

TABLE IV. HTLV-I Proviral Loads and HTLV-I Tax-Specific CD8+ Cells in PBMCs and Bronchoalveolar Fluid Cells

No.	PBMCs					
	CD4+ (%)	CD8+ (%)	CD4/8	PVL ^a	PVL/CD4 ^b	Pentamer+/CD8 ^c (%)
1	30.64	13.71	2.23	16.37	82.89	0.90
2	29.52	57.30	0.52	3.92	16.80	2.78
3	32.66	29.56	1.10	3.91	15.29	0.15
4	19.53	14.99	1.30	7.25	86.72	0.96
5	54.83	17.19	3.18	4.94	11.02	3.54
6	41.43	32.34	1.28	39.60	129.38	0.64
Mean	34.77	27.52	1.60	12.70	57.02	1.50
SD	12.07	16.55	0.95	14.00	19.52	1.34

Bronchoalveolar fluid cells ^d						
CD4+ (%)	CD4+ (%)	CD4/8	PVL	PVL/CD4	Pentamer+/CD8 (%)	Pentamer+ ratio ^d
69.02	10.65	6.48	22.75	66.34	2.90	3.22
72.09	9.27	7.86	2.40	8.58	18.99	6.83
24.24	14.99	1.61	3.53	37.91	0.16	1.07
23.26	63.74	0.36	3.57	33.47	2.52	2.63
68.71	25.00	2.75	29.18	68.76	19.56	5.52
37.85	39.50	0.96	27.92	93.24	1.61	2.52
49.20	27.19	3.34	14.90	51.38	7.62	3.63
23.33	21.15	3.10	13.00	30.37	9.08	2.13

^aHTLV-I proviral loads (PVL) in total cells (copy/10² cells).

^bHTLV-I PVL in CD4+ cells (copy/10² CD4+ cells).

^cPercentage of pentamer+ HTLV-I Tax-specific CD8+ cells in CD8+ cells.

^dRatio of percentage of pentamer+ HTLV-I Tax-specific CD8+ cells in bronchoalveolar fluid cells to that in PBMCs.

was within the normal range. Lymphocytosis was observed in five of six patients except in patient 3 (Table III). Percentages of CD4+ and CD8+ cells were analysed by flow cytometry, and CD4/8 ratios in PBMCs and bronchoalveolar lavage fluid cells were 1.60 and 3.34, respectively (Table IV).

HTLV-I Proviral Loads in Bronchoalveolar Lavage Fluid Cells and PBMCs

The HTLV-I proviral load in PBMCs and bronchoalveolar lavage fluid cells of the patients was determined by quantitative PCR. HTLV-I was detected in all tested samples (Table IV). No difference was observed in the HTLV-I proviral load in the DNA extracted from PBMCs and bronchoalveolar lavage fluid cells (12.70 and 14.90 copies/10² cells, respectively; $P = 0.92$). The cellular composition differs between PBMCs and bronchoalveolar lavage fluid cells, and HTLV-I predominantly infects CD4+ cells in vivo [Richardson et al., 1990]. Therefore, the HTLV-I proviral load was calculated in 10² CD4+ cells from PBMCs and bronchoalveolar lavage fluid cells according to the following formula: (proviral load in 10² total cells)/[(percentage of lymphocytes in total cells) × (percentage of CD4+ cells in lymphocytes)] × 10⁴. No significant difference was observed in the proviral load in 10² CD4+ cells from PBMCs and bronchoalveolar lavage fluid cells (57.02 and 51.38 copies, respectively; $P = 0.75$; Table IV).

Increased HTLV-I-Specific CD8+ Cells in Bronchoalveolar Lavage Fluid Cells Than in PBMCs

Whether HTLV-I-specific CD8+ cells were increased in the lungs of patients infected with HTLV-I was investigated using HTLV-I Tax epitope/HLA pentamers. Figure 1 shows the results of a representative flow cytometric analysis in which the frequency of HTLV-I Tax-specific CD8+ cells was determined in CD8 high lymphocytes. The frequency of the CD8+ cells in bronchoalveolar lavage fluid cells was increased compared with that in PBMCs (Fig. 2A, $P = 0.028$). The mean frequency of HTLV-I-specific CD8+ cells was 1.50% in PBMCs and 7.62% in bronchoalveolar lavage fluid cells (Table IV). The frequency in bronchoalveolar lavage fluid cells was 5.1 times higher than that in PBMCs. In addition, the frequency of HTLV-I Tax-specific CD8+ cells in bronchoalveolar lavage fluid cells correlated well with that in PBMCs (Fig. 2B, $P = 0.035$).

Correlation of HTLV-I Proviral Loads With HTLV-I-Specific CD8+ Cells

Whether the HTLV-I proviral load correlated with HTLV-I-specific CD8+ cell responses was investigated. The frequency of HTLV-I-specific CD8+ cells tended to correlate negatively with the proviral load in both PBMCs and bronchoalveolar lavage fluid cells, although this was not statistically significant (Fig. 3).

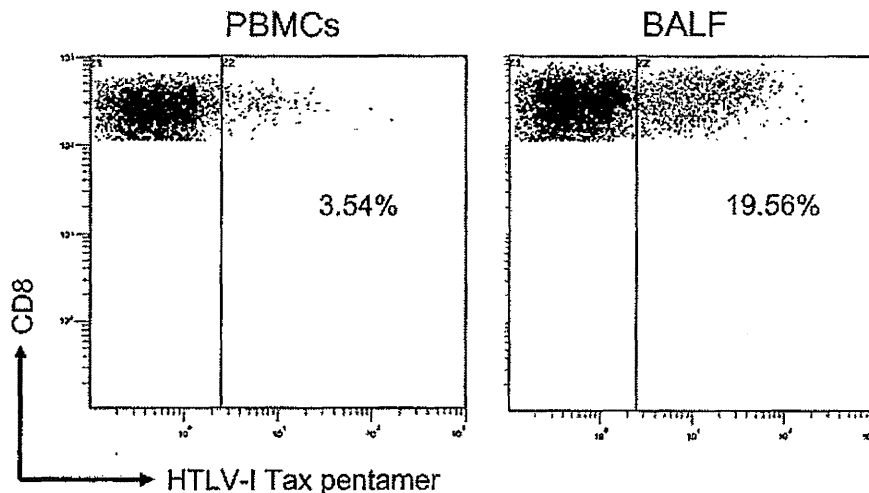


Fig. 1. Representative flow cytometric detection of HTLV-I Tax-specific CD8+ cells in PBMCs and bronchoalveolar lavage fluid cells. PBMCs and bronchoalveolar lavage fluid cells (BALF) were obtained from six subjects with HTLV-I infection. Lymphocytes were gated on the basis of forward and side scatter, and the CD8 high population was gated. The frequency of HTLV-I Tax pentamer-positive cells was determined in the CD8+ cells in PBMCs and bronchoalveolar lavage fluid cells. The figures are representative data from patient 5, and the numbers indicate the pentamer-positive cells as a percentage of total CD8 high cells.

In situ Tetramer Staining of HTLV-I Tax-Specific CD8+ Cells

In situ detection of CD8+ cells using HTLV-I Tax tetramer was performed using lung tissue from a patient with HAM/TSP and bronchiolitis obliterans. CD4+ and CD8+ cells were distributed in the alveolar septa. As shown in Figure 4, HLA-A*2402/HTLV-I Tax301-309-specific CD8+ cells detected in lung tissue formed clusters. Tetramer staining was co-localized with CD8 molecules in the positive cells from the

tissue (Fig. 4A). In contrast, no cells in lung tissue from the same patient were stained with the negative control HLA-A*2402/HIV Gag tetramer (Fig. 4B). In addition, no cells were stained with HLA-A*2402/HTLV-I Tax301-309 tetramer in lung tissue from a patient who was HLA-A*2402 positive but HTLV-I negative (Fig. 4C). Whether HTLV-I proteins could be detected in lung tissue was examined using anti-HTLV-I Tax and Gag antibodies. However, no HTLV-I proteins were found (data not shown).

DISCUSSION

In this study, HTLV-I-specific CD8+ cells were present at a markedly higher frequency in bronchoalveolar lavage fluid cells than in PBMCs of all patients studied (Fig. 2A and Table IV). In terms of bacterial species cultured from sputum samples, patient 2 was positive for *Mycobacterium intracellulare*, patient 4 was positive for *Haemophilus influenzae*, and the other patients were positive for normal oral flora (Table II). HTLV-I-specific CD8+ cells were found to be circulating in the body of a patient infected with HTLV-I. A small minority of the CD8+ cells may infiltrate non-specifically inflamed tissues, even if inflammation was caused by microorganisms other than HTLV-I. However, in this study, HTLV-I-specific CD8+ cells accumulated at a 5.1 times higher frequency in bronchoalveolar lavage fluid cells than in PBMCs (Table IV), which suggests that the CD8+ cells selectively infiltrated the lungs and that HTLV-I-specific immune responses occurred in the lungs of these patients. In situ tetramer staining

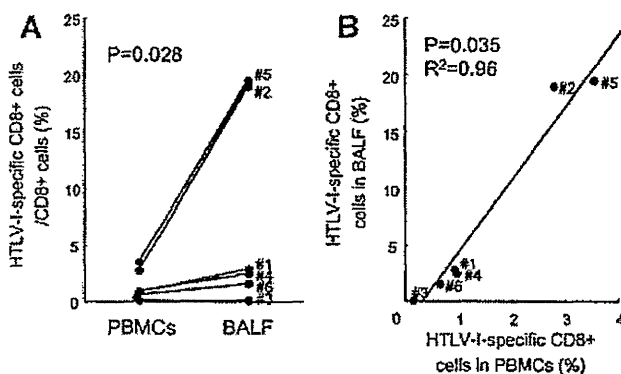


Fig. 2. Frequency of HTLV-I Tax-specific CD8+ cells in PBMCs and bronchoalveolar lavage fluid cells. A: The frequency of HTLV-I Tax-specific CD8+ cells was significantly increased in bronchoalveolar lavage fluid cells (BALF) than in PBMCs according to Wilcoxon signed-rank test. B: The frequency of HTLV-I Tax-specific CD8+ cells in bronchoalveolar lavage fluid cells significantly correlated with that in PBMCs according to Spearman's rank correlation test.

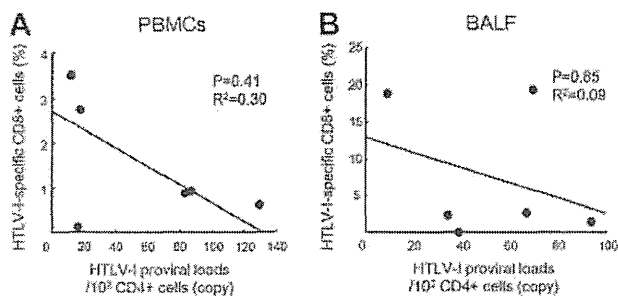


Fig. 3. The relationship between HTLV-I proviral load and frequency of HTLV-I Tax-specific CD8+ cells in PBMCs and bronchoalveolar lavage fluid cells. The frequency of HTLV-I Tax-specific CD8+ cells in CD8+ cells was plotted against HTLV-I proviral loads in CD4+ cells in PBMCs (A) and in bronchoalveolar lavage fluid cells (BALF) (B). Negative correlations were observed in both PBMCs and bronchoalveolar lavage fluid cells, but these were not statistically significant according to Spearman's rank correlation test.

revealed that HTLV-I-specific CD8+ cells formed clusters in the lung tissue, further supporting the above proposition (Fig. 4). In addition, the frequency of HTLV-I-specific CD8+ cells in the bronchoalveolar lavage fluid cells correlated with that in PBMCs (Fig. 2B), which suggests that the increased frequency of HTLV-I-specific CD8+ cells in peripheral blood drives an efficient infiltration of these CD8+ cells into the lungs.

