the HAM/TSP group (1.607% mean value) compared to the HAC group (0.731% mean value) ($p = 0.0294$).

SAGE library analysis

To access the identity of $CD8^+$ T cell libraries, we used hierarchical cluster analysis to compare the gene expression profile of our libraries with data from libraries of different leukocyte types. The dendrogram revealed that lymphoid and myeloid cells clustered separately. Notably, $CD8^+$ T cell libraries derived from HTLV-1-infected individuals were grouped together in the lymphoid cluster as shown in Fig. 1, indicating similarity in the gene expression patterns with lymphoid cells. HAC and HAM/TSP libraries also showed a tight correspondence, branching together.

A total of 62,432 and 60,620 tags were sequenced from HAC and HAM/TSP libraries, respectively. These tags yielded 12,262 and 13,025 unique tags mapped to known genes. Most identified and mapped tags (60%) appeared only once. Both libraries showed a large number of ribosomal protein tags. Supplementary Tables S1 and S2 (Supplementary Data are available online at www.liebertpub.com/aid) list the 50 most abundant tags (ribosomal tags were excluded) found in the HAC and HAM/TSP libraries, respectively. The most abundant tag identified in HAC and HAM/TSP libraries was the heterogeneous nuclear ribonucleoprotein A1 pseudogene 5 (HNRPA1P5).

Gene expression patterns observed in HAC versus HAM/TSP groups

We used SAGE libraries of pooled HAC and HAM/TSP $CD8^+$ T cells to screen for differentially expressed pathways involved in HAM/TSP development. Comparison of gene expression profiles between the HAC and HAM/TSP groups showed 285 differentially expressed tags. Tables 3 and 4 show the top 20 increased tag-associated transcripts in the HAC and in the HAM/TSP libraries, respectively. Of the 285 deregulated tags observed between HAC and HAM/TSP, 174 were overexpressed in the HAM/TSP group. To investigate the role of those differentially expressed tag-associated genes they were classified according to their biological function. We performed Ingenuity Pathways Analysis, which resulted in the annotation of the following most represented functional categories: apoptosis, cytolysis, cytotoxicity, cellular development, growth and proliferation, immunological and neurological diseases, inflammatory response, and diseases and infection. Additionally, we classified the differentially expressed genes into a number of functional categories according to Gene Ontology terms of interest (Table 5) to search for genes involved in HAM/TSP development. We found molecules related to immune response, such as zeta-chain (TCR)-associated protein kinase 70 kDa (ZAP70), leukocystatin (CST7), and linker for activation of T cells (LAT); apoptosis,

Table 3. Top 20 Increased Transcripts Expressed in the Human T Lymphotropic Virus Type I-Asymptomatic Carriers Library

Tag	Symbol	Description	Normalized frequencies		FC	p value ≤ 0.05	FDR
			HAM/TSP	HAC			
CTGATCTGTG	HLA-B	Major histocompatibility complex, class I, B	0	54	54.0	Yes	0.00
AGCTGCAATC	EEF1G	Eukaryotic translation elongation factor 1 gamma	1	17	17.0	Yes	0.00
AATCCAGGAG	DDOST	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase	1	16	16.0	Yes	0.00
CTGGCGCGAG	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	4	57	14.3	Yes	0.00
CTGAGACGAA	BTF3	Basic transcription factor 3	0	14	14.0	Yes	0.00
TGTGGGAACC	SMAP1L	Stromal membrane-associated protein 1-like	0	12	12.0	Yes	0.00
ATCCGCAAGA	ACO1	Aconitase 1, soluble	1	12	12.0	Yes	0.00
GAATTTGTGT	EFCAB5	EF-hand calcium binding domain 5	1	12	12.0	Yes	0.00
GAGCAGGAGC	DIS3L2	DIS3 mitotic control homolog (*S. cerevisiae*)-like 2	1	12	12.0	Yes	0.00
GCCAAGGGGC	OGDH	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	1	12	12.0	Yes	0.00
TAGAAAAATA	GPI	Glucose phosphate isomerase	1	12	12.0	Yes	0.00
TGTTCCACTC	ENTPD6	Ectonucleoside triphosphate diphosphohydrolase 6 (putative function)	1	12	12.0	Yes	0.00
TTGAGCCAGC	KHSRP	KH-type splicing regulatory protein (FUSE binding protein 2)	1	12	12.0	Yes	0.00
GGGCCCCGCA	PMPCA	Peptidase (mitochondrial processing) alpha	0	11	11.0	Yes	0.00
GTGGTGTACG	RBM3	RNA binding motif (RNP1, RRM) protein 3	1	11	11.0	Yes	0.00
GTTCTCCCAC	SEC61A1	Sec61 alpha 1 subunit (*S. cerevisiae*)	1	11	11.0	Yes	0.00
TGGGCTGGGG	ADFP	Adipose differentiation-related protein	1	11	11.0	Yes	0.00
AAGATCAAGA	ACTA1	Actin, alpha 1, skeletal muscle	1	11	11.0	Yes	0.00
CTTGCCTGAA	BIN1	Bridging integrator 1	3	27	9.0	Yes	0.00
GGGGCTGGGG	EGLN2	Egl nine homolog 2 (*C. elegans*)	4	35	8.8	Yes	0.00
CTGACTTGTG	HLA-B	Major histocompatibility complex, class I, B	21	156	7.4	Yes	0.00

HAC, HTLV-1-asymptomatic carriers; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; FC, fold change; FDR, false discovery rate. The tag sequence represents the 10-bp SAGE tag. Normalized frequencies were obtained using SAGE software by calculating the total number of 300,000.

TABLE 4. TOP 20 INCREASED TRANSCRIPTS EXPRESSED IN THE HUMAN T LYMPHOTROPIC VIRUS TYPE I-ASSOCIATED MYELOPATHY/TROPICAL SPASTIC PARAPARESIS LIBRARY

Tag	Symbol	Description	Normalized frequencies		FC	p value ≤ 0.05	FDR
			HAM/TSP	HAC			
CGAGGGGCCA	ACTN4	Actinin, alpha 4	18	1	18.0	Yes	0.00
GAAGCAATAA	ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	16	0	16.0	Yes	0.00
AGTCGGGAGC	HNRPK	Heterogeneous nuclear ribonucleoprotein K	14	1	14.0	Yes	0.00
CGGCCTCACC	TTYH2	Tweety homolog 2 (*Drosophila*)	14	0	14.0	Yes	0.00
GCAGGGTACA	KIAA1949	KIAA1949	14	0	14.0	Yes	0.00
TCTGAAGTCA	ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	14	0	14.0	Yes	0.00
TGTAAGTCTG	KHDRBS1	KH domain containing, RNA binding, signal transduction associated 1	14	0	14.0	Yes	0.00
CAAAAAAAAA	OCIAD1	OCIA domain containing 1	13	1	13.0	Yes	0.00
GGCCAGCAAT	GLIPR1	GLI pathogenesis-related 1 (glioma)	13	1	13.0	Yes	0.00
GCGTCCTGCC	LAT	Linker for activation of T cells	13	0	13.0	Yes	0.00
GCTAAAAAAA	FBF1	Fas (TNFRSF6) binding factor 1	13	0	13.0	Yes	0.00
TCTGCTAAAG	HMGB1	High-mobility group box 1	13	0	13.0	Yes	0.00
AATGCTGGCA	DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	11	1	11.0	Yes	0.00
ACCCTCTTCC	HLA-A	Major histocompatibility complex, class I, A	11	1	11.0	Yes	0.00
CTGGCCATCG	EHBP1L1	EH domain binding protein 1-like 1	11	1	11.0	Yes	0.00
GAGCGGGATC	SFRS1	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	11	1	11.0	Yes	0.00
GGGGGCCCCG	YIF1A	Yip1 interacting factor homolog A (*S. cerevisiae*)	11	1	11.0	Yes	0.00
AGATGAGATG	KLF6	Kruppel-like factor 6	11	0	11.0	Yes	0.00
GGGAATCAAA	CNO	Cappuccino homolog (mouse)	11	0	11.0	Yes	0.00
GTAGCAGGTG	M6PRBP1	Mannose-6-phosphate receptor binding protein 1	11	0	11.0	Yes	0.00

HAC, HTLV-1-asymptomatic carriers; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; FC, fold change; FDR, false discovery rate. The tag sequence represents the 10-bp SAGE tag. Normalized frequencies were obtained using SAGE software by calculating the total number of 300,000.

such as granzyme H (GZMH), integrin beta 2 (ITGB2), and programmed cell death 4 (PDCD4); and inflammatory response, such as chemokine (C–C motif) ligand 5 (CCL5) and natural cytotoxicity triggering receptor 3 (NCR3), among others (Table 5).

Gene expression patterns observed in CT versus HTLV-1-infected groups (HAC or HAM/TSP)

We searched for differentially expressed tags between a public library of CD8+ T cells from normal healthy volunteers (CT) and HAC and also between CT and HAM/TSP in order to identify genes deregulated in HTLV-1 infection. Although this comparison has some drawbacks due to methodological and ethnical differences among samples, we could identify 899 differentially expressed tags from the comparison between CT and HAC, whereas CT and HAM/TSP revealed 855. From these tags we determined that many cytokine-related genes including chemokine (C–X–C motif) receptor 4 (CXCR4), interleukin 8 (IL-8), chemokine (C–C motif) ligand 20 (CCL20), and chemokine (C–C motif) receptor 7 (CCR7) were decreased in HTLV-1-infected groups (HAC and HAM/TSP), whereas IL-23, alpha (IL23A), lymphotoxin beta (LTB), IL-24, and CCL5 expressions were increased, when compared to the CT library.

Genes related to cytotoxicity were also identified as differentially expressed between CT and HTLV-1-infected libraries. For example, GZMH and granzyme A (GZMA) have a higher expression in HTLV-1-infected individuals than in the CT group.

Expression levels of cytotoxicity and cytokines genes were different between HAC and HAM/TSP patients

To further investigate the involvement of specific genes in HTLV-1 infection based on fold change in SAGE libraries and on their biological function, we selected the CCL5, GZMH, and ZAP70 genes to assess their gene expression levels by real time PCR in 55 samples. We also tested the cytolytic genes granzyme B (GZMB) and PRF1 and the cytoskeletal adaptor paxillin (PXN) to better explore their associated pathways.

In SAGE libraries, CCL5 was 3.5 times higher, GZMH was 2 times higher, and ZAP70 was 5.8 times higher in the HAM/TSP group than in HAC (Table 5). When we compared the expression levels of GZMB, PRF1, and PXN between the HAC and HAM/TSP libraries, we found an expression ratio lower than 2.0.

Relative quantification of CCL5 and GZMH by qRT-PCR corroborated SAGE data showing that their expression levels were significantly higher in the HAM/TSP than in the HAC group and also than in the CT group (Fig. 2A and B). When we tested ZAP70 by qRT-PCR we found a different result from SAGE, since no difference was observed between the HAC and HAM/TSP groups. Interestingly, we found that all infected patients (HAC + HAM/TSP) showed a significantly higher expression level of ZAP70 than the CT group (Fig. 2E). Expression of the cytotoxicity gene PRF1 was significantly higher in the HAM/TSP group than in the CT group (Fig. 2C). Analyzing gene expression of PXN by qRT-PCR, we found a significant increase in the HAM/TSP group compared to the CT group and also compared to the HAC groups (Fig. 2D).

TABLE 5. COMPARISON OF GENES DIFFERENTIALLY EXPRESSED BETWEEN HUMAN T LYMPHOTROPIC VIRUS TYPE I-ASYMPTOMATIC CARRIERS AND HUMAN T LYMPHOTROPIC VIRUS TYPE I-ASSOCIATED MYELOPATHY/TROPICAL SPASTIC PARAPARESIS $CD8^+$ T CELL LIBRARIES FOR SELECTED TERMS OF GENE ONTOLOGY

