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In vivo and *in vitro* immunogenicity of novel MHC class I presented epitopes to confer protective immunity against chronic HTLV-1 infection

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1) has infected approximately 20 million people worldwide. While 90% are asymptomatic, 5% develop severe diseases including adult T-cell leukemia/lymphoka (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). No vaccine against HTLV-1 exists, and screening programs are not universal. However, patients with chronic HTLV-1 infection have high frequencies of HTLV-1-activated CD8+ T cells, and the two main HLA alleles (A2, A24) are present in 88% of infected individuals. We thus utilized an immunoproteomics approach to characterize MHC-I restricted epitopes presented by HLA-A2+, A24+ MT-2 and SLB-1 cell lines. Unlike traditional motif prediction algorithms, this approach identifies epitopes associated with cytotoxic T-cell responses in their naturally processed forms, minimizing differences in antigen processing and protein expression levels. Out of nine identified peptides, we confirmed six novel MHC-I restricted epitopes that were capable of binding HLA-A2 and HLA-A24 alleles and used *in vitro* and *in vivo* methods to

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Conflict of Interest

The authors declare that they have no competing interests.

generate CD8+ T cells specific for each of these peptides. MagPix MILLIPLEX data showed that *in vitro* generated epitope-specific CD8+ T cells secreted IFN-γ, granzyme B, MIP-1α, TNF-α, perforin and IL-10 when cultured in the presence of MT-2 cell line. Degranulation assay confirmed cytotoxic response through surface expression of CD107 on CD8+ T cells when cultured with MT-2 cells. A CD8+ T-cell killing assay indicated significant antiviral activity of CD8+ T cells specific against all identified peptides. *In vivo* generated CD8+ T cells similarly demonstrated immunogenicity on ELISpot, CD107 degranulation assay, and MagPix MILLIPLEX analysis. These epitopes are thus candidates for a therapeutic peptide-based vaccine against HTLV-1, and our results provide preclinical data for the advancement of such a vaccine.

Keywords

HTLV-1; Vaccine; Epitopes; Immunoproteomics; ATLL; HAM/TSP

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus in the genus *Deltaretrovirus* of the subfamily Orthoretrovirinae (1). There are seven reported subtypes of HTLV-1 (A through G), although no evidence suggests that the pathogenic capacity of HTLV-1 differs between subtypes (2). Presently, subtype A has spread worldwide and is responsible for the majority of HTLV-1 infections (2). HTLV-1 is prominent in most regions of Africa, and recent data have described communities in Australia, Japan, and Brazil where as many as 45% of individuals are infected (3–5). HTLV-1 is estimated to have infected 20 million people, of which 90% are asymptomatic carriers (ACs) (6). ACs facilitate the silent transmission of HTLV-1, predominantly through modes such as blood contact, breastfeeding, and sexual intercourse (6). In symptomatic infected individuals, HTLV-1 is causally associated with numerous pathologies that are neither curable nor effectively treatable (7). HTLV-1 can cause a range of symptoms, including sicca syndrome, arthropathy, chronic periodontal disease, erectile dysfunction, and overactive bladder (8). Other inflammatory diseases caused by HTLV-1 include Sjören's syndrome, polymyositis, infective dermatitis, and HTLV-1 associated arthropathy (9). 5% of infected individuals develop severe diseases such as adult T-cell leukemia/lymphoma (ATLL) or HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (7). ATLL is associated with a median survival of less than one year (10), and HAM/TSP is characterized by debilitating symptoms such as dysfunction of the bladder and bowel and spastic paraparesis (11).

The mechanism by which HTLV-1 causes disease is not well understood (12). Once the virus has invaded a host cell, it uses its retroviral machinery to transcribe genes that promote its proliferation (13). HTLV-1 can spread within a host via one of two pathways: *de novo* infection, whereby the virus is transmitted from infected to uninfected cells, or the more commonly observed clonal expansion, whereby infected cells can minimize their expression of viral proteins and make themselves less susceptible to immune attack (13). HTLV-1 further evades the host immune system by infecting CD4 T cells and inherently weakening host immunity (13). HTLV-1 viral expression is known to be low in individuals with ATLL, due to methylation or deletion of the 5′ long terminal repeat (14). Among HTLV-1 infected

individuals, high proviral load (PVL) is more commonly observed in individuals with conditions such as HAM/TSP and infective dermatitis (15). In some instances, high PVL has been observed in ACs and been associated with a heightened inflammatory response (15).

Vaccines against HTLV-1 are not currently available, and screening programs are not universal (6). Recent treatments aimed at HTLV-1 carriers who might be at risk for developing disease attempt to improve PVL by administration of reverse transcriptase in combination with histone deacetylase inhibitors (14). However, most treatments, when delivered to patients afflicted with ATLL or HAM/TSP, do not yield satisfactory success (14). The median survival time of ATLL, despite vigorous treatment attempts with chemotherapy, interferon therapy, and antiviral drugs, continues to be measured in months (16). Thus, developing a therapeutic vaccine against HTLV-1 presents a unique set of challenges, given its tremendous genetic stability and its ability to hide from the immune system through modification of its genetic material, such as in ATLL patients (14).

In order to design a vaccine or therapeutic treatment for HTLV-1, it is essential to understand the difference in immune response that leaves 95% of individuals asymptomatic and the remaining 5% with chronic disease. The viral transactivator protein Tax is a key protein that has been implicated in the viral pathogenesis of HTLV-1 infected patients with HAM/TSP (6, 17, 18). This viral gene product has been observed to promote proliferation of infected cells, inhibit their apoptosis, and also activate a vigorous cytotoxic T lymphocyte (CTL) response (6). However, the CD8 T-cell response against the Tax antigen has not been shown to diminish PVL and is in fact associated with detrimental HAM/TSP pathogenesis (19, 20). Further studies have implicated the PD-1/PD-L1 pathway as causative of the high PVL and high Tax levels in HAM/TSP patients that lead to disease progression (21). This evidence suggests that, by activating specific mechanisms of tolerance and immune suppression, HTLV-1 promotes the exhaustion of HTLV-specific CTLs to facilitate establishment of chronic infection (21).

