DOI: 10.1111/hiv.12751

HIV Medicine (2019), **20**, 513–522

ORIGINAL RESEARCH

Increased monocyte activation with age among HIV-infected long term non-progressor children: implications for early treatment initiation

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Objectives

The key to newer therapeutic and eradication approaches often lies in understanding slow disease progression in HIV infection. The paediatric population has been poorly studied in this regard. We aimed to describe a cohort of perinatally infected long-term nonprogressor (LTNP) children living with HIV in India and to evaluate the immune biomarkers of disease progression.

Methods

LTNPs (ART-naïve, with a CD4 count ≥ 500 cells/μL at age ≥ 7 years) among the cohort of HIV-infected children were identified and monitored longitudinally, and their CD4 T-cell counts and plasma viral loads were measured every 6 months. The plasma monocyte/macrophage activation markers, namely soluble CD14 (sCD14), soluble CD163 (sCD163) and interferon-inducible protein-10 (IP-10) were measured by enzyme-linked immunosorbent assay (ELISA) in LTNPs and progressors. The Mann–Whitney *U*-test was used to compare the two groups and *P* values < 0.05 were considered statistically significant. Spearman's rank or Pearson's correlation coefficient (*r*) was calculated to determine the associations between variables.

Results

Among 378 children living with HIV-1 surveyed in our cohort, 40 (10.6%) were LTNPs. Longitudinal analysis of the LTNP data showed that both CD4 count and viral load declined significantly with age ($P < 0.0001$ for both). Plasma sCD14 levels were significantly ($P < 0.005$) higher in progressors and sCD163 levels were significantly ($P < 0.0001$) higher in LTNPs.

Conclusions

The prevalence of LTNPs in our cohort of perinatally infected children living with HIV was 10.6%. We observed a trend for associations between the increasing sCD163 monocyte/macrophage activation marker levels, declining CD4 counts and the gradual loss of nonprogressor status with age in the LTNPs. These findings underscore the need for early antiretroviral therapy in those children with proven slow disease progression.

Keywords: children, HIV-1, immune biomarkers, long-term nonprogressors, monocyte activation

Accepted 13 March 2019

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Introduction

Globally, 36.9 million people are living with HIV; among them, 2.1 million are children below the age of 15 years [1]. In India, 2.11 million people living with HIV were registered up to March 2015; of these, 137 994 (6.54%) were children under the age of 15 years [2]. The HIV

epidemic in India is attributed mainly to HIV-1 subtype C, the viral clade that accounts for approximately half of all global infections [3,4]. Multiple lines of evidence suggest that subtype C may have greater replicative fitness than other subtypes [5], with a greater magnitude of transcription [6] and high transmissibility [7,8].

Long-term nonprogressors (LTNPs) are defined as children with perinatally acquired HIV-1 infection who are antiretroviral therapy (ART) naïve, maintain normal-for-age CD4 counts ($\geq$ 500 cells/μL at age $\geq$ 7 years) and remain free of manifestations of disease progression. Several large paediatric LTNP cohorts similar to our cohort have been described in South Africa/Durban [9,10], Uganda [11], Thailand and Cambodia [12], France and seven other European countries [13], Brazil [14], Puerto Rico and the USA [15]. In India, there are limited reports on paediatric LTNP cohorts and analysis of their immune biomarkers [16–18].

Host genetics, immune markers and viral factors play a role in nonprogression, yet these components fail to provide a comprehensive explanation. In paediatric HIV infection, despite successful treatment with ART, there is persistent elevated immune activation directly linked to disease progression. The systemic immune activation levels in LTNPs are lower than in progressors, but remain significantly higher compared with uninfected individuals irrespective of their viral loads [19,20]. Hence, in the long run, understanding the mechanism of slow disease progression and its effects on the developing immune system by studying LTNPs is of particular importance. Additionally, evaluating the key biomarkers of immune activation and disease progression is fundamental in view of their association with an increased incidence of non-AIDS-related mortality and morbidities [21–23]. Studies have implicated monocyte and macrophage-related inflammatory biomarkers such as soluble CD14 (sCD14), soluble CD163 (sCD163) and interferon-inducible protein-10 (IP-10) as predictors and probable causes of disease progression in addition to viral load and CD4 count [24–26].

