High frequencies of Th1-type CD4+ T cells specific to HTLV-1 Env and Tax proteins in patients with HTLV-1–associated myelopathy/tropical spastic paraparesis


CD4+ T cells are critical for inducing and maintaining efficient humoral and cellular immune responses to pathogens. The CD4+ T-cell response in human T-lymphotropic virus 1 (HTLV-1) infection has not been studied in detail. However, CD4+ T cells have been shown to predominate in early lesions in HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). We present direct estimates of HTLV-1 Env- and Tax-specific CD4+ T-cell frequencies in patients infected with HTLV-1. We first showed that there was a strong bias toward the Th1 phenotype in these HTLV-1–specific CD4+ T cells in patients with HAM/TSP. We then demonstrated significantly higher frequencies of HTLV-1–specific Th1-type CD4+ T cells in HAM/TSP patients than in asymptomatic HTLV-1 carriers. The majority of these HTLV-1–specific CD4+ T cells did not express HTLV-1 Tax and were therefore unlikely to be infected by HTLV-1. High frequencies of activated HTLV-1–specific CD4+ T cells of the Th1 phenotype might contribute to the initiation or pathogenesis of HAM/TSP and other HTLV-1–associated inflammatory diseases.

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and that HTLV-1–specific CD4+ T cells are selectively infected with HTLV-1.

Our results showed significantly higher frequencies of HTLV-1 Env- and Tax-specific CD4+ T cells in patients with HAM/TSP compared to ACs. Patients with HAM/TSP also showed a significant predominance of the Th1 phenotype in these HTLV-1–specific CD4+ T cells. Furthermore, most HTLV-1–specific CD4+ T cells did not express Tax and were therefore not susceptible to lysis by HTLV-1–specific CTLs. These results have implications for the initiation or pathogenesis of HTLV-1–associated inflammatory diseases.

Patients, materials, and methods

Subjects and cells

Subjects were asymptomatic HTLV-1 carriers and patients with HAM/TSP attending the HTLV-1 clinic at St Mary’s Hospital. Infection with HTLV-1 was confirmed by the presence of antibodies to HTLV-1 gag (p19 and p24) and env (gpg21 and gpg46)-antigens in sera by Western blot (Genelabs HTLV 2.4, Singapore). The diagnosis of HAM/TSP was made according to World Health Organization criteria. All patients gave informed consent.

The PBMCs were isolated via density gradient centrifugation on Histopaque-1077 (Sigma, Dorset, United Kingdom) and washed 3 times with phosphate-buffered saline (PBS). Cells were then stored frozen in liquid nitrogen in fetal calf serum (FCS; Sigma) supplemented with 10% dimethyl sulphoxide (DMSO; Sigma).

After thawing and washing in cold sterile PBS (2 times), cells were cultured in complete medium (CM), which is RPMI 1640 medium (Sigma) supplemented with 10% FCS, 2 mM glutamine (Gibco, Paisley, United Kingdom), 100 IU/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). All cultures were undertaken in this medium unless stated otherwise. To induce nonspecific cytokine production by PBMCs, the combination of 0.1 ng/mL phorbol myristate acetate (PMA; Sigma) and 0.5 nM oktide and 0.1 μg/mL anti-CD8 T cells using magnetic microbeads (Miltenyi Biotec, Surrey, United Kingdom) according to the manufacturer’s instructions. Fifty thousand cells from the CD8-depleted PBMCs were also added to the culture medium.

The PBMCs were depleted of CD8+ T cells using magnetic microbeads (Miltenyi Biotec, Surrey, United Kingdom) according to the manufacturer’s instructions. Fifty thousand cells from the CD8-depleted PBMCs were also stained and analyzed by flow cytometry to measure the percentage positivity for the required surface markers of CD4 and CD8; to derive the numbers of CD4+ T cells (responding CD4+ T cells, which corresponds to the CD4+, CD3+ population [data not shown]), and CD8+ cells left after CD8+ depletion. Typically, there were less than 3% CD8+ cells left after depletion.