A possible method to counteract this immunosuppressive action of HTLV-1 is to introduce a novel immunotherapeutic approach of peptide-based vaccines. If a vaccine can be formulated to display a collection of epitopes found on chronically infected cells, it may be possible to stimulate a polyclonal T-cell response in HTLV-1 infected patients with chronic disease to eradicate infected cells (22). The immunoproteomics approach is markedly advantageous to the traditional method of using motif prediction algorithms, because it is capable of identifying epitopes associated with CTL responses in their naturally processed forms and minimizing differences in antigen processing and protein expression levels (23). Furthermore, peptide based vaccines are able to accommodate several distinct peptide epitopes in one dose; thus an immunotherapeutic agent formulated using this strategy can be effective for a wide range of individuals with diverse MHC alleles (23). Published and ongoing clinical trials support the use of immunotherapy for chronic HTLV-1 infection, including anti-inflammatory drugs (G.Taylor, NCT00773292) and monoclonal Ab-based therapies (S.Jacobson, NCT00076843).

In this study, we performed systematic immunoproteomics analyses of MHC class I:peptide complexes on HTLV-1 infected cells to identify T-cell epitopes that might be used to restore

CTL activity in chronically infected patients. After selecting specific epitopes that were abundant in chronically infected cells, we performed immunogenicity testing to identify those candidates that would be best suited for inducing a polyclonal T-cell response against HTLV-1. Demonstration of *in vitro* and *in vivo* efficacy of a multi-epitope peptide vaccine will provide preclinical data for the advancement of an anti-HTLV-1 therapeutic vaccine.

Materials and Methods

Cell lines

HepG2, hepatoma cells, MT2, HTLV-1 virion expressing cells, and T2, TAP deficient lymphoblasts, were obtained from ATCC. HepG2 were maintained in DMEM medium (Corning, NY) while MT2 and T2 cells were maintained in RPMI 1640 (Corning, NY). All culture medium was supplemented with 10% fetal bovine serum, L-glutamine (300 mg/mL), non-essential amino acids (1x), 0.5 mM sodium pyruvate, penicillin and streptomycin (1x), supplements were purchased from Corning). All cell lines were maintained at 37°C in a humidified incubator with 5% CO2.

Mice

Six to eight-week old female HLA-A2 transgenic mice were obtained from Taconic (Strain HLA-A2.1, CB6F1-Tg(HLA-A*0201/H2-Kb)A*0201). Mice were housed at Lampire Biologicals (Pipersville, PA), and all experiments were conducted in adherence to the Guide for Care and use of Laboratory Animals of the NIH. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Lampire Biologicals.

Human subjects

We obtained biological specimens from two subjects with HAM/TSP and with HTLV-1 infection without neurological disease from former blood donors in the multi-center US HTLV Outcomes Study (HOST) of the University of California San Francisco. This study was approved by the Institutional Review Boards and Ethical Committees of the University of California, San Francisco and University of Hawaii.

Immunoproteomics approach to isolate, purify, and extract MHC associated peptides

MHC class I restricted peptides were isolated as previously described (24). Briefly, cell lysates were prepared by subjecting 5×10^8 cells to three freeze/thaw cycles in a buffer of 150 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, and 1.0% NP40. Lysates were cleared by centrifugation, and peptide/MHC complexes were subsequently isolated by immunoaffinity chromatography using UltraLink Immobilized Protein A/G beads (Pierce, ThermoFisher Scientific, Inc., Waltham, MA) coated with monoclonal antibody W632 that recognizes pan class I molecules. The bound peptide/MHC complexes were eluted from the beads by adding 0.1% trifluoroacetic acid, and the eluate was heated to 85°C for 15 minutes to dissociate peptides bound to the heavy chain of MHC molecules. The solution was cooled to room temperature, and peptides were separated from the antibody by centrifugation using an Amicon Ultra-3kDA filter (Millipore, Billerca, MA). The antibody-free peptide mixture was fractionated by utilizing C-18 reverse-phase column on an offline 3000 HPLC (Dionex, Sunnyvale, CA).

Mass spectrometry analysis to identify peptide/protein

The Orbitrap (ThermoFisher) instrument interfaced with nano ultimate HPLC (Dionex) was utilized for mass spectrometry experiments. Purified peptide fractions were individually injected into the LC-MS/MS system to identify sequences of the MHC peptides. Peptides were first concentrated with a 300 μm ID x 5 mm C-18 trap column (ThermoFisher) and then separated with a 75 μm ID x 15cm C18 analytical column (ThermoFisher) equilibrated in 4% ACN/0.1% formic acid (FA) at a flow rate of 250 nL/min. Mobile phase A was 2% ACN and 0.1% FA in water, while mobile phase B was 0.1% FA and 90% ACN in water. Gradients of 4% to 50% B in 60 minutes and 50% to 80% in 90 minutes were used to separate peptides. Peptides were directly eluted into the mass spectrometer and analyzed in a data-dependent manner. Automatic switching between MS and MS/MS modes was controlled by Xcalibur (ThermoFisher) software. MHC peptides and corresponding proteins were identified by searching raw MS/MS data in the NCBI HTLV-1 database on the Proteome Discoverer software (ThermoFisher). The database search parameters were enzyme-no enzyme, threshold-100, peptide tolerance-20ppm, the fragment ion tolerance-0.8 Da. The search results were filtered with XCorr according to individual peptide charge status (+1:1.6, +2:1.8, and +3:2.0). Search results were further verified manually and with their synthetic analogues to confirm correct peptide sequence, using identical conditions to the aforementioned experiment.

Generation of epitope-specific CTLs *in vitro*

Epitope-specific CTLs were generated as previously described (25). Briefly, heparinized blood samples from healthy HLA-A2+ and HLA-A24+ donors were subjected to differential centrifugation using lymphocyte separation medium (Mediatech, Prince William County, VA) to purify samples of PBMCs. 20×10^6 PMBCs in 2 mL RPMI-1640 medium were cultured overnight at 37°C with 5% CO_2 in 6-well plates. Following this period, non-adherent cells were harvested from the sample and saved. Epitope-specific CD8+ T cells were selected for proliferation by pulsing adherent cells with 50 μg/mL MHC class I restricted synthetic peptides and 1.5 μg/mL β2-microglobulin (Sigma-Aldrich) for 2 hours. Non-adherent cells were subsequently resuspended at a volume of 1 mL/well in 4 mL RPMI-1640 medium with 5 ng/ml IL-7, 25 ng/mL Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), 50 ng/mL IL-4, and 5 μg/mL keyhole limpet hemocyanin (KLH; Sigma-Aldrich, St. Louis, MO), and then restored to the 6-well plates. Plates at a final volume of 5 mL/well were incubated at 37°C with 5% CO_2 for 12 days. After the incubation period, T cells in culture were restimulated with autologous CD4+ and CD8+ depleted PBMCs pulsed with 10 μg/mL synthetic peptide and 1.5 μg/mL β2-microglobulin. 5 days after restimulation, 2.5 mL of medium from each well was replaced with complete medium supplemented with IL-2 to a final concentration of 10 U/mL and incubated for 2 additional days. The restimulation and subsequent IL-2 stimulation procedure was further repeated thrice using the protocol described above and the resultant samples were used for CTL functional assays. A diagrammatic representation of this process is depicted in Supplemental Figure 1A.