We first identified children with this rare LTNP phenotype, with no clinical progression to AIDS for the first one or two decades of life, among the overall cohort of children living with HIV-1 subtype C. Current implementation of the new protocol for universal treatment of LTNPs may preclude elucidation of the immune mechanism and viral control in this group, and hence we found that samples collected prior to ART initiation were of most value for our purpose. Moreover, non-AIDS-related events, including those involving the cardiovascular and central nervous systems and cancers, were frequently described in the LTNPs/controllers with no relevant clinical signs readily detected by the patient or the clinician. Hence, after conducting a literature review, we determined that sCD14, sCD163 and IP-10 were suitable biomarkers of immune activation and, in order to elucidate disease progression trends, we compared levels of these plasma biomarkers in paediatric LTNPs and progressors using cross-sectional analysis.

Methods

Study population

Since 2010, we have maintained a clinical cohort and sample biorepository of perinatally infected children aged between 1 month and 18 years at St John's Hospital, Bangalore, India [17,27,28]. All LTNPs selected for this study had been followed in the cohort for a minimum of 7 years, with at least eight longitudinal consecutive blood samples available up to March 2017 for confirmation of stable CD4 counts. To allow comparison of their immune biomarkers with those of progressors, we also included ART-experienced progressors in the study. Progressors were defined as those who manifested HIV-related clinical features at World Health Organization (WHO) stage 3 or 4, and/or demonstrated rapid deterioration of CD4 count to < 350 cells/μL prior to ART initiation. Among the cohort, we randomly picked those progressors who were > 2 years of age and had at least eight longitudinal consecutive blood samples available up to March 2017. Those younger than 2 years were excluded as their immune system was thought to be relatively immature [29–31]. Biomarker assays were performed on the most recent sample from LTNPs and on a sample obtained from progressors at least 1 year after ART initiation. At the time of sampling, all LTNPs were ART-naïve as the treatment guidelines were reflective of CD4 counts. At the time of writing, all of these paediatric LTNPs have been initiated on ART as per the revised Indian national guidelines (April 2017).

Ethics statement

Written informed consent was obtained from all patients and/or their primary caregivers. All the investigations were conducted with the approval of the institutional ethical review board of St John's Hospital, Bangalore (IRB No: 32/2012).

Viral load and CD4 T-cell counts

A peripheral blood sample was obtained from each study subject during a routine clinic visit every 6 months. Plasma HIV-1 RNA was measured by real-time

polymerase chain reaction using a sensitive quantitative HIV-1 RNA assay (Abbott m2000rt system; Abbott Molecular Diagnostics, Wiesbaden, Germany) with a detection limit of 50 HIV-1 RNA copies/mL. CD4 count was measured with the dual-platform flow cytometer FACS Calibur (Becton-Dickinson Biosciences, San Jose, CA).

Quantification of immune biomarkers

Plasma samples were obtained from blood collected in EDTA tubes. After centrifugation (800 × *g* for 10 min), plasma was separated and stored at −80°C. The soluble immune activation markers sCD14, sCD163 and IP-10 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN). Plasma was diluted 1:200 for sCD14, 1:10 for sCD163 and 1:10 for IP-10. The reported minimum detectable doses of sCD14, sCD163 and IP-10 were 0.125 ng/mL, 0.177 ng/mL and 1.67 pg/mL respectively.

Statistical analysis

Data were expressed as the median value [with interquartile range (IQR)]. The Mann–Whitney *U*-test was used to compare the two groups and Spearman's rank or Pearson's correlation coefficient (*r*) was calculated to determine the associations between the variables. P values < 0.05 were considered statistically significant. Analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

Results

Of the initial cohort of 378 children living with HIV-1 in 2010, there were 71 children who could not be included because of loss to follow-up (34; 9.0%), transfer out (22; 5.8%) or death (15; 4.0%). Among the remaining 307 HIV-1-infected children in the cohort as of March 2017, we identified 40 children who fulfilled the LTNP definition, indicating a prevalence of 10.6% (40/378). Longitudinal analysis of the LTNP data indicated significant CD4 count and viral