No bronchoalveolar lavage fluid was obtained from patients infected with HTLV-I without pulmonary involvement. Therefore, whether accumulation of HTLV-I-specific CD8+ cells in the lungs is associated with pulmonary involvement or a common phenomenon in patients infected with HTLV-I remains unclear. Further studies are needed to elucidate this point. Cells stained with antigen/HLA class I complex multimers contain not only effector/memory cells but also naïve cells; therefore, some multimer-positive

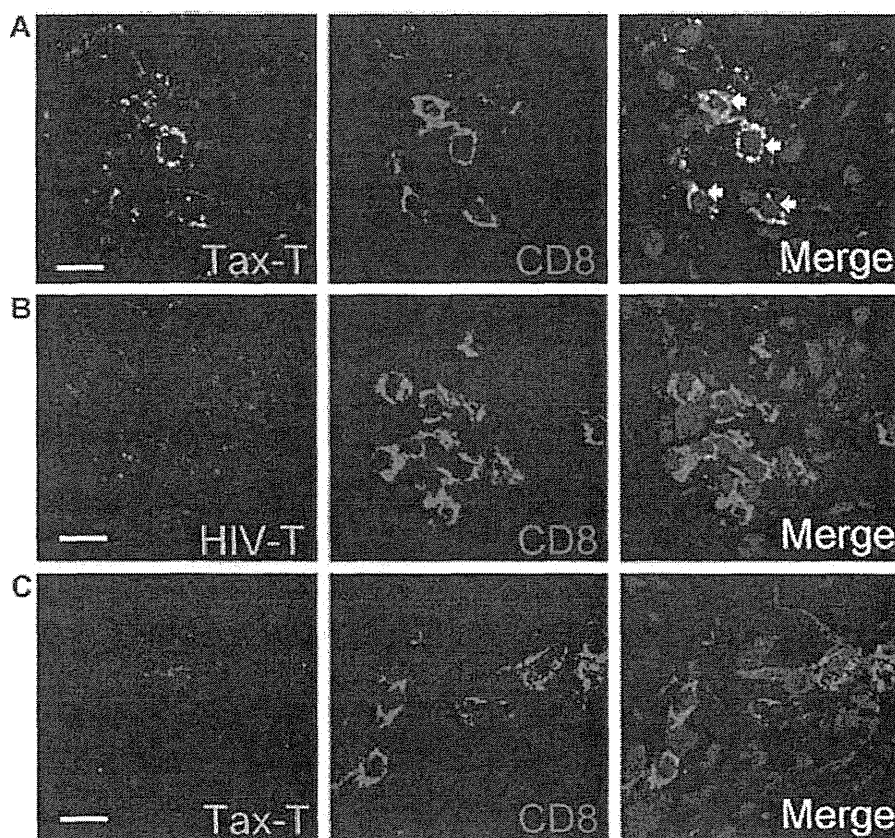


Fig. 4. In situ detection of HTLV-I Tax-specific CD8+ cells in a patient with HAM/TSP and bronchiolitis obliterans. A video-assisted thoracoscopic biopsy was performed in a 65-year-old woman with HAM/TSP and bronchiolitis obliterans who was HLA-A*2402 positive. Sections from the frozen lung tissue were stained with tetramers (Tax-T; green) and anti-CD8 antibody (red). A: Staining with HLA-A*2402/HTLV-I Tax301–309 tetramer overlapped with CD8 staining, yielding a yellow color in the merged image (left, arrowed). Tetramer-positive cells were observed in the alveolar septa localized within a cluster. B: No cells were stained with HLA-A*2402/HIV Gag tetramer (HIV-T) in the serial section from the same patient. C: No cells were stained with HLA-A*2402/HTLV-I Tax301–309 tetramer (Tax-T) in the control sample from a patient positive for HLA-A*2402 but negative for HTLV-I infection. Original magnification is $\times 400$. The bars indicate 10 μm .

cells are inert cytotoxic T lymphocytes [Goulder et al., 2000]. It would be important to know how many HTLV-I-specific CD8+ cells are functional cytotoxic T lymphocytes against HTLV-I and whether the cytotoxic activity of the cells differs between bronchoalveolar lavage fluid cells and PBMCs. However, because the sample number was limited, this could not be determined in this study. Further analysis including cytotoxic activity is needed to understand the role of accumulation of the CD8+ cells in the lungs.

A range of pulmonary involvement in patients infected with HTLV-I has recently been described in a study using computed tomography to analyse samples from 320 HTLV-I carriers [Okada et al., 2006]. The pulmonary manifestations included centrilobular nodules, thickening of bronchovascular bundles, ground-glass opacity, bronchiectasis, interlobular septal thickening, and consolidation. Pathologically, these findings correspond to lymphocytic infiltration along respiratory bronchioles and bronchovascular bundles. In the present study, all patients showed changes in the peripheral bronchioles in computed tomography images. Peripheral bronchioles and alveoli have been emphasized as an important lesion site in HTLV-I-associated lung disease, probably because the marked lymphocytosis in bronchoalveolar lavage fluid is a characteristic of the disease [Sugisaki et al., 1998]. However, the respiratory tract may be another affected lesion in HTLV-I-associated lung disease.

HTLV-I-infected cells are activated and express various chemokine receptors and increased adhesion molecules on the surface in peripheral blood, suggesting that the cells have an enhanced ability to migrate into tissue sites [Seki et al., 1999; Yamazato et al., 2003]. Previous studies reported that lymphocytosis in bronchoalveolar lavage fluid is observed not only in HTLV-I-infected patients with abnormal lung images but also in infected individuals with apparently normal lungs [Seki et al., 2000a]. In the present study, the patients who had less abnormal lung images showed an accumulation of HTLV-I-specific CD8+ cells in the lungs (patients 1, 5, and 6 in Table IV). Taken together, these results suggest that inflammation can be induced in the lung by HTLV-I infection even in the absence of apparent clinical manifestations [Sugisaki et al., 1998].

In summary, HTLV-I-specific CD8+ cells accumulate and HTLV-I-infected cells exist in the lungs of patients infected with HTLV-I with pulmonary involvement. These results suggest that an interaction between HTLV-I-infected cells and HTLV-I-specific CD8+ cells may occur in the lung of patients infected with HTLV-I. Further studies to clarify clinical features of the lung specifically associated with HTLV-I infection are needed.

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Positive feedback loop via astrocytes causes chronic inflammation in virus-associated myelopathy

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Human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare neurodegenerative disease characterized by chronic inflammation in the spinal cord. We hypothesized that a positive feedback loop driven by chemokines may be responsible for the chronic inflammation in HAM/TSP. We aimed to determine the identity of these chemokines, where they are produced, and how they drive chronic inflammation in HAM/TSP. We found that patients with HAM/TSP have extraordinarily high levels of the chemokine CXCL10 (also known as IP-10) and an abundance of cells expressing the CXCL10-binding receptor CXCR3 in the cerebrospinal fluid. Histological analysis revealed that astrocytes are the main producers of CXCL10 in the spinal cords of patients with HAM/TSP. Co-culture of human astrocytoma cells with CD4⁺ T cells from patients with HAM/TSP revealed that astrocytes produce CXCL10 in response to IFN- γ secreted by CD4⁺ T cells. Chemotaxis assays results suggest that CXCL10 induces migration of peripheral blood mononuclear cells to the central nervous system and that anti-CXCL10 neutralizing antibody can disrupt this migration. In short, we inferred that human T-lymphotropic virus type 1-infected cells in the central nervous system produce IFN- γ that induces astrocytes to secrete CXCL10, which recruits more infected cells to the area via CXCR3, constituting a T helper type 1-centric positive feedback loop that results in chronic inflammation.

Keywords: HTLV-1; HAM/TSP; CXCL10; CXCR3; astrocyte

Abbreviation: HAM/TSP = human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis

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Introduction

The rise of chronic inflammatory disorders has prompted researchers to reconsider the classical concept of inflammation, which dates back to ancient Roman times when inflammation was first defined as redness, swelling, heat and pain in response to injury or infection. In general, inflammation is an adaptive immune response to tissue malfunction that ideally neutralizes the source of the disturbance and restores tissue homeostasis. Paradoxically, a prolonged state of inflammation has been implicated in the pathogenesis of various diseases characterized by the loss of homeostasis, such as autoimmune diseases, cancers and neurodegenerative diseases (Libby, 2002; Mantovani *et al.*, 2008; Medzhitov, 2008, 2010). To produce effective therapies for these debilitating disorders, we must first elucidate the mechanisms by which this maladaptive chronic inflammatory state develops.

Although there are many chronic inflammatory disorders for which the initiating trigger is ill-defined or unknown, human T-lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare neurodegenerative chronic inflammatory disease clearly caused by HTLV-1 retroviral infection (Gessain *et al.*, 1985; Osame *et al.*, 1986). In other words, the HTLV-1-infected cells in patients with HAM/TSP represent a useful starting point from which to investigate the origins of chronic inflammation.

HTLV-1 infects 10–20 million people worldwide, some of whom develop serious conditions such as adult T cell leukaemia (Hinuma *et al.*, 1981) and up to 2–3% of whom develop the debilitating inflammation in the spinal cord that characterizes HAM/TSP (Gessain *et al.*, 1985; Osame *et al.*, 1986). Evidence has accumulated to support the theory that infected CD4⁺ T cells (as opposed to infected neuronal cells or non-infected peripheral blood mononuclear cells) are primarily responsible for this transition to the HAM/TSP disease state: HTLV-1 primarily infects CD4⁺ T cells (Richardson *et al.*, 1990); levels of infected CD4⁺ T cells circulating in the blood of patients with HAM/TSP are higher than those in the blood of asymptomatic carriers (Nagai *et al.*, 1998; Yamano *et al.*, 2002), the levels in the CSF surrounding the spinal cord are higher still (Nagai *et al.*, 2001a); and these infected CD4⁺ T cells have also been detected in the spinal cord lesions themselves (Moritoyo *et al.*, 1996; Matsuoka *et al.*, 1998). There are many cell types capable of producing an inflammatory response upon contact with viral antigens, and it is true that the cases where these antigen-specific cells are most abundant are indeed in patients with HAM/TSP, but there is a large range of overlap in which patients with HAM/TSP and asymptomatic carriers have the same amount of antigen-specific cells in their peripheral blood mononuclear cells (Jacobson *et al.*, 1990; Jeffery *et al.*, 1999; Kubota *et al.*, 2000; Yamano *et al.*, 2002). Therefore, we hypothesized that their presence may not be the key factor that determines a patient's fate to experience the disease or not, and that perhaps there might be another cell type responsible for initiating the chronic inflammation in HAM/TSP through a more unique pathway. Research shows that infected CD4⁺ T cells are indeed capable of migrating across the blood–brain barrier into the CNS (Furuya *et al.*, 1997) and secreting proinflammatory

cytokines such as interferon-gamma (IFN- γ) (Hanon *et al.*, 2001; Yamano *et al.*, 2005, 2009). We guessed that these cells might even be capable of producing IFN- γ spontaneously due only to intracellular activation of transcription factors by the invading HTLV-1 virus, which has been shown to be capable of such potent effects (Waldmann, 2006).

