Leukotrienes Are Upregulated and Associated with Human T-Lymphotropic Virus Type 1 (HTLV-1)-Associated Neuroinflammatory Disease

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Abstract

Leukotrienes (LTs) are lipid mediators involved in several inflammatory disorders. We investigated the LT pathway in human T-lymphotropic virus type 1 (HTLV-1) infection by evaluating LT levels in HTLV-1-infected patients classified according to the clinical status as asymptomatic carriers (HACs) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients. Bioactive LTBA and CysLTs were both increased in the plasma and in the supernatant of peripheral blood mononuclear cell cultures of HTLV-1-infected when compared to non-infected. Interestingly, CysLT concentrations were increased in HAM/TSP patients. Also, the concentration of plasma LTBA and LTC4 positively correlated with the HTLV-1 proviral load in HTLV-1-infected individuals. The gene expression levels of LT receptors were differentially modulated in CD4⁺ and CD8⁺ T cells of HTLV-1-infected patients. Analysis of the overall plasma signature of immune mediators demonstrated that LT and chemokine amounts were elevated during HTLV-1 infection. Importantly, in addition to CysLTs, IP-10 was also identified as a biomarker for HAM/TSP activity. These data suggest that LTs are likely to be associated with HTLV-1 infection and HAM/TSP development, suggesting their putative use for clinical monitoring.

Introduction

Human T-lymphotropic virus type 1 (HTLV-1), a complex retrovirus, is the causal agent of adult T cell leukemia (ATL), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and other inflammatory disorders that develop after a variable period of latency ranging between months and decades [1,2]. Although the majority of HTLV-1-infected individuals remain asymptomatic carriers (HACs), the lifetime risk of developing HTLV-1-associated diseases may be close to 10%, and the incidence of HAM/TSP ranges from 0.3% to 4% [3].

HAM/TSP is a neuroinflammatory disease characterized by a chronic progressive myelopathy with infiltrating mononuclear cells in the areas of demyelination and axonal dystrophy [4,5]. It is not clear how HTLV-1 causes neurological damage, but spontaneous T cell proliferation and proinflammatory responses characterized by elevated ex vivo production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α by peripheral blood mononuclear cells (PBMCs) are associated with HAM/TSP [6,7]. In addition, patients with HAM/TSP display an increased proviral burden when compared to HACs, and high proviral loads have been associated with rapid disease progression [8–10]. Thus, few disease markers and prognostic predictors have been described for HAM/TSP.

Leukotrienes (LTs) are bioactive lipid mediators involved with inflammatory conditions [11] that may represent candidate biomarkers for HAM/TSP. Biosynthesis of LTs is triggered by stimuli such as antigen, cytokines, microorganisms and immune complexes [12]. Just after stimulation, arachidonic acid (AA) that is liberated from cellular membrane phospholipids through the action of phospholipase A2 (PLA2) is oxidized by 5-lipoxygenase (5-LO) in combination with 5-LO-activating protein (FLAP) to generate the leukotriene A4 (LTA4). The downstream enzymes LTA4 hydrolase (LTA4H) and LTC4 synthase (LTC4S) give rise to leukotriene B4 (LTB4) and leukotriene C4 (LTC4). LTC4 is further converted to LTD4 and LTE4, which are collectively termed cysteinyl leukotrienes (CysLTs) respectively. LTB4 and CysLTs signal through distinct cell surface receptors named BLT1 and BLT2 and CysLT1 and CysLT2, respectively [13]. Functionally, LTB4 is recognized as a potent leukocyte chemoattractant that...
also displays leukocyte activating functions, whereas the CysLT1
are better known for leading to airway constriction, increased
vascular permeability, mucus secretion and cell trafficking [14].
In addition, LTs have been shown to improve the host defense
against pathogens [15–18].