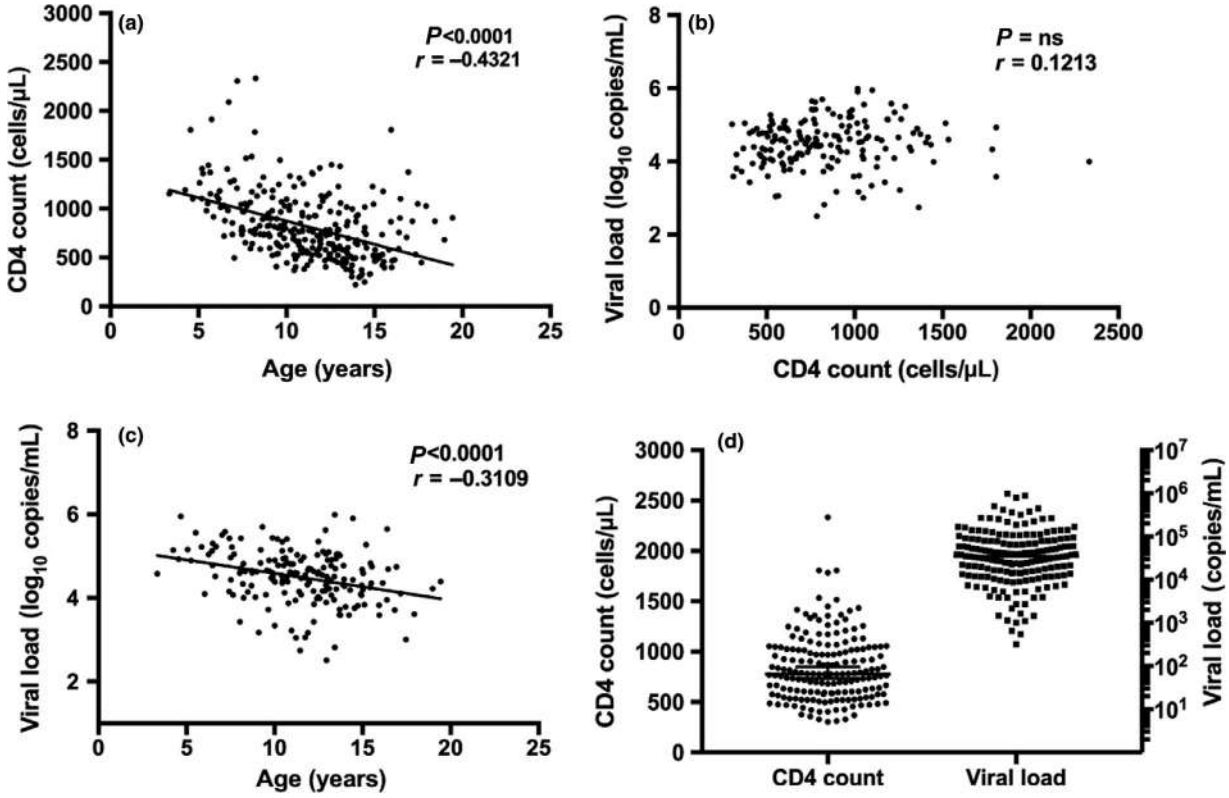

Fig. 1 Analysis of longitudinal data for 40 long-term nonprogressors (LTNPs) over a period of 18 years. (a, c) Longitudinal decline of the CD4 count and viral load with age in the LTNPs; (b) lack of correlation between CD4 count and viral load in LTNPs; (d) Longitudinal CD4+ T cell count and viral load in the LTNPs. ns, not significant.

load declines with age ($P < 0.0001$ for both) (Fig. 1a,c) and no correlation was observed between the CD4 count and viral load (Fig. 1b). Longitudinal CD4 count and viral load data for these 40 LTNPs indicated median values of 779.5 cells/μL (IQR 587.3–1039 cells/μL) and 34 813 copies/mL (IQR 13 038–80 873 copies/mL), respectively (Fig. 1d).

