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Use of human antigen presenting cell gene array profiling to examine the effect of human T-cell leukemia virus type 1 Tax on primary human dendritic cells

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Human T-cell leukemia virus type 1 (HTLV-1) is etiologically linked to adult T-cell leukemia and a progressive demyelinating disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). One of the most striking features of the immune response in HAM/TSP centers on the expansion of HTLV-1-specific CD8^{+} cytotoxic T lymphocyte (CTL) compartment in the peripheral blood and cerebrospinal fluid. More than 90% of the HTLV-1-specific CTLs are directed against the viral Tax (11–19) peptide implying that Tax is available for immune recognition by antigen presenting cells, such as dendritic cells (DCs). DCs obtained from HAM/TSP patients have been shown to be infected with HTLV-1 and exhibit rapid maturation. Therefore, we hypothesized that presentation of Tax peptides by activated DCs to naïve CD8^{+} T cells may play an important role in the induction of a Tax-specific CTL response and neurologic dysfunction. In this study, a pathway-specific antigen presenting cell gene array was used to study transcriptional changes induced by exposure of monocyte-derived DCs to extracellular HTLV-1 Tax protein. Approximately 100 genes were differentially expressed including genes encoding toll-like receptors, cell surface receptors, proteins involved in antigen uptake and presentation and adhesion molecules. The differential regulation of chemokines and cytokines characteristic of functional DC activation was also observed by the gene array analyses. Furthermore, the expression pattern of signal transduction genes was also significantly altered. These results have suggested that Tax-mediated DC gene regulation might play a critical role in cellular activation and the mechanisms resulting in HTLV-1-induced disease. *Journal of NeuroVirology* (2006) **12,** 47–59.

Keywords: dendritic cells; HTLV-1 Tax; HTLV-1; microarray

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of two major diseases; a progressive lymphoma designated adult T cell leukemia (ATL) and a debilitating neurologic disease known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Uchiyama, 1997). HAM/TSP is manifested by an increased HTLV-1 proviral load and a hyperstimulated immune response including the expression of inflammatory cytokines and HTLV-1-specific antibodies. Accumulation of CD4^{+} T

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cells and anti-HTLV-1 cytotoxic $CD8^+$ T lymphocytes (CTLs) in the cerebrospinal fluid (CSF) and spinal cord lesions of HAM/TSP patients represents a pivotal event in the acute stage of this disease (Kannagi *et al*, 1992; Umhera *et al*, 1993). However, the most prominent characteristic of the cellular immune response in chronic disease is the oligoclonal expansion of HTLV-1 transactivator protein Tax-specific $CD8^+$ CTLs in both peripheral blood and CSF of infected individuals (Jacobson *et al*, 1992; Levin and Jacobson, 1997; Osame, 2002). In some HAM/TSP patients carrying the HLA-A*201 allele, 30% of all $CD8^+$ T cells in peripheral blood (Yamano *et al*, 2002) and an even greater frequency of $CD8^+$ T cells in the CSF (Elovaara *et al*, 1993; Nagai *et al*, 2001) are highly specific for the HTLV-1 Tax (11–19) peptide (LLFGYPVYV) which is defined as the immunodominant epitope (Koenig *et al*, 1993).

The 40 kDa HTLV-1 transactivator protein Tax plays an important role in disease pathogenesis and has been central to many theories regarding the progression of HAM/TSP. One such theory involves the presence of a Tax-specific CTL response implying that Tax is available for immune recognition by antigen-presenting cells (APCs) such as dendritic cells (DCs). DCs obtained from the peripheral blood of HAM/TSP patients have been found to be infected with HTLV-1 (Macatonia *et al*, 1992) and exhibit rapid maturation (Ali *et al*, 1993). DCs have also been shown to be susceptible to infection *in vitro* (Makino *et al*, 1999) and studies examining HTLV-1 infection of DCs confirmed that DCs co-cultured with an HTLV-1-producing cell line stimulated autologous T cell proliferation, suggesting that HTLV-1-infected DCs play a crucial role in the production of autoreactive T cells in HAM/TSP patients (Makino *et al*, 2001). Other theories focusing on the role of Tax in progression of HAM/TSP have suggested that the immune response mounted against the viral transactivator protein may promote molecular mimicry involving antibodies that recognize both Tax and the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) (Levin and Jacobson, 1997; Levin *et al*, 2002). It has also been postulated that HTLV-1-induced damage to the central nervous system (CNS) is not a direct consequence of viral infection of neuronal cells but rather involves an immune-mediated disease in which Tax can impart some of its pathologic effects by functioning as an extracellular cytokine (Brady, 1992; Lindholm *et al*, 1990, 1992; Marriott *et al*, 1991; Marriott *et al*, 1992). The presence of cell-free Tax in CSF has been demonstrated during progression of HAM/TSP (Cartier Rovirosa and Ramirez, 2005) that may be the result of apoptosis or necrosis of HTLV-1-infected cells. Tax can also be released by secretion from Tax-expressing cells. Secretion of full-length Tax was demonstrated from both Tax-transfected cells (Alefantis *et al*, 2005a) as well as HTLV-1-infected cells (Lindholm *et al*, 1990; Marriott *et al*, 1991).

Extracellular Tax has been shown to induce production and secretion of TNF-α from a human neuronal cell line (Cowan *et al*, 1997). Primary human microglial cells have also been shown to respond to extracellular Tax by secreting inflammatory cytokines TNF-α, IL-1β, and IL-6 (Dhib-Jalbut *et al*, 1994). The effects of extracellular Tax are not limited to the CNS since Tax-stimulated primary human peripheral blood macrophages have also been shown to produce and secrete TNF-α, IL-1β, and IL-6 (Dhib-Jalbut *et al*, 1994). Soluble Tax treatment has been shown to induce the nuclear accumulation of NF-κB (Brady, 1992; Lindholm *et al*, 1990), as well as the expression of the immunoglobulin light chain (Brady, 1992; Lindholm *et al*, 1992), TNF-β (Brady, 1992), and IL-2 receptor in lymphoid cells (Marriott *et al*, 1992). In a separate study focused on the effect of HTLV-1 Tax on the functions of primary human monocyte-derived dendritic cells (MDDCs), we have performed extensive biochemical and immunological analyses including imaging of Tax binding, entry, and intracellular trafficking in DCs, kinetics and dose-dependence of Tax-induced production of Th1 cytokines (IL-12 and TNF-α) and β-chemokines (MIP-1α, MIP-1β, and RANTES) at the protein level, phenotypic analysis of MHC and costimulatory molecules on DCs at cell-surface, and the ability of Tax-treated DCs to simulate autologous and antigen-specific T cell proliferation (Jain and Wigdahl, unpublished observations). However, the molecular pathways whose expression is differentially regulated during the process of Tax-mediated DC activation and maturation remain undefined.