Flow cytometry analysis to detect *in vitro* generated epitope-specific CD8+ T cells

Epitope-specific CTLs generated as described earlier were incubated for 18 hours with either MT2 cells or T2 cells left unpulsed, or pulsed with HTLV-1 specific peptides (2ug/mL), or with PBMCs from one HAM/TSP patient, and PBMCs from one asymptomatic carrier. 18 hours later, cells were washed and stained with anti-CD107a-PE (BD Biosciences, San Jose, CA) and anti-CD8α-FITC antibodies (ThermoFisher) for 30 minutes at room temperature at 4°C to detect degranulation in CD8+ T cells. Cells were again washed and resuspended in flow cytometry staining buffer. Flow cytometry events were acquired using FACS Calibur (BD Biosciences), and data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Killing assay for *in vitro* generated CTLs

CTLs generated *in vitro* were further assayed using a CD8+ T-cell killing assay to determine antiviral efficacy of the peptide-specific CTLs, as previously described (26). 7 days after the final restimulation, CTLs generated *in vitro* were divided into two groups, high and low/medium binders, based on binding specificity. These cells were then co-cultured with target cells that were either MT-2 cells, or PBMCs from one HAM/TSP patient or asymptomatic carrier at 3–4 different ratios (lower, including, and higher than normal patient ratios), keeping the target cell count constant, and incubated for 18 hours at 37°C, 5% CO_2. After 18 hours in culture, cells were washed in flow cytometry staining buffer, and surface stained for CD4 and CD8 antigens using 15 μg/mL PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 (BD Biosciences). Cells were washed twice, fixed with fixation buffer for 20 minutes at room temperature in the dark. Cells were then permeabilized and stained intracellularly for HTLV-1 early protein Tax (anti-Tax antibody kindly provided by Professor Yuetsu Tanaka at the University of the Ryukyus, Japan). Flow cytometry events were acquired using FACS Calibur (BD Biosciences), and data was analyzed using FlowJo software (Tree Star). Subsequent analysis of CD8+ T cell efficacy was performed by non-linear least squares regression using the equation $dy/dt = c - \varepsilon yz$, where y = Tax+CD4+ cells/CD4+ cells, c = rate of increase of Tax expression, z = proportion of CD8+ lymphocytes, and ε = CD8+ cell mediated antiviral efficacy using IBM SPSS Statistics for Macintosh, Version 25.0. (IBM Corp., Armonk, NY) and Microsoft Excel. A diagrammatic representation of this process is depicted in Supplemental Figure 1B.

Generation of epitope-specific CTLs *in vivo*

HLA-A2 transgenic mouse experiments were conducted at Lampire Biologicals. Their initial inoculation consisted of a mixture of pooled free peptide in PBS plus Montanide ISA 51 (Seppic, Paris, France) (50:50 emulsion), PBS alone, or two independent, individual free peptide in PBS plus Montanide ISA 51 (50:50 emulsion). Mice were injected at two sites; i.d. near the base of the tail and s.c. on the flank. Injections were repeated two more times at 10-day intervals. One week after the third inoculation, mice were sacrificed, and spleens were harvested for analysis.

ELISpot assays for *in vivo* generated CTLs

Antigen stimulated interferon-γ (IFN-γ) release as a measure of CTL activation was assayed using an ELISpot assay kit (BD) according to the manufacturer's instructions.

Briefly, 96 well PVDF-membrane plates (Millipore) were coated with murine IFN-gamma capture antibody overnight at 4°C. On the day of the assay, the plates were blocked for 2 hours in RPMI-1640 complete medium and washed prior to use in the ELISpot assay. Then, a fixed number of various target cells and effector cells at an effector to target ratio of 10:1 for murine splenocytes were cultured in replicate wells overnight. Spots were quantitated using an ELISpot Reader System (AID, Strasburg, Germany). Results are presented as the number of interferon-γ producing cells (spot forming units; SFU) per 1×10^6 splenocytes. Error bars represent standard deviation of experimental replicates.

Flow cytometry analysis for *in vivo* generated CTLs

Epitope specific $CD8^+$ T cells generated innoculating mice were assessed for cytotoxic capabilities via CD8, CD107a staining. Briefly, PBMCs, in the presence of murine anti-CD107a antibody, were incubated for 5 hours with MT2 cells and T2 cells left unpulsed, or pulsed with HTLV-1 specific peptides (2ug/mL). After the co-incubation, cells were washed and stained with anti-CD8a (TheromoFisher). Cells were washed extensively and resuspended in PBS/0.1%BSA for analysis. All flow cytometry was performed using Guava 8HT EasyCYTE system (Millipore) and data analyzed using the InSight software (Millipore).

MagPix cytokine detection for *in vitro* and *in vivo* generated CTLs

The MILLIPLEX magnetic bead assay was used as per manufacturer's instructions (Millipore) to measure cytokine secretion from activated CD8+ T cells. Briefly, *in vitro* generated CD8+ T cells stimulated were co-cultured for 18 hours with MT-2 cells. For the *in vivo* analysis, supernatants were harvested from splenocytes stimulated overnight with HepG2 cells pulsed with HTLV-1 specific peptides or MT-2 cells. Supernatants were harvested and cleared of cellular debris by centrifugation. 96-well plates containing assay buffer diluted at a 1:1 ratio were loaded with 25 μL of samples, standards, and controls. Magnetic beads coated with antibodies against IFN-γ, Granzyme B, MIP-1α, TNF-α, Perforin and IL-10 were added to each well before the plate was sealed and incubated overnight at 4°C with shaking. The following day, the plate was washed twice and biotinylated detection antibodies were added before it was sealed again and incubated at room temperature with rocking for 1 hour. Streptavidin-PE was then added and the plate was rocked at room temperature for another 30 minutes. The plate was washed twice again and loaded with sheath fluid to be read on the MAGPIX system. The Milliplex Analyst software was used as per manufacturer's instructions (Luminex, Austin, TX) for data analysis.