Clinical and demographic characteristics

The demographics and laboratory parameters of the LTNPs and progressors included in the immune biomarker analysis are summarized in Table 1. LTNPs were older than progressors, with median ages of 10.9 and 4.6 years, respectively, at the time of sampling. Opportunistic infections (OIs) were common at the time of diagnosis of HIV infection and prior to treatment initiation in our paediatric cohort—the most common OIs were oral candidiasis, moluscum contagiosum skin infections, pyoderma and tuberculosis. LTNPs typically did not manifest any OIs. ART was not initiated in LTNPs in accordance with the existing national guidelines for treatment at the time of sample collection (before April 2017), which was only based on clinical stage and CD4 threshold and not viral load. The progressors were on recommended ART regimens [two nucleoside reverse transcriptase inhibitors (NRTIs) + one nonnucleoside reverse transcriptase inhibitor (NNRTI) or two NRTIs + one protease inhibitor (PI)] and adherence details are shown in Supporting Information - Data S1. Adherence to therapy among the progressors was 100% in 29 children, 95–99% in nine children, and 80–90% in the remaining two children. Both the groups had normal-for-age CD4 counts and CD4 percentages (CD4%). LTNPs had high levels of HIV-1 RNA in plasma (median 38 119 copies/mL).

Immune biomarker levels in LTNPs and progressors

kPlasma sCD14 levels were lower in the LTNPs compared with the progressors, with a median value of 2982 ng/mL (IQR 2794–3235 ng/mL) for LTNPs and 3372 ng/mL (IQR 2892–3617 ng/mL) for progressors ($P < 0.005$). The levels of sCD163 were significantly higher in the LTNPs than in the progressors, with a median value of 1535 ng/mL (IQR 1183–1789 ng/mL) in the LTNPs and 1058 ng/mL (IQR 830–1423 ng/mL) in the progressors ($P < 0.0001$). There was no statistically significant difference in plasma levels of IP-10 between the two groups, with a median value of 59.61 pg/mL (IQR 38.56–113.3 pg/mL) in the LTNPs and 76.39 pg/mL (IQR 37.81–134.9 pg/mL) in the progressors (Fig. 2).

Table 1 Demographic and laboratory parameters of long-term nonprogressor (LTNP) and progressor groups

Parameter	LTNPs ($n = 40$)	Progressors ($n = 40$)
Age (years) [median (IQR)]	10.9 (8.85–12.43)	4.62 (3.55–6.0)
Sex [*n* (%)]		
Male	20 (50)	26 (65)
Female	20 (50)	14 (35)
CD4 count (cells/uL) [median (IQR)]	780 (686–960)	1283 (760–2025)
CD4% [median (IQR)]	24.5 (20.25–29.75)	32.5 (26.25–38.75)
HIV RNA (copies/mL) [median (IQR)]	38 119 (16 419–794 574)	<50 (<50–150)

IQR, interquartile range.

Correlation between immune biomarkers and age and viral load

Among LTNPs, sCD14 levels showed a negative correlation with age ($r = -0.298$; $P < 0.001$) and sCD163 levels showed a strong positive correlation with age ($r = 0.443$; $P < 0.0001$) (Fig. 3). There was no significant correlation between immune biomarker levels and the viral load. As expected, we also observed a negative correlation of CD4 count and CD4% with age ($P < 0.0001$ and $P = 0.0008$, respectively) (Fig. 3).

Discussion

Our finding that over a tenth of children living with HIV were likely to have slow disease progression indicates a significant prevalence of LTNPs in this cohort. The prevalence rates of paediatric LTNPs previously reported across the globe have varied between 5% and 24%, possibly as a result of the variable criteria used to define LTNPs, different cohort sizes and different populations included. The prevalence of paediatric LTNPs was 5–10% in South Africa [9], 13.6% in US cohorts [32,33], and 24% in cohorts from France and seven other European countries [13]. Our LTNP cohort had a median viral load of 34 813 copies/mL (IQR 13 038–80 873 copies/mL), whereas the median viral loads were 26 000 copies/mL in South Africa [10], 367 854 copies/mL in Brazil [14] and 118 716 copies/mL in the USA [32]. Interestingly, we observed a significant decline in the viral load with age over a period of 18 years ($P < 0.0001$) (Fig. 1c), possibly as a consequence of viral adaptation within the host [34]. Although sustained high CD4 counts are typically found in association with low viral loads in ART-naïve adult nonprogressors [35], in our study in paediatric LTNPs, we found no association between absolute CD4 count and viral load. Our findings of no correlation of viral load and CD4 count in LTNPs, and lower plasma sCD14 levels in LTNPs are consistent

