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Dynamic acquisition of HTLV-1 tax protein by mononuclear phagocytes: role in neurologic disease

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Abstract

Pathology of HTLV-1 associated myelopathy/Tropical spastic paraparesis (HAM/TSP) is believed to be the result of “bystander damage” involving effector CD8 (+) T lymphocytes (CTLs) killing of virus infected cells. But the specific cellular events leading up to tissue injury are still unclear. Here, we developed the Microscopy Imaging of Cytotoxic T lymphocyte assay with Fluorescence emission (MI-CaFé), an optimized visualization analysis to explore the interactions between CTLs and virus infected or viral antigen presenting target cells. Various cell-to-cell formations can be observed and our results demonstrate elevated frequencies of CTL-target cell conjugates in HAM/TSP patient PBMCs compared to control PBMCs. Furthermore, HTLV-1 Tax protein expression can be localized at the cell-cell junctions and also tracked moving from an infected cell to a CD14 (+) mononuclear phagocyte (MP). Activation of CD14 (+) MPs in HAM/TSP patient PBMCs and antigenic presentation of HTLV-1 Tax by MPs can be inferred by their spontaneous cytotoxicity after 18 hours of in vitro culture. Given that CD4 (+) T lymphocytes are the primary reservoirs of HTLV-1 and MPs are scavenger cells responsible for pathogen clearance, spontaneous cytotoxicity against MPs in HAM/TSP PBMCs suggests a mechanism of chronic inflammation, secondary to low level of persistent virus infection within the central nervous system.

Graphical abstract

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CNS Inflammation with HTLV-1 Tax

Keywords

HAM/TSP; HTLV-1; CTL; Tax

INTRODUCTION

The most widely accepted hypothesis suggests that tissue injury in HTLV-1 associated myelopathy/Tropical spastic paraparesis (HAM/TSP), a chronic progressive inflammatory neurologic disorder, is the result of “bystander damage”, but precise mechanisms sustaining this virus-mediated immunity remain controversial ((Yamano and Sato, 2012) for a comprehensive review of HAM/TSP).

Perivascular cuffing and parenchymal lymphocytic cell infiltration affecting both white and gray matters of the middle and lower thoracic spinal cord are hallmarks of HAM/TSP pathology (Levin et al., 1997) (Umehara et al., 1993). CD4 (+) T lymphocytes, in particular, are the primary *in vivo* reservoirs of the virus, although macrophages, CD8 (+) T and B-lymphocytes are also susceptible to HTLV-1 infection, albeit, to a much lower extent. Virus-infected CD4 (+) T lymphocytes are activated and therefore are more equipped to cross the blood-brain-barrier, but the high level of HTLV-1 Tax expression also makes them targets of cytotoxic T lymphocytes (CTLs). This view of disease pathogenesis is most consistent with data generated from immunohistochemistry studies which demonstrate both CD4 (+) and CD8 (+) T lymphocytic infiltrates in spinal cord tissues during early stages of disease, with increasing dominance of CD8 (+) T lymphocytes and macrophages over the course of the illness (Umehara et al., 1993) (Abe et al., 1999) (Kubota et al., 1998). Recently, specific interactions between HTLV-1 Tax positive CD4 (+) T lymphocytes and virus-specific CTLs were demonstrated in spinal cord tissues of HAM/TSP patients using confocal laser scanning microscopy (Matsuura et al., 2015).