Results

Identification of MHC class I presented epitopes from HTLV-1 infected cells by Nano-LC/MS/MS methods

A peptide based vaccine is a strong candidate for immunotherapeutic treatment of patients chronically infected with HTLV-1 virus. Such a vaccine can only generate an effective T-cell response if it includes antigens that are naturally processed and presented by HTLV-1 infected cells. We therefore sought to identify naturally processed and presented HTLV-1 specific MHC class I restricted epitopes via an immunoproteomics approach.

Our studies utilized MT-2 and SLB-1 cells, which are both HLA-A2 and A24 positive (Figure 1A). These cells were subjected to the immunoprecipitation of peptide/MHC-I complexes followed by elution of peptides and LC-MS/MS analysis. This led to identification of two A2-specific, six A2 and A24 double binding, and two A24-specific epitopes with high XCorr values (XCorr > 1.5, high confidence/high level) (Figure 1B). From this subset, we validated 6 MHC class I epitopes from HTLV-1 infected cells using an immunoproteomics approach (22). In this approach, peptides associated with MHC class I molecules were isolated from the infected cells and identified using nano-LC/MS/MS analysis. We then confirmed the sequence identity of these peptides using their synthetic analogs. The fragment mass spectra of the synthetic peptides matched the spectra of the experimentally identified peptides nearly identically (Figure 2). The intensity differences between the tandem mass spectra of experimental and synthetic peptides may be attributed to the expression level of those peptides in infected cells and instrument reproducibility such as collision energy and gas pressure in the ion trap as we ran the confirmation experiments at different times. In addition, we further verified the MS/MS spectra manually to confirm the identity of all the experimentally observed peptides.

Peptide epitopes identified by immunoproteomics analysis activate HTLV-1 specific CTLS *in vitro*

Once sequences were confirmed for the experimentally identified peptides, we ascertained whether these epitopes could activate CD8+ T cells. We used synthetic peptide versions to generate epitope-specific CTLs from PBMCs of healthy HLA-A2+ and HLA-A24+ donors. *In* vitro generated CD8+ T cells, when cultured with MT-2 cells, were found to upregulate CD107a, a classical marker of degranulation (Figure 3) (27). Peptides induced cytokine expression, antiviral activity, and CD107a upregulation independent of the HLA molecule tested, suggesting that these HLA-A2 and A24 double binding peptides are able to activate both HLA-A2 and A24 specific T-cell responses. Additionally, epitope specific CD8+ T cells were found to express IFN-γ, granzyme B, MIP-1α, TNF-α, perforin and IL-10 when cultured in the presence of MT-2 cells (Figure 3C). Epitope specific CD8+ T cells were also shown to express IFN- γ by FACS analysis (Supplemental Figure 2). Except in the case of IL-10, these responses showed specificity to HTLV-1 infected cells, as these cytokines were observed only at background levels when T cells were cultured alone. We next performed a killing assay to measure the antiviral efficacy of *in vitro* generated CD8+ T cells, as previously defined, the *ex vivo* clearance rate of HTLV-1 infected Tax+CD4+ cells CD8+ cells (26).. MHC binding scores of the six confirmed peptides were calculated using the SYFPEITHI prediction algorithm (Figure 4A). This program evaluates the binding capacity of each amino acid within a certain peptide to assign a score representing the peptide's binding capacity (28). Among those peptides, five of them have a binding motif to both HLA-A2 and HLA-A24 (FLN, FTD, IIN, ITN, PLL) and one is HLA-A24 binding peptide (LFA). It was found that percentage of Tax+CD4+ cells decreased with increasing proportions of CD8+ T cells, with no significant differences between CD8+ T cells generated against high MHC scoring epitopes versus those against low/medium MHC scoring epitopes (Figure 4B). A mathematical model reflecting the clearance of Tax+CD4+ cells by CD8+ T cells was fitted to the data using non-linear regression analysis (Figure 4C). Based on this model, antiviral efficacy of CD8+ T cells generated against both high binding

and medium binding peptides was calculated with no significant difference between the two groups (Figure 4D). *In vitro* generated CD8+ T cells were similarly shown to demonstrate cytotoxicity against patient samples of HAM/TSP patients as well as asymptomatic carriers via degranulation assay (Figure 5A). Killing assay confirmed CD8+ T cells have antiviral efficacy against HTLV+ patient samples. This was especially seen within the HAM/TSP patient with CD8+ T cells that were primed with the low/medium binders where the data from the assay fit the regression model (E=0.015, 95% confidence interval 1.465–2.395) (Figure 5B).

Peptide epitopes identified by immunoproteomics analysis activate HTLV-1 specific CTLs *in vivo*

We wanted to determine if the experimentally identified HTLV-1 peptides could stimulate $CD8^+$ T cells *in vivo*. Synthetic versions of the HTLV-1 peptides were injected into HLA-$A2^+$ transgenic mice emulsified with Montanide ISV-51 adjuvant, with a total of three injections. One week after the third injection, splenocytes were harvested and cultured with HepG2 cells pulsed individually with peptides alone or HTLV-1 infected cells in an IFN-γ ELISPot assay. As shown in Figure 6A, $CD8^+$ T cells generated *in vivo* specifically recognized peptide loaded HepG2 cells as well as HTLV-1 infected MT2 cells. In addition, $CD8^+$ T cells generated *in vivo* also upregulated a degranulation marker (CD107a) after stimulation with both peptide pulsed T2s and HTLV-1 MT2 cells (Figure 6B).

As degranulation is associated with delivery of perforin and granzyme to target cells, we next checked the levels of granzyme B being secreted by the peptide activated $CD8^+$ T cells. Supernatants were collected from the stimulated cells and the levels of granzyme B were detected using Luminex MAGPIX magnetic bead technology. Most $CD8^+$ T cells from immunized HLA-A2 mice secreted high levels of granzyme-B in response to peptide stimulation and HTLV-1 infected MT2 cell stimulation compared to their naïve counterparts indicating the activation of a specific cytotoxic response (Supplemental Figure 3).