		Normalized frequencies			
Tag	*FC*	*HAM/TSP*	*HAC*	*Gene symbol*	*Description*
Immune response					
CTGATCTGTG	54.0	1	54	HLA-C	Major histocompatibility complex, class I, C
CTGGCGCGAG	14.3	4	57	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta
CTGACTTGTG	7.4	21	156	HLA-C	Major histocompatibility complex, class I, C
TGACCCACCA	8.0	1	8	HLA-C	Major histocompatibility complex, class I, C
TGGGAGCTCA	8.0	1	8	NCR3	Natural cytotoxicity triggering receptor 3
CTGACCTGTG	−2.0	483	240	HLA-C	Major histocompatibility complex, class I, C
GGCTCCTCGA	−2.1	19	9	TAPBP	TAP binding protein (tapasin)
GCTTAATGCT	−2.2	24	11	CD8B	CD8b molecule
GTAGCACCTC	−2.3	69	30	CST7	Cystatin F (leukocystatin)
TTCCCTTCTT	−2.4	26	11	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1
CCACTACACT	−3.5	14	4	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
CCTCTAGAGG	−3.5	14	4	CCL5	Chemokine (C–C motif) ligand 5
GCTGAACGCG	−3.0	9	3	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
AGGGGCTGCC	−4.8	19	4	HLA-C	Major histocompatibility complex, class I, C
CGAGCCTGTT	−5.8	23	4	ZAP70	Zeta-chain (TCR)-associated protein kinase 70 kDa
GCGTCCTGCC	−13.0	13	1	LAT	Linker for activation of T cells
Apoptosis					
CAAGATAAAT	9.0	1	9	MAGED1	Melanoma antigen family D, 1
GTGGACCCCA	8.0	1	8	PUF60	Poly-U binding splicing factor 60 kDa
AGACTAACCT	−2.0	32	16	GZMH	Granzyme H (cathepsin G-like 2, protein h-CCPX)
GAGACTTGAG	−2.6	23	9	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
GACTCTGGGA	−2.8	11	4	CLPTM1L	CLPTM1-like
GGGGGCGCCT	−3.0	9	3	SLC25A6	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
TGTGTGGGGC	−3.0	9	3	RHOT2	Ras homolog gene family, member T2
GTGACAACAC	−8.0	8	1	VDAC1	Voltage-dependent anion channel 1
TGAAGCAGTA	−8.0	8	1	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
TGCCCCTGAA	−8.0	8	1	XAF1	XIAP-associated factor 1
Cell adhesion					
GAAGAGTTCC	5.7	3	17	LEF1	Lymphoid enhancer-binding factor 1
ATAGGTCAGA	4.0	3	12	CLSTN1	Calsyntenin 1
TGGAACTGTG	−2.0	16	8	SIGLEC8	Sialic acid binding Ig-like lectin 8
GAGACTTGAG	−2.6	23	9	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
TTGCCCAGCA	−2.7	16	6	CERCAM	Cerebral endothelial cell adhesion molecule
CCTCTAGAGG	−3.5	14	4	CCL5	Chemokine (C–C motif) ligand 5
CAAAAAAAAA	−13.0	13	1	ADA	Adenosine deaminase
Inflammatory response					
TGGGAGCTCA	8.0	1	8	NCR3	Natural cytotoxicity triggering receptor 3
TACCTGCAGA	−2.2	54	25	S100A8	S100 calcium binding protein A8
GAGACTTGAG	−2.6	23	9	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
CCTCTAGAGG	−3.5	14	4	CCL5	Chemokine (C–C motif) ligand 5
GCTGAACGCG	−3.0	9	3	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
Cytokine activity					
TAGAAAAATA	12.0	1	12	GPI	Glucose-6-phosphate isomerase
TTTATCTGCT	8.0	1	8	HMGB1	High-mobility group box 1
CCACTACACT	−3.5	14	4	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
GAAATTTAAA	−3.0	9	3	HMGB1	High-mobility group box 1
TCTGCTAAAG	−13.0	13	1	HMGB1	High-mobility group box 1

HAC, HTLV-1-asymptomatic carriers; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; FC, fold change. The tag sequence represents the 10-bp SAGE tag. Normalized frequencies were obtained using SAGE software by calculating the total number of 300,000.

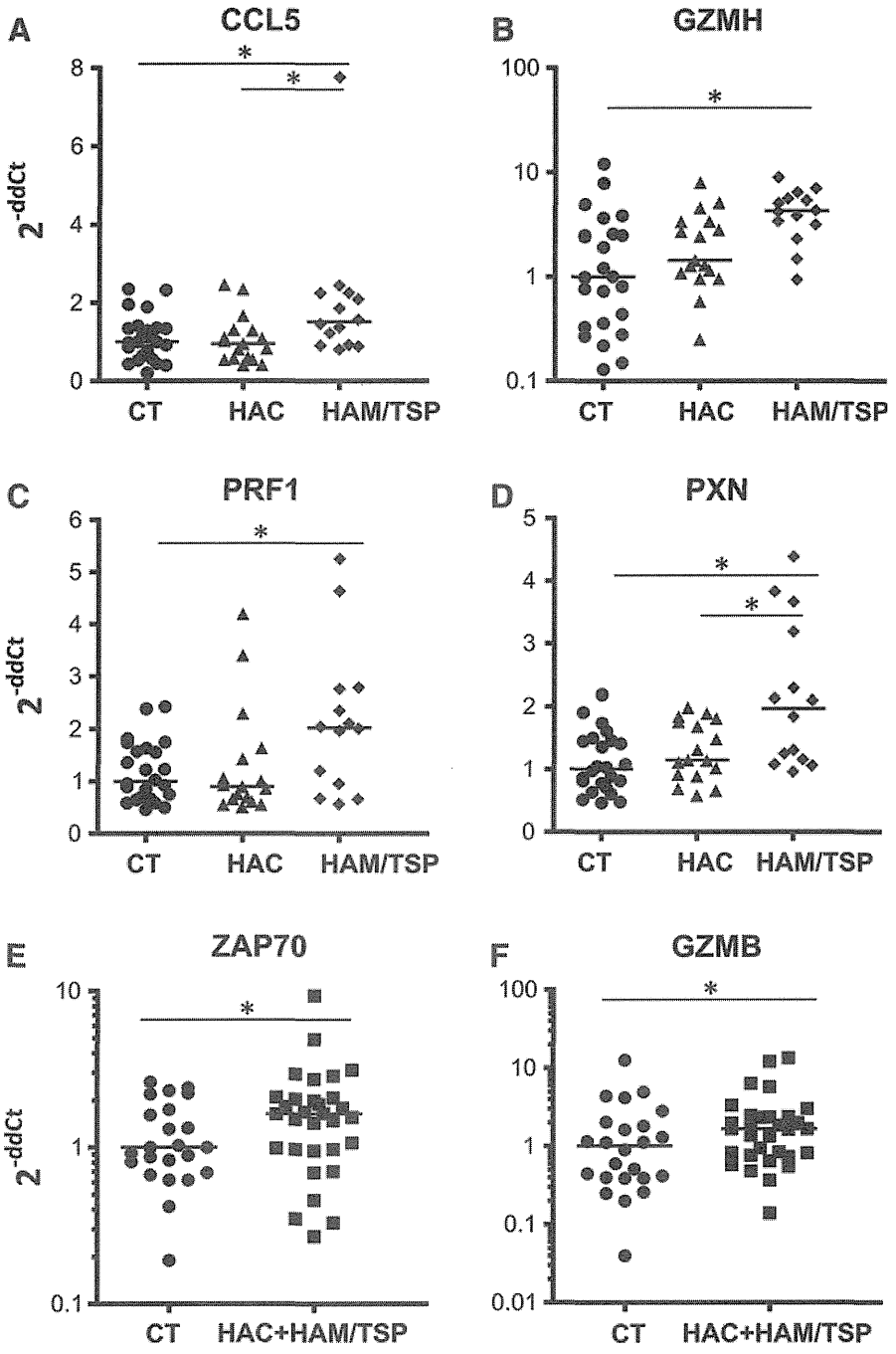

FIG. 2. Gene expression in CT, HAC, and HAM/TSP CD8+ T cells using qRT-PCR. **(A)** CCL5, **(B)** GZMH, **(C)** PRF1, **(D)** PXN, **(E)** ZAP70, and **(F)** GZMB. The relative level of gene expression was obtained from the CT ($n=24$), HAC ($n=17$), and HAM/TSP ($n=14$) groups. Bars indicate the median of each group. The median of the CT group was used as a calibrator. Asterisks indicate p values <0.05 (*) compared with ANOVA. The Mann–Whitney test was used for comparison between the two groups. CT, control group; HAC, HTLV-1-asymptomatic carriers; and HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis.

We also tested expression levels of GZMB and although no difference was observed among the CT, HAC, and HAM/TSP groups, we found a significant increase in whole infected patients (HAC+HAM/TSP) compared to the CT group (Fig. 2F).

PRF1 and GZMB protein levels were differentially expressed among HAC and HAM/TSP groups

To more directly confirm the results of gene expression data, we quantified proteins level of PRF1 and GZMB by quantitative flow cytometry. When we performed intracellular staining of PRF1, we detected significantly higher MFI in the HAM/TSP group compared to the HAC group (Fig. 3A). Moreover, the GZMB intracellular expression also revealed a significant increase of MFI in HAM/TSP patients than the HAC group, as shown in Fig. 3B.

Distinct correlation between gene expression and PVL

We considered if there was an association between PVL and the gene expression data and we saw no correlation among PVL and PRF1, GZMB, ZAP70, or PXN ($p>0,05$). However, PVL correlated positively with GZMH ($r=0.3947$, $p=0.0345$) and CCL5 ($r=0.5257$, $p=0.0060$).

Discussion

CD8+ T cells play an important role in HTLV-1 infection and their frequency and efficiency are related to PVL levels,

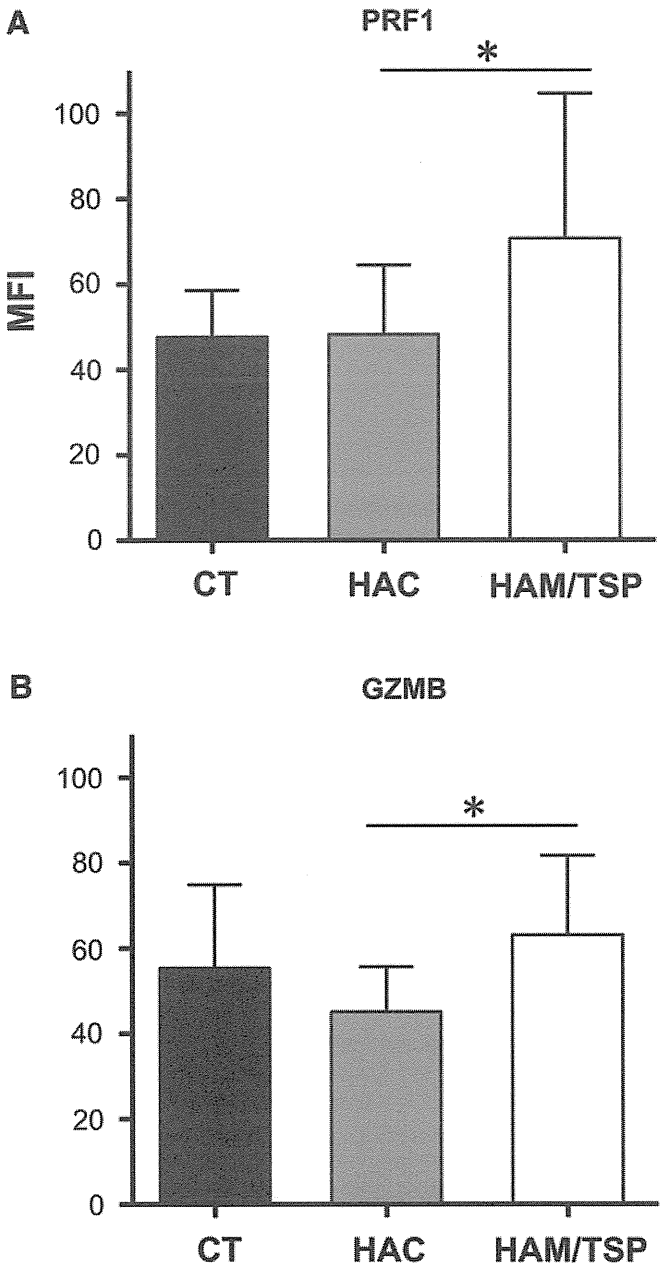

FIG. 3. Quantitative flow cytometry of PRF1 **(A)** and GZMB **(B)** in $CD8^+$ T cell populations from HTLV-1-infected patients and controls. MFI indicates the mean fluorescence intensity. Bars indicate the mean of each group with standard deviation. Asterisks indicate p values < 0.05 (*) compared with ANOVA. CT, control group; HAC, HTLV-1-asymptomatic carriers; and HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis.

which in turn might be associated with HAM/TSP development. In our study, we found that a distinct gene expression profile of $CD8^+$ T cells between the HAC and HAM/TSP groups exists. Using SAGE methodology, we screened for some differentially expressed genes associated with a number of functional categories, including apoptosis, cytolysis, cytotoxicity, cellular development, growth and proliferation, immunological and neurological diseases, inflammatory response, and diseases and infection. Due to their potential biological role in HTLV-1 infection, we focus on cytotoxicity and cytokine-related genes and assessed the levels of expression of GZMB, GZMH, PRF1, ZAP70, PXN, and CCL5.

Our results showed that patients with HAM/TSP have high expression levels of genes related to cell-mediated lysis, namely GZMH and PRF1. Moreover, GZMH expression showed a significant positive correlation with PVL. In addition, we found that HTLV-infected patients (HAC + HAM/TSP) expressed higher levels of GZMB than the noninfected group (CT). In agreement with mRNA measurements, we detected higher protein expression levels of PRF1 and also of GZMB in HAM/TSP patients, compared to the HAC group.

$CD8^+$ T cells are responsible for combatting target cells bearing antigens recognized by the T cell receptor (TCR). When $CD8^+$ T cells encounter cells presenting HTLV antigens, they are supposed to release specialized granules containing cytolytic molecules, including perforin and granzymes, and to kill them. During HTLV-1 infection, overexpression of these cytolytic molecules, as we found here, might be explained as an effect of the vast antigen stimulus due to circulating infected cells, which is more prominent in HAM/TSP patients who have a high PVL. These findings suggest that $CD8^+$ T cells are activated during HTLV-1 infection, mainly in HAM/TSP patients, although they are not able to limit viral replication and control disease development.

In contrast to our results, Sabouri *et al.*[32] detected that HTLV-1-infected individuals express lower protein levels of PRF1 as compared to CT. The same study found that GZMB shows higher levels in the HAM/TSP group than in HAC, corroborating our data. Vine *et al.*[33] compared the gene expression of $CD8^+$ T cells from HTLV-1-infected individuals with low and high levels of PVL. They found that a number of genes related to cell-mediated lysis (including PRF1 and GZMB) were overexpressed in patients with low PVL, suggesting that there is an association between a strong cytotoxic $CD8^+$ T cell activity and an effective HTLV-1 suppression, which diverges from our findings.