Since the discovery of HAM/TSP, efforts to understand the disease pathogenesis have focused on the interplay between virus-specific T lymphocytes (effectors) and virus-infected cells (targets). Immunological control of virus transmission is, in part, accomplished by

effector CD8 (+) T lymphocytes, which mediate lytic reactions of infected cells, via the release of perforin molecules followed by degranulation of granzymes. Tissue damage in HAM/TSP has been suggested to be the result of an overzealous CD8 (+) cytotoxic T lymphocyte (CTL) response driven by HTLV-1 infected CD4 (+) T lymphocytes. This idea is further supported by strong correlations between proviral load, HTLV-1 Tax (a viral transactivator protein) expression and frequencies of HTLV-1 specific CTLs in HAM/TSP patient PBMCs (Daenke et al., 1996) (Jacobson et al., 1990) (Nagai et al., 1998). With an expanded population of HTLV-1 Tax-specific CTLs, elevated levels of cytolytic granules (i.e. perforin and granzymes), TNF- α and IFN- γ are secreted thereby creating an inflammatory milieu within the central nervous system (CNS). In addition to mediating cytotoxic killing, the cascading flow of inflammatory chemicals are also signals to recruit other immune cells such as granulocytes, NK cells, B and T lymphocytes as well as tissue macrophages to the site of inflammation. Mononuclear phagocytes (MPs) are scavenger cells at the first line of immune defense to clear any foreign substances or cellular debris and they are highly plastic. MPs can adapt to a pro-inflammatory or anti-inflammatory phenotype depending on environmental cues. Based on their functional diversity, three main types of MPs that have been described: host defense macrophages that are associated with tissue damaged are driven by exposure to proinflammatory cytokines, while cytokines such as IL-4 or IL-10 differentiate macrophages into wound healing or regulatory cells, respectively (Mosser and Edwards, 2008).

Why does CNS inflammation continue to persist in HAM/TSP even after the numbers of infected CD4 (+) T cells have subsided in the spinal cord over the course of disease? Our laboratory has previously reported that MPs can also carry provirus and demonstrated activated or infected MP induced CD8+ T cell activation in HAM/TSP through IL-15 (Enose-Akahata et al., 2008). An interesting characteristic of MPs is their ability to bridge both the innate and acquired immunity; they are known to contribute to host defense by sampling cellular debris, foreign pathogens, or abnormal cancerous cells and alert the acquired immune system of potential danger. HTLV-1 infected MPs have especially high levels of activation marker HLA-DR, fractalkine receptor CX₃CR1 (Enose-Akahata et al., 2012), and secrete T cell chemoattractant CXCL9 and CCL5 (Amorim et al., 2014). Other immune cells including NK cells, B lymphocytes, CD4 (+), and CD8 (+) T lymphocytes are thought to follow the gradient of increasing concentration of chemoattractants and arrive to the CNS at which point they can either be activated or suppressed, depending upon the nature of antigens presented by MPs. Observing the dynamics between MPs and T lymphocytes may provide insights to the question we are addressing in this study: how does HTLV-1 manipulate the interactions between MPs, CD4 (+) and CD8 (+) T lymphocytes to facilitate its transmission?

RESULTS

Immunohistochemistry analysis of HAM/TSP spinal cord tissue

We obtained spinal cord tissue from the thoracic level of a patient with long history of HAM/TSP and studied the cellular infiltrates associated with the disease course. Briefly, the clinical record indicates she was a female patient with over 10 years of disease and exhibited

symptoms typical of HAM/TSP, which included spastic paraparesis with pathological reflexes such as Babinski's and Chaddock's sign, urinary dysfunction and loss of sweating in her lower extremities. Muscle relaxant (Dantrolene Sodium) and anticonvulsant (Carbamazepine) were prescribed to manage her symptoms of spasticity. The patient died of pneumonia at the age of 58 years old.

The formalin-fixed and paraffin embedded spinal cord tissue was used in our immunohistochemical study. As described in the methods, 8mm thick sections were stained with anti-CD4, anti-CD8, and anti-CD68 antibodies. Figure 1 shows the characterization of cellular infiltrates in the cord tissue. The results demonstrate that although an abundance of both CD8 (+) and CD68 (+) cells can be seen in the parenchyma and meninges of the cord tissue, very few CD4 (+) T lymphocytes are observed. This finding is in agreement with previous immunohistochemistry study showing an increasing dominance of CD8 (+) T lymphocytes over the course of HAM/TSP (Umehara et al., 1993).

