Journal of NeuroVirology, 10: 358–371, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490521104

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Human T-cell leukemia virus type I Tax induces the expression of dendritic cell markers associated with maturation and activation

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Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of both adult T-cell leukemia (ATL) and HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the genesis of HAM/TSP likely involves several steps, the generation of a highly specific and effective population of Tax-specific CD8$^+$ cytotoxic T lymphocytes (CTLs) that migrate to the central nervous system (CNS) is of pivotal importance in this neuropathologic process. Presentation of Tax peptides by activated dendritic cells (DCs) to naïve CD8$^+$ T cells likely plays an important role in the induction of a Tax-specific CTL response and the eventual neurologic dysfunction observed in HAM/TSP. The immune response mounted during HTLV-I infection is primarily targeted against Tax with both Tax-specific antibodies and CTLs found in HTLV-I–infected individuals, indicating that Tax is available for immune recognition. Studies have suggested that Tax may be secreted from HTLV-I–infected cells and act as an extracellular cytokine, be internalized and processed for presentation, or be transported to the nucleus where it may act as a transcriptional activator. The authors report in this article that purified Tax induces DC activation involving an increase in the production of CD80 and CD86 mRNA in the absence of corresponding protein synthesis. Furthermore, intracellular Tax down-regulates the protein expression of molecules involved in antigen presentation. This implies a difference in the mechanism of Tax activity depending upon its location. Additionally, treatment of JAWS II DCs with extracellular Tax decreases the ability of DCs to present a major histocompatibility complex (MHC) class I–restricted peptide, indicating that Tax likely matures the DCs to the point where presentation of a secondary antigen is restricted. The implication of the experimental results with respect to the generation of a Tax-specific CTL compartment that participates in the genesis of HAM/TSP is discussed. *Journal of NeuroVirology* (2004) **10,** 358–371.

Keywords: ATL; CTL; dendritic cells; HAM/TSP; HTLV-I; Tax

Introduction

Human T-cell leukemia virus type I (HTLV-I) is known to be the etiologic agent of several disease states, the most notable being adult T-cell leukemia (ATL) and HTLV-I–associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is a progressive neurodegenerative disease that primarily affects the spinal cord and brain. Clinical pathogenesis of HAM/TSP includes the presence of multiple white matter lesions in both the spinal cord and brain. In addition, HAM/TSP is characterized by a highly stimulated immune response, including the expression

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These studies were supported by the United States Public Heath Service/National Insitutes of Health (USPHS/NIH CA54559 awarded to B. Wigdahl; USPHS/NIH NS044801-01 awarded to K. Mostoller; USPHS/NIH AI056094 awarded to C. Norbury). These studies were also supported by funding provided by the Pennsylvania State University College of Medicine Tobacco Settlement Block Grant under the Department of Health's Health Research Formula Funding Program (State of PA, Act 2001-77—part of the PA Tobacco Settlement Legislation).

Received 17 February 2004; revised 14 June 2004; accepted 21 July 2004.

of elevated levels of inflammatory cytokines and HTLV-I–specific antibodies, an increase in the level of activated T cells, and the oligoclonal expansion of HTLV-I Tax-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) in both the peripheral blood and cerebrospinal fluid (CSF) of HAM/TSP patients (Levin and Jacobson, 1997). Activated lymphocytes including Tax-specific $CD8^+$ CTLs are present in white matter lesions of HAM/TSP patients and may contribute to neuropathology (Jacobson *et al*, 1988; Kubota *et al*, 1994; Levin and Jacobson, 1997; Moritoyo *et al*, 1996; Umehara *et al*, 1993). Activated lymphocytes having a high specificity for Tax protein have also been identified in the CSF of HAM/TSP patients (Jacobson *et al*, 1992; Levin and Jacobson, 1997). Several mechanisms of central nervous system (CNS) damage can be hypothesized based on experimental observations that HTLV-I–infected lymphocytes infiltrate the CNS, as well as the fact that CNS-resident cells are susceptible to HTLV-I infection (Barmak *et al*, 2003a, 2003b).

Although the viral protein Tax is known to play a critical role in the genesis of ATL by functioning as a transcriptional activator and an oncoprotein (Franklin *et al*, 1993; Lenzmeier *et al*, 1998; Tillmann *et al*, 1994; Tillmann and Wigdahl, 1994; Uchiyama, 1997; Wessner *et al*, 1995, 1997; Wessner and Wigdahl, 1997; Zhao and Giam, 1992), its contribution to the genesis of HAM/TSP has not been clearly delineated. Studies have defined the number of Tax-producing cells during the course of HAM/TSP and have shown that between 0.04% and 1.16% of CSF cells, and between 0.02% and 0.54% of peripheral blood mononuclear cells (PBMCs) contain detectable levels of Tax protein (Moritoyo *et al*, 1999). Theoretically, Tax may be released from these cells through apoptosis, necrosis, or secretion. It is of interest to examine the effect released Tax has on cells relevant to the genesis of HTLV-I–induced neurologic dysfunction, fueled by the recent observation that Tax contains a nuclear export signal (NES) that mediates its localization to the cytoplasm (Alefantis *et al*, 2003) where it may be targeted for secretion. As the immune response to HTLV-I infection has garnered much attention in terms of the outcome of disease progression, it is important to delineate the effect of Tax on immune cell populations. Several cell populations are crucial with respect to generating an efficient immune response against the virus, including $CD4^+$ T cells, $CD8^+$ T cells, monocytes/macrophages, and dendritic cells (DCs). The immune response in HAM/TSP patients may transition from a beneficial response aimed at controlling the viral infection to a detrimental response that participates in mediating pathology observed in HAM/TSP (Barmak *et al*, 2003b; Grant *et al*, 2002; Wigdahl and Brady, 1996). Of the many components of the immune system, DCs are critical mediators of the immune response. DCs are professional antigen-presenting cells (APCs) that initiate and regulate adaptive immune responses. When antigen is captured by immature DCs, the cells traffick to lymphoid tissue during which they undergo a maturation process. Once in the lymphoid tissue, mature DCs present the processed antigens via peptide–major histocompatibility complex (MHC) to T cells. Effective DC/T cell interactions result in the activation and expansion of antigen-specific T cells. Importantly, DCs are the only APC capable of initiating a primary immune response against antigens. The ability of DCs to regulate adaptive immunity is dependent on the process of DC maturation. Only mature DCs are competent to prime and activate $CD8^+$ T cells, as the process of maturation results in the expression of cell surface molecules involved in antigen presentation and T-cell activation. DC maturation may be induced by a variety of factors, including whole bacteria or bacterial-derived antigens such as lipopolysaccharide (LPS) (Rescigno *et al*, 1999; Verhasselt *et al*, 1997), inflammatory cytokines (De Smedt *et al*, 1997; Jonuleit *et al*, 1996), ligation of cell surface receptors such as CD40 (Buelens *et al*, 1997; Canque *et al*, 1998; Morel *et al*, 2001), and viral products (Fanales-Belasio *et al*, 2002; Giovanna Quaranta *et al*, 2002; Williams *et al*, 2002). We hypothesize that the HTLV-I Tax protein may also serve a similar function.