Considering the importance of LTs as powerful mediators of
inflammation, the present study was undertaken to test the
hypothesis that HTLV-1 infection leads to an exacerbation of the
5-LO products formation and LT signaling in patients with
HAM/TSP. We examined LT concentrations in plasma, the
ability of PBMCs to produce LTs and LT receptor expression in
lymphocytes from HTLV-1 patients. We also investigated the
overall plasma LT, chemokine and cytokine signatures of HACs
and HAM/TSP patients. Moreover, we investigated the correla-
tions between LTs, chemokines and cytokines in HTLV-1-infected
individuals and the capacity of LTs to modulate cytokine
production. Our results demonstrate for the first time that LTs
are upregulated during HTLV-1 infection, suggesting a role for
LTs in HAM/TSP pathogenesis and presenting them as potential
biomarkers for monitoring HAM/TSP development.

Results

CysLT is Upregulated in HTLV-1-associated
Neuroinflammatory Disease

LTs have been shown to function as inflammatory mediators
[11]. To investigate whether HAM/TSP disease is characterized
by elevated levels of LTs, we measured the amount of these
mediators in the plasma of HTLV-1 patients. LTB4 was increased
in the plasma of HACs and HAM/TSP patients when compared
to that of NI donors; however, no difference was observed in LTB4
levels between HACs and HAM/TSP patients (Figure 1A).
Interestingly, HACs and HAM/TSP patients displayed increased
amounts of CysLTs when compared with NI donors, but CysLT
amounts were higher in the plasma of HAM/TSP patients than in
the plasma of HACs (Figure 1B). Thus, although HTLV-1 induces
increased concentrations of LTs in the plasma of both HACs
and HAM/TSP patients, these results associate increased CysLT
concentrations with HAM/TSP. In addition, we explored the
correlation between HTLV-1 proviral load and plasma LTB4
(Figure 1C) or CysLTs (Figure 1D) and found a positive correlation.
Thus, in infected persons, the plasma LTs are associated with the
HTLV-1 proviral load in PBMCs.

HTLV-1 Enhances LT Generation

HTLV-1-induced LT generation was examined in PBMCs of
NI donors. We found increased production of LTB4 (Figure 2A)
and LTC4 (Figure 2B) when cells were challenged with cell-free
virus. Next, because we found increased levels of LTs in the
plasma of HTLV-1 patients, we measured LT generation by
PBMCs from HTLV-1 patients. We observed increased produc-
tion of LTB4 by cells from HACs and HAM/TSP patients when compared
to those from NI donors, with the highest amount of LTB4 in
the supernatant of HACs and HAM/TSP patients (Figure 2C).
Moreover, as expected, the generation of CysLTs was increased
in PBMCs from HACs and HAM/TSP patients when compared
to those from NI donors, with the highest amount of CysLTs in
cells from HAM/TSP patients (Figure 2D). Next, we assessed 5-
LO and LTC4 synthase expression in PBMCs. Our results
demonstrated that cells from HAM/TSP patients expressed
higher levels of 5-LO than cells from NI donors or HACs
(Figure 2E); however, no differences in the expression of LTC4
synthase were observed between the groups (Figure 2F).

Lymphocytes from HTLV-1 Patients have Altered LT
Receptor Gene Expression

We next analyzed the gene expression of LT receptors by
detecting BLT1 and CysLT1 expression in both CD4+ and CD8+
T cells (Figure 3). In CD4+ T cells, BLT1 expression was increased
only in HAM/TSP patients than in NI donors (Figure 3A) but
CysLT1 was expressed at higher amounts in HACs and HAM/
TSP patients than in NI donors (Figure 3B). Analysis of CD8+ T
cells showed no differences in BLT1 gene expression among all of
the groups (Figure 3C), whereas decreased CysLT1 gene
expression was detected in HAC and HAM/TSP patient CD8+ T
cells when compared to NI donor CD8+ T cells (Figure 3D).