Discussion

The peptides described in this study are among the first ever identified class I-associated peptides presented by HTLV-1 infected cells and, to our knowledge, have not been previously reported. Because these peptides derive from several viral proteins, they increase the number of targets for T cells and potentiate a broad response to facilitate viral clearance. Two of the peptides (FLN and LFA) are derived from Tax protein, one (FTD) is from the Gag-Pro-Pol polyprotein, one (IIN) from the envelope glycoprotein, one (ITN) from the Gp21 ectodomain, and one (PLL) from the P12 protein. The proteins of origin corresponding to these peptides are immunodominant in the pathogenesis of HTLV-1, and they have been previously identified as targets to reduce both risk of HAM/TSP and proviral load (7, 29). The epitopes identified using Nano-LC/MS/MS methods did not always correspond to previously identified immunodominant epitopes. For example, the well-studied HLA-A2-specific Tax(11–19) epitope which has previously been shown to exhibit immunodominance (16) was found only in the low confidence/low level MS/MS data (Xcorr < 1.5). One potential explanation for this observation is difference in epitope specificity in

acute versus chronic stages of infections (22). Such differences have been documented for other chronic infections including lymphocytic choriomeningitis virus (LCMV) (30, 31) and human immunodeficiency virus-1 (HIV-1) (32). Additionally, low levels of expression of these epitopes on the surface of chronically infected cells would explain their low representation during MS/MS analysis. Furthermore, transient but not sustained increases in certain low confidence epitope-specific CD8+ T-cell responses may be explained a number of reports demonstrating expansion of naïve but not memory CD8+ T-cell expansion in response to low antigen levels (33–35). Low confidence peptides are thus undesirable targets for therapeutic vaccines against HTLV-1, and it is most advantageous to identify targets through analysis of chronically infected cells.

In this study, we utilized an immunoproteomics approach to identify nine epitopes that are naturally processed and presented by cells chronically infected with HTLV-1. Using synthetic analogs, we confirmed a total of six novel MHC class I restricted epitopes against that derive from five different proteins of the HTLV-1 genome. These six peptides (FTD, IIN, ITN, PLL, LFA, and FLN) demonstrated ability to activate CD8+ T cells based on ELISpot assay results measuring IFN-γ production. Notably, T cells could recognize naturally processed and presented epitopes on various HTLV-1 infected cells in spite of different HLA binding affinities (Table 1). Of further relevance, five out of six peptides characterized in this study contain motifs for both HLA-A2 and HLA-A24 supertypes. The HLA-A2 supertype is present in over 40% of the entire world population (www.allelefrequencies.net), and HLA-A2 and HLA-24 together have been found in 88% of HTLV-1 infected individuals. (36, 37).

We next measured antiviral efficacy of eptiope-specific T cells using the CD8+ T-cell killing assay (26) and found that both high MHC scoring and low/medium MHC scoring epitopes showed increased clearance of Tax+CD4+ cells in response to increased proportion of CD8+ T cells. The killing assay, as previously described, is meant to provide a physiological index of antiviral efficacy by measuring clearance of natural target cells in HTLV-1 infected individuals by autologous CD8+ T cells (26). It is advantageous in that it will not be biased by redundant CD8+ T-cell clones against viral epitopes that have since mutated or epitopes that are immunodominant but not necessarily important in the *in vivo* control of viral infection (26). We have repurposed this assay to measure antiviral efficacy of peptide-specific CTLs that we generated *in vitro* against HTLV-1 infected cells. Our data demonstrated that peptides with both high and low/medium affinity for HLA-A2 produced CTLs with reasonable antiviral efficacy that increased with increasing proportions of CTLs. These findings were corroborated by other measures of T-cell activation, including IFN-γ, granzyme B, MIP-1α, TNF-α, perforin and IL-10 secretion and CD107 upregulation. Epitope-specific CTLs generated *in vivo* demonstrated successful stimulation and proliferation as well based on expression of CD107a, and secretion of IFN-γ, granzyme B, MIP-1α, TNF-α, perforin and IL-10. Interestingly, the *in vivo* data showed that epitopes derived from Tax protein did not elicit superior CTL responses, but epitopes derived from envelope glycoprotein incited the greatest response against peptide-loaded target. This finding corroborates previous findings suggesting that Tax protein may be more relevant to the acute stage of infection rather than the chronic (12).

Previous efforts at epitope discovery for generation of a vaccine against HTLV-1 have utilized peptide motif prediction algorithms. However, these methods have not been successful at identifying epitopes which are naturally presented by MCH class I molecules in individuals with chronic HTLV-1 infections. The immunoproteomics approach is superior in this regard, and our study represents a preliminary effort to identify naturally presented epitopes by MHC class I molecules in chronic infection with HTLV-1. The immunodominant epitopes we have described in this study are unique from those that have been previously described and as such may significantly contribute to the generation of an effective vaccine against HTLV-1.

Thus far, from two infected cell lines, we have identified and confirmed six epitopes presented on the cell surface that are otentially representative of the immune response in chronically infected patients. Interestingly, epitopes identified by immunoproteomics were not the same as those reported by motif-prediction algorithms, suggesting that direct identification of epitopes form the cell surface may be a superior approach for the generation of a vaccine. This study demonstrated immunogenicity of identified epitopes in *in vitro* and *in vivo* generated epitope-specific CD8+ T cells. The killing assay further confirmed epitopes as future vaccine candidates by demonstrating antiviral efficacy of CD8+ T cells generated against high and medium binding epitopes. CD8+ T cells generated *in vivo* similarly demonstrated immunogenicity on ELISpot, CD107 degranulation assay, and MagPix MILLIPLEX analysis. This study demonstrates pre-clinical data for the advancement of a therapeutic multi-epitope peptide vaccine against HTLV-1.

In future studies, we hope to expand our epitope repertoire by utilizing additional infected cells. We would like to generate tetramer constructs for identified epitopes to further analyze epitope-specific CD8+ T-cells. We would also like to perform *ex vivo* functional analysis in clinical samples to further assess cytotoxic potential of neo-epitope specific CD8+ T-cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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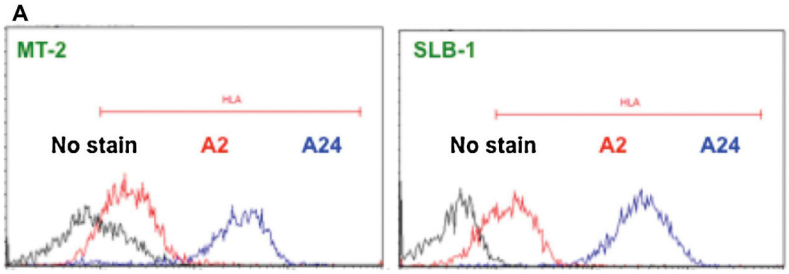
Highlights

- HTLV-1 is a potentially seriously disabling virus for which there is currently no cure or vaccine.
- Immunoproteomics can identify naturally presented epitopes in infected individuals.
- We confirmed 6 novel epitopes presented in chronically infected patients.
- *In vitro* and *in vivo* generated epitope-specific CD8 cells showed immunogenicity.
- Results contribute to creation of a therapeutic peptide-based vaccine for HTLV-1.

