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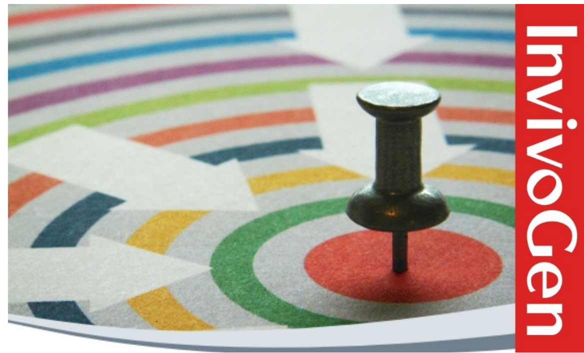

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Human T Cell Lymphotropic Virus Type I (HTLV-I)-Specific CD4$^+$ T Cells: Immunodominance Hierarchy and Preferential Infection with HTLV-I[1]

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CD4$^+$ T cells predominate in early lesions in the CNS in the inflammatory disease human lymphotropic T cell virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP), but the pathogenesis of the disease remains unclear and the HTLV-I-specific CD4$^+$ T cell response has been little studied. We quantified the IFN-γ-producing HTLV-I-specific CD4$^+$ T cells, in patients with HAM/TSP and in asymptomatic carriers with high proviral load, to test two hypotheses: that HAM/TSP patients and asymptomatic HTLV-I carriers with a similar proviral load differ in the immunodominance hierarchy or the total frequency of specific CD4$^+$ T cells, and that HTLV-I-specific CD4$^+$ T cells are preferentially infected with HTLV-I. The strongest CD4$^+$ T cell response in both HAM/TSP patients and asymptomatic carriers was specific to Env. This contrasts with the immunodominance of Tax in the HTLV-I-specific CD8$^+$ T cell response. The median frequency of HTLV-I-specific IFN-γ^+ CD4$^+$ T cells was 25-fold greater in patients with HAM/TSP ($p = 0.0023$, Mann-Whitney) than in asymptomatic HTLV-I carriers with a similar proviral load. Furthermore, the frequency of CD4$^+$ T cells infected with HTLV-I (expressing Tax protein) was significantly greater ($p = 0.0152$, Mann-Whitney) among HTLV-I-specific cells than CMV-specific cells. These data were confirmed by quantitative PCR for HTLV-I DNA. We conclude that the high frequency of specific CD4$^+$ T cells was associated with the disease HAM/TSP, and did not simply reflect the higher proviral load that is usually found in HAM/TSP patients. Finally, we conclude that HTLV-I-specific CD4$^+$ T cells are preferentially infected with HTLV-I. ***The Journal of Immunology,*** **2004, 172: 1735–1743.**

Human T cell lymphotropic virus type I (HTLV-I)[3] was the first human retrovirus discovered, in 1980 (1). The virus is endemic in many tropical regions particularly Melanesia, the Caribbean, West Africa, Central/South America, and in Southern Japan and Iran. It is estimated to infect between 10 and 20 million people worldwide (2). Unlike HIV, HTLV-I does not cause disease in the majority of infected subjects (asymptomatic carriers (AC)). Approximately 2–3% develop adult T cell leukemia/lymphoma and another 2–3% develop chronic inflammatory disease, of which HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the most commonly recognized but polymyositis, alveolitis, arthritis, thyroiditis, uveitis, and other end-organ inflammatory diseases have also been described (3).

Like other exogenous replication-competent retroviruses, HTLV-I possesses the three main genomic regions of *env*, *gag*, and *pol*, but unlike other leukemia viruses, it has an additional region called *pX* that encodes the proteins Tax, Rex, Tof (including p12^{I}), Rof (including p13^{II}) and possibly others by variant mRNA splicing. The Tax protein is crucial to virus dynamics, because as well as transactivating viral transcription, it is thought to drive host cell proliferation. The Tax protein is also the dominant target Ag recognized by HTLV-I-specific CTL in most responding individuals (4–6) although CTLs have also been detected against Env, Rex, Gag, Rof, and Tof proteins (5–7). So far, most investigations of the specific cellular immune response to HTLV-I have focused on the CD8$^+$ CTL response.

However, the CD4$^+$ T cell response to HTLV-I is also important for the following reasons: 1) CD4$^+$ T cells are the predominant subset of lymphocytes infiltrating the CNS early in HAM/TSP (8). 2) CD4$^+$ T cell help is necessary for optimal CTL and Ab responses in both animal and human infections. 3) CD4$^+$ T cells carry most of the HTLV-I proviral load burden in vivo (9, 10) and a high proviral load is associated with the development of HAM/TSP. 4) HTLV-I-infected CD4$^+$ T cells spontaneously secrete proinflammatory, neurotoxic cytokines such as IFN-γ, TNF-α, and GM-CSF (11, 12) which are found in high levels in the cerebrospinal fluid and spinal cord lesions of HAM/TSP (13–15).

In a previous study (16), we showed that a proportion of CD8$^+$ T cells are also infected with HTLV-I in vivo, and we obtained evidence of preferential infection of HTLV-I-specific CD8$^+$ T cells. Infection of T cells with HTLV-I renders them susceptible to lysis by the abundant, activated HTLV-I-specific CTLs, and preferential infection of HTLV-I-specific CTLs is likely to diminish the efficiency of the anti-HTLV-I CTL response (17). Preferential infection of HTLV-I-specific CD4$^+$ T cells might similarly impair the immune response to HTLV-I.

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[3] Abbreviations used in this paper: HTLV-I, human T cell lymphotropic virus type I; AC, asymptomatic carrier; HAM/TSP, HTLV-I-associated myelopathy/tropical spastic paraparesis; CM, complete medium; RT, room temperature; PB, permeabilization buffer; SFC, spot-forming cell.