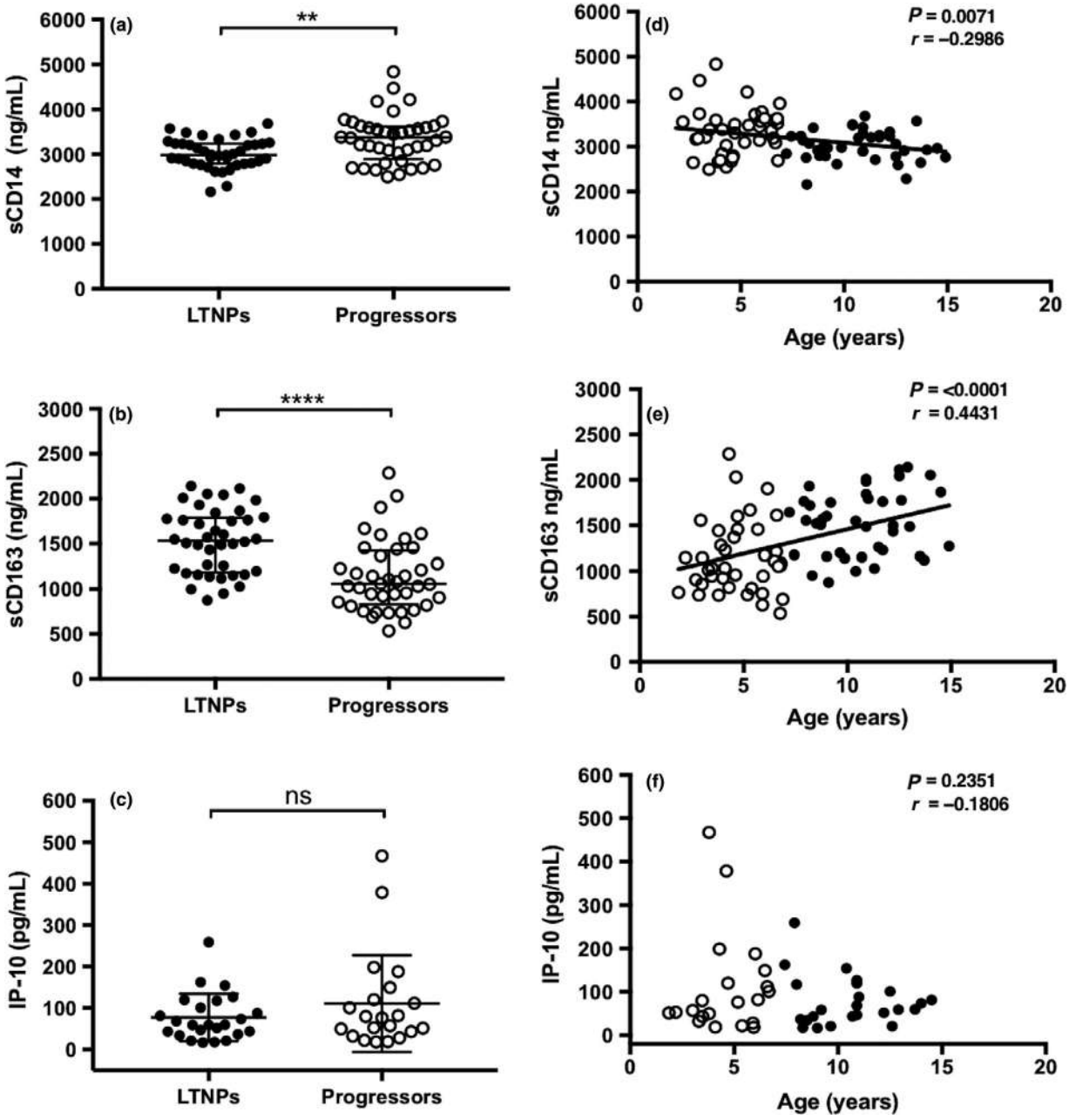

Fig. 2 Plasma levels of immune activation biomarkers and their correlation with age. (a) Soluble CD14 (sCD14); (b) soluble CD163 (sCD163); (c) interferon-inducible protein-10 (IP-10); (d) correlation between sCD14 and age ($r = -0.298$; $P = 0.007$); (e) correlation between sCD163 and age ($r = 0.443$; $P < 0.0001$) and (f) correlation between IP-10 and age ($r = -0.1806$; $P = 0.2351$). This bivariate correlation was performed using Pearson/Spearman rank correlation analysis. Filled circles represent the long-term nonprogressors (LTNPs) and open circles the progressors. Each filled/open circle represents one subject. Significant differences in each biomarker between the LTNP and progressor groups are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). ns, not significant.

with the findings of a larger cohort of paediatric LTNPs ($n = 275$) from Durban [10].

Although the subject has not been well studied, there are reports indicating higher levels of naïve cells in slow progressors and LTNPs compared with progressors [36,37]. The immune response in LTNPs and adult controllers may be mainly attributable to the immune homeostasis that is resistant to HIV and an immune response leading to viral control [38], but the mechanism is still not clearly understood. Paediatric LTNPs show strong