Microarray technology serves as a powerful means to extensively and systematically assess entire transcriptomes and has previously been used to analyze expression of transcription factors, cell cycle-regulated kinases, and DNA repair genes in HTLV-1-infected T cells (de La Fuente *et al*, 2000). Studies using cDNA microarrays to examine intracellular Tax-mediated transcriptional changes in human Jurkat T cell lines have previously been performed (Ng *et al*, 2001). In addition, the use of DNA microarrays to study the effect of the HIV-1 transactivator protein Tat on the gene expression program of immature DCs has also been reported (Izmailova *et al*, 2003). Utilizing a genome-wide mouse microarray, we have previously shown that purified HTLV-1 Tax protein induced the mRNA expression of many genes associated with activation and maturation in a murine dendritic cell line JAWS II. A component of these results has been reported (Mostoller *et al*, 2004). Due to the considerable differences between mouse and human DCs, we have proceeded to examine the effects of Tax on human DCs. Therefore, in the present study, pathway-specific cDNA microarrays have been used to examine transcriptional changes in human MDDCs stimulated with extracellular HTLV-1 Tax. The antigen presenting cell gene arrays have been spotted with 192 cDNA fragments specific for genes

involved in dendritic cell activation and maturation. The microarray-based analyses presented herein provide initial clues with respect to the genes involved in the Tax-mediated effects on DCs and provide novel insights into Tax-induced signaling in DCs.

Results and discussion

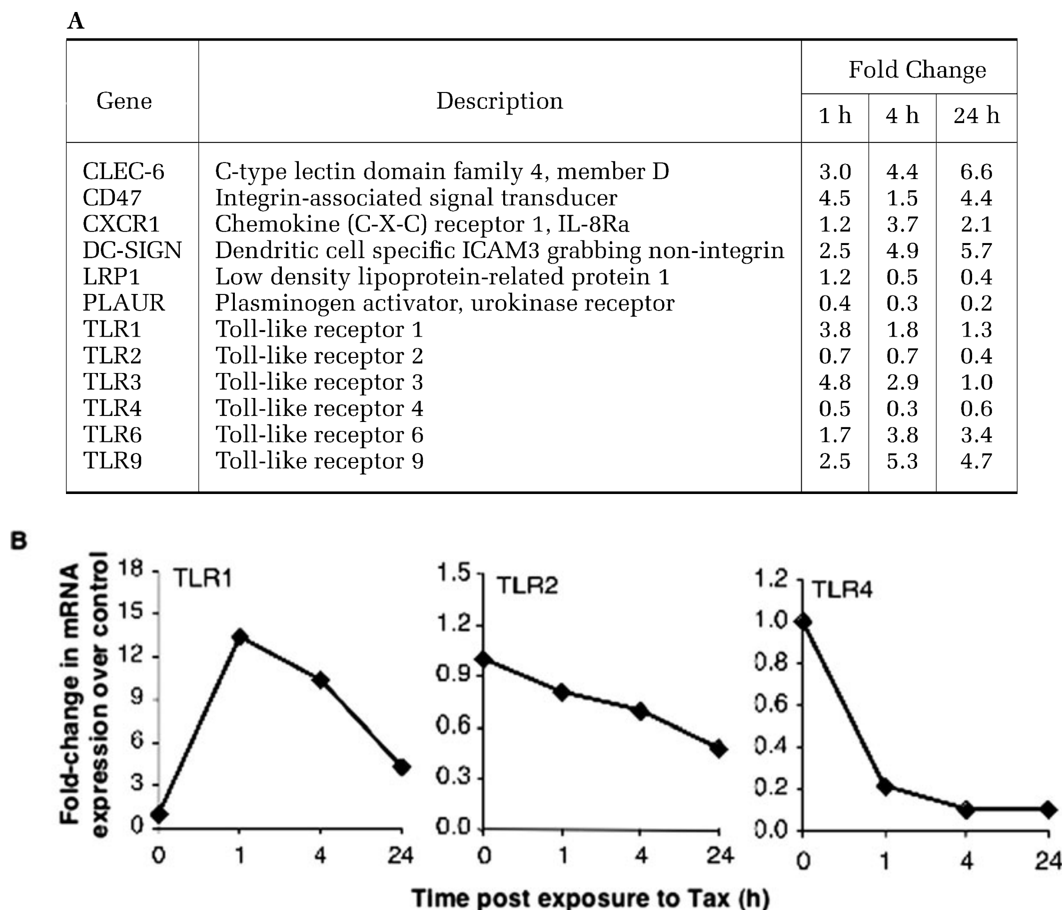
Of the two major diseases associated with HTLV-1 infection, ATL is characterized by transformation of $CD4^+$ T-cells while HAM/TSP is categorized as an autoimmune disease manifested by an extensive proliferation of Tax-specific $CD8^+$ T cells. T-cell proliferation in both cases is triggered by antigen presentation, costimulation, and cytokine production by antigen presenting cells such as DCs. DCs are of particular significance in the pathogenesis of HTLV-1-associated neurologic disease, as the development of HAM/TSP is associated with their rapid maturation (Ali *et al*, 1993). Furthermore, HTLV-1 is known to infect DCs both *in vitro* (Ali *et al*, 1993; Makino *et al*, 1999) and *in vivo* (Macatonia *et al*, 1992), suggesting that HTLV-1-infected DCs play a crucial role in the production of the autoreactive T-cells observed in HAM/TSP patients. This study was performed in order to establish the role of DCs in HTLV-1-associated neurologic disease, with particular emphasis on characterizing the genesis of Tax (11–19)-specific CTL response. The results reported herein have significant physiological implications and support the general hypothesis that DCs once exposed to Tax, either by infection or uptake from the extracellular environment, undergo activation with processing and presentation of Tax to T-cells in the context of MHC class molecules ultimately initiating an intense Tax-specific CTL response, characteristic of HAM/TSP.

As a component of previous investigations concerning DC function, a genome-wide mouse microarray (13,443 mouse genes) analysis was performed using RNA derived from a mouse dendritic cell line JAWS II (Mostoller *et al*, 2004). After categorizing 200 differentially expressed genes based on their function, the most distinguished category was comprised of genes associated with the process of DC activation and maturation. Mouse microarray analyses also revealed several genes related to cell cycle and DNA repair, cytoskelton/matrix remodeling genes, and genes encoding growth factors that were significant, but may not be DC-specific. In view of the fact that mouse and human DCs represent significant differences, we have now moved on to the utilization of primary human DCs. However, the use of primary cells has presented some limitations with respect to obtaining sufficient starting genetic material for the rapid completion of a genome-wide array analysis including duplicate arrays coupled with dye-flips, with each array requiring at least 20 μg of total RNA. Therefore, a human antigen presenting cell cDNA array has provided an effective option since it focused on a specific set of genes associated with DC activation and maturation, enabling us to simultaneously work with limited amounts of mRNA from four different time points after Tax exposure.

