The Journal of Immunology

High Frequencies of Functionally Competent Circulating Tax-Specific CD8 $^{+}$ T Cells in Human T Lymphotropic Virus Type 2 Infection

André L. A. Oliveira, Hitoshi Hayakawa, Doris Schor, Ana Claudia C. B. Leite, Otávio M. Espíndola, Allison Waters, Jonathan Dean, Derek G. Doherty, Abelardo Q.-C. Araújo and William W. Hall

J Immunol 2009; 183:2957-2965; Prepublished online 5 August 2009;
doi: 10.4049/jimmunol.0900508
http://www.jimmunol.org/content/183/5/2957

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

High Frequencies of Functionally Competent Circulating Tax-Specific CD8$^+$ T Cells in Human T Lymphotropic Virus Type 2 Infection

André L. A. Oliveira,* Hitoshi Hayakawa,* Doris Schor,‡ Ana Claudia C. B. Leite,§ Otávio M. Espíndola,‡ Allison Waters,† Jonathan Dean,* Derek G. Doherty,¶‖ Abelardo Q.-C. Araújo,*§ and William W. Hall[1]*†

Human T lymphotropic virus type 2 (HTLV-2) is characterized by a clinically asymptomatic persistent infection in the vast majority of infected individuals. In this study, we have characterized for the first time ex vivo specific CTL responses against the HTLV-2 Tax protein. We could detect CTL responses only against a single HLA-A*0201-restricted Tax2 epitope, comprising residues 11–19 (LLYGYPVYV), among three alleles screened. Virus-specific CTLs could be detected in most evaluated subjects, with frequencies as high as 24% of circulating CD8$^+$ T cells. The frequency of specific CTLs had a statistically significant positive correlation with proviral load levels. The majority of virus-specific CD8$^+$ T cells exhibited an effector memory/terminally differentiated phenotype, expressed high levels of cytotoxicity mediators, including perforin and granzyme B, and lysed in vitro target cells pulsed with Tax2$_{(11–19)}$ synthetic peptide in a dose-dependent manner. Our findings suggest that a strong, effective CTL response may control HTLV-2 viral burden and that this may be a significant factor in maintaining persistent infection and in the prevention of disease in infected individuals. *The Journal of Immunology,* **2009, 183: 2957–2965.**

The human T lymphotropic viruses type 1 (HTLV-1) and type 2 (HTLV-2)[2] are closely related members of a family of retroviruses that have a tropism for mature T lymphocytes. HTLV-1 is endemic in a number of well-defined geographic areas, and as many as 10 million individuals are thought to be infected worldwide. While the vast majority of HTLV-1-infected individuals are clinically asymptomatic, some 5% will develop either a hematological malignancy, adult T cell leukemia/lymphoma (ATLL), or an inflammatory neurological disease, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). All HTLV-1-infected individuals remain persistently infected throughout their lives, and provirus can be readily detected in circulating lymphocytes. In contrast to HTLV-1, the number of HTLV-2-infected individuals is unknown, but there is significant endemic infection in indigenous groups in the Americas and Central Africa, and high levels of epidemic infection in injecting drug users in the Americas, Southern Asia, and parts of Europe (1). HTLV-2 infection is similar to HTLV-1, in that the vast majority of individuals remain persistently infected through their lifetimes. However, HTLV-2 appears to be less pathogenic. To date, no hematological malignancies have been associated with infection, and while there have been a number of reports describing neurological diseases similar to HAM/TSP, these are very rare, and in some instances it is unclear if they can in fact be attributed to HTLV-2 infection (2).

To date, limited information is available on the immunological features of HTLV-2 infection. The virus has a preferential in vivo tropism for CD8$^+$ T lymphocytes (3), but it has also been shown to infect CD4$^+$ T cells, monocytes, and B cells in certain patients at lower levels (4, 5). This contrasts with HTLV-1, where the virus preferentially infects CD4$^+$ T lymphocytes in vivo, although, in certain individuals, CD8$^+$ cells may also be infected (6). Spontaneous proliferation of naive and memory subsets of both CD4$^+$ and CD8$^+$ lymphocytes from HTLV-2-infected individuals has also been described, at significantly lower rates when compared with HTLV-1 infection (7). Additionally, PBMCs from HTLV-2 infected individuals spontaneously produce C-C chemokines, including MIP-1α, MIP-1β, and RANTES (8).

CTL responses play a pivotal role in controlling proviral load (PVL) in HTLV-1 infection. Large numbers of Tax1-specific CD8$^+$ T cells can be detected in most infected individuals, both in HAM/TSP patients and in asymptomatic carriers (9, 10). The HTLV-1 Tax protein is immunodominant for circulating specific CTLs, but responses to other viral Ags have also been detected (11–13). The exact pathogenic role of the CTL responses remains unclear. While it has been suggested that the increased numbers of chronically activated Tax1-specific CTLs might be responsible for triggering neurological damage, leading to HAM/TSP (14), increasing experimental evidence supports the hypothesis that such a strong CTL response could certainly contribute to the control of viral spread and, subsequently, disease development (15).

The present study describes the first characterization of HTLV-2-specific CTL responses in infected individuals, specifically those

*Centre for Research in Infectious Diseases and †National Virus Reference Laboratory, University College Dublin, Dublin, Ireland; ‡Viral Pathogenesis Research Laboratory and §Laboratory for Research in Neuroinfections, Evandro Chagas Institute for Clinical Research, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil; ¶Cellular Immunology Laboratory, Department of Biology, National University of Ireland, Maynooth, Ireland; and ‖Department of Immunology, Trinity College Dublin, St. James's Hospital, Dublin, Ireland

Received for publication February 17, 2009. Accepted for publication June 24, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Prof. W. Hall, The Centre for Research in Infectious Diseases, University College Dublin, Belfield, Dublin 4, Ireland. E-mail address: william.hall@ucd.ie

[2] Abbreviations used in this paper: HTLV, human T lymphotropic virus; ATLL, adult T cell leukemia/lymphoma; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; PD-1, programmed death 1; PVL, proviral load; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{EMRA}, terminally differentiated memory T cell; T_N, naive T cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900508

directed toward the Tax protein (Tax2). We have mapped responses against Tax2-derived peptides restricted by three alleles, and we found a single HLA-A*0201-restricted epitope recognized by specific CTLs. The frequency of specific CTLs is high in infected individuals and had a significant positive correlation with the PVL levels. Most of these virus-specific CTLs constitute an immediate effector cell population, with a strong cytotoxicity profile, and they are capable of killing HLA-matched target cells in vitro, suggesting that the CTL response might play a direct role in the control of HTLV-2 infection.

Materials and Methods

Subjects and controls

Nine HLA-A*0201 HTLV-2-infected individuals and three HLA-matched and three HLA-mismatched healthy uninfected control subjects were enrolled in this study after informed consent was given. Diagnosis of HTLV-2 infection was made according to standard ELISA (Vironostika HTLV-I/II; Organon Teknika) and Western blot (HTLV blot 2.4; Genelabs Diagnostics) criteria. All individuals were seronegative for HTLV-1, HIV, hepatitis B virus, and hepatitis C virus infections. The study was approved by the human research ethics committees in Brazil and in Ireland. PBMCs were isolated from whole blood by Ficoll density gradient and cryopreserved until use. HLA typing was performed using sequence-specific primers, as previously described (16).

Epitope mapping

A library of 348 overlapping 9-mers with 1 amino acid offset, spanning the entire Tax2 sequence (PEPscreen; ProImmune), was analyzed in HLA binding assays. The binding affinity of each peptide to the alleles HLA-A*0201, HLA-B*3501, and A*0301 was assessed in binding assays (REVEAL binding assay; ProImmune). Peptide-MHC complexes with a high binding affinity were then selected for the synthesis of MHC class I-peptide pentamer complexes (ProVE pentamers; ProImmune) for epitope validation.

Epitope validation

Frozen PBMCs were thawed and washed twice in ice-cold staining buffer (PBS supplemented with 0.1% BSA and 0.1% sodium azide). The cells (1 to 2×10^6) were incubated with one test of unlabelled pentamers for 10 min at room temperature, washed, and then stained with PE-conjugated Pro5 Fluorotag (ProImmune), FITC-conjugated anti-human CD8 (ProImmune) for 20 min on ice. Finally, cells were washed and resuspended in fixative solution (1% FCS, 2.5% paraformaldehyde in PBS).