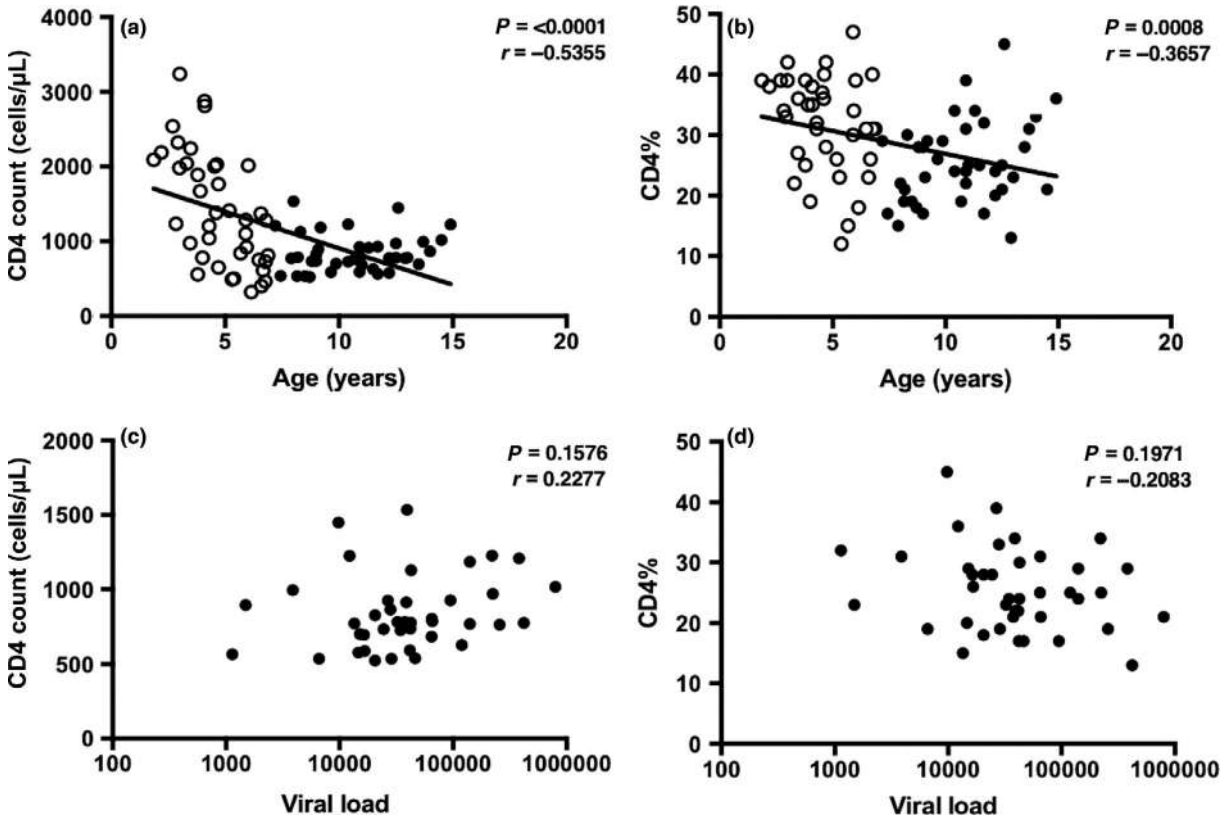

Fig. 3 Correlation analysis for laboratory parameters in long-term nonprogressor (LTNP) and progressor samples used for immune biomarker analysis. (a) Correlation between CD4 count and age; (b) correlation between CD4% and age; (c) correlation between CD4 count and viral load (only LTNPs); (d) correlation between CD4% and viral load (only LTNPs). Regression lines are shown only for significantly correlated variables. Filled circles represent the LTNPs and open circles the progressors. Each filled/open circle represents one subject.

virus-specific immune responses, decreased C-C chemokine receptor type 5 (CCR5) expression on long-lived central memory CD4 cells, and low levels of infection (viral reservoirs) in long-lived central memory (Tcm) and stem cell memory (Tscm) CD4 T cells [10,39]. However, there are several reports of gradual loss of the nonprogressor status in LTNPs being observed with declining CD4 cell count, which may be attributable to reduced thymic output with age as well as possibly slow disease progression. This corresponds well with our finding that CD4 counts gradually began to drop with age, starting at around 13–14 years of age (Fig. 1a). Therefore, understanding the mechanisms of this long-term maintenance of normal immunology in LTNPs, with low immune activation particularly during the early years of life, is of prime importance in preventing the growing burden of non-AIDS-associated morbidities and mortality in children. The role played by cytokines, chemokines and markers of immune activation in paediatric LTNPs has not been well defined, even in the context of the current new ART era test-and-treat policy for all HIV-positive individuals. Our assessment of the levels of immune biomarkers sCD14, sCD163 and IP-10 in LTNPs and progressors showed that significantly lower levels of sCD14 occurred in LTNPs, indicating limited microbial translocation. Muenchhoff *et al.* showed that paediatric LTNPs from Durban had significantly lower plasma sCD14 levels than progressors, which was consistent with the lower plasma intestinal fatty-acid binding protein levels in these children [10]. These data are also consistent with those for simian immunodeficiency virus (SIV) infection in sooty mangabeys, which have persistent high viral loads, like these LTNP children, and low levels of plasma sCD14 [40]. However, the higher level of sCD163 in our LTNP children is suggestive of a possible risk of developing non-AIDS-associated complications and of CD4 count decline with age in the near future when left untreated [41,42].

