Attenuation of immune activation in an open-label clinical trial for HIV–AIDS using a polyherbal formulation

Mangaiarkarasi Asokan · Vijaya Sachidanandam ·
Kadappa Shivappa Satish · Udaykumar Ranga

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Abstract To explain a stable clinical outcome observed in a previous pilot clinical trial using a polyherbal formulation (PHF) for HIV–AIDS, we, in the present study, evaluated the T cell functions from fresh and stored blood samples. In three clinical groups—the anti-retroviral therapy, PHF and control arms—we compared the circulating levels of lipopolysaccharide, LPS-binding protein, soluble CD14, aspartate transaminase (AST) and alanine transaminase (ALT). Additionally, we evaluated the expression of T cell markers and gag-specific immune responses. The PHF treatment significantly reduced the levels of sCD14, AST and ALT. In a cross-sectional analysis at 30 months post-treatment, in comparison to the control group, the PHF arm showed significantly low per-cell expression of PD1, CD95 and HLA-DR. The PHF treatment appears to have attenuated general immune activation and hepatic inflammation in the study participants. Targeting the mediators of immune activation must be pursued as a useful strategy for HIV–AIDS management.

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M. Asokan · U. Ranga (✉)
HIV–AIDS Laboratory, Molecular Biology and Genetics Unit,
Jawaharlal Nehru Centre for Advanced Scientific Research,
Jakkur, Bangalore 560 064, India
e-mail: udaykumar@jncasr.ac.in

V. Sachidanandam
Microbiology and Cell Biology Department, Indian Institute of
Science, Bangalore 560 012, India

K. S. Satish
Chest and Maternity Centre, Rajaji Nagar, Bangalore 560 010,
India

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Introduction

Chronic immune activation (CIA), the hallmark of viral pathogenesis in AIDS, underlies severe immune dysfunction and gradual CD4 T-cell loss in the infection with human immunodeficiency virus Type I (HIV-1). The CIA is characterized by high rates of T-cell turnover, activation-induced T-cell death and the presence of high levels of proinflammatory cytokines in the body fluids among others [22]. Although the causes of CIA in HIV–AIDS are not completely understood, two major factors, the viral products directly and the mucosal microbiota and their products indirectly, are believed to underlie this phenomenon. The activation of the Toll-like receptors by viral RNA or RNA–DNA hybrids, env-mediated signaling through the coreceptor molecules especially CXCR4 on the target cell and powerful immunomodulatory activities of the viral products such as Tat and Nef—all play a significant role in modulating CIA. The depletion of certain T-cell subsets, especially the Th17 CD4 T-cells, critical for maintaining the integrity of the mucosal barrier, especially during acute viremia, has a lasting and crippling impact on the immune system [16]. The loss of the Th17 lymphocyte subset in the gut in turn is believed to cause ‘microbial translocation’ which is the consequence of the compromised mucosal barrier [6]. The compromised mucosal barrier permits leaking of microbial products such as LPS into the systemic compartment leading to generalized systemic immune activation including the activation of various cell types such as the T-cells and monocytes [9]. The CIA also causes induced secretion of soluble CD14 (sCD14) into the

blood by activated monocytes. Importantly, sCD14, but not LPS itself, was recently found to be an independent predictor of mortality in AIDS [46].

The prognostic significance of CIA is superior even to that of the plasma viral load [15, 17, 23]. Although the significance of the microbial translocation for CIA is appreciated broadly, a few reports from Africa fail to detect a direct association between the microbial products in blood and the chronic immune activation in the viral infection [43, 44]. The model proposing an association between microbial translocation and disease progression in HIV–AIDS thus remains controversial. Furthermore, although intensified anti-retroviral therapy successfully controls the plasma viral load, the level of immune activation persists, though reduced to a great extent [9]. The residual immune activation in ART-treated subjects is indicative of an incomplete immune reconstitution in the gastrointestinal tract [29]. Furthermore, the residual immune activation is associated with several long-term sequelae which in turn may be associated with the continued leaking of the microbial products into the systemic compartment thus emphasizing on the significance of the association between microbial translocation and CIA in the viral infection. This observation is also suggestive that efficient management of the CIA, rather than suppressing the plasma viral load alone, is important for disease management.

Since the plasma viral load is an important factor contributing to disease progression [31, 51], an intervention strategy reducing the viral load should offer a significant clinical benefit. This assumption, however, was not realized when anti-retroviral therapy (ART) successfully controlled viral proliferation and reconstituted CD4 cells, but failed to reduce immune activation to normal levels [9, 12] probably as the residual viral proliferation was sufficient to ensure continued immune activation [7]. Despite several months of successful viral suppression by ART, the expression of the activation markers such as CD38 and HLA-DR on the T-cells was significantly higher as compared to healthy controls [19]. Only a partial immune reconstitution was observed in the gastrointestinal tract, despite 1–7 years of ART [30]. Furthermore, investigations with intensified highly active ART (HAART) were inconclusive where the viral load reduction was associated with a concomitant drop in the magnitude of the immune activation, although only to a moderate level [21]. Considering the failure of the suppressive anti-retroviral therapy to resolve the magnitude of the chronic immune activation to normal levels, attempts have been made to attenuate the residual immune activation using suppressive chemotherapy, either independently or in combination with HAART. A therapeutic strategy that could alleviate immune activation could confer significant clinical benefits in HIV–AIDS.