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The $CD4^+$ T cell response to HTLV-I is difficult to quantify by conventional T cell proliferation assays, because of the antigenic and mitogenic effects of spontaneously expressed HTLV-I Ags, chiefly Tax (18). However, we recently showed that a short-term (6 h) ELISPOT assay can be used to identify specific $CD4^+$ T cells, before the HTLV-I protein expression significantly increases IFN-γ expression (19). In the same study, we found that the frequency of $CD4^+$ T cells specific to HTLV-I Env and Tax proteins was significantly greater in patients with HAM/TSP than in ACs. In the present study, we quantified the frequency of $CD4^+$ T cells specific to Gag, Pol, Rex, Tof, and Rof in addition to Env and Tax, in a group of patients with HAM/TSP and in a group of ACs of HTLV-I whose proviral load was not significantly different from that of the HAM/TSP group.

The aims of the present study were 1) to compare the total frequency and the immunodominance hierarchy of IFN-γ-producing HTLV-I-specific $CD4^+$ T cells between patients with HAM/TSP and ACs, and 2) to test the hypothesis that HTLV-I-specific $CD4^+$ T cells are preferentially infected with HTLV-I.

We assayed the HTLV-I-specific $CD4^+$ T cell response using overlapping peptides spanning the HTLV-I proteome. The total frequency of HTLV-I-specific $CD4^+$ T cells was significantly greater in HAM/TSP patients than in ACs of the virus and was predominantly directed to the major retroviral proteins Env, Gag, and Pol although responses to other viral proteins were detected in certain individuals. The immunodominance of Env in the HTLV-I-specific $CD4^+$ response in both HAM/TSP patients and ACs contrasts with the typically strong immunodominance of the Tax protein in the anti-HTLV-I $CD8^+$ T cell response.

Furthermore, we show that these HTLV-I-specific $CD4^+$ T cells were preferentially infected with HTLV-I, by direct comparison with CMV-specific $CD4^+$ T cells in the same HTLV-I-infected subjects. Preferential infection and consequent CTL-mediated destruction of HTLV-I-specific Th cells may impair the efficiency of the immune response to HTLV-I.

Materials and Methods

Subjects and cells

Subjects were informed and consenting asymptomatic HTLV-I-infected ACs and patients with HAM/TSP attending the HTLV-I clinic at St. Mary's Hospital (London, U.K.). Uninfected controls were derived from healthy uninfected staff from Imperial College (London, U.K.). HTLV-I infection was confirmed by the presence of Abs to HTLV-I Gag (p19 and p24) and Env (rgp21 and rgp46-I) Ags in sera by Western blot (Genelabs HTLV 2.4, Singapore). The diagnosis of HAM/TSP was made according to World Health Organization criteria (20). PBMC were isolated via density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich, Dorset, U.K.) and washed three times with PBS. Cells were then preserved in liquid nitrogen in FCS (Sigma-Aldrich) supplemented with 10% DMSO (Sigma-Aldrich).

After thawing and washing in cold sterile PBS (two times), cells were cultured in complete medium (CM) which is RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS, 2 mmol/L glutamine (Life Technologies, Paisley, U.K.), 100 IU/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies). All cells were cultured in this medium unless stated otherwise. To induce nonspecific cytokine production by PBMCs, the combination of 0.1 ng/ml PMA (Sigma-Aldrich) and 0.5 μg/ml A23187 (Sigma-Aldrich) was added to the culture medium. For certain experiments, 20 nM concanamycin A (an inhibitor of perforin release) was added to the culture medium to prevent CTL-mediated lysis via the perforin-dependent cytotoxic pathway (10).

PBMC were depleted of $CD8^+$ T cells using magnetic microbeads (Miltenyi Biotec, Surrey, U.K.) according to the manufacturer's instructions, and run through two successive columns to achieve maximum depletion. Fifty thousand cells from the CD8-depleted PBMC were also stained and analyzed by flow cytometry to measure the percentage positivity for the surface markers CD4, CD8, and CD3. Typically, there were <1% $CD8^+$ T cells left after depletion (data not shown).

HTLV-I proviral DNA quantification

HTLV-I viral DNA was quantified by real-time PCR using the Roche LightCycler (Mannheim, Germany).

Genomic DNA extracted from 1×10^3 PBMCs (21) was used as a template for amplification. In addition, the reaction mixture contained 2 μl of 10× LightCycler-DNA Master SYBR Green 1(Roche, Mannheim, Germany), 0.5 μM of each HTLV-I primer (SK43 and SK44; Ref. 22), and a final $MgCl_2$ concentration of 3.5 mM, made up in water to a volume of 20 μl. After denaturation at 95°C for 30 s, the DNA was amplified for 40 cycles: denaturation at 95°C for 0 s, annealing at 58°C for 5 s, and extension at 72°C for 8 s. At the end of each cycle, the SYBR Green 1 incorporated into dsDNA was quantified by measurement of fluorescence at 525 nm. Fluorescence was detected at 85°C, below the melting temperature of the specific product but above the melting temperature of primer-dimers. The β-globin copy number of each sample was similarly quantified, using primers PC03 and PC04 (23) and a final $MgCl_2$ concentration of 4 mM.

Standard curves were generated for both PCRs using genomic DNA from C10-PBL cells, which contains 1 Tax copy per cell. Reactions containing 10^4, 10^3, 10^2, and 10^1 copies were used to generate the standard curves. The copy numbers in the samples were estimated by interpolation from the standard curves.

The HTLV-I viral load was then calculated as: [tax copies/10^3 cells]/[0.5 β-globin copies/10^3 cells] $\times$ 100 = tax copies/100 PBMCs.

Synthetic peptides

Peptide libraries spanning the entire length of HTLV-I proteins (strain ATK; Ref. 24) were commercially synthesized by Mimotopes (formerly Chiron Mimotopes, Chiron Technologies, Victoria, Australia). Purity was checked by reverse phase HPLC and ion spray mass spectrometry and was always >84%.

Env peptides − 20-mer peptides offset by 5 (n = 95).
Gag peptides − 20-mer peptides offset by 6 (n = 70).
Pol peptides − 20-mer peptides offset by 6 (n = 147).
Pro peptides − 20-mer peptides offset by 6 (n = 37).
Tax peptides − 13-mer peptides offset by 4 (n = 86).
Rex peptides − 20-mer peptides offset by 6 (n = 30).
Tof (including $p12^I$) peptides − 20-mer peptides offset by 6 (n = 38).
Rof (including $p13^{II}$) peptides − 20-mer peptides offset by 6 (n = 23).
Full details of peptide sequences are available on request.