Characterization of the cells in CTL assay coupled with Fluorescence emission

The immunohistochemistry observation of increased number of CD8 (+) T lymphocytes and CD68 (+) macrophages in the spinal cord tissue led us to question whether mononuclear phagocytes (MPs) such as macrophages and monocytes could be targets of CTLs. To address this question, we first developed the **Microscopy Imaging of Cytotoxic T cells assay coupled with Fluorescence emission (MI-CaFé, pronounced AM-I-CaFé)** to visualize autologous CTL-target conjugate formations in HAM/TSP patient PBMCs. Perforin polarization, a cellular response of immune cytotoxicity requiring movement of vesicles to one side of the cell accompanied by cytoskeletal reorganization, is indicative of effector function and used as a surrogate marker to identify CTLs. HTLV-1 Tax protein expression is used as an indicator of HTLV-1-infected cells. By empirical observations, the optimal time to detect perforin and HTLV-1 Tax protein expression using MI-CaFé was determined to be 8-hours (hrs) in culture. Figure 2 shows the results of perforin and HTLV-1 Tax protein staining with MI-CaFé. Perforin are detected in all CD8 (+) cells (Figure 2A). HTLV-1 Tax proteins are primarily contained within CD4 (+) T lymphocytes (Figure 2B-D). HTLV-1 Tax (+) cell is in contact with perforin (+) cell (Figure 2E-1). 3D rendering of serially captured images of the cells revealed HTLV-1 Tax protein in cytoplasm (Figure 2E-2). However, HTLV-1 Tax expression can be also detected in CD14 (+) cells (Figure 2F).

Visualization of CTL-Target conjugates with MI-CaFé

Using MI-CaFé, various combinations of autologous cell conjugates can be observed. Cell-cell communications are presumed to occur at the junctions of these conjugates either via the virological or immunological synapses. To understand the significance of CTL-target conjugates in disease pathogenesis, we enumerated their frequencies with MI-CaFé. As experimental control, we first determined the percentage of CTL:target conjugates in the PBMCs from normal healthy donors (NDs) (Figure 3). However, since the frequencies of CTLs or target cells may vary between individuals therefore can bias our comparative analysis. Prior to applying statistical comparison, we normalized the observed frequency with a special formula as described in the method section. As shown in figure 3, there is no significant difference between the frequencies of CD4 (+) and CD14 (+) target cells

conjugated with CTLs in ND PBMCs. By contrast, the frequency of CTL:target cell conjugates is significantly elevated in HAM/TSP patient PBMCs. Interestingly, there are more CTL:CD14 (+) conjugates than CTL:CD4 (+) conjugates in HAM/TSP patient PBMCs ($p>0.05$). We also evaluated the percentages of Tax expressing target cells among the conjugates in 4 HAM/TSP patients (Table 1). Based on our observations, both CD4 (+) and CD14 (+) cells expressed high level of HTLV-1 Tax protein, although CTLs conjugated with CD14 (+) cells more than CD4 (+) cells, suggesting that CTLs might be able to recognize MPs more than CD4+ T cells as target cells in HAM/TSP patients. Moreover, in figure 4, HTLV-1 Tax protein can be seen at the cell-cell junction between an infected cell and a CD14 (+) mononuclear macrophage (MP). 3D rendering of serially captured images demonstrates extension of the pseudopodia from the MP followed by transferring of HTLV-1 Tax from the infected cell to an uninfected cell (Figure 4B, C, D).