A few studies previously examined the influence of generic immune suppressive agents such as the glucocorticoids, cyclosporine A, mycophenolic acid, rapamycin, hydroxyurea, TNF inhibitors and chloroquine, thalidomide and others, to control CIA but without much success [9, 22]. With the advent of ART, the cytokine-mediated immune reconstitution was conceived as an option to improvise the treatment regimens. The common γ -chain family cytokines, IL-2, IL-7 and IL-15, IFN- α , GM-CSF, and growth hormone have been tested either alone or in conjunction with ART. The administration of IL-2 to more than 5,000 subjects in the ESPRIT and SILCAAT clinical trial, though improved the peripheral CD4 cell count, surprisingly, did not contribute to clinical benefit [1]. Recently, synthetic immunomodulators—including several statins, leflunomide, chloroquine, celecoxib etc.—have gained attention and are being tested in pilot clinical trials. The statins are potential candidates due to their anti-inflammatory and anti-viral activities in vitro although their efficacy against HIV–AIDS has been controversial. At least four different statins have been tested with the results varying from partial control of the plasma viral load to no changes in any of the biomarkers tested [8, 13, 32, 41, 49, 54, 55]. Other immunomodulatory agents such as leflunomide [42], chloroquine [33] and celecoxib [40] have also been tested with varying results on immune activation, the clinical significance of which needs to be ascertained.

Although natural products and polyherbal medicines are a promising source of immunomodulators, their potential has not been adequately explored [24, 38]. A large number of plants and herbal products have been evaluated for immunomodulatory properties both in vitro and in vivo [26] although clinical trials are limited. In a recent clinical trial from our laboratory, we reported a stabilized clinical profile in the arm of a polyherbal formulation (PHF) in 13 participants over a period of 24 months [2]. In the pilot clinical trial registered with the Clinical Trials Registry of India (CTRI/2008/091/000021), we evaluated a PHF, for its safety and efficacy in treating HIV–AIDS. As expected, the control HAART arm showed a significant improvement in the CD4 T-cell count ($154.4 \text{ cells}/\mu\text{l}/\text{year}$, $p < 0.001$) and suppression in the viral load ($-0.431 \pm 0.004 \log_{10} \text{ IU}/\text{month}$, $p < 0.001$, in 3–6 months). The clinical benefits observed in the PHF arm included the slow kinetics of CD4 T-cell reduction ($-14.3 \pm 6.1 \text{ cells}/\mu\text{l}/\text{year}$, $p = 0.021$), a stabilized, but not suppressed, plasma viral load ($-0.003 \pm 0.016 \log_{10} \text{ IU}/\text{ml}$, $p = 0.877$) and the absence of a significant difference in the incidence of AIDS-associated clinical manifestations between the PHF and HAART arms over the study period of 24 months. Furthermore, in the PHF arm, at month 1, we observed a statistically significant increase in the CD4 cell count and a concomitant decrease in the viral load suggesting

immunomodulation. These changes were, however, transient and returned to the basal level at month 3. Further analysis suggested a compartmental redistribution of the lymphocytes as the primary cause of the transient increase in the number of the T-cells in the periphery. While the PHF did not demonstrate anti-viral properties, a potential for immunomodulation could not be ruled out. Several technical limitations associated with the study design, however, imposed restrictions on drawing a meaningful interpretation from these observations. The present study was therefore undertaken to provide an immunological explanation for the apparent clinical stabilization observed in the clinical trial. We reasoned that if the PHF indeed improved the quality of life of the study participants, such a benefit was likely to be reflected in the improved quality of the T-cells and their functions. Nearly 6 months after the original clinical trial ended in August 2009, a fresh permission was obtained for the present study from the institutional bioethics committees. A single blood sample was collected from each of the study participants available at this time under both the arms of the study, PHF and HAART. We show that a minimum of four independent immune markers were significantly modulated in the PHF arm suggesting attenuation of the T-cell activation in this clinical group.

Materials and methods

Study participants

Permission for the present study was granted by two different ethics committees. The Institutional Ethics Committee of *Samraksha*, the NGO that monitored the original clinical trial, permitted collection of a fresh blood sample from the original participants of the trial. The permission to collect a single blood sample from volunteers that constituted additional groups of the present study was obtained from the *Bangalore Central Ethics Committee* of the *Chest and Maternity Centre*, Bangalore. All the samples were obtained with a written informed consent. We used a total of six different clinical groups in the present analysis of which three groups were derived from the original clinical trial—HAART ($n = 17$, CD4 cell count 862.1 ± 268.8), PHF ($n = 9$, 242.2 ± 54.3) and PHF-to-HAART ($n = 8$, 629.4 ± 149.0). Three additional control groups of fresh volunteers were added to the study. While two of the three groups consisted of drug-naïve seropositive subjects with the CD4 cell count matched to that of the PHF (low CD4, 245.9 ± 117.7 , $n = 13$), or the HAART (high CD4, 668.7 ± 106.1 , $n = 9$) groups, respectively. The sixth group consisted of healthy controls (control, $1,023.0 \pm 314.4$, $n = 13$). Although we performed the

