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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 LTB_4 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 LTB_4 and LTC_4 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.

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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 A_2 (PLA_2) is oxidized by 5-lipoxygenase (5-LO) in combination with 5-LO-activating protein (FLAP) to generate the leukotriene A_4 (LTA_4). The downstream enzymes LTA_4 hydrolase (LTA_4H) and LTC_4 synthase (LTC_4S) give rise to leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4). LTC_4 is further converted to LTD_4 and LTE_4, which are collectively termed cysteinyl leukotrienes (CysLTs) respectively. LTB_4 and CysLTs signal through distinct cell surface receptors named BLT_1 and BLT_2 and $CysLT_1$ and $CysLT_2$, respectively [13]. Functionally, LTB_4 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 HACs 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 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. LTB_4 was increased in the plasma of HACs and HAM/TSP patients when compared to that of NI donors; however, no difference was observed in LTB_4 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 LTB_4 (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 LTB_4 (Figure 2A) and LTC_4 (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 LTB_4 by cells from HACs and HAM/TSP patients when compared to those from NI donors, with the highest amount of LTB_4 in the supernatant of cells from HACs (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 LTC_4 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 LTC_4 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 BLT_1 and $CysLT_1$ expression in both $CD4^+$ and $CD8^+$ T cells (Figure 3). In $CD4^+$ T cells, BLT_1 expression was increased only in HAM/TSP patients than in NI donors (Figure 3A) but $CysLT_1$ 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 BLT_1 gene expression among all of the groups (Figure 3C), whereas decreased $CysLT_1$ 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; LTB_4 = 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 mediators in each group. The analysis of the HAC group demonstrated positive correlation between the concentrations of CysLTs with the concentrations of LTB_4 and IL-13 (Figure 5A). In contrast to our findings in HACs, CysLT concentrations were not correlated with the amounts of other mediators, but LTB_4 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

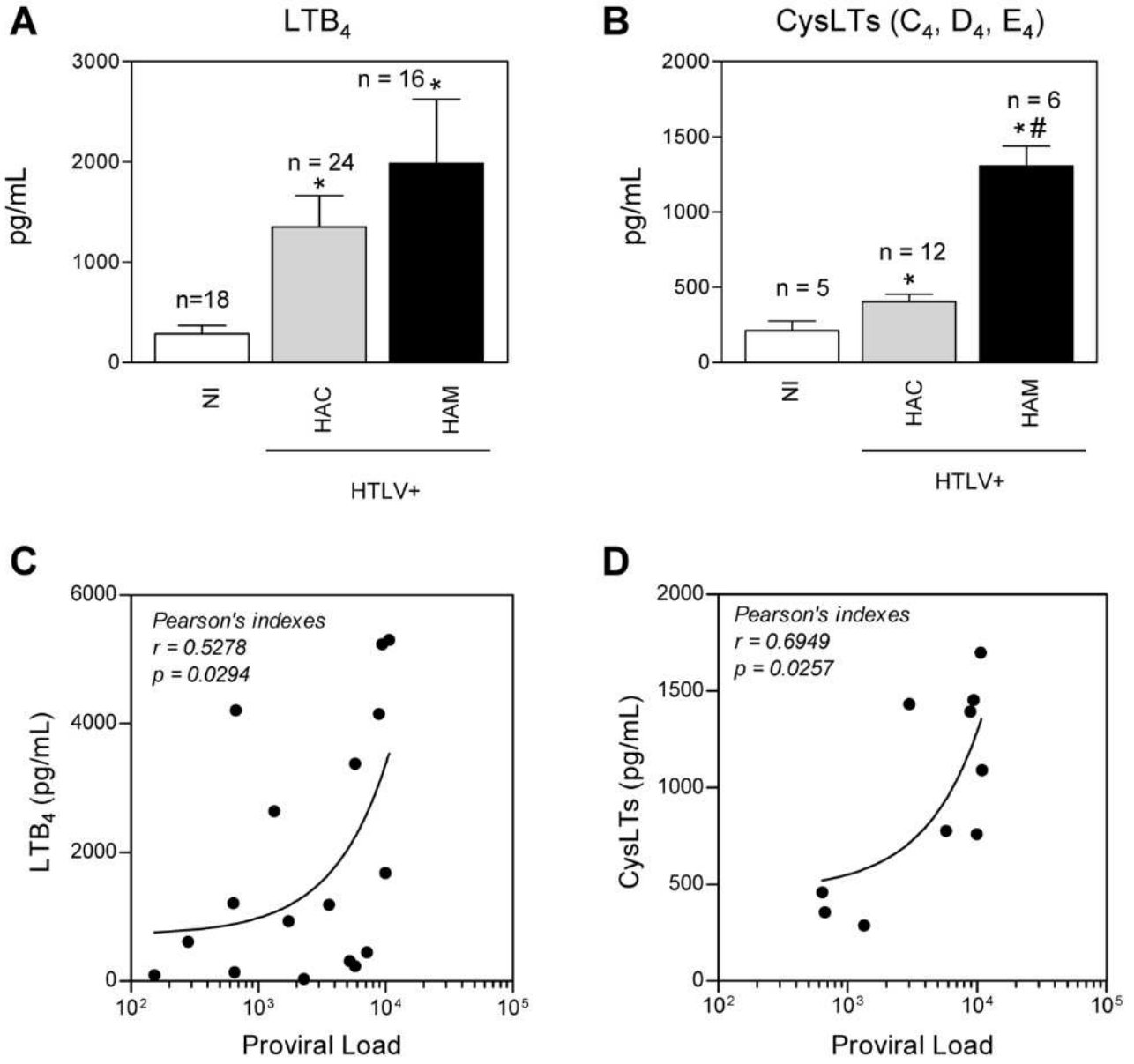

Figure 1. Leukotrienes are increased in the plasma of HTLV-1-infected subjects and correlate with the proviral load. LTB_4 (A) and CysLTs (B) were measured by EIA after sample purifications on Waters C18 Sep-Pak cartridges. Plasma from non-infected healthy donors (NI), HTLV-1 asymptomatic carriers (HAC) and HAM/TSP subjects (HAM) were used. (C, D) A correlation is shown between the plasma levels of LTB_4 (C), CysLTs (D) and the DNA proviral load in peripheral blood mononuclear cells of HTLV-1-infected individuals (HAC and HAM/TSP) using Pearson rank correlation test. The data are presented as means ± SEM. *p, 0.05, compared with control; # p, 0.05, compared with HAC (one-way ANOVA). Statistically significant correlations at p, 0.05 are displayed in the graphs along with Pearson's coefficient (r).