In addition to the Tax2-derived pentamers, we used the following pentamers as controls: A*0201/NLVPMVATV and B*3501/IPSINVHHY, derived from CMV pp65 Ag (17, 18); and HLA-A*0301/ILRGSVAHK, derived from influenza virus A nucleoprotein (all from ProImmune) (19).

Samples were analyzed using a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Pentamer-positive cells were quantified by gating first on live cells using the forward and side scatter discriminators. A minimum of 10^5 live events were acquired. Pentamer-positive and -negative $CD8^+$ cells were identified by plotting pentamer fluorescence against CD8 fluorescence.

PVL quantitation assay

Measurement of the HTLV-2 PVL was conducted by a multiplex quantitative real-time PCR assay, adapted from a previously published assay (20). For quantitation of HTLV-2 *Tax* gene, the primers Tax2C-S (5′-CGATTGTGTACAGGCCGATTG-3′, positions 7275–7295), Tax2C-AS (5′-CAGGAGGGCATGTCGATGTAG-3′, positions 7350–7330), and the probe Tax2CP (5′-FAM-TGTCCCGTCTCAGGTGGTCTATGTTC CA-BHQ-3′, positions 7297–7324) were designed using the software Primer Express 3.0 (Applied Biosystems), based on a previously published sequence from a Brazilian HTLV-2c isolate (GenBank accession no. AF139382) (21). Quantification of a human albumin gene fragment was used as a reference gene. The reaction was carried on in a 7500 Fast real-time PCR system (Applied Biosystems). Each sample was analyzed in duplicate, and PVL was expressed as Tax copies per 100 PBMCs.

Phenotypic and functional analysis of $CD8^+$ T cells

Four subsets of $CD8^+$ T cells have been identified based on the expression of CCR7 and CD45RA: naive T cells (T_N cells), $CCR7^+/CD45RA^+$; central memory T cells (T_{CM} cells), $CCR7^+/CD45RA^-$; effector memory T cells (T_{EM} cells), $CCR7^-/CD45RA^-$; and terminally differentiated memory T cells (T_{EMRA} cells), $CCR7^-/CD45RA^+$ (22).

Frozen PBMCs were thawed and washed twice in ice-cold staining buffer. Subsequently, a $CD8^+$-enriched fraction was isolated using anti-CD8 microbeads, in an autoMACS Pro instrument (Miltenyi Biotec). The $CD8^+$ T cell-enriched fraction (1 to 2×10^6) was washed with staining buffer and incubated with unlabelled pentamer for 10 min at room temperature, washed, and then incubated for 20 min on ice with a combination of PerCP-conjugated anti-HLA-DR, PE-Cy7-conjugated anti-CCR7, Pacific Blue-conjugated anti-CD8 (all from BD Biosciences), PE-conjugated Pro5 Fluorotag (ProImmune), and PE-Texas Red-conjugated anti-CD45RA (Invitrogen). The samples were further fixed and permeabilized using Cytofix/Cytoperm Plus (BD Biosciences) and stained with FITC-conjugated anti-perforin, allophycocyanin-conjugated anti-IFN-γ, and Alexa Fluor 700-conjugated anti-granzyme B (all from BD Biosciences). Cells were then washed and resuspended in fixative solution before analysis.

Functional profile analysis was conducted using a modified version of previously published methods (23). In brief, frozen PBMCs (1 to 2×10^6 cells) were thawed, washed twice, and resuspended in RPMI 1640 medium supplemented with 10% pooled human AB serum. Cells were stimulated with 2 μg/ml of $Tax2_{(11-19)}$ synthetic peptide (ProImmune) in the presence of costimulatory Abs (anti-CD28 and anti-CD49d, 1 μg/ml each), 20 μl of FITC-conjugated anti-CD107a, and the protein transport inhibitors brefeldin A and monensin (all from BD Biosciences). For some experiments, cells were also stimulated with a peptide pool derived from CMV, EBV, and influenza proteins (ProMix CEF peptide pool; ProImmune). Unstimulated controls consisted of cells incubated in the aforementioned conditions but in the absence of synthetic peptides. A positive control stimulated with 250 ng/ml PMA and 1 μg/ml ionomycin was included in the assays. Following a 4-h incubation, the cells were washed with staining buffer and then incubated for 15 min with PerCP-conjugated anti-CD8 (BD Biosciences) and VioBlue-conjugated anti-CD3 (Miltenyi Biotec). The samples were further fixed and permeabilized using Cytofix/Cytoperm Plus (BD Biosciences) and stained with PE-conjugated anti-MIP-1β, PE-Cy7-conjugated anti-TNF-α, allophycocyanin-conjugated anti-IL-2, and Alexa Fluor 700-conjugated anti-IFN-γ (all from BD Biosciences). Cells were then washed and resuspended in fixative solution before analysis.

To assess the frequency of programmed death-1 (PD-1) in HTLV-2-specific $CD8^+$ T cells, frozen PBMCs (1 to 2×10^6) were thawed and washed twice in ice-cold staining buffer. Cells were then incubated with unlabelled pentamer for 10 min at room temperature, washed, and then incubated for 20 min on ice with a combination of FITC-conjugated anti-CD3, PerCP-conjugated anti-CD8, allophycocyanin-conjugated anti-PD-1 (all from BD Biosciences), and PE-conjugated Pro5 Fluorotag (ProImmune). Cells were then washed and resuspended in fixative solution before analysis. In addition to the Tax2-derived pentamer, we used the A*0201/NLVPMVATV pentamer as a control.

Samples were analyzed in a CyAn ADP analyzer using Summit software (Beckman Coulter). Tax2-specific $CD8^+$ T cells and the nonspecific $CD8^+$ T cells were detected and quantified by gating first on live cells using the forward scatter and side scatter discriminators, followed by gating on the $CD8^+$ population. Results for memory subsets, functional analysis, and PD-1 expression were derived by plotting pentamer fluorescence against CD8 fluorescence.

CTL cytotoxicity assays

$CD8^+$ T cell cytotoxic function was assessed in 4-h ^{51}chromium release assays. The target cells used in this assay were T2 cells (ATCC no. CRL-1992) pulsed overnight with 20 μg/ml synthetic peptide. T2 cells without the synthetic peptide were used as target controls. Effector cells were $CD8^+$ cells isolated using CD8 microbeads from freshly thawed PBMCs. The effectors were then incubated with target cells labeled with sodium [^{51}Cr]chromate (PerkinElmer), at E:T ratios of 5:1, 25:1, 50:1, and 100:1 in triplicate wells of 96-well plates. Chromium release was measured by scintillation counting. Percentage specific lysis was expressed as: [(cpm of sample − cpm of spontaneous release)]/[(cpm of maximum release − cpm of spontaneous release)].

Statistical analysis

Statistical significance was calculated by the nonparametric Spearman's rank correlation test. A two-tailed p value of <0.05 was deemed significant.