At this point we cannot explain these apparently contrasting results. In fact, the investigation of PRF1 and granzyme expression is complex. $CD8^+$ T cell degranulation does not require new gene transcription and can occur within 30–60 min of stimulation[34] making mRNA and mainly protein measurement a challenge.

We also detected ZAP70 increased expression in HTLV-infected patients (HAC + HAM/TSP) compared to the noninfected group (CT), suggesting that $CD8^+$ T cells of HTLV-1-infected individuals are activated. ZAP70 is a protein tyrosine kinase associated with TCR, which is involved in signal transduction that leads to cell response upon TCR activation.[35] The high ZAP70 expression can also be an effect of the vast antigens circulating during infection, as we proposed for cytolytic altered genes.

Another cytokine investigated in our study was CCL5 (RANTES), with higher expression in the HAM/TSP group as compared to the HAC and CT groups. This cytokine is associated with immunomodulation and inflammatory processes. In agreement with our findings, several studies have demonstrated that elevation of CCL5 expression is observed in HTLV-1-infected cell lines, in peripheral blood cells, and in lymph nodes from HAM/TSP and ATLL patients.[36–38] High levels of this chemokine were also found in the cerebrospinal fluid from HTLV-1 patients.[38] It is likely that $CD8^+$ T cells from HTLV-1-infected individuals are somehow chronically activated,

probably in response to HTLV-1 antigens. Additionally, mediating leukocyte recruitment and T cell stimulation, CCL5 might play a role in HAM/TSP physiopathology.

At last, we detected an increase of PXN in the HAM/TSP group compared to the CT and to HAC groups. Paxillin is a cytoskeletal adaptor protein that plays an important role in cell adhesion and motility in adherent cells. During HIV-1 infection, PXN was described as a positive regulator of viral infection. In other words, PXN is involved in host cytoskeleton organization that allows viral entry in the target cell during virological synapse.[39] Additionally, in human cytomegalovirus infection, PXN regulation plays a role in the process of viral entry into monocytes and consequently in viral dissemination.[40] It is not clear what role PXN plays in T cells, but there is evidence that PXN is involved in $CD8^+$ T cell degranulation in immunological synapse. Once TCR is activated, there is a microtubule organizing center reorientation, movement, and fusion of the cytolytic granules with the plasma membrane, with PXN involvement.[34] In this way, although there is no report of PXN participation in HTLV infection, as we found a high expression level of ZAP70 and of the cytolytic genes PRF and granzymes, we propose that PXN is overexpressed in HAM/TSP patients due to the intense granules releasing in the immunological synapses. PXN could also be involved in HTVL spread, although $CD8^+$ T cells are not the main infected cells during the infection.

To investigate whether the gene expression differences found between the HAC and HAM/TSP groups were caused by the differences in PVL, we assessed the correlation between PVL and the gene expression data. We could not answer this question since half of the genes showed a correlation with PVL whereas the rest of them did not. The analysis of data with similar PVL and different clinical status would be useful to address the gene expression differences between the HAC and HAM/TSP conditions. However, in our cohort, we could not identify a group of HAC and HAM/TSP patients with similar PVL.

In this study, we analyzed the whole $CD8^+$ T cell population, which comprises specific $CD8^+$ T cells to a variety of antigens, including HTLV-1-specific $CD8^+$ T cells, and also includes $CD8^+$ T cells infected by HTLV-1. To identify genes involved in HTLV-1 infection, it would be preferable to study purified HTLV-1-specific $CD8^+$ T cells; however, these cells were not isolated here because current quantification techniques of antigen-specific cells may modify gene expression. It is known that 10% of total circulating $CD8^+$ T cells are HTLV-1-specific $CD8^+$ T cells.[41] Thus, we believe that the gene expression profile generated here is most likely the result of virus-activated cells.

We also performed Tax protein quantification, which is a marker of proviral expression, to estimate the frequency of HTLV-1-infected $CD8^+$ T cells capable of expressing Tax protein. We found that this frequency, despite being higher in HAM/TSP, was low in both groups, compared to the frequency of HTLV-1-infected $CD4^+$ T cells (data not shown). Since HTLV-1-infected $CD8^+$ T cells are not the main reservoir of HTLV-1 *in vivo*, we believe that the frequency of these cells did not undermine our results.

We found some discordant results among the different employed methodologies: SAGE, qRT-PCR, and flow cytometry. We understand that each methodology has its particularities and some inherent drawbacks, and we believe the main reason for our discordant findings is that we could not perform SAGE, qRT-PCR, and flow cytometry with the same number of samples.

Our findings showed that $CD8^+$ T cells of HAM/TSP patients have an inflammatory and active profile. PXN and ZAP70 overexpression in HTLV-1-infected patients was described for the first time here and reinforces this concept. However, although active and abundant $CD8^+$ T cells exist, they are not able to completely eliminate infected cells and prevent the development of HAM/TSP. Moreover, these active cells might contribute to the pathogenesis of the disease by migrating to the CNS, as we found deregulation of CCL5 expression in infected patients.

Our results provide a large-scale perspective of gene expression that should be further tested with biological functional assays to increase our understanding of the role these molecules play in the development of HTLV-1-related diseases.

Sequence Data

SAGE data have been deposited in the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) (GEO ID: GSM641893 and GSM641894).

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Author Disclosure Statement

No competing financial interests exist.

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Potential Contribution of a Novel Tax Epitope–Specific CD4$^+$ T Cells to Graft-versus-Tax Effect in Adult T Cell Leukemia Patients after Allogeneic Hematopoietic Stem Cell Transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for adult T cell leukemia/lymphoma (ATL) caused by human T cell leukemia virus type 1 (HTLV-1). We previously reported that Tax-specific CD8$^+$ cytotoxic T lymphocyte (CTL) contributed to graft-versus-ATL effects in ATL patients after allo-HSCT. However, the role of HTLV-1–specific CD4$^+$ T cells in the effects remains unclear. In this study, we showed that Tax-specific CD4$^+$ as well as CD8$^+$ T cell responses were induced in some ATL patients following allo-HSCT. To further analyze HTLV-1–specific CD4$^+$ T cell responses, we identified a novel HLA-DRB1*0101–restricted epitope, Tax155–167, recognized by HTLV-1–specific CD4$^+$ Th1-like cells, a major population of HTLV-1–specific CD4$^+$ T cell line, which was established from an ATL patient at 180 d after allo-HSCT from an unrelated seronegative donor by in vitro stimulation with HTLV-1–infected cells from the same patient. Costimulation of PBMCs with both the identified epitope (Tax155–167) and known CTL epitope peptides markedly enhanced the expansion of Tax-specific CD8$^+$ T cells in PBMCs compared with stimulation with CTL epitope peptide alone in all three HLA-DRB1*0101$^+$ patients post–allo-HSCT tested. In addition, direct detection using newly generated HLA-DRB1*0101/Tax155–167 tetramers revealed that Tax155–167-specific CD4$^+$ T cells were present in all HTLV-1–infected individuals tested, regardless of HSCT. These results suggest that Tax155–167 may be the dominant epitope recognized by HTLV-1–specific CD4$^+$ T cells in HLA-DRB1*0101$^+$–infected individuals and that Tax-specific CD4$^+$ T cells may augment the graft-versus-Tax effects via efficient induction of Tax-specific CD8$^+$ T cell responses. *The Journal of Immunology*, **2013, 190: 4382–4392.**

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of a highly aggressive CD4$^+$ T cell malignancy, adult T cell leukemia/lymphoma (ATL) (1, 2). This virus has infected 10–20 million people worldwide, especially in southern Japan, the Caribbean basin, South America, Melanesia, and equatorial Africa (3). Approximately 5% of HTLV-1–seropositive individuals develop ATL, and another 2–3% develop a slow progressive neurologic disorder known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) or various chronic inflammatory diseases (4). The majority of HTLV-1–infected individuals remain asymptomatic throughout their lives.

ATL is characterized by extremely poor prognosis, mainly because of intrinsic drug resistance to cytotoxic agents. It has been reported that allogeneic hematopoietic stem cell transplantation (allo-HSCT), but not autologous HSCT, improved the outcome of ATL (5, 6). In previous clinical studies carried out by the ATL allo-HSCT Study Group, the overall survival rate within 3 y after allo-HSCT with reduced intensity conditioning (RIC) was 36% (7). HTLV-1 proviral load became and remained undetectable in some ATL patients with complete remission after allo-HSCT, suggesting that it is an effective treatment for ATL (7–9). In these studies, we reported that donor-derived HTLV-1 Tax-specific CD8$^+$ CTLs were induced in some ATL patients who achieved complete remission after allo-HSCT (10). These CTLs were able to lyse recipient–derived HTLV-1–infected T cells in vitro, suggesting potential contributions to graft-versus-leukemia effects. CD8$^+$ T cells, especially CTLs, generally play an important role in controlling viral replication in various infections, such as those

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Abbreviations used in this article: AC, asymptomatic carrier; allo-HSCT, allogeneic stem cell transplantation; ATL, adult T cell leukemia/lymphoma; HAM/TSP, HTLV-1–associated myelopathy/tropical spastic paraparesis; HTLV-1, human T cell leukemia virus type 1; ILT, IL-2–dependent T cell line; LCL, lymphoblastoid B cell line; rhIL-2, recombinant human IL-2; RIC, reduced intensity conditioning; Treg, regulatory T.

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involving HIV, hepatitis B virus, and hepatitis C virus. In HTLV-1 infection, HTLV-1–specific $CD8^+$ T cells predominantly recognize the Tax Ag and are believed to contribute to controlling infected cells (11, 12). A high frequency of functional Tax-specific $CD8^+$ T cells can be detected in HAM/TSP patients and some asymptomatic carriers (ACs), whereas most ATL patients and a small population of ACs show severely reduced Tax-specific $CD8^+$ T cell responses (13, 14). The mechanism underlying the suppression of HTLV-1–specific $CD8^+$ T cell responses in these patients has not yet been fully elucidated.

For induction and maintenance of virus-specific CTLs, virus-specific $CD4^+$ Th cell responses are required in many virus infections (15–19). However, there are only a few reports of HTLV-1–specific Th cell responses (20–23), presumably because of their susceptibility to HTLV-1 infection in vivo and in vitro (24). Preferential HTLV-1 infection in HTLV-1–specific $CD4^+$ T cells could be one of the reasons for immune suppression in ATL patients. In addition, it has been reported that a higher frequency of $CD4^+FOXP3^+$ regulatory T (Treg) cells is observed in infected individuals compared with uninfected healthy donors. The frequency of Tax^- Treg cells, which are a major population of Treg cells in infected individuals, is negatively correlated with HTLV-1–specific CTL responses (25). HTLV-1 basic leucine zipper factor might also be involved in immune suppression, because HTLV-1 basic leucine zipper was constitutively expressed in infected cells (26) and inhibited the activity of IFN-γ promoters by suppressing NFAT and AP-1 signaling pathways, resulting in the impaired secretion of Th1 cytokines from $CD4^+$ Th cells in a transgenic mouse model (27) These reports suggest that both the dysfunction of HTLV-1–specific $CD4^+$ Th cells and the increased number of uninfected Treg cells might be implicated in the immunosuppression observed in ATL patients. Conversely, in HAM/TSP patients, $CD4^+$ T cells are predominantly found in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory, neurotoxic cytokines, such as IFN-γ and TNF-α (30), suggesting their contributions to the pathogenesis of HAM/TSP. However, the precise roles of HTLV-1–specific $CD4^+$ T cells in HTLV-1 infection remain unclear.

In some ATL patients who achieved complete remission after allo-HSCT, it has been suggested that donor-derived HTLV-1 Tax-specific CTLs may contribute to elimination of ATL cells (graft-versus-Tax effects) (10). We believe that $CD4^+$ T cells also play a critical role in the graft-versus-ATL effects because $CD4^+$ T cells are required for induction and maintenance of optimal CTL responses (15–19). It therefore is important to clarify the role of HTLV-1–specific $CD4^+$ T cells in the effects for understanding HTLV-1–specific T cell immunity in ATL patients after allo-HSCT and for developing new vaccine strategies to prevent recurrence of ATL.

Several studies have reported some HTLV-1–specific $CD4^+$ T cell epitopes restricted by different HLA haplotypes (20–23). The helper functions of these epitopes in HTLV-1–specific CTL responses in HTLV-1–infected individuals have not been well understood. However, Jacobson et al. (20) showed that $CD4^+$ T cells specific for Env gp46 196–209, an epitope restricted by HLA-DQ5 or -DRw16, exhibited a cytotoxic function by directly recognizing HTLV-1–infected cells. This observation raises the possibility that some HTLV-1–specific $CD4^+$ T cells may contribute to the graft-versus-ATL effects through their cytotoxic function in ATL patients after allo-HSCT.