A



B

Peptide Epitope	HTLV-1 Protein of Origin	HLA Binding Motif
SLF	TAXREB302	A2
VQL	Env glycoprotein	A2
FLN	Tax-1	A24
FTD	Gag-Pro-Pol polyprotein	A2 and A24
IIN	Envelope glycoprotein	A2 and A24
ITN	Gp21 Ectodomain	A2 and A24
KLL	Env glycoprotein	A2 and A24
PLL	P12	A2 and A24
LFA	Tax protein	A24
PFQ	P12	A24

Figure 1. HLA-A2 and A24 restricted peptides

(A) HLA typing of MT-2 and SLB-1 cells shows these cells expressing HLA-A2 and HLA-A24 alleles. (B) Immunoproteomics analysis (XCorr > 1.5) to identify MHC class I-associated peptides presented by HTLV-1 infected cells.

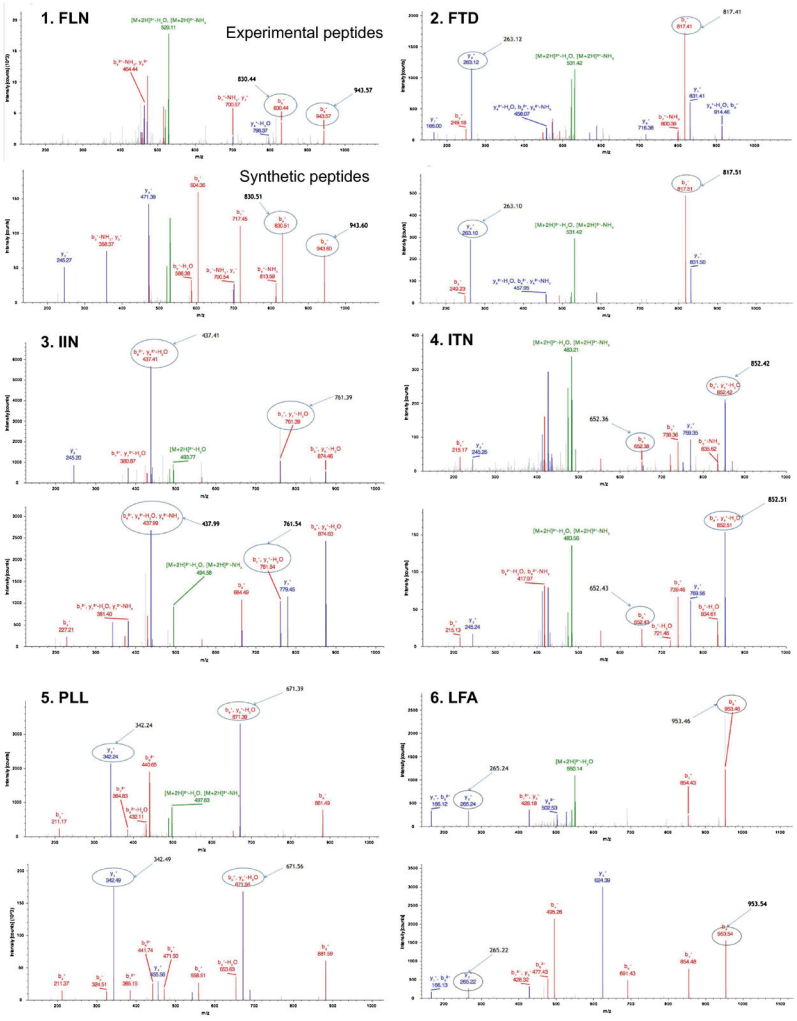
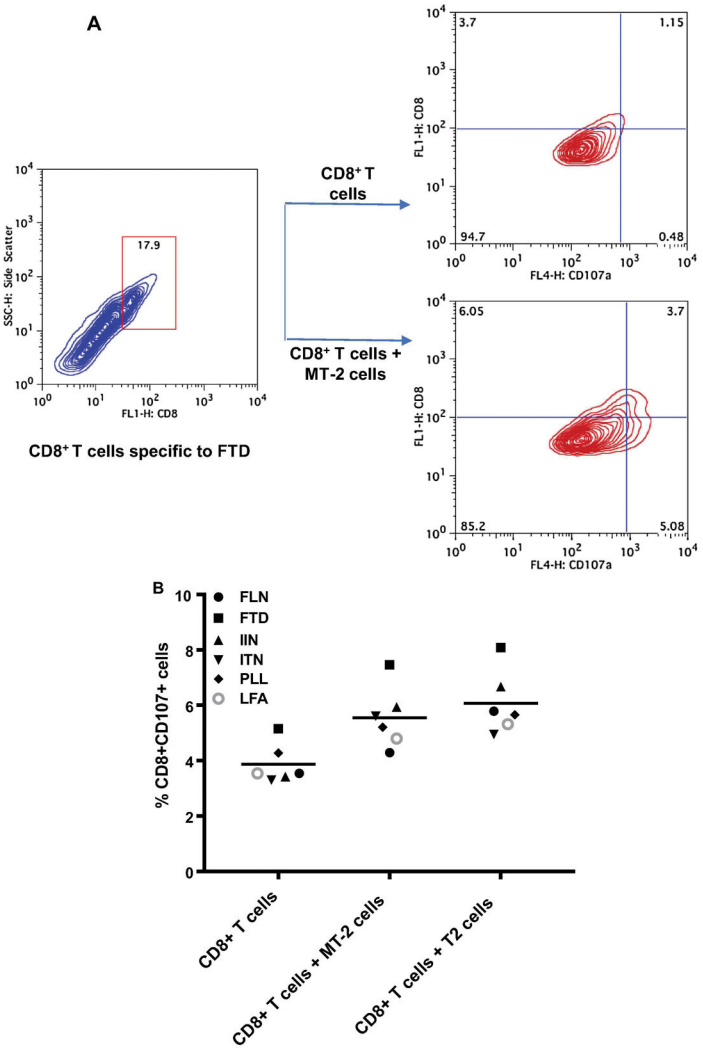


Figure 2. Validation of naturally presented MHC class I epitopes from HTLV-1 infected cells

Mass spectra (MS/MS) of the experimentally identified peptides (top panel) versus their synthetic analogs (bottom panel). Fragment masses that match are denoted.