Peptides were grouped in pools of 15–30 (mean of 22, to ensure maximal equivalence but certain pools, i.e., Gag p15, p19, p24, were kept intact for ease of analysis), and added to the cell culture medium to achieve a final concentration of 1 μg/ml of each peptide, before incubation (either ELISPOT or flow cytometric assays) at 37°C.

ELISPOT assays for IFN-γ

Method and analysis used have been previously described (19). The nonspecific T cell mitogens, PMA, and A23187 (an ionomycin analog) (both from Sigma-Aldrich) were always included as positive controls, in concentrations described earlier. Stimulatory mAbs to CD28 (clone CD28.2; BD PharMingen/BD Biosciences, Oxford, U.K.) and CD49d (clone HP2/1; Serotec, Oxford, U.K.) (each at 0.5 μg/ml) were added (25). All assays were conducted in duplicate wells. SFC stands for spot-forming cell.

Flow cytometry

Cell preparation and Ag stimulation. For Ag-specific cytokine responses, 1×10^6 cells (PBMCs) were placed in 16 × 125-mm round-bottom polystyrene tissue culture tubes (Corning Costar, Cambridge, MA) with 1 ml of CM, supplemented with peptides and costimulatory mAbs as above. Culture tubes were incubated at 37°C in a humidified 5% CO_2 atmosphere for a total of 6 h, with the last 5 h including a final concentration of 10 μg/ml brefeldin A (Sigma-Aldrich) to inhibit secretion of cytokines from the cell. After incubation, the cells were harvested for subsequent staining.

Peptide stimulation protocol is as for ELISPOT but typically all peptides were grouped into one pool and then used for stimulation of PBMCs.

Cell surface staining. Harvested cells were washed in PBS containing 7% normal goat serum (Sigma-Aldrich) and incubated with FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 4°C to block the FcR 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, i.e., energy-coupled dye-labeled anti-CD4, PC5-labeled anti-CD8, FITC-labeled anti-CD3 (Beckman Coulter, Bedfordshire, U.K.) for 30 min at 4°C. The stained cells were washed and fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) for 5 min at room temperature (RT) and washed again. Cells were then resuspended in PBS

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at 4°C awaiting analysis (if surface staining alone was required) or processed further for intracellular cytokines and/or HTLV-I Tax protein detection.

Detection of intracellular Tax protein and cytokines. Cells were permeabilized with PBS/7% normal goat serum containing 0.2% saponin (permeabilization buffer (PB)) for 10 min at RT and then washed. The cells were resuspended in this solution with PE-labeled or FITC-labeled anti-IFN-γ mAb (Beckman Coulter), anti-Tax mAb (LT4), or isotype controls as appropriate for 20 min at RT. The cells were washed twice with PB and resuspended in PB with FITC or PE-labeled goat $F(ab')_2$ anti-mouse IgG3 (Southern Biotechnology Associates, Birmingham, AL) as appropriate for 20 min at RT. Finally, the cells were washed twice with PB, resuspended in PBS, and analyzed by flow cytometry on a Coulter Epics XL (Beckman Coulter) with Coulter Expo 32 software.

MACS IFN-γ cytokine secretion assay. Full protocol details are obtained from datasheets from MACS (Miltenyi Biotec). PBMCs depleted of $CD8^+$ cells were cultured for 5 h with the respective Ags, i.e., peptides or viral lysate. Costimulatory mAbs anti-CD28 and anti-CD49d were also added at the start of culture, as detailed previously. After 5 h, the cells were harvested and then labeled with anti-CD45 linked to anti-IFN-γ fusion Ab (Catch Reagent) for 5 min on ice (20 μl of catch reagent per 80 μl of cold CM per 10^7 cells). This cell suspension was then diluted with 10 ml of warm CM, transferred into a Petri dish, and incubated for 1 h at 37°C, 5% CO_2. Cells were then harvested and washed with 10 ml of cold buffer (PBS/10% FCS) twice and then resuspended in 80 μl of cold buffer per 10^7 cells. Twenty microliters of anti-IFN-γ-PE or FITC detection Ab were then added per 10^7 cells and the suspension was incubated for 10 min on ice. The cells were then washed with 10 ml of cold buffer twice and the cell pellet was resuspended in 80 μl of cold buffer per 10^7 cells and 20 μl of anti-PE or anti-FITC magnetic microbeads were added (per 10^7 cells) and incubated for 15 min at 6–12°C. Finally, the cells were washed twice with 10 ml of cold buffer and put through two magnetic columns sequentially, to select a highly pure population of IFN-γ^+ Ag-specific $CD4^+$ T cells.

Statistical analysis

We relied on nonparametric statistical tests, because such tests involve fewer assumptions about the distribution of the data than do parametric tests. To compare the median frequency of HTLV-I-specific $CD4^+$ T cells between two subject groups, we used the Mann-Whitney *U* test. To test for evidence of a correlation between specific $CD4^+$ T cell frequency and proviral load, we used Spearman's rank-order correlation.

All data were stored and manipulated in Microsoft Excel databases (Microsoft, Redmond, WA). Statistical analysis was performed using Excel and GraphPad InStat (San Diego, CA).

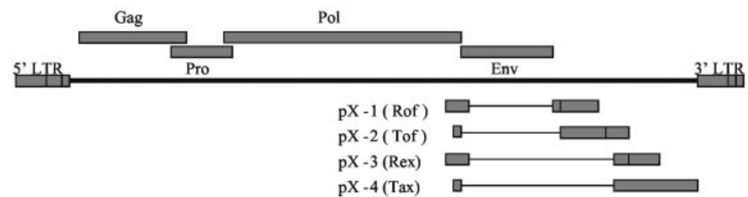
Results

Immunodominance hierarchy of the HTLV-I-specific $CD4^+$ T cell response

We used libraries of overlapping synthetic peptides spanning all major HTLV-I proteins, to quantify the total frequencies of HTLV-I- specific IFN-γ^+ $CD4^+$ T cells regardless of the subject's MHC haplotype because the libraries would contain all possible T cell epitopes. This strategy has also been used successfully by groups studying other viral infections (26–29).