In the current study, we demonstrated that both $CD4^+$ and $CD8^+$ Tax-specific T cell responses were induced in patients after allo-HSCT with RIC for ATL. To further analyze HTLV-1–specific $CD4^+$ T cell responses in ATL patients after allo-HSCT, we determined a novel HLA-DRB1*0101–resricted epitope, Tax155–167, recognized by HTLV-1–specific $CD4^+$ Th1-like cells, a major population of HTLV-1–specific $CD4^+$ T (T4) cell line, which was established from a patient in complete remission following allo-HSCT with RIC. Costimulation with oligopeptides corresponding to the Th1 epitope, Tax155–167, together with a known CTL epitope led to robust expansion of Tax-specific $CD8^+$ T cells in PBMCs from three HLA-DRB1*0101^+ patients after allo-HSCT tested. Furthermore, Tax155–167-specific $CD4^+$ T cells were found to be maintained in all HTLV-1–infected HLA-DRB1*0101^+ individuals tested, regardless of HSCT, by direct detection with newly generated HLA-DRB1*0101/Tax155–167 tetramers. Our results suggest that Tax155–167 may be a dominant epitope recognized by HTLV-1–specific $CD4^+$ T cells in HTLV-1–infected individuals carrying HLA-DRB1*0101 and that Tax-specific $CD4^+$ T cells may strengthen the graft-versus-ATL effects through efficient induction of Tax-specific CTL responses.

Materials and Methods

Subjects

A total of 18 ATL patients who underwent allo-HSCT with RIC regimen, and one HTLV-1–seronegative (#365) and two seropositive donors (one AC #310 and one HAM/TSP patient #294) carrying HLA-DRB1*0101 donated peripheral blood samples after providing written informed consent. Approximately one-half of these patients received allogeneic peripheral blood stem cell transplantation from HLA-A-, B-, and -DR–identical sibling donors. The other half received allogeneic bone marrow cells from HLA-A–, B–, and DR–identical seronegative unrelated donors (Table I). These patients were the participants of clinical studies organized by the ATL allo-HSCT Study Group, supported by the Ministry of Health, Welfare, and Labor of Japan. This study was also reviewed and approved by the Institutional Ethical Committee Review Board of the Tokyo Medical and Dental University.

Generation of cell lines derived from patients and donors

PBMCs were isolated using Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, U.K.) density gradient centrifugation and stored in liquid nitrogen in Bambanker stock solution (NIPPON Genetics, Tokyo, Japan) until required. These were used in part to obtain HTLV-1–infected IL-2–dependent T cell lines (ILT) and EBV-transformed lymphoblastoid B cell lines (LCL). ILT-#350 was spontaneously immortalized during long-term culture of PBMCs from patient #350 before allo-HSCT and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 20% FCS (Sigma Aldrich, St. Louis, MO) and 30 U/ml recombinant human IL-2 (rhIL-2; Shionogi, Osaka, Japan). LCL-#307, -#341, and -#350 were established by maintaining PBMCs from ATL patients #307, #341, and #350, respectively, after allo-HSCT. These PBMCs were maintained in RPMI 1640 medium containing 20% FCS, following infection with the EBV-containing culture supernatant of the B95-8 cell line, LCL-Kan, derived from a healthy individual was also used.

Synthetic peptides

A total of 18 overlapping peptides, 12- to 25-mer in length, spanning the central region of Tax (residues 103–246) were purchased and used for epitope mapping (Scrum Tokyo, Japan) (Table II). HLA-A*2402–restricted CTL epitopes (Tax301–309, SFHSLHLLF) (10) were used for in vitro stimulation of Tax-specific CTLs (Hokudo, Sapporo, Japan).

GST–Tax fusion protein–based immunoassay

HTLV-1 Tax-specific T cell responses were evaluated using GST–fusion proteins of the N-terminal (residues 1–127), central (residues 113–237), and C-terminal (residues 224–353) regions of HTLV-1 Tax (GST–Tax-A, -B, and -C, respectively) as described previously (13, 31). PBMCs (1×10^6 cells/ml) were incubated with or without a mixture of GST–Tax-A, -B, and -C proteins (GST–TaxABC) in 200 μl RPMI 1640 medium supplemented with 10% FCS. After 4 d, the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined using an OptiEIA Human IFN-γ ELISA Kit (BD Biosciences, San Jose, CA). The minimum detectable dose for this assay was determined to be 23.5 pg/ml IFN-γ. $CD8^+$ cells were depleted from PBMCs by negative selection using Dynabeads M-450 CD8 (Invitrogen, Carlsbad, CA), according to the

manufacturer's instructions. For cytokine profiling of a HTLV-1–specific $CD4^+$ T cell line, cells were stimulated with formaldehyde-fixed ILT-#350 for 48 h. Culture supernatant was collected, and various cytokines were measured using a Human Th1/Th2/Th17 Cytokine Kit for a Cytokine Beads Array (BD Biosciences).

Induction of HTLV-1–specific $CD4^+$ T cell line (T4 cells)

PBMCs (1×10^6 cells/ml) from patient #350, in complete remission at 180 d after allo-HSCT, were cultured for 2 wk with 100 nM Tax301–309 peptide in 96-well round-bottom tissue culture plate (BD Biosciences) in a final volume of 200 μl RPMI 1640 medium with 20% FCS and 10 U/ml rhIL-2. $CD4^+$ cells were then isolated by negative selection using a Human CD4 T lymphocyte Enrichment Set-DM (BD Biosciences) and maintained in RPMI 1640 medium with 20% FCS and 100 U/ml rhIL-2. Cells (1×10^6 cells/ml) were stimulated with formaldehyde-fixed ILT-#350 (2.5×10^5 cells/ml) every 2–3 wk. After multiple rounds of stimulation, the resulting $CD4^+$ T cell line was assessed for HTLV-1 specificity by comparing IFN-γ production against ILT-#350 to that against an HTLV-1–negative cell line, LCL-#350.

RT-PCR

Total RNA from cells was isolated using Isogen (Nippon Gene, Tokyo, Japan) and Turbo DNA-free (Life Technologies). First-strand cDNA was prepared from 0.5 μg RNA using ReverTra Ace and $Oligo(dT)_{20}$ primers provided in a ReverTra Ace-α-kit (Toyobo, Osaka, Japan). PCRs were performed in 50 μl reaction mixture containing ReverTra Dash (Toyobo), 0.5 μM of each HTLV-1 pX-specific primer (pX1, 5′-CCA CTT CCC AGG GTT TAG ACA GAT CTT C-3′ and pX4, 5′-TTC CTT ATC CCT CGA CTC CCC TCC TTC CCC-3′), and 2 μl cDNA. GAPDH-specific primers (GAPDH5′, 5′-ACC ACA GTC CAT GCC ATC AC-3′; GAPDH3′, 5′-TCC ACC ACC CTG TTG CTG TA-3′) were used as an internal control. The thermal cycling conditions comprised an initial activation step at 94°C for 1 min, followed by 30 cycles of denaturation (98°C, 10 s), annealing (60°C, 2 s), and extension (74°C, 30 s). The PCR amplicons were visualized by ethidium bromide staining following 2% (w/v) agarose gel electrophoresis.

Flow cytometry

For cell surface staining, the following fluorochrome-conjugated mouse anti-human mAbs were used: CD3-FITC (UCHT1; BioLegend, San Diego, CA), CD4-FITC (RPA-T4; BioLegend), CD8-FITC (RPA-T8; BioLegend), and CD8-PE-Cy5 (HIT8a; BD Biosciences, San Jose, CA). For tetramer staining, PE-conjugated HLA-A*0201/Tax11–19, HLA-A*1101/Tax88–96, HLA-A*1101/Tax272–280, and HLA-A*2402/Tax301–309 tetramers were purchased from Medical & Biological Laboratories (Nagoya, Japan). PE-conjugated HLA-DRB1*0101/Tax155–167 tetramer were newly generated through the custom service of Medical & Biological Laboratories. Whole-blood or cultured cells were stained with PE-conjugated Tax/HLA tetramer in conjunction with CD3-FITC and CD8-PE-Cy5 or CD4-PE-Cy5. For whole-blood samples, RBCs were lysed and fixed in BD FACS lysing solution (BD Biosciences) before washing. Samples were analyzed on a FACSCalibur (BD Biosciences), and data analyses were performed using FlowJo software (Tree Star, Ashland, OR).

Epitope mapping

T4 cells (3×10^5 cells/ml) were stimulated with LCL-#350, pulsed with various concentrations of synthetic peptides for 1 h at 37°C, at a responder/stimulator (R/S) ratio of 3. The culture supernatant was collected at 6 h poststimulation, and peptide-specific IFN-γ production from T4 cells was determined by ELISA.

HLA class II restriction assay

T4 cells (5×10^5 cells/ml) were cocultured for 6 h with ILT-#350 (1×10^5 cells/ml) in the presence or absence of anti-human HLA-DR (10 μg/ml; L243; BioLegend), anti-human HLA-DQ (10 μg/ml; SPVL3; Beckman Coulter, Fullerton, CA), or anti–HLA-ABC (10 μg/ml; W6/32; BioLegend). The IFN-γ in the supernatant was measured by ELISA.

To identify a HLA class II molecule responsible for Ag presentation to T4 cells, Tax155–167 peptide-specific IFN-γ responses were evaluated using various HLA-typed LCLs (LCL-#350, LCL-#341, LCL-#307, and LCL-Kan). These LCLs (1×10^5 cells/ml) were pulsed with 100 ng/ml Tax155–167 peptide for 1 h, fixed with 2% formaldehyde, and then cultured with T4 cells (3×10^5 cells/ml) for 6 h. The culture supernatant was collected, and IFN-γ in the supernatant was measured by ELISA.

Tetramer-based proliferation assay

PBMCs (1.0×10^6 cells/ml) were cultured for 13 or 14 d with or without 100 nM antigenic peptides in the presence of 10 U/ml rhIL-2. Cells were stained with HLA/Tax tetramer-PE, CD3-FITC, and CD8-PE-Cy5 or CD4-PE-Cy5 and then analyzed by flow cytometry.

Statistic analysis

Statistical significance was evaluated with the unpaired t test using Graphpad Prism 5 (Graphpad Software, La Jolla, CA). In all cases, two-tailed p values <0.05 were considered significant.

Results

Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC

We previously reported that Tax-specific $CD8^+$ T cells were induced in some ATL patients after allo-HSCT with RIC from HLA-identical sibling donors (10). In this study, we examined the Tax-specific T cell response in a larger number of ATL patients who received allo-HSCT with RIC. Table I provides a summary of the

Table I. Clinical information and summary for Tax-specific $CD8^+$ T cells in 18 ATL patients at 180 d post–allo-HSCT with RIC

ID (Age, Sex)	ATL Subtype	Type of Donor	Donor-HLA	Donor HTLV-1 Sero Status	Chimerism (%)[a]	Tetramer (%)[b]	Proviral Load[c]
239 (55, M)	Lymphoma	r-PB	A 26/33, DR 4/13	(−)	<5	NT	0.1
241 (61, F)	Acute	r-PB	A 2/26, DR 10/18	(−)	<5	0.00	0.1
247 (52, F)	Lymphoma	r-PB	A 24/−, DR 9/15	(−)	<5	0.07	0.1
270 (57, M)	Lymphoma	r-PB	A 24/33, DR 13/15	(−)	<5	0.00	0.0
300 (53, F)	Lymphoma	r-PB	A 24/26, DR 4/15	(+)	<5	1.34	4.8
301 (57, F)	Acute	ur-BM	A 24/33, DR 13/15	(−)	<5	0.72	0.0
307 (68, F)	Acute	r-PB	A 2/11, DR 14/15	(+)	<5	0.10	5.4
317 (60, M)	Acute	ur-BM	A 2/24, DR 14/15	(−)	<5	0.92	0.0
328 (62, M)	Acute	ur-BM	A 11/24, DR 8/9	(−)	<5	0.75	NT
340 (50, M)	Acute	r-PB	A 2/24, DR 4/8	(−)	<5	1.40	0.7
341 (61, F)	Acute	ur-BM	A 24/33, DR 1/15	(−)	<5	0.45	0.1
344 (58, M)	Lymphoma	ur-BM	A 2/24, DR 4/−	(−)	<5	0.44	0.0
349 (53, M)	Acute	r-PB	A 24/−, DR 8/15	(+)	<5	0.00	0.0
350 (60, F)	Acute	ur-BM	A 24/26, DR 1/14	(−)	<5	0.59	0.6
351 (57, F)	Acute	ur-BM	A 24/26, DR 9/12	(−)	<5	0.45	0.0
358 (63, F)	Lymphoma	r-PB	A 2/11, DR 4/14	(−)	<5	0.42	0.0
352 (61, M)	Acute	ur-BM	A 11/26, DR 8/15	(−)	<5	0.14	0.0
364 (52, M)	Acute	r-PB	A 24/26, DR 1/−	(−)	<5	0.11	0.0

[a]Indicates percentage of recipient-derived T cell chimerism.
[b]Indicates percentage of tetramer$^+$ cells among $CD8^+$ T cells in PBMCs.
[c]Indicates copy number per 1000 PBMCs.
F, Female; M, male; NT, not tested; r-PB, related donor-derived peripheral blood stem cell; ur-BM, unrelated donor-derived bone marrow cell.

results of Tax-specific $CD8^+$ T cell detection by flow cytometry, using the Tax/HLA tetramers, in the peripheral blood of 18 ATL patients at 180 d after allo-HSCT, together with clinical information. During this period, all patients achieved a complete chimera state consisting of >95% of donor-derived hematopoietic cells. By using four available tetramers (HLA-A*0201/Tax11–19, HLA-A*2402/Tax301–309, HLA-A*1101/Tax88–96, and HLA-A*1101/Tax272–280), Tax-specific $CD8^+$ T cells were found in 14 patients. Because the donors were uninfected individuals in the majority of cases (Table I), induction of the Tax-specific donor-derived $CD8^+$ T cells in recipients indicated the presence of newly occurring immune responses against HTLV-1 in the recipients. This evidence strengthens our previous observation (10, 32).