Studies have indicated that among the CD4⁺ T cell subtypes, immune responses by CD4⁺ T helper type 1 (Th1)-like cells may be dominant in patients with HAM/TSP (Goon *et al.*, 2002; Narikawa *et al.*, 2005), leading to the theory that the Th1 axis should be the primary focus in the study of HAM/TSP. These Th1 cells express both the CC chemokine receptor type 5 (CCR5) and CXC motif receptor 3 (CXCR3), which respond to the presence of CC motif ligand (CCL) 3, 4 and 5 and CXC motif ligand (CXCL) 9, 10 and 11, respectively. These ligands are chemokines, a subclass of cytokines that stimulate directed chemotaxis in responsive cells, and it is known that chemokine receptor–ligand interactions play an important role in recruiting immune cells to inflammatory sites (Luster, 1998; Qin *et al.*, 1998). Of particular interest are the CXCR3 agonists, which are regulated by the aforementioned proinflammatory cytokine IFN- γ and carry this relationship in the alternative nomenclature: monokine induced by gamma interferon (MIG/CXCL9), IFN- γ -inducible protein 10 (IP-10/CXCL10), and interferon-inducible T cell alpha chemoattractant (I-TAC/CXCL11) (Proost *et al.*, 2001, 2003). We and others have shown that CCL5, CXCL9, and especially CXCL10, are elevated in the CSF of patients with HAM/TSP (Teixeira *et al.*, 2004; Narikawa *et al.*, 2005; Tanaka *et al.*, 2008; Sato, *in press*).

We hypothesized that these chemokines play a key role in the pathogenesis of HAM/TSP by recruiting more cells infected with HTLV-1 to the inflammation site and potentially initiating a positive feedback loop. We first compared the levels of several chemokines in the serum and CSF of patients with HAM/TSP and asymptomatic carriers and found that CXCL10 was the most closely associated with known features of HAM/TSP pathogenesis, namely increased CSF cell count. We then analysed samples of peripheral blood mononuclear cells and CSF cells along with images of the spinal cord tissue to demonstrate that CD4⁺ cells expressing CXCL10-binding CXCR3, namely cells of the Th1 subtype, are indeed infected with HTLV-1, do migrate across the blood–brain barrier into the CNS, and do produce IFN- γ in patients with HAM/TSP. We demonstrated that this IFN- γ production can occur in the absence of external stimuli. Immunohistochemical analysis of the spinal cord tissue not only confirmed that CXCL10 production is elevated in patients with HAM/TSP but also revealed that astrocytes may be the main producers of CXCL10 in the spinal cord. We used novel techniques to demonstrate that these astrocytes likely represent the missing piece of the puzzle in the positive feedback loop: infected CD4⁺ T cells produce IFN- γ , which stimulates astrocytes to produce CXCL10, which recruits more CD4⁺CXCR3⁺ Th1 cells to the CNS. Finally, chemotaxis assays were used to compare the inhibitory potentials of anti-CXCL10 and anti-CXCR3 neutralizing antibodies on this positive feedback loop as the first step toward the development of an effective therapy.

Materials and methods

Patient selection and sample preparation

Written informed consent was obtained from all patients before the study, which was reviewed and approved by the Institutional Ethics Committee (St. Marianna University) and conducted in compliance with the tenets of the Declaration of Helsinki. The study included 26 HTLV-1 non-infected healthy donors (14 females and 12 males; mean age, 49 years), 29 asymptomatic carriers (21 females and eight males; mean age, 50 years), 17 patients with adult T cell leukaemia with no history of chemotherapy (eight females and nine males; mean age, 68 years), and 58 patients with HAM/TSP (47 females and 12 males; mean age, 62 years). Diagnosis of adult T cell leukaemia was based on the criteria established by Shimoyama (1991). HTLV-1 seropositivity was determined by a particle agglutination assay (Serodia-HTLV-1) and confirmed by western blot (SRL Inc.). HAM/TSP was diagnosed according to WHO guidelines (Osame, 1990).

Samples of peripheral blood mononuclear cells were prepared using density gradient centrifugation (Pancoll; PAN-Biotech) and viably cryopreserved in liquid nitrogen with freezing medium (Cell Banker 1; Mitsubishi Chemical Medience Corporation). Plasma and serum samples were obtained from 16 healthy donors, 26 asymptomatic carriers, 30 patients with HAM/TSP and 14 patients with adult T cell leukaemia (six smouldering type and eight chronic type). Multiple serum and CSF samples were taken within a 1-h window for each of 32 patients with HAM/TSP. A Fuchs–Rosenthal chamber (Hausser Scientific Company) was used for CSF cell counts, after which the cells were isolated by centrifugation and cryopreserved in the aforementioned freezing medium. A medulla oblongata tissue sample from one patient with HAM/TSP as well as thoracic spinal cord tissues from four patients with HAM/TSP and six control individuals with no spinal cord lesions (numbered controls 1–6; one female and five males; mean age, 67 years) were obtained post-mortem, fixed in 10% formalin, and embedded in paraffin. Clinical characteristics of the patients with HAM/TSP who underwent post-mortem examination are shown in Supplementary Table 1.

Cell culture

Before culture, peripheral blood mononuclear cells from patients with HAM/TSP, asymptomatic carriers and healthy donors were sorted using MACS beads (Miltenyi Biotec) according to the manufacturer's instructions; CD4⁺ T cells and CD8⁺ T cells were separated negatively, and CD14⁺ cells were separated positively, and the purity of all cell populations exceeded 95%. The isolated cells were seeded at 1×10^5 cells/200 μ l/well in 96-well round-bottom plates in RPMI 1640 medium (Wako Pure Chemical Industries Ltd.) supplemented with 10% heat-inactivated human serum (Wako Pure Chemical Industries Ltd.), and 1% penicillin/streptomycin antibiotic solution (Wako Pure Chemical Industries Ltd.) without any stimuli. The culture supernatants were collected after incubating at 37°C for 24, 48 and 72 h in 5% CO₂.

U251 human astrocytoma cells were cultured in Dulbecco's minimal essential medium (Wako Pure Chemical Industries Ltd.) supplemented with 10% heat-inactivated foetal bovine serum (Gibco-Invitrogen) and 1% penicillin/streptomycin. In total, 2×10^4 U251 cells were then co-cultured in 48-well flat-bottom plates at 37°C for 48 h in 5% CO₂ with 0, 2×10^2 , 2×10^3 or 2×10^4 CD4⁺ T cells isolated

from peripheral blood mononuclear cells of patients with HAM/TSP or healthy donors using MACS beads. A control group of 2×10^4 CD4⁺ T cells was single-cultured under the same conditions. The U251 cells were also cultured with and without 1 ng/ml recombinant human IFN- γ (285-IF, R&D Systems). After culture for 48 h, CD4⁺ T cells were removed by washing with PBS and the U251 cells were then cultured for an additional 24 h before collecting the culture supernatants.

For the experiment investigating the inhibitory potential of neutralizing antibodies, 2×10^4 CD4⁺ T cells isolated from peripheral blood mononuclear cells of patients with HAM/TSP using MACS beads were cultured in 96-well round-bottom plates for 72 h under the same conditions, and the culture supernatant was collected after centrifugation. Then, in this supernatant, 2×10^4 U251 cells were cultured in 48-well flat-bottom plates with 10 μ g/ml monoclonal neutralization antibodies: anti-IFN- γ antibody (MAB285, R&D Systems), anti-tumour necrosis factor (TNF)- α antibody (MAB610, R&D Systems), or isotype control antibody (MAB002 and MAB003, R&D Systems). The U251 cells were cultured for additional 24 h before collecting the culture supernatants for assay.

Measurement of chemokines, IFN- γ , IL-17A and sIL-2 receptor

The concentrations of four chemokines (CCL4, CCL5, CXCL9 and CXCL10) in the serum and CSF samples and levels of CXCL10, IFN- γ and IL-17A in the culture supernatants were measured with a cytometric bead array kit (BD Biosciences) using a FACSCalibur flow cytometer (BD Biosciences) according to the manufacturer's instructions. It should be noted that the cytometric bead array kit measures the total concentrations of all chemokine isoforms irrespective of aminoterminal variation (Proost *et al.*, 2001, 2003). The sIL-2R in the serum was measured using an ELISA (Cell-free N IL-2R, Kyowa Medex).

Flow cytometric analysis

Peripheral blood mononuclear cells and CSF cells, which were obtained on the same day, were immunostained with various combinations of the following fluorescence-conjugated antibodies: anti-CD3 (UCHT1), anti-CD4 (OKT4), anti-CD8 (RPA-T8), anti-CD19 (HIB19), anti-CD14 (61D3) (all from eBioscience), and anti-CXCR3 (1C6; BD Biosciences). The cells were stained with a saturating concentration of antibody in the dark (4°C, 30 min) and washed twice before analysis using FACSCalibur (BD Biosciences). Data were processed using FlowJo software (TreeStar). For cell sorting, JSAN (Bay Bioscience,) was used, and the purity exceeded 95%.

Real-time polymerase chain reaction

The HTLV-1 proviral DNA load was measured using ABI Prism 7500 SDS (Applied Biosystems) as described previously (Yamano *et al.*, 2002). In brief, DNA was extracted and 100 ng samples were analysed per well. The proviral DNA load was calculated using the following formula: copy number of HTLV-1 (pX) per 100 cells = (copy number of pX) / (copy number of β -actin / 2) \times 100.

Tissue staining

Formalin-fixed thoracic spinal cord and medulla oblongata tissue sections were deparaffinized in xylene and rehydrated in a series of

graded alcohols and distilled water. The antigenicity of the tissue sections was recovered using a standard microwave heating technique. For immunohistochemistry, the slides were incubated with anti-CXCL10/IP-10 antibody, followed by detection with streptavidin-biotin-horseradish peroxidase and diaminobenzidine (DakoCytomation Japan Co. Ltd.). The CXCL10⁺ cells in the spinal cord were also counted under the microscope; the data show the mean number of cells in three random 1-mm² fields per sample. Haematoxylin and eosin staining was conducted to detect inflammatory cells that had invaded the tissue samples. For immunofluorescence (thoracic spinal cord sections only), the slides were incubated in phosphate-buffered saline with 10% goat serum for 1 h at room temperature, in anti-CXCR3 antibody (Abcam), anti-CXCL10/IP-10 antibody (Santa Cruz Biotechnology), and anti-glial fibrillary acidic protein (GFAP) antibody (DakoCytomation Japan Co. Ltd) overnight at 4°C, labelled with Alexa Fluor® 488 or Alexa Fluor® 594 conjugated secondary antibody (Invitrogen), and examined under a fluorescence microscope (Nikon eclipse E600 with fluorescence filter Nikon F-FL; Nikon Instech) with rabbit or mouse immunoglobulin G (IgG) as the negative control.