Spontaneous monocyte cytotoxicity in HAM/TSP PBMCs

We were intrigued by the elevated frequencies of CTL: CD14 (+) conjugates observed in PBMCs of HAM/TSP patients and extended our study to examine their functional relevance. PBMCs of 10 NDs and 10 HAM/TSP patients were cultured without any exogenous stimulation for 18 hrs, and the cell populations including CD3+ T cells and CD14+ cells were analyzed by flow cytometry. Figure 5A is the SSC/FSC profile of a representative HAM/TSP patient and shows significant changes in cell populations after 18 hrs of culture. Phenotypic analysis of cell populations with surface markers anti-CD14 and anti-CD3 antibodies demonstrates the disappearance of CD14 (+) cells post-culture (Figure 5C). The frequency of CD3 (+) T lymphocytes, however, remains unchanged. As demonstrated by our evaluation of percentage of cell numbers within Gate M pre and post-culture in both ND and HAM/TSP patient PBMCs, the drastic decrease in cell number is most significant in HAM/TSP patients PBMCs (Figure 5B). Since monocytes are known to differentiate into macrophages or dendritic cells (DCs) with corresponding increase in MHC class-I molecules and loss of CD14 expression, we performed phenotypic analysis to ensure the spontaneous loss of cell population within Gate M was not due to cell differentiation (data not shown). When the cell population within Gate M was analyzed with anti-CD14 cell surface marker, CD14+ cells were significantly decreased in HAM/TSP patients after the culture, suggesting a mechanism of spontaneous cytotoxicity against MPs specifically in HAM/TSP (Figure 5C).

DISCUSSION

In the present study, we developed the MI-CaFé to visualize *ex-vivo* effector-target conjugate formations in PBMCs. CTL killing of infected or abnormal target cells is a natural host defense which typically subsides following the clearance of foreign substances. But prolonged inflammation which suggests a failure to heal damaged tissues may set the stage for the underlying pathology of chronic inflammatory neurodegenerative disorders. In HAM/TSP, the role of CTLs has been extensively studied due to the extraordinarily high frequencies of HTLV-1 specific CTLs that are found in both PBMCs and CSF. Cytolytic potential of HTLV-1 specific CTLs have been inferred by CTL assays and their functional activity was determined to be comparable to that of Flu-HA or other virus-specific CTLs (Utz and Biddison, 1992) (Hayashi et al., 2008). However, *in vitro* CTL assays require

pulsing of exogenous peptides and effector:target ratios that are well beyond normal physiological conditions. MI-CaFé, on the other hand, has been optimized to allow visualization of effector:target interactions at a single cell level and thus opens the opportunity to examine some previously unresolved questions.

I. Can HTLV-1 exploit the immunological synapse (IS) for viral progeny propagation?

Perforin are molecules secreted by CTLs and inserted into the plasma membrane of target cells to create pores for passage of cytolytic granzyme granules. The effector cell mediates its cytotoxicity on the target cell by establishing a molecular bridge--immunological synapse (IS) through ligation of a cluster of cell surface molecules collectively known as Supramolecular Activation Complex (SMAC) which includes CD2, CD3, CD8, TCR, MHC, ICAM, LFA-1 and Talin. In contrast to neuronal synapse, IS connections are much more dynamic and can assemble and disassemble rapidly to facilitate pairing with multiple targets. Within this synaptic space, targeted signaling events, such as exocytosis of lytic granules perforin/granzymes can take place (Piguet and Sattentau, 2004). Synthesized in the ER, perforin is shuttled to the Golgi and carefully packaged into secretory vesicles. Perforin polarization which involves moving the vesicles to one side of the cell also occurs during IS engagement. In our experiments, elevated frequencies of CTLs conjugated with target cell were specifically observed in PBMCs from HAM/TSP patients, suggesting a heightened immune response that is consistent with observations from other studies (Jeffery et al., 1999) (Kubota et al., 2000).