Results

*A single Tax2 epitope is recognized by ex vivo CTLs in the context of HLA-A*0201*

To map the epitopes recognized by specific CTLs, we initially screened a library of nonameric peptides, spanning the entire Tax2

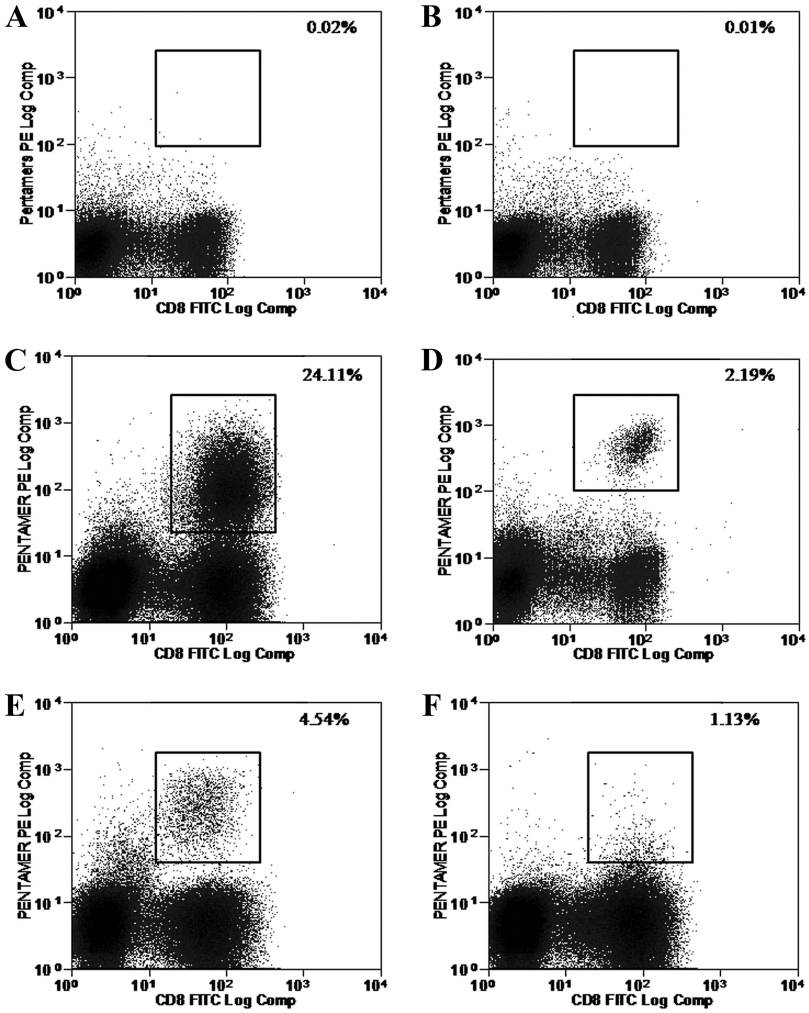


FIGURE 1. Representative data of pentamer staining of PBMCs from HTLV-2-seropositive and -seronegative individuals. Representative results of Tax2-specific $CD8^+$ T lymphocytes identified by fluorescent-labeled pentamer staining of PBMC samples from HLA-A*0201-infected individuals. PBMCs from HTLV-2-seropositive and -seronegative subjects were tested for their ability to bind the pentamer complexes used in this study. The percentage of all $CD8^+$ T cells binding the pentamers is indicated in each panel. *A*, The background level was set by staining HLA-mismatched PBMC samples. *B*, Representative plot of a HLA-A*0201 uninfected individual, showing a frequency of pentamer-binding $CD8^+$ cells below background level. *C* and *D*, PBMCs from two selected HLA-A*0201 individuals infected with HTLV-2, stained with HLA-A*0201/$Tax2_{(11–19)}$ pentamer. *E* and *F*, Samples from HLA-matched, HTLV-2-seropositive individuals stained with A*0201/NLVPMVATV and HLA-A*0301/ILRGSVAHK control pentamer complexes, respectively.

sequence, against three HLA alleles: HLA-A*0201, HLA-A*0301, and HLA-B*3501. These alleles were chosen based on their prevalence in the Brazilian population. The binding assays revealed 37 peptides binding to HLA-A*0201, 18 binding to HLA-A*0301, and 12 binding to HLA-B*3501 (data not shown). All peptides identified in the screening binding assays were synthesized as MHC class I/peptide pentamers for further validation as specific CTL epitopes.

To identify the Tax2 epitopes recognized by specific $CD8^+$ T cells, we stained PBMCs from the asymptomatic HTLV-2 carriers employing fluorescent-labeled pentamers. The analysis was conducted using freshly thawed cryopreserved PBMCs, providing a more accurate pattern of the CTL in vivo specificities compared with cultured and/or stimulated PBMCs. For initial screening, PBMCs were stained with pools of up to six pentamers. When positive responses were detected, PBMCs were further stained with each individual pentamer to permit fine mapping. Background levels of pentamer staining were established by analysis of HLA-mismatched samples from uninfected individuals, where the frequency of pentamer-positive cells was found to be $<0.02\%$ $CD8^+$ T cells (Fig. 1*A*).

Among the three HLA alleles screened against Tax2-derived peptide sequences, the only response detected was against a single HLA-A*0201-restricted peptide, corresponding to the $Tax2_{(11–19)}$ sequence (LLYGYPVYV). Representative plots are shown in Fig. 1, *C* and *D*. The average frequency of $Tax2_{(11–19)}$-specific CTLs was 3.92% of $CD8^+$ T cell lymphocytes, ranging from 0 to 24.11% (Fig. 1*C*), as shown in Table I. Only one out of nine evaluated individuals had undetectable virus-specific CTLs. Fig. 1*B* shows a representative plot of an HLA-matched uninfected individual; as expected, none of the evaluated uninfected control subjects showed a positive staining with the Tax2-derived pentamers.

Three subjects with HLA-A*0301 and two subjects with HLA-B*3501 were also evaluated. None of the samples was stained by the Tax2-derived pentamers restricted to these alleles. To demonstrate responses to other viral infections restricted to these alleles, we also stained the cells with HLA-matched control pentamers

Table I. *Frequencies of HTLV-2-specific CTLs and proviral load levels in nine HTLV-2 infected individuals*

Subject ID	Frequency of Specific CTLs[a]	PVL[b]
2AC02	24.11	20.99
2AC07	6.49	4.29
2AC03	2.19	4.8
2AC09	1.19	2.34
2AC01	0.67	3.15
2AC06	0.33	2.26
2AC08	0.19	0
2AC05	0.12	0
2AC04	0	0

[a] Percentage of $Tax2_{(11–19)}$-positive cells among $CD8^+$ cells.
[b] PVL expressed as Tax copies per 100 PBMCs.

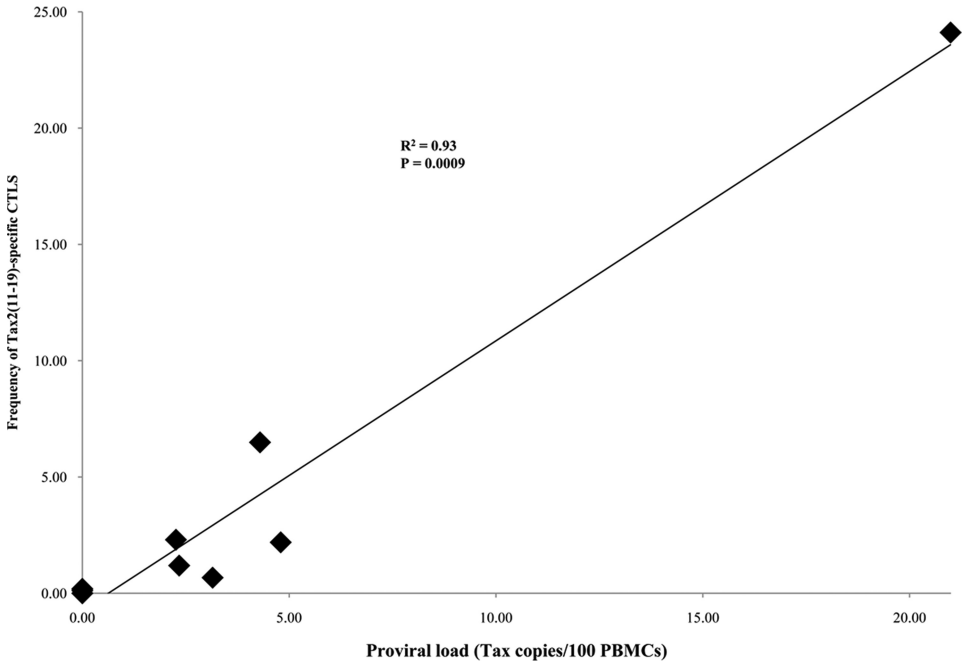


FIGURE 2. Correlation between frequency of $Tax2_{(11–19)}$-specific $CD8^+$ lymphocytes and PVL. The percentage of $Tax2_{(11–19)}$ pentamer-positive cells in $CD8^+$ lymphocytes correlated with the PVL levels in 9 HTLV-2-infected asymptomatic carriers. The *p* value was calculated by a two-tailed Spearman test.