Chemotaxis assay

Peripheral blood mononuclear cells from patients with HAM/TSP were washed and then suspended (at 1×10^7 cells/ml) in 37°C serum-free RPMI 1640 medium containing 1 mg/ml bovine serum albumin (Wako Pure Chemical Industries, Ltd.), hereafter 'chemotaxis medium'. The lower wells of a 96-well chemotaxis chamber (MBA96; Neuroprobe) were filled with chemotaxis medium containing 0.25 µg/ml recombinant human CXCL10 protein (266-IP; R&D Systems). For the negative control, the lower wells were filled with only the chemotaxis medium. For chemotaxis assays using neutralizing monoclonal antibodies, peripheral blood mononuclear cells were pretreated (room temperature, 30 min) with 10 µg/ml of anti-CXCL10 antibody (MAB266; R&D Systems), 10 µg/ml of anti-CXCR3 antibody (MAB160; R&D Systems), or 10 µg/ml of isotype control antibody (MAB002; R&D Systems). A polyvinylpyrrolidone-free micropore polycarbonate filter (PFD5; Neuroprobe) with 5-µm pores was placed over the lower chamber. The upper wells were filled with 1×10^6 peripheral blood mononuclear cells in 100 µl of chemotaxis medium. The chamber was incubated for 120 min at 37°C in a humidified 5% CO₂ atmosphere. After incubation, the fluid in the lower chambers was collected and cell counts were determined using FACSCalibur. To compare results across all chemotaxis assays, a chemotactic index was calculated using the following formula (Nie *et al.*, 2009):

$$\text{Chemotactic index} = \frac{\text{(number of migrated cells in a test sample well)}}{\text{(number of migrated cells in a negative control well)}}$$

To determine the inhibitory effect of neutralizing antibodies, an inhibitory efficiency scale was calculated using the following formula:

$$\text{Inhibitory efficiency (\% inhibition)} = \frac{\left[\frac{\text{[(chemotactic index of isotype control) - 1]} - \text{[(chemotactic index of neutralizing antibody) - 1]}}{\text{[(chemotactic index of isotype control) - 1]} \times 100} \right]}{100}$$

Proliferation assay

The migrated cells in the lower chamber after the chemotaxis assay were collected and washed with RPMI 1640 medium supplemented with 5% foetal bovine serum and 1% penicillin/streptomycin. Those

cells were then plated on 96-well round-bottom plates and cultured in the same medium without any mitogenic stimuli in 5% CO₂ at 37°C. Cell proliferation was measured using a ³H-thymidine incorporation assay as described previously (Yamano *et al.*, 2009).

Statistical analysis

Correlation analysis was assessed using Spearman's rank test. The paired *t*-test was used for within-group comparisons, and the *t*-test or the Mann-Whitney U-test was used for comparisons between groups. One-way ANOVA was used for multiple comparisons followed by Tukey's test. The Friedman test was used for paired multiple comparisons, followed by the Dunn test. Statistical analyses and graphs were performed using Graphpad Prism 5 and Prism statistics (GraphPad Software, Inc), and statistical significance was set at $P < 0.05$.

Results

Significantly higher levels of cerebrospinal fluid CXCL10 compared with serum CXCL10 in patients with HAM/TSP

To determine whether the aforementioned chemokines were involved in the migration of cells to the CNS, we first compared the levels of these chemokines with CSF cell counts in patients with HAM/TSP ($n = 29$). CSF cell counts significantly correlated with levels of CXCL10 and CXCL9 but not with those of CCL5 or CCL4, the negative control (Fig. 1A). In addition, the correlation was clearly stronger with CXCL10 than with CXCL9. Following this, we compared the CSF and serum levels of these chemokines. Interestingly, only CXCL10 levels were higher in the CSF than the serum, although serum CXCL10 levels were also high to some extent (Fig. 1B, $P < 0.0001$). Next, to investigate whether these high CXCL10 levels were a HAM/TSP-specific phenomenon within HTLV-1-associated disorders, we tested for a correlation between CXCL10 and soluble interleukin-2 receptor (sIL-2R), a marker for adult T cell leukaemia (Yasuda *et al.*, 1988). As expected, serum sIL-2R levels were the highest in patients with adult T cell leukaemia. By contrast, plasma CXCL10 levels were significantly higher in patients with HAM/TSP than in those with adult T cell leukaemia, asymptomatic carriers or healthy donors (Supplementary Fig. 1A). This higher concentration of plasma CXCL10 in patients with HAM/TSP was observed even when compared to asymptomatic carriers with equivalently high proviral loads (Supplementary Fig. 1B).

Existence of abundant CXCR3⁺ cells in the spinal cords of patients with HAM/TSP

Because CXCL10 is a ligand of CXCR3, we investigated the possibility of CXCL10 recruiting proinflammatory CXCR3⁺ cells into the CSF by measuring the presence of CXCR3⁺ cells in the CSF and spinal cord lesions of patients with HAM/TSP (Fig. 2A–C). Flow cytometric analysis revealed that the average percentage of

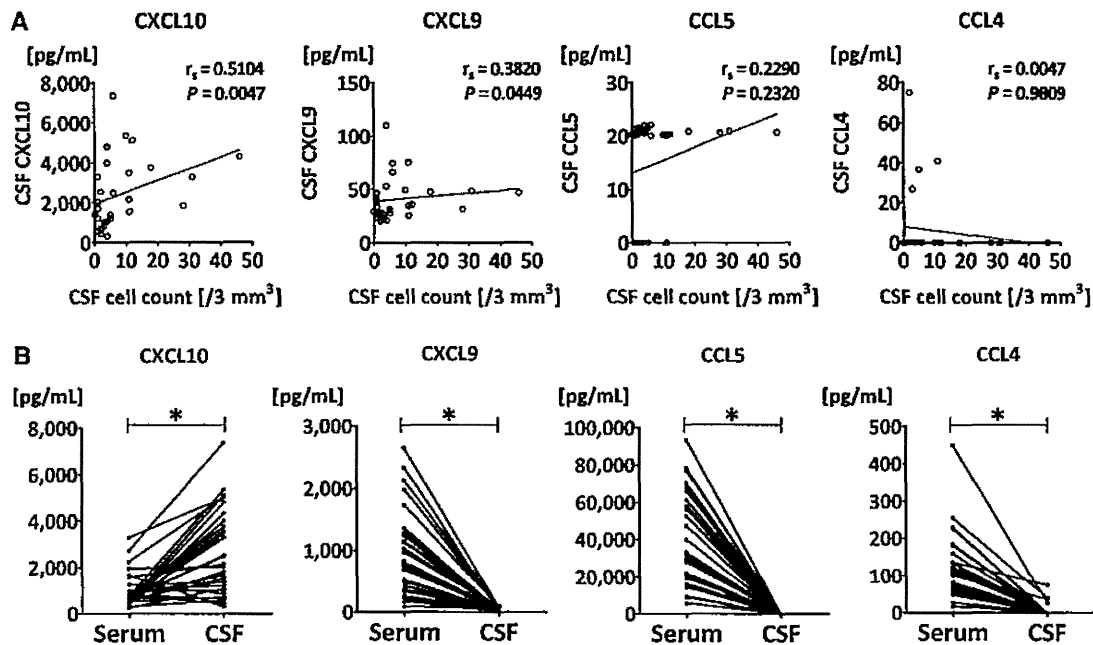


Figure 1 CXCL10 levels in CSF of patients with HAM/TSP were correlated with CSF cell counts and were significantly higher than those in serum of patients with HAM/TSP. (A) Correlation analysis between CSF levels of four chemokines (CXCL10, CXCL9, CCL5 and CCL4) and CSF cell counts in patients with HAM/TSP ($n = 29$). Statistical analysis was performed using Spearman's rank test. The linear regression line is indicated by a straight line in each graph. (B) Comparison of concentrations of four chemokines (CXCL10, CXCL9, CCL5, and CCL4) in CSF and serum samples obtained from patients with HAM/TSP such that all samples from a given patient were taken within a 1-h window of the first sample taken from that patient ($n = 32$). * $P < 0.0001$ by the paired t -test.

CXCR3⁺ cells among CSF cells was $92.4 \pm 7.0\%$, whereas the average percentage of CXCR3⁺ cells among peripheral blood mononuclear cells was $9.9 \pm 8.2\%$ ($P < 0.0001$, Fig. 2B). Immunofluorescence staining revealed abundant CXCR3⁺ cell infiltrate around small vessels in the leptomeninges of spinal cord lesions in patients with HAM/TSP (Fig. 2C). We examined the types of CXCR3⁺ cells in the CSF using flow cytometry and found that CSF CXCR3⁺ cells mainly consist of CD3⁺ cells (>90%) and small populations of CD14⁺ and CD19⁺ cells (Fig. 2D, left). Uniquely, the percentage of CXCR3⁺ cells was extremely high in all CSF cell populations under study, especially CD4⁺ ($94.33 \pm 2.95\%$), CD8⁺ ($98.64 \pm 1.05\%$), and even CD14⁺ ($84.97 \pm 18.49\%$) and CD19⁺ ($76.38 \pm 17.35\%$) cells (Supplementary Fig. 2). Our data show that the ratio of CD4⁺ to CD8⁺ cells in the CSF was $\sim 1:1$ in patients with HAM/TSP (Fig. 2D, right). In both these cell populations, the rate of CXCR3 positivity was higher in CSF cells than in peripheral blood mononuclear cells (Supplementary Fig. 2). The percentage of CXCR3⁺ cells in peripheral blood mononuclear cells of patients with HAM/TSP was lower than those in peripheral blood mononuclear cells of asymptomatic carriers as well as healthy donors; however, there were no significant differences between patients with adult T cell leukaemia and patients with HAM/TSP (Supplementary Fig. 3A). This lower percentage of CXCR3⁺ cells in patients with HAM/TSP was observed even when compared with asymptomatic carriers with equivalently high proviral loads (Supplementary Fig. 3B). Finally, to support our hypothesis that HTLV-1-infected T cells (the majority

of which are known to be CD4⁺) migrate from the circulating blood to the spinal cord tissue through CXCL10–CXCR3 interaction, we confirmed that there does exist a subset of peripheral CD4⁺CXCR3⁺ T cells infected with HTLV-1 (Fig. 2E).

Numerous CXCL10-producing cells in inflamed spinal cords of patients with HAM/TSP

To quantitatively compare the level of expression of CXCL10, we microscopically counted the number of CXCL10⁺ cells in the spinal cord tissue and found a larger number of CXCL10⁺ cells in the spinal cord lesions of patients with HAM/TSP than in control patients (Fig. 3A, $P = 0.0095$). In addition, we compared tissue sections from the thoracic spinal cord (a region of high inflammation) and the medulla oblongata (comparatively very low inflammation) from a single patient with HAM/TSP, and we observed a much larger CXCL10 presence in the thoracic spinal cord region (Supplementary Fig. 4).