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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 LTB_4 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

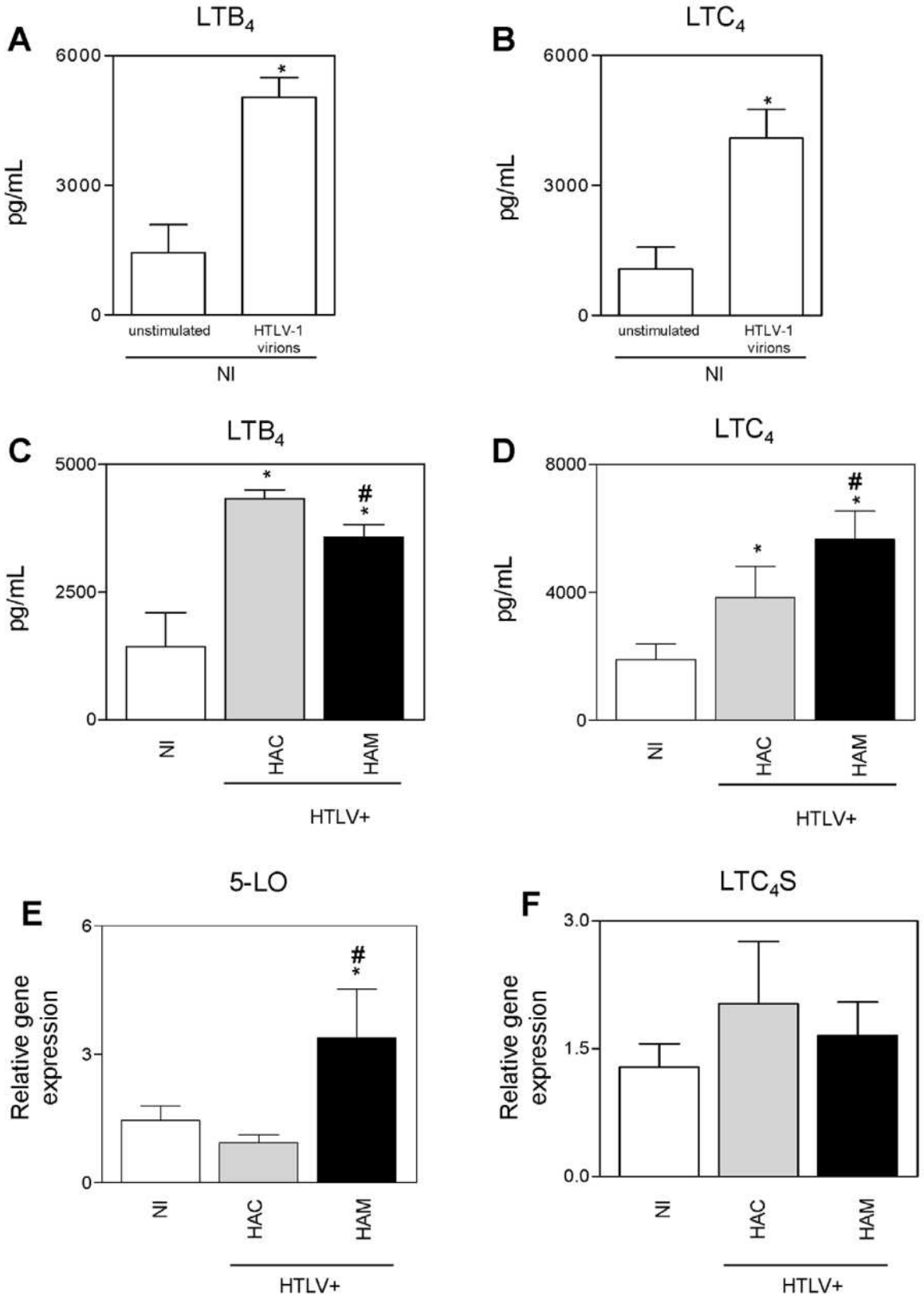

Figure 2. HTLV-1 primes cells for leukotriene generation. (A–D) 10^6 peripheral blood mononuclear cells were cultured for 48 hours and then treated for 30 min with A23187 (0.5 M) before detection of LTB_4 (A, C) and LTC_4 (B, D) by EIA (n = 7 per group). (A, B) Cells from non-infected healthy donors 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-1 asymptomatic carriers (HAC) and HAM/TSP patients (HAM) were cultured. (E,F) Quantitative PCR (qPCR) was performed to detect 5-LO (E) and LTC_4 synthase (F). The relative expression levels of these genes were determined in PBMCs from NI donors, HACs and HAM/TSP patients (n = 15 per group). Gene expression levels were normalized to the expression level of GAPDH mRNA in the same real-time PCR reaction. The data are presented as means 6 SEM. *p, 0.05, compared with unstimulated samples or NI donors; # p, 0.05, compared with HACs (t-test or one-way ANOVA as appropriate).
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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_4 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 HLTV 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 HLTV infection, as further studies will be necessary to validate this hypothesis.