Our laboratory had previously demonstrated antigen dependent killing by HTLV-1 specific CTLs using a GFP labelled target cell system (Tomaru et al., 2003). Using this system, Tax protein loaded MHC complexes could be tracked moving from a target cell to a CTL. This observation raised a seminal question on whether HTLV-1 could benefit from using the specialized IS for viral spread. HTLV-1 infection is mainly cell-associated and its transmission is accomplished most efficiently by way of the virological synapse (VS) (Pique and Jones, 2012). HTLV-1 Env binding to cell surface membrane molecules is required for viral entry. We had reported the preferential use of heparan sulfate proteoglycans (HSPG) as a HTLV-1 viral receptor (Pinon et al., 2003) (Takenouchi et al., 2007) whereas Manel et al. reported GLUT-1 transporter (Manel et al., 2003) to be the Env binding protein in host cells. Discoveries of both HSPG and GLUT-1 as receptors of HTLV-1 could suggest a stepwise process for viral entry. Although VS may be functionally divergent from IS, they do share some elements in common: (1) both are specialized cell to cell connections that catalyze the transfer of cellular materials, (2) ICAM, LFA-1 and Talin are molecules found in both structures. As we demonstrated with MI-CaFé, Tax proteins in target cell co-localized with CD3 molecules in the CTL (Figure 3), suggesting the close proximity of HTLV-1 Tax or virions to an IS. Moreover, Tax expression at the cell-cell junction has been shown to facilitate viral spread (Chevalier et al., 2014).

II. Does detection of Tax expression suggest internal infection of MPs?

Although MPs including monocytes, macrophages and DCs of HAM/TSP patients were shown to be infected with HTLV-1, the estimated percent of infected MPs were as high as up to 5% in PBMCs and up to 95 to 99% in the MP enriched fractions contained provirus after

long term in vitro culture (Koyanagi et al., 1993) (Macatonia et al., 1992) (Venkateshan et al., 1996). In situ PCR or hybridization pathological studies have revealed that HTLV-1 preferentially infect CD4 (+) T lymphocytes but other cell types at the site of the inflammation of human spinal cord and muscle tissues (Higuchi et al., 1996) (Matsuoka et al., 1998) (Moritoyo et al., 1996) (Matsuura et al., 2008). Conversely, our result showing high percentage of CD14 (+) target cells expressing HTLV-1 Tax and a large amount of HTLV-1 Tax being transferred from a presumably infected CD4 (+) T lymphocyte to a CD14 (+) monocyte suggest a possible mechanism whereby viral antigen can be spread to uninfected cells that can be potential targets for CTLs (Figure 3). Moreover, Jaworski et al. suggested exosomes derived from HTLV-1-infected cells contain viral proteins that contribute to HTLV-1-induced pathogenesis (Jaworski et al., 2014). Therefore, Tax positivity in the targeted CD14 (+) monocytes doesn't necessary mean its internal infection. However, some infected MPs may transfer virus to autologous primary CD4 (+) T lymphocytes by a mechanism known as *trans*-infection (Jones et al., 2008).

III. What implications does acquisition of HTLV-1 Tax by MPs have on pathogenesis of neurologic diseases?

An unexpected pathology we described in this report was the apparent lack of CD4 (+) T lymphocytes among high frequencies of CD8 (+) T lymphocytes and macrophages in the spinal cord tissue of a HAM/TSP patient. It is axiomatic that HTLV-1 infected CD4 (+) T lymphocytes cross the blood-brain-barrier and trigger an overwhelming CTL response against the virus thus causing tissue damage and further recruitment of macrophages into the CNS.

Therefore, in the absence of Tax positive CD4 (+) T lymphocytes, what factors could be responsible for maintaining the persistent CNS inflammation? An explanation maybe derived from two of our experiments. First, we demonstrated the transfer of HTLV-1 Tax from infected CD4 (+) T lymphocytes to MPs using MI-CaFé (Figure 4). Second, when PBMCs were cultured, spontaneous cytotoxicity against MPs was specifically observed in HAM/TSP patients, but not in NDs (Figure 5). Spontaneous MPs cytotoxicity following antigen capture suggests a strong HTLV-1 CTL response, which is consistent with HAM/TSP neuropathology. Previous study by our laboratory showed that MPs play an important role in pathogenesis of HAM/TSP by accelerating HTLV-1 Tax expression in CD4 (+) T lymphocytes and also activating CTL degranulation (Enose-Akahata et al., 2008). Collectively, these results suggest a possible mechanism that fosters chronic CNS inflammation after the initial infection has subsided.