In order to analyze kinetics of gene expression induced by HTLV-1 Tax, MDDCs were treated with purified Tax protein for 1, 4, and 24 h. Cells treated with mock fluid served as negative control. Approximately 100 genes were differentially regulated subsequent to Tax stimulation. Fold changes in gene expression were obtained relative to mock-treated cells (the negative control). To fulfill the criterion of differential expression, selected genes were reproducibly up- or down-regulated more than two-fold. For comparative purposes, genes were categorized into functional groups including cell surface receptors, signal transduction genes, activation and maturation genes, and antigen uptake and presentation genes. Results of the microarray analyses were verified by real-time polymerase chain reaction (PCR) for genes of interest and representative genes from each category have been examined.

Cell surface receptors

Immature DCs capture antigen through phagocytosis, pinocytosis, or endocytosis via different groups of receptor families (Banchereau and Steinman, 1998). Treatment of MDDCs with HTLV-1 Tax resulted in the differential expression of a number of cell surface receptors, the kinetic expression of which is listed in Figure 1A. One such family of cell surface receptors included toll-like receptors (TLRs). Antigen recognition by TLRs triggers signaling cascades that result in subsequent immune activation (Underhill and Ozinsky, 2002). The recognition of invading pathogens by TLRs on DCs is also known to induce proinflammatory cytokine production and enhanced antigen presentation to naïve T-cells, ultimately activating antigen-specific adaptive immune responses (Bowie and Haga, 2005). Among the TLRs, TLR2 and TLR4 were down-regulated by 1 h and the expression level of these genes decreased continually. In contrast, TLR1 and TLR3 were maximally expressed at 1 h (3.8- and 4.8-fold, respectively), and TLR6 and TLR9 were up-regulated at the 4 h time-point (3.8- and 5.3-fold, respectively). The differential expression for these TLRs was confirmed by real-time PCR using gene-specific primers (Figure 1B) and was found to be in good correlation with the microarray results; TLR1 being expressed maximally at 1 h (13-fold), and TLR2 and TLR4 being down-regulated at increasing times of Tax exposure (0.8-, 0.7-, and 0.5-fold for TLR2 and 0.2-, 0.1-, and 0.1-fold for TLR4 at 1, 4, and 24 h, respectively). The differences in expression levels of the TLRs examined was not surprising since every TLR has been shown to recognize molecules derived from a unique class of microbial agents (Takeda *et al*, 2003). TLR2, for example, is known to sense certain bacterial pathogens by cooperation with TLR1 and TLR6



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Gene	Description	Fold Change		
		1 h	4 h	24 h
CLEC-6	C-type lectin domain family 4, member D	3.0	4.4	6.6
CD47	Integrin-associated signal transducer	4.5	1.5	4.4
CXCR1	Chemokine (C-X-C) receptor 1, IL-8Ra	1.2	3.7	2.1
DC-SIGN	Dendritic cell specific ICAM3 grabbing non-integrin	2.5	4.9	5.7
LRP1	Low density lipoprotein-related protein 1	1.2	0.5	0.4
PLAUR	Plasminogen activator, urokinase receptor	0.4	0.3	0.2
TLR1	Toll-like receptor 1	3.8	1.8	1.3
TLR2	Toll-like receptor 2	0.7	0.7	0.4
TLR3	Toll-like receptor 3	4.8	2.9	1.0
TLR4	Toll-like receptor 4	0.5	0.3	0.6
TLR6	Toll-like receptor 6	1.7	3.8	3.4
TLR9	Toll-like receptor 9	2.5	5.3	4.7

Figure 1 Expression of cell surface receptors on MDDCs exposed to Tax. MDDCs were treated with Tax (1 μg/ml) for 1, 4, and 24 h after which time the mRNA was harvested, converted to a biointylated cDNA probe, and hybridized onto a human dendritic cell gene array spotted with 192 gene-specific fragments on a nylon membrane. (**A**) mRNA expression levels of cell surface receptors that were differentially regulated following treatment of MDDCs with Tax are listed. (**B**) Real-time PCR analysis using gene-specific primers confirmed the expression of Toll-like receptors TLR1, TRL2, and TLR4. The threshold cycle (C_T) values obtained for triplicate samples were averaged, normalized to levels of β-actin, and fold-change in mRNA expression with respect to control was calculated as described in the Materials and Methods.

(Bowie and Haga, 2005), TLR4 is known to recognize lipopolysaccharide (LPS) (Poltorak *et al*, 1998), TLR3 has been shown to recognize double-stranded RNA from double-stranded and single-stranded viruses of negative polarity (Alexapoulou *et al*, 2001), and the role of TLR9 has been implicated in the recognition of unmethylated CpG DNA found in prokaryotic genomes (Hemmi *et al*, 2000). Microarray analyses followed by real-time PCR validation demonstrated a decreased expression of TLR4, suggesting that the pattern of Tax-mediated DC maturation differs from the effect of LPS on DC maturation. In contrast, the expression of TLR1, TLR3, TLR6, and TLR9 was up-regulated, suggesting that signaling pathways triggered via these receptors from the membrane may play a role in Tax-mediated DC activation and maturation.

Treatment of MDDCs with HTLV-1 Tax also resulted in the differential expression of another important family of receptors known as C-type lectin receptors (CLRs). C-type lectins represent a recently identified group of cell surface molecules involved in the recognition of carbohydrate structures present on antigens (Figdor *et al*, 2002). In addition to functioning as adhesion receptors, CLRs have recently been shown to play a role in antigen processing and presentation in DCs (Figdor *et al*, 2002). Microarray analyses demonstrated a time-dependent increase in the expression of two CLRs: CLEC-6 (3-, 4.4-, and 6.6-fold at 1, 4, and 24 h, respectively) and dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN) (2.5-, 4.9-, and 5.7-fold at 1, 4 and 24 h, respectively), suggesting a role for these C-type lectins in Tax-induced DC activation by functioning as adhesion molecules and/or antigen receptors. Uptake of Tax following interaction with these cell surface receptors may activate signaling cascades, resulting in a number of events that culminate with DC activation and maturation.