Table 1 Baseline characteristics of the study participants used in the analysis of the chronic immune activation

Characteristic	Healthy	HAART	PHF	p^*
Sample size	23	12	12	–
Age (years)	29 (23–52)	36 (27–50)	34 (28–41)	0.351
Female gender	8 (35 %)	7 (58 %)	4 (33 %)	0.414
CD4 cell count (cells/ μ l)	1,074 (585–1,708)	236 (145–322)	245 (182–306)	0.660
Plasma viral load ($\log_{10}$ IU/ml)	Nd	4.4 (2.3–5.6)	4.7 (3.7–6.6)	0.479

The p value was determined using an unpaired t test or a Fisher's exact test. Data in parentheses are percent of number of participants or range of the mean values. *nd* not determined; * comparison between the HAART and PHF arms

Table 2 Baseline characteristics of the study participants used in the immunophenotyping analysis

Characteristic	ART-	HAART	PHF	p^*
Sample size	13	17	9	–
Age (years)	42 (14–63)	38 (29–52)	37 (31–48)	0.578
Female gender	5 (38 %)	9 (53 %)	2 (22 %)	0.217
CD4 cell count (cells/ μ l)	246 (95–421)	862 (230–1,531)	242 (177–341)	<0.001
Plasma viral load ($\log_{10}$ IU/ml)	4.5 (3.7–5.2)	nd ^a	5.1 (3.9–6.6)	–

The p value was determined using an unpaired t -test or a Fisher's exact test. Data in parentheses are percent of number of participants or range of the mean values. *nd* not detectable. ^aOne participant had unsuppressed viral load of 160,000 copies/ml. *comparison between the HAART and PHF arms

comparisons using all the 6 clinical groups in all the assays, data comparing only 3 of the primary groups (HAART, PHF and low CD4/ART-) have been presented as inclusion of the three additional groups of controls (the low CD4, the high CD4 and the control groups) did not add to the significance of the findings. The baseline characteristics of the study participants have been summarized (Tables 1, 2). The HAART and PHF groups were administered respective treatments originally between November 2006 and July 2007. A longitudinal analysis of the microbial translocation was performed on plasma samples stored during this period, corresponding to baseline, month-12 and month-24 post treatment initiation. The cross-sectional analyses of T-cell properties were performed on fresh blood samples collected between June 2009 and August 2010. While the subjects of the original HAART group continued to receive uninterrupted ART, those of the PHF group did not receive any additional treatment after the initial 4-month PHF administration as part of the original clinical trial. At the time of the present analysis, there was an average delay of 32.5 months for the PHF subjects between the original PHF administration and sample collection. The average time delay for the HAART group was 31.3 months, that is

Table 3 The four-color antibody panels used for the staining of the whole blood or the PBMC

Panel	FITC	PE	PerCP	APC
Subset distribution-1	CD3	CD62L	CD4/ CD8	CD45RA
Subset distribution-2	CD27	Ki67	CD4/ CD8	CD45RO
Immune activation	CD3	HLA- DR	CD4/ CD8	CD38
Apoptosis susceptibility	CD4	CD95	CD8	CD3
Costimulation	CD4	CD28	CD8	CD3
Exhaustion	CD4	PD1	CD8	CD3
Regulatory T cells	CD3	CD25	CD4	FoxP3 ^a
Natural killer cells	CD3	CD16	–	CD56
ICS–CD4 T cells	IFN- γ^b	IL-2	CD4	TNF- α
ICS–CD8 T cells	CD3	CD107 ^a	CD8	TNF- α

^a Alexa 647 used instead of APC and ^b Alexa 488 used instead of FITC

the gap between the antiretroviral therapy initiation and the clinical analysis for the present study.

Medicines and treatment administration

The HAART arm was administered 30 mg of Stavudine, 150 mg of Lamivudine and 200 mg of Nevirapine, twice a day as per the 2004 guidelines of The National AIDS Control Organization (NACO), India. Alternative regimens, with Nevirapine substituting for Efavirenz/Indinavir or Stavudine replacing Zidovudine were used as required. A complete description of the PHF preparation, the plant components, extraction procedures and dosage regimen etc., has been reported previously [2]. The PHF was prepared by *Vedic Drugs Pvt. Ltd.*, India. The PHF consisted of ingredients from 58 different plant species and formulated into 6 primary formulations (F_{BP}, F_{TP}, F_{KC}, F_{MC}, F_{CK} and F_{PB}), and five supplementary formulations (F_{AJ}, F_{AD}, F_{AZ}, F_{OT} and F_{SWT}). The polyherbal formulations were administered orally only for 4 months following recruitment.