MATERIALS AND METHODS

Patients and cell preparation

HAM/TSP diagnosis was assessed according to the World Health Organization guidelines (Osame, 1990). HTLV-1 seropositivity was determined by Western blot analysis (MP Biomedicals, CA). Blood samples were obtained after written informed consent and this study was approved by the National Institute of Neurologic Disorders and Stroke (NINDS) Institutional Review Board.

Cryopreserved PBMCs were thawed and washed twice with PBS containing 1% fetal calf serum (FCS) and 0.1% NaN₃ before use. Culture media used was RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Lonza, Walkersville, MD), and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA).

Immunohistochemistry (IHC)

Paraffin embedded block of the spinal cord from the patient with HAM/TSP was cut in 8mm thick sections and placed on glass slides. After a process of deparaffinization, prepared slides were heated in a glass container filled with Target Retrieval Solution, pH9 (Dako, CA, USA) for 10 minutes (min) at 121 °C by autoclave machine, Tuttnauer 3850-M (Tuttnauer, NY, USA). After cooling for 30 min and rinsing in water, sections were immersed in 0.03% hydrogen peroxide solution (Dako) for 5 min to quench endogenous peroxidase activity. Primary antibodies, mouse anti-CD4 antibody (1F6, AbD Serotec, Oxford, UK), anti-CD8 antibody (4B11, AbD Serotec) or anti-CD68 (PG-M1, Dako) were incubated with sections for 1-hour at room temperature (RT), rinsed with PBS prior to addition of HRP-labeled polymer-conjugated anti-mouse antibody reagent (EnVision™+, Dako) for 30 min at RT and rinsed again. Finally, peroxidase was visualized by 3-Amino-9-ethylcarbazole; AEC (Dako), yielding a red color. The sections were counterstained with hematoxylin and analyzed with light microscopy.

Flow cytometry study

PBMCs from 10 patients with HAM/TSP and 10 normal healthy donors (NDs) were cultured in 24 well culture plate at a concentration of 1×10^6 cells/ml in RPMI-1640 at 37 °C. PBMCs were collected after 18 hours (hrs) of culture and washed in PBS containing 1% FBS and 0.1% NaN₃. Cells were stained with antibodies for surface markers, PE-conjugated anti-CD14 antibody (M5E2; BD biosciences, San Jose, CA), PE-Cy5 conjugated anti-CD4 antibody (RPA-T4; BD biosciences) and APC-conjugated anti-CD3 antibody (UCHT-1; BD biosciences) for 30 min at RT and washed prior to acquisition and analysis on the FACS Calibur (BD Biosciences). CD14 (+) cell population (%) of each sample cultured for 18 hrs was estimated. For comparison with the result of immunocytochemistry (ICC), PBMCs from 5 patients with HAM/TSP and 5 NDs were cultured on the chambered glass slide for 8 hrs and stained with antibodies for surface markers, followed by fixation with 4 % paraformaldehyde for 15 min at 4 °C and intracellular staining with FITC-conjugated anti-HTLV-1 Tax antibody (Lt-4). The frequencies (%) of CD4 (+) cells, HTLV-1 Tax positive CD4 (+) cells, and CD14 (+) cells in the PBMC were evaluated.

Antibody staining in MI-CaFé

Four well chamber slide glasses (Thermo Scientific, MA) were doubly coated with poly-L-lysine (Sigma, St. Lois, MO) before use. PBMCs from 5 HAM/TSP patients and 5 NDs were cultured in triplicates at a concentration of 1×10^6 cells/ml for 8 hrs at 37 °C. One of the 3 wells was used for FACS to evaluate the frequency of the CD4 (+) and CD14 (+) cells in the PBMC. The other one was used for **MI-CaFé** with anti-perforin (50x)(dG9; Santa Cruz), anti-HTLV-1 Tax (500x) (Lt-4) and anti-CD4 antibody (50x)(MT-310; DAKO) and the last one was for ICC with anti-perforin, anti-HTLV-1 Tax and anti-CD14 antibody (25x)