Assays used for detecting microbial translocation during HIV infection using other markers such as lipopolysaccharide (LPS), lipopolysaccharide-binding protein, lipoteichoic acid and endotoxin core antibody (EndoCAb) are

contentious as a consequence of their divergent, inconsistent results in comparison to immune activation and/or clinical outcome [40,43–47]. Considering the challenges in reproducibility, contamination and heterogeneity in the previously reported studies [45,47,48], we did not use LPS and instead focused on comparing systemic monocyte/macrophage activation markers in LTNPs and progressor children in India. An elevated plasma level of sCD14 is a strong predictor of unfavourable prognosis in HIV infection [48,49] and moderately increased levels persist in HIV-infected nonprogressors and also in patients on long-term ART with controlled viral replication [50]. In our study, we observed that plasma sCD14 levels were significantly lower in LTNPs, indicating relatively restored integrity of the mucosal barrier, potentially limited microbial translocation and possibly reduced immune activation in the LTNPs. We also observed the highest sCD14 levels in the youngest children between 1.86 and 2.3 years old (average value of 4494.66 ng/mL). The sCD14 marker showed a significant negative correlation with age, indicating an impact of age on measures of microbial translocation in young children irrespective of therapy status. This further emphasizes the influence of age on sCD14 levels, as a consequence of the physiological increase in microbial translocation in neonates and the effect of ongoing transition of the gut microbiota (as a result of antibiotic prophylaxis, change in diet, probiotic usage, rotavirus vaccination, etc.) since birth on overall immune activation [44,51,52]. These reports are supported by our current findings, where sCD14 levels were highest in the youngest children and lower in older children.