Synthetic peptides

Peptide libraries spanning the entire length of HTLV-1 Env and Tax proteins (strain ATK) were synthesized by Mimotopes Pty (formerly Chiron Mimotopes, Chiron Technologies, Victoria, Australia). Purity was checked by reverse-phase high-performance liquid chromatography and ion spray mass spectrometry and was more than 84%. Env peptides: 20mer peptides offset by 5 (total of 95). Tax peptides: 13mer peptides offset by 4 (total of 86). (Full details are available on request.)

Peptides were grouped in pools of 20, and added to the cell culture medium to achieve a final concentration of 1 μM for each peptide, prior to incubation (either Elispot or flow cytometric assays) at 37°C.

Elispot assays for IFN-γ and interleukin 4

Flat-bottomed 96-well polystyrene microtiter plates (MAIPS45, Millpore, Bedford, United Kingdom) were first washed with sterile PBS. Each well was then precoated with the primary capture antibody to IFN-γ or interleukin-4 (IL-4) (respectively clones 1-D1K or IL-4-1, Mabtech, Nacka, Sweden) at a concentration of 15 μg/mL in sterile PBS. Antibody was allowed to bind to the membrane overnight at 4°C.

Plates were then washed 6 times with sterile PBS. CM was then added to block nonspecific binding of cytokines and antibodies, and incubated at 37°C for 3 hours.

The blocking solution was then discarded and cells were then dispensed at predefined input cell numbers per well.

Stimulatory mAbs to CD28 (clone CD28.2, Pharmingen, Becton Dickinson, Oxford, United Kingdom) and CD49d (clone HP2/1, Serotec), both at 0.5 μg/mL, were then added for CD4+ T-cell assays. Peptides were then added directly to the supernatant at a final concentration of 1 μM each and the plates incubated for 6 hours at 37°C in 5% CO2.

The cells were then discarded and the plate washed 6 times with PBS/0.05% Tween 20. Plates were then incubated at room temperature for 2 hours with the second-layer biotinylated antibody to IFN-γ or IL-4 (7-B6-1-biotin or IL-4-biotin, Mabtech). The medium was then discarded and plates washed 6 times with PBS/0.05% Tween 20. Chromogenic alkaline phosphatase conjugate (diluted 1:1000 in sterile PBS) was added and incubated at room temperature for 1 hour. This solution was then discarded and the plates washed 6 times with PBS/0.05% Tween 20. The PBMCs were then exposed to 1-D1K or IL-4-I, Mabtech, Nacka, Sweden) at a concentration of 15 μg/mL, were then added for CD4+ T-cell assays. Peptides were then added directly to the supernatant at a final concentration of 1 μM each and the plates incubated for 6 hours at 37°C in 5% CO2.

Flow cytometry

Cell preparation and antigen stimulation. For antigen-specific cytokine responses, 1 × 106 cells (PBMCs depleted of CD8+ cells) were placed in 16 × 125-mm round-bottom polystyrene tissue culture tubes (Corning Costar, Cambridge, MA) with 1 mL CM, supplemented with peptides each at 1 μM final concentration and costimulatory mAbs as above. Culture tubes were incubated at 37°C in a humidified 5% CO2 atmosphere for a total of 6 hours, with the last 5 hours including a final concentration of 10 μg/mL. Brefeldin A (Sigma) to inhibit secretion of cytokines from the cell. After incubation, the cells were harvested for subsequent staining.