Signal transduction genes

Having identified the differential expression of TLRs in MDDCs activated with HTLV-1 Tax, signaling events triggered via these receptors were subsequently examined. The kinetic expression levels for

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Gene	Description	Fold Change		
		1 h	4 h	24 h
ATF 4	Activating transcription factor 4 (CREB-2)	2.7	3.5	1.8
Cystatin C	Amyloid angiopathy and cerebral hemorrhage	5.2	4.6	4.9
EB13	Epstein-Barr virus induced gene 3	0.6	4.7	3.1
ISG15$^+$	(GIP2) Interferon, alpha-inducible protein	1.4	3.8	1.8
IFI1616$^+$	(GIP3) Interferon, alpha-inducible protein	1.8	3.2	4.7
IFI16$^+$	Interferon, gamma-inducible protein 16	4.1	3.8	4.2
IFI27$^+$	Interferon, gamma-inducible protein 27	2.4	1.8	2.2
IFITM3$^+$	Interferon induced transmembrane protein 3	2.8	3.6	3.6
ISG20$^+$	Interferon stimulated gene 20 kDa	2.5	4.4	2.4
NF-κB1	NFκB subunit 1, p105	3.5	6.4	3.2
NF-κB3	NFκB subunit 3, p65; Rel A	1.1	5.4	3.3
Rel B	v-rel reticuloendotheliosis viral oncogene homolog B	1.3	2.9	8.6
S100A4	S100 calcium binding protein A4	4.3	2.8	2.9
S100B	S100 calcium binding protein, beta	3.8	3.6	1.5
ZNF398	Zinc finger protein 398	2.6	3.0	1.0

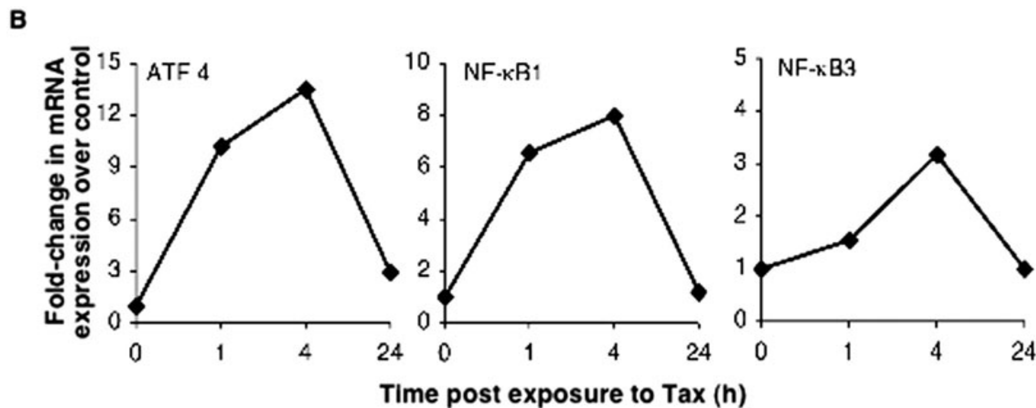

Figure 2 Tax modulates the expression of genes via cellular signaling cascades and transcription factors. (**A**) Microarray analyses revealed a number of signal transduction genes as listed. (**B**) At various times (1, 4, and 24 h) post-treatment, mRNA from Tax-treated MDDCs was isolated and transcribed to cDNA. Real-time PCR analysis using gene-specific primers for ATF-4, NF-κB1, and NF-κB3 validated the microarray data. The results are represented as fold change in mRNA expression of indicated genes with respect to control, calculated from threshold cycle values obtained from triplicate samples.

a number of signal transduction genes are listed in Figure 2A. Microarray analyses demonstrated a significant increase in the expression of genes of the NF-κB family including NF-κB1 (3.5-, 6.4-, and 3.2-fold at 1, 4, and 24 h, respectively), NF-κB3/Rel A (1.1-, 5.4-, and 3.3-fold at 1, 4, and 24 h, respectively), and Rel B (1.3-, 2.9-, and 8.6-fold at 1, 4, and 24 h, respectively). These results were confirmed by real-time PCR (Figure 2B). The NF-κB/Rel family of enhancer binding proteins play a central role in cell growth and survival (Jeang, 2001). Activation of the NF-κB pathway has been demonstrated by both endogenous (Kanno *et al*, 1994; Kanno *et al*, 1995; Harhaj *et al*, 2005) and exogenous Tax (Lindholm *et al*, 1990; Lindholm *et al*, 1992). Extracellular Tax protein stimulates nuclear NF-κB DNA binding activity and the expression of NF-κB-responsive Ig kappa and TNF-β genes in lymphoid cells (Brady, 1992; Lindholm *et al*, 1990; Lindholm *et al*, 1992). It was also shown that Tax activated RANTES mRNA expression via NF-κB (Mori *et al*, 2004). More recently, the role of HTLV-1 and Tax in modulating the expression of CD40 and other members of the TNF/TNFR family of genes via the NF-κB pathway has also been implicated (Harhaj *et al*, 2005). Activation of the NF-κB pathway initiated by TLRs may provide an autocrine loop for continuous DC activation and the release of chemokines and cytokines in order to maintain extensive T cell proliferation and a hyper-stimulated immune response characteristic of HAM/TSP.

HTLV-1 Tax is also known to alter cellular gene expression by interacting with a variety of cellular transcription factors and cell cycle control elements. One such transcription factor is the cAMP responsive element binding protein 2 (CREB-2, also called ATF-4). Several studies have indicated that Tax can trans-activate viral gene expression by interacting with

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Gene	Description	Fold Change		
		1 h	4 h	24 h
IFN-α1	Interferon, alpha 1	1.7	4.9	3.2
IFN-β1	Interferon, beta 1, fibroblast	3.5	7.2	2.8
IFN-γ	Interferon, gamma	8.9	3.7	4.3
IL-12p35	Interleukin 12, CTL maturation factor 1	2.1	4.0	2.8
IL-12p40	Interleukin 12, CTL maturation factor 2	2.5	3.3	2.3
IL-15	Interleukin 15, T cell activation and proliferation	2.6	3.2	3.9
IL-17	Interleukin 17	2.2	1.0	1.6
IL-2	Interleukin 2, T cell proliferation	3.0	2.0	1.5
IL-6	Interleukin 6, Interferon, beta 2	3.4	4.9	4.4
CCL3	Macrophage inflammatory protein 1-α, MIP-1α	2.1	2.1	2.1
CCL5	Regulated upon activation, normally T-cell expressed, RANTES	7.8	5.1	4.6
CCL17	Thymus activation-regulated chemokine, TARC	1.1	2.3	3.4
CCL18	Pulmonary & activation regulated-chemokine, PARC	1.1	2.6	1.9
CCL19	Macrophage inflammatory protein 3-β, MIP-3β	1.5	6.7	2.8
CCL20	Macrophage inflammatory protein 3-α, MIP-3α	2.0	5.1	1.8
CCL21	Secondary lymphoid tissue chemokine, SLC	1.1	3.2	2.3
TNF-α	Tumor necrosis factor	3.6	2.8	2.3
TNFSF4	TNF (ligand) superfamily, member 4	3.9	4.6	2.1
TNFSF11	TNF superfamily (ligand), member 11	4.9	3.4	3.7
TNFSF14	TNF superfamily (ligand), member 14	4.5	4.2	4.1