We also used a GST–Tax fusion protein-based assay to evaluate Tax-specific T cell responses. The tetramer-based assay was limited to four kinds of epitopes and restricted by three HLA alleles but did not detect T cells directed to other epitopes or HLAs. The GST–Tax fusion protein-based assay can detect both $CD4^+$ and $CD8^+$ T cell responses, irrespective of HLA types. However, this sensitivity is not as good as single-cell analysis by flow cytometry (31). As shown in Fig. 1A, there was a wide variation in the IFN-γ responses to the Tax protein in the PBMCs among the 16 patients tested. In five patients (#247, #270, #328, #340, and #349), IFN-γ production of PBMCs against GST–TaxABC proteins was very low or not specific for the Tax protein. PBMCs from the other 11 patients (#239, #241, #301, #317, #341, #344, #350, #351, #352, #358, and #364) produced higher amounts of IFN-γ in response to GST–TaxABC proteins compared with GST. However, the levels of IFN-γ production varied among the patients.

We also evaluated the extent to which Tax-specific $CD4^+$ T cells were responsible for IFN-γ in the GST–Tax-based immunoassay system. We used PBMCs from patients #350 and #341, who showed high Tax-specific T cell responses. $CD8^+$ cell–depleted PBMCs from patient #350 and #341 showed a reduced but still significant level of Tax-specific IFN-γ–producing response compared with whole PBMCs (Fig. 1B). These results indicate that not only $CD8^+$ but also $CD4^+$ T cells against Tax are present in the peripheral blood from patient #350 and #341 after allo-HSCT with RIC.

Induction of an HTLV-1–specific $CD4^+$ T cell line from patient #350

We next attempted to induce HTLV-1–specific $CD4^+$ T cells from the PBMCs of patient #350 at 180 d after allo-HSCT, using an HTLV-1–infected T cell line (ILT-#350) as APCs. Freshly isolated PBMCs were stimulated for 2 wk with Tax301–309, a dominant CTL epitope presented by HLA-A*2402, to eliminate HTLV-1–infected cells, which potentially existed in PBMCs. The $CD4^+$ cells were then isolated from the cultured cells and stimulated with formaldehyde-fixed ILT-#350 every 2–3 wk. The established cell line was found to be a $CD4^+$ T cell line (designated as T4 cells thereafter) because cells expressed CD3 and CD4 but not CD8

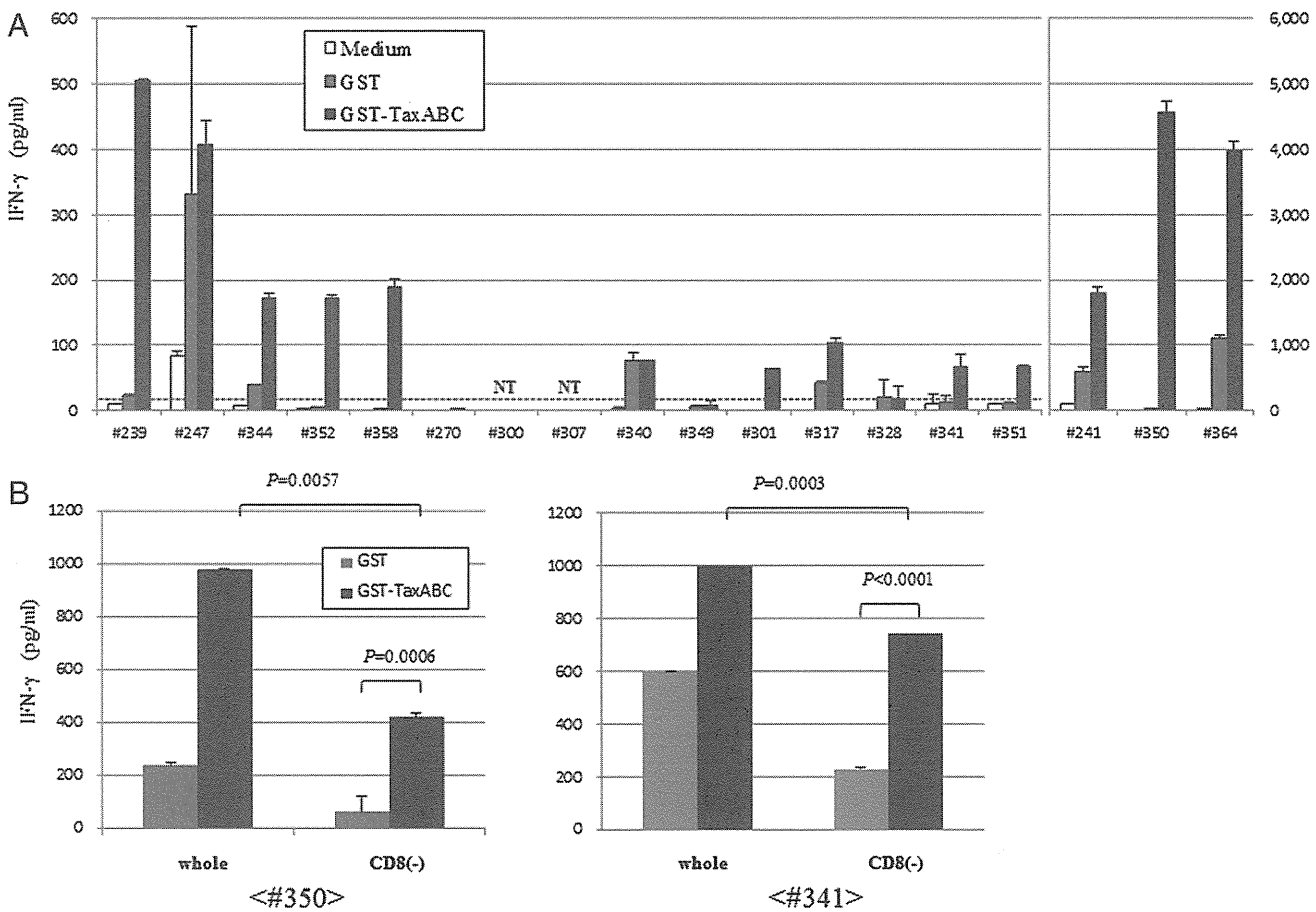


FIGURE 1. Diversity of Tax-specific T cell responses in ATL patients who received allo-HSCT with RIC. (**A** and **B**) PBMCs from 18 ATL patients at 180 d after allo-HSCT (A) or whole and $CD8^+$ cell–depleted PBMCs from two patients at 540 d after allo-HSCT (#350 and #341) (B) were cultured for 4 d in the absence (open square) or presence of GST (gray square), or GST–Tax (black square) proteins. The concentration of IFN-γ in the supernatant was determined by ELISA. The *y*-axis on the right side indicates the results from three patients (#241, #350, and #364). The dotted horizontal line indicates the detection limit (23.5 pg/ml). The error bars represent SD of duplicated wells. The representative result of two independent experiments is shown in (B).

(Fig. 2A). Because HTLV-1 has been shown to preferentially infect $CD4^+$ T cells in vivo and in vitro (24), we examined HTLV-1 expression in T4 cells by RT-PCR (Fig. 2B). As expected, the T4 cells did not express HTLV-1 Tax, indicating that the cells were not infected with HTLV-1. We assessed expression of various cytokines in T4 cells (Fig. 2C). The T4 cells were stimulated with formaldehyde-fixed ILT-#350 or LCL-#350. The cells produced large amounts of IFN-γ and TNF-α and small amounts of IL-2, IL-4, and IL-10 in response to ILT-#350 but not against LCL-#350. IL-6 and IL-17A were not detected in the culture supernatant. These data indicate that T4 cells are mainly HTLV-1–specific $CD4^+$ Th1-like cells but contain minor populations to produce Th2 cytokines.

Determination of the minimum epitope recognized by T4 cells

Freshly isolated PBMCs in the patient #350 produced IFN-γ in response to GST–Tax (Fig. 1A). We expected that the epitope recognized by the T4 cells should be present in the Tax protein. We therefore examined whether the T4 line responded to Tax using LCL-#350 pulsed with GST–Tax proteins as APCs. As shown in Fig. 3A, the T4 cells produced significantly higher amounts of IFN-γ in response to GST–TaxABC and GST–Tax-B (residues 113–237) (31) but not GST–Tax-A (residues 1–127) (31) and -C (residues 224–353 (31), when compared with the GST control protein, indicating that the T4 cells recognized the central region (residues 113–237) of the Tax Ag. We next synthesized eight overlapping 25-mer peptides spanning the central region of Tax (residues 103–246) and analyzed their abilities to stimulate T4 cells (Table II). The cell line produced high amounts of IFN-γ only when stimulated with Tax154–178 (Fig. 3B). We then prepared four overlapping 15-mer peptides, covering residues 154–178 of Tax, to examine the IFN-γ responses of the T4 cells (Table II). Both Tax151–165 and Tax156–170-stimulated cells to induce IFN-γ responses but not at a comparable level to Tax154–178 (Fig. 3C). These results suggest that the epitope recognized by T4 cells might be present in the N-terminal half of Tax154–178. We therefore stimulated the cells with Tax154–168, Tax155–169, or Tax156–170. The cells showed higher IFN-γ responses against Tax154–168 and Tax155–169 than Tax156–170, indicating that the minimum epitope might be within residues 155–168 of Tax (Fig. 3D). To identify the minimum epitope recognized by T4 cells, we next synthesized three overlapping peptides of 12- to 14-mer lengths beginning at residue 155 of Tax (Table II). Tax155–167 induced IFN-γ responses in cells at a similar level to Tax155–169 and Tax155–168, although Tax155–166 did not (Fig. 3E). Moreover, IFN-γ production of cells in response to various concentrations of Tax155–167 was comparable to that against Tax155–169 and Tax155–168 (Fig. 3F). These data clearly show that the minimum epitope recognized by the T4 cells is Tax155–167.

*HLA-DRB1*0101 restriction of Tax-specific T4 cells*

To analyze HLA class II molecules involved in the presentation of the minimum epitope, T4 cells were stimulated with ILT-#350 in the presence or absence of anti–HLA-DR, -DQ, and anti-HLA class I blocking Abs. As shown in Fig. 4A, the addition of an anti–HLA-DR blocking Ab abrogated IFN-γ responses of the T4 cells against ILT#-350, indicating that the epitope was HLA-DR restricted.

We further investigated the HLA-DR alleles responsible for the presentation of the minimum epitope by using four HLA-typed LCLs displaying different HLA-DRs. As shown in Fig. 4B, the T4 cells responded by producing IFN-γ when Tax155–167 was presented by autologous LCL-#350 (DR1/14) and allogeneic LCL-#341 (DR1/15). These results clearly indicate that this epitope is presented by HLA-DRB1*0101 on APCs. We searched for a known HLA-DRB1*0101 motif in the identified epitope Tax155–167 and found that this epitope contained the HLA-DRB1*0101 motif (Fig. 4C) (33).

Enhancement of Tax-specific $CD8^+$ T cell expansion by Tax155–167-specific $CD4^+$ T cell help

As T4 cells were established from PBMCs of an HTLV-1–infected patient #350, it is suggested that Tax155–167-specific $CD4^+$ T cells may be maintained in the HLA-DRB1*0101^+ patient #350.

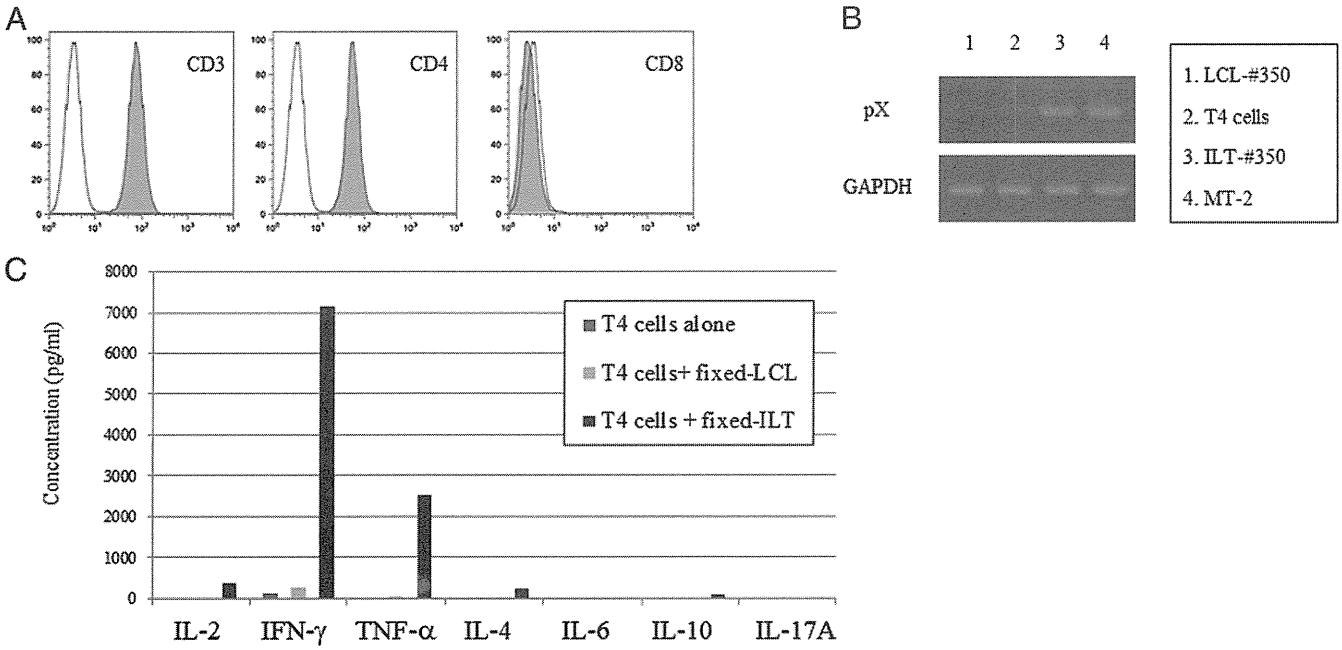


FIGURE 2. Phenotype and function of $CD4^+$ T cell line (T4) generated from patient #350. (**A**) Cell surface phenotype of T4 cells was analyzed by flow cytometry. (**B**) Total RNA was extracted from LCL-#350 (*lane 1*), T4 cells (*lane 2*), ILT-#350 (*lane 3*), and MT-2 (*lane 4*). Tax mRNA expression for each cell type was analyzed by RT-PCR. GAPDH was used as an internal control. (**C**) T4 cells were stimulated for 24 h with or without formaldehyde-fixed ILT-#350 or LCL-#350 cells. The concentration of indicated cytokines in the supernatants was measured using a cytometric bead array system.