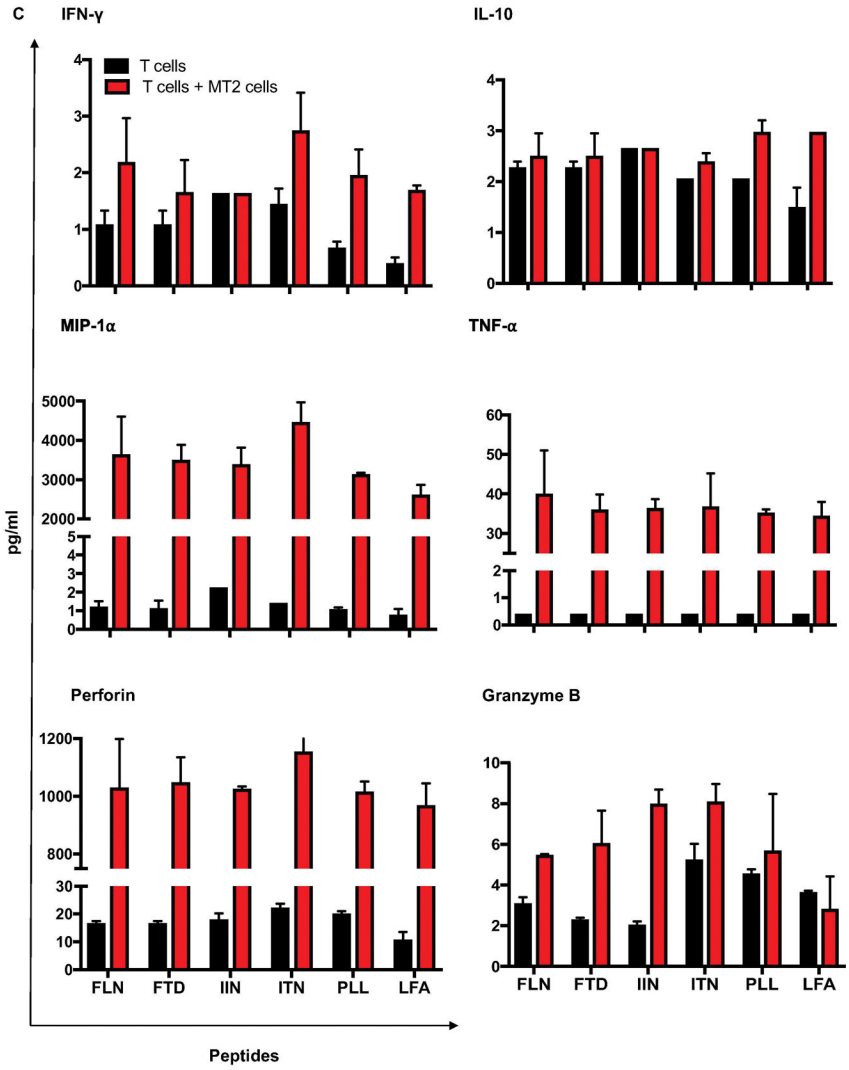
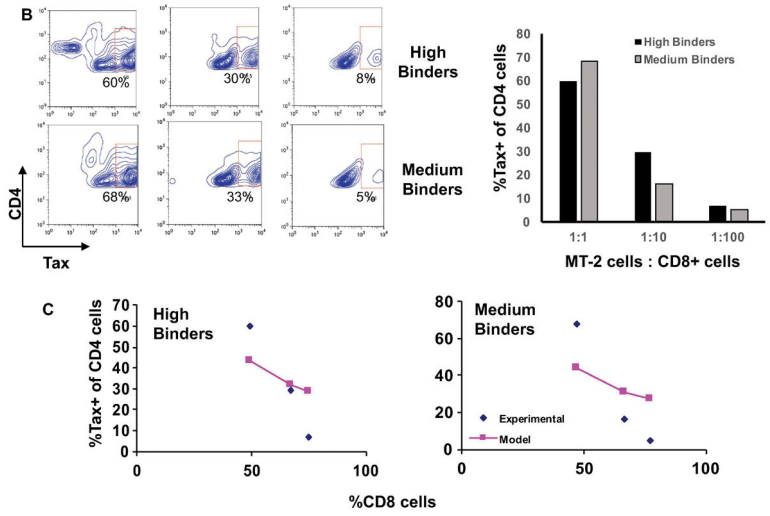


Figure 3. T-cell activation measured by CD107a secretion and cytokine secretion

(A) Degranulation assay of CD8+ T cells was conducted using flow cytometry. (B) Degranulation marker CD107a was released by *in vitro* generated CD8+ T cells in the presence of MT-2 cells + peptide as well as in the presence of MT-2 cells alone. (C) Secretion of IFN-γ, granzyme B, MIP-1α, TNF-α, perforin and IL-10 by *in vitro* generated CD8+ T cells was measured by MagPix MILLIPLEX assay.

A

Peptide	Protein	HLA binding motif	Xcorr	MHC binding score	Group
FTD	Gag-Pro-Pol polyprotein	A2/24	2.93	A2:11, A24:1	Medium Binder
IIN	Envelope glycoprotein	A2/24	2.05	A2:26, A24:12	High Binder
ITN	Gp21	A2/24	2.64	A2:21, A24:12	Medium Binder
PLL	P12	A2/24	2.04	A2:23, A24:14	High Binder
LFA	Tax protein	A24	2.13	A24:17	Medium Binder
FLN	Protein Tax-1	A24	2.01	A2:26, A24:10	High Binder



D

	T:E ratios	%CD8+	%CD4+Tax+	CD8+ Efficacy
High Binders	1:1	49.5	60	0.5577
	1:10	67.4	29.5	
	1:100	74.8	6.84	
Medium Binders	1:1	47.3	68.3	0.5954
	1:10	66.8	16.1	
	1:100	77.2	5.01	

Figure 4. CD8+ T-cell antiviral efficacy

(A) Peptides were divided into two groups based on their A2 MHC binding score. High binders (FLN, IIN, PLL) and low/medium binders (FTD, ITN, LFA) were grouped together and aliquoted with MT-2 cells in three different ratios. (B) Flow cytometry was used to determine percentage of Tax+CD4+ cells with increasing proportions of CD8+ T cells. (C) Percentage of Tax+CD4+ cells with increasing proportions of CD8+ T cells was plotted in comparison to that predicted by the model of nonlinear regression analysis. (D) Calculation of antiviral efficacy as previously described by Asquith *et. al.* (2005).