Utilizing the JAWS II cell line, which represents a model of immature DCs derived from the bone marrow of p53-deficient mice (MacKay and Moore, 1997), the effect of purified extracellular Tax on DC maturation and activation has been evaluated. Studies presented herein demonstrate that purified Tax protein induces morphologic changes in JAWS II cells that are consistent with DC maturation, including the formation of dendrites, although other phenotypic characteristics such as antigen uptake remain unchanged. In addition, soluble Tax protein potently induces the mRNA expression of some but not all molecules that are up-regulated during DC maturation and function in antigen presentation, including CD80 and CD86, but not CD40. However, cell surface protein levels of these molecules are unchanged by Tax treatment. It has also been demonstrated that Tax produced intracellularly as a result of retroviral delivery results in differential mRNA expression of genes up-regulated during DC maturation as opposed to treatment of cells with extracellular Tax. In addition, intracellular Tax significantly down-regulates detectable cell surface proteins. These results have implications with respect to the ability of extracellular Tax secreted or otherwise released from HTLV-I–infected cells, either in the periphery or in the CNS, to affect the maturation and thus the immunologic function of cells comprising the immune system, including the DC compartment. Alterations in DC function may affect the efficiency of the priming of HTLV-I–specific $CD8^+$ CTLs and, ultimately, the generation of a Tax-specific $CD8^+$ CTL population that participates in the genesis of HAM/TSP. With respect to immune function, studies presented in this

report demonstrate that pretreatment of JAWS II DCs with purified Tax results in a decrease in the ability of these cells to activate the B3Z hybridoma cell line via presentation of an MHC class I–restricted peptide. These studies have indicated that pretreatment with Tax likely results in maturation of JAWS II DCs accompanied by an inability to efficiently present a secondary antigen via MHC class I to T cells. This again suggested that Tax contributes to the functional maturation of DCs.

Results

Soluble Tax induces phenotypic alterations consistent with DC maturation

In patients infected with HTLV-I, HAM/TSP is characterized by the presence of a large population of Tax-specific CTLs (Elovaara *et al*, 1993; Jacobson *et al*, 1990). Activation of virus-specific $CD8^+$ T cells requires presentation of viral peptides in the context of MHC class I by DCs, and only mature DCs are competent to prime and activate $CD8^+$ T cells. Phenotypic changes associated with the transition from an immature to a mature state include morphologic changes such as the formation of dendrites, redistribution of MHC molecules from intracellular compartments to the cell surface, down-regulation of antigen capture, secretion of chemokines and cytokines, and an increase in the expression of cell surface molecules involved in antigen presentation and T-cell activation (Gunzer *et al*, 2000; Kohrgruber *et al*, 1999; Tureci *et al*, 2003). Viral products including HIV-1 Tat, Nef, and gp120 are among the stimuli that are capable of inducing DC maturation (Fanales-Belasio *et al*, 2002; Giovanna Quaranta *et al*, 2002; Williams *et al*, 2002). The HTLV-I Tax protein may also serve a similar function, thereby impacting the strength of the immune response mounted against HTLV-I.

One of the phenotypic changes associated with the process of DC maturation is the formation of dendrites, which parallels the acquisition of high cellular motility (Winzler *et al*, 1997), a necessary precursor to the ability of DCs that have captured antigen to migrate from peripheral tissues to the draining lymph organs to activate T cells. To determine whether Tax could induce the formation of dendrites, immature JAWS II DCs were exposed to purified Tax protein. Immature JAWS II DCs have a predominantly rounded morphology (Figure 1A and B), which is altered during their maturation. JAWS II DCs treated with LPS demonstrated an increase in the formation of long processes (Figure 1C and D). This effect was also apparent, although to a lesser degree, during cytokine-induced maturation of JAWS II DCs, where there was a similar formation of dendrites (Figure 1E and F). Tax-treated JAWS II DCs exhibit the formation of dendrites to a similar degree when compared to DCs treated with the cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-4, and interferon (IFN)-γ (Figure 1G and H). In addition, Tax-treated JAWS II DCs displayed an increase in number of cells possessing dendrites, paralleling dendrite formation in cytokine-treated cells. Both the number of dendrites per cell and the average length of the dendrites as measured in microns is similar to that observed following cytokine treatment of JAWS II DCs (Table 1).

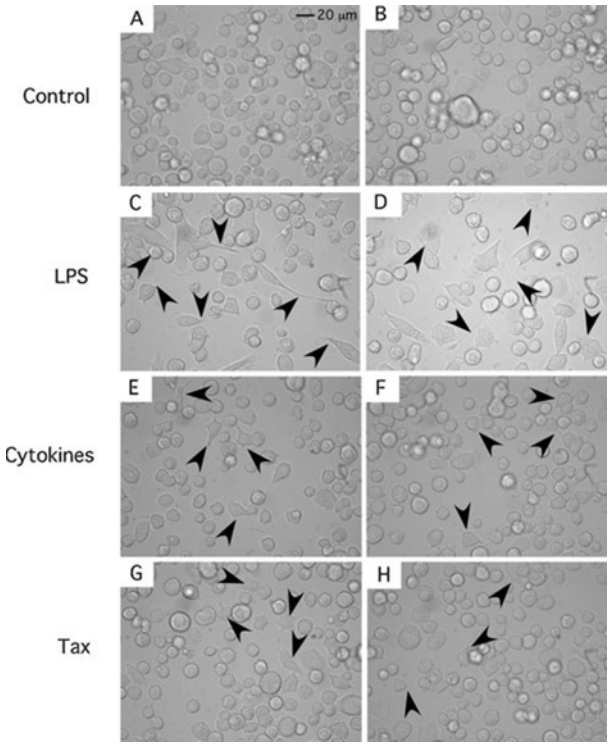

Figure 1 Tax protein induces the formation of dendrites in JAWS II DCs. (**A** and **B**) Immature JAWS II DCs were characterized morphologically by a predominantly rounded phenotype. (**C** and **D**) JAWS II DCs treated with LPS (1 mg/ml, 48 h) displayed an increase in both the number and length of processes extending from the central cell body. (**E** and **F**) JAWS II DCs treated with cytokines (IFN-γ [100 U/ml], TNF-α [10 ng/ml], and IL-4 [10 ng/ml], 24 h) also exhibited an increase in the number and length of dendrites. (**G** and **H**) JAWS II DCs treated with Tax (500 ng/ml, 24 h) display morphology similar to that of cytokine-treated cells. Phase contrast images represent 40× magnification.

Table 1 Analysis of DC morphology following exposure to extracellular Tax

Treatment	*Avg of cells with dendrites/50 cells*	*Average no. of dendrites/cell*‡	*Average dendrite length*
Control	10 ± 1.4	2.5 ± 1.5	2.3 ± 0.6
LPS	31 ± 1.0*	7.0 ± 3.0	23.3 ± 15.3*
Cytokines	25 ± 4.0*	5.7 ± 3.5	11.7 ± 2.9*
Tax	25 ± 2.6*	6.0 ± 4.0	11.7 ± 2.8*

*Denotes statistically significant difference from control with $P < .05$.

‡Average no. of dendrites/cell represents the average number of dendrites per dendrite-bearing cell.

In addition to dendrite formation, DC maturation is accompanied by an increase in the expression of a number of cell surface molecules involved in the process of antigen presentation. Expression of the costimulatory molecules CD80 and/or CD86 is necessary for DCs to become mature and competent to activate $CD8^+$ T cells. In addition to APCs, *in vivo* priming of $CD8^+$ CTLs usually requires the participation of $CD4^+$ T cells (Bennett *et al*, 1997; Husmann and Bevan, 1988). CD40 ligand (CD40L) expressed on activated $CD4^+$ T cells interacts with CD40 expressed on APCs such as DCs. Consequently, the ability of extracellular Tax to induce the expression of mRNA encoding the cell surface markers CD80, CD86, and CD40 was examined by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Bacterial LPS and the cytokine cocktail previously described have both been shown to be potent inducers of DC maturation, and were therefore utilized as controls. Following each treatment, RNA was isolated and subjected to RT-PCR analysis to determine expression levels of CD80, CD86, and CD40. In the absence of LPS or cytokine treatment, Tax was shown to be a very potent inducer of mRNAs corresponding to the costimulatory molecules CD80 and CD86, and caused an increase in their expression to much greater levels than either of the known inducers of DC maturation (Figure 2A and B). However, Tax failed to induce CD40 mRNA expression, although the expression level of this molecule was significantly increased following treatment with either LPS or the cytokine cocktail (Figure 2C). This demonstrated that exogenous Tax was capable of enhancing the mRNA expression of CD86 and CD80, but not CD40. This was confirmed by a real-time approach to RNA analysis (microarray), which demonstrated that Tax-treated JAWS II DCs displayed an increase in the mRNA expression of CD80 and CD86, but not CD40 (Table 2). This effect is both time and dose dependent, with exogenous Tax increasing the mRNA expression of CD80 and CD86, but not CD40, in a concentration-dependent manner (Figure 3) and over a time span of 1 to 48 h (Figure 4).

