} expansion, survival and turnover with regard to viraemia.

In our study, we focused only on the total population of T_{regs} within CD4$^+$ T cells based on total FoxP3 expression and segregated the T_{regs} based on Helios expression. Nevertheless, our findings are similar to previous studies, with some using additional markers for their T_{reg} quantification, such as CD25 and CD45RA [20,31–33]. As T_{reg} numbers decrease with CD4 depletion, their percentages increase within the CD4$^+$. This observation is most evident in the chronically infected viraemic patients. When the patients are better controlled with good CD4 counts and undetectable HIV, their T_{reg} percentages decrease to within the reported 5–10% in healthy donors [34]. Even though our patient population was heterogeneous we were able to find correlations and differences, indicating the significance of our findings.

Although T_{regs} are critical for regulating immune homeostasis and activation it appears that, at a certain threshold, they lose control. While there was a selective increase in the proportion of T_{regs} during CD4 lymphopenia and HIV viraemia, there was also an increase in CD8$^+$ expansion and activation based on up-regulation of CD38 and HLA-DR within CD8$^+$ T cells. This increased turnover of T_{regs} correlated with hyperactivation and disease progression, as demonstrated by another study [35]. The functions that T_{regs} and their Helios subsets were playing in this setting were unclear at this point. While their numbers were low, they might be selectively preserved to prevent or control the clinical manifestation of autoimmunity and inflammation. A recent study revealed that, in their HIV group with CD4 <15% that had low T_{reg} numbers but increased frequencies, there was increased autoantibody production but no clinical autoimmune diseases [36]. One possibility is that T_{regs} negotiate for the host survival by accepting HIV chronicity and progression. It is currently not possible to test whether, in their absence, the HIV disease course would be better or worse. It would be interesting to investigate whether immunotherapy with *ex-vivo* expanded autologous T_{regs} would be beneficial in patients with HIV progression.

In conclusion, our study is the first to characterize the Helios subsets of T_{regs} in the paediatric population with perinatal-acquired HIV infection. Within the T_{reg} population, the percentage of Helios$^+$ subset does not fluctuate with CD4 depletion or HIV viraemia but appears to have selective preservation. Future studies will examine why some patients have Helios$^+$ T_{regs} below 50%, while others are above 80%. A longitudinal study will contribute to a better understanding of the role of T_{regs} and the Helios subsets.

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Disclosures

The authors have no financial conflicts of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Difference in the ratio of regulatory T cells (T_{reg}) and Helios$^+$ T_{reg} to (a) CD8, (b) CD8$^+$CD38$^+$ and (c) CD8$^+$human leucocyte antigen D-related (HLA-DR)$^+$ within the poor, intermediate and good HIV status groups. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}001$.