The quantitation of the plasma markers of microbial translocation

The plasma endotoxin levels were measured using the Endpoint Chromogenic LAL kit (Lonza, Basel, Switzerland). Samples were diluted tenfold in endotoxin-free water (W50-100, Lonza), heat-inactivated at 85 °C for 12 min and assayed in endotoxin-free plates (655180, Lonza). The plasma samples were diluted 1,000-fold using PBS supplemented with 0.1 % Triton X-100, and the

concentrations of the LBP (Hycult Biotechnology, Fronts-straat, The Netherlands) and sCD14 (R&D Systems, Minneapolis, USA) were quantified using ELISA as per the manufacturers' instructions. All the measurements were made in duplicate wells and the mean values were used in the statistical analyses.

Immunophenotyping using flow cytometry

All the assays were performed within 6 h from the time of blood collection. The fluorochrome conjugated antibodies and buffers were procured from Becton and Dickinson (New Jersey, USA). The immunophenotyping for different cell markers and immune responses was performed using an array of 4-color antibody panels as listed in Table 3.

- The whole blood staining: One hundred μ l of whole blood was stained with appropriate antibody combinations for 15 min at room temperature and lysed with 1X FACS Lysis buffer (349202, BD) for 15 min. Cells were washed once in the FACS buffer (2 % FCS, 0.02 % sodium azide in PBS) and resuspended in 300 μ l of the same buffer. For the analysis of Ki67 expression, the cells were permeabilized in the Perm-Wash buffer (554723, BD) and then stained for Ki67.
- PBMC staining: PBMCs were isolated from whole blood using Ficoll density-gradient centrifugation and 1×10^6 cells were stained in 50 μ l final volume using appropriate antibody combinations. The cells were washed once with the FACS buffer and fixed in 1 % paraformaldehyde. The regulatory T cells were stained using the FoxP3 buffer Set (560098, BD) as per the manufacturer's instructions.
- The intracellular cytokine staining: PBMCs were rested overnight in RPMI medium supplemented with 10 % fetal calf serum, glutamine and antibiotics. The cells were replenished with fresh medium on the following day. Subsequently, 1×10^6 cells were stimulated with a pool of 121 overlapping gag peptides representing the Subtype C consensus sequence (#8118, NIH AIDS Reagent Program, 1.65 μ g/ml/peptide) for 6 h. Anti-CD28 and anti-CD49d antibodies were added each at a final concentration of 1 μ g/ml. Brefeldin A (10 μ g/ml for CD4 and 5 μ g/ml for CD8) and Monensin (5 μ g/ml for CD8) were added after the initial stimulation for 1 h. For the analysis of the CD8 T-cells, the PBMCs were incubated with anti-CD107 α antibodies throughout the period of stimulation. All the assays contained a 'DMSO negative control' and a 'Staphylococcus enterotoxin B (SEB) positive control' (1 μ g/ml) for cell stimulation. The cells were stained

for surface CD4 or CD8, fixed in 4 % paraformaldehyde, permeabilized with Perm-Wash buffer, stained intra-cellularly for IFN- γ , IL-2 and TNF- α or CD3 and TNF- α , and fixed in 300 μ l of 1 % paraformaldehyde.

- (d) The data acquisition: The samples were acquired using a time-based acquisition on a BD FACSCalibur and analyzed using CellQuest Pro software. Compensations were performed manually in every run, using single-stained controls. FMO controls were used to set gates.

Statistical analyses

Comparisons between groups were made using one way ANOVA with adjustments performed for multiple comparisons by Bonferroni method. The longitudinal analyses were performed using repeated measures one way ANOVA. All statistical analyses were performed using Graph Pad Prism version 5.02. p values <0.05 were considered to be significant.

Results

Reduced levels of markers of the microbial translocation in the HAART and PHF arms

Given the apparently stabilized clinical profile in the PHF arm of the previous clinical trial [2], and our perception that efficient management of the CIA, in addition to suppressing the plasma viral load, is critical for HIV disease management, we asked if microbial translocation and its control could underlie the clinical observation. Since no reports from India previously examined the significance of the microbial translocation for HIV-AIDS, we first quantified the levels of a few components associated with the microbial translocation in the plasma samples of different clinical groups using a cross-sectional analysis. HIV seropositive subjects, all drug-naïve, were classified into 3 groups based on the CD4 cell count: >500 (I, $n = 25$), $500-250$ (II, $n = 25$) and <250 (III, $n = 26$) and each arm was compared with a group of healthy volunteers ($n = 23$). The levels of three different components, bacterial lipopolysaccharide (LPS), LPS-binding protein (LBP) and soluble CD14 (sCD14), were determined in the plasma samples of each of these subjects (Fig. 1a). A progressively increasing association between the circulating LPS concentration and disease progression ($p = 0.005$) was evident in this analysis. There were significantly higher levels of LPS in groups II (0.27 ± 0.53 EU/ml, $p < 0.05$) and III (0.32 ± 0.46 EU/ml, $p < 0.01$) as compared to the healthy

controls (0.13 ± 0.62 EU/ml). Although the difference was statistically insignificant, the group I contained higher levels of LPS (0.24 vs 0.13 EU/ml) as compared to the control group. Likewise, the levels of LBP were significantly higher in groups II ($p < 0.05$) and III ($p < 0.01$) but not in group I as compared to the control group. The levels of sCD14 were significantly higher in all the stages of disease progression. Collectively, these data are suggestive that microbial translocation could serve as an important factor modulating chronic immune activation in Indian seropositive subjects.