Having shown that Tax treatment of JAWS II DCs resulted in an increase in the mRNA level of molecules known to be up-regulated during DC maturation, we proceeded to examine the treated cells for expression of the corresponding cell surface proteins. Specifically, the ability of Tax to increase the cell surface protein expression of CD80, CD86, and CD40 was analyzed in JAWS II DCs. Although statistically significant increases in expression of CD86 were detected with LPS or cytokine treatment, and CD80 expression was observed with cytokine treatment, no increases in the cell surface expression of any of the proteins examined were observed (Figure 5). In addition, treatment of the cells with Tax did not enhance mean cell fluorescence relative to untreated cells for any of the molecules examined (data not shown). Western immunoblot analyses also indicated that there were no

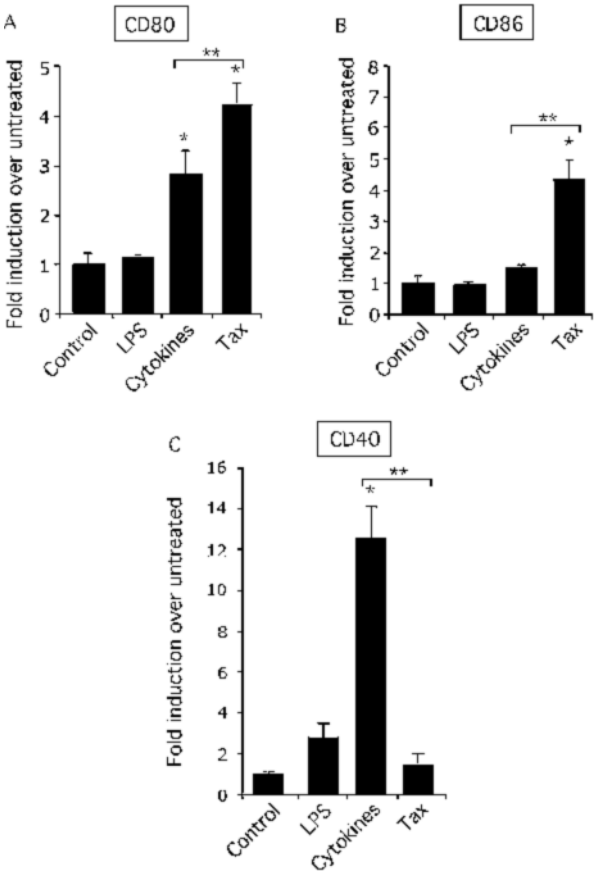

Figure 2 Tax potently induces the transcription of CD80 and CD86, but not CD40. JAWS II DCs were either incubated in the absence or presence of LPS (1 mg/ml, 48 h), cytokines (TNF-α [10 ng/ml], IFN-γ [100 U/ml], and IL-4 [10 ng/ml) 24 hr, or Tax (500 ng/ml, 24 hr). RNA was isolated from treated cells and subjected to RT-PCR. Expression levels of CD80, CD86, and CD40 were normalized to expression levels of GAPDH. Tax was able to induce the expression of both CD80 and CD86 to levels greater than that observed following the addition of known inducers of DC maturation. However, Tax protein was unable to similarly induce the transcription of CD40. The asterisk (*) denotes a statistically significant increase in expression as compared to control cells, with $P \leq .05$. The double asterisk (**) indicates a statistically significant difference between cytokine-treated and Tax-treated cells, with $P \leq .05$. Results are representative of three independent experiments.

Table 2 Up-regulation of DC activation markers following exposure to extracellular Tax

Gene	*Fold upregulation*
Costimulatory/T-cell activation/DC maturation	
CD80	2.0*
CD86	4.0*
CD40	1.8
CD83	2.2*
MHC class II	
H2-T23	2.5*
H2-T24	2.2*
H2-DMA	2.0*

*Statistically significant increase.

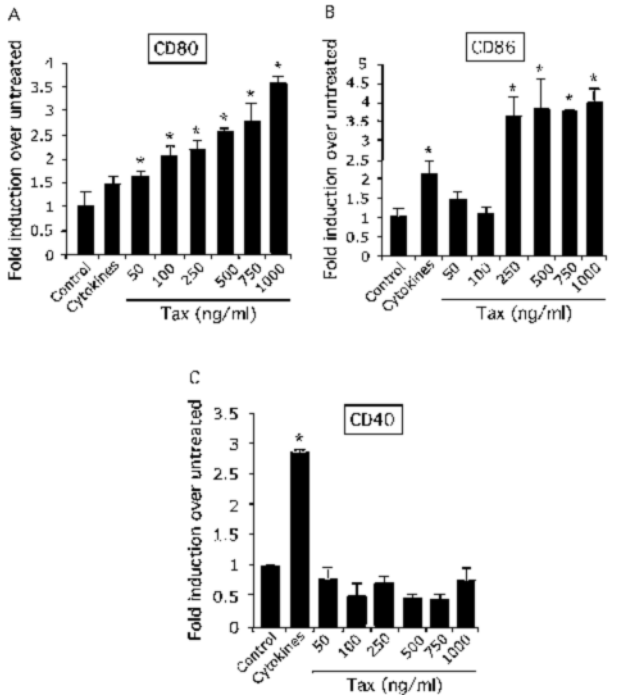

Figure 3 Tax increases the mRNA expression of CD80 and CD86, but not CD40, in a dose-dependent manner. JAWS II DCs were either incubated in the absence or presence of cytokines (TNF-α [10 ng/ml], IFN-γ [100 U/ml], and IL-4 [10 ng/ml], 24 h), or Tax (50 to 1000 ng/ml). RNA was isolated from treated cells and subjected to RT-PCR. Expression levels of CD80, CD86, and CD40 were normalized to expression levels of GAPDH. Tax was able to induce the mRNA expression of CD80 and CD86 in a dose-dependent manner, whereas the mRNA expression of CD40 remained unchanged with an increasing concentration of exogenous Tax. The asterisk (*) denotes a statistically significant increase in expression as compared to control cells, with $P \leq .05$. Results are representative of three independent experiments.

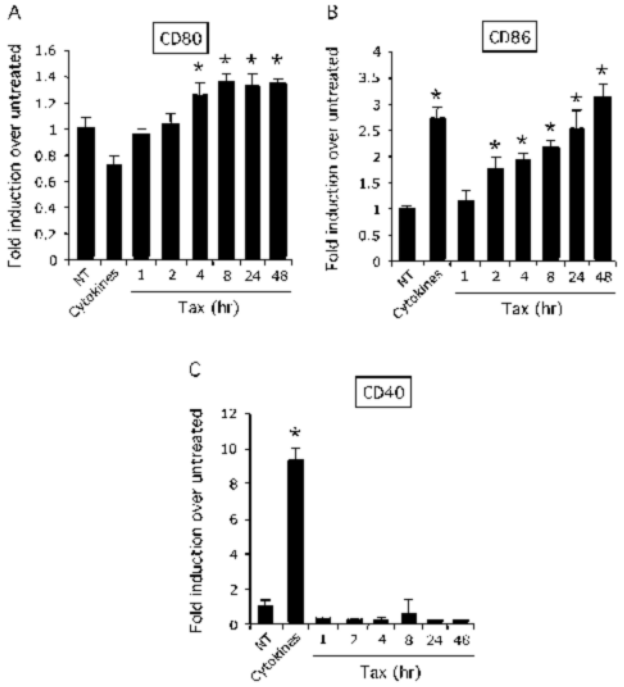

Figure 4 Tax increases the mRNA expression of CD80 and CD86, but not CD40, in a time-dependent manner. JAWS II DCs were incubated in the absence or presence of cytokines (TNF-α, IL-4, IFN-γ; 24 h) or Tax (500 ng/ml) for increasing amounts of time ranging from 1 to 48 h. Following treatment, RNA was isolated and subjected to RT-PCR. All values obtained were normalized to expression levels of GAPDH and represent triplicates. **A**, The addition of Tax to JAWS II DCs caused an increase in the mRNA expression of CD80 beginning at 4 h post-treatment and peaking at 8 h post-treatment. Expression remained steady through 48 h of Tax treatment. **B**, Enhanced mRNA expression of CD86 began by 2 h post-treatment and continually rose through 48 h post-treatment. **C**, Over the course of 48 h, Tax caused no increase in the mRNA expression of CD40. The asterisk (*) denotes a statistically significant increase in expression as compared to control cells, with $P \leq .05$. Results are representative of three independent experiments.