Astrocytes as the main producers of CXCL10 in the spinal cords of patients with HAM/TSP

To identify which cell populations are the main CXCL10 producers, we immunostained thoracic spinal cord tissues from patients with

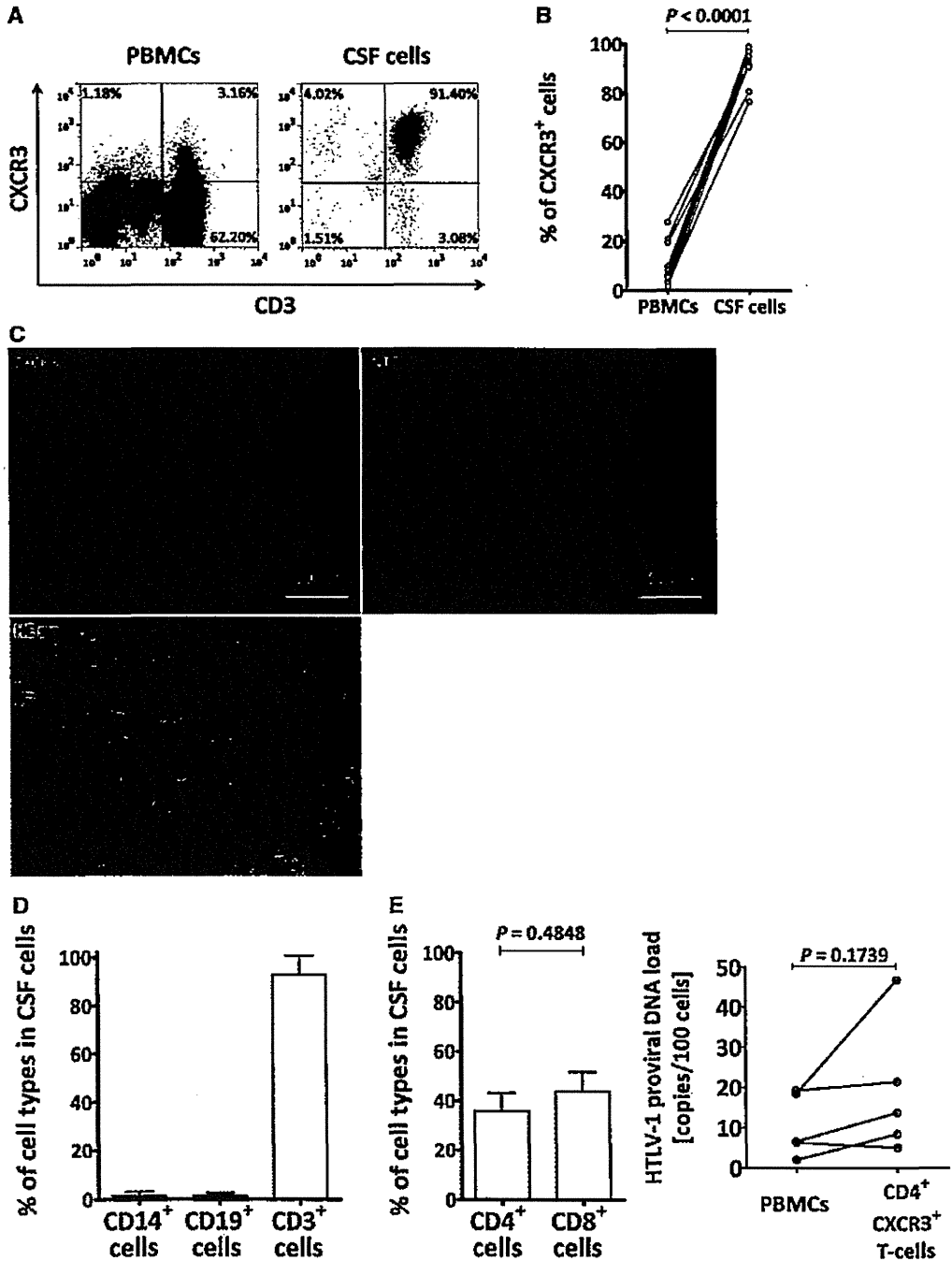


Figure 2 Abundant CXCR3⁺ cells in the CSF and spinal cord tissue of patients with HAM/TSP. (A) Representative dot plots of CD3 and CXCR3 expression in peripheral blood mononuclear cells (PBMCs, *left*) and CSF cells (*right*) from a patient with HAM/TSP measured using flow cytometry. (B) Comparison of the percentages of CXCR3⁺ cells in peripheral blood mononuclear cells and CSF cells, samples of which were obtained from 12 patients with HAM/TSP such that all samples from a given patient were taken within a 1-h window of the first sample taken from that patient. Statistical analysis was performed using the paired *t*-test. See also Supplementary Fig. 2. (C) Representative images of immunofluorescent detection of CXCR3, shown in green (*upper panels*), and haematoxylin-eosin (HE) staining for inflammatory cells, shown in blue (*lower panel*), in the thoracic spinal cords of patients with HAM/TSP. Rabbit IgG antibody used as the negative control (NTC). (D) *Left*: Percentages of CD3⁺, CD19⁺, and CD14⁺ cells in CSF cells derived from patients with HAM/TSP (*n* = 6). *Right*: Percentages of CD4⁺ cells and CD8⁺ cells. Statistical analysis was performed using the Mann–Whitney U-test. Error bars represent the mean ± SD. (E) The HTLV-1 proviral DNA loads of CD4⁺CXCR3⁺ T cells with peripheral blood mononuclear cells as the control. This result confirms the non-negligible existence of HTLV-1-infected CD4⁺CXCR3⁺ T cells, which may migrate to the CNS. Cells are from patients with HAM/TSP (*n* = 5). Statistical analysis was performed using the paired *t*-test.

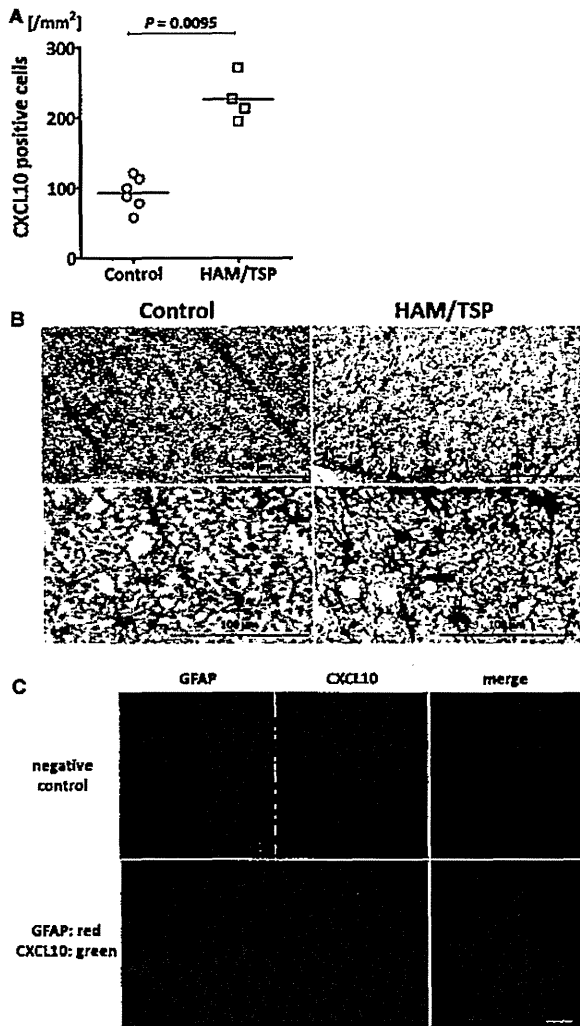


Figure 3 Astrocytes are the major CXCL10-producing cells in the spinal cords of patients with HAM/TSP. (A) Quantitative histological analysis (control: $n = 6$, HAM/TSP: $n = 4$). The numbers of CXCL10⁺ cells in the spinal cord sections were counted under a microscope. The data represent the mean number of CXCL10⁺ cells in three random fields of 1 mm² per sample. Horizontal bars represent the mean. Statistical analysis was performed using the Mann–Whitney U-test. (B) Representative immunohistochemical images of CXCL10 in the thoracic spinal cord tissues from control individuals ($n = 6$) and patients with HAM/TSP ($n = 4$). CXCL10-positive cells are brown. *Upper panel*: low magnification; *Lower panel*: high magnification. (C) Representative immunofluorescent images of GFAP (red), a marker for astrocytes, and CXCL10 (green) in the thoracic spinal cord tissues from a control individual and a patient with HAM/TSP patient. Similar results were observed in images of spinal cord tissues obtained from two other patients with HAM/TSP.

HAM/TSP ($n = 4$) and control individuals ($n = 6$). CXCL10-positive staining was mainly observed in star-shaped cells with extensive and radiating cytoplasmic processes, indicating that CXCL10 is expressed in activated astrocytes in the thoracic spinal cord of

patients with HAM/TSP (Fig. 3B). We also used immunofluorescence to confirm that CXCL10 is mainly expressed in astrocytes (GFAP⁺ cells) (Fig. 3C).

Co-culture with CD4⁺ T cells from patients with HAM/TSP enhances CXCL10 production in U251 human astrocytoma cells

CXCL10, also known as an IFN- γ -inducible protein 10, is mainly produced in response to IFN- γ stimulation (Muller *et al.*, 2010). We used this fact to investigate the events leading to CXCL10 production by astrocytes in the spinal cords of patients with HAM/TSP. First, we compared the capacities of several purified cell populations within peripheral blood mononuclear cells to produce IFN- γ spontaneously, i.e. without any stimulation. We found that CD4⁺ T cells exhibited the highest production of IFN- γ among peripheral blood mononuclear cells isolated from patients with HAM/TSP, and CD4⁺ T cells from patients with HAM/TSP produced more IFN- γ than those from asymptomatic carriers (Fig. 4A and B left). No peripheral blood mononuclear cells isolated from healthy donors displayed any detectable level of IFN- γ production (data not shown). Interestingly, CD4⁺ T cells from patients with HAM/TSP did not produce IL-17A, a proinflammatory cytokine known to play a key role in the pathogenic inflammatory response that characterizes multiple sclerosis (Fig. 4B, right) (Matusevicius *et al.*, 1999). Next, we used a co-culture system to confirm that CD4⁺ T cells induce astrocytes to produce CXCL10 by releasing IFN- γ . CD4⁺ T cells from patients with HAM/TSP induced CXCL10 production in U251 astrocytoma cells in a cell number-dependent manner (Fig. 4C), whereas CD4⁺ T cells from healthy donors did not induce CXCL10 production (data not shown). Importantly, in the presence of anti-IFN- γ neutralizing antibodies, the supernatant from HAM/TSP patient CD4⁺ T cell cultures stimulated significantly less CXCL10 production in U251 cells (Fig. 4D).