Cell surface staining. Harvested cells were washed in PBS containing 7% normal goat serum (NGS; Sigma) and then incubated with NGS for 30 minutes at 4°C to block the Fc receptor sites on cells of the monocyte/macrophage lineage. After one wash, cells were incubated with the relevant mAbs (each at 15 μg/mL) to surface markers, that is, phycoerythrin-cyanine 5.1 (PC5)–labeled anti-CD4, energy-coupled dye (ECD)–labeled anti-CD8 (ECD = phycoerythrin [PE] + Texas red), PE-labeled anti-CD69 (Beckman Coulter, Becton Dickinson, Oxford, United Kingdom) for 30 minutes at 4°C.

The stained cells were washed and fixed with 4% paraformaldehyde (Sigma) in PBS (pH 7.4) for 5 minutes at room temperature and washed again. Cells were then resuspended in PBS at 4°C awaiting analysis (if surface staining alone was required) or processed further for intracellular cytokines and HTLV-1–Tax protein detection.

Detection of intracellular Tax protein and cytokines. Cells were permeabilized with PBS/7% NGS containing 0.2% saponin (permeabilization buffer [PB]) for 10 minutes at room temperature and then washed. The cells were then resuspended in this solution with PE-labeled or fluorescein isothiocyanate (FITC)–labeled anti-IFN-γ mAb (Beckman Coulter), anti-Tax mAb (LT4), isotype controls, or mAbs to other cytokines, for example, IL-4, as appropriate for 20 minutes at room temperature. The cells were washed twice with PB and then resuspended in PB with FITC- or
Results

Detection of HTLV-1–specific CD4+ T cells by in vitro activation with HTLV-1 Env and Tax peptides

There has been no previous report of experimental estimates of HTLV-1–specific CD4+ T-cell frequencies in HTLV-1–infected subjects. Conventional proliferation assays cannot be used to quantify HTLV-1–specific CD4+ T-cell frequencies because of the high level of spontaneous cytokine production and proliferation in PBMCs from HTLV-1–infected people when cells are cultured in vitro. We have recently shown that HTLV-1–infected CD4+ T cells spontaneously produce detectable cytokines (initially IFN-γ) only after more than 6 hours of in vitro cultivation (no cytokines are detectable ex vivo). We have therefore devised flow cytometric and Elispot assays to directly detect HTLV-1–specific CD4+ T cells by short-term (6 hours) in vitro stimulation with Env and Tax peptides. We used peptide libraries encompassing the full-length sequences of Env and Tax to ensure that CD4+ T cells would be detected, regardless of their class II MHC specificity.

Figure 1A shows that HTLV-1 peptides induced detectable IFN-γ production only in CD4+ lymphocytes from the patient with HAM/TSP and not in cells from an uninfected control subject. The absence of IFN-γ+ cells in the uninfected control demonstrates the specificity of the assay. The use of an isotype control mAb also confirmed the specificity of IFN-γ staining (data not shown). This suggested that the 6-hour assay could be used for detection of HTLV-1 peptide-specific CD4+ T cells. When cultivation was extended to 12 hours or longer for samples from infected patients, the background IFN-γ staining became unacceptably high and meaningful results could not be obtained because over 10% of Tax-expressing CD4+ T cells expressed IFN-γ at 12 hours and this figure rose to about 20% at 24 hours.13

Elispot assays produced an equivalent result, that is, only CD4+ T cells cultivated with HTLV-1–derived peptides for 6 hours in 96-well plates showed positive responses, defined as a spot-forming cell (SFC) count that significantly exceeded the negative controls (see “Patients, materials, and methods”).

Comparison of independent replicate Elispot assays on cells from a single blood sample, performed on different dates, showed high reproducibility (Figure 1B). We then compared data from both Elispot and intracellular cytokine staining assays on cells obtained from single blood samples. Figure 1C shows that the results were similar.

To further confirm that the peptide-induced responses were MHC class II mediated, we added anti-MHC class I and II mAbs to the culture medium to inhibit class I and II restricted responses, respectively. As expected, the responses were inhibited by anti–class II MHC antibodies but not anti–class I MHC antibodies (Figure 2).