increase in intracellular synthesis of CD80, CD86, or CD40 (data not shown), thereby precluding the possibility that these molecules were synthesized but not transported to the cell surface.

Retroviral delivery of Tax alters the expression of genes involved in antigen presentation

Although HTLV-I primarily targets $CD4^+$ T cells, other human cells including $CD8^+$ T cells, immature bone marrow cells, monocytes, cells of neural origin, and DCs have been shown to be infected by the virus (Hoffman *et al*, 1992; Knight *et al*, 1993; Nagai *et al*, 2001; Yamada *et al*, 1991). In the case of DCs, it has been demonstrated that peripheral blood DCs are infected with HTLV-I in HAM/TSP patients, and these DCs stimulate persistent T-cell activity (Knight *et al*, 1993). Additional studies have demonstrated that 0.4% to 5.1% of DCs from patients with HAM/TSP are infected with HTLV-I and stimulate autologous lymphocyte proliferation. In HAM/TSP patients, depletion of DCs and purification of T cells abolished spontaneous lymphocyte proliferation, a hallmark of HAM/TSP pathogenesis. Reinstating DCs, but not B cells or macrophages, restored proliferation, suggesting that spontaneous *in vivo* proliferation of T cells may occur following presentation of HTLV-I antigens by APCs (Macatonia *et al*, 1992).

To determine if Tax produced intracellularly altered DC characteristics associated with maturation, JAWS II DCs were mock-infected, or infected with a retroviral vector encoding Tax or the control vector expressing green fluorescent protein (GFP). Tax produced within DCs as opposed to being added exogenously may be able to function as a transcriptional activator within the cell to activate the expression of genes known to be upregulated during DC maturation. Following infection, RNA was isolated from the cells and analyzed by semiquantitative RT-PCR. Whereas the mRNA expression of CD86 and CD40 was enhanced following infection with pCLXSN-Tax (Figure 6B, C), there was no increase in the mRNA expression of CD80 (Figure 6A). Based on the strong induction of CD40 and CD86 following infection with pCLXSN-Tax, an analysis was performed

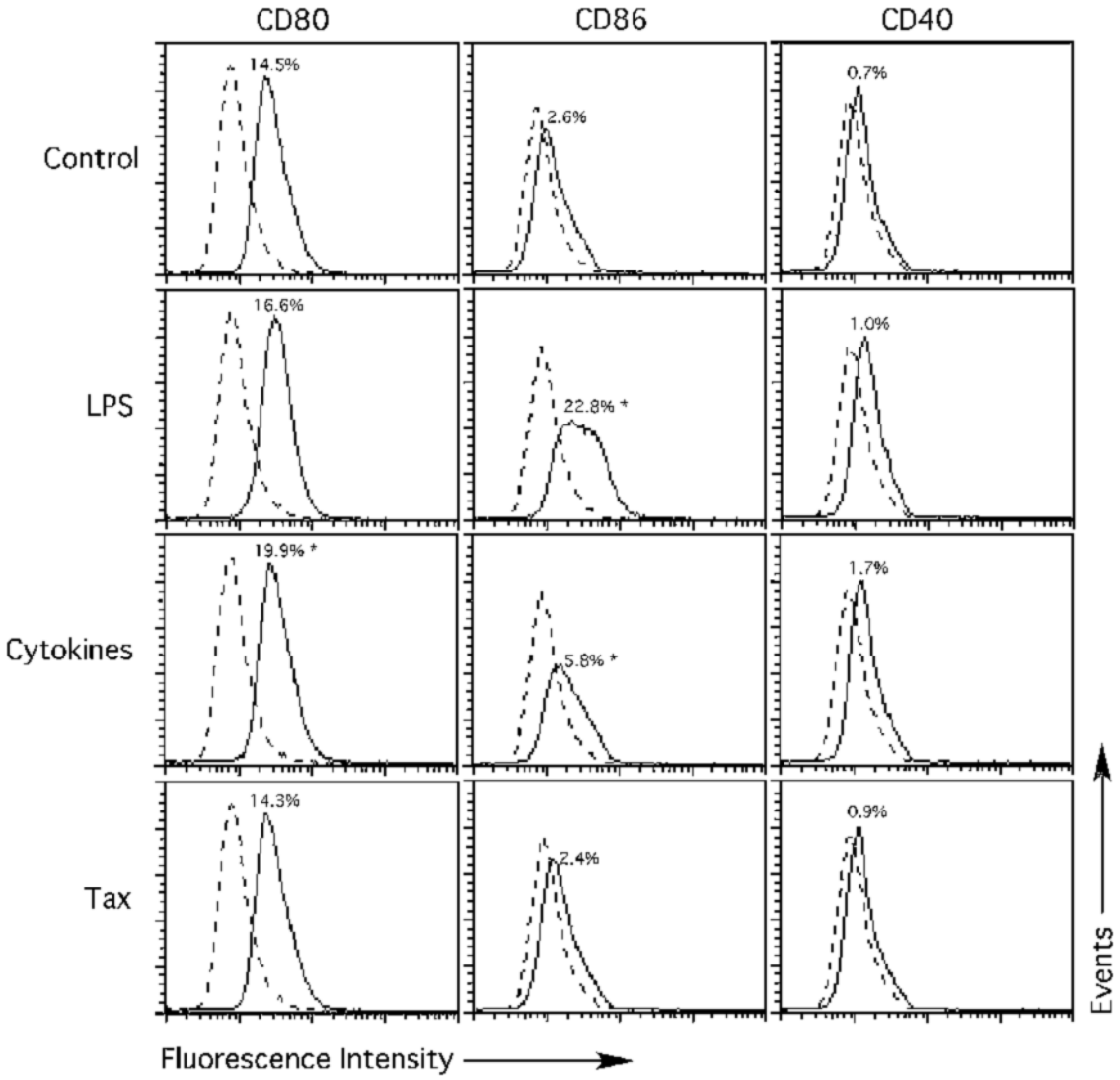

Figure 5 Tax is unable to mediate a detectable increase in cell surface protein expression of molecules known to be upregulated during DC maturation as determined by FACS analysis. JAWS II DCs were treated with a number of stimuli, including those known to mediate DC maturation, including LPS and a cytokine cocktail comprised of IFN-γ, IL-4, and TNF-α. Cytokine treatment resulted in a small increase in the expression of CD80, whereas the addition of LPS or Tax did not cause an increase in CD80 expression. Treatment of cells with LPS caused an increase in the number of CD86-positive cells (2.6% for untreated cells versus 22.8% for treated cells). Cytokine treatment caused a small increase in CD86 expression (5.8%), whereas treatment with Tax had no effect on CD86 expression. All stimuli examined failed to mediate a detectable increase in cell surface CD40 expression. The asterisk (*) denotes a statistically significant increase in expression as compared to control cells, with $P \leq .05$.

concerning the ability of infection with this retroviral vector to enhance the cell surface expression of the corresponding proteins. JAWS II DCs were either mock-infected, or infected with a retroviral vector encoding Tax. Cells were harvested post-infection and assessed for the presence of cell surface CD80, CD86, and CD40 by fluorescence activated cell sorting (FACS) analysis. Rather than increasing the expression of any of these proteins, infection with retroviral vectors expressing Tax caused a statistically significant decrease in the cell surface protein expression of CD80, CD86, and CD40 (Figure 7). This is somewhat surprising considering the strong induction of both CD86 and CD40 expression at the mRNA level following infection with pCLXSN-Tax. However, these results again parallel studies demonstrating that increases in gene expression at the RNA level are not accompanied by increases in corresponding protein expression in JAWS II DCs as assessed by FACS analysis (Jorgensen *et al*, 2002).