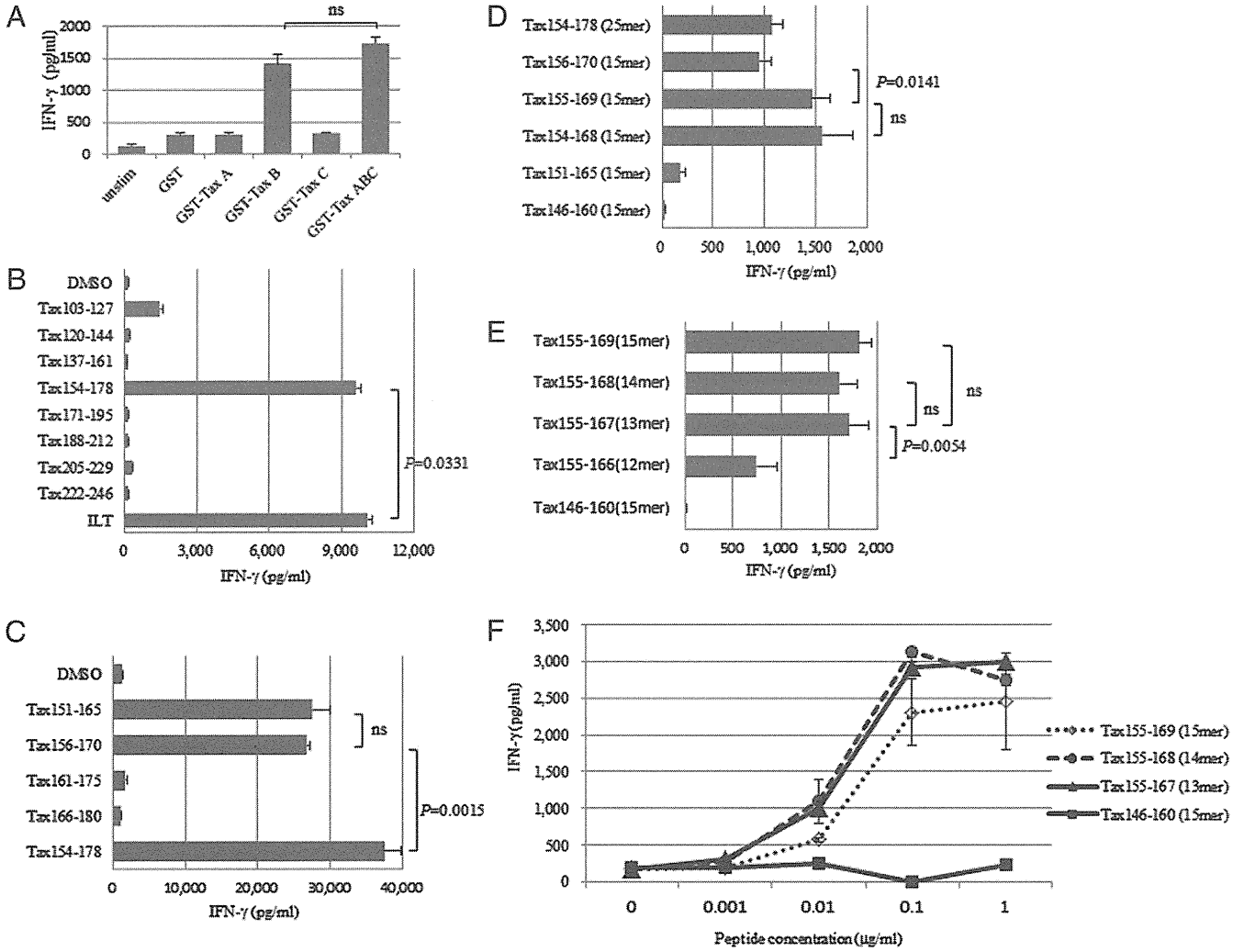


FIGURE 3. Identification of the dominant Tax-derived epitope recognized by established T4 cells. (**A**) Donor-derived LCL-#350 was pulsed with GST, GST–Tax-A, GST–Tax-B, GST–Tax-C, or a mixture of GST–Tax-A, -B, and -C (GST–TaxABC) for 24 h and then cocultured for 24 h with the T4 cells at a responder/stimulator (R/S) ratio of 3. IFN-γ production from T4 cells was analyzed by ELISA. (**B** and **C**) LCL-#350 was pulsed with the indicated overlapping 25-mer-long (B) or 15-mer-long (C) synthetic peptides (10 μg/ml) within the Tax-B region for 1 h. Formaldehyde-fixed ILT-#350 cells were cocultured with T4 cells for 6 h. IFN-γ in the supernatant was measured by ELISA. (**D** and **E**) IFN-γ responses of T4 cells were assessed using the indicated overlapping 12- to 25-mer-long synthetic peptides (100 ng/ml). (**F**) IFN-γ responses of T4 cells against indicated concentrations of 13- to 15-mer-long peptides were assessed as in (B) and (C). (A–F) Results are representative of two or three independent experiments. The error bars represent SD of triplicate wells. Statistical significance was analyzed by the unpaired *t* test.

We therefore evaluated the helper function of Tax155–167-specific $CD4^+$ T cells on the expansion of dominant Tax-specific CTLs in fresh PBMCs of the patient #350. Freshly isolated PBMCs from patient #350 (A24/26, DR1/14) at 540 d after allo-HSCT were stimulated for 13 d with the HLA-A24–restricted CTL epitope peptide (Tax301–309) in the presence or absence of the HLA-DRB1*0101–restricted $CD4^+$ Th epitope peptide (Tax155–167), and Tax-specific $CD8^+$ T cell expansion was evaluated using the HLA-A*2402/Tax301–309 tetramer. As shown in Fig. 5, Tax301–309-specific $CD8^+$ T cells proliferated to 9.26% of $CD8^+$ T cells when stimulated with Tax301–309 alone. Surprisingly, a highly elevated frequency (62.3%) of tetramer-binding $CD8^+$ T cells was detected by in vitro costimulation with Tax301–309 and Tax155–167, suggesting the presence of Tax155–167-specific $CD4^+$ Th cells in patient #350.

We examined whether Tax155–167-specific $CD4^+$ T cells existed and functioned as helper cells in the other two HTLV-1–infected HLA-DRB1*0101^+ patients after allo-HSCT (day 360 for patient #341 and day180 for #364). These patients had detectable levels of HLA-A*2402/Tax301–309 tetramer-binding $CD8^+$ T cells in the peripheral blood (Fig. 5). In patients #341 and #364, the tetramer-binding cells expanded to 7.7 and 0.849% of $CD8^+$ T cells at 13 d of culture when stimulated with the CTL epitope peptide, Tax301–309, alone. Costimulation of PBMCs with both peptides Tax155–167 and Tax301–309 led to a vigorous proliferation of tetramer-binding $CD8^+$ T cells (59.6% for patient #341 and 15.5% for patient #364) as observed in patient #350 (Fig. 5). These results indicate that Tax155–167-specific $CD4^+$ T cells may be present and contribute to enhancing $CD8^+$ T cell responses in HTLV-1–infected HLA-DRB1*0101^+ individuals after allo-HSCT.

*Tax155–167-specific $CD4^+$ T cells were maintained in HTLV-1–infected HLA-DRB1*0101^+ individuals*

We next generated the HLA-DRB1*0101/Tax155–167 tetramer to directly detect Tax155–167-specific $CD4^+$ T cells and examined the presence of Tax155–167-specific $CD4^+$ T cells in the PBMCs freshly isolated from two HLA-DRB1*0101^+ patients after allo-HSCT (day 180 for patient #350 and day 360 for patient #364).

Table II. Synthetic oligopeptides used in this study

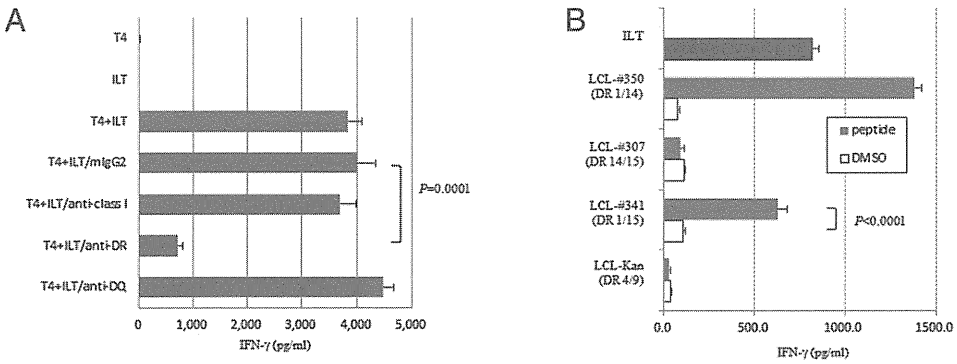
Peptide	Sequence
Tax103–127	P S F L Q A M R K Y S P F R N G Y M E P T L G H
Tax120–144	M E P T L G Q H L P T L S F P D P G L R P Q N L Y
Tax137–161	G L R P Q N L Y T L W G G S V V C M Y L Y Q L S P
Tax154–178	M Y L Y Q L S P P I T W P L L P H V I F C H P G Q
Tax171–195	V I F C H P G Q L G A F L T N V P Y K R I E E L L
Tax188–212	Y K R I E E L L Y K I S L T T G A L I I L P E D C
Tax205–229	L I I L P E D C L P T T L F Q P A R A P V T L T A
Tax222–246	R A P V T L T A W Q N G L L P F H S T L T T P G I
Tax146–160	L W G G S V V C M Y L Y Q L S
Tax151–165	V V C M Y L Y Q L S P P I T W
Tax154–168	M Y L Y Q L S P P I T W P L L
Tax155–169	Y L Y Q L S P P I T W P L L P
Tax156–170	L Y Q L S P P I T W P L L P H
Tax161–175	P P I T W P L L P H V I F C H
Tax166–180	P L L P H V I F C H P G Q L G
Tax155–168	Y L Y Q L S P P I T W P L L
Tax155–167	Y L Y Q L S P P I T W P L
Tax155–166	Y L Y Q L S P P I T W P

Tax155–167-specific CD4$^+$ T cells were detected ex vivo in the patient #350 (0.11%) and proliferated to 11.6% among CD4$^+$ T cells at 13 d poststimulation with Tax155–167 peptide. In the patient #364, tetramer-binding CD4$^+$ T cells were undetectable in fresh PBMCs but expanded to 0.37% by in vitro stimulation with Tax155–167 peptide (Fig. 6A). In an HLA-DRB1*0101$^+$–seronegative donor #365, Tax155–167-specific CD4$^+$ T cells were not found in fresh PBMCs and did not become detectable at 13 d after stimulation with Tax155–167 peptide (Fig. 6A). This result indicates that Tax155–167-specific CD4$^+$ T cells are maintained and possesses the abilities to proliferate in response to HTLV-1 Tax in these patients.

We further examined whether Tax155–167-specific CD4$^+$ T cells existed in two HTLV-1–infected individuals carrying HLA-DRB1*0101, an AC #310 and a HAM/TSP patient #294, and detected 0.18 and 0.31% of tetramer-binding cells in peripheral CD4$^+$ T cells, respectively (Fig. 6B). These results suggest that Tax155–167-specific CD4$^+$ T cells are maintained in HTLV-1–infected individuals expressing an HLA-DRB1*0101 allele, regardless of HSCT.