The CTL response to HTLV-I appears to be chiefly directed toward the protein Tax (4–6). However, the majority of the $CD4^+$

A The integrated HTLV-I genome and associated major proteins



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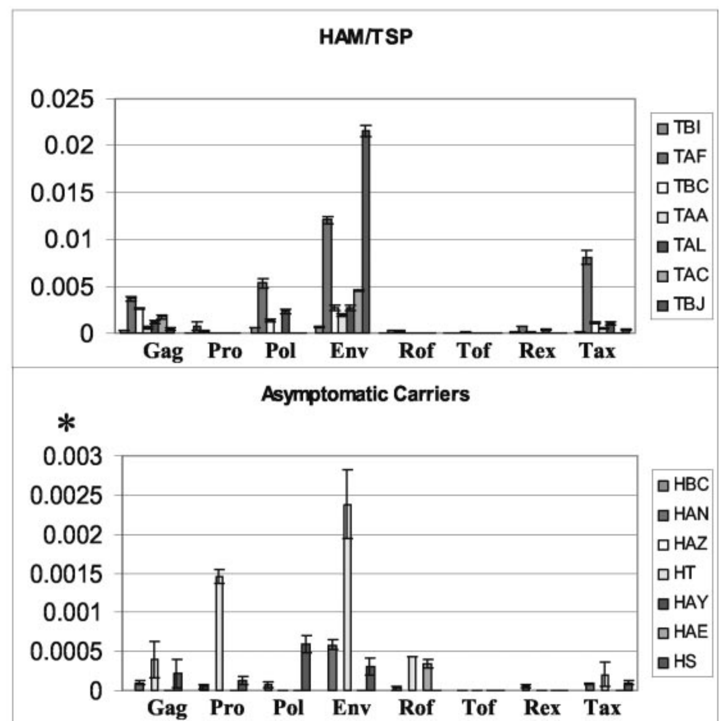

FIGURE 1. Detection of HTLV-I-specific $CD4^+$ T cells by in vitro activation with peptide pools covering all major proteins from the entire HTLV-I genome. 1) Genetic structure of the HTLV-I proviral genome and the associated major proteins derived from the respective open reading frames. 2) ELISPOT data showing the mean frequencies of HTLV-I peptide-specific $CD4^+$ T cells responding to different HTLV proteins. Seven HAM/TSP patients and seven ACs were analyzed. All assays were done in duplicate; error bars indicate ±SD (see Table I). The anonymized code for each subject is shown. The data show the immunodominance hierarchy for the individual proteins in the $CD4^+$ T cell response.

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T cell response was skewed toward the major structural and enzymatic proteins of the virus, consistent with the processing of exogenous proteins via the MHC class II presentation (endocytic) pathway. These data are illustrated in Fig. 1 and in Table I. Similar results have been obtained on the CD4$^+$ T cell response to other viruses such as HIV, where the main response is toward major viral proteins such as Env, Gag, and Pol (26). These results indicate that any HTLV-I viral protein can be the target of virus-specific CD4$^+$ T cells although the largest responses are directed toward Env, Gag, Tax, and Pol. The most frequently recognized proteins were Env and Gag (10 of 14 subjects each), then Tax (9 of 14 subjects), Pol (6 of 14), then Pro, Rof, and Rex (each 5 of 14) and lastly Tof (1 of 14). The frequency of Env-specific CD4$^+$ T cells (median frequency = 0.0025 of total circulating CD4$^+$ T cells) was significantly higher than those that recognized Gag (median frequency = 0.0005) or Tax (median frequency = 0.0004) (Mann-Whitney U test two-tailed $p = 0.0288$, $p = 0.0089$, respectively). The frequency of anti-Gag CD4$^+$ T cells was not significantly higher than that of anti-Tax cells ($p = 0.3154$). Thus, Env was clearly the immunodominant target Ag for the HTLV-I-specific CD4$^+$ T cell response.

Mapping of an immunodominant epitope in Env

We identified a novel immunodominant epitope in Env, restricted by the class II MHC allele DRB1*1303/33. These data are shown in Table II. The 15-mer amino acid sequence was IAQYAAQN-RRGLDLL, which corresponds to aa 371–385 and is located in the gp21 transmembrane region. This region also contains several epitopes restricted by DRB1*0101 (Ref. 30). In the two HAM/TSP patients (TBJ and TAT) who possessed this allele, the CD4$^+$ T cell response to this epitope constituted >90% of their total IFN-γ spot-forming cell (SFC) response (data not shown). Mapping of other class II MHC-restricted epitopes is underway.

High total HTLV-I-specific CD4$^+$ T cell frequencies were associated with HAM/TSP

There was a significant difference between the total frequencies of HTLV-I-specific IFN-γ-secreting CD4$^+$ T cells in seven HAM/TSP patients compared with seven ACs and four uninfected controls (Fig. 2 and Table I). Total responses ranged from 0.17% to 3.09% of CD4$^+$ T cells (median = 0.76%) in HAM/TSP patients compared with 0% (undetectable) to 0.46% of circulating CD4$^+$ T cells (median = 0.03%) in the ACs. Uninfected controls showed

Table I. *Frequencies of HTLV-I Ag-specific IFN-γ^+ CD4$^+$ T cells by ELISPOT in HAM/TSP patients, ACs, and uninfected controls*[a]