A limitation of the study is that there are other likely drivers of immune activation that are more important than microbial translocation [10,42], including HIV itself, via proteins such as Negative Regulatory Factor (Nef) and Envelope Glycoprotein 120 (gp120) [53,54], malnutrition [55], and coinfections (both acute and chronic) such as cytomegalovirus infection, malaria, tuberculosis, *Candida* infection, herpes zoster and visceral leishmaniasis [56–58].

We observed significantly higher levels of sCD163 in LTNPs than in progressors, reflecting macrophage activation [59,60]. Lower plasma levels of sCD163 indicate delayed or negligible non-AIDS-related comorbidities [61–63]. In our study, the plasma sCD163 levels showed a strong positive correlation with age, indicating immunological aging in HIV-infected children leading to the gradual development of non-AIDS-related pathologies associated with age. This may be attributable to the exhaustion of lymphopoietic capacity during the course of infection, which eventually affects all the compartments of the immune system [64].

IP-10 [C-X-C motif chemokine 10 (CXCL10)] is highly expressed in monocytes and mature dendritic cells, and helps to recruit stimulated natural killer cells, CD4 T cells and monocytes to the target site [65,66]. IP-10 is rapidly produced at high levels during the acute phase of HIV infection, and a high IP-10 level is associated with a low CD4 count during early infection [67,68]. Elevated blood IP-10 levels in HIV infection are also associated with rapid disease progression, pre-ART (pre-AIDS) seropositivity, persistent immune activation and coronary atherosclerosis [41,42,69]. IP-10 levels detected in our study did not differ significantly between the LTNPs and progressors and were comparable with those found in several previous studies. Those studies found a similar range of plasma IP-10 levels in LTNPs, viraemic ART-naïve individuals and progressors on ART whose levels were close to those of healthy donors [41,58,70,71]. Moreover, except for four progressors and two LTNPs, the observed IP-10 levels in our cohort were below the recommended cut-off of 161 pg/mL, as this cut-off provided a sensitivity of 95.5% and specificity of 76.5% to detect acute HIV infection using the Luminex assay and ELISA [72]. IP-10 levels are significantly higher in the group of patients with greatly reduced CD4 counts (< 350 cells/μL) and normalization of IP-10 levels seems to depend on the CD4 count nadir at ART initiation [71]. Grinztejn and colleagues showed that better results are obtained when ART is initiated early and that it takes about 1–2 years for proinflammatory cytokine/chemokine levels to normalize after ART initiation [73]. Our progressor children had been on ART for a minimum of 1 year (median 1.46 years; IQR 1.0–1.55 years) and this could possibly be the reason that no change in IP-10 levels was observed in our study participants, which further suggests that IP-10 is an interesting marker of the general clinical state of HIV-positive patients.

Conclusions

This study described an Indian paediatric LTNP cohort that constituted a tenth of a cohort of children living with HIV. It provides further evidence that, although the immunology and disease progression of pediatric LTNPs are different from those of pediatric progressors in the early years, there are similarities in disease progression patters as age advances among the LTNPs. These observations support current guidelines that recommend starting ART in all children irrespective of clinical manifestations or disease markers. The study of the slow disease progression phenotype of LTNPs offers a chance to understand the critical mechanisms underlying the maintenance of normal immunology and low immune activation during

the early years of life despite high viraemia, which may open up new avenues of research in the area of paediatric HIV vaccines and therapeutics.

Acknowledgements

We thank and acknowledge all the children who participated in this study, paediatric cohort study nurse Ms Bincy Antony and the data collectors for their invaluable cooperation with and support of this study.

Financial disclosure: The study was supported by a Wellcome Trust/Department of Biotechnology India Alliance Senior Fellowship awarded to AS (grant number IA/S/13/2/501017) (http://www.wellcomedbt.org/).

Conflicts of interest: The authors declare that they have no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Data for long-term nonprogressors (LTNPs; perinatally infected, CD4 count > 500 cells/μL and age ≥ 7 years) and progressors (perinatally infected, > 1.0 year on ART and age ≥ 7 years).