Pretreatment of JAWS II DCs with soluble Tax results in decreased MHC class I–mediated antigen presentation

Studies presented herein indicate that although purified Tax protein induces the expression of mRNA encoding cell surface markers associated with DC

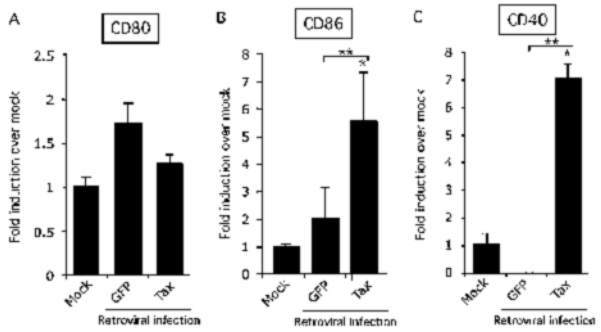

Figure 6 Retroviral delivery of Tax potently induces the mRNA expression of CD86 and CD40, but not CD80. JAWS II DCs were either mock-infected, or infected with a retroviral vector encoding either GFP or Tax. RNA was isolated from the cells and evaluated for levels of mRNA encoding CD80, CD86, and CD40 by RT-PCR. Expression levels were normalized to levels of GAPDH mRNA and represent triplicate determinations. (**A**) mRNA expression levels of CD80 did not change significantly upon infection with GFP or Tax. Infection with GFP caused no significant increase in the RNA expression of (**B**) CD86 or (**C**) CD40, whereas infection with Tax caused an approximately 5.5-fold induction of CD86 mRNA expression and a 7-fold induction of CD40 expression. The asterisk (*) denotes a statistically significant increase in expression as compared to control cells, with $P \leq .05$. The double asterisk (**) indicates a statistically significant difference between GFP-infected and Tax-infected cells. Results are representative of three independent experiments.

maturation and activation, the expression of the corresponding proteins was not detected. Although Tax induced the formation of dendrites and enhanced the expression of genes known to be up-regulated during DC activation, Tax failed to mediate a detectable increase in the cell surface protein expression of molecules such as CD80, CD86, or CD40. However, previous studies have suggested that despite only limited increases in the expression of cell surface molecules involved in aiding antigen presentation, treatment of JAWS II DCs with maturation-inducing stimuli resulted in functional alterations in the DCs (Jorgensen *et al*, 2002). Based on these studies, experiments were performed to examine the effect of Tax treatment on the ability of JAWS II DCs to activate T cells via MHC class I–mediated antigen presentation. To this end, JAWS II DCs were treated with Tax (500 ng/ml). Following Tax treatment, and presumably maturation of the DCs, JAWS II DCs were incubated with the OVA peptide SIINFEKL, followed by detection of peptide presentation via MHC class I by B3Z hybridoma cells. B3Z hybridoma cells have been previously described (Karttunen *et al*, 1992) and represent a T cell hybridoma specific for the OVA peptide $OVA_{257–264}/K^b$ complex ($OVA_{257–264}$ denotes the SIINFEKL peptide) transfected with CD8 and a construct encoding lacZ under the direction of the NFAT responsive element of the IL-2 promoter. Nuclear factor of activated T cells (NFAT)-mediated transcriptional activity has been shown to be an early event in T-cell activation (Durand *et al*, 1987; Shaw *et al*, 1988). The interaction of the peptide-MHC complex with the T cell receptor (TCR) results in the expression of NFAT activity, which activates the expression of IL-2 via the NFAT-responsive element within the IL-2 promoter. Therefore, B3Z activation through

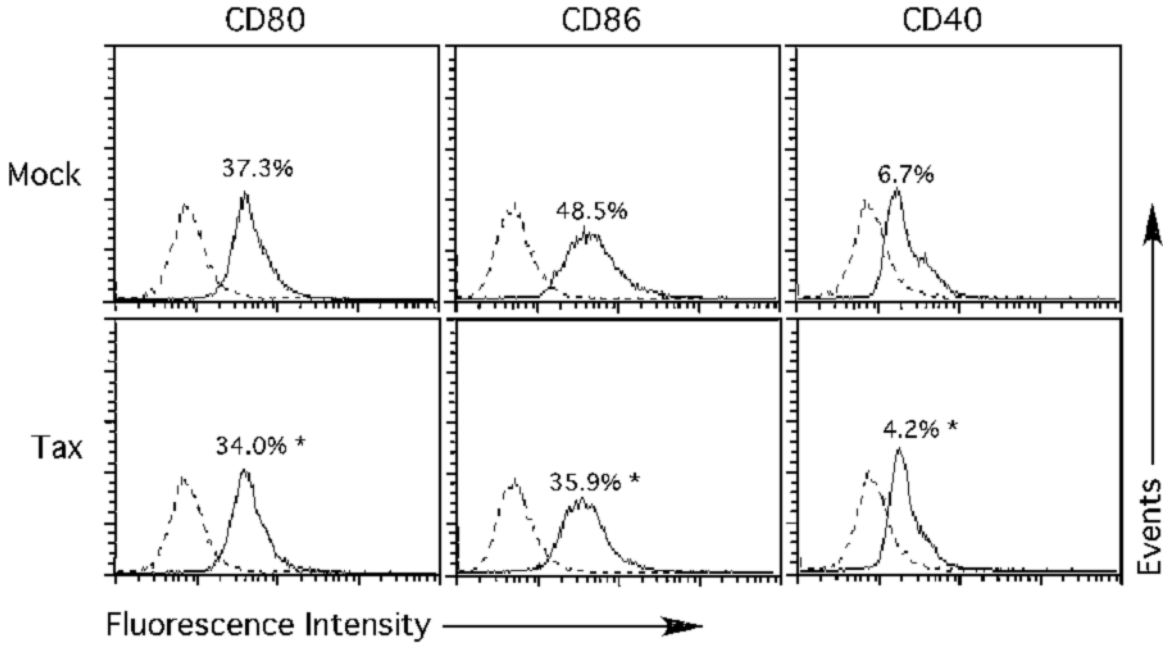

Figure 7 Retroviral infection with pCLXSN-Tax decreases the cell surface expression of molecules involved in DC maturation and activation. JAWS II DCs were infected with a retroviral vector encoding Tax and assessed for the presence of cell surface molecules associated with DC maturation and activation by FACS analysis. The cell surface expression of CD80, CD86, and CD40 decreased following infection with the retroviral vector encoding Tax. The asterisk (*) denotes a statistically significant increase in expression as compared to control cells, with $P \leq .05$.

