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Induction of proinflammatory cytokines by human T cell leukemia virus type 1 Tax protein as determined by multiplexed cytokine protein array analyses of human dendritic cells

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Summary

Human T cell leukemia virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is characterized by a hyperstimulated immune response including elevated levels of inflammatory cytokines/chemokines, and the oligoclonal expansion of virus-specific CD8⁺ T cells in the cerebrospinal fluid. Studies have shown that the HTLV-1 transactivator protein Tax is available for immune recognition by antigen presenting cells (APCs) such as dendritic cells (DCs). DCs are relevant to the pathogenesis of HAM/TSP since the presentation of Tax peptides by activated DCs to naïve CD8⁺ T cells may play an important role in the induction of a Tax-specific immune responses observed in HAM/TSP. In this study, a human cytokine protein array was used to study the secretion of cytokines by monocyte-derived DCs (MDDCs) exposed to Tax. Of the 16 cytokines analyzed, 6 cytokines were secreted in significantly high amounts (≥ 2 -fold) including Th1 cytokines (IFN- γ , IL-12, and TNF- α) and C-C chemokines (Eotaxin, MCP-1, and MCP-3). Selected cytokines were further examined at two concentrations of Tax at two time periods. Furthermore, a transient exposure to Tax did not result in any cytokine production when examined at three different time points after exposure, indicating that the prolonged presence of Tax was required for its activity. Finally, inhibition of the NF- κ B signaling pathway by specific inhibitors abrogated Tax-mediated cytokine secretion. Collectively, these investigations suggest a role for Tax-induced cytokine secretion from MDDCs that may be critical for cellular activation and tissue damage observed in HAM/TSP.

Keywords

HTLV-1 Tax; dendritic cells; protein array

1. Introduction

Human T cell leukemia virus type 1 (HTLV-1), an exogenous type C retrovirus, infects 10 – 20 million people worldwide [1] and is endemic in southern Japan, Caribbean, Central and South America, Middle East, and Africa [2]. While majority of the infected individuals are asymptomatic carriers, about 2 – 3% develop a progressive lymphoma designated adult T cell leukemia (ATL) while an additional 0.5 – 3% develop a debilitating neurologic disease known

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as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3]. HAM/TSP is a chronic progressive disease of the central nervous system (CNS) characterized by weakness and stiffness in the lower extremities, lower back pain, urinary dysfunction, thoracic myelopathy, and paraplegia. Pathologic observations indicate a symmetrical loss of myelin and axonal dystrophy in the thoracic and lumbar regions of the spinal cord [4].

It is assumed that CNS tissue damage observed in HAM/TSP is the result of a hyperstimulated immune response and a number of theories on how this immune response may contribute to tissue damage have been proposed [5]. One such theory suggests that HTLV-1 may infect neurons or CNS resident cells and these infected cells may, in turn, be subject to HTLV-1-specific immune responses. Other theories focusing on the mechanism of CNS damage assume that antibodies or T cells that recognize certain HTLV-1 epitopes may cross-react with host cell heterogeneous nuclear ribonucleoprotein A1 to promote antigen mimicry [6,7]. An additional pathogenic mechanism likely operative in HAM/TSP involves the process of “bystander” or “collateral” damage caused by inflammatory cytokines released by HTLV-1-infected cells that may have crossed the blood-brain barrier (BBB) [8].