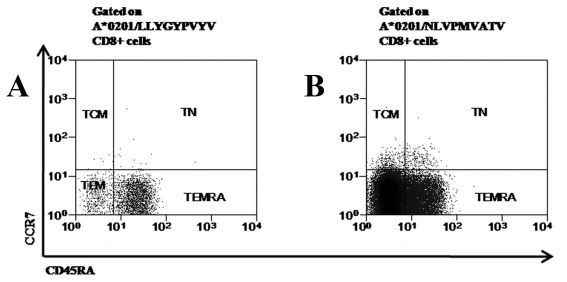
derived from immunodominant peptides from CMV and influenza virus. In all cases, $CD8^+$ cells stained with the control pentamers could be readily detected. The frequencies of the CMV-specific A*0201/NLVPMVATV-positive $CD8^+$ cells ranged from 0.5 to 4.54%; CMV-specific B*3501/IPSINVHHY-positive $CD8^+$ cells ranged from 0.13 to 0.85%; and the influenza A-specific HLA-A*0301/ILRGSVAHK ranged from 1.13 to 4.6%. Fig. 1*E* shows an example of staining with the A*0201/NLVPMVATV pentamer; an example of HLA-A*0301/ILRGSVAHK pentamer staining is shown in Fig. 1*F*.

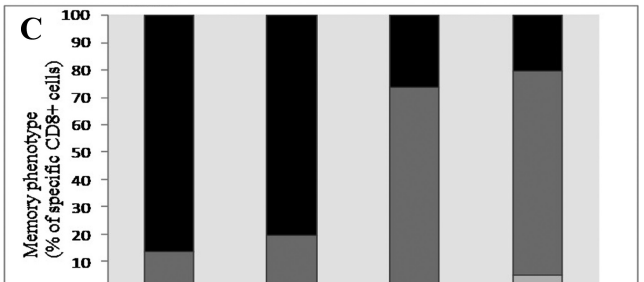
The high frequency of $Tax2_{(11–19)}$-specific $CD8^+$ T cells has a significant positive correlation with PVL

PVL has been shown to be an important risk factor in disease development in HTLV-1 infection (24). We investigated if the frequency of Tax2-specific CTLs correlated with the PVL levels in HTLV-2 infection. The average PVL was 4.20 copies/100 PBMCs, with three of the nine evaluated subjects having undetectable PVL levels (Table I). As can be seen in Fig. 2, there was a significant positive correlation between the frequency of specific CTLs and the PVL levels ($R^2 = 0.93$, $p = 0.0009$, Spearman rank correlation test). One individual (2AC02) exhibited remarkably high frequencies of specific CTLs and PVL levels when compared with the others, and could therefore be considered as an outlier. However, even after excluding this subject from analysis, we could still find a significant positive correlation between both variables ($R^2 = 0.92$, $p = 0.0138$, Spearman rank correlation test).

$Tax2_{(11–19)}$-specific CTLs consist primarily of terminally differentiated and effector memory cells, with a strong cytotoxicity profile

The assessment of the memory phenotype of $CD8^+$ lymphocytes provides information on their potential functional characteristics, as well as their ability to control viral infection. To verify the memory phenotype and functional profile of HTLV-2-specific CTLs, we performed polychromatic ex vivo flow cytometric analysis. Representative data on the differentiation phenotype of $Tax2_{(11–19)}$-specific CTLs are shown in Fig. 3. Although there was some variation between infected individuals, the vast majority of these cells (94.65–99.63%) exhibited either a T_{EM} or T_{EMRA} phenotype, which are known to be associated with immediate effector functions.





ID	2AC2	2AC7	2AC3	2AC9
TEMRA	86.26	80.47	26.61	20.44
TEM	12.82	19.16	72.45	74.21
TCM	0.92	0.37	0.75	5.35
TN	0	0.01	0.19	0

FIGURE 3. Phenotypic analysis of $Tax2_{(11–19)}$-specific $CD8^+$ T cell subsets. Representative data on the memory phenotype of virus-specific memory $CD8^+$ T cells in four HTLV-2-infected individuals. Subsets of $CD8^+$ T cells have been identified based on the coexpression of CCR7 and CD45RA: T_N cells, $CCR7^+/CD45RA^+$; T_{CM} cells, $CCR7^+/CD45RA^-$; T_{EM} cells, $CCR7^-/CD45RA^-$; and T_{EMRA} cells, $CCR7^-/CD45RA^+$. *A*, Representative plot showing the memory phenotype of $Tax2_{(11–19)}$-specific $CD8^+$ T cells. For comparison, the memory phenotype of CMV-specific $CD8^+$ T cells from a representative subject is shown in *B*. *C*, Frequency of memory subsets of HTLV-2-specific $CD8^+$ T cells in four HTLV-2 infected individuals. Percentages of each subset are shown below each bar.

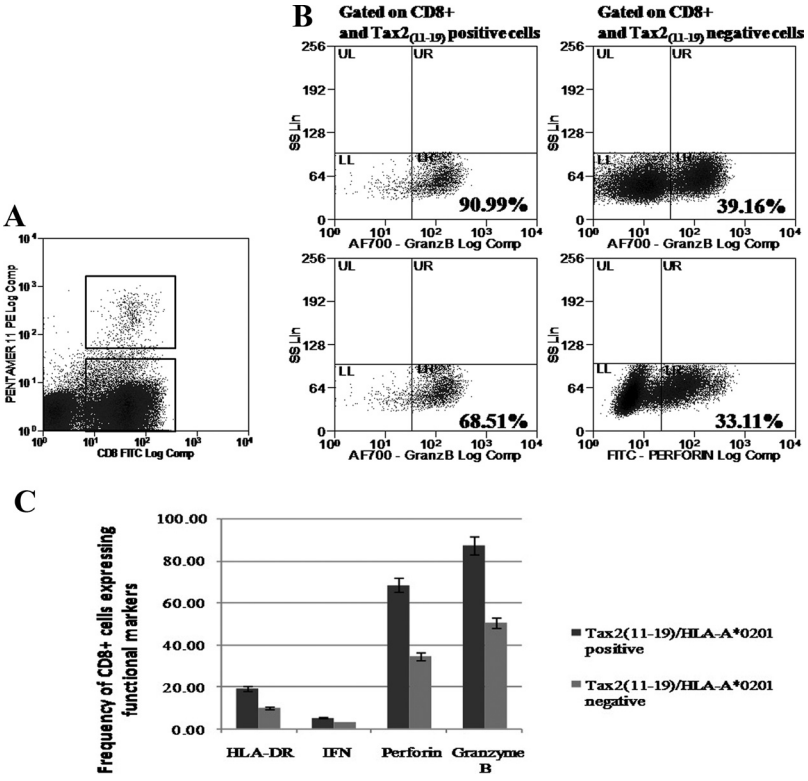


FIGURE 4. Functional profile of $Tax2_{(11–19)}$-specific $CD8^+$ lymphocytes. To assess the functional profile of HTLV-2-specific CTLs, cells from HLA-A*0201-infected individuals were analyzed by multicolor surface and intracellular staining. *A*, For the analysis, cells were gated on the $CD8^+/Tax2_{(11–19)}$ pentamer-positive (upper box) or pentamer negative (lower box) populations. *B*, Representative plots showing the differential levels of expression of perforin and granzyme B in $Tax2_{(11–19)}$ pentamer binding $CD8^+$ cells and in pentamer-negative $CD8^+$ cells from an infected individual. The percentage of cells expressing each marker is indicated in each plot. *C*, Frequency of $CD8^+$ cells expressing functional markers in the evaluated individuals. Error bars with SEs are shown.

To assess the functional properties of HTLV-2 Tax-specific CTLs, we verified the expression of HLA-DR, granzyme B, perforin, and IFN-γ both in the $CD8^+/Tax2_{(11–19)}$ pentamer-positive and pentamer-negative populations. As shown in Fig. 4, most pentamer-positive $CD8^+$ T cells showed low levels of expression of HLA-DR and IFN-γ. A markedly higher expression of perforin and granzyme B was found in the virus-specific subset compared with the pentamer-negative $CD8^+$ population. Neither the memory phenotype nor the frequency of perforin/granzyme B-expressing cells correlated with the PVL (data not shown).