presentation of the SIINFEKL peptide via MHC class I leads to expression of the lacZ gene under the direction of the NFAT-responsive element of the IL-2 promoter. Lysis of cells and release of lacZ is achieved by using a solution of chlorophenol red β-galactosidase (CPRG) containing NP-40. Cleavage of CPRG by lacZ then results in the production of chlorophenol red, which can be quantitated by measuring absorbance. If Tax alters the process of DC maturation, it is expected that pretreatment with Tax would mature the JAWS II DCs to the point where internalization of exogenous antigen is down-regulated, because mature DCs lose this capability. If this were to occur, Tax-treated JAWS II DCs would fail to internalize the $OVA_{257–264}$ peptide, and therefore the presentation of this peptide to the $OVA_{257–264}$-specific lacZ-inducible B3Z T-cell hybrid would not occur. This would be represented as a decrease in T-cell activation as measured by production of chlorophenol red following cleavage of CPRG by lacZ. As expected, pretreatment of JAWS II DCs with Tax resulted in a decrease in the efficiency of B3Z activation, as evidenced by comparing control and Tax-treated cells at peptide dilutions of 10^{-11} to 10^{-9} (Figure 8). At low and high concentrations of peptide, it was expected that there would be no difference in lacZ expression because there exists a threshold at which T cells were activated. At the low and high concentrations, there was not enough peptide and too much peptide, respectively, resulting in an "all or nothing" response by the responding T cells. However, at the middle concentrations of peptide, there was a statistically significant decrease in the activation of B3Z hybridoma cells, whereas the addition of an irrelevant K^b-restricted peptide, DAPITYNV (β-$gal_{96–103}$) produced no signal (data not shown). Pretreatment of JAWS II DCs with Tax clearly decreased the efficiency of peptide presentation to B3Z hybridoma cells, suggesting the possibility that upon Tax treatment, the DCs failed to internalize a secondary antigen (SIINFEKL) for presentation by MHC class I.

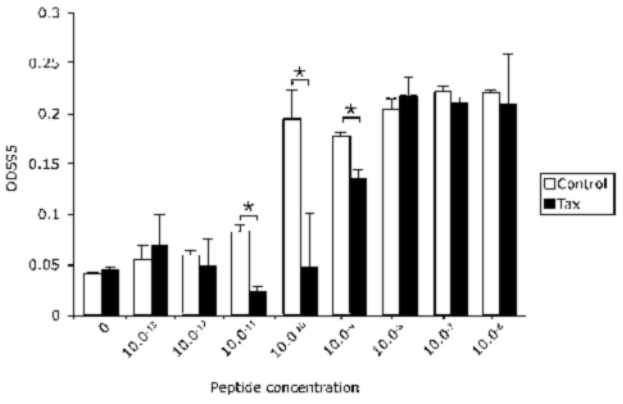

Figure 8 Tax treatment of JAWS II DCs results in a decreased in presentation of an MHC class I–restricted peptide. JAWS II DCs were incubated in either the presence or absence of Tax (500 ng/ml, 16 h) prior to the addition of the MHC class I–restricted peptide SIINFEKL. Pretreatment of cells with Tax resulted in a decrease in the ability of JAWS II DCs to effectively present this peptide to T cells as detected by lacZ expression in the B3Z hybridoma cell line. The asterisk (*) denotes a statistically significant difference between control and Tax-treated cells with $P \leq .05$.

Alternatively, a phenomenon known as "peptide exchange" exists, in which short exogenous peptides may associate with cell surface MHC class I molecules without first being internalized into the APC (Chefalo and Harding, 2001). If exogenous Tax added to DCs was processed and presented via MHC class I molecules, it would be expected that the number of secondarily applied SIINFEKL peptides that were capable of associating with cell surface MHC class I in the absence of peptide internalization would decrease, as Tax peptides would likely be associated with cell surface MHC class I molecules. Whether the SIINFEKL peptide was internalized and subsequently transported to the cell surface in association with MHC class I or whether the participated in peptide exchange, the biological function of Tax remained the same. Specifically, pretreatment of DCs with Tax lead to a decrease in the ability of SIINFEKL to associate with MHC class I. Whether peptide exchange occurred or whether the cells were internalizing, processing, and presenting Tax peptides also represents an intriguing explanation of the results. These possibilities will be examined in greater detail in future studies.

Discussion

In addition to performing vital functions intracellularly, it has been postulated that Tax may mediate some of its pathologic effects as an extracellular protein. Supporting this hypothesis is the observation that Tax was secreted from HTLV-I–transformed cells in culture (Brady, 1992; Lindholm *et al*, 1990). In studies performed *in vivo*, Tax was present in cells contained within the serum and CSF of HAM/TSP patients (Moritoyo *et al*, 1999), and has been recently demonstrated to be present in a cell-free form within the CSF (Cartier Rovirosa *et al*, 2003). This has suggested the possibility that Tax may be secreted or otherwise released from HTLV-I–infected cells, most likely $CD4^+$ T cells because this is the cellular compartment that is primarily targeted for infection.

HAM/TSP is characterized by a strong immune response targeted at HTLV-I, including the presence of CTLs specific for the immunodominant Tax protein. To be capable of inducing the Tax-specific cytolytic activity of $CD8^+$ T lymphocytes, APCs such as DCs mature phenotypically and present Tax peptides in the context of MHC class I to $CD8^+$ T cells. The maturation of DCs is accompanied by characteristic increases in the expression of several cell surface molecules including MHC molecules, the costimulatory molecules CD80 and CD86, as well as CD83, CD40, and others. Studies presented in this report

demonstrate that purified Tax protein enhances the expression of CD80, CD86, and CD83 mRNAs, but not CD40. Whether this was due to an increase in transcription or stabilization of RNA is yet to be determined. However, it seems likely that the increased mRNA expression was due to an increase in transcription. Cytokine signaling through CD40 has been shown to up-regulate nuclear factor (NF)-κB activation (Schonbeck and Libby, 2001; Wesemann *et al*, 2002). The expression of CD40 itself, as well as CD80, CD86, and CD83, are all regulated at least in part through NF-κB binding sites (Dudziak *et al*, 2003; Hinz *et al*, 2001; Li *et al*, 2000; Zhao *et al*, 1996). A number of molecules, including TNF-α and Tax, have been shown to induce translocation of NF-κB to the nucleus, and NF-κB was constitutively activated in HTLV-I–infected cells (Ballard *et al*, 1988; Yao and Wigdahl, 2000). The possibility that Tax enhanced the mRNA expression of these molecules through activation of the NF-κB pathway is currently under investigation.

Despite increases in CD80 and CD86 mRNA expression, Tax protein was unable to mediate a detectable increase in the cell surface expression of the corresponding proteins. However, Tax-treated DCs displayed altered functional activity with regard to activation of antigen-specific T cells. This suggests that despite the lack of detectable alterations in the cell surface protein expression of molecules involved in T-cell activation, Tax treatment caused functional alterations in the JAWS II DCs. There are a number of possible explanations for these observations, which are currently under investigation. Treatment of DCs with Tax may result in the stabilization of cell surface molecules involved in T-cell activation, thereby allowing for an enhancement of their function by preventing protein turnover. Furthermore, because DCs are such potent APCs, it is possible that undetectable alterations in cell surface protein expression or cell surface protein stabilization could result in dramatic functional changes. It has been demonstrated that between 0.4% and 5.1% of DCs in HAM/TSP patients are infected with HTLV-I (Knight *et al*, 1993). Studies have also shown that peripheral blood DCs are infected with HTLV-I and stimulate persistent T-cell activity (Knight *et al*, 1993). DCs from HAM/TSP patients were found to more strongly express CD86 as compared to DCs from uninfected patients (Makino *et al*, 1999). We hypothesize that there is a link between infection of DCs and stimulation of T-cell activity. We have evaluated whether Tax produced within JAWS II DCs impacts the expression of molecules associated with DC activation. Although endogenous Tax caused a significant increase in the mRNA expression of both CD86 and CD40, no increase in the corresponding cell surface protein expression was apparent. Significantly, the observed increase in mRNA expression of CD40 and the lack of an increase in CD80 mRNA expression differed from results obtained following treatment of DCs with exogenous Tax. This implicates different mechanistic pathways of Tax activity, depending on whether the protein is acting as an extracellular mediator or an endogenously produced protein. For example, exogenous Tax added to DCs may activate a signaling cascade following interaction with a cellular receptor, resulting in a number of events that culminated with activation of cellular transcription factors that can then mediate an increase in the expression of genes relevant to DC maturation. However, Tax produced within infected cells may itself function either directly as a transcriptional activator, or may interact with cellular transcription factors to activate cellular gene expression. Complexes formed within the nucleus will necessarily differ, depending on whether Tax is present or not. In this respect, there may be differences in genes that are activated, depending on whether Tax is present in nuclear transcription factor complexes. However, caveats exist when attempting to compare the outcome of adding purified Tax to DCs versus expressing Tax as an endogenous protein following retroviral infection. First, the process of infecting the cells with Tax through the use of a retroviral vector has a certain amount of mitogenic activity. Second, the amount of exogenous and endogenous Tax are likely quite different, as it is difficult to control the expression levels of endogenous Tax in the infected cells.