Overall Plasma Signatures of LTs, Chemokines and
Cytokines in HTLV-1 Infection

We next sought to characterize the immune and inflammatory
mediators in the plasma of NI donors to allow for further
comparative analysis of HACs and HAM/TSP patients. We
assessed the overall LT, chemokine and cytokine signatures by
categorizing volunteers as “low-” or “high-” mediator producers
to minimize the impact of individual concentrations on the final
analysis and to make the data more homogeneous. The global
median index of each mediator was calculated (CysLTs = 438.9;
LTB4 = 402.6; IP-10 = 91.4; MCP-1 = 75.8; MIP1-α = 41.2; IL-
8 = 0; IL-17 = 0; IL-23 = 0; IL-1 = 0; IL-4 = 0; IL-10 = 0; TNF-
α = 0; IL-12 = 0; IFN-γ = 0; IL-6 = 0; IL-5 = 0; GM-CSF = 9.2; IL-
13 = 26.2) (data not shown), and based on these values, each
volunteer was classified as a low- (□) or high ■-mediator
producer (upper panels in Figures 4 A, B, C). An assembly of
the frequency of high-mediator producers among NI donors in
ascendant fashion is shown in Figure 4A. The mediator signature
curves of NI donors were used as a reference to identify changes in
the overall mediator signatures of HACs and HAM/TSP patients.
Analysis of the HAC signatures demonstrated that LTs, the
majority of chemokines (MCP-1, IL-8 and MIP1-α) and some
cytokines (IL-17, IL-23, IL-4, TNF-α and IL-12) are increased
when compared to the values observed in NI donors (Figure 4B).
We also examined the signatures of HAM/TSP patients
(Figure 4C) and found that LTs and chemokines (MCP-1 and
IP-10) were increased, and in contrast to our findings in HACs,
cytokines were decreased when compared to the values observed
in NI donors. Additionally, high producers of CysLTs and IP-10
were more frequent in HAM/TSP group than in HACs. In
contrast, the frequency of high cytokine producers was lower in
HAM/TSP patients than in HACs. Thus, our findings showed
that LTs and chemokines are the prominent mediators in HACs
and HAM/TSP patients.

Association between Immune and Inflammatory
Mediators in HTLV-1 Infection

The differences in the concentrations of LTs, chemokines and
cytokines between HACs and HAM patients prompted us to
investigate the correlation between the concentrations of media-
tors in each group. The analysis of the HAC group demonstrated
positive correlation between the concentrations of CysLTs with
the concentrations of LTB4 and IL-13 (Figure 5A). In contrast to
our findings in HACs, CysLT concentrations were not correlated
with the amounts of other mediators, but LTB4 concentrations
were positively correlated with the levels of some chemokines,
including MCP-1 and IP-10, and cytokines, including IL-17, IL-23
and IL-10 in HAM/TSP patients (Figure 5B). Meanwhile,
although no specific pattern associated with any kind of immune
or inflammatory response was observed, the expression levels of
several chemokines and cytokines were correlated in HACs and HAM/TSP patients (Figure 5).