Chemotaxis of peripheral blood mononuclear cells from patients with HAM/TSP due to CXCL10 and inhibition of chemotaxis by anti-CXCL10 neutralizing antibodies

To investigate the potential role of CXCL10 or CXCR3 as a therapeutic target for inhibiting the migration of proinflammatory cells into the CNS, we assessed whether neutralizing antibodies against CXCL10 or CXCR3 could inhibit the migration of peripheral blood mononuclear cells in patients with HAM/TSP through the use of an *in vitro* chemotaxis assay system. Human CXCL10 increased the chemotactic activity of peripheral blood mononuclear cells from patients with HAM/TSP by ~ 1.7 -fold (Fig. 5A). Compared with isotype control monoclonal antibodies, the chemotactic activity due to CXCL10 was inhibited by anti-CXCL10 neutralizing antibodies (Fig. 5A; 65.9% inhibition, $P < 0.01$) but not by anti-CXCR3 antibodies (Fig. 5A; 9.2% inhibition, $P > 0.05$: not

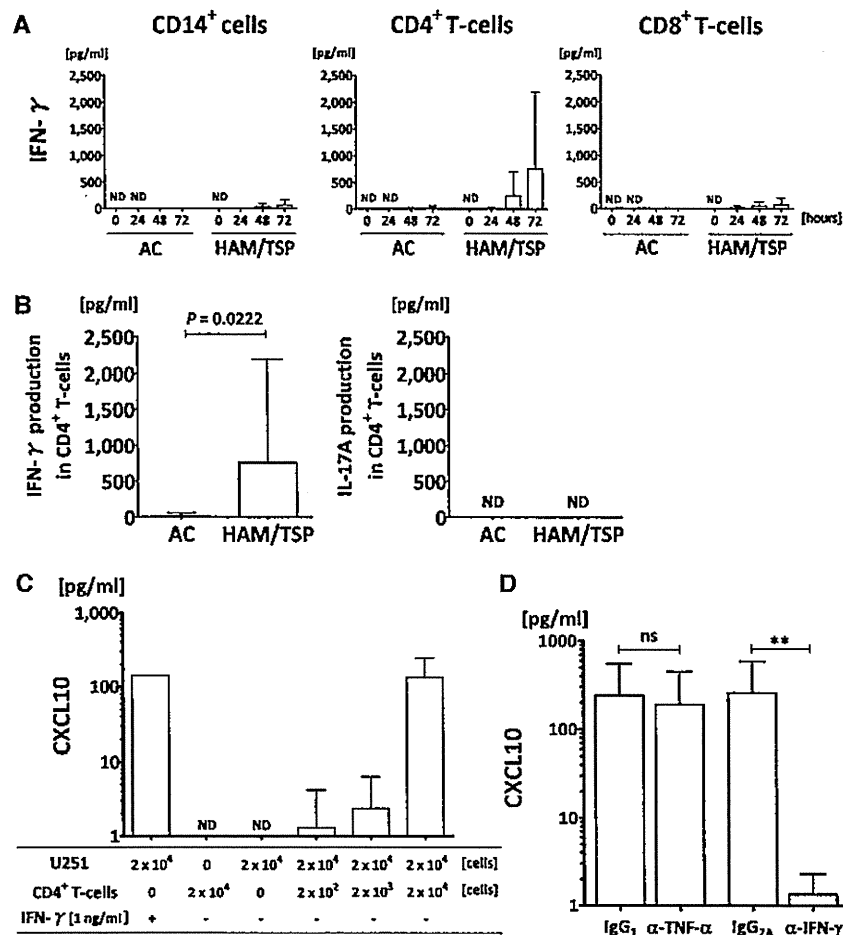


Figure 4 Co-culturing with CD4⁺ T cells from patients with HAM/TSP increases CXCL10 production in a U251 human astrocytoma cell line. (A) Concentration of IFN-γ in supernatants of cultured CD4⁺, CD8⁺, or CD14⁺ cells in peripheral blood mononuclear cells (PBMCs) from patients with HAM/TSP ($n = 6$) compared with that in peripheral blood mononuclear cells from asymptomatic carriers (AC, $n = 5$). These cells were cultured without any stimuli for 24, 48 and 72 h. ND = not detected. (B) Concentrations of IFN-γ (*left*) and IL-17A (*right*) in culture supernatants of cultured CD4⁺ T cells for 72 h from patients with HAM/TSP compared with the concentrations in those from asymptomatic carriers. Patients with HAM/TSP: $n = 6$, asymptomatic carrier: $n = 5$. ND = not detected. Statistical analyses were performed using the Mann-Whitney U-test. (C) Concentration of CXCL10 produced by U251, a human astrocytoma cell line, co-cultured with CD4⁺ T cells from patients with HAM/TSP ($n = 5$). ND = not detected. (D) Concentration of CXCL10 produced by U251 stimulated by the supernatant of cultured CD4⁺ T cells of patients with HAM/TSP ($n = 5$) in the presence of neutralizing antibodies against IFN-γ and TNF-α, and isotype control antibodies for each. NS = not significant. ** $P < 0.01$. Error bars represent the mean \pm SD.

significant). Next, we investigated whether or not this decreased migration would also be reflected in the absolute number of HTLV-1-infected cells among migrated cells. Chemotaxis assays revealed that the addition of human CXCL10 (0.25 μ g/ml) increased the absolute number of HTLV-1-infected cells by ~2.1-fold (Fig. 5B) compared with isotype control monoclonal antibodies, and that this increase was largely inhibited by anti-CXCL10 neutralizing antibodies (Fig. 5B; 101.1% inhibition, $P < 0.01$) but only slightly by anti-CXCR3 antibodies (Fig. 5B; 65.7% inhibition, $P > 0.05$; not significant). Finally, we evaluated the degree to which the migrated cells were proliferating spontaneously, where spontaneous proliferation is defined as proliferation in the absence of exogenous antigens or stimulants (Itayama et al., 1988; Ijichi et al., 1989). This is important because the

level of spontaneous proliferation of peripheral blood mononuclear cells in patients with HAM/TSP is believed to reflect the cell proliferation that occurs in the CNS (Itayama et al., 1988; Ijichi et al., 1989). Significantly less ³H-thymidine uptake, an assay for cell proliferation, was detected in the lower chemotaxis assay chamber following administration of anti-CXCL10 antibody than isotype control antibodies (Fig. 5C; 33.8% inhibition, $P < 0.05$).

Discussion

Previous studies of HAM/TSP pathogenesis have revealed that chronic inflammation occurs in the spinal cords of patients with HAM/TSP (Saito and Bangham, 2012; Yamano and Sato, 2012);

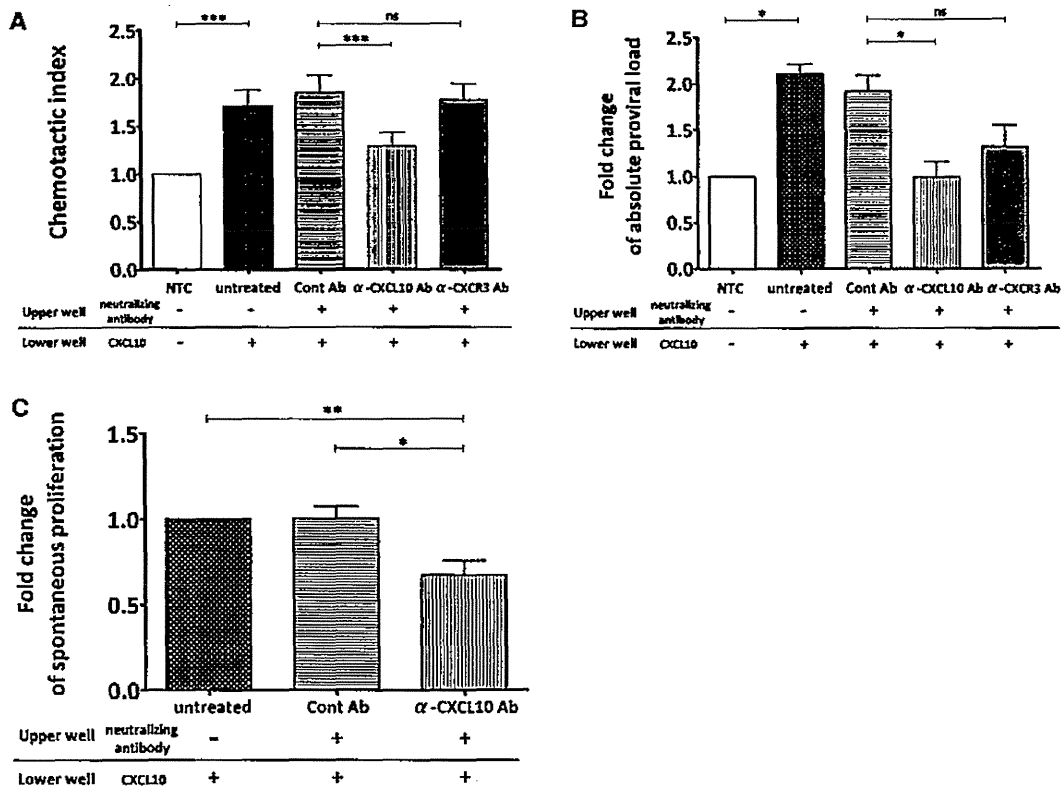


Figure 5 Chemotaxis of peripheral blood mononuclear cells due to CXCL10 and inhibition of chemotaxis by anti-CXCL10 neutralizing antibody in cells from patients with HAM/TSP. (A) The migration-inducing effect of CXCL10 and the inhibitory effect of neutralizing antibody against CXCL10 versus its receptor, CXCR3. Peripheral blood mononuclear cells from patients with HAM/TSP ($n = 21$) migrated into the lower well in response to CXCL10, and treatment with anti-CXCL10 antibody significantly reduced the migration of peripheral blood mononuclear cells, as compared to anti-CXCR3 antibody and control antibody. (B) The inhibition of cell migration led to an overall decrease in migrated cells including HTLV-1-infected cells, effectively decreasing the absolute proviral load. Peripheral blood mononuclear cells used were collected from patients with HAM/TSP; $n = 4$. (C) The inhibition of cell migration led to an overall decrease in migrated cells which also means less spontaneous proliferation. Peripheral blood mononuclear cells used were collected from patients with HAM/TSP; $n = 7$. Error bars represent the mean \pm SD. Statistical analyses were performed using the Friedman test followed by the Dunn test for multiple comparison. NS = not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NTC = negative control; Cont Ab = isotype control monoclonal antibody; α -CXCL10 Ab = anti-CXCL10 monoclonal antibody; α -CXCR3 Ab = anti-CXCR3 monoclonal antibody.

however, the precise mechanisms by which these inflammatory lesions are formed and maintained remain unclear. We hypothesized that a positive feedback loop driven by chemokines may be responsible for the chronic inflammation associated with HAM/TSP. We identified CXCL10 as the principal chemokine responsible for inducing this chronic inflammation. We found for the first time astrocytes to be the main producers of CXCL10. Our data suggest that these astrocytes are stimulated to produce CXCL10 initially by IFN- γ released by infected T cells, where the infection appears to have produced changes in the cells that promote spontaneous IFN- γ production. In short, we inferred that spinal cord lesions found in patients with HAM/TSP arise when IFN- γ produced by HTLV-1-infected T cells induces astrocytes to secrete CXCL10, which attracts CXCR3 $^+$ T cells, including more T cells infected with HTLV-1, thereby continuing the cycle. Furthermore, we demonstrated that an interruption of this pathway represents a promising strategy for treating HAM/TSP.

First, we identified the key chemokine involved in inducing the migration of cells to sites of inflammation. We compared the CSF and serum levels of several chemokines and demonstrated for the first time that CXCL10 is the only chemokine of those studied that is present at a significantly higher concentration in the CSF than in the sera of patients with HAM/TSP. Although previous reports indicate that CCL5 and CXCL9 levels are also elevated in the CSF of patients with HAM/TSP (Teixeira *et al.*, 2004; Tanaka *et al.*, 2008), we showed that these two chemokines exhibit a concentration gradient in the opposite direction (Fig. 1B). We previously measured the levels of other chemokines such as CCL3, CCL4, CXCL11, CCL17, CCL20 and CCL22 in the CSF and found that the levels of these chemokines are negligible in patients with HAM/TSP (Sato, *in press*). Importantly, we also previously demonstrated that CSF CXCL10 levels are correlated with the rate of disease progression (Sato, *in press*). These findings suggest that CXCL10 is crucial for the development of chronic inflammation in patients with HAM/TSP.