Cytokines play pivotal roles in the regulation of the immune response and the HTLV-1 transactivator protein Tax has been postulated to contribute to the inflammatory response observed in HAM/TSP as a result of the aberrant regulation of host cell cytokines [9]. Elevated levels of proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin 1 (IL-1), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been detected in the cerebrospinal fluid of HAM/TSP patients [10–12]. Supporting the hypothesis of “bystander” damage, it has been suggested that HTLV-1-induced damage to the CNS involves an immune-mediated disease in which Tax can impart some of its pathologic effects by functioning as an extracellular cytokine [13–17]. The presence of cell-free Tax in cerebrospinal fluid (CSF) has been demonstrated during progression to HAM/TSP [18] and may be the result of apoptosis or necrosis of HTLV-1-infected cells, or the secretion of Tax from Tax-expressing cells [19]. Extracellular Tax has been shown to induce production and secretion of TNF- α from a human neuronal cell line [20]. Primary human microglial cells have also been shown to respond to extracellular Tax by secreting inflammatory cytokines TNF- α , IL-1 β , and IL-6 [21]. Tax-stimulated primary human peripheral blood macrophages have also been shown to produce and secrete TNF- α , IL-1, and IL-6 [21] and soluble Tax treatment has been shown to induce the nuclear accumulation of NF- κ B as well as the expression of the immunoglobulin μ light chain [13,15], TNF- β [13], and IL-2 receptor in lymphoid cells [17].

Bystander damage to the CNS tissue in HAM/TSP likely involves the concerted activity of several infiltrating immune cell populations. Although CD4⁺ T cells, dendritic cells (DCs), and monocytes/macrophages contribute to the pathogenesis of HAM/TSP, the CD8⁺ T cell compartment plays an important role in neurodegeneration as reflected by the oligoclonal expansion of Tax-specific cytotoxic CD8⁺ T cells (CTLs) in the CSF of infected individuals [6]. Since the induction of a CTL response is strictly dependent on antigen presentation and costimulation provided by antigen presenting cells (APCs), it is hypothesized that these immune cell components are critical for the genesis of a Tax-specific host immune response. In support of this hypothesis, the most potent APCs, dendritic cells, obtained from HAM/TSP patients have been shown to be infected with HTLV-1 and stimulate autologous T cell proliferation [22–24]. DCs exposed to extracellular Tax have also been shown to undergo activation and maturation [25,26] and may thereby facilitate the entry of HTLV-1-infected cells into the brain by compromising the integrity of the BBB through the secretion of inflammatory cytokines and chemokines, contributing to tissue damage observed in the CNS of HAM/TSP patients. We have previously shown utilizing gene expression analyses with a pathway-specific gene array a range of proinflammatory cytokines and chemokines that were differentially expressed following treatment of DCs with extracellular Tax [25]. However, very

few protein-based investigations of the proinflammatory cytokine response of DCs exposed to cell-free Tax have been reported. In this regard, a human cytokine protein array has been utilized for the detection of cytokines in supernatants of Tax-stimulated DCs. This array is composed of 16 wells, each well containing 16 different cytokine-specific antibodies spotted in triplicate. The multiplexed protein array-based analyses provide initial clues with respect to the proinflammatory cytokines involved in the Tax-mediated effects on DCs and may provide insights into mechanisms of HTLV-1-induced disease pathogenesis.

2. Materials and Methods

2.1 Generation of monocyte-derived dendritic cells (MDDCs)

MDDCs were generated from peripheral blood mononuclear cells (PBMCs) as described [25]. Briefly, PBMCs were labeled with anti-CD14-coated magnetic beads (Miltenyi Biotec Inc., Auburn, CA) and monocytes were magnetically purified as described by the manufacturer (VarioMACS; Miltenyi Biotec). Purified monocytes were differentiated to immature DCs by culture in complete medium comprised of BioWhittakerTM RPMI-1640 with L-glutamine (Cambrex, Walkersville, MD) supplemented with 10% FBS (Hyclone, Logan, UT) and 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 rHuIL-4 (10 ng/ml; PeproTech) for 5 days at 37°C and 5% CO₂. Cells were replenished with a new supply of cytokines every third day. Differentiation to DCs was confirmed by phenotypic analyses of specific surface markers (CD11c, CD40, CD80, CD83, CD86, HLA-ABC, and HLA-DR) by flow cytometry.

2.2 HTLV-1 Tax expression and purification

HTLV-1 Tax protein was prepared as described [25,26] using a pTax-His₆ expression vector (provided by Dr. Chou-Zen Giam, Uniformed Services University of the Health Sciences, Bethesda, MD) in *E. coli* HB101 followed by Ni²⁺ chromatography (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 specificity of purified protein 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 the Tax responsive element 1. Mock bacterial extracts were prepared in an identical manner from *E. coli* cells not expressing the Tax gene. In both cases, the 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).