Discussion

The participation of LTs in several infections [19,20] and inflammatory disorders [21,22] has long been appreciated; however, the involvement of these lipid mediators in HTLV-1 infection and HAM/TSP development has not been studied previously. Here, we report for the first time that HTLV-1 infection dysregulates the LT pathway. Our results demonstrate increased LTB₄ and CysLT plasma concentrations in HTLV-1 patients, suggesting a role for LTs in several HTLV-1-associated inflammatory diseases. Furthermore, a key finding in our study was the association between plasma CysLT concentrations and HAM/TSP. The concentration of plasma CysLTs was increased more than 3-fold in HAM/TSP patients when compared to HACs. Studies have detected LTs in the central nervous system of patients with autoimmune diseases [23] and infectious diseases [24,25] and have suggested a potential pathophysiological role for these molecules. Specifically, the inhibition of 5-LO activity during experimental demyelination attenuates neuroinflammation and axonal damage [26]. Together, these observations are consistent
Figure 2. HTLV-1 primes cells for leukotriene generation. (A–D) $10^6$ peripheral blood mononuclear cells were cultured for 48 hours and then treated for 30 min with A23187 (0.5 μM) before detection of LTB₄ (A, C) and LTC₄ (B, D) by EIA (n = 7 per group). (A, B) Cells from non-infected healthy donors were seeded alone (unstimulated) or in the presence of cell-free HTLV-1 (HTLV-1 virions). (C, D) Cells from non-infected healthy donors (NI), HTLV+.
with our results demonstrating that HAM/TSP patients display enhanced CysLT production, suggesting that these mediators contribute to HAM/TSP pathogenesis. As there is no effective therapy for HAM/TSP [27], CysLT signaling may represent a new therapeutic target. Although many investigators have concentrated their efforts on the discovery of HAM/TSP markers, previous studies have relied on ex vivo culture, and few associations have been established in vivo [28,29]. Thus, our work extends to the knowledge of in vivo HAM/TSP markers by presenting CysLTs as a putative biomarker of HAM/TSP. Therefore, we next tested the hypothesis that HTLV-1 proviral load is correlated with the concentration of plasma LTs. Using Pearson’s correlation, we observed a positive correlation between LTs or CysLTs and proviral load indicating that concentrations of LTs in plasma of infected individuals reflect proviral load. However, in the present study, our data did not demonstrate a strong association between LTs and disease activity or even clinical progression in HAM/TSP patients. In this pioneering investigation, we explored the complex pro-inflammatory network underlying the immunological profile of HTLV infected patients to find potential biomarkers of disease activity or even prognostic markers for monitoring purposes. We believe that LTs could be putative immunological biomarkers that could serve as prognostic markers or could be associated with disease activity. It is important to mention that the present investigation should be considered the first step toward the discovery of LT biomarkers for HTLV infection, as further studies will be necessary to validate this hypothesis.

**Figure 3. Leukotriene receptor mRNA expression in lymphocytes of HTLV-1 patients.** Quantitative PCR (qPCR) was performed for BLT1 (A, C) and CysLT1 (B, D), and their relative expression levels were determined in CD4+ (A, B) and CD8+ (C, D) T cells from twenty non-infected healthy donors (NI), twenty asymptomatic carriers (HAC) and seventeen HAM/TSP patients (HAM). Gene expression levels were normalized to the gene expression levels of ACTB, GAPDH, B2M and RPL13a for CD4+ T cells and of ACTB for CD8+ T cells in the same real-time PCR reaction. The data are presented as means ± SEM. *p<0.05, compared with NI donors; #p<0.05, compared with HACs (one-way ANOVA). doi:10.1371/journal.pone.0051873.g003
Leukotriene Pathway in HTLV-1 Infection

A

B

HAC

C

HAM

Percentages of high producers

Leukotrienes Chemokines Cytokines

Leukotrienes Chemokines Cytokines

Leukotrienes Chemokines Cytokines

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Leukotriene Pathway in HTLV-1 Infection