(UCHM-1; Santa Cruz). Cultured PBMCs for FACS were collected after 8-hr culture and stained as described before. Cultured PBMCs for MI-CaFé were gently washed with PBS containing 1% FBS and 0.1% NaN₃ several times. Cells were then stained with mouse anti-CD4 or CD14 antibody for 15 min at RT and washed again. After fixation with 4 % paraformaldehyde for 15 min at 4 °C, PBS containing 0.01% Triton-X (PBS-T) was used for washing. Cells were stained with the two antibodies, mouse anti-HTLV-1 tax antibody and mouse anti-perforin antibody diluted with PBS-T for 30 min at RT and washed again. Subsequently, a cocktail of antibodies were used as secondary detection at 200x dilution with PBS-T: Alexa488-conjugated goat anti-mouse IgG3 (Life technologies, CA) for tax detection, Alexa594-conjugated goat anti-mouse IgG2b (Life technologies) for perforin detection and Alexa647-conjugated goat anti-mouse IgG1 or IgG2a (Life technologies) for CD4 or CD14 detection. After incubation with these antibodies for 30 min at RT, cells were washed again. The chamber wall was removed and cells were mounted with Prolong Gold with DAPI (Life technologies). Observation was performed with Zeiss Axiovert 200M equipped with mercury lamp house HBO-100 and four appropriate dichroic filters (Carl Zeiss MicroImaging Inc, NY).

Phenotypic analysis of target cell by MI-CaFé

To determine the target population of cytotoxic T cells in the PBMC, we looked for conjugates exhibiting polarized perforin (perforin cell) that was in contact with another cell (target cell) using fluorescence microscopy. The phenotype of target cells were characterized as follow: At first, we looked for a perforin cell in contact with a target cell using just two filters for perforin and DAPI (counter stain) of fluorescence microscopy. To avoid difficulty in deciphering perforin and target cell-cell conjugates, we excluded scenarios when a perforin positive cell was found within a cell clot. Images of the remaining cell conjugates were captured with four filters for perforin, DAPI, CD4 or CD14 and HTLV-1 Tax. Acquired images were then deconvoluted mathematically using the imaging software, Volocity (Improvision Inc, NY) and modified into 2-dimensional or 3-dimensional images for further evaluation. The frequencies of various combinations of perforin containing cell conjugates were evaluated: (1) CD4 (+) cell, (2) CD14 (+) cell, (3) HTLV-1 Tax positive CD4 (+) cell, (4) HTLV-1 Tax positive CD14 (+) cell. Prior to applying statistical comparison, we needed to normalize the observed frequency of CTL:target with the following formula: Normalized Value (NV) = $[(F - AF)/AF] \times 100\%$. F equals the frequency of CTL:target conjugates observed by MI-CaFé and AF represents the actual frequency of CD4 (+) or CD14 (+) cells detected by FACS analysis.

HTLV-1 proviral DNA load measurement

DNA was extracted from the cells using QIAamp DNA Blood Mini Kit (Qiagen). HTLV-1 proviral tax DNA load was measured using real-time PCR with an ABI PRISM 7700 Sequence Detector (life technologies), as previously described (Oh et al., 2005).

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Highlight

- An optimized visualization analysis to explore the interactions between CTLs and virus infected or viral antigen presenting target cells.
- Visualization of HTLV-1 Tax protein localized at the cell-cell junctions and also tracked moving from an infected cell to a CD14 (+) mononuclear phagocyte (MP).
- Antigenic presentation of HTLV-1 Tax by MPs can be inferred by their spontaneous cytotoxicity in vitro culture.
- Spontaneous cytotoxicity against MPs in HAM/TSP PBMCs suggests a mechanism of chronic inflammation, secondary to low level of persistent virus infection within the central nervous system

CNS Inflammation with HTLV-1 Tax

Figure 1. Immunohistochemistry staining of HAM/TSP spinal cord tissue

Infiltrating cells were characterized with antibodies against CD4 (A), CD8 (B) and CD68 (C) with formalin-fixed paraffin embedded spinal cord tissue from a patient with HAM/TSP. Although, very few CD4+ T lymphocytes were observed among the inflammatory cell infiltrates, CD8+ T lymphocytes and CD68+ macrophages, by comparison, were the predominate cell populations found in the parenchyma and meninges.