To further explore the functional attributes of specific $CD8^+$ T cells, we also measured five independent $CD8^+$ T cell functions simultaneously: CD107a mobilization and MIP-1β, TNF- α, IL-2, and IFN-γ production. Cells were stimulated in vitro for 4 h with $Tax2_{(11–19)}$ synthetic peptide, together with costimulatory CD28 and CD49d Abs, in the presence of anti-CD107a and protein transport inhibitors. After incubation, cells were further stained and submitted to surface marker and intracellular staining for polychromatic flow cytometry analysis. Samples were analyzed using Boolean gates to assess the full array of possible combinations of response patterns for five functions. Upon Ag-specific stimulation, the frequencies of $CD8^+$ T cells expressing any of the functional markers increased in all evaluated samples. Representative plots are shown in Fig. 5*A*. The analysis of each possible combination of these five functions is shown in Fig. 5. As can be seen, most specific $CD8^+$ T cells exhibited a monofunctional profile (89.78% of responders), followed by bi-, tri-, tetra- and pentafunctional cells (9.23, 0.77, 0.20, and 0.01%, respectively). Monofunctional $CD8^+$ T cells expressing IFN-γ or MIP-1β were found to be the most frequently identified subsets.

We also evaluated the expression of PD-1 in $Tax2_{(11–19)}$-specific $CD8^+$ T cells as a marker of immune exhaustion. Representative subjects are depicted in Fig. 5*D*. None of the samples showed an increased expression of PD-1 in specific $CD8^+$ T cells when compared with $Tax2_{(11–19)}$ pentamer-negative $CD8^+$ T cells and A*0201/NLVPMVATV pentamer-positive $CD8^+$ T cells.

Tax2-specific $CD8^+$ T cell lymphocytes are capable of specifically killing target cells

To verify the cytolytic activity of virus-specific CTLs, we performed conventional 4-h chromium release assays. $CD8^+$ lymphocytes isolated from total PBMCs were used as effectors, and targets were T2 cells pulsed with $Tax2_{(11–19)}$ synthetic peptide. Control experiments were performed using effectors incubated with T2 cells in the absence of $Tax2_{(11–19)}$ peptide. We could detect specific lysis in all HTLV-2-infected individuals in a dose-dependent manner (Fig. 6). Strikingly, there was a moderate inverse correlation between the frequency of specific lysis and the PVL levels, although this association was not statistically significant (data not shown; $R^2 = -0.71$, $p = 0.0768$, Spearman rank correlation test).

Discussion

This study provides the first characterization of specific cellular immune responses in HTLV-2 infection. We detected high frequencies of circulating $Tax2_{(11–19)}$-specific $CD8^+$ T cells in almost all of the asymptomatic carriers, and our findings are similar to those observed in HTLV-1 infection. Interestingly, responses against this $Tax2_{(11–19)}$ epitope have already been detected in HTLV-1-infected individuals (25). The sequences of $Tax2_{(11–19)}$ and $Tax1_{(11–19)}$ peptides are highly conserved, differing only by a tyrosine, in Tax2, and a phenylalanine in Tax1 at position 13. Taking into account the binding motifs of both peptides, it is likely that Tax2-specific CTLs would also recognize the $Tax1_{(11–19)}$ epitope.

One of the most remarkable findings in this study is that, among the three alleles screened, the CTL response against Tax2 protein was found to be confined to a single epitope in the context of HLA-A*0201. This contrasts with that observed in HTLV-1 infection, where multiple Tax1 epitopes are recognized in the context of HLA-A*02 (12, 26). HLA-A*02 is of major importance in HTLV-1 infection, being associated with lower PVL levels and protection against HAM/TSP development in different populations (27, 28).

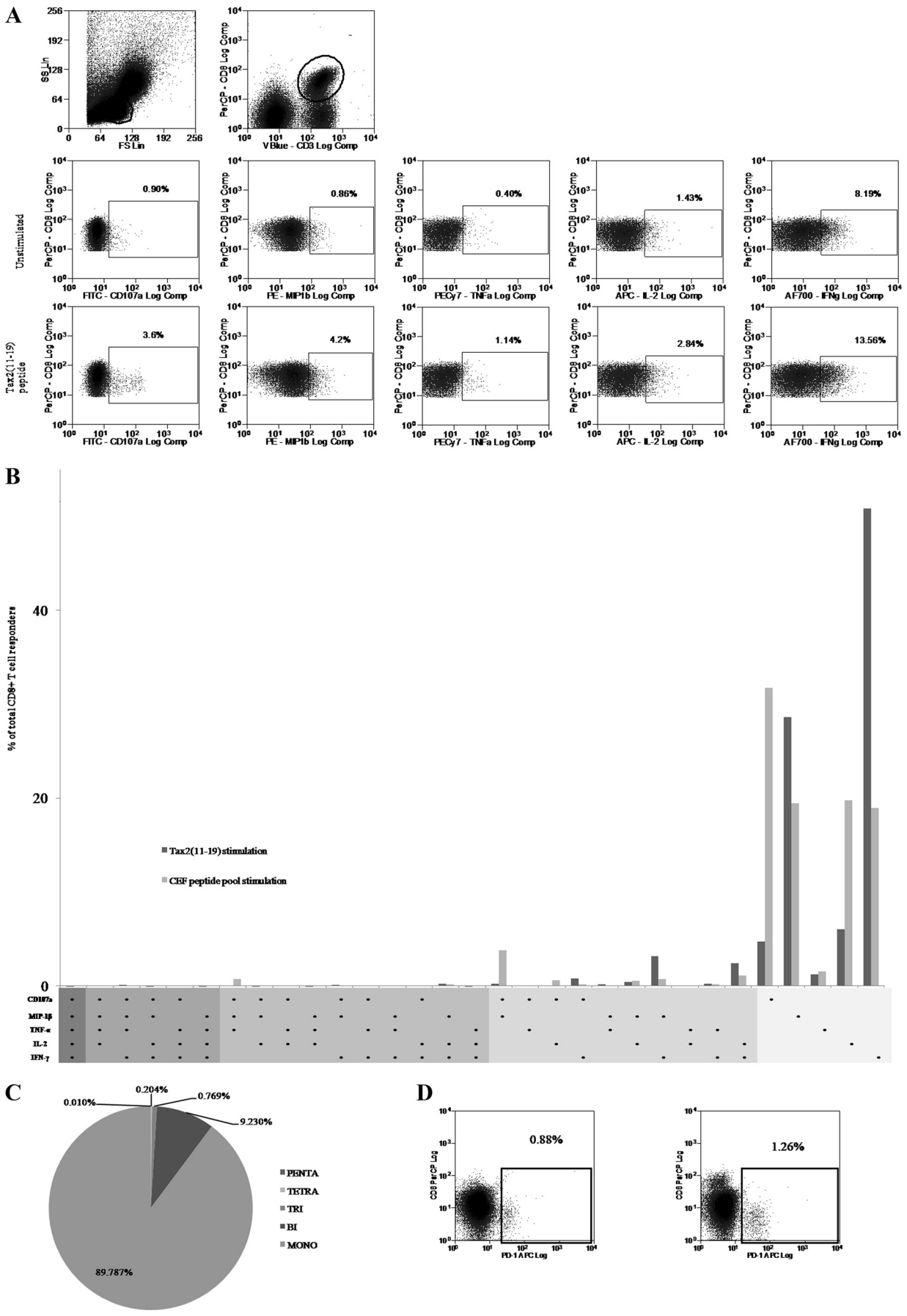


FIGURE 5. Polyfunctional profile of specific CD8$^+$ T cell responses in HTLV-2 infected individuals. *A*, Representative plots of gating strategy for polyfunctionality analysis. Cells were initially gated on size-scatter discriminators, followed by gating on CD3$^+$ and CD8$^+$ cells. Subsequently, single function gates were consistently placed across samples, based on unstimulated control samples. The frequency of responders for each single function is