LTs are produced primarily by neutrophils, eosinophils, mast cells and monocytes/macrophages [30]. Among PBMCs, it is assumed that monocytes are largely responsible for LT generation. Moreover, B cells express 5-LO but do not synthesize LTs upon A23187 stimulation [31]. Notably, we found that cell-free viral particles induce LT generation by PBMCs after A23187 stimulation, suggesting that monocytes are a significant source of LTs during HTLV-1 infection. This also suggests that LT production is increased in the central nervous system of HAM/TSP patients because infiltrating monocytes are found in the areas of demyelination [4] and may be stimulated by the virus to release lipid mediators. Importantly, HTLV-1 and HTLV-1-Tax antigen induce another lipid mediator, PGE2 [32], demonstrating that HTLV-1 regulates both the 5-LO and the cyclooxygenase pathway of the AA cascade. Moreover, LTC4 generation by PBMCs supports elevated concentrations of plasma CysLTs in HAM/TSP patients. However, despite the low number of HTLV-1 virions in vivo, a DNA insertion analysis showed that the virions can induce the production of LTs in PBMCs in vitro without infecting these cells (data not shown). On the other hand, LTs are involved in the control of host defense, including defense against HIV [33,34]; thus, the decreased LTB4 generation observed in the PBMCs of HAM/TSP patients when compared to HACs may not represent a good method for controlling the high HTLV-1 set points seen in HAM/TSP patients. In addition, it was interesting to note the lack of correlation between 5-LO and LTC4S gene expression and LT generation by human PBMCs. Despite this disassociation between changes in 5-LO mRNA levels and protein expression, which has been reported previously [35,36], increased 5-LO mRNA expression indicates a positive regulation of the LT pathway as evidenced by the increased mRNA expression of 5-LO in HAM/TSP PBMCs.

The upregulation of LT receptors has been noted previously in experimental neuroinflammatory disease and is thought to be involved in the pathogenesis of this disease [37,38]. It is noteworthy that LT receptor expression has been detected in both CD4+ and CD8+ T cells [12]. These cells are found in inflamed areas in HAM/TSP patients. Thus, we hypothesized that LT receptor expression may be increased in T cells in HAM/TSP patients. Our data demonstrate that gene expression of LT receptors is modulated by HTLV-1 infection. Specifically, BLT1 was upregulated in CD4+ T cells from HAM/TSP patients. Meanwhile, CysLT1 was upregulated in CD4+ T cells but downregulated in CD8+ T cells of HACs and HAM/TSP patients. In animal models, deletion of BLT1 [39] and inhibition of CysLT1 [40] signaling can suppress the recruitment of inflammatory cells into the central nervous system and thus inhibit experimental autoimmune encephalomyelitis. In this regard, we speculate that high LT amounts and high LT receptor expression levels in CD4+ T cells may bias the host toward cellular infiltration of inflamed tissues, worsening the HAM/TSP disease. Moreover, as HTLV-1 preferentially affects CD4+ T cells, the migration of non-infected CD4+ T cells to inflamed sites containing HTLV-1-infected lymphocytes could facilitate cell-cell contact and consequently the spread of infection.

Studying the immunological response to HTLV-1 infection is important for the understanding of HAM/TSP pathogenesis. Others have shown that CXCL9 [41], CXCL10 [42], CCL22 [43], IP-10 [28], sCD30 [28] and IFN-γ [28] are increased in the systemic circulation of HTLV-1-infected individuals. We hypothesized that dysregulation of the immune system is likely to be involved in the pathogenesis of HTLV-1 infection and that the clinical presentation of HAM/TSP may result from multifactorial immunological mechanisms. Impairment in the cytokine network has been found to be one of the determining factors in several human diseases. Because conventional strategies may not be suitable to capture minor changes in the immunological profile and because of the wide range of chemokines/ cytokines/LTs, in this study, we employed an alternative strategy to assess the biomarker signature and to describe the dominant profiles associated with asymptomatic presentation and HAM/TSP caused by chronic HTLV-1 infection. This panoramic overview offers additional insight into the immunological events that are relevant for clinical studies of HTLV-1 infection. This approach may allow for a better understanding of the immunological parameters that control disease outcome and provide useful tool for prognostic monitoring. We therefore examined the concentrations of several chemokines and cytokines in the plasma of our cohort to further establish a signature curve for LTs, chemokines and cytokines. Using a signature curve of non-infected individuals as a reference, we demonstrated that LTs and chemokines are increased in the plasma of both HACs and HAM/TSP patients. The signature curves both confirm CysLT and identify IP-10 as biomarkers of HAM/TSP. The Th1-associated chemokine IP-10 belongs to the CXC chemokine superfamily. IP-10 has been shown to be a potential marker of inflammation and diseases [43–45] including HAM/TSP [28]. Furthermore, our results show that although the plasma concentrations of some cytokines are increased in HACs, the majority of the analyzed cytokines are decreased in the plasma of HAM/TSP patients. The low levels of these cytokines observed in HAM/TSP patients may reflect the attenuated inflammatory response observed in the central nervous system after a long period of HAM/TSP manifestation [46]. In contrast to these decreased cytokine levels, however, our work clearly demonstrates some plasma mediators of inflammation remain elevated even after a long period of disease manifestation. In addition, as a trigger for positive feedback regulation, LTs, chemokines and cytokines have been shown to influence the production of each other [16,18,47,48]. Supporting these findings, we have shown positive correlations between plasma LT, chemokine and cytokine concentrations but in the absence of a singular pattern of immune response. Importantly, CysLTs are increased in HAM/TSP patients, but no positive correlation was detected between CysLT levels and the concentrations of other mediators, presenting this family of lipid mediators as an independent biomarker of HAM/TSP.