B

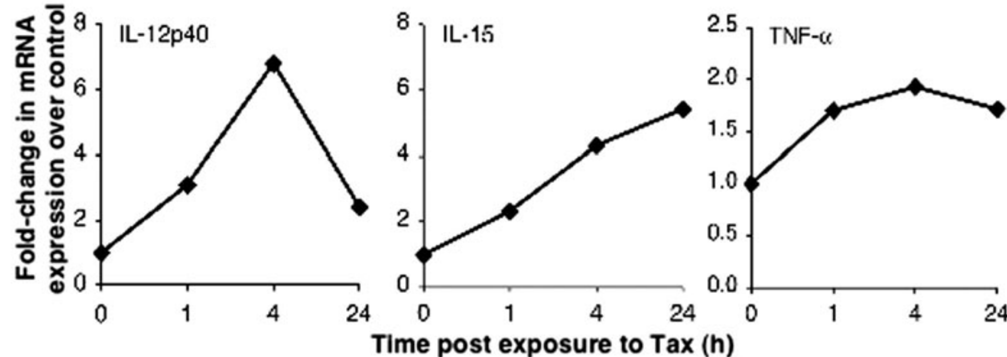


Figure 3 HTLV-1 Tax induces an increase in the expression of genes associated with DC maturation. (**A**) Microarray analyses revealed differential expression of genes that play a role in DC maturation. (**B**) Real-time PCR analysis using gene-specific primers confirmed the indicated genes encoding cytokines IL-12p40, IL-15 and TNF-α. The threshold cycle (C_T) values obtained for triplicate samples were averaged, normalized to levels of β-actin, and fold-change in mRNA expression respective to control was calculated as described in the Materials and Methods.

host transcriptional machinery, especially to facilitate binding of cellular transcription factors to the viral long terminal repeat (Azran *et al*, 2004). The effect of HTLV-1 Tax on transcript levels of ATF-4 in MDDCs was then examined by microarray analyses. Indeed the expression of ATF-4 was up-regulated (2.7-, 3.5-, and 1.8-fold at 1, 4, and 24 h, respectively). These results were in good correlation with real-time PCR analysis where a 10-, 14-, and 2-fold increase in the mRNA expression of ATF-4 over control was observed. An important downstream effect of the ATF/CREB pathway centers on the activation of the IL-17 promoter as reported in a recent study by Dodon *et al.* (2004). The microarray results demonstrated an increased expression of IL-17 (2.2-fold at 1 h post-stimulation, Figure 3A). As IL-17 up-regulates the expression of several pro-inflammatory cytokines (Dodon *et al*, 2004), these observations have provided new insights into the involvement of the HTLV-1 Tax activated ATF/CREB pathway in the pathophysiology of HTLV-1-associated inflammatory disorders.

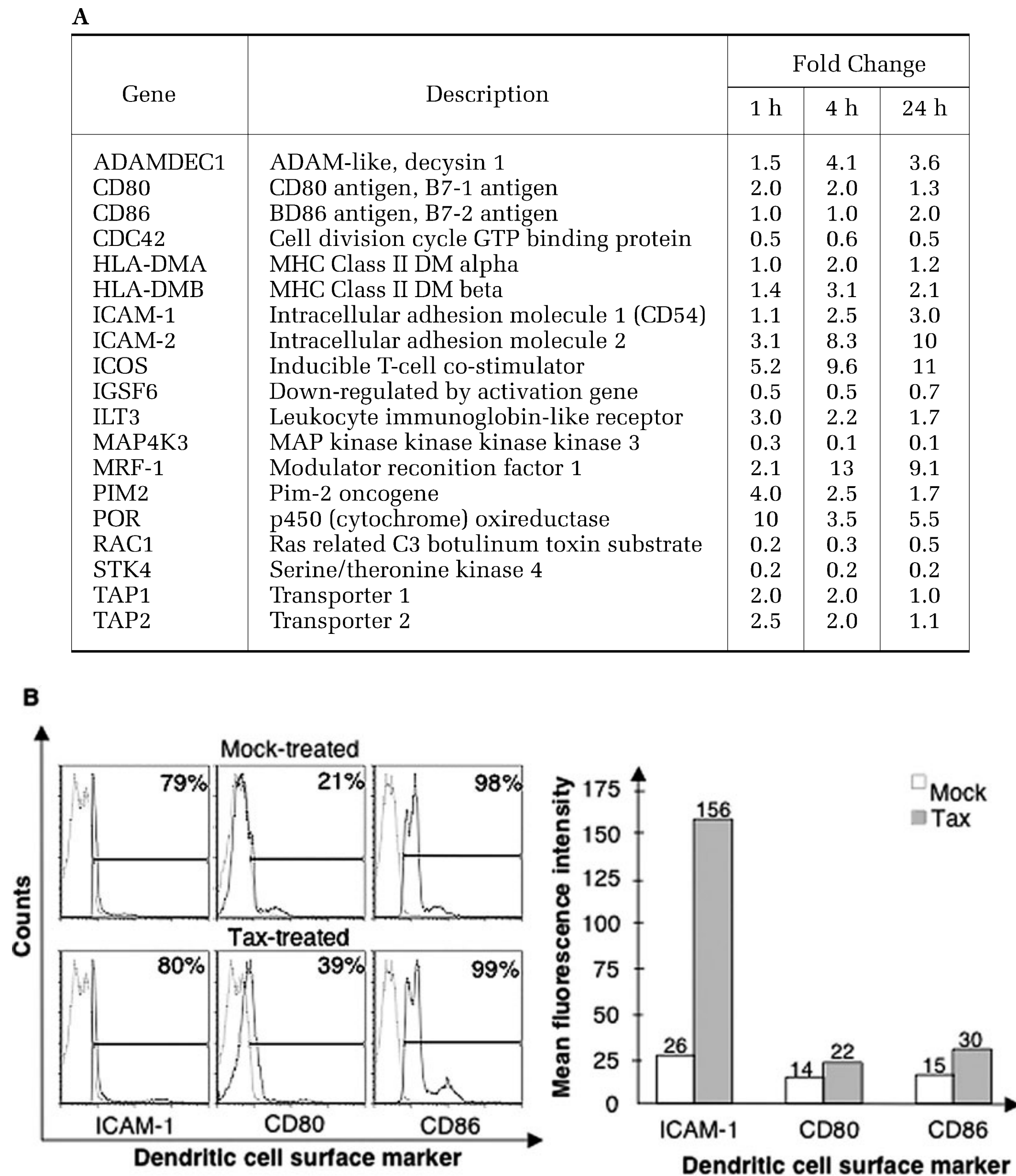
At 1 and 4 h post Tax stimulation, an apparent differential regulation of a number of interferon inducible genes indicated by asterisk (*) (Figure 2A) was also detected. This response likely represented an early host defense gene expression profile that occurs prior to the onset of an immune response (Samuel, 2001).