Figure 2. Detection of effector:target conjugates with MI-CaFé

PBMCs from HAM/TSP patients were cultured on glass-chambered slides for 8 hrs; perforin, and HTLV-1 tax expression were detected with MI-CaFé. A. All the perforin (red) containing cell were positive for CD8 (green), B.C. HTLV-1 tax (green) was expressed on CD4+ cell (purple), D. HTLV-1 tax expression did not co-localize with CD14+ cell (red), E-1. Tax positive CD4+ cells in contact with a perforin cell. E-2. 3-D image of the picture E-1, F-1. Tax positive CD14+ cell in contact with perforin cell, F-2.3.4. 3-D image of the picture F-1. 3-D image revealed tax inside of CD14 molecules.

Figure 3. Elevated frequencies of effector:target conjugates in HAM/TSP patients

A. Frequencies of CTL:CD4+ or CTL:CD14+ cell conjugates in PBMCs of NDs and HAM/TSP patients, B. Effector:target conjugates after normalization using the formula specified in the Methods section. There was no significance difference between the frequencies of CTL:CD4+ or CTL:CD14+ cells in ND PBMCs. By contrast, after normalization, CTL:CD14+ conjugate formations were statistically elevated in HAM/TSP patients compared to NDs ($p < 0.05$). Moreover, there was also a significant difference between CTL:CD4+ and CTL:CD14+ conjugates in HAM/TSP PBMCs ($p < 0.05$).

Figure 4. Transfer of HTLV-1 Tax from an infected cell to CD14+ MP

Polarized HTLV-1 Tax at the cell-cell junction. B. 3D rendering of HTLV-1 Tax transfer between an infected cell and a CD14+ MP. C-1. Visualization of antigenic capture by CD14+ MP cell. C-2,3,4. 3D rendering of antigen capture shown in C-1. D. HTLV-1 Tax positive MP in contact with CD3+ T lymphocytes. D-1. Surface molecule CD3 (red) of the contacted cell is replaced by tax in MP at the contact site (arrow head). D-2. Differential interference contrast image of D-1. D-3. Three dimensional image of D-1 revealed interaction of CD3+ T lymphocyte with pseudopodia of HTLV-1 Tax positive MP.

Figure 5. Spontaneous MP cytotoxicity in HAM/TSP patient PBMC after 18 hours of culture
PBMCs of 10 HAM/TSP patients and 10 NDs were cultured for 18 hours without any stimulation and analyzed by FACS. **A.** Representative dot plots of the cell population in HAM/TSP patient pre/after culture. FACS analysis demonstrating disappearance of cell population circumscribed within Gate M from HAM/TSP patient PBMCs after culture. Surface marker staining with anti-CD14 and anti-CD3 antibodies showed CD14+ and CD3+ cells in live cells. **B.** Cell frequencies of M population in total lymphocytes were significantly decreased after 18 hours culture. **C.** Cell frequencies of CD14+ cells in M population were significantly decreased after 18 hours culture in HAM/TSP patient PBMCs.

Table 1
The frequencies of HTLV-1 infected cells in HAM/TSP patients

The frequencies of HTLV-1-infected cells in fresh PBMC, in CD4 or CD14 positive target cell in contact with CTL after 18hours culture.

Patient ID	Proviral load (%) in patient's PBMC	%Tax-positive cell in target CD4 cells	%Tax-positive cell in target CD14 cells
A	23.2 %	18.6%	9.1%
H	9.2%	12.5%	2.9%
D	19.2%	35.3%	12.5%
M	68.0%	15.4%	4.0%