Figure 4. Signature curves of high biomarker producers during HTLV-1 infection. (A) Representative scattergraphs were used to establish the concept of low biomarker producers (white) and high biomarker producers (black). The results from all groups studied (non-infected donors, HACs and HAM/TSP patients) were assembled to calculate the global median for each biomarker. Low biomarker producers were defined as having values lower than the global median, whereas high biomarker producers were defined as having values greater than or equal to the global median cut-off. Data from 3 of 18 molecules analyzed are shown. (B–D) The diagrams were plotted using the global median index of plasma biomarkers (measured by ELISA) as the cut-off to identify each volunteer as a low (□) or high (■) producer. The ascendant frequency of high producers found in the Ni group was established as a reference curve (– – –) to identify changes in the overall biomarker signature of the other groups. Significant differences were defined as a shift to a distinct 25% quantile interval between the studied groups. *, significantly different values compared with Ni donors; †, significantly different values compared with HACs. Ni – non-infected healthy donors; HAC – HTLV-1 asymptomatic carrier; HAM – HAM/TSP subjects. doi:10.1371/journal.pone.0051873.g004
Figure 5. Correlation of plasma biomarkers in HTLV-1 infection. Samples from HTLV-1-infected subjects were used to detect leukotrienes, chemokines and cytokines by ELISA. A correlation analysis was performed to analyze biomarkers levels. The results of a non-parametric Spearman's test and \( r \) index are provided in the figure. Filled squares indicate positive correlations. HAC – HTLV-1 asymptomatic carriers; HAM – HAM/TSP subjects.

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In this report, we demonstrate that HTLV-1 dysregulates the LT pathway. This finding has important implications for the understanding of HAM/TSP development. First, CysLTs could be used as a biomarker for HAM/TSP development. Second, the fact that LTs are mediators of inflammation suggests that the LT pathway might be involved in HAM/TSP pathogenesis. Therefore, further experiments will be required to elucidate a potentially pathogenic function of LTs in HAM/TSP. Moreover, it will be interesting to determine whether drugs targeting the LT pathway ameliorate HAM/TSP symptoms.

Patients, Materials and Methods

Study Population

The study subjects were classified as asymptomatic HTLV-1 carriers or HAM/TSP patients in accordance with the criteria proposed by the World Health Organization. Non-infected (NI) healthy volunteers were included as a control (Table 1). Biological specimens were obtained from HTLV-1 patients from the clinical cohort of the Neurology Department of Ribeirão Preto University Hospital, Brazil. Diagnosis of HTLV-1 infection was established by enzyme-linked immunosorbent assay (ELISA) and confirmed by PCR (tax and LTR regions). Subjects with HAM/TSP were selected from among a heterogeneous disease progression group. Individuals receiving therapies were excluded. All procedures were approved by the Ethical Committee of the University Hospital, School of Medicine of Ribeirão Preto, University of São Paulo (process number 1108/2008), and all subjects provided written informed consent.