Figure 1. Detection of HTLV-1–specific CD4+ T cells by in vitro activation with Env and Tax peptides. (A) Dot plots showing intracellular cytokine staining for IFN-γ production in CD4+ T cells from a patient with HAM/TSP (TAF) and an uninfected control (UN). PBMCs were depleted of CD8– T cells, cultivated with Env and Tax peptides for 6 hours in vitro, returned and stained. One representative experiment from 3 patients with HAM/TSP is shown. Numbers in brackets show actual numbers of events acquired. (B) Comparison of HTLV-1–specific CD4+ T-cell frequencies determined by independent Elispot assays at different time points from a single blood sample. One representative experiment from 3 patients with HAM/TSP is shown. IFN-γ SFCs were divided by the number of CD4+ T cells present in each well, then multiplied by 100 to provide the data shown. A response was defined as positive if the number of spots exceeded the mean + 2 SDs of the spot count in the negative control wells (no peptides added). (C) Comparison of HTLV-1–specific CD4+ T-cell frequencies determined by Elispot and intracellular cytokine staining from the same blood sample. Results from 3 patients with HAM/TSP (TAT, TAF, TW) are shown. IFN-γ SFCs are divided by the number of CD4+ T cells present in each well, then multiplied by 100 to provide the Elispot data shown. Flow cytometric data shown is derived from IFN-γ+CD4+ T cells divided by the total number of CD4+ T cells present, then multiplied by 100.
The failure to detect IL-4 could be due to poor sensitivity of the Elispot assay used. To investigate this possibility, PBMCs depleted of CD8+ cells from all the patients were stimulated with polyclonal activators (PMA and A23187) and spots detected with our IL-4 Elispot assay. Responses ranged from 0.01% to 0.82% of CD4+ T cells (median frequency, 0.21%). This showed that a significant proportion of the CD4+ T cells retained the ability to secrete IL-4 and that these responses were detectable. The spontaneous IL-4 responses to peptides were detectable in 5 of 9 patients and ranged from 0.0020% to 0.17% of their CD4+ T cells (median, 0.10%).

High frequencies of HTLV-1 Env- and Tax-specific CD4+ T cells in HAM/TSP patients

Having established that the Th1 phenotype is predominant among HTLV-1–specific CD4+ T cells in patients with HAM/TSP, we determined the frequencies of IFN-γ–secreting HTLV-1 Env- and Tax–specific CD4+ T cells in 9 patients with HAM/TSP, 7 ACs, and 3 uninfected controls.

The results (Figure 4) show that patients with HAM/TSP had a significantly higher frequency of HTLV-1–specific CD4+ T cells than both healthy ACs and uninfected controls. These responses varied from 0.12% to 0.93% (median, 0.23%) of circulating CD4+ T cells for patients with HAM/TSP, compared to ACs in whom the frequency varied from undetectable to 0.34% (median frequency, 0.038%). Uninfected controls showed a basal level of response (median frequency, 0.035%). There was no significant difference between the responses seen in ACs and the basal level seen in uninfected controls. Experiments are now under way to define the precise peptides recognized by each individual.

Detection of Tax expression in PBMCs from HTLV-1–infected patients

We have recently devised a sensitive flow cytometric assay to detect intracellular Tax protein expression.21 We have now further characterized the sensitivity and specificity of the assay. Figure 5A shows the result of Tax staining in MT-2 cells (chronically infected with HTLV-1). Jurkat cells (uninfected), and a patient with HAM/TSP’s PBMCs (cultivated in vitro for 6 hours). Figure 5B shows the result of Tax staining in MT-2 cells (chronically infected with HTLV-1). Jurkat cells (uninfected), and a patient with HAM/TSP’s PBMCs (cultivated in vitro for 6 hours).
shows the results of staining differing ratios of MT-2 cells with Jurkat cells. These data confirmed that the Tax staining assay was able to detect one MT-2 cell among 10,000 uninfected cells.