Discussion

In this study, we demonstrated Tax-specific CD4$^+$ T cell responses in some ATL patients post–allo-HSCT and identified a novel HLA-DRB1*0101–restricted CD4 T cell epitope, Tax155–167, which was recognized by HTLV-1–specific CD4$^+$ T cells and consequently led to robust Tax-specific CD8$^+$ T cell expansion. We also found that Tax155–167-specific CD4$^+$ T cells existed in all HTLV-1–infected HLA-DRB1*0101$^+$ individuals tested, regardless of HSCT, by newly generated HLA-DRB1*0101/Tax155–167 tetramers. These results suggest that Tax155–167 might be a dominant epitope recognized by HTLV-1–specific CD4$^+$ T cells



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	Relative position												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Tax155-167	Y	L	Y	Q	L	S	P	P	I	T	W	P	L
Anchor residues	Y,V, L,F, I,A M,W	*	*	L,A, I,V, M,N, Q	*	A,G, S,T, P	*	*	L,A, I,V, N,F, Y				

FIGURE 4. HLA-DRB1*0101 restriction of Tax155–167 recognition by established T4 cells. (**A**) T4 cells were cocultured for 6 h with ILT-#350 in the presence or absence of the following blocking Abs (10 μg/ml): anti-human HLA-DR; anti-human HLA-DQ; anti–HLA-class I; or isotype control. IFN-γ production from T4 cells was measured by ELISA. (**B**) The T4 cells were cocultured for 6 h with autologous (#350) or allogeneic (#307, #341, and Kan) LCLs pulsed with (closed bar) or without (open bar) Tax155–167 for 1 h or with recipient-derived ILT-#350. The HLA-DR alleles of each LCL line are indicated in parentheses. IFN-γ production of T4 cells was assessed by ELISA. (A and B) Representative data of three independent experiments are shown. The error bars represent SD of triplicate wells. Statistical significance was analyzed by the unpaired *t* test. (**C**) The amino acid sequence between residues 155 and 167 of Tax contained a putative HLA-DRB1*0101 anchor motif (33).

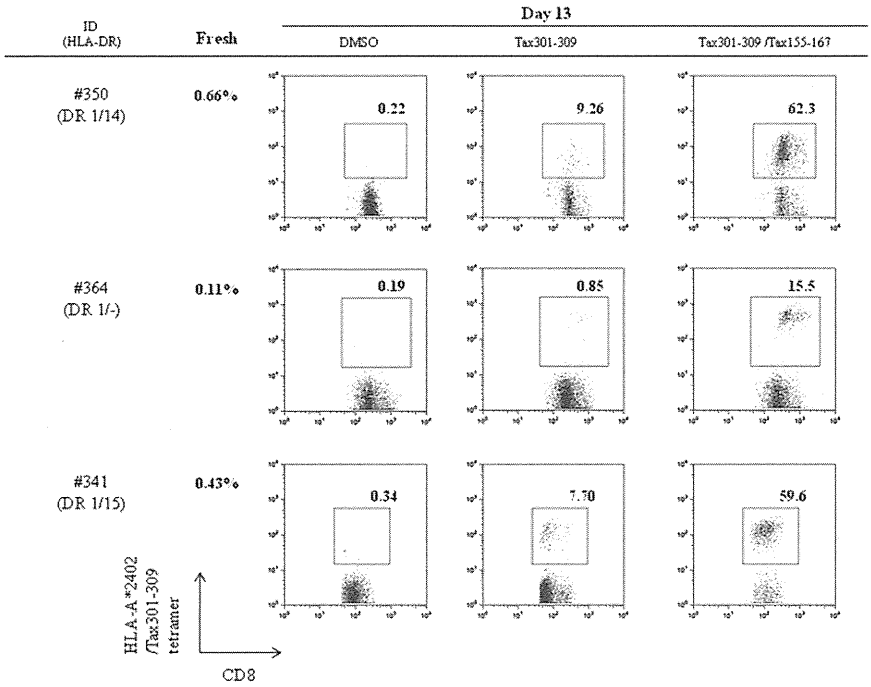

FIGURE 5. Augmentation of Tax-specific $CD8^+$ T cell expansion by costimulation with CTL epitope and Tax155–167 peptides. PBMCs from HLA-DRB1*0101– and HLA-A24–expressing ATL patients (#350, #364, and #341) who underwent allo-HSCT with RIC were cultured for 13 d in the presence of DMSO, 100 nM CTL epitope (Tax301–309), or a mixture of Tax301–309 (100 nM) and Tax155–167 (100 nM) peptides. Data indicate percentages of HLA-A*2402/Tax301–309 $tetramer^+$ cells among $CD3^+CD8^+$ T cells. Fresh indicates frequency of HLA-A*2402/Tax301–309 $tetramer^+CD8^+$ T cells detected in fresh peripheral blood.

in HTLV-1–infected individuals expressing HLA-DRB1*0101 and that Tax-specific $CD4^+$ T cells might efficiently induce HTLV-1–specific CTL expansion to strengthen the graft-versus-ATL effects in ATL patients after allo-HSCT.

In HTLV-1 infection, analysis of virus-specific $CD4^+$ T cell responses appears to be limited because $CD4^+$ T cells are preferentially infected with HTLV-1 (24, 34, 35), and HTLV-1 Ags are produced from infected cells at a few hours postculture (34, 36). In this study, we used blood samples from 18 ATL patients after allo-HSCT with RIC and from HLA identical–related or unrelated donors and found that these recipients had undetectable or very low proviral loads (Table I), as previously shown (7–9). We previously reported that Tax-specific CTLs were induced in some patients with complete remission after allo-HSCT for ATL and might contribute to the graft-versus-leukemia effect (10). In the current study, Tax-specific T cell responses or tetramer-binding $CD8^+$ T cells were detected in 68.8% (11 of 16) or 82.4% (14 of 17) of patients tested, respectively (Fig. 1A, Table I). In addition, helper function of Tax-specific $CD4^+$ T cells to enhance Tax-specific $CD8^+$ T cell expansion was observed in PBMCs from all three HLA-DRB1*0101^+ patients tested (Fig. 5). These data suggest that both $CD8^+$ and $CD4^+$ Tax-specific T cell responses might contribute to elimination of remaining leukemic and/or infected cells in some patients having T cell responses against Tax. However, given the fact that not all ATL patients who achieved complete remission after allo-HSCT had Tax-specific $CD8^+$ T cells, graft-versus-host reaction may mainly contribute to achieve complete remission after allo-HSCT. It is of note that Tax-specific

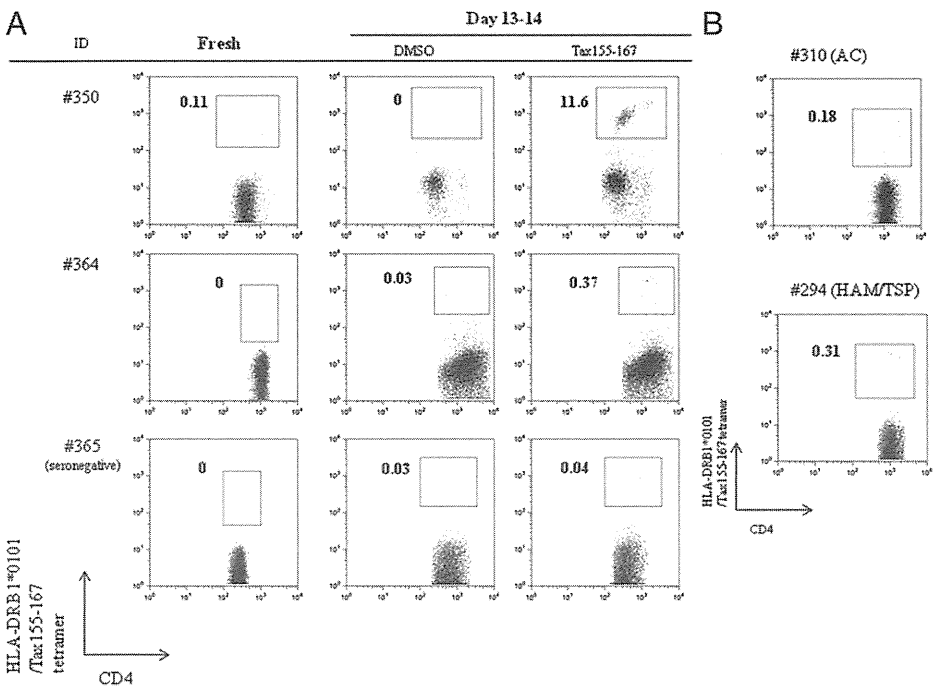

FIGURE 6. Detection of Tax155–167-specific $CD4^+$ T cells in HTLV-1–infected HLA-DRB1*0101^+ individuals. (**A**) In two ATL patients after allo-HSCT (#350 and #364) and an HLA-DRB1*0101^+–seronegative donor (#365), frequency of HLA-DRB1*0101/Tax155–167 tetramer-binding $CD4^+$ T cells was analyzed in fresh PBMCs and PBMCs cultured for 13–14 d in the presence of Tax155–167 (100 nM) peptide. Data indicate percentages of $tetramer^+$ cells in $CD3^+CD4^+$ T cells. (**B**) Frequency of HLA-DRB1*0101/Tax155–167 tetramer-binding $CD4^+$ T cells in fresh PBMCs from an AC #310 and an HAM/TSP patient #294 was analyzed. Data indicate percentages of $tetramer^+$ cells in $CD3^+CD4^+$ T cells.

T cell responses were detected in 57.1% (four of seven) or 87.5% (seven of eight) of the patients after allo-HSCT with RIC from HTLV-1–seronegative sibling or unrelated donors, respectively. A Tax-specific T cell response was not detected in three patients who underwent allo-HSCT from seropositive donors (Fig. 1, Table I).

It has been proposed that CTLs are the main effector cells against many pathogenic viruses, including HTLV-1. To date, many CTL epitopes recognized by HTLV-1–specific CTLs have been identified, some of which are thought to be the candidates of peptide-based T cell immunotherapy (10, 20, 32, 37–40). $CD4^+$ T cells have also been known to be critical for induction and maintenance of Ag-specific $CD8^+$ T cells (15–19). With respect to HTLV-1 infection, there are several reports identifying HLA-DRB1*0101–restricted epitopes recognized by $CD4^+$ T cells against Env or Tax (Env380–394 (21), Env436–450, Env451–465, Env456–470 (23), and Tax191–205 (22)), which were established by stimulating PBMCs from uninfected or infected individuals with synthetic peptides. In this study, for determination of an epitope recognized by HTLV-1–specific $CD4^+$ T cells, we established an HTLV-1–specific $CD4^+$ T cell line from the patient #350 at 180 d after allo-HSCT by several stimulations with an HTLV-1 Ags-expressing T cell line (ILT-#350) from the same patient. In addition, we found that Tax155–167-specific $CD4^+$ T cells were present in peripheral blood from patient #350 at 180 and 540 d after all-HSCT, indicating that the epitope, Tax155–167, identified in this study is naturally presented on HTLV-1–infected cells and predominantly recognized by HTLV-1–specific $CD4^+$ Th cells in the patient #350 at least within 540 d after allo-HSCT. Another HLA-DRB1*0101–restricted Tax epitope, Tax191–205, has been reported previously (22). In this study, the amino acid sequence within this region was revealed to be conserved in the infected T cell line, ILT-#350 established from the patient #350 (data not shown), indicating that Tax191–205 can be presented on APCs and Tax191–205-specific $CD4^+$ T cells may be induced in patient #350. However, Tax155–167-specific but not Tax191–205-specific $CD4^+$ T cells were revealed to predominantly appear in the HTLV-1–specific T4 cell line, established from PBMCs in the patient #350 at 180 d after allo-HSCT. This suggests that in the case of patient #350 at 180 d after allo-HSCT, Tax191–205-specific $CD4^+$ T cells may not be the most frequent population among HTLV-1–specific $CD4^+$ T cells.

It has been known that Ag-specific effector and memory $CD4^+$ T cells are typically present at much lower frequencies than their $CD8^+$ counterparts and that MHC class II tetramer might have a weak TCR–MHC affinity (41). Although this limited affinity of MHC class II tetramer might preclude detection of Ag-specific low-affinity $CD4^+$ T cells, the low-affinity $CD4^+$ T cells, below detection with MHC class II tetramers, were also proved to be critical effectors in Ag-specific responses (42). In the current study, MHC class II tetramer analysis revealed that Tax155–167-specific $CD4^+$ T cells were present in HLA-DRB1*0101$^+$ HTLV-1–infected individuals: two ATL patients after allo-HSCT (day 180 for #350 and day 360 for #364), an AC #310, and a HAM/TSP patient #294 (Fig. 6). Because of a shortage of blood sample from patient #341, we could not perform the direct detection for Tax155–167-specific $CD4^+$ T cells by the MHC class II tetramers. However, enhanced expansion of Tax301–309-specific $CD8^+$ T cells was observed in patient #341 at 360 d after allo-HSCT when PBMCs were stimulated with Tax301–309 in the presence of Tax155–167 (Fig. 5). So far, Tax155–167-specific $CD4^+$ T cells were detected in fresh and/or Tax155–167-stimulated PBMCs of all HTLV-1–infected HLA-DRB1*0101$^+$ individuals tested, although their frequencies were various. These results suggest that Tax155–167 may be the dominant epitope recognized by Tax-specific $CD4^+$ T cells in HTLV-1–infected HLA-DRB1*0101$^+$ individuals. In ATL patients after HSCT, the donor-derived T cells reconstituted in recipients will first encounter HTLV-1 Ags, because HTLV-1 still persists in the patients even though proviral loads become undetectable in the peripheral bloods. Indeed, we found that donor-derived Tax155–167-specific $CD4^+$ T cells were present in three ATL patients after allo-HSCT from seronegative donors. This finding also suggests that Tax155–167-specific naive $CD4^+$ T cells may pre-exist in HLA-DRB1*0101$^+$ individuals and can be primed with HTLV-1 Ags during the primary infection. In this study, Tax155–167-specific $CD4^+$ T cells were also detected in an AC and a HAM/TSP patient (Fig. 6B), suggesting that Tax155–167-specific $CD4^+$ T cells may be maintained in some HLA-DR1$^+$ individuals during the chronic phase of HTLV-1 infection. However, it has been reported that epitope hierarchies may change because of T cell escape mutants (43, 44) and unresponsiveness or deletion of epitope-specific T cells because of prolonged Ag stimulation during chronic infection (45, 46). Further longitudinal studies with a number of samples will be required to confirm that Tax155–167 is a dominant epitope of HTLV-1–specific $CD4^+$ T cells in HLA-DRB1*0101$^+$–infected individuals in the course of HTLV-1 infection.