Isolation of Blood Leukocytes

After separation of plasma from the heparinized venous blood, PBMCs were isolated using Ficoll-Paque (GE Healthcare) density gradient centrifugation. To isolate lymphocytes from PBMCs, magnetic beads conjugated with anti-CD4 or anti-CD8 antibodies (Mini-Macs Micro-Beads, Miltenyi Biotec) were used to separate CD4$^+$ and CD8$^+$ T cells by positive selection using the manufacturer’s protocol. Phenotypic analysis performed by flow cytometry (BD-FACSCanto) using anti-CD4-FITC, anti-CD8-PE and anti-CD3-PE antibodies (BD Biosciences) demonstrated a minimum of 80% purity of CD4$^+$ and CD8$^+$ lymphocytes. A hemocytometer chamber was used to obtain absolute cell counts, and cell viability was determined by trypan blue exclusion.

HTLV-1 Proviral load

HTLV-1 proviral load was quantified as previously described [49]. Genomic DNA samples isolated from peripheral blood of the HTLV-1 infected individuals were used to perform quantitative RT-PCR with the SYBR Green system (Applied Biosystems). The single-sample reactions for human β-actin and HTLV-1 tax were performed in duplicate on the same plate. The HTLV-1 proviral load was calculated using the following equation: average of tax/average of β-actin×2×10$^7$. The values obtained were Log10 transformed for the correlation analysis.

Detection of Leukotriene Pathway Transcripts

Total RNA was extracted from PBMCs and lymphocytes with TRIzol (Invitrogen) according to the manufacturer’s instructions, and reverse transcription was carried out with 2 μg of total cellular RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Thereafter, quantitative RT-PCR was performed using ABI 7500 Sequence Detection (Applied Biosystems). The reaction was performed in duplicate using TaqMan assay reagents (Applied Biosystems) and analyzed using 7500 System SDS software. The relative mRNA expression was determined using the ΔΔCT method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH: 4310884-E) was used as an internal control for PBMCs. The geometric means of the values obtained for β-actin (ACTB: 4326315E), GAPDH, β2 microglobulin (B2M: 4333776-0710013) and ribosomal protein L13a (RPL13a: 185720330-7) were used as internal controls for CD4$^+$ T cells, and ACTB was used as an internal control for CD8$^+$ T cells.

Production of Cell-free Virus

The human T cell line MT-2 was used as a source of HTLV-1 producing cells. For preparation of cell-free HTLV-1, cells were seeded at 5×10$^5$/mL and incubated at 37°C in a humidified CO$_2$ atmosphere for 2 days in RPMI supplemented with 10% FBS. The supernatants were passed through a 0.45-μm filter (Millipore) to remove cells and debris, and the virions were concentrated 10 times by ultracentrifugation for 2 hours at 100,000 g. The pellet containing virus particles was resuspended in RPMI and quantified before being subjected to HTLV-1 p19 ELISA (ZeptoMetrix).

Stimulation and Culture of Cells

We plated PBMCs (10$^6$/well) in forty eight-well plates and maintained them overnight at 37°C and 5% CO$_2$. The cells were provided with fresh RPMI 1640 containing 5% AB human serum (Sigma) and 100 U/mL penicillin and cultured for an additional 48 hours. For analysis of the effects of cell-free HTLV-1, cell cultures from healthy donors were challenged with 10 ng of virions particles (p19 equivalent) prior additional culture. For leukotriene detection, the supernatants were removed, and the cells were resuspended in HBSS containing Ca$^{2+}$ and Mg$^{2+}$ and stimulated

| Table 1. Demographical and Clinical Characteristics of the Study Groups. |
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| **Group** | **Number** | **Age, mean years (± SD)** | **Sex (M/F)** | **Provincial load mean/10$^5$ cell (± SD)** | **First reported symptoms, mean years (± SD)** |
| Non-infected | 31 | 45.7 (± 12.2) | 9/22 | N/A$^b$ | N/A |
| HAC$^a$ | 26 | 42.9 (± 12.9) | 11/15 | 2,105.4 (± 2,312.2) | N/A |
| HAM/TSP$^a$ | 19 | 54.9 (± 9.1) | 4/15 | 6,180.9 (± 3,544.6) | 13 (± 9.7) |