Genes involved in DC activation and maturation
DCs are powerful producers of key inflammatory and immunoregulatory cytokines and chemokines when activated by antigens (Banchereau and Steinman, 1998). Microarray analyses revealed the differential expression of several proinflammatory cytokines (Figure 3A) including IFN-γ (8.9-fold at 1 h), IFN-α (4.9-fold at 4 h), and IFN-β (7.2-fold at 4 h). Interferons are transcriptionally regulated cytokines that have been shown to be key players in innate antiviral immune responses (Bonjardim, 2005). The production of IFN-γ by infiltrating lymphocytes has been reported in the CNS of patients with HAM/TSP (Watanabe *et al*, 1995) and it was also reported that CSF IFN-γ concentrations were elevated in HAM/TSP patients (Biddison *et al*, 1997). IFN-γ is also known to play a role in inducing the production of MHC class I and II molecules, and costimulatory molecules by APCs thereby promoting a cell-mediated immune response (Sztein *et al*, 1984). More importantly, IFN-γ has been shown to stimulate the production of IL-12, a critical immunoregulatory cytokine (Trinchieri and Gerosa, 1996). The up-regulated expression of mRNA encoding both subunits of IL-12: IL-12p35 (4-fold at 4 h) and IL-12p40 (3.9-fold at 1 h) was observed. IL-12 is the most important cytokine for the polarization of T-cells to Th1 cells (Pulendran *et al*, 1999), and has been considered to be involved in the progression of a number of autoimmune diseases (Gately *et al*, 1998). Production of IL-12 induces rapid production of IFN-γ that in turn stimulates the increased production of IL-12, thus forming a positive feedback loop essential in the generation of Th1 immune responses (Trinchieri and Gerosa, 1996). In addition, IL-12 augments the production of TNF-α also demonstrated by microarray analyses (3.6-fold increase at 1 h post-stimulation). TNF-α is a neurotoxic cytokine partially responsible for demyelination and axonal degeneration observed in HAM/TSP (Sharief and Thompson, 1992). The elevated expression of other members of the TNF superfamily (TNFSF4, TNFSF11, and TNFSF14) were also demonstrated by microarray analyses (Figure 3A). TNFSF4, for example, was shown to be up-regulated by 4.6-fold at 4 h post-Tax stimulation. This gene plays a critical role in T cell-APC interactions and studies using a human TNF/TNFR superfamily gene array have demonstrated increased mRNA transcripts for TNFSF4 in cells transfected with a Tax-encoding retroviral vector (Harhaj *et al*, 2005).

An early increase in the mRNA expression levels for IL-2 (3.0-fold at 1 h), IL-15 (2.6-fold at 1 h), and IL-6 (4.9-fold at 4 h) was also detected. Previous studies using cDNA microarrays have detected up-regulation of IL-2 in a Tax-immortalized human T cell line T1 (Fung *et al*, 2005). IL-2 has been shown to induce T cell proliferation and is required for development of effective immune responses (Smith 1984). IL-15 is another proinflammatory cytokine that has functional characteristics similar to IL-2. Elevated expression levels of IL-15 have been demonstrated in HTLV-1-associated diseases, including HAM/TSP (Azimi *et al*, 2000). IL-15 has previously been shown stimulate the proliferation of peripheral blood leukemic cells in ATL patients (Mori *et al*, 1998). This proinflammatory cytokine also plays a key role in the spontaneous proliferation of PBMCs in HAM/TSP patients and has a preferential effect in stimulating $CD8^+$ T cells (Zhang *et al*, 1999). Transcript levels of IL-12p40, IL-15, and TNF-α were validated by real-time PCR and correlated with microaray data (IL-12p40: 3.1-, 6.8-, and 2.4-fold at 1, 4, and 24 h, respectively; IL-15: 2.3-, 4.3-, and 5.4-fold at 1, 4, and 24 h, respectively; TNF-α: 1.7-. 2.0, and 1.7-fold at 1, 4, and 24 h, respectively).

Chemokines play a crucial role in DC migration to the lymph node and in the effector phase of the lymphocyte response (Sallusto *et al*, 1998). Inflammatory chemokines CCL3 (macrophage inflammatory protein 1 alpha), CCL5 (RANTES), CCL17 (thymus and activation-regulated chemokine), CCL18 (pulmonary and activation-regulated chemokine), CCL19 (macrophage inflammatory protein 3 beta), CL20 (macrophage inflammatory protein 3 alpha) and CCL21 (secondary lymphoid tissue chemokine) were all highly expressed following exposure of DCs to HTVL-1 Tax. The expression levels of chemokines CCL5 and CCL19 were confirmed by real-time PCR and correlated with the microarray data (data not shown). Endogenous Tax has been shown to play a role in the expression and secretion of CCL3 and CCL5 from Tax-transfected T cell lines (Venkatanarayan and May, 1999; Mori *et al*, 2004). CCL3 has been detected in the CSF of patients with HAM/TSP (Miyagishi *et al*, 1995). The expression of CCL5 was also reported in HTLV-1-infected T cell lines and leukemic cells from ATL patients (Mori *et al*, 2004). The role of CCL19 and CCL20 secreted by activated DCs has been implicated in patients with multiple sclerosis where maintenance of chronic neuroinflammation was attributed to significant expression of these chemokines in the CSF of affected individuals (Pashenkov *et al*, 2003). It is possible that production of these chemokines by Tax-stimulated DCs may also contribute to the neurologic damage observed in HAM/TSP.

Antigen uptake and presentation genes
The unique capacity of DCs to induce immune responses is dependent on their ability to uptake and process antigen and migrate to the lymph node to present the antigen to T-cells (Banchereau and Steinman, 1998). Antigen uptake by immature DCs was characterized by the expression of adhesion molecules, MHC class I and II molecules, costimulatory molecules, and additional genes listed in Figure 4A. Among the adhesion molecules essential for optimal T-cell activation, a time-dependent increase (1.1-, 2.5-, and 3.0-fold at 1, 4, and 24 h, respectively) in expression of intracellular adhesion



A

Gene	Description	Fold Change		
		1 h	4 h	24 h
ADAMDEC1	ADAM-like, decysin 1	1.5	4.1	3.6
CD80	CD80 antigen, B7-1 antigen	2.0	2.0	1.3
CD86	BD86 antigen, B7-2 antigen	1.0	1.0	2.0
CDC42	Cell division cycle GTP binding protein	0.5	0.6	0.5
HLA-DMA	MHC Class II DM alpha	1.0	2.0	1.2
HLA-DMB	MHC Class II DM beta	1.4	3.1	2.1
ICAM-1	Intracellular adhesion molecule 1 (CD54)	1.1	2.5	3.0
ICAM-2	Intracellular adhesion molecule 2	3.1	8.3	10
ICOS	Inducible T-cell co-stimulator	5.2	9.6	11
IGSF6	Down-regulated by activation gene	0.5	0.5	0.7
ILT3	Leukocyte immunoglobin-like receptor	3.0	2.2	1.7
MAP4K3	MAP kinase kinase kinase kinase 3	0.3	0.1	0.1
MRF-1	Modulator reconition factor 1	2.1	13	9.1
PIM2	Pim-2 oncogene	4.0	2.5	1.7
POR	p450 (cytochrome) oxireductase	10	3.5	5.5
RAC1	Ras related C3 botulinum toxin substrate	0.2	0.3	0.5
STK4	Serine/theronine kinase 4	0.2	0.2	0.2
TAP1	Transporter 1	2.0	2.0	1.0
TAP2	Transporter 2	2.5	2.0	1.1