HAM	PL (%)		Gag	Pro	Pol	Env	Rof	Tof	Rex	Tax	Total
TBI	19.4		0.000304	0	0.00059	0.000615	0	0	0.0000759	0.000143	0.0017279
		±SD	0.000049	0	0.000046	0.000088	0	0	0.0000417	0.000062	0.000134
TAF	8.3		0.0037	0.000761	0.00536	0.0121	0.000263	0	0.000714	0.008038	0.030936
		±SD	0.00024	0.00043	0.00052	0.00039	0	0	0.000027	0.00074	0.00111
TBC	30.4		0.00261	0.000214	0.00133	0.00274	0.000177	0.000181	0.00011	0.00116	0.008522
		±SD	0.00012	0.000077	0.00014	0.00031	0.000052	0.00000473	0.000014	0.00009	0.000385
TAA	3.5		0.000555	0	0	0.00195	0	0	0	0.000497	0.003002
		±SD	0.00015	0	0	0.00015	0	0	0	0.0001	0.000241
TAL	4.6		0.00119	0	0.00238	0.00269	0	0	0.00036	0.00101	0.00763
		±SD	0.000095	0	0.000215	0.000332	0	0	0.000049	0.00015	0.000416
TAC	14.3		0.00173	0	0	0.0046	0	0	0	0	0.00633
		±SD	0.00026	0	0	0.0001	0	0	0	0	0.000281
TBJ	5.8		0.00039	0	0	0.0215	0	0	0	0.00038	0.02227
		±SD	0.00015	0	0	0.00059	0	0	0	0.000062	0.000619
AC											
HBC	0.1		0	0	0	0	0	0	0	0	0
		±SD	0	0	0	0	0	0	0	0	0
HAN	41.5		0.0000948	0.0000482	0.000073	0.000583	0.0000295	0	0.0000513	0.000087	0.0009668
		±SD	0.0000237	0.0000267	0.0000395	0.000614	0.000023	0	0.0000175	0.00000983	0.0000926
HAZ	3.2		0	0	0	0	0	0	0	0	0
		±SD	0	0	0	0	0	0	0	0	0
HT	3.9		0.000397	0.00145	0	0.00238	0.00043	0	0	0.000205	0.004862
		±SD	0.000236	0.0000899	0	0.000435	0	0	0	0.000153	0.000526
HAY	5		0	0	0	0	0	0	0	0	0
		±SD	0	0	0	0	0	0	0	0	0
HAE	0.007		0	0	0	0	0.000337	0	0	0	0.000337
		±SD	0	0	0	0	0.0000511	0	0	0	0.0000511
HS	4.4		0.000222	0.00013	0.000598	0.000309	0	0	0	0.0000916	0.0013506
		±SD	0.000178	0.0000545	0.000107	0.000107	0	0	0	0.0000272	0.000241
Uninfected											
Un1	NA		0	0	0	0	0	0	0	0.000168	0.000168
		±SD	0	0	0	0	0	0	0	0.00000758	0.00000758
Un2	NA		0	0	0	0	0.000145	0	0	0	0.000145
		±SD	0	0	0	0	0.00000895	0	0	0	0.00000895
Un3	NA		0	0	0	0	0	0	0	0	0
		±SD	0	0	0	0	0	0	0	0	0
Un4	NA		0	0	0	0	0	0	0	0	0
		±SD	0	0	0	0	0	0	0	0	0

[a] Mean frequencies of responding CD4$^+$ cells to different pools of HTLV-1 peptides corresponding to the major viral proteins. 0, Below limit of detection. PL, Proviral load shown in percent of PBMC; HAM, HAM/TSP patients; anonymized codes are shown. Occasional very low frequency responses to single Ags can be detected in uninfected (seronegative) controls (Ref. 19).

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Table II. *CD4$^+$ T cell response to Env transmembrane peptide IAQYAAQNRRGLDLL is restricted by DRB1*1303/33*[a]

Subject	DRB1*1303/33	DRB3*0101	SFCs to Peptide
TAT	+	+	+++
TBJ	+	+	+++
TAA	−	+	−
HAZ	−	+	−

[a] ELISPOT assays were undertaken to define the class II MHC allele which restricted the response to this peptide. TAT and TBJ had only the above two HLA alleles in common, and controls (TAA, HAZ), who were negative for DRB1*1303/33 but positive for DRB3*0101, showed no detectable SFC response to this peptide.

negligible responses (median = 0.01%). There was no significant difference in the median frequency of HTLV-I-reactive CD4$^+$ T cells seen between the ACs and uninfected controls.

Correlation of HTLV-I proviral load with CD4$^+$ T cell frequencies

We quantified proviral load using real-time PCR in aliquots of cells from the same blood samples to test the possibility that the frequency of HTLV-I-specific CD4$^+$ T cells is correlated with proviral load as reported for the CD8$^+$ T cell response for HTLV-I (31, 32). The data showed no correlation between the HTLV-I-specific CD4$^+$ T cells of HAM/TSP patients or ACs with the respective proviral loads. Spearman rank correlation coefficients and p values were $r = -0.2387$, $p > 0.99$ for HAM/TSP patients and $r = -0.0866$, $p = 0.96$ for ACs, respectively. Furthermore, the median proviral load of the HAM/TSP patients (8.3%) did not differ significantly from the median proviral load of ACs (3.9%) ($p = 0.5781$, Mann-Whitney) in this study. This observation suggests that the high frequency of HTLV-I-specific CD4$^+$ T cells observed in the HAM/TSP patients was associated with the disease, and was not caused by a higher proviral load in the HAM/TSP patients than the ACs.

Preferential infection of HTLV-I-specific CD4$^+$ T cells compared with CMV-specific CD4$^+$ T cells

Previous work from this laboratory suggested that HTLV-I-specific CTLs were preferentially infected with HTLV-I when compared with EBV-specific CTLs (16). Preliminary work also indicated that CD4$^+$ Th cells specific to Env and Tax might also be preferentially infected, at least in some infected subjects (19). However, the possibility remained that CD4$^+$ T cells specific for other HTLV-I Ags (Gag, Pol, Rex, Tof, and Rof) were not infected. Therefore, we quantified Tax protein expression in total HTLV-I-specific and CMV-specific CD4$^+$ T cells. Other groups have used flow cytometry and intracellular cytokine staining to define the total frequencies of CMV-specific CD4$^+$ Th cells (25, 33). PBMCs were stimulated with appropriate viral Ag preparations in vitro for 6 h and then assayed for surface CD4$^+$ and intracellularly for IFN-γ and Tax protein by flow cytometry as previously described (18, 19).