We next examined whether the markers of the microbial translocation were modulated in 12 of the 13 participants of the PHF arm. These subjects continued in the PHF arm until the end of the clinical trial and were available for the present study nearly 6 months after the original clinical trial ended. We used a subset of an equal number of randomly selected HAART arm participants for comparison. Baseline clinical parameters of these subjects have been summarized (Table 1). The subjects in the control group are marginally younger and contained a mean CD4 cell count of 1,074 (with a range of 585–1,708). The participants in the HAART and the PHF groups did not differ significantly from each other with respect to the sample size, age, gender distribution, CD4 cell count and PVL (Table 1). The mean CD4 cell count of the HAART group was 236 (145–322) and that of the PHF group 245 (182–306, $p = 0.660$). In a longitudinal analysis, we measured the markers of microbial translocation in the plasma samples at three different time points: baseline, month (M) 12 and M24 (Fig. 1b). The levels of LPS reduced progressively with the inception of HAART at baseline (0.40 ± 0.21 EU/ml), decreasing to 0.21 ± 0.13 EU/ml at M12 ($p < 0.001$) and 0.28 ± 0.18 EU/ml at M24 ($p = \text{ns}$). Interestingly, a concomitant reduction in the levels of LBP ($p = 0.018$), but not sCD14 ($p = 1.000$), was observed in this arm. In contrast, the PHF treatment did not show a significant reduction in the circulating levels of LPS or LBP as a function of time ($p = 0.264$ and 0.472 , respectively). Importantly, the PHF administration resulted in a statistically significant and progressive reduction of sCD14 concentration in the plasma. The plasma concentration of sCD14 reduced from 3.45 ± 0.92 μ g/ml to 2.73 ± 1.84 at M12 ($p < 0.01$) and 2.47 ± 1.30 at M24 ($p < 0.001$). A recent study identified that sCD14 alone could serve as an independent correlate of mortality and disease progression in HIV infection, but not any of the other markers tested including LPS and LBP [46]. Collectively the data suggested a significant impact of the PHF administration on the levels of sCD14 in the blood suggesting a possible attenuation of general immune activation in the study participants.

Fig. 1 The markers of microbial translocation in seropositive subjects. **a** In a cross-sectional analysis, circulating levels of the markers of microbial translocation (LPS, LBP and sCD14) were measured in the plasma samples from healthy controls ($n = 23$), and HIV-1 seropositive subjects stratified into three groups based on the CD4 cell count, >500 (I, $n = 25$), 500-250 (II, $n = 25$) and <250 CD4 cells (III, $n = 26$). Each dot represents an individual participant and the horizontal line denotes the median value of the group. All the subjects were drug-naïve at the time of the analysis. **b** The markers of

microbial translocation in the study participants of the clinical trial. In a longitudinal analysis, the levels of LPS, LBP and sCD14 were measured in the plasma samples of 12 participants each of the HAART or PHF clinical groups at three different time points—baseline, month 12 and month 24—after treatment initiation. Inter-group and within-group comparisons were made using non-parametric one-way ANOVA and non-parametric repeated measures ANOVA, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *ns* non-significant

The markers of liver injury in the PHF arm

The finding that the levels of sCD14, but not those of the causative factor LPS, were significantly down modulated in the PHF arm in the longitudinal analysis was perplexing. Thus, given the prognostic value of the sCD14 for HIV-1 disease progression [46], the liver serving as the major source of the sCD14 in the plasma and the observation that the sCD14 levels were found significantly reduced in the PHF arm of our study, we wondered if the reduced levels of the sCD14 represent an improved hepatoprotection. To this end, we compared the concentration of the liver enzymes

AST and ALT in the plasma samples of 11 study participants each under the HAART and PHF arms at three different time points: baseline, month 15 and month 24 (Fig. 2). In the PHF arm, a significant reduction in the concentration of AST was identified from the base level (28.8 ± 4.9 IU/L), at M15 (20.1 ± 7.7 IU/L, $p < 0.001$) and M24 (22.8 ± 5.4 IU/L, $p < 0.01$), the differences being statistically significant at both the time points (Fig. 2, lower left panel). Furthermore, a trend of reduced ALT concentrations was evident at M15 a difference that was significant (19.9 ± 8.9 IU/L, $p < 0.05$) and at M24 (22.7 ± 6.7 IU/L, $p = \text{ns}$) as compared to the baseline

Fig. 2 Plasma concentration of the hepatic enzymes in the study participants The AST and ALT levels in fresh plasma samples of 11 each of the study participants under the HAART and PHF arms were quantified at three different time points—baseline, month 15 and month 24—after treatment initiation. Statistical significance was tested using repeated measures ANOVA. * $p < 0.05$, ** $p < 0.01$, ns: non-significant

levels (30.5 ± 10.8 IU/L) (Fig. 2, lower right panel). Importantly, HAART administration did not seem to have an impact on the plasma levels of either of the enzymes in the longitudinal analysis ($p = 0.298$ and 0.296 for AST and ALT, respectively) (Fig. 2, upper panels). Furthermore, improved hepatoprotection was suggestive also in a rat model used for the sub-chronic oral toxicity testing of the PHF administered orally at three different doses (data not shown). Collectively, two independent lines of evidence appear to suggest a reduced hepatic inflammation in the PHF arm—a significant reduction in the plasma levels of sCD14 and a concomitant reduction in at least one of the liver enzymes AST. The HAART administration does not appear to confer this clinical benefit on the study participants as neither the sCD14 levels nor the plasma concentrations of liver enzymes changed at any of the time points. In HAART, a prolonged drug administration is required to achieve a significant clinical benefit as the residual viraemia could ensure continued immune activation [9].