Studies presented herein have begun to provide a potential link between the presence of detectable extracellular Tax in the CSF and peripheral blood of HAM/TSP patients, and the genesis of a hyperstimulated immune response that is characteristic of this neurologic disorder. Several studies have demonstrated the effect of extracellular Tax on a number of cell populations including the induction of cytokine expression in microglia (Dhib-Jalbut *et al*, 1994), induction of TNF-β expression, Igκ light chain expression, and NF-κB activity in lymphoid cells (Lindholm *et al*, 1990, 1992), and induction of IL-2Rα expression in lymphocytes (Marriott *et al*, 1992). Studies presented in this report demonstrate that extracellular Tax reduced the ability of JAWS II DCs to activate a T-cell hybrid via presentation of an MHC class I–restricted peptide. Several potential explanations are possible. This includes the possibility that extracellular Tax interacts with a cell surface receptor or receptors to initiate signaling cascades resulting in the maturation of DCs. This may then inhibit antigen uptake, or render the DCs incapable of presenting an internalized antigen if MHC molecules have been redistributed to the cell surface as a consequence of maturation prior to the cells encountering the SIINFEKL peptide. It remains possible that exogenous SIINFEKL peptide that had not been internalized may have been able to associate with cell surface MHC class I molecules, either through peptide exchange or by associating with free MHC class I molecules that were not associated with a peptide (Chefalo and Harding, 2001). If this were the case, it

would be expected that pretreatment of the DCs with Tax would necessarily decrease the presence of free cell surface MHC class I molecules, as they would likely be associated with Tax peptides. Therefore, whether SIINFEKL was internalized for presentation by MHC class I or whether the peptide participated in peptide exchange at the cell surface, the biological activity of Tax in terms of reducing the presentation of SIINFEKL would be the same. This possibility is currently under investigation.

Alternatively, it is possible that Tax was internalized, causing the maturation the DCs to such a degree that they were no longer capable of antigen uptake. This would prevent activation of B3Z cells because the DCs would be incapable of internalizing the SIINFEKL peptide. This is consistent with the concept that Tax contributes to the maturation of DCs, as mature DCs lose the ability to internalize antigen as they shift to an antigen presentation function. In addition, previous studies have shown that Tax can be internalized by cells (Lindholm *et al*, 1990; Marriott *et al*, 1991). An intriguing possibility is that the JAWSII DCs may have internalized Tax, which could then itself be processed for presentation by MHC class I. In the context of HAM/TSP, this would be consistent with the abundance of Tax-specific CTLs in the CSF and peripheral blood of HAM/TSP patients (Jacobson *et al*, 1990, 1992). Studies have shown that DCs from HAM/TSP patients are infected with HTLV-I and stimulate the proliferation of autologous lymphocytes (Macatonia *et al*, 1992). Although this may result simply from the DCs being infected with HTLV-I and expressing Tax, it also remains possible that extracellular Tax initiates or stimulates the processing and presentation of endogenous Tax. In the event that extracellular Tax encounters DCs that are not infected with HTLV-I, it is possible that Tax is internalized, processed, and presented via MHC class I to T cells. This hypothesis is supported by evidence indicating that soluble Tax can be internalized by cells (Marriott *et al*, 1991).

Studies presented herein extend observations concerning a number of emerging functions of extracellular Tax. Observations made begin to explore the connection between the presence of extracellular Tax in the peripheral blood and CSF of HAM/TSP patients and the hyperstimulated immune response characteristic of this HTLV-I–induced disease state. As the repertoire of functions ascribed to Tax expands, it will be critical to delineate the mechanisms by which Tax performs such functions.

Materials and methods

Cell culture

JAWS II, an immortalized C57BL/6 murine bone marrow–derived DC line (ATCC no. CRL-11904) were grown in endotoxin-free RPMI 1640 containing sodium bicarbonate (2.0 mg/ml; Mediatech, Herndon, VA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), HEPES (18 mM; Mediatech), sodium pyruvate (1 mM; Mediatech), recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (5 ng/ml; R&D Systems, Minneapolis, MN), and the antibiotics penicillin and streptomycin (0.1 mg/ml each; Mediatech). 293 T cells (ATCC no. CRL-1573) were grown in endotoxin-free Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc) supplemented with 10% heat-inactivated bovine serum and the antibiotics penicillin and streptomycin (0.1 mg/ml each). B3Z hybridoma cells specific for the OVA peptide $OVA_{257–264}/K^b$ complex were maintained in RPMI 1640 supplemented with 5% FBS and the antibiotics penicillin and streptomycin (0.1 mg/ml each). All cells were maintained at 37°C in 5% CO_2 at 90% relative humidity. JAWS II DCs were stimulated to mature with the addition of a cytokine cocktail comprised of recombinant murine IL-4 (10 ng/ml), recombinant murine IFN-γ (100 U/ml), and recombinant murine TNF-α (10 ng/ml) for 12 to 24 h (R&D Systems). In some experiments, cells were also stimulated to mature with the addition of lipopolysaccharide from *Escherichia coli* (*E. coli*) O26:B6 (1 μg/ml) for 24 to 48 h (Sigma, St. Louis, MO), or purified Tax-His6x at the indicated concentrations and times.

HTLV-I Tax expression, purification, and treatment

Tax protein was expressed in *E. coli* HB101 by the pTax-His6x expression vector kindly provided by Dr. Chou-Zen Giam (Uniformed Services University, Bethesda, MD). Tax protein was purified by Ni^{2+} chromatography (Novagen, Madison, WI) following sonication, and was dialyzed overnight at 4°C in endotoxin-free dialysis buffer containing HEPES (40 mM), glycerol (20%), potassium chloride (7.5 mg/ml), EDTA (0.2 mM), phenylmethylsulfonyl fluoride (PMSF; 34.8 μg/ml), and dithiothreitol (DTT; 78 μg/ml). Silver staining was performed by separating proteins on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, which was then fixed and stained utilizing the Silver Stain Plus kit as described by the manufacturer (Bio-Rad, Hercules, CA). The gel was dried overnight with the DryEase Mini-Gel Drying System as described by the manufacturer (Novex, San Diego, CA) and subjected to densitometry to assess the concentration of Tax. Purified Tax was then analyzed by electrophoretic mobility shift assay (EMSA) to determine the activity of the protein as assessed by its ability to enhance CAMP-responsive element binding protein (CREB) (kindly provided by Dr. Jennifer Nyborg, Colorado State University, Ft. Collins, CO) binding to the 21–base pair HTLV-I promoter proximal repeat of tax-responsive element (TRE)-1. For experiments designed to determine the effect of exposure to Tax, cells were treated with TaxH6 (500 ng/ml).