In the present study, we found a positive correlation between CSF CXCL10 levels and CSF cell counts (Fig. 1A), a high percentage of CXCR3-positive cells in the CSF (Fig. 2A and B; $92.4 \pm 7.0\%$), and perivascular accumulation of CXCR3⁺ cells in spinal cord lesions of patients with HAM/TSP (Fig. 2C). These results strongly indicate that a high concentration of CXCL10 in the spinal cord attracts CXCR3⁺ cells that include proinflammatory cells (Qin *et al.*, 1998; Sallusto *et al.*, 1998; Thomas *et al.*, 2003). Intriguingly, the percentage of CXCR3⁺ cells among peripheral blood mononuclear cells from patients with HAM/TSP was significantly lower than that observed in asymptomatic carriers and healthy donors, but not patients with adult T cell leukaemia (Supplementary Fig. 3). CXCR3⁺ peripheral blood mononuclear cells are relatively few in patients with adult T cell leukaemia, perhaps because of an increase in CCR4⁺CXCR3⁻ tumour cells in the peripheral blood (Ishida *et al.*, 2003). Although the precise mechanism by which peripheral CXCR3⁺ cells in patients with HAM/TSP become diminished remains unclear, we believe that many of these cells migrate into the CNS and contribute to the formation of spinal cord lesions. Other possible mechanisms include migration to lymphoid organs such as lymph nodes or the spleen. Because lymph nodes are important organs for CXCL10–CXCR3 interactions in patients suffering from various diseases (Groom *et al.*, 2012; Sung *et al.*, 2012), future studies analysing the lymph nodes of patients with HAM/TSP may provide a more complete understanding of HAM/TSP pathogenesis.

The discovery that CXCL10–CXCR3 interactions represent an important pathway for recruiting cells to the CNS in patients with HAM/TSP prompted us to search the spinal cord lesions of patients with HAM/TSP and identify the CXCL10-producing cells. Firstly, we confirmed that CXCL10-producing cells are more numerous in the spinal cords of patients with HAM/TSP than control individuals (Fig. 3A). We also compared high and low inflammatory regions within a single patient with HAM/TSP and found more CXCL10-producing cells in the more inflamed region (Supplementary Fig. 4), although the limitation of sampling from only a single individual prevents us from extrapolating too freely on the significance of this result. Although CXCL10 is secreted by several cell types such as monocytes, endothelial cells, fibroblasts and astrocytes in response to IFN- γ (Luster and Ravetch, 1987; Lee *et al.*, 2009), our study demonstrated that astrocytes are the major CXCL10-producing cells in thoracic spinal cord lesions in patients with HAM/TSP (Fig. 3). Notably, the astrocytes examined in this study were star-shaped with radiating cytoplasmic processes, indicating high cytological activity (Fig. 3B and C). In the CNS, CXCL10 is mainly produced by astrocytes; however, CXCL9 is primarily a product of microglial cells (Muller *et al.*, 2010). Therefore, the finding that CXCL10 production is substantially higher than CXCL9 production in the CSF (Fig. 1) suggests that astrocytes are very active in HAM/TSP. This finding supports a previous finding that gliosis is one of the main pathological features of HAM/TSP (Iwasaki, 1990; Izumo *et al.*, 1992).

Next, we investigated the mechanism by which astrocytes produce CXCL10 in patients with HAM/TSP. CXCL10 is generally not detectable in most non-lymphoid tissues under physiological conditions; however, its synthesis is easily induced by cytokines, particularly IFN- γ . Therefore, it was important to determine the source of

IFN- γ that stimulates astrocytes to produce CXCL10 in patients with HAM/TSP. Interestingly, we have shown that CD4⁺ T cells from patients with HAM/TSP spontaneously produce IFN- γ and induce CXCL10 production by U251 human astrocytoma cells via IFN- γ (Fig. 4), whereas CD4⁺ T cells from healthy donors do not induce CXCL10 production (data not shown). These results support the hypothesis that there are interactions between HTLV-1-infected CD4⁺ T cells and astrocytes in patients with HAM/TSP *in vivo* that may possibly initiate the first wave of CXCL10 production. Moreover, this CXCL10 production may further induce the trafficking of peripheral CXCR3⁺ T cells. Importantly, we demonstrated that a number of peripheral CXCR3⁺ T cells are infected with HTLV-1 (Fig. 2E), indicating that migration of peripheral CXCR3⁺ T cells into the CNS can induce further secretion of IFN- γ that continues the vicious cycle. In fact, HTLV-1-infected CD4⁺ T cells and IFN- γ -producing T cells have been detected in HAM/TSP spinal cord lesions (Umehara *et al.*, 1994; Moritoyo *et al.*, 1996; Matsuoka *et al.*, 1998). Notably, more than half of the CXCR3⁺ T cells in the CSF of patients with HAM/TSP are CD8⁺ T cells (Fig. 2D). It has been shown that CD8⁺ cytotoxic T lymphocytes (CTLs), particularly HTLV-1-specific CTLs, have a high potential for secreting IFN- γ (Kubota *et al.*, 2000; Hanon *et al.*, 2001) and are abnormally elevated in the CSF and spinal cord lesions (Nagai *et al.*, 2001a, b; Matsuura *et al.*, 2010). Therefore, CXCL10 production by astrocytes may further boost the trafficking of CXCR3⁺-infected CD4⁺ T cells as well as CXCR3⁺CD8⁺ CTLs that secrete IFN- γ , leading to a positive feedback-driven chronic inflammatory loop.

The results of the present study and other studies show that the pathology of HAM/TSP is unique among immune disorders. Unlike other inflammatory disorders such as multiple sclerosis or rheumatoid arthritis that exhibit Th17 as well as Th1 involvement (Matusevicius *et al.*, 1999; Kirkham *et al.*, 2006), HAM/TSP pathogenesis appears to be dominated by the Th1 axis, particularly CXCL10–CXCR3 interactions. Our research indicates that the characteristics of HTLV-1-infected T cells may be responsible for the emphasis on the Th1 axis in HAM/TSP pathogenesis. We have reported that cultured CD4⁺ T cells from patients with HAM/TSP clearly exhibit detectable production of IFN- γ (a Th1 cytokine) but not IL-17 (a Th17 cytokine) (Fig. 4B), and we previously demonstrated that HTLV-1-infected T cells in patients with HAM/TSP exhibit elevated IFN- γ and reduced IL-17 production (Yamano *et al.*, 2009). Furthermore, HAM/TSP peripheral blood contained more CXCL10 (Supplementary Fig. 1B) and fewer CXCR3⁺ cells (Supplementary Fig. 3B) than asymptomatic carrier blood, suggesting that a greater number of CXCR3⁺ cells had migrated out of the periphery due at least in part to chemotaxis induced by elevated CXCL10 production in the CNS. As the proviral loads of all the samples used in the above experiment were roughly identically high, it can be assumed that these characteristics are indeed features of HAM/TSP pathogenesis as opposed to simple consequences of having a high proviral load.

We suspect that a genetic predisposition for higher IFN- γ or CXCL10 production in response to HTLV-1 may exist. Recently, systems biology approaches were used to show that a subset of IFN-stimulated genes, including the gene encoding CXCL10, is overexpressed in peripheral blood mononuclear cells of patients with HAM/TSP compared with asymptomatic carriers

(Tattermusch *et al.*, 2012). It will be important to test for an association between genetic polymorphisms in interferon-associated genes and the presence of HAM/TSP in future studies. The existence of this genetic predisposition would strengthen the argument for Th1-dominance and explain why some infected individuals develop HAM/TSP, whereas others remain life-long asymptomatic carriers. Because it is well-known that interferons and products of interferon-stimulated genes mediate antiviral responses (Randall and Goodbourn, 2008), IFN- γ and CXCL10 production in HTLV-1-infected patients (Supplementary Fig. 1) may be considered a normal immune response. However, once the production levels surpass threshold and a CXCL10–CXCR3 amplification loop develops, it may begin to cause tissue damage. Possible reasons for CXCL10 overproduction in HAM/TSP include the presence of a high number of HTLV-1-infected T cells (Nagai *et al.*, 1998; Yamano *et al.*, 2002) and a genetic predisposition for higher IFN- γ and/or CXCL10 production in response to HTLV-1.

The ideal therapeutic strategy for treating HAM/TSP would be eradication of HTLV-1-infected cells, but this has yet to be proven possible. Another promising approach would be a receptor blockade using anti-CXCR3 neutralizing antibody, which has been reported to be effective at blocking CXCR3 activity (Van den Steen *et al.*, 2008). Although we were unable to validate this effect using our commercially available antibody, this certainly does not rule out a receptor blockade as a therapeutic candidate. Our relative success at disrupting inflammatory cell migration using anti-CXCL10 neutralizing antibodies (Fig. 5) suggests that targeting CXCL10 to interrupt the positive feedback loop may be the more promising new strategy for effectively treating HAM/TSP. A noteworthy potential advantage of anti-CXCL10 over anti-CXCR3 is that it may yield less severe side effects as only interactions with CXCL10 rather than all CXCR3 agonists would be blocked.

In conclusion, our data revealed novel insights into the pathogenic processes of HAM/TSP. Our results suggest that CXCL10 plays a pivotal role in the development of chronic inflammatory lesions where HTLV-1-infected T cells produce IFN- γ , which induces astrocytes to secrete CXCL10. This further boosts the trafficking of CXCR3⁺-infected T cells that secrete IFN- γ , leading to a virus-induced CXCL10–CXCR3 inflammatory loop. Thus, HAM/TSP represents a pathological consequence of interactions that occur between the immune system and CNS. Understanding these complex interactions should provide new insights into the functional regulation of both systems and help uncover new therapeutic targets.

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Supplementary material

Supplementary material is available at *Brain* online.

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CSF CXCL10, CXCL9, and Neopterin as Candidate Prognostic Biomarkers for HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis

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Abstract

Background: Human T-lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare chronic neuroinflammatory disease. Since the disease course of HAM/TSP varies among patients, there is a dire need for biomarkers capable of predicting the rate of disease progression. However, there have been no studies to date that have compared the prognostic values of multiple potential biomarkers for HAM/TSP.

Methodology/Principal Findings: Peripheral blood and cerebrospinal fluid (CSF) samples from HAM/TSP patients and HTLV-1-infected control subjects were obtained and tested retrospectively for several potential biomarkers, including chemokines and other cytokines, and nine optimal candidates were selected based on receiver operating characteristic (ROC) analysis. Next, we evaluated the relationship between these candidates and the rate of disease progression in HAM/TSP patients, beginning with a first cohort of 30 patients (Training Set) and proceeding to a second cohort of 23 patients (Test Set). We defined "deteriorating HAM/TSP" as distinctly worsening function (≥ 3 grades on Osame's Motor Disability Score (OMDS)) over four years and "stable HAM/TSP" as unchanged or only slightly worsened function (1 grade on OMDS) over four years, and we compared the levels of the candidate biomarkers in patients divided into these two groups. The CSF levels of chemokine (C-X-C motif) ligand 10 (CXCL10), CXCL9, and neopterin were well-correlated with disease progression, better even than HTLV-1 proviral load in PBMCs. Importantly, these results were validated using the Test Set.