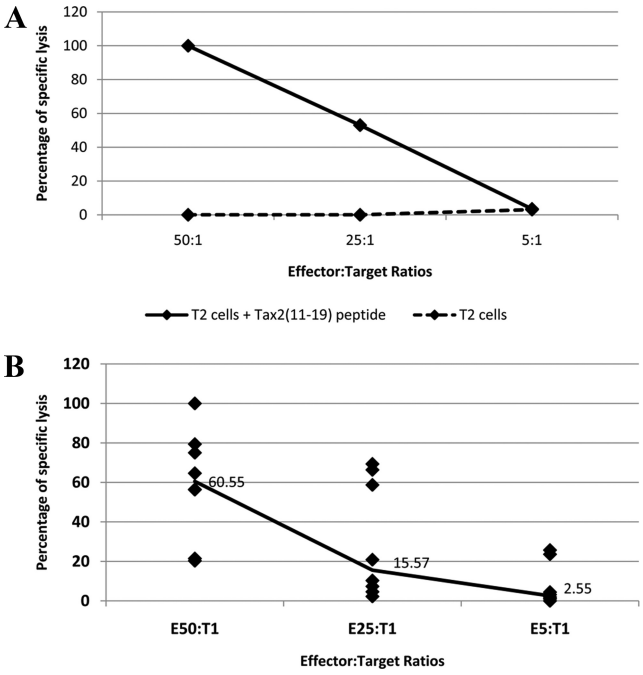


FIGURE 6. Cytotoxicity of $Tax2_{(11–19)}$-specific specific $CD8^+$ lymphocytes from infected individuals. $CD8^+$ T cells isolated by immunomagnetic separation from freshly thawed PBMCs were used as effectors in a 4-h ^{51}Cr-release cytotoxicity assay. Targets labeled with ^{51}Cr consisted of T2 cells alone, or pulsed with $Tax2_{(11–19)}$ synthetic peptide. Cytolytic activity was assayed at various E:T ratios. *A*, Comparative percentage of specific lysis from a representative HTLV-2-infected individual. *B*, Percentage of specific lysis from all evaluated subjects; the line represents the median values at different E:T ratios.

Taking into consideration that HTLV-2 is less pathogenic than HTLV-1, this highly focused response restricted to HLA-A*0201 could well represent an effective immune control mechanism. Indeed, Kozako et al. reported that a reduced frequency and diversity, associated with impaired function, of specific anti-Tax1 CTLs could represent a risk for ATLL development (29). In other viral infections, high levels of CTL responses against single epitopes can represent effective control of viral burden and a relative protection against disease development. During acute HIV infection, a strong response against a single B*27-restricted epitope is observed, with a marked suppression in viral load and delayed progression to AIDS (30). Moreover, high numbers of specific CTLs against a single epitope are also observed in healthy CMV seropositive individuals (31).

We could not detect responses in the context of HLA-A*0301 and HLA-B*3501. HLA-B*35-restricted CTL responses against Tax protein peptides have already been described in HTLV-1 infection (12, 32). In our study population, these alleles were codominant in the evaluated subjects. It has been shown that other HLA class I alleles can present Tax1 epitopes simultaneously with HLA-A*02 in the same individual (26). Whether CTL responses restricted to HLA-A*0201 predominate over other alleles remains to be clarified, and this will require an evaluation of CTL responses in a larger group of infected individuals, including subjects with alleles other than HLA-A*02. As we evaluated the ex vivo pentamer staining of PBMCs, this could have led to an underestimation memory virus-specific CTL populations. These cells might circulate at frequencies below the threshold of the assay, and may only be detected after in vitro antigenic stimulation. Further studies will be required to investigate this.

Although CTL responses against other HTLV-1 Ags have been described, we have focused our initial characterization of CTL responses against the Tax2 protein because of the immunodominance of Tax1 in HTLV-1-specific CTL responses. Further investigation of CTL epitopes derived from other viral proteins will be necessary to fully assess the CTL responses in HTLV-2 infection. It would be also of interest to evaluate HTLV-2-infected individuals with suspected clinical manifestations. However, in HTLV-1 infection, there is no difference between the frequency or immunodominance of specific CTLs between asymptomatic carriers and HAM/TSP patients (13, 32).

In our study, we could demonstrate a significant positive correlation between the frequency of circulating $Tax2_{(11–19)}$-specific CTLs and PVL levels. This finding may solely reflect a frequent Ag encounter in infected individuals due to persistent viral replication, rather than a failure of specific CTLs to control infection. The PVL levels in HTLV-2 infection have been reported to be lower than in HTLV-1 carriers, and this might also explain the differences in pathogenicity of the viruses (33, 34). In HTLV-1 infection, several studies have failed to show a consistent correlation between HTLV-1-specific CTL frequency and PVL levels (15). In fact, the rate of $CD8^+$ cell-mediated lysis of HTLV-1-infected cells in vitro (i.e., the efficiency of virus-specific CTLs) shows a significant negative correlation with PVL in vivo (35).

In our study, we used the classification of memory T cells proposed by Sallusto et al. (36), which permitted a direct comparison with the memory phenotype previously described in HTLV-1 infection. Indeed, we found a HTLV-2-specific $CD8^+$ T cell memory phenotype similar to that observed in HAM/TSP patients and in CMV infection, with high percentages of T_{EMRA} cells (37, 38). Whether this represents a particular differentiation phenotype associated with HTLV-2 infection or reflects a persistent activation and expansion of virus-specific CTLs remains to be determined. Moreover, as HTLV-2 infects predominantly $CD8^+$ lymphocytes, the frequencies of a particular phenotype might also be influenced by cellular proliferation known to be associated with infection itself. Further studies are necessary to address this possibility.

The differentiation phenotype of virus-specific CTLs might not correlate directly with the functional status of a particular cell subset (39). The use of unstimulated, freshly thawed cryopreserved PBMCs in our study had the advantage that the phenotypes of Ag-stimulated $CD8^+$ T lymphocytes may not be accurately reflected following in vitro culture (40). Therefore, the relatively lower levels of IFN-γ observed does not necessarily indicate impairment in production by HTLV-2 Tax-specific CTLs. It is likely that the use of unstimulated PBMCs in our experiments may have led to an underestimation of their ability to produce cytokines in

shown both in the unstimulated and $Tax2_{(11–19)}$-stimulated samples (second and third rows, respectively). *B*, Phenotypic and functional assessment of $Tax2_{(11–19)}$-specific $CD8^+$ T cells. Every possible combination of responses is shown in the graph. For comparison, the functional profile after stimulation with a pool containing CMV, EBV, and influenza peptides is shown. Bars represent the mean percentages of cells displaying a given combination of functions with the responder $CD8^+$ T cells. *C*, Pie chart depicting the background-adjusted multifunctional behavior (one to five functions) of $Tax2_{(11–19)}$-specific $CD8^+$ T cells in HTLV-2-infected individuals. Responses are grouped by the number of functions (indicated in the legend). The percentages of each group within the total responders are shown. *D*, Representative plots showing PD-1 expression in $Tax2_{(11–19)}$ pentamer-positive $CD8^+$ T cells (*left*) and CMV pentamer-positive $CD8^+$ T cells (*right*).

response to specific Ag stimulation. This became evident in our polyfunctionality studies, where a marked increase in the frequency of IFN-γ-producing cells could be detected after Ag-specific stimulation in vitro. In fact, such cells constitute the major subpopulation of responders upon stimulation, suggesting that specific CTLs are capable of producing IFN-γ in vivo.

Polyfunctionality has emerged as a key concept in protective immunity against viral infections. Polyfunctional T cells have been shown to be associated with a superior control of viral replication and vaccine efficacy (41, 42). In our study, we found an epitope-specific response biased to bi- or monofunctional responses. Although the polyfunctional attributes of specific $CD8^+$ T cells have not been described in HTLV-1 infection, our findings are similar to what have been demonstrated in chronic HIV-1 infection, where deterioration in the number of functions exhibited by specific $CD8^+$ T cells was markedly higher among untreated patients (23). Whether this profile observed in our study represents a dysfunction of HTLV-2-specific $CD8^+$ T cells still remains to be clarified. However, virus-specific $CD8^+$ T cells would appear to preserve their cytolytic activity, therefore being capable, at least partially, of controlling viral replication.