Proportion of HTLV-1–specific CD4+ T cells that express Tax protein

We wished to know whether HTLV-1–specific CD4+ T cells were selectively infected with HTLV-1 because infection has been shown to alter the function of T-helper cells.19,20 Recent data from our laboratory suggested that there is preferential infection of HTLV-1–specific CTLs.30

Therefore, staining for intracellular Tax expression was combined with the 6-hour flow cytometric assay for intracellular IFN-γ. One representative experiment from 5 subjects is shown in Figure 6.

Table 1 shows that Tax expression was less frequent in HTLV-1–specific CD4+ T cells than CD4+ T cells of other specificities, in 2 patients with HAM/TSP, whereas Tax expression was more frequent in HTLV-1–specific CD4+ T cells in both ACs and a third patient with HAM/TSP. This contrasts with our previous finding that Tax expression in CD8+ T cells was detected only among HTLV-1–specific cells (in 5 of 5 individuals) and not among Epstein-Barr virus (EBV)–specific cells (0 of 4 individuals).30

The fact that most HTLV-1–specific CD4+ T cells were not infected with transcriptionally active virus, also implies that they were not susceptible to CTL-mediated killing in vivo. Thus, the majority of circulating HTLV-1–specific CD4+ T cells remain capable of producing proinflammatory cytokines on encountering their cognate antigen.
associated with a strong antiviral CTL response and therefore with effective control of HTLV-1 infection, or harmful because the production of proinflammatory cytokines could lead to “bystander damage” to uninfected cells. It is possible that the balance of good and harm done by HTLV-1–specific CD4+ T cells depends on factors such as the proviral load, in a manner analogous to the mechanism we have proposed in the CD8+ T-cell response to HTLV-1.34

Furthermore, we showed that the frequencies of HTLV-1 Env- and Tax-specific CD4+ T cells are significantly greater in HAM/TSP patients than in ACs. Activated Th1 cells (but not Th2) are reported to have a strong tendency to cross vessel walls (including the blood-brain barrier) and infiltrate areas of inflammation.35 Therefore, we suggest that these abundant Th1-type HTLV-1–specific CD4+ T cells are likely to encounter cognate antigen in the tissues (eg, the CNS) when the proviral load is high (as in patients with HAM/TSP), because the frequencies of both the Th1 CD4+ T cells and infected CD4+ T cells are high. The high frequencies of specific CD4+ T cells suggest that there is continuous antigen stimulation in vivo, implying in turn that the virus is not latent.

It is well established that HTLV-1 infection is associated with a high proviral load in peripheral blood.36 It is also known that the main burden of HTLV-1 infection in vivo is carried by the activated memory subset of CD4+ T cell (CD3+, CD4+, CD45RO+).10,31 Furthermore, we have previously shown that a majority of these infected cells were clustered in the effector/memory CD4+ T cell.10,11 The paucity of data on the HTLV-1–specific CD4+ T-cell response reflects the problems encountered with the phenomena of spontaneous lymphocyte proliferation and cytokine production seen in PBMCs from these infected patients.

Using short (6-hour) cultures we have eliminated the effect of spontaneous proliferation of lymphocytes from our assays. This has enabled us to demonstrate HTLV-1 peptide–specific CD4+ T-cell responses in subjects infected with HTLV-1. We have found that these cells exhibit a Th1 phenotype and we therefore postulate a role for these cells in the pathogenesis of HTLV-1–associated inflammatory disease, especially in HAM/TSP. Pathologic examination of the spinal cord has shown that in patients with HAM/TSP with a short duration of symptoms, the characteristic perivascular lymphocyte infiltrate consists predominantly of CD4+ T cells; only later do CD8+ T cells predominate, and finally an atrophic picture is seen.31