Among three patients (#241, #350, and #364) showing high T cell responses against recombinant Tax protein, two patients (#350 and #364) were found to carry HLA-DRB1*0101 and have efficient $CD4^+$ Th cell responses against Tax155–167. Intriguingly, it has been reported that HLA-DRB1*0101 is associated with susceptibility to HAM/TSP (47, 48). In addition, $CD4^+$ T cells have been shown to be the dominant cells infiltrating in early active inflammatory spinal cord lesions (28, 29) with spontaneous production of proinflammatory cytokines (30). These observations suggest that HLA-DRB1*0101 might be associated with susceptibility to HAM/TSP via an effect on high $CD4^+$ T cell activation. Further studies are needed to clarify whether HLA-DRB1*0101 is associated with high Tax-specific $CD4^+$ T cell responses in HTLV-1–infected individuals.

Early studies using lymphocytic choriomeningitis virus showed that $CD4^+$ T cell help is critical for maintenance of $CD8^+$ T cell function during chronic infections (18). It has also been suggested that $CD4^+$ T cells are required for optimal CTL responses during HTLV-1 infection (49). Aubert et al. (50) showed that both Ag-specific naive and effector $CD4^+$ T cell help rescued exhausted $CD8^+$ T cells in vivo, resulting in a decrease in viral burden. In the current study, we determined a novel HLA-DRB1*0101–restricted Th epitope, Tax155–167, which was capable of augmenting Tax-specific $CD8^+$ T cell expansion by stimulating Tax155–167-specific $CD4^+$ T cells. This epitope would be a useful tool for investigating the roles of HTLV-1–specific $CD4^+$ T cells in antitumor immunity and in pathogenesis of HTLV-1–related inflammatory diseases such as HAM/TSP and developing novel vaccines to prevent progression or recurrence of ATL.

Disclosures

The authors have no financial conflicts of interest.

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HIV-1 Vpr Accelerates Viral Replication during Acute Infection by Exploitation of Proliferating CD4$^+$ T Cells *In Vivo*

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Abstract

The precise role of viral protein R (Vpr), an HIV-1-encoded protein, during HIV-1 infection and its contribution to the development of AIDS remain unclear. Previous reports have shown that Vpr has the ability to cause G_2 cell cycle arrest and apoptosis in HIV-1-infected cells *in vitro*. In addition, *vpr* is highly conserved in transmitted/founder HIV-1s and in all primate lentiviruses, which are evolutionarily related to HIV-1. Although these findings suggest an important role of Vpr in HIV-1 pathogenesis, its direct evidence *in vivo* has not been shown. Here, by using a human hematopoietic stem cell-transplanted humanized mouse model, we demonstrated that Vpr causes G_2 cell cycle arrest and apoptosis predominantly in proliferating CCR5$^+$ CD4$^+$ T cells, which mainly consist of regulatory CD4$^+$ T cells (Tregs), resulting in Treg depletion and enhanced virus production during acute infection. The Vpr-dependent enhancement of virus replication and Treg depletion is observed in CCR5-tropic but not CXCR4-tropic HIV-1-infected mice, suggesting that these effects are dependent on the coreceptor usage by HIV-1. Immune activation was observed in CCR5-tropic wild-type but not in *vpr*-deficient HIV-1-infected humanized mice. When humanized mice were treated with denileukin diftitox (DD), to deplete Tregs, DD-treated humanized mice showed massive activation/proliferation of memory T cells compared to the untreated group. This activation/proliferation enhanced CCR5 expression in memory CD4$^+$ T cells and rendered them more susceptible to CCR5-tropic wild-type HIV-1 infection than to *vpr*-deficient virus. Taken together, these results suggest that Vpr takes advantage of proliferating CCR5$^+$ CD4$^+$ T cells for enhancing viremia of CCR5-tropic HIV-1. Because Tregs exist in a higher cycling state than other T cell subsets, Tregs appear to be more vulnerable to exploitation by Vpr during acute HIV-1 infection.

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Introduction

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), encodes four viral accessory proteins: Vif, Vpu, Nef, and Vpr. Vpr is a small (96 amino acids) but multipotent protein which is known to induce G_2 cell cycle arrest, apoptosis, and the enhancement of HIV-1 long terminal repeat (LTR)-driven transcription in infected cells [1]. Previous *in vitro* studies have reported that *vpr*-deficient HIV-1 is less replicative in CD4$^+$ T cell lines [2] and cycling primary CD4$^+$ T cells [3]. On the other hand, *vpr* deficiency modestly affects viral replication kinetics in tonsil histocultures in which resting CD4$^+$ T cells dominantly reside [4]. *In vivo*, *vpr*-deficient SIV is less replicative but induces AIDS in macaque monkeys [5]. However, although the underlying molecular mechanisms of Vpr function have been widely investigated, the significance and the precise role(s) of Vpr *in vivo* remain unclear.

The main target of HIV-1 *in vivo* is CD4$^+$ T cells. Based on their function and phenotype, primary CD4$^+$ T cells are classified into three subsets: naive CD4$^+$ T cells (Tns), memory CD4$^+$ T cells (Tms), and regulatory CD4$^+$ T cells (Tregs). It is speculated that such phenotypic and functional differences among these subsets

Author Summary

HIV-1 encodes nine genes, five of which (*gag*, *pol*, *env*, *tat*, and *rev*) are essential for viral replication, and four, termed accessory genes (*vif*, *vpu*, *nef*, and *vpr*), appear to aid virus infection. Of the four accessory proteins, Vpr is the most enigmatic. It is well known that Vpr has the potential to cause G_2 cell cycle arrest and apoptosis *in vitro*. Moreover, it has been reported that Vpr-mediated G_2 arrest increases HIV-1 production *in vitro*. However, the role of Vpr in HIV-1 propagation *in vivo* remains unclear. Here, by using a humanized mouse model, we demonstrate that Vpr enhances CCR5-tropic but not CXCR4-tropic HIV-1 replication *in vivo* by exploiting Tregs during acute infection. In CCR5-tropic HIV-1-infected humanized mice, Vpr-dependent G_2 cell cycle arrest and apoptosis are predominantly observed in infected Tregs, and wild-type but not *vpr*-deficient HIV-1-infected mice displayed acute Treg depletion. This Vpr-dependent Treg depletion may lead to immune activation and provide a pool of activated/proliferating $CD4^+$ T cells, which supports subsequent HIV-1 expansion *in vivo*. This is the first report demonstrating the role of Vpr in HIV-1 infection *in vivo*.

closely associates with the infectivitiy, productivity, and replicativity of HIV-1 [6]. However, since cultured primary $CD4^+$ T cell subsets do not retain all of their *in vivo* attributes, the dynamics of each subset on HIV-1 infection are poorly understood.

Among the $CD4^+$ T cell subsets, Tregs constitute 5–10% of all $CD4^+$ T cells in human, monkey, and mouse species [7]. The potential and phenotype of Tregs are under the control of a transcription factor called forkhead box P3 (FOXP3), which is exclusively expressed in Tregs [8]. Tregs are more actively proliferating *in vivo* than the other $CD4^+$ T cell subsets [9–11]. It is well known that Tregs play a central role in the maintenance of self-tolerance and immune homeostasis [7]. In addition, it is implicated that Tregs are closely associated with immunopathological events such as autoimmune diseases [7] and infectious diseases [12–14]. In particular, there are lines of reports showing that HIV-1/SIV infection decreases Tregs in HIV-1-infected patients [15–17] and simian immunodeficiency virus (SIV)-infected macaque monkeys [18–20].

In this study, we infect a human hematopoietic stem cell (HSC)-transplanted humanized mouse model [21–25] with wild-type (WT) and *vpr*-deficient HIV-1 and investigate the fundamental role of Vpr in HIV-1 infection *in vivo*. Our findings suggest that Vpr plays a crucial role in accelerating CCR5-tropic (R5) but not CXCR4-tropic (X4) HIV-1 propagation during acute infection by utilizing $CCR5^+$ proliferating $CD4^+$ T cells including Tregs.

Results

Tregs are depleted during the acute phase of R5 HIV-1 infection

We first characterized the profile of human $CD4^+$ T cell subsets, including Tns, Tms, and Tregs, in human peripheral blood mononuclear cells (PBMCs) isolated from HIV-1-negative healthy donors and in the spleen of humanized mice [21–23]. As shown in Figure 1A, we detected 6.3±0.2% $FOXP3^+$ $CD4^+$ T cells in splenic human $CD4^+$ T cells of humanized mice, which was comparable to those in human peripheral $CD4^+$ T cells (5.4±0.6%; Figure 1B). Consistent with previous reports [26–29], we also confirmed that the phenotypes of Tregs including the expression levels of CD25, CD127, and cytotoxic T-lymphocyte

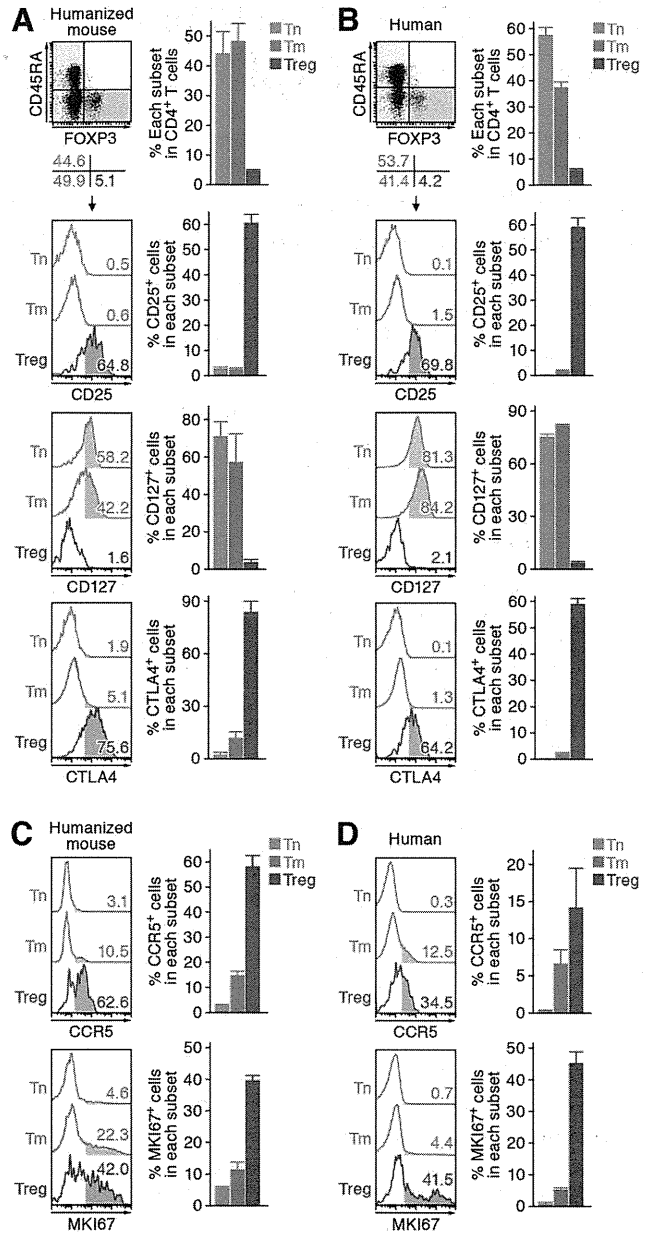

Figure 1. Comparison of the profile of $CD4^+$ T cell subsets between human and humanized mouse. Human $CD4^+$ T cells isolated from the spleen of humanized mice (A and C, n = 8) and the PB of HIV seronegative humans (B and D, n = 6) and were classified into Tn ($CD45^+$ $CD3^+$ $CD4^+$ $CD45RA^+$ $FOXP3^-$ cells), Tm ($CD45^+$ $CD3^+$ $CD4^+$ $CD45RA^-$ $FOXP3^-$ cells), and Treg ($CD45^+$ $CD3^+$ $CD4^+$ $CD45RA^-$ $FOXP3^+$ cells) by flow cytometry. Representative dot plots and histograms are shown on the left panels. The percentage of each subset in $CD4^+$ T cells (A and B, top) and the percentages of the cells positive for CD25, CD127, CTLA4, CCR5, and MKI67 in each subset are respectively shown on the right panels. In the left panels, the numbers under the dot plots (A and B, top) indicate the percentage of the cells in each quadrant, and the numbers in each histogram indicate the positivity. Data represent mean ± SEM.

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associated protein 4 (CTLA4; also known as CD152) in humanized mice (Figure 1A) were similar to those in humans (Figure 1B). Since the suppressive function of the Tregs differentiated in humanized mouse models has been demonstrated previously [26–29], our results strongly suggest that the majority of