Figure 4 Tax mediates an increase in expression of molecules expressed during DC antigen uptake and activation. (**A**) Microarray analyses to demonstrate mRNA expression levels of antigen uptake and presentation genes following treatment of MDDCs with purified Tax (1 μg/ml) at 1, 4, and 24 h post Tax stimulation. (**B**) Phenotypic analysis of MDDCs for the cell surface expression of adhesion (ICAM-1) and costimulatory (CD80 and CD86) molecules by FACS analysis. Immature DCs were treated with mock fluid or Tax (1 μg/ml) and subsequently incubated for 24 h. At the end of incubation, cells were harvested, washed, and stained with DC markers (CD11c, ICAM-1, CD80, and CD86). Left panel depicts phenotypic changes in DCs represented by histogram gated on live CD11c^{+} cells. The *x* axis represents DC maturation marker staining. The isotype control is represented by the dotted line while the solid line in the graph represents specific antibody staining. The number represents percentage of corresponding marker-positive cells. The right panel indicates quantitation of mean fluorescence intensity (MFI) of the positive population. The *y*-axis represents MFI and *x*-axis represents DC markers associated with antigen uptake and presentation.

molecule 1 (ICAM-1) or CD54 and ICAM-2 (3.1-, 8.3-, and 10-fold at 1, 4, and 24 h, respectively) was observed. These adhesion molecules may serve to initiate contact between DCs and T cells to facilitate recognition of peptide-MHC complexes by the T cell receptor (de la Fuente *et al*, 2005), and may thus play a role in Tax presentation by activated DCs to CD8^{+} T cells. Microarray analyses also demonstrated the expression of the co-stimulatory molecule CD80 that was amplified approximately 2-fold at 1 h after Tax stimulation and also displayed a two-fold increase in CD86 transcripts, indicating the antigen presentation function of DCs 24 h post Tax-stimulation (Figure 4A).

The microarray data for adhesion and costimulatory molecules was confirmed by real-time PCR (data

not shown). Since these represent key molecules associated with phenotypic alterations in DCs undergoing activation and maturation, a cell-surface phenotypic analysis of these molecules was performed. MDDCs treated with Tax for 24 h were analyzed for the expression of ICAM-1, CD80, and CD86 by flow cytometry (FACS) (Figure 4B). Since ICAM-1 and CD86 have been shown to be present on 80–90% of DCs, a large change in the number of cells expressing these molecules was not anticipated. However, flow cytometry also enables the determination of change in expression of a specific cell surface marker on a per cell basis using the mean fluorescence intensity (MFI) measurement. As expected, even though there was no detectable increase in the percentage of cells expressing ICAM-1 and CD86 following Tax treatment, a significant increase (6-fold for ICAM-1 and 2-fold for CD86) in the MFI was observed. With respect to CD80, a 2-fold increase in the percentage of cells expressing CD80 following Tax treatment was observed (Figure 4B).

Following antigen binding, antigen-receptor complexes relocate to intracellular compartments of DCs for further processing (Mellman and Steinman, 2001) and DCs display large amounts of MHC-peptide complexes (Banchereu and Steinman, 1998). Interestingly, a number of genes encoding proteins involved in antigen processing were also identified as a result of microarray analyses (Figure 4A). These include transporter 1 (TAP1) and transporter 2 (TAP2) associated with antigen processing. TAP1 is an MHC class I molecule while TAP2 is an MHC class II molecule. The early (1 h) expression of TAP1 and TAP2 (2-fold for TAP1 and 2.5-fold for TAP2) is suggestive of a possible role of these proteins in transporting Tax between the cytosol and the endoplasmic reticulum for association with MHC molecules. The formal cellular pathway associated with Tax transport has been reviewed (Alefantis *et al*, 2005b) and detailed studies focusing on interactions between Tax and proteins of this pathway are currently under investigation.

Differentiation of DCs following Tax stimulation was also accompanied by an upregulation of MHC class II molecules HLA-DMA (2.0-fold) and HLA-DMB (3.1-fold) at 4 h after Tax stimulation and a number of supplementary genes associated with DC antigen uptake and presentation (Figure 4A). These include inducible T cell co-stimulator (ICOS), with an increase in expression of 5.2-, 9.6-, and 11-fold at 1, 4, and 24 h, respectively, and ADAMDEC1 (Decysin 1), with an upregulation of 1.5-, 4.1-, and 3.6-fold at 1, 4, and 24 h, respectively. ICOS has been shown to regulate DC-induced T cell activation and stimulation and has previously been demonstrated to be functionally important for the costimulatory capacity of DCs (Richter *et al*, 2001). Decysin is believed to play an important role in DC function and their interactions with T cells (Mueller *et al*, 1997). Microarray analyses has thus revealed a number of genes that may play a role in Tax-mediated DC activation, providing information concerning the operative pathways involved in Tax antigen presentation.

Microarray analyses presented herein were performed to examine Tax-mediated effects on the DC activation process at the genomic level and have identified a number of differentially regulated genes. The results indicate that Tax can activate DCs and this process could be initiated from the membrane by toll-like receptors. The involvement of NF-κB in this process leads to the secretion of cytokines and chemokines essential for DC activation and T cell proliferation. HTLV-1 is known to infect DCs in a natural infection (Macatonia *et al*, 1992) and we hypothesize that HTLV-1-infected DCs may express Tax. Alternately, DCs could be exposed to Tax in the extracellular environment. Whatever the source of Tax, the results indicate that DCs once exposed to Tax, undergo activation, process and may present Tax to T cells, ultimately initiating a Tax-specific CTL response, characteristic of HAM/TSP. Current investigations are aimed at examining whether these observations can be translated to an infection system.

Materials and methods

Generation of monocyte-derived dendritic cells
MDDCs were generated from peripheral blood mononuclear cells (PBMCs) as described previously (Fanales-Belasio *et al*, 2002). Briefly, PBMCs isolated from heparinized blood using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation were magnetically labeled with anti-CD14-coated microbeads (Miltenyi Biotec Inc., Auburn, CA) and monocytes were purified by sorting with a magnetic separator device as described by the manufacturer (VarioMACS; Miltenyi Biotec). The purity of monocytes was always >95%, as assessed by flow cytometry (FACScan; BD Biosciences, San Jose, CA).