2.3 Multiplexed cytokine detection using human cytokine protein array

Immature DCs were treated with mock fluid (negative control), LPS (positive control), or purified Tax protein (1 µg/ml). Cell supernatants were collected after 24 hours and analyzed for cytokine secretion using a human cytokine antibody array (Clontech Laboratories Inc., Mountain View, CA). Briefly, a supernatant sample (100 µl) or a recombinant cytokine cocktail dilution (100 µl; Clontech) was added to each well of the array chamber slide that had been pre-coated with 16 monoclonal antibodies (each spotted in triplicate) specific to the cytokines listed in Figure 1A. The slide was washed to remove any unbound proteins and incubated with a mixture of biotinylated detector antibodies (Clontech) for 1 hour. Secreted cytokines were detected using a fluorescence-based technique by exposing the slide to a mixture of a dye reagent and an enhancer reagent (Clontech) for 1.5 hours. The slide was washed several times, spin-dried by centrifugation at 1,500 rpm, and scanned by a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Image analysis was performed with GenePix software

(version 5.0) which provides a tool to generate a GenePix Array List (GAL) file from a plain text file describing the position and fluorescence intensity of each spot. Data was analyzed to generate standard curves for each cytokine using a data analysis workbook (www.bioinfo.clontech.com). The concentrations of cytokines in the test samples were extrapolated from the standard curves to generate a final report.

2.4 Enzyme-linked immunosorbent assays for cytokines

For experiments designed to determine the effect of a transient exposure to Tax, immature DCs were treated with Tax (1 $\mu\text{g/ml}$) for either 4, 8, or 24 hours, thereafter, cells were washed three times with phosphate-buffered saline (PBS) and re-incubated in culture media without Tax protein. In each case, culture supernatants were collected at 1, 4, and 24 hours following re-incubation in the absence of Tax and analyzed for the secretion of cytokines (IL-12 and TNF- α) by ELISA as described by the manufacturer (eBioscience, San Diego, CA).

To examine the role of NF- κB in Tax-mediated cytokine secretion, immature DCs were pre-treated with 10 μM of PDTC (pyrrolidine dithiocarbamate, Sigma, St Louis, MO) or TLCK (N α -p-Tosyl-L-Lysine Chloromethyl Ketone; Biomol, Plymouth Meeting, PA), potent inhibitors of NF- κB , for 30 minutes, followed by treatment with Tax (1 $\mu\text{g/ml}$) for 24 hours. Culture supernatants, collected 24 hours post-Tax treatment, were analyzed for the secretion of cytokines (IL-12 and TNF- α) using Ready Set Go! ELISAs (eBioscience). All experiments were performed in triplicate and the results were statistically analyzed by Student's *t*-test. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained.