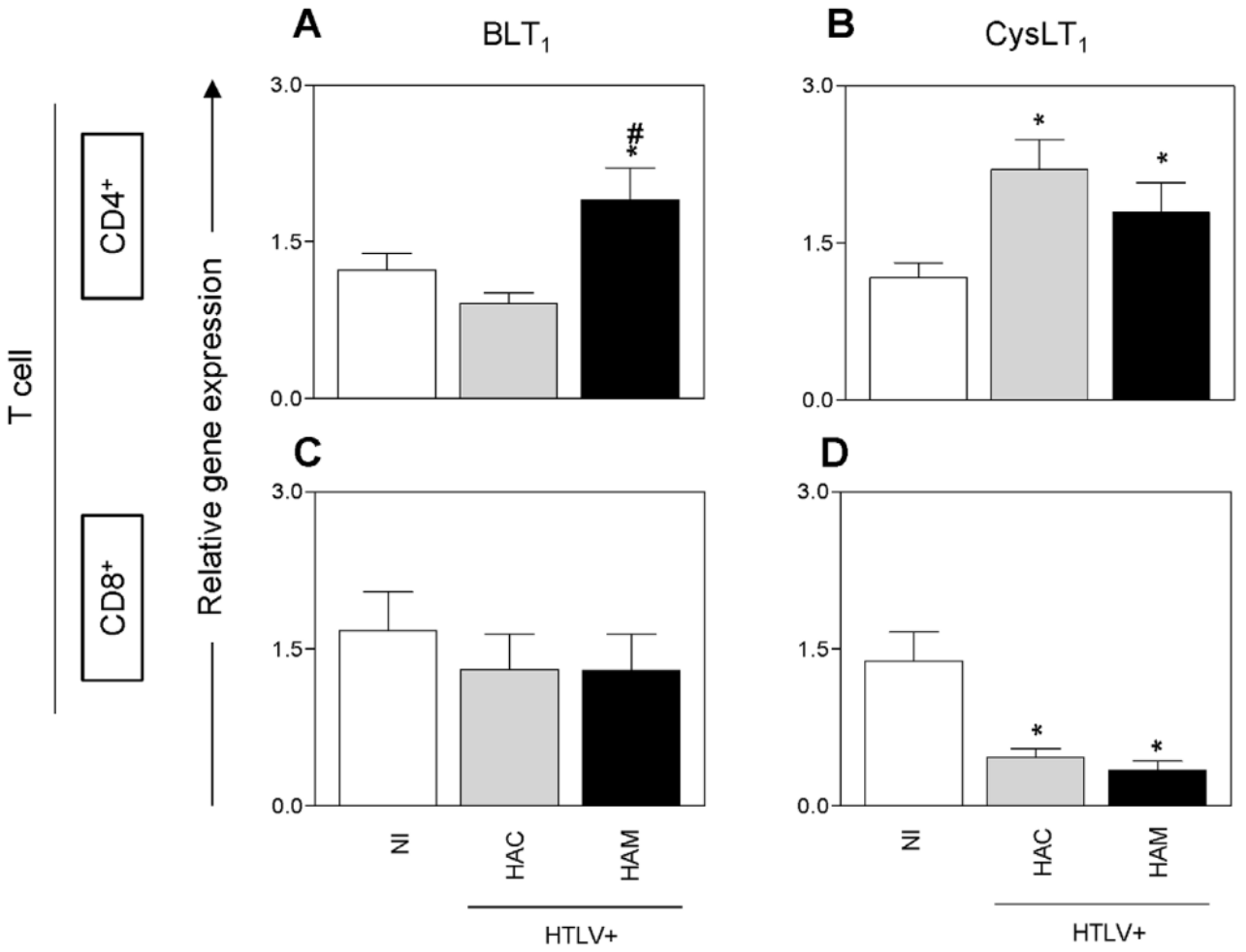

Figure 3. Leukotriene receptor mRNA expression in lymphocytes of HTLV-1 patients. Quantitative PCR (qPCR) was performed for BLT_1 (A, C) and $CysLT_1$ (B, D), and their relative expression levels were determined in CD4 (A, B) and CD8 (C, D) T cells from twenty non-infected healthy donors (NI), twenty 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 6 SEM. *p, 0.05, compared with NI donors; # p, 0.05, compared with HACs (one-way ANOVA).
