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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 as many as 10 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 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.

1. 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 10 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

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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 is also capable of infecting CD4 T cells [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.

2. Materials and methods

2.1. 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 (1×), 0.5 mM sodium pyruvate, penicillin and streptomycin (1×), supplements were purchased from Corning). All cell lines were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.2. 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.

2.3. 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.

2.4. 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 min to dissociate

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