Morphologic analysis of JAWS II DCs

JAWS II DCs were seeded at 2×10^6 cells in 100-mm tissue culture plates. Cells were either incubated in the absence or presence of LPS (1 μg/ml, 48 h; Sigma), a cytokine cocktail comprised of recombinant murine IL-4 (10 ng/ml), recombinant murine IFN-γ (100 U/ml), and recombinant murine TNF-α (10 ng/ml) for 24 h, or Tax (24 h). Following stimulation, cell morphology was analyzed by high-magnification phase-contrast microscopy. Images were obtained with a Cook CCD Sensicam digital camera controlled by Slidebook software (Intelligent Imaging Innovations, Denver, CO). All components of the microscopy system were controlled using an Apple Macintosh G4 dual 1-GHz processor computer.

Retroviral vector infections

Indicated retroviral vector constructs (pCLXSN-GFP or pCLXSN-Tax, 1 μg) were cotransfected with pCL-Ampho (1 μg) and VSV-g (0.15 μg) into 293 T cells (3×10^5) in 6-well plates with FuGene6 (Roche, Indianapolis, IN). After 48 h, viral supernatant was filtered through a polysulfone filter (0.45 μM) and used to resuspend JAWS II cells (1×10^6), which were then distributed into 6-well plates. Polybrene (8 μg/ml; Sigma) was added to each well, and the cells were centrifuged at 1800 rpm for 45 min. Cells were subsequently incubated at 37°C, washed with RPMI the following day, and expanded for 3 to 7 days in culture.

Analysis of RNA production by RT-PCR

Total RNA was isolated from JAWS II DCs utilizing an RNeasy kit as described by the manufacturer (Qiagen, Valencia, CA). Total RNA (2 μg) was converted into single-stranded cDNA by reverse transcription using Omniscript Reverse Transcriptase (4 U; Qiagen) in the presence of dNTP mix (0.5 mM; Qiagen), oligo-dT_{15} primer (1 μM; Invitrogen, Carlsbad, CA), and RNase inhibitor (10 U; Roche Molecular Biochemicals, Indianapolis, IN). The reverse transcription reaction was allowed to proceed for 1 h at 37°C. A total of 2.5 μl of cDNA was then used for each PCR. Conditions for the PCR were as follows: after initial denaturation at 94°C for 2 min, 35 amplification cycles were conducted in a DNA thermocycler (model 9600, Perkin Elmer). Each cycle consisted of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min and 30 s. PCR components included PCR buffer (1×; Invitrogen), $MgCl_2$ (1.5 mM; Invitrogen), dNTP mix (0.2 mM; Invitrogen), Platinum Taq DNA polymerase (0.625 U; Invitrogen), and upstream and downstream primers (1.0 μM of each; IDT, Coralville, IA) in a 50-μl reaction volume. PCR products were separated by agaorse gel electrophoresis and bands were quantitated by densitometry.

Microarray analysis

Total RNA was isolated from JAWS II DCs utilizing an RNeasy Midi kit as described by the manufacturer (Qiagen). Mouse chip microarrays comprised of Qiazen's array-ready oligo set (13,443; 70-mer probes) spotted on poly-L-lysine–coated glass slides (Corning) were obtained from the institutional Molecular and Genomics Laboratory (Drexel University College of Medicine). For each hybridization, 20 μg of total RNA from Tax (500 ng/ml)-treated and mock-treated samples was converted into cDNA and labeled with either Cy3 or Cy5 fluorescent dye (Amarsham Pharmacia Biotech, Piscataway, NJ) using a superscript indirect cDNA labeling system (Invitrogen). A 60-μl hybridization buffer was prepared containing 20 mM HEPES buffer (pH 7.0 to 7.2), 3× SSC (Fisher), 0.2% SDS (Sigma), 10 μg poly(A) RNA (Sigma), 10 μg mouse Cot1 DNA(Sigma), and 15 to 20 pM of dye-labeled probe(s). The mixture was heated for 2 min at 95°C to 100°C, cooled at room temperature, subjected to centrifugation, and applied to the slide by pipetting under one corner of the lifter slip (25 × 44 mM, Erie Scientific, Portsmouth, NH). The slide was placed in a DeRisi hybridization chamber (DieTech, part no. DT1001) and hybridization was performed at 65°C for 18 h. The array was washed with decreasing concentrations of SSC (1× SSC + 0.03% SDS, 0.2× SSC, 0.05× SSC, 0.01× SSC), dried with a stream of nitrogen gas and scanned by a GenePix 4000B Microarray Scanner (Axon Instrument, Foster City, CA). Image analysis was performed with GenePix Pro 5.0 software, which provides the tool to generate a GenePix Array List (GAL) file from a plain text file describing the position and content of each spot (detailed information can be found at www.axon.com). Each hybridization was prepared in duplicate with the cyanine fluors reversed to eliminate any false signal and the resultant data are represented as the average fold change from four different arrays. Fold up-regulation or down-regulation equal to or greater than 2 is considered to be significant.

Flow cytometric analyses

Cells were isolated and resuspended in phosphate-buffered saline (PBS) containing 20% normal mouse serum (Sigma) and Fc Block (BD PharMingen San Diego, CA). After 20 min on ice, specific fluorochrome-conjugated monoclonal antibodies were added and the cells were incubated in the presence of antibody for 45 min on ice. Prior to analysis, cells were fixed in 2% paraformaldehyde. For analysis of cell surface expression of proteins associated with DC maturation, the following monoclonal antibodies were utilized: fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD40, R-phycoerythrin (R-PE)–conjugated mouse anti-mouse $I\text{-}A^b$, FITC-conjugated hamster anti-mouse CD80 (B7.1), FITC-conjugated rat anti-mouse CD86 (B7.2), and R-PE–conjugated rat anti-mouse CD86 (B7.2) (BD PharMingen). Monoclonal isotype control antibodies utilized were: FITC-conjugated rat

$IgG_{2a,\kappa}$, FITC-conjugated Armenian hamster $IgG_{2*\kappa}$, R-PE–conjugated mouse $IgG_{2a,\kappa}$, and R-PE–conjugated rat $IgG_{2a,\kappa}$ (BD PharMingen). Flow cytometric analysis was performed using a FACScan (Becton Dickinson). JAWS II DCs were first gated by forward and side scatter, and nonviable cells were gated out on the basis of their light scatter. Data from 10,000 to 100,000 cells was collected and analyzed by CELLQuest software.

Analysis of antigen presentation by JAWS II DCs
Following 16 h in either the absence or presence of Tax (500 ng/ml), 5×10^4 JAWS II DCs were plated per well in a 96-well plate. Following centrifugation (2000 rpm, 5 min), medium was removed and replaced with medium containing 10-fold dilutions of the MHC class I–restricted $OVA_{257-264}$ peptide SIINFEKL at concentrations ranging from 10^{-6} to 10^{-13} M either in the absence or presence of Tax (500 ng/ml). After 40 min at room temperature, 5×10^4 B3Z T-cell fusion cells in medium either lacking or containing Tax (500 ng/ml) were added to each well. Following 16 h incubation at 37°C, the plate was centrifuged (2000 rpm, 5 min), medium was removed, and cells were lysed. LacZ expression was detected by cleavage of chlorophenol red β-galactosidase (CPRG; 0.15 mM containing $MgCl_2$ [1 mM] and NP-40 [0.125%]) by lacZ, resulting in the production of chlorophenol red. After 4 h incubation at 37°C, stop buffer (300 mM glycine and 15 mM EDTA in water) was added to each well and absorption of each well was read using a 96-well plate reader (MRX Revelation, Dynex Tech., Chantilly, VA) at 595 nm with a 630-nm reference wavelength.

Statistical analyses
All statistical analyses were performed using JMP version 3.0 (SAS Institute, Cary, NC). Briefly, each set of data was imported into JMP and an analysis of variance (ANOVA) was performed. The statistical signfificance of each comparison was determined using Student's *t* test with a *P* value of .05.

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