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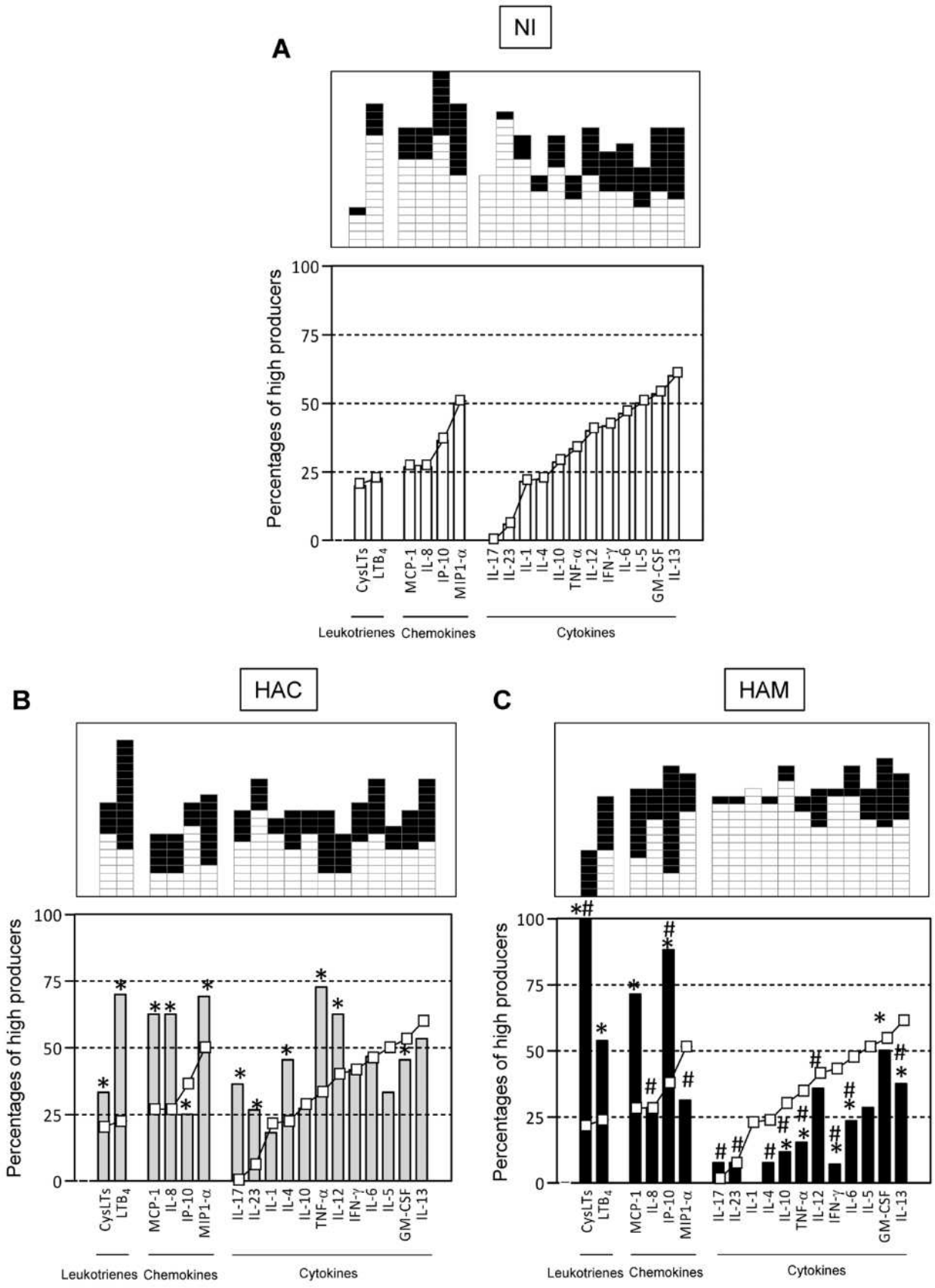
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NI
Percentages of high producers
100
75
50
25
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CysLTs
LTB4
MCP-1
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IL-23
IL-1
IL-4
IL-10
TNF-α
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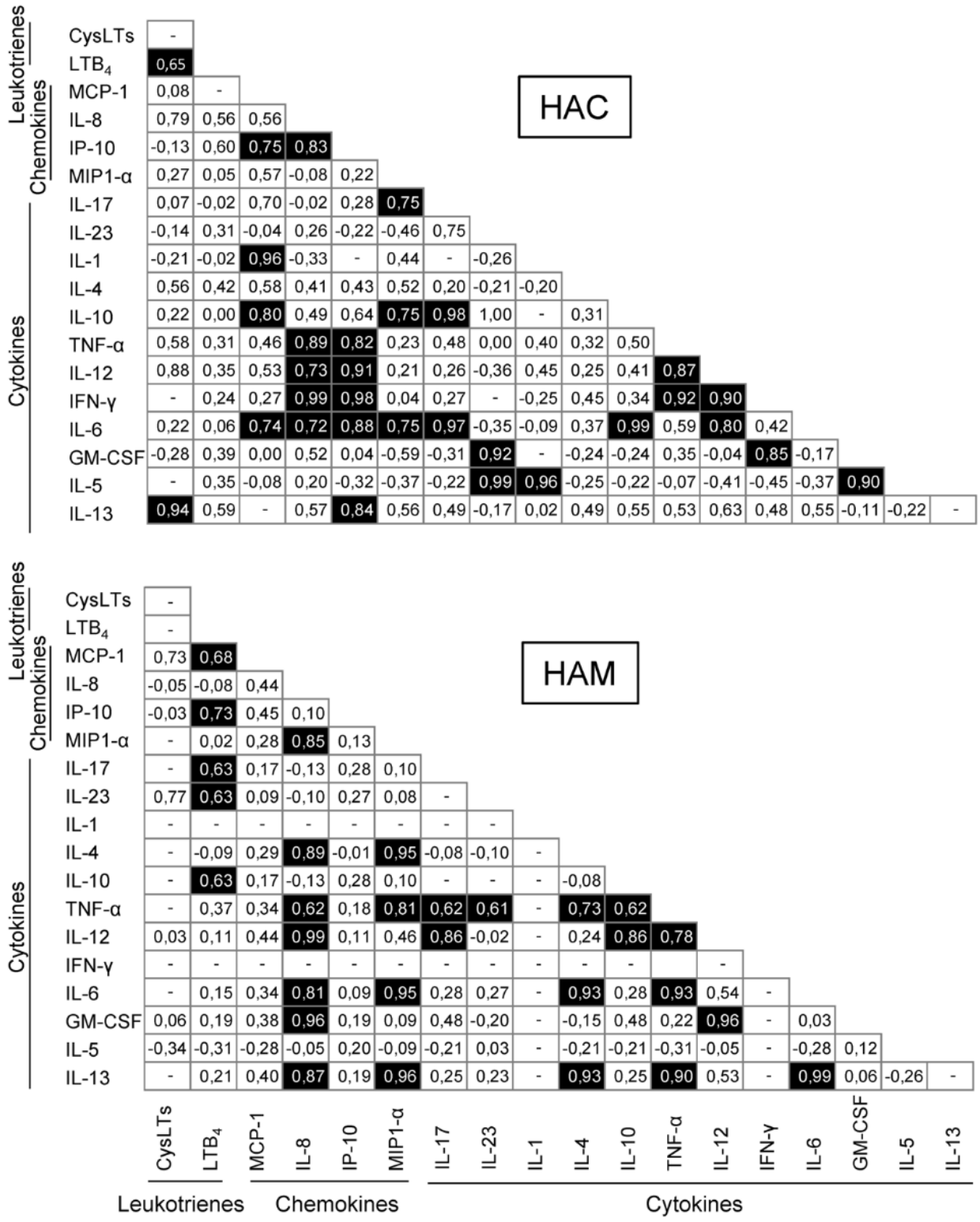

Figure 5. Correlation of plasma biomarkers in HTLV-1 infection. Samples from HTLV-1-infected subjects were used to detect leukotrienes, chemokines and cytokines by ELISA. A correlation analysis was performed to analyze biomarkers levels. The results of a non-parametric Spearman's test and 'r' index are provided in the figure. Filled squares indicate positive correlations. HAC – HTLV-1 asymptomatic carriers; HAM – HAM/TSP subjects.

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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 LTB_4 and LTC_4 in cell-free supernatants and LTB_4 and CysLT in plasma per the manufacturer's instructions. For plasma measurements, samples stored at 2 70uC 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-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 6 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 LDFN TMM MTP OMT. Analyzed the data: BCT OAMF SK OMT LHF. Contributed reagents/materials/analysis tools: OAMF SK DTC. Wrote the paper: BCT LHF SK OAMF.

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