Note. $^a$M/F: male/female; $^b$N/A: not applicable; $^a$HAC: HTLV-1 asymptomatic carriers; $^a$HAM/TSP: HTLV-1 associated myelopathy/tropical spastic paraparesis.

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for 30 minutes with 0.5 μM of the calcium ionophore A23187 (Sigma), and then, reactions were stopped using ice.

Measurement of Leukotrienes, Chemokines and Cytokines

A specific enzyme immunoassay (Cayman) was used to quantify LTB4 and LTC4 in cell-free supernatants and LTB4 and Cy5LT in plasma per the manufacturer’s instructions. For plasma measurements, samples stored at −70°C were purified on Waters C18 Sep-Pak cartridges (Waters Associates) prior to performing the assay. Moreover, the cell-free supernatants were tested for IP-10 and TNF-α, and the plasma samples were tested for MCP-1, MIP1-α, IP-10, IL-8, IL-5, IL-4, IL-13, IL-1β, IL-6, GM-CSF, TNF-α, IL-12, IFN-γ, and IL-10 using a Duoset ELISA Development kit (R&D Systems) and for IL-17 and IL-23 using an OptEIA ELISA kit (BD Bioscience) in accordance with the manufacturer’s instructions. The reactions were performed in 96-well ELISA plates (Corning), and the optical densities were determined at 450 nm using a microplate reader. The cytokine concentration in each sample was estimated by interpolation of sample optical densities with the cytokine standard using a four-parameter curve-fitting program.

Leukotriene, Chemokine and Cytokine Signature Analysis

A method for identifying low and high producers of mediators by analyzing cytokine profiles was previously reported by Luiza-Silva et al. [50]. The concentrations of LTs, chemokines and cytokines (pg/mL) were assembled to calculate the global median index (values of NI donors+HACs+HAM/TSP patients)/number of samples, and plasma samples were characterized as low- or high-mediator producers. Low-mediator producers were defined as having values lower than the global median, whereas high-mediator producers were defined as having values greater than or equal to the global median cut-off. The percentage of high producers was calculated for each analyzed molecule, and the ascendant frequency of the non-infected group was used as the reference curve to identify changes in the overall mediator patterns from all the groups.

Statistics

The data are presented as means ± SEM of values determined from the indicated number of samples. The data were analyzed by Student's t-tests or ANOVA with Bonferroni’s post-test as appropriate to identify significant differences between group means using GraphPad Prism version 5 (GraphPad Software).

Spearman's correlation test was performed to assess the association between the levels (pg/mL) of LTs, chemokines and cytokines while Person’s test was used to analyze the association of LTs and the HTLV-1 proviral load. In all cases, statistical significance was defined as p≤0.05. The cytokine signatures analyses were performed using the non-infected signature as the reference curve, and differences were considered significant when the values fell outside of the quartile of the reference signature. The use of the 50th percentile as the limit to identify relevant differences in the chemokine/cytokine/LT signatures between the groups has been adapted from a pioneering study by Luiza-Silva et al. [50]. This approach has been shown to be relevant to detect, with high sensitivity, putative minor changes in the cytokine signatures that are not detectable by conventional statistical approaches.

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Author Contributions

Conceived and designed the experiments: BCT CAS DTC OAMF SK LHF. Performed the experiments: BCT CAS DTC OAMF SK LHF. Contributed reagents/materials/analysis tools: OAMF SK DTC. Wrote the paper: BCT LHF.

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