Purified monocytes were differentiated to DC (MDDC) by culture in complete medium comprising of BioWhittaker RPMI-1640 with L-glutamine (Cambrex, Walkersville, MD) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% of a stock of penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml) (Mediatech Inc., Herndon, VA) in the presence of recombinant human GM-CSF (50 ng/ml; PeproTech, Rocky Hill, NJ) and rhIL-4 (10 ng/ml; PeproTech) for 6 days at 37°C and 5% CO_2. Cells were replenished with fresh cytokines every third day. Differentiation to DC was confirmed by morphologic observation and phenotypic analysis of specific surface markers (CD11c, CD40, CD80, CD83, CD86, HLA-ABC, and HLA-DR) by flow cytometry as described previously (Izmailova *et al*, 2003).

HTLV-1 Tax expression and purification
Highly purified Tax protein was prepared as described previously (Mostoller *et al*, 2004). Briefly,

Tax was expressed in *Escherichia coli* HB101 by the pTax-His6x coding sequence (kindly provided by Dr. Chou-Zen Giam, Uniformed Services University of the Health Sciences, Bethesda, MD) and purified by Ni^{2+} chromatography using a His-bind purification kit (Novagen, Madison, WI). The presence of Tax protein in the preparations was confirmed by western immunoblot analysis using an anti-Tax monoclonal antibody (1:50; TAB 170, provided by Dr. Fatah Kashanchi, George Washington University School of Medicine, Washington, DC). The specific functionality of purified Tax was assessed by electrophoretic mobility shift (EMS) analysis by its ability to enhance binding of the cAMP responsive element binding (CREB) protein (provided by Dr. Jennifer Nyborg, Colorado State University, Ft Collins, CO) to the 21-bp HTLV-1 promoter proximal repeat of Tax responsive element-1. Mock bacterial extracts were prepared in an identical fashion from *E. coli* cells not expressing the Tax gene. In both cases, endotoxin concentration was below the detection limit (<0.016 EU/μg), as determined by the Limulus Amebocyte Lysate analysis (Associates of Cape Cod, Falmouth, MA).

mRNA isolation and microarray hybridization
MDDCs were treated with purified Tax protein (1 μg/ml), or mock fluid (negative control) for three different lengths of time (1, 4, and 24 h). Cells were harvested post-treatment and processed to isolate mRNA using oligo-$(dT)_{14}$-coated magnetic beads as described by the manufacturer (Array Grade mRNA Purification Kit, SuperArray Bioscience Corporation, Frederick, MD). For each hybridization, purified mRNA (0.3 μg) from control and treated samples was converted to cDNA, labeled with biotin-16-dUTP (Roche, Indianapolis, IN) and amplified using the AmpoLabeling-LPR Kit (SuperArray). GEArray S Series human dendritic and antigen presenting cell gene arrays spotted with 192 gene-specific cDNA fragments on nylon membrane (SuperArray) were prehybridized with continuous agitation at 5 to 10 rpm at 60°C for 2 h with GEAprehyb (SuperArray), a mixture of denatured sheared salmon sperm DNA (100 μg/ml, SuperArray) and GEAhyb Hybridization Solution (SuperArray). A 750 μl volume of solution comprised of denatured probe and GEAprehyb was used to hybridize the membranes for 12–18 h at 60°C with continuous agitation at 5 to 10 rpm. The membranes were washed with decreasing concentrations of saline sodium citrate (SSC): $2 \times$ SSC + 1% sodium dodecyl sulfate (SDS), $0.1 \times$ SSC + 0.5% SDS, and blocked for 45 min with GEAblocking Solution Q (SuperArray). Alkaline phosphatase-conjugated streptavidin (SuperArray) was used to detect hybridization of the membrane-bound biotinylated cDNA probes. The membranes were washed and rinsed with phosphatase reaction buffer and detected using Chemiluminescent Detection Kit (SuperArray). Images of the membranes were acquired on x-ray films and data was analyzed using the web-based GEArray Expression Analysis Suite (SuperArray). Following background correction and normalization with respect to appropriate housekeeping genes, changes in gene expression of Tax-treated cells were obtained relative to the negative control. Data were examined for genes having at least a two-fold increase or decrease relative to the untreated sample.

Flow cytometry
In order to examine the role of Tax in DC activation and maturation, MDDCs were exposed to mock fluid or Tax (1 μg/ml) for 24 h. Cell suspensions (0.5–1×10^6 cells/ml in PBS with 3% FBS and 0.02% sodium azide with more than 90% viability as determined by trypan blue exclusion) were kept at 4°C during the entire procedure. Cell suspensions were incubated for 15 min with anti-CD16/CD32 in order to block the non-specific binding of the antibodies to Fc receptors. Fluorochrome-conjugated antibodies were added directly to the suspension at the appropriate titer, mixed, and incubated on ice for 30 min before being washed three times with staining buffer. Cells were either directly analyzed or fixed with 2% paraformaldehyde (Alfa Aesar, Ward Hill, MA). The expression of cell surface antigen on the DC population was determined using a FACScan (Becton Dickinson). Live DCs were gated and analyzed using FlowJo software (Tree Star, San Carlos, CA). Two or more color immunofluorescence analysis was performed after appropriate color compensation for each marker utilized, with a Coulter EPICS profile analyser. The percent positive cells and mean fluorescence intensity (MFI) of the test sample were calculated as described by the manufacturer.

Verification of gene-array data using real-time PCR
mRNA isolated from mock- or Tax-treated MDDCs was transcribed to cDNA using Omniscript Reverse Transcriptase (4U, Qiagen, Valencia, CA) in the presence of dNTP mix (0.5 mM each dNTP; Qiagen) oligo dT_{15} primer (1 μM, Promega, Madison, WI) and SUPERaseIn RNase Inhibitor (10U, Ambion, Austin, TX) for 1 h at 37°C. Real-time PCR was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The standard real-time PCR reaction using SYBR Green I consisted of 15 μl SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μM each of forward and reverse primers (IDT, Coralville, IA), and 5 μl of cDNA in a total volume of 30 μl. The thermal cycling conditions comprised an initial activation step at 95°C for 10 min followed by 40 cycles including denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. Experiments were performed in triplicate for each data point. Dissociation or melting curve analysis was implemented to ensure the presence of a single peak at the correct melting temperature. Gene expression was measured by quantifying cDNA transcribed from mRNA for the Tax-treated samples relative to

mock-treated samples. Even though the C_T values for the endogenous control β-actin were identical among various samples, we still normalized the C_T values for target genes with those of actin for each sample to rule out any minor differences. The mean threshold cycle (C_T) value for each target gene was subtracted from the mean C_T value for β-actin for each sample to obtain the ΔC_T value. The difference $\Delta\Delta C_T$ was calculated from the ΔC_T values of the sample and the mean C_T value of mock-treated sample. Fold changes in mRNA expression for genes were expressed as $2^{-\Delta\Delta CT}$.

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