We first tested the specificity of the CMV-specific CD4$^+$ T cell detection assay (Fig. 3). The data showed that only CMV seropositive controls demonstrated detectable responses to the CMV Ag, with no responses seen to the control Ag. Seronegative controls showed no response to either the CMV or control Ags, as expected. We then directly compared the frequency of Tax expression in HTLV-I-specific and CMV-specific CD4$^+$ T cells. These data are shown in Fig. 4 and the analysis is summarized in Table III. The results show that Tax protein expression was significantly greater in the HTLV-I-specific CD4$^+$ Th cell population than in

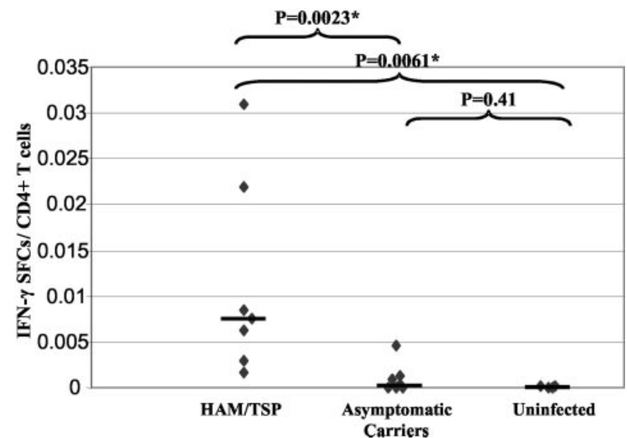

FIGURE 2. The frequency of HTLV-I-specific CD4$^+$ T cells is associated with HAM/TSP disease status. Total frequencies of HTLV-I-specific CD4$^+$ T cell were derived by ELISPOT assay for seven HAM/TSP patients, seven ACs, and four uninfected controls. All assays were done in duplicate. Horizontal bar indicates the median frequency. *, A significant difference. Statistical analysis was performed using the Mann-Whitney U test.

the CMV-specific population (Mann-Whitney two-tailed, $p = 0.0152$) after 6 h of culture in vitro. There was no significant correlation between the degree of preferential infection of HTLV-I-specific CD4$^+$ T cells (% Tax$^+$ in HTLV-I-specific CD4$^+$/% Tax$^+$ in CMV-specific CD4$^+$) and the proviral load (data not shown).

Although the FACS data were suggestive of preferential infection in HTLV-I-specific CD4$^+$ T cells by HTLV-I, it was possible that CMV-specific CD4$^+$ T cells harbored silent or latent provirus. To exclude this possibility, we obtained fresh PBMCs ex vivo from two HAM/TSP patients and positively selected IFN-γ-secreting CD4$^+$ T cells after 5 h of stimulation with HTLV-I peptides or CMV Ag (using MACS IFN-γ cytokine secretion assay (cell capture technique); Miltenyi Biotec). We then extracted genomic DNA and quantified HTLV-I proviral DNA using real-time PCR as before. Table IV shows these data. The data showed that there was increased detection of HTLV-I Tax in HTLV-I-specific CD4$^+$ cells compared with CMV-specific CD4$^+$ cells in both patients. This therefore excluded the possibility of latent provirus being present in CMV-specific CD4$^+$ T cells but which was not detected using a protein detection assay such as our FACS assay. Taken together, the data from our FACS and real-time quantitative PCR assays suggested strongly that there was indeed preferential infection of HTLV-I-specific CD4$^+$ T cells occurring in vivo.

Discussion

Studies of the CD4$^+$ T cell response to HTLV-I infection have hitherto been limited by the inherent difficulties of conventional CD4$^+$ T cell assays in this system. In particular, the spontaneous proliferation (34–36) and spontaneous cytokine production (12, 15, 18) associated with HTLV-I infection complicate assays of both T cell proliferation and cytokine detection. To overcome these problems, we recently established 6-h ELISPOT and intracellular cytokine staining assays and detected significant frequencies of Env and Tax-specific CD4$^+$ T cells in infected subjects (19).

Using these assays, we have now measured total HTLV-I-specific IFN-γ-secreting CD4$^+$ T cell frequencies in HAM/TSP patients, ACs, and uninfected controls using peptide libraries that span the entire genome of the virus. The data show that the CD4$^+$ T cell response to the virus was not restricted to any single protein:

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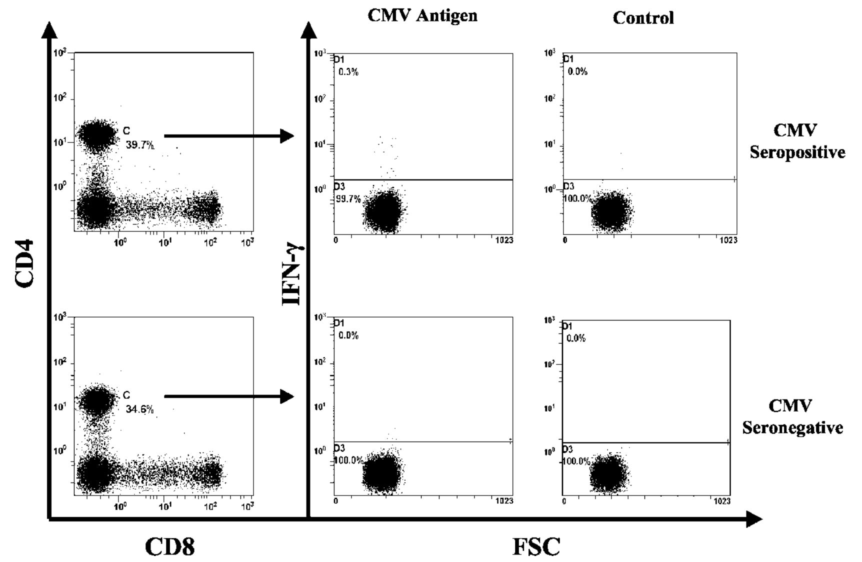

FIGURE 3. Detection of CMV-specific CD4$^+$ T cells by stimulation with CMV Ag. CMV Ag or control Ag was added to the culture medium for a 6-h culture in vitro with brefeldin A added after 1 h to inhibit protein secretion, and intracellular staining for IFN-γ was performed after cell harvesting. The dot plots were gated on CD4$^+$ T cells and 50,000 events were collected. The data show that only seropositive controls mounted a detectable response to CMV Ag. This was representative of four independent experiments. FSC, forward scatter.