Immune and functional analyses of the T-cell subsets using flow cytometry

In a continued effort to understand the apparent clinical stabilization observed in the PHF arm of the study, we evaluated various cell lineage and functional markers and cell-mediated immune functions in 9 PHF subjects (mean CD4 cell count 242.2 ± 54.34) and 17 HAART participants (CD4 862.1 ± 268.8). These groups were compared with 13 HIV-1 seropositive drug-naïve subjects (ART-, CD4 cell count 245.9 ± 117.7). Note that the mean CD4

cell count of the ART- arm was comparable to that of the PHF subjects (Table 2). The cross-section analysis was performed using fresh blood sample drawn between June 2009 and August 2010 (average of 32.5 months after PHF- and 31.3 months after HAART-initiation) and four-color flow cytometry (Table 3). Representative cell gating strategies have been depicted (Fig. S1). The analyses consisted of staining for T-cell immune activation (HLA-DR and CD38, Fig. 3), exhaustion (PD1, Fig. 4a), apoptosis (CD95, Fig. 4b), subset distribution (CD62L/CD45RA, CD27/CD45RO, Fig. S2), cell proliferation (Fig. S3), costimulatory molecules (CD28, Fig. S4a), regulatory T-cells (CD25^{hi}FoxP3⁺, Fig. S4b) and natural killer cells (CD16 and CD56, Fig. S4c). Furthermore, we also evaluated the polyfunctional potential of the CD4 or CD8 cells following activation with a pool of gag peptides representing the subtype C consensus sequence or activation of the CD4 cells with a super antigen, Staphylococcus enterotoxin B (SEB, Fig. S5). These analyses collectively suggested a possible reduction in the immune activation as represented by three different biomarkers, HLA-DR, PD1 and CD95, on the T-cells in the PHF arm.

In comparison to the ART- arm, the HAART arm demonstrated a significant downregulation of the activation markers, HLA-DR and CD38 (Fig. 3a and b, upper panels) on both the CD4 (15.8 ± 10.9 vs 7.4 ± 5.1 , $p < 0.05$ and 51.0 ± 11.8 vs 37.0 ± 12.3 , $p < 0.01$, respectively) and the CD8 T cells (31.2 ± 19.1 vs 12.8 ± 8.8 , $p < 0.01$ and 55.8 ± 19.8 vs 18.5 ± 12.4 , $p < 0.001$, respectively). In contrast, in the PHF arm, significant differences were observed in the expression of only HLA-DR, but not CD38 on both the CD4 and the CD8 T Cells (Fig. 3a, b, upper panels). This difference was restricted to the mean fluorescence intensity, but not the percent of cells, suggesting an overall reduction in the HLA-DR surface expression in individual cells (252.3 ± 106.0 vs 133.0 ± 38.6 , $p < 0.01$ on CD4 and 197.7 ± 94.4 vs 105.4 ± 24.4 , $p < 0.05$) of the CD8 phenotype (Fig. 3a, b, lower panels). The MFI of the HLA-DR expression in the PHF arm was comparable to that of the HAART arm (133.0 ± 38.6 vs 126.1 ± 37.1 , $p = \text{ns}$ on the CD4 and 105.4 ± 24.4 vs 104.4 ± 48.4 , $p = \text{ns}$ on the CD8 cells). Of note, a significant negative correlation between the CD4 cell count and the MFI of HLA-DR was found only in the PHF arm, but not HAART or ART- groups, on the CD4 (Fig. 3c, left panel) and the CD8 T-cells (Fig. 3c, right panel). The inverse relationship between the HLA-DR MFI and the CD4 cell count in the PHF arm could be suggestive of attenuated immune activation [27, 34].

PD1, the marker for cell exhaustion, was significantly down-regulated in the HAART arm, on both the CD4 and CD8 cells (88.7 ± 29.2 in ART- arm vs 50.6 ± 13.2 , $p < 0.01$ and 49.6 ± 6.9 vs 39.2 ± 4.6 , $p < 0.01$,

Fig. 3 The expression of immune activation markers on T lymphocytes. The expression of CD38 and HLA-DR was evaluated on the **a** CD4 and **b** CD8 T lymphocytes derived from subjects of three different study arms—HIV-1 seropositive and drug-naïve (ART-, $n = 13$), anti-retroviral therapy (HAART, $n = 17$) and polyherbal formulation (PHF, $n = 9$). Each dot represents an individual subject and the horizontal line denotes the median value of the group.