Conclusions/Significance: As the CSF levels of CXCL10, CXCL9, and neopterin were the most strongly correlated with rate of disease progression, they represent the most viable candidates for HAM/TSP prognostic biomarkers. The identification of effective prognostic biomarkers could lead to earlier detection of high-risk patients, more patient-specific treatment options, and more productive clinical trials.

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Introduction

Human T-lymphotropic virus type 1 (HTLV-1) is a human retrovirus associated with persistent infection of T-cells [1]. While the majority of HTLV-1-infected individuals remain asymptomatic, approximately 2.5–5% develop an aggressive T-cell malignancy, termed adult T-cell leukemia (ATL) [2,3] and 0.3–3.8% develop a serious chronic neuroinflammatory disease, termed HTLV-1-associated myelopathy/tropical spastic paraparesis

(HAM/TSP) [4–6]. Aside from Japan, endemic areas for this virus and the associated disorders are mostly located in developing countries in the Caribbean, South America, Africa, the Middle East, and Melanesia [7,8], which may explain why these conditions have remained ill-defined and virtually untreatable for so long [9].

HAM/TSP is characterized by unremitting myelopathic symptoms such as spastic paraparesis, lower limb sensory disturbance, and bladder/bowel dysfunction [10,11]. Although

Author Summary

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare neurodegenerative disease caused by infection with human T-lymphotropic virus type 1 (HTLV-1). HTLV-1 infects 10–20 million people worldwide, and, depending on the region, 0.25–3.8% of infected individuals develop HAM/TSP. As the disease progresses, chronic inflammation damages the spinal cord and lower limb and bladder function gradually decline. In the worst cases, even middle-aged patients can become perpetually bedridden. Today, there are treatments that may alleviate the symptoms to a certain degree, but there is no cure that can halt disease progression, and there are no known biomarkers to indicate the level and speed of disease progression. In this study, we successfully identified three promising candidate biomarkers. We believe that the use of these biomarkers could lead to more accurate prognoses and more prudent, patient-specific treatment plans. We not only hope that these biomarkers are sensitive enough to use as selection criteria for clinical trials, but also that measurements of these biomarkers can be used to accurately evaluate drug effectiveness. In short, the biomarkers we identified have the potential to help more effectively treat current HAM/TSP patients and to pave the way for new drugs to potentially cure future HAM/TSP patients.

the symptoms of HAM/TSP have been well documented for quite some time, the rate at which these symptoms progress has only recently become a point of interest. The clinical course of HAM/TSP has classically been described very simply as insidious onset and continuous progression [12], but recent reports have hinted at a more complex, heterogeneous pool of patients with differing clinical needs. Recent studies have shown that although HAM/TSP usually progresses slowly and without remission as per the classical description, there is a subgroup of patients whose conditions decline unusually quickly and who may be unable to walk within two years of onset and another subgroup whose conditions decline unusually slowly and who may only display very mild symptoms [13–15]. It is only logical that these patients should receive treatments tailored to suit their individual needs rather than identically aggressive treatments. Unfortunately, clinicians are currently only able to distinguish between these different groups by observing the way a patient's disease progresses over time, usually years; clinicians often decide to treat the patients immediately and identically rather than wait and allow the disease to progress further. Therein lies the dire need for biomarkers with the power to forecast the rate and extent of disease progression and enable clinicians to make more accurate prognoses and prescribe the most appropriate and effective treatments in a timely manner.

Several candidate prognostic biomarkers with elevated levels in HAM/TSP patients have already been identified in the peripheral blood and cerebrospinal fluid (CSF). In the peripheral blood, such candidates include the HTLV-1 proviral load in peripheral blood mononuclear cells (PBMCs) and serum levels of the soluble IL-2 receptor (sIL-2R) [16,17]. The level of neopterin in the CSF has been reported to be a useful parameter for detecting cell-mediated immune responses in the spinal cord of HAM/TSP patients and the CSF anti-HTLV-1 antibody titer has been shown to be associated both with CSF neopterin levels and the severity of clinical symptoms [18–20]. In addition, several cytokines have been detected in the CSF and/or spinal cord of HAM/TSP patients, including interleukin (IL)-1 β , granulocyte-macrophage

colony-stimulating factor (GM-CSF), interferon (IFN)- γ , and tumor necrosis factor (TNF)- α [21–24]. Some chemokines, such as chemokine (C-X-C motif) ligand (CXCL) 9, CXCL10, and chemokine (C-C motif) ligand (CCL) 5, have been shown to be substantially elevated in both the blood and the CSF with respect to asymptomatic carriers (ACs) or patients with other neurological diseases such as multiple sclerosis [25–28]. This is the first study to compare the adequacies of several of these candidate biomarkers for forecasting the rate of disease progression.

We hypothesized the existence of biomarkers capable of differentiating stable and deteriorating HAM/TSP patients. In this retrospective study, a preliminary experiment was first conducted to select the most promising candidate biomarkers by comparing blood and CSF levels in HAM/TSP patients and control subjects (Figure S1). Four candidate blood markers (sIL-2R, CXCL9, CXCL10, and proviral load) and five candidate CSF markers (CXCL9, CXCL10, neopterin, cell count, and anti-HTLV-1 antibody titer) were selected. To evaluate the relative effectiveness of these candidate biomarkers for predicting rate of disease progression, a classification system was created and HAM/TSP patients were designated as either deteriorating or relatively stable. The levels of candidate biomarkers were then compared between the two patient groups. In the current study, we identified three viable candidates for HAM/TSP prognostic biomarkers that could lead to more accurate prognoses and more prudent, patient-specific treatment plans.

Materials and Methods

Ethical considerations

The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki. The protocol in this study was approved by the Ethics Review Committee of St. Marianna University School of Medicine (No. 1646). Prior to the collection of blood or CSF samples, all subjects gave written informed consent permitting the analysis of their samples for research purposes as part of their clinical care.

Subjects

Between April 2007 and February 2013, we enrolled 53 HAM/TSP patients according to the inclusion and exclusion criteria shown in Table 1, and divided them into two cohorts based on the chronological order of their doctor's visits: a 30-patient Training set and a 23-patient Test set. Demographics and clinical characteristics of the Training set and Test set are shown in Table 2 and Table 3, respectively. Between April 2007 and December 2009, we enrolled 22 HTLV-1-infected ACs as control subjects for blood analysis and eight HTLV-1-infected subjects (seven ACs, one patient with smoldering ATL) as control subjects for CSF analysis according to the inclusion and exclusion criteria shown in Table 1. These two groups were not mutually exclusive; some ACs donated both blood and CSF to this study. Demographics of control subjects as compared to the HAM/TSP patients are shown in Table S1.

Sample preparation

Blood and/or CSF samples were obtained within a one-hour window for each subject. Peripheral blood samples were collected in heparin-containing blood collection tubes and serum-separating tubes. Plasma and PBMCs were obtained from the former tubes and serum was obtained from the latter. PBMCs were isolated with standard procedures using Pancoll[®] density gradient centrifugation (density 1.077 g/mL; PAN-Biotech GmbH, Aidenbach, Germany). Plasma and serum samples were stored at -80°C until

Table 1. Inclusion and exclusion criteria for this study.

	HAM/TSP	Control for Blood	Control for CSF
Inclusion Criteria	Willing and able to give informed consent		
	HTLV-1 seropositive individuals conformed by CLEIA and Western blot		
	Diagnosed with HAM/TSP as defined by WHO criteria		Choose to provide CSF for the purposes of differential diagnosis
Exclusion Criteria	History of treatment with corticosteroids or other immunomodulating drugs (interferon, cyclosporin, methotrexate, etc.)		
	Diagnosed with an autoimmune disease or other chronic inflammatory disorder aside from HAM/TSP		
	Diagnosed with additional disease affecting gait disturbance (e.g. parkinsonism, rheumatoid arthritis, cervical spondylosis, brain infarction, etc.)		
	History of severe urinary infection, decubitus sores, pneumonia, deep venous thrombosis, or other condition potentially affecting disease course within the last four years	Diagnosed with HAM/TSP as defined by WHO criteria	
	Diagnosed with adult T-cell leukemia (ATL)		

CLEIA = chemiluminescent enzyme immunoassay.
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use. CSF was collected in polypropylene tubes. A small amount of CSF was used for routine laboratory tests, which included total protein, cell count, and IgG level. The remaining CSF was aliquoted into cryotubes and stored at -80°C until undergoing further analysis. All tests in this study were performed on samples from these frozen stocks.

Measurement of blood candidate markers

The serum concentration of sIL-2R was determined using an ELISA (Cell Free N IL-2R; Kyowa Medex Ltd., Tokyo, Japan). HTLV-1 proviral load was measured using real-time PCR, following DNA extraction from PBMCs, as previously described [29–31]. Plasma levels of IL-1 β , TNF- α , and IFN- γ were measured using a cytometric bead array (CBA) (BD Biosciences, Franklin Lakes, NJ USA), which was used according to the manufacturer's instructions. Plasma concentrations of CXCL9, CXCL10, CXCL11, and CCL5 were also measured using a CBA (BD Biosciences).

Measurement of CSF candidate markers

CSF cell count was determined using the Fuchs-Rosenthal chamber (Hausser Scientific Company, Horsham PA USA). Total protein and IgG levels in the CSF were measured using a pyrogallol red assay and a turbidimetric immunoassay, respectively. The anti-HTLV-1 antibody titer was determined using the gelatin particle agglutination test (Serodia-HTLV-1; Fujirebio, Tokyo, Japan). CSF concentration of sIL-2R was determined using an ELISA (Cell Free N IL-2R; Kyowa Medex). CSF neopterin level was measured using high-performance liquid chromatography. IFN- γ and six chemokines (CXCL9, CXCL10, CXCL11, CCL3, CCL4, and CCL5) were measured using a CBA (BD Biosciences). The CSF concentrations of three chemokines (CCL17, CCL20, and CCL22) and IL-17A were measured using commercially available ELISA kits (CCL17, CCL20, and CCL22: TECHNE/R&D Systems, Minneapolis, MN USA; IL-17A: Gen-Probe, San Diego, CA USA). All assays were conducted according to the respective manufacturers' instructions.

Table 2. Demographics and clinical characteristics of HAM/TSP patients (Training Set).

	Total n = 30	Stable HAM/TSP n = 14	Deteriorating HAM/TSP n = 11	p-value*
Demographics				
Age, y**	58 [37–75]	54.5 [39–75]†	62 [53–72]	0.0183†
Female sex	80.0%	64.3%	90.9%	0.1696‡
Clinical characteristics				
Age of onset, y**	48 [20–70]	33 [20–58]	57 [40–70]	0.0021†
Disease duration, y**	12.5 [1–33]	19 [7–33]	9 [1–13]	0.0021†
OMDS**	6 [2–11]	5 [2–9]	8 [5–11]	0.0065†

In the Training set, deteriorating patients were significantly older, experienced disease onset later in life, had been living with the disease for shorter periods, and were more severely disabled (OMDS).

*Stable HAM/TSP vs Deteriorating HAM/TSP.

**Data are expressed as median [range].

†By Mann-Whitney test.

‡By Fisher's exact test.

OMDS = Osame's Motor Disability Score.

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