In our study, we could not demonstrate an increased expression of PD-1 in HTLV-2-specific $CD8^+$ T cells when compared with either pentamer-negative or CMV-specific $CD8^+$ T cells. Our findings contrast with what has been shown in HTLV-1 infection, where a marked increase of PD-1 expression could be found both in asymptomatic carriers and in patients with ATLL (43). Although these findings would not suggest immune exhaustion of virus-specific CTLs in HTLV-2 infection, this will require further studies.

Recently, it has been shown that there is a significantly lower frequency of $CD8^+$ T cells expressing perforin and granzyme B in both HAM/TSP patients and HTLV-1 asymptomatic carriers, compared with uninfected healthy subjects. A significant inverse correlation has also been demonstrated between the PVL and the frequency of perforin-positive $CD8^+$ T cells, and this correlation was stronger in HLA-A*02-positive individuals. Moreover, virus-specific CTLs in HAM/TSP patients have been reported to have a poor lytic ability, as evidenced by CD107a mobilization assays (44). Whether this lower frequency of cytotoxicity mediators would represent a dysfunction of the virus-specific CTLs or merely the result of continuous antigenic stimulation, with consequent degranulation of the CTLs (45), remains to be clarified. The situation might be somewhat different in HTLV-2 infection. In our study, we could show that most of circulating $Tax2_{(11–19)}$-specific CTLs not only exhibit a T_{EM} and T_{EMRA} phenotype, but also have a higher expression of cytotoxicity mediators such perforin and granzyme B, compared with the $Tax2_{(11–19)}$-negative $CD8^+$ population. These features are characteristic of CTLs with immediate effector functions and low proliferative potential (22). Taken together, these data strongly suggests that HTLV-2-specific cells should be capable of killing virus-infected cells.

Indeed, $Tax2_{(11–19)}$-specific CTLs seem to be functional with regard to cytolytic activity in our HTLV-2 infected population. We observed that $Tax2_{(11–19)}$-specific CTLs were capable of killing in vitro HLA-matched target cells loaded with $Tax2_{(11–19)}$ synthetic peptide. The use of enriched $CD8^+$ cells as effectors reduced the influence of at least two confounding factors: a likely control of cytolytic function by regulatory T cells and the unspecific lysis of targets by NK cells. Since $CD8^+$ T lymphocytes are the preferential reservoir of HTLV-2 in vivo, the T2 cell line was used as target to clearly distinguish between target and effector populations. T2 cells do not process Ags and express only HLA-A*02, which is stabilized on the cell surface by the synthetic peptide loading (46).

Our data suggest that $Tax2_{(11–19)}$-specific CTLs might be capable of recognizing and lysing naturally infected cells in vivo. Due to technical limitations, we were unable to determine whether virus-specific CTLs are also infected in vivo, which could result in a "$CD8^+$ T lymphocyte fratricide" phenomenon, as has been described in HTLV-1 infection (6). This interesting question will be addressed in further studies.

The differences in tropism to HTLV-1, where there is a preferential infection of $CD4^+$ T lymphocytes, may also contribute to the differences in the pathogenic properties of the two viruses. CTLs are of major importance in limiting the viral burden, although they are not the only component of the antiviral immune response. The investigation of other key mediators of the innate and adaptative immune response against HTLV-2 should be performed to fully address the dynamics of this longstanding, complex host-pathogen interaction.

One limitation of the present study is the relatively low number of enrolled HTLV-2 infected individuals. However, it is difficult to identify large numbers of HTLV-2-infected subjects who are not actively coinfected with other blood-borne viruses, which might influence immunological responses. However, attempts to recruit larger numbers of singly infected individuals are underway.

In summary, we provide the first characterization of specific CTL responses against the HTLV-2 Tax protein. We could clearly demonstrate high frequencies of $CD8^+$ T cell responses against a single, HLA-A*0201-restricted Tax2 derived epitope in HTLV-2 asymptomatic carriers. Moreover, we found a positive correlation between the magnitude of the CTL response and the PVL, suggesting that persistent viral replication activates and expands the pool of specific CTLs. Most HTLV-2 Tax-specific CTLs are immediate effector cells, as demonstrated by their phenotype, expression of cytotoxicity mediators, and ability to lyse peptide-pulsed targets. Our results suggest that the CTL response against HTLV-2 in vivo is capable of limiting PVL levels, at least partially, and may be important in the prevention of disease development in infected individuals.

Acknowledgments

We particularly thank the patients, whose trust and cooperation were essential for this study. We thank Drs. Barbara Roe and Noreen Sheehy for the helpful discussions and comments on the manuscript, and Dr. Alfonso Blanco for flow cytometry assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Roucoux, D. F., and E. L. Murphy. 2004. The epidemiology and disease outcomes of human T-lymphotropic virus type II. *AIDS Rev.* 6: 144–154.
2. Araújo, A., and W. W. Hall. 2004. Human T-lymphotropic virus type II and neurological disease. *Ann. Neurol.* 56: 10–19.
3. Ijichi, S., M. B. Ramundo, H. Takahashi, and W. W. Hall. 1992. In vivo cellular tropism of human T cell leukemia virus type II (HTLV-II). *J. Exp. Med.* 176: 293–296.
4. Lal, R. B., S. M. Owen, D. L. Rudolph, C. Dawson, and H. Prince. 1995. In vivo cellular tropism of human T-lymphotropic virus type II is not restricted to $CD8^+$ cells. *Virology* 210: 441–447.
5. Casoli, C., A. Cimarelli, and U. Bertazzoni. 1995. Cellular tropism of human T-cell leukemia virus type II is enlarged to B lymphocytes in patients with high proviral load. *Virology* 206: 1126–1128.
6. Hanon, E., J. C. Stinchcombe, M. Saito, B. E. Asquith, G. P. Taylor, Y. Tanaka, J. N. Weber, G. M. Griffiths, and C. R. Bangham. 2000. Fratricide among $CD8^+$ T lymphocytes naturally infected with human T cell lymphotropic virus type I. *Immunity* 13: 657–664.
7. Prince, H. E., J. York, S. M. Owen, and R. B. Lal. 1995. Spontaneous proliferation of memory ($CD45RO^+$) and naive ($CD45RO^-$) subsets of CD4 cells and CD8 cells in human T lymphotropic virus (HTLV) infection: distinctive patterns for HTLV-I versus HTLV-II. *Clin. Exp. Immunol.* 102: 256–261.
8. Lewis, M. J., V. W. Gautier, X. P. Wang, M. H. Kaplan, and W. W. Hall. 2000. Spontaneous production of C-C chemokines by individuals infected with human

