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 LTB4 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 LTB4 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 chemokines 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.

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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 LTA₄ (LTA₄). The downstream enzymes LTA₄ hydrolase (LTA₄H) and LTA₄ synthase (LTA₄S) give rise to leukotriene B₄ (LTB₄) and leukotriene C₄ (LTC₄). LTC₄ is further converted to LTD₄ andLTE₄, 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 CysLT 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 HAGs and HAM/TSP patients. Moreover, we investigated the correlations 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. Herein, we present a 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 HAGs and HAM/TSP patients when compared to that of NI donors; however, no difference was observed in LTB4 levels between HAGs and HAM/TSP patients (Figure 1A). Interestingly, HAGs 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 HAGs (Figure 1B). Thus, although HTLV-1 induces increased concentrations of LTs in the plasma of both HAGs 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 production of LTB4 by cells from HAGs and HAM/TSP patients when compared to those from NI donors, with the highest amount of LTB4 in the supernatant of cells from HAGs (Figure 2C). Moreover, as expected, the generation of CysLTs was increased in PBMCs from HAGs 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 HAGs (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 HAGs and HAM/TSP patients than in NI donors (Figure 3B). Analysis of CD8+ T cells showed no differences in BLT1 gene expression among all 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 HAGs 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 (CysLT1 = 138.9; LTB4 = 402.6; IP-10 = 91.4; MCP-1 = 75.8; MIP1α = 41.2; IL-8 = 0; IL-17 = 0; IL-23 = 0; IL-1 = 0; IL-4 = 0; TNF-α = 0; IL-12 = 0; IFN-γ = 0; IL-6 = 0; IL-5 = 0; GM-CSF = 0.2; 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- and low-producer 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 HAGs and HAM/TSP patients. Analysis of the HAG signatures demonstrated that LTs, the majority of chemokines (MCP-1, IL-8 and MIP1α) 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 HAGs, 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 HAGs. In contrast, the frequency of high cytokine producers was lower in HAM/TSP patients than in HAGs. Thus, our findings showed that LTs and cytokines are the prominent mediators in HAGs 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 HAGs and HAM patients prompted us to investigate the correlation between the concentrations of mediators in each group. The analysis of the HAG group demonstrated a positive correlation between the concentrations of CysLTs with the concentrations of LTB4 and IL-13 (Figure 5A). In contrast to our findings in HAGs, 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 infectious [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 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-LT 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) 10⁶ peripheral blood mononuclear cells were cultured for 48 hours and then treated for 30 min with A23187 (0.3 μM) before detection of LTB₄ (A, C) and LTC₄ (B, D) by ELISA (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),
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 LTB, or CysLT, 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 BLT, (A, C) and CysLT, (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, EMD, and PRL13e for CD4 T cells and of ACT3 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).
### 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 asymptomatically carriers; HAM = HAM/TSP subjects.

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for 30 minutes with 0.5 M 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 LTD$_4$ and LTE$_4$ in cell-free supernatants and LTD$_4$ and CysLT$_1$ in plasma per the manufacturer’s instructions. For plasma measurements, samples stored at 2-7°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-1, 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-25 using an OpgELIA ELISA kit (BD Bioscience) in accordance with the manufacturer’s instructions. The reactions were performed in 96-well ELISA plates (Coming) 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 density 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 Liu-Zamora et al. [50]. The concentrations of LTs, chemokines and cytokines (pg/mL) were assembled to calculate the global median index (values of N donors HAGCs 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 ascending frequency of the non-infected group was used as the reference curve to identify changes in the mediator profiles 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-test 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 Pearson’s test was used to analyze the association of LTs and the HLV-I 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 quartiles 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 Liu-Zamora 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. Analyzed the data: BCT OAMF SK OMI. Contributed reagents/materials/analysis tools: OAMF SK DTC. Wrote the paper: BCT LHF SK OAMF.

References

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