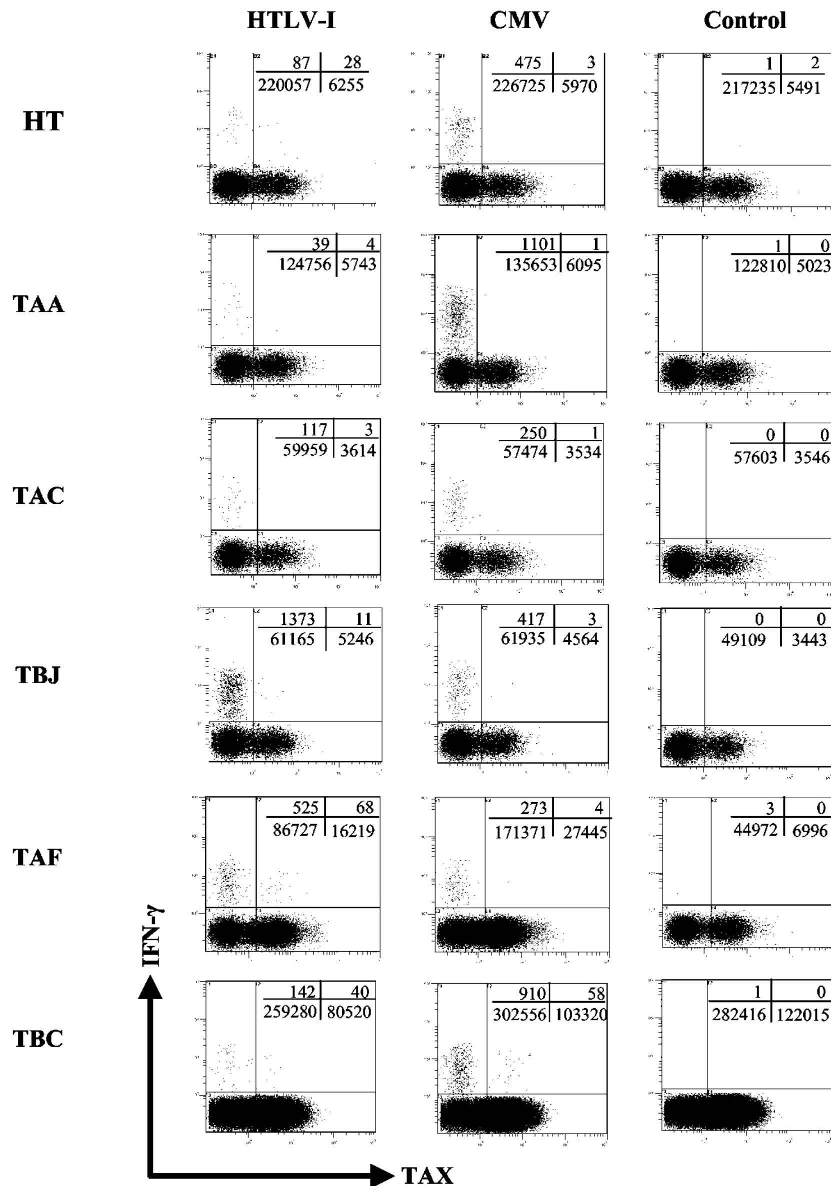

FIGURE 4. Increased frequency of Tax expression in HTLV-I-specific CD4$^+$ T cells compared with CMV-specific CD4$^+$ T cells. This figure shows concomitant detection of intracellular Tax and IFN-γ expression in CD4$^+$ T cells after 6 h of in vitro activation with CMV Ag or all available HTLV-I peptides. Dot plot data gated on CD4$^+$ T cells on all six subjects are shown. There was significantly increased expression of Tax protein in the HTLV-I-specific population (refer to Table III). Data shown are the numbers of events (cells) collected in the individual quadrants. Anonymized codes on the left begin with H for ACs and T for HAM/TSP patients.

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Table III. *Detection of Tax protein expression in virus-specific CD4$^+$ T cells from HTLV-I-infected subjects*[a]

Patient	Proviral Load (%)	% *Tax*-Expressing Cells in	
		HTLV-I-specific CD4$^+$ T cells	CMV-specific CD4$^+$ T cells
HT	1.3	24.3	0.6
TAA	3.0	9.3	0.09
TAC	10.9	2.5	0.4
TBJ	5.8	0.8	0.7
TAF	8.3	11.5	1.4
TBC	30.4	22.0	6.0

[a] PBMCs were stimulated for 6 h with either total HTLV-I peptides or CMV Ag and then stained for Tax protein expression. Mann-Whitney two-tailed, $p = 0.0152$.

Table IV. *Quantitative real-time PCR for HTLV-I proviral DNA in virus-specific CD4$^+$ T cells*[a]

Subject	Mean Tax Copy Number Per 100 CD4$^+$ Cells		
	Anti-HTLV-I	Anti-CMV	Control
TBU	12.0	1.3	0.7
TAQ	20.6	1.3	0.8

[a] Data from two HAM/TSP patients. Ex vivo PBMCs were CD8-depleted and cultured for 5 h with total HTLV-I peptides, CMV Ag, or complete medium alone. IFN-γ^+ CD4$^+$ were then positively selected and analyzed for HTLV-I proviral DNA.

the immunodominant Ag was Env, with smaller responses to Gag, Tax, and Pol. Responses toward protease or other pX proteins such as Tof, Rof, and Rex were also found but were less common. These results are consistent with published work on other viruses such as influenza A, HIV, and CMV showing responses to several different proteins (26, 37, 38). The data also contrast strongly with the immunodominance of Tax in the CTL response (4–6). Recent work on the CD8$^+$ T cell immunodominance hierarchy in this laboratory has confirmed that Tax is strongly immunodominant (59).