Whether the CD4+ T cells present in HAM/TSP lesions are predominately HTLV-1 infected or HTLV-1–specific has not been shown. However, if HTLV-1–specific CD4+ T cells of the Th1 phenotype are also frequent among the CD4+ T cells that infiltrate CNS lesions (as they are in peripheral blood, which we show in this paper), then such cells may be important in the pathogenesis of HAM/TSP, particularly in early lesions, through the secretion of neurotoxic cytokines such as IFN-γ. A similar role has been suggested for HTLV-1 Tax–specific CTLs,32 which predominate later in disease. However, relatively little is known of the functions of the infiltrating lymphocytes; both cell types may contribute to pathogenesis, in varying proportions, throughout the course of the disease.

This observation that HTLV-1–specific CD4+ T cells in HAM/TSP patients are mainly of the Th1 phenotype is important because a dominant Th1 response could be either beneficial, harmful, or both. A strong Th1 response could be beneficial because it is

### Table 1. Detection of Tax protein expression in CD4+ T cells from HTLV-1–infected subjects

<table>
<thead>
<tr>
<th>Percentage of Tax-expressing cells</th>
<th>IFN-γ− cells</th>
<th>IFN-γ+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAF</td>
<td>3.3</td>
<td>5.8</td>
</tr>
<tr>
<td>TAT</td>
<td>0.4</td>
<td>6.1</td>
</tr>
<tr>
<td>TW</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>HAY</td>
<td>10.7</td>
<td>0.4</td>
</tr>
<tr>
<td>HT</td>
<td>5.9</td>
<td>0.8</td>
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The 6-hour flow cytometric assay for intracellular IFN-γ was combined with staining for Tax protein expression. Patients with HAM/TSP have codes beginning with T and ACs have codes beginning with H.

These data do not exclude the possibility that some infected HTLV-1–specific CD4+ cells are unable to produce cytokines when activated with their respective antigen and thus escape detection in these assays. Testing of this point may have to await the advent of better techniques.

### Discussion

The role of the cellular immune response in HTLV-1 infection is not fully understood. To date, the focus has been on the CTL response to the virus and there are few published data on the CD4+ T-cell response to HTLV-1. However, the importance of the CD4+ T-cell response should not be underestimated, because it has been shown to be essential for efficient humoral and cellular immune responses, including the CTL response.17-18 The situation is complicated further by the fact that the main subset of lymphocytes infected with HTLV-1 in vivo is the effector/memory CD4+ T cell.10,11 The paucity of data on the HTLV-1–specific CD4+ T-cell response reflects the problems encountered with the phenomena of spontaneous lymphocyte proliferation and cytokine production seen in PBMCs from these infected patients.

Infection of HTLV-1–specific CD4+ T cells would have the following implications: (1) disruption of normal in vivo function by up-regulation and transactivation of cellular gene expression by Tax, and (2) viral protein expression in infected T cells would render them susceptible to killing by autologous CTLs. Taken together, these could seriously impair or weaken the CD4+ T-cell immune response to HTLV-1. Using our recently devised sensitive and specific flow cytometric assay, we have shown that the majority of these HTLV-1–Env- and Tax–specific CD4+ T cells did not express the Tax protein after 6 hours of culture in vitro. This observation suggested that most HTLV-1–specific CD4+ T cells were not infected by transcriptionally active HTLV-1 and therefore remained capable of normal cytokine production on encountering antigen.

In conclusion, we demonstrate significantly higher frequencies of Env- and Tax–specific CD4+ T cells in patients with HAM/TSP than in ACs. We also show that the Th1 phenotype is dominant among these cells in patients with HAM/TSP. The majority of these Th1-type HTLV-1–specific CD4+ T cells are also shown not to express Tax protein after short in vitro culture and thus remain capable of responding normally when encountering cognate antigen. We suggest that these cells are involved in the initiation and pathogenesis of inflammatory disease in patients with HAM/TSP by the production of proinflammatory cytokines when activated by HTLV-1 antigens in the CNS and other end organs.

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