Statistical testing was performed using one-way ANOVA. **c** Correlation between the CD4 cell count and HLA-DR MFI on the CD4 and CD8 T lymphocytes. Correlations were performed using the Pearson method. ** $p < 0.01$, *** $p < 0.001$, *ns* non-significant. Samples were phenotyped at an average of 32.5 or 31.3 months after initiation of PHF or HAART treatment

respectively) and in the PHF arm only on the CD4 cells (58.1 ± 11.6 , Fig. 4a, lower panel). Importantly, PD1 expression in the PHF arm was seen in a significantly higher percentage of the CD4 cells (6.9 ± 4.6 in ART-arm vs 26.9 ± 9.2 , $p < 0.01$), however, the mean MFI of PD1 was significantly lower, suggesting an overall reduction of the PD1 expression on a per cell basis. Furthermore, CD95, a pro-apoptotic marker, was similar between ART- and PHF arms, but lowered in the HAART arm (Fig. 4a). However, on a per cell basis, CD95 found significantly down regulated in both the HAART and PHF arms on both the CD4 and CD8 cells (ART— 712.9 ± 180.2 vs HAART 325.7 ± 92.0 , $p < 0.001$ and PHF 427.0 ± 209.0 ,

$p < 0.001$ on the CD4 T cells and ART— 387.2 ± 89.0 vs HAART 161.4 ± 51.3 , $p < 0.001$ and PHF 181.3 ± 67.9 , $p < 0.001$ on the CD8 T cells, Fig. 4b).

No other significant differences were observed in the PHF arm for the other markers that we tested and the cellular immune responses to the gag peptide pool or SEB (Fig. S2-5). All the three groups contained comparable levels of CD4 T cell subsets, regardless of the phenotypic marker combinations used, CD45RA and CD62L or CD27 and CD45RO. Among the CD8 T cells, however, the HAART arm showed elevated levels of naïve cells and contracted magnitude of central memory cells (Fig. S2). Furthermore, in the HAART arm, a significant down

Fig. 4 Expression of the exhaustion and apoptotic markers on T lymphocytes. The expression of **a** PD1 and **b** CD95 was evaluated on the CD4 and CD8 T lymphocytes derived from subjects of three different study arms. Statistical analysis was performed using one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns non-significant. Samples were phenotyped at an average of 32.5 or 31.3 months after initiation of PHF or HAART treatment

regulation of Ki67 was evident in total CD4 and CD8 cells and in several of the T cell subsets (Fig. S3). Additionally, the expression of CD28 was increased in the HAART arm (Fig. S4a). In contrast, in the PHF arm, the expression of the above markers remained comparable to that in the ART-group. The PHF and HAART arms also differed in percentage regulatory T cells and NK cell subsets, though similar comparisons with the ART-group failed to identify any PHF-related effects. In comparison to HAART, the PHF arm had significantly higher CD4⁺ CD25^{high} FoxP3⁺ T cells, though differences were only marginal (Fig S4b). Also the PHF arm had higher CD16⁺ CD56[−] NK cells and correspondingly lower CD16⁺ CD56⁺ NK cells. There were no differences in the CD16[−]CD56⁺ subset (Fig. S4c). The three arms did not differ significantly in the cytokine response to a consensus subtype C Gag peptide pool (Fig. S5a and c). The HAART arm exhibited better responses,

notably IL-2, to the superantigen SEB. The PHF arm remained similar to the ART- group (Fig. S5b).

Discussion

The present study was undertaken to examine if there was a concomitant improvement in the quality of the T-cell function in the study participants of the PHF arm that demonstrated an apparent stabilized clinical profile. We previously observed four different lines of evidence to this effect in the PHF arm of the clinical trial over the study period of 24 months. The present analysis currently identified four independent markers—sCD14, HLA-DR, PD1, and CD95, in addition to a suggestive hepatoprotection—all pointing at reduced levels of immune activation in the PHF arm. Collectively, the data from the present analysis appear to offer an explanation at the immunological level for the apparently stable clinical profile observed in the PHF arm of the clinical trial.

In our analysis, however, it appears paradoxical that the plasma levels of the causative agent LPS did not change, but those of sCD14, a host factor secreted by the monocytes in response to LPS, were significantly down modulated in the PHF arm in comparison to the control drug-naïve group or even the HAART arm (Fig. 1). This paradoxical observation could be explained in the light of a recent clinical report that sCD14, but not LPS, could be an independent prognostic marker in HIV-AIDS [46]. The reduced levels of sCD14 in plasma in the PHF arm are strongly suggestive of either a concomitant reduction in the magnitude of microbial translocation and/or a kind of immune modulation protecting the monocytes and as an extension other lymphocytes. The presence of sCD14 in plasma could be ascribed to two different and independent sources, the activated monocyte and the liver. CD14 is expressed as a membrane-anchored receptor on various cell types including the monocytes. Additionally, sCD14 is also found in the body fluids at moderate levels under normal conditions. Under the conditions of stress, such as an inflammation, a dramatic increase in the level of CD14 secretion is observed from the monocytes [5, 10]. sCD14 therefore may serve as a marker for the activation of the monocytes in various infections including HIV [35] and may play a role in the pathogenesis of the viral infection [25]. Importantly, sCD14 is also known to be an acute-phase protein produced by the hepatocytes especially under the conditions of toxicity, stress and inflammation [25, 36, 52]. The liver serves not only as the major source of the sCD14 present in the plasma but the secretion of sCD14 from the liver is elevated several fold under the conditions of hepatotoxicity and infectious diseases [3, 4]. The exposure of the hepatocytes to microbial products such as