3. Results

3.1 HTLV-1 Tax induces the production of cytokines from primary human MDDCs

To examine whether extracellular Tax induces the production of cytokines from MDDCs, the optimal dose of protein essential to detect cytokine secretion was previously determined by exposing cells to an increasing concentration of Tax (Jain and Wigdahl, unpublished observations). As a result of these studies, a concentration of 1 $\mu\text{g/ml}$ of Tax was sufficient to induce detectable cytokine secretion and this concentration of Tax was used for all subsequent experiments. Immature DCs were exposed to mock fluid (a negative control), LPS (a positive control) or purified Tax protein. Supernatants harvested 24 hours post-stimulation were analyzed for the production of cytokines using a human cytokine protein array as described in Materials and Methods. Figure 1A displays a panel of cytokines and control proteins as imprinted on the slide. The normalized amount (pg/ml) as well as the fold-changes (over the negative control) of cytokines produced following treatment of MDDCs with Tax are listed in Figure 1B. Cytokines produced in significantly high amounts (≥ 2 -fold) included Eotaxin (692 pg/ml), IFN- γ (1462 pg/ml), IL-12 (126 pg/ml), MCP-1 (98 pg/ml), MCP-3 (1799 pg/ml), and TNF- α (7839 pg/ml). Some cytokines were produced in moderate amounts (≥ 1.5 -fold) including IL-2 (117 pg/ml), IL-10 (2233 pg/ml), and TNF- β (2814 pg/ml). As indicated in Figure 1B, extracellular Tax failed to induce the secretion of IL-5, IL-6, IL-7, IL-8, and IP-10. Of the 16 cytokines analyzed using the protein array, IL-4 and GM-CSF were detectable due to the fact that these cytokines were added to the culture medium to generate DCs. The cytokine profile following LPS-stimulation is also shown (Figure 1B) and it was observed that while LPS, a conventional DC stimulant, induced the secretion of IL-7 in moderate amounts (1.6-fold), Tax did not induce any IL-7 production indicating differences in the cytokine production profile following Tax or LPS stimulation of MDDCs.

3.2 Validating cytokine secretion profile

Having examined the cytokine secretion profile following treatment of MDDCs with extracellular Tax protein, we proceeded to further validate the production of cytokines produced in significant amounts with two doses of Tax at two different time periods. Immature DCs were treated with mock fluid (negative control), LPS (positive control), or Tax (0.1 and 1 $\mu\text{g/ml}$). Culture supernatants, harvested 24 and 48 hours post-stimulation, were analyzed for the secretion of cytokines using the protein array as described above. As indicated in Figure 2, Tax significantly induced the secretion of cytokines compared to the mock protein extract in a dose- and time-dependent manner. With the exception of TNF- α , the cytokines examined were produced in detectable amounts with a Tax dose of 0.1 $\mu\text{g/ml}$. All cytokines tested in maximal amounts at the higher Tax dose of 1 $\mu\text{g/ml}$. LPS, the positive control, induced the secretion of cytokines by 24 hours, however, with the exception of Eotaxin, cytokine secretion remained unchanged at 48 hours post-stimulation.

3.3 Effect of transient exposure of Tax on cytokine production

Of the cytokines detected using the protein array, IL-12 has been shown to be the most important cytokine for induction of Th1 cells and is considered to be involved in the onset of various autoimmune diseases [27,28]. Furthermore, IL-12 augments T-cell mediated cytotoxicity and stimulates the production of TNF- α , a neurotoxic cytokine [27]. Both IL-12 and TNF- α are important in disease pathogenesis as elevated levels of these inflammatory cytokines have been detected in HAM/TSP patients [29]. Consequently, it was of interest to determine whether a short exposure to Tax was sufficient to induce a sustained release of the cytokines. To this end, cells were exposed to Tax for three different time periods (4, 8, and 24 hours) after which Tax was removed by extensive washing. Tax-pulsed DCs were cultured in fresh medium and culture supernatants harvested at three different time points (1, 4, and 24 hours) were analyzed for the secretion of cytokines by antigen-specific ELISAs. As indicated in Figure 3, moderate levels of IL-12 (280 pg/ml) were detected in the culture medium only at 24 hours post-Tax stimulation. With respect to TNF- α , protein production was observed at 4 hours after stimulation with Tax and peaked to 300 pg/ml by 24 hours. In both cases, the continued presence of Tax was critical to observe sustained production of these cytokines and the removal of Tax subsequent to brief treatment pulse, resulted in undetectable levels of cytokine production, indicating that continuous Tax stimulation is essential to observe cytokine secretion. Additionally, the levels of cytokines as determined by ELISA, were lower than those obtained by protein array analyses due to differences in the sensitivity of the two systems.