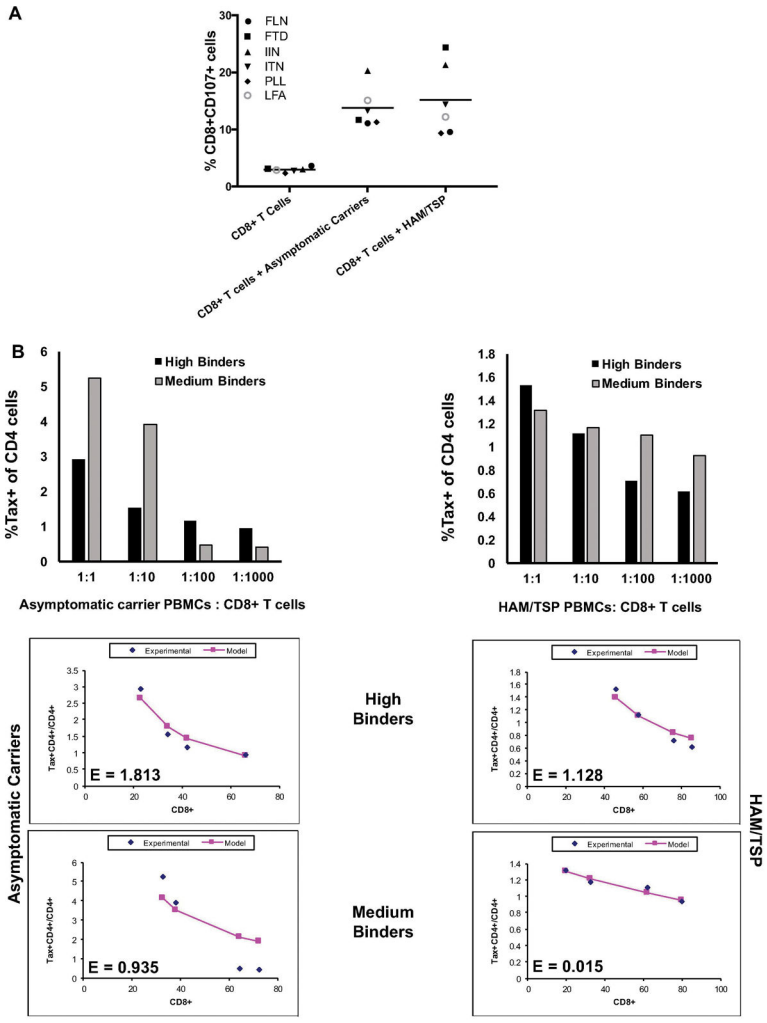


Figure 5. CD8+ T-cell activation and efficany against HTLV+ patient samples

(A) T-cell activation was assayed by the detection of classical degranulation marker CD107a by *in vitro* generated CD8+ T cells in the presence of cells of patients with HAM/TSP as well as those of asymptomatic carriers. (B) CD8+ T cell antiviral efficacy was determined as previously described by Asquith *et. al.* (2005) by incubating various concentrations of CTLs with patient samples. Above, CD4+Tax+ T cell numbers in response to increasing numbers of peptide-specific CTLs. Below, experimental data fitted to non-linear regression model estimating CD8+ T cell efficacy.

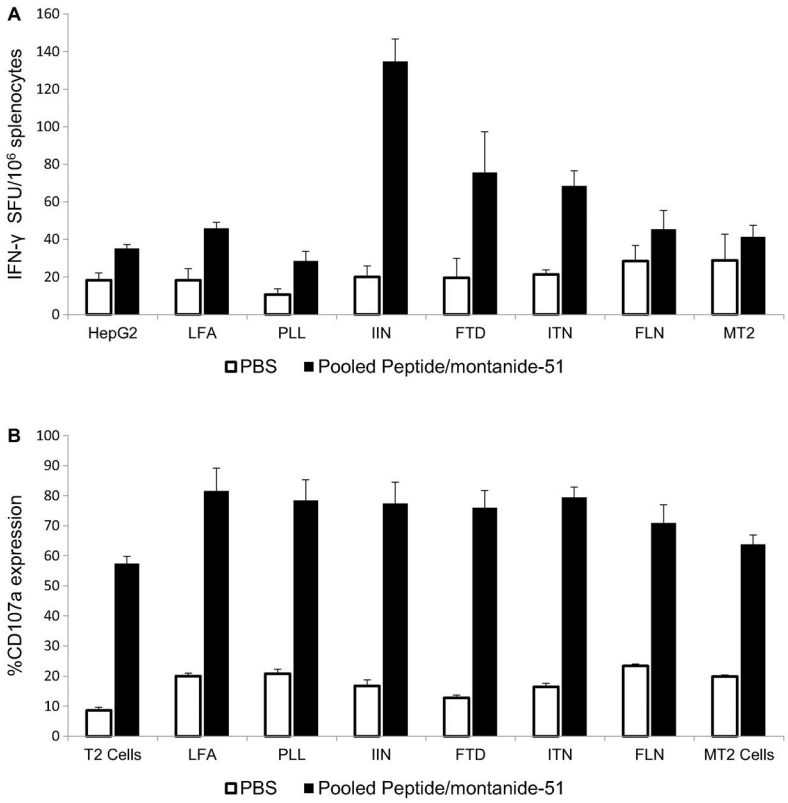


Figure 6. HTLV-1 specific peptides are able to activate CD8$^+$ T cells *in vivo*

(A) HLA-A2 transgenic mice were primed and boosted with peptides as previously described. Spleens were harvested, homogenized into single cell suspensions, and cultured with peptide pulsed HepG2 cells or HTLV-1 infected cells overnight in an IFN-γ ELISpot assay. (B) T-cell activation was also measured by examining expression of CD107a degranulation marker on HLA-A2 CD8$^+$ T cells. Splenocytes were cultured for 5 hours with peptide pulsed T2 cells or HTLV-1 infected cells in the presence of anti-CD107a and subsequently stained for CD8$^+$ expression. Data represents the percent expression of CD107a based off the CD8+ cells in culture.

Table 1

Peptide Epitope	HTLV-1 Protein of Origin	HLA Binding Motif
SLF	TAXREB302	A2
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LFA	Tax protein	A24
PFQ	P12	A24