Until recently, it was generally believed that HTLV-I is latent in vivo (39–41). However, we have argued that there is significant evidence to the contrary (42, 43). The presence of high frequencies of HTLV-I-specific Th cells and activated CTLs (44, 45) in the peripheral blood supports the hypothesis that the virus is not latent, i.e., there is some ongoing viral transcription and that this is greater in HAM/TSP patients than in ACs. This conclusion has important implications for the pathogenesis of HAM/TSP. Similar work in HIV found that HIV-specific CD4$^+$ Th cells diminished in frequency on suppression of viraemia by antiretroviral therapy (46).

Our work also shows that the frequency of HTLV-I-specific IFN-γ^+ Th cells was substantially greater in HAM/TSP patients than in ACs. Indeed, the median frequency of virus-specific CD4$^+$ T cells in HAM/TSP patients was >25 times greater than that in ACs (0.76 vs 0.03%), and there was little overlap between the distributions. This finding is consistent with the hypothesis that these CD4$^+$ Th cells are involved in the initiation or pathogenesis of inflammatory CNS disease. A high proviral load is associated with a higher risk of HAM/TSP (47) and in our cohort of patients here in London, the average proviral load is 10-fold higher in patients with HAM/TSP than in ACs (21). It was therefore possible that the higher frequency of HTLV-I-specific CD4$^+$ T cells observed here in HAM/TSP patients resulted from a higher proviral load in these patients. However, the ranges of proviral loads documented in patients with HAM/TSP and ACs overlap considerably (47) and in the two groups of subjects studied here the median proviral loads did not differ significantly. We conclude that the high frequency of HTLV-I-specific CD4$^+$ T cells was associated with the disease HAM/TSP.

This conclusion is corroborated by evidence from several sources: 1) the predominant T cell in early HAM/TSP lesions is the CD4$^+$ T cell (8); 2) HTLV-I-infected CD4$^+$ T cells spontaneously produce proinflammatory cytokines (11, 12, 18); 3) activated CD4$^+$ Th1 type cells are reported to cross the blood-brain barrier and infiltrate areas of inflammation (48). Secretion of cytokines such as IFN-γ by these CD4$^+$ T cells, caused either by local viral Ag stimulation or induced by Tax expression in the CD4$^+$ T cell itself (18), could then cause damage to bystander cells in the CNS. This hypothesis has been proposed as a possible pathogenetic mechanism in HAM/TSP (49, 50).

Interestingly, the two patients with HAM/TSP (TAF, TBJ) and the highest detectable frequencies of HTLV-I-specific CD4$^+$ T cells were those who showed the greatest degree of clinical progression of their HAM/TSP over the 18 months of observation (data not shown). Further longitudinal studies are required in patients with active disease to ascertain whether the circulating HTLV-I-specific CD4$^+$ T cell frequency is indeed correlated with disease activity. A recent report suggested that HLA-A2/Tax$_{11-19}$ specific CTLs are correlated with disease severity (32). At present, the best marker of disease activity is thought to be the level of neopterin in the cerebrospinal fluid (47, 51). However, lumbar punctures are highly invasive and inconvenient for repeated clinical sampling.

In this study, there was no significant correlation between proviral load and the frequencies of Th cells from the same blood samples. However, we have recently shown (43) that the frequency of virus-specific CTLs in persistent infection at equilibrium is not a useful measure of the efficiency of that T cell response. It will be still harder to infer the efficiency of HTLV-I-specific CD4$^+$ T cells from the frequency of such cells in the circulation, because the effects of CD4$^+$ T cells on the virus are less direct (being exerted mainly through CTLs and Ab) than those of CTLs. Also, HTLV-I drives CD4$^+$ T cells to proliferate, through antigenic and mitogenic stimulation, and finally the virus also renders infected CD4$^+$ T cells susceptible to CTL-mediated lysis.

We have previously published evidence for an apparent preferential infection of HTLV-I-specific CTLs by HTLV-I compared with EBV-specific CTLs (16). Preliminary work raised the possibility that the same phenomenon was occurring in the HTLV-I-specific CD4$^+$ Th cell population (19). This possibility was intriguing because of the implications for the immune control of HTLV-I: 1) it has been shown that infection of T cell clones by HTLV-I impairs their function (52, 53). This suggested that in vivo infection of T cells would impair their function as well. 2) Infection with HTLV-I would make the CD4$^+$ T cells susceptible to CTL surveillance and destruction (10). 3) Infection may also inhibit apoptosis of the infected cell (54).

What are the possible consequences of the observed preferential infection of specific CD4$^+$ T cells for the pathogenesis of HAM/TSP? CD4$^+$ T cells predominate in early HAM/TSP lesions (8), and may contribute to the tissue damage in the CNS, for example by secreting neurotoxic cytokines such as IFN-γ (18). Activation of specific CD4$^+$ T cells by HTLV-I Ags may cause these T cells to enter the CNS (48); if such cells are also preferentially infected with HTLV-I, this could increase the chance of establishing a self-perpetuating inflammatory focus in the CNS (55).

We have previously shown that a majority of HTLV-I-specific Th cells were not infected with HTLV-I in vivo (19). However, the question of preferential infection of these virus-specific cells was not answered. A recent study has uncovered evidence that HIV preferentially infects HIV-specific CD4$^+$ T cells (56). In the present study, we have shown that there is apparent preferential

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infection of HTLV-I-specific compared with CMV-specific Th cells in vivo. We consider here two possible causes of the observed preferential infection. First, HTLV-I-specific CD4$^+$ T cells might be especially susceptible to HTLV-I infection, for example because a specific activated T cell remains in contact for a longer time with an APC (57), or because the T cell becomes activated on contact with the APC. Both prolonged cell contact and T cell activation may favor direct intercellular transfer of HTLV-I through the recently described HTLV-I "viral synapse" (58). Alternatively, HTLV-I-infected HTLV-I-specific CD4$^+$ T cells might have a growth advantage over other T cells, especially if the antigenic and mitogenic effects on HTLV-I on T cells are synergistic. At present, neither of these possibilities can be excluded.

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