the LPS by means of microbial translocation is believed to be a major pathway leading to the secretion of large quantities of the sCD14 by the liver [22, 25, 47]. HIV infection and the chronic immune activation have a significant impact on the function of the liver which can be evaluated by monitoring the hepatic enzymes AST and ALT, the levels of which in the blood increase in correlation with the viral load [28]. Importantly, of the various markers, only the sCD14 was found to be significantly associated with the disease outcome in HIV infection thus serving as an efficient prognostic marker [46].

The present study, however, could not delineate which of these two sources, the activated monocyte and the liver exposed to the microbial products, was mainly responsible for the reduced concentration of sCD14 in the PHF arm. The reduction in the liver enzymes AST and ALT, however, is suggestive of an overall improvement in hepatic inflammation, which in turn, is associated with disease prognosis [53]. Of note, the reduced surface expression of the activation marker HLA-DR on both the CD4 and CD8 cells in the PHF arm is also suggestive of attenuated immune activation (Fig. 3).

While anti-retroviral therapy reduced the surface expression of both CD38 and HLA-DR on both the CD4 and CD8 cells, as reported previously [50], in the PHF arm, the HLA-DR mean intensity alone, and not that of CD38, was down modulated on both the CD4 and CD8 cells. In comparison to the ART-arm, the HLA-DR MFI of the PHF arm was 1.9-fold lower on both CD4 and CD8 T cells, while the CD38 MFI remained comparable. Thus, we observed a novel discordant activation marker phenotype with respect to the expression levels of HLA-DR and CD38, in the PHF arm. The pathological significance of both HLA-DR and CD38 activation markers has been extensively evaluated in the viral infection. Although the prognostic value of CD38 is relatively well appreciated as compared to that of HLA-DR [48], the pathological significance of the individual activation markers could be variable and may have different consequences between the CD4 and CD8 cells. Several studies previously identified that the CD8 cells are characterized by a pattern of discordant expression for CD38 and HLA-DR [14, 18, 20, 45]. While the double positive CD38⁺ HLA-DR⁺ CD8 T-cells typically demonstrate the highest anti-viral cytotoxic activity, the single positive CD8 cells CD38⁺ HLA-DR⁻ or CD38⁻ HLA-DR⁺ too show a potential anti-viral activity although at relatively lower levels unlike the double negative cells which are devoid of this function [14, 45]. Evidence exists that the single positive CD8 cells are not anergic but are capable of demonstrating anti-viral functions [20]. Importantly, the CD8⁺ HLA-DR⁺ cells, but not the CD8⁺ HLA-DR⁻ cells, are refractory to diverse cell activation stimuli and display a defective clonogenic

potential at different stages of the viral infection suggestive of a beneficial outcome of the HLA-DR down-regulation on the CD8 T-cells [37]. In this context, further analysis would be required to understand the functional significance of the discordant expression of CD38 and HLA-DR we observed in the PHF arm and ask if it is likely to possess unique anti-viral properties. It is possible that the continued high level CD38 expression on the CD8 cells could signify a potential anti-viral function and the reduced HLA-DR expression a partial attenuation of the cellular activation.

In the PHF arm, the PD1 expression was found in a larger percentage of CD4 cells but at a lower MFI (Fig. 4a). In the SIV model, resolution of immune activation during the acute infection was associated with the PD1 up-regulation [11]. It is therefore possible that the low level PD1 expression on the CD4 T-cells could be protective by minimizing the magnitude of the activation-induced cell death. The PD1 expression has indeed been associated with exhaustion and apoptosis but only at higher levels. We also observed CD95 down regulation on both the CD4 and CD8 cells in the PHF arm (Fig. 4b). It is possible that the reduced surface expression of PD1 and CD95 collectively could have protected the CD4 cells by minimizing apoptosis [39] thus at least partially explaining the statistically stable CD4 cell profile in the PHF arm.

In summary, the detailed and extensive immune profiling of the T-cells from the PHF arm identified four independent and potential biomarkers (PD1, CD95, HLA-DR and sCD14) in addition to the reduction in hepatic inflammation (ALT and AST) that are collectively suggestive of attenuation of the chronic immune activation in HIV-AIDS, which could be beneficial. Importantly, this analysis is only a cross-sectional evaluation performed more than 2 years following the PHF administration. It is possible that some of the positive effects of the PHF treatment have diminished as a consequence of the average time delay between the original PHF administration and the current phenotypic analyses which is approximately 32.5 months, and compounded by the continued viral replication during this period. The stabilized immune and viral profiles as well as the validity of the biomarkers identified in this study must be confirmed in the future clinical trials designed to rectify the several limitations of the present study. This includes incorporating a placebo control, double-blinding and randomization of the participants.

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