T lymphotropic virus type II (HTLV-II) alone and HTLV-II/HIV-1 coinfected individuals. *J. Immunol.* 165: 4127–4132.
9. Jacobson, S., H. Shida, D. E. McFarlin, A. S. Fauci, and S. Koenig. 1990. Circulating CD8$^+$ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease. *Nature* 348: 245–248.
10. Parker, C. E., S. Daenke, S. Nightingale, and C. R. Bangham. 1992. Activated, HTLV-1-specific cytotoxic T-lymphocytes are found in healthy seropositives as well as in patients with tropical spastic paraparesis. *Virology* 188: 628–636.
11. Kannagi, M., S. Harada, I. Maruyama, H. Inoko, H. Igarashi, G. Kuwashima, S. Sato, M. Morita, M. Kidokoro, M. Sugimoto, et al. 1991. Predominant recognition of human T cell leukemia virus type I (HTLV-I) pX gene products by human CD8$^+$ cytotoxic T cells directed against HTLV-I-infected cells. *Int. Immunol.* 3: 761–767.
12. Hollsberg, P. 1999. Mechanisms of T-cell activation by human T-cell lymphotropic virus type I. *Microbiol. Mol. Biol. Rev.* 63: 308–333.
13. Goon, P. K., A. Biancardi, N. Fast, T. Igakura, E. Hanon, A. J. Mosley, B. Asquith, K. G. Gould, S. Marshall, G. P. Taylor, and C. R. Bangham. 2004. Human T cell lymphotropic virus (HTLV) type-1-specific CD8$^+$ T cells: frequency and immunodominance hierarchy. *J. Infect. Dis.* 189: 2294–2298.
14. Jacobson, S. 2002. Immunopathogenesis of human T cell lymphotropic virus type I-associated neurologic disease. *J. Infect. Dis.* 186(Suppl. 2): S187–S192.
15. Bangham, C. R., and M. Osame. 2005. Cellular immune response to HTLV-1. *Oncogene* 24: 6035–6046.
16. Bunce, M., C. M. O'Neill, M. C. Barnardo, P. Krausa, M. J. Browning, P. J. Morris, and K. I. Welsh. 1995. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens* 46: 355–367.
17. Wills, M. R., A. J. Carmichael, K. Mynard, X. Jin, M. P. Weekes, B. Plachter, and J. G. Sissons. 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J. Virol.* 70: 7569–7579.
18. Gavin, M. A., M. J. Gilbert, S. R. Riddell, P. D. Greenberg, and M. J. Bevan. 1993. Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. *J. Immunol.* 151: 3971–3980.
19. DiBrino, M., T. Tsuchida, R. V. Turner, K. C. Parker, J. E. Coligan, and W. E. Biddison. 1993. HLA-A1 and HLA-A3 T cell epitopes derived from influenza virus proteins predicted from peptide binding motifs. *J. Immunol.* 151: 5930–5935.
20. Deheé, A., R. Cesaire, N. Desire, A. Lezin, O. Bourdonne, O. Bera, Y. Plumelle, D. Smadja, and J. C. Nicolas. 2002. Quantitation of HTLV-I proviral load by a TaqMan real-time PCR assay. *J. Virol. Methods* 102: 37–51.
21. Lewis, M. J., P. Novoa, R. Ishak, M. Ishak, M. Salemi, A. M. Vandamme, M. H. Kaplan, and W. W. Hall. 2000. Isolation, cloning, and complete nucleotide sequence of a phenotypically distinct Brazilian isolate of human T-lymphotropic virus type II (HTLV-II). *Virology* 271: 142–154.
22. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22: 745–763.
23. Streeck, H., Z. L. Brumme, M. Anastario, K. W. Cohen, J. S. Jolin, A. Meier, C. J. Brumme, E. S. Rosenberg, G. Alter, T. M. Allen, B. D. Walker, and M. Altfeld. 2008. Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8$^+$ T cells. *PLoS Med.* 5: e100.
24. Nagai, M., K. Usuku, W. Matsumoto, D. Kodama, N. Takenouchi, T. Moritoyo, S. Hashiguchi, M. Ichinose, C. R. Bangham, S. Izumo, and M. Osame. 1998. Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP. *J. Neurovirol.* 4: 586–593.
25. Koenig, S., R. M. Woods, Y. A. Brewah, A. J. Newell, G. M. Jones, E. Boone, J. W. Adelsberger, M. W. Baseler, S. M. Robinson, and S. Jacobson. 1993. Characterization of MHC class I restricted cytotoxic T cell responses to tax in HTLV-1 infected patients with neurologic disease. *J. Immunol.* 151: 3874–3883.
26. Parker, C. E., S. Nightingale, G. P. Taylor, J. Weber, and C. R. Bangham. 1994. Circulating anti-Tax cytotoxic T lymphocytes from human T-cell leukemia virus type I-infected people, with and without tropical spastic paraparesis, recognize multiple epitopes simultaneously. *J. Virol.* 68: 2860–2868.
27. Catalan-Soares, B. C., A. B. Carneiro-Proietti, F. G. Da Fonseca, R. Correa-Oliveira, D. Peralva-Lima, R. Portela, J. G. Ribas, D. U. Goncalves, and F. A. Proietti. 2009. HLA class I alleles in HTLV-1-associated myelopathy and asymptomatic carriers from the Brazilian cohort GIPH. *Med. Microbiol. Immunol.* 198: 1–3.
28. Jeffery, K. J., K. Usuku, S. E. Hall, W. Matsumoto, G. P. Taylor, J. Procter, M. Bunce, G. S. Ogg, K. I. Welsh, J. N. Weber, et al. 1999. HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. *Proc. Natl. Acad. Sci. USA* 96: 3848–3853.
29. Kozako, T., N. Arima, S. Toji, I. Masamoto, M. Akimoto, H. Hamada, X. F. Che, H. Fujiwara, K. Matsushita, M. Tokunaga, et al. 2006. Reduced frequency, diversity, and function of human T cell leukemia virus type 1-specific CD8$^+$ T cell in adult T cell leukemia patients. *J. Immunol.* 177: 5718–5726.
30. Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, et al. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat. Med.* 3: 212–217.
31. Gillespie, G. M., M. R. Wills, V. Appay, C. O'Callaghan, M. Murphy, N. Smith, P. Sissons, S. Rowland-Jones, J. I. Bell, and P. A. Moss. 2000. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8$^+$ T lymphocytes in healthy seropositive donors. *J. Virol.* 74: 8140–8150.
32. Daenke, S., A. G. Kermode, S. E. Hall, G. Taylor, J. Weber, S. Nightingale, and C. R. Bangham. 1996. High activated and memory cytotoxic T-cell responses to HTLV-1 in healthy carriers and patients with tropical spastic paraparesis. *Virology* 217: 139–146.
33. Murphy, E. L., T. H. Lee, D. Chafets, C. C. Nass, B. Wang, K. Loughlin, and D. Smith. 2004. Higher human T lymphotropic virus (HTLV) provirus load is associated with HTLV-I versus HTLV-II, with HTLV-II subtype A versus B, and with male sex and a history of blood transfusion. *J. Infect. Dis.* 190: 504–510.
34. Hisada, M., W. J. Miley, and R. J. Biggar. 2005. Provirus load is lower in human T lymphotropic virus (HTLV)-II carriers than in HTLV-I carriers: a key difference in viral pathogenesis? *J. Infect. Dis.* 191: 1383–1385; author reply 1385–1386.
35. Asquith, B., A. J. Mosley, A. Barfield, S. E. Marshall, A. Heaps, P. Goon, E. Hanon, Y. Tanaka, G. P. Taylor, and C. R. Bangham. 2005. A functional CD8$^+$ cell assay reveals individual variation in CD8$^+$ cell antiviral efficacy and explains differences in human T-lymphotropic virus type 1 proviral load. *J. Gen. Virol.* 86: 1515–1523.
36. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
37. Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, et al. 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 410: 106–111.
38. Johnson-Nauroth, J. M., J. Graber, K. Yao, S. Jacobson, and P. A. Calabresi. 2006. Memory lineage relationships in HTLV-1-specific CD8$^+$ cytotoxic T cells. *J. Neuroimmunol.* 176: 115–124.
39. Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, et al. 2002. Memory CD8$^+$ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* 8: 379–385.
40. Geginat, J., A. Lanzavecchia, and F. Sallusto. 2003. Proliferation and differentiation potential of human CD8$^+$ memory T-cell subsets in response to antigen or homeostatic cytokines. *Blood* 101: 4260–4266.
41. Almeida, J. R., D. A. Price, L. Papagno, Z. A. Arkoub, D. Sauce, E. Bornstein, T. E. Asher, A. Samri, A. Schnuriger, I. Theodorou, et al. 2007. Superior control of HIV-1 replication by CD8$^+$ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J. Exp. Med.* 204: 2473–2485.
42. Precopio, M. L., M. R. Betts, J. Parrino, D. A. Price, E. Gostick, D. R. Ambrozak, T. E. Asher, D. C. Douek, A. Harari, G. Pantaleo, R. Bailer, B. S. Graham, M. Roederer, and R. A. Koup. 2007. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8$^+$ T cell responses. *J. Exp. Med.* 204: 1405–1416.
43. Kozako, T., M. Yoshimitsu, H. Fujiwara, I. Masamoto, S. Horai, Y. White, M. Akimoto, S. Suzuki, K. Matsushita, K. Uozumi, et al. 2009. PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients. *Leukemia* 23: 375–382.
44. Sabouri, A. H., K. Usuku, D. Hayashi, S. Izumo, Y. Ohara, M. Osame, and M. Saito. 2008. Impaired function of human T-lymphotropic virus type 1 (HTLV-1)-specific CD8$^+$ T cells in HTLV-1-associated neurologic disease. *Blood* 112: 2411–2420.
45. Bangham, C. R. 2008. HTLV-1 infection: role of CTL efficiency. *Blood* 112: 2176–2177.
46. Salter, R. D., and P. Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J.* 5: 943–949.