3.4 Role of NF- κ B activation in Tax-mediated production of cytokines

Having identified the cytokines produced following treatment of MDDCs with Tax, we proceeded to examine the signaling pathways that may play a significant role in this process. Extracellular Tax protein is known to stimulate nuclear NF- κ B DNA binding and the expression of NF- κ B-responsive Ig kappa and TNF- β genes in lymphoid cells [13–15]. Therefore, experiments were performed to determine whether the inhibition of NF- κ B signaling could abrogate Tax-mediated cytokine secretion. MDDCs were pre-treated (30 minutes) with conventional inhibitors of the NF- κ B pathway (PDTC and TCLK) prior to the addition of Tax for additional 24 hours. As shown in Figure 4, inhibitor treatment blocked the effect of Tax with no secretion of cytokines observed in the presence of these inhibitors. This was a strong indication that Tax-mediated effects were regulated by a NF- κ B signaling pathway; however, the role of other signaling cascades such as protein kinases may not be ruled out and is currently being examined.

4. Discussion

Recent studies denote a role for dendritic cells in a number of inflammatory and neurodegenerative conditions including bacterial and parasitic infections [30,31], amyotrophic lateral sclerosis [32], multiple sclerosis [33], and in the animal model of experimental autoimmune encephalitis [31]. 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 [22]. Furthermore, HTLV-1 is known to infect DCs both in vitro [22, 24] and in vivo [23], suggesting that HTLV-1-infected DCs play a crucial role in the production of the autoreactive T cells observed in HAM/TSP patients. Previous studies using gene array analyses demonstrated that DCs, once exposed to extracellular Tax, undergo activation and maturation [25,26]. We have also performed extensive biochemical and immunological assays in order to examine the effect of extracellular Tax on the functions of DCs (Jain and Wigdahl, unpublished observations). Studies reported herein further characterize the role of DCs in HTLV-1-associated neurologic disease, with particular emphasis on proinflammatory cytokine production. The results have significant physiological implications and support our general hypothesis that DCs once exposed to Tax, either by infection or uptake from the extracellular environment, secrete inflammatory and immunoregulatory cytokines, thereby compromising the integrity of the BBB and playing a crucial role in initiating Tax-specific CTL responses.

In this study, the production of cytokines in Tax-stimulated DCs was examined using a high throughput multiplatform cytokine protein array system that enabled the simultaneous detection of 16 cytokines using an ELISA-based system. Of the 16 cytokines spotted on the antibody array, 10 were highly expressed subsequent to Tax or LPS (known stimulator of DCs) treatment. IL-4 and GM-CSF were highly expressed probably due to the fact that these cytokines were added to the culture medium to generate DCs. An increased secretion of IFN- γ was noted in the presence of Tax, the concentrations of which are reported to be elevated in HAM/TSP patients [34]. IFN- γ is also known to play a role in inducing the production of MHC and co-stimulatory molecules by APCs thereby promoting a cell-mediated immune response [35]. Furthermore, IFN- γ has been shown to stimulate the production of IL-12, a critical immunoregulatory cytokine [28]. IL-12 is the most important cytokine for the polarization of T cells to Th1 cells [36], and has been considered to be involved in the progression of a number of autoimmune diseases [27]. 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 [28]. In addition, IL-12 augments the production of TNF- α also demonstrated by the cytokine protein array analyses. The presence of TNF- α , a neurotoxic cytokine, has been demonstrated in spinal cord lesions of HAM/TSP patients and is reported to be responsible for the observed demyelination and axonal degeneration [37]. Cytokine array analyses also demonstrated an increase in the level of TNF- β , a cytokine known to mediate inflammatory and antiviral responses. The constitutive expression of this cytokine has been previously demonstrated in HTLV-1-infected T cells lines [38] and the role of soluble Tax in stimulating the production of TNF- β from lymphoid cells has been reported previously [15].

The cytokine array also enabled the detection of chemokines, a group of chemotactic cytokines that play a crucial role in DC migration to the lymph node and in the effector phase of the lymphocyte response [39]. Two major families of chemokines are distinguished by whether the first two of the four cysteine residues are separated by one amino acid (C-X-C; α -chemokines) or occur adjacent to each other (C-C; β -chemokines). While the C-X-C chemokines (IP-10 and IL-8) were not detected, the C-C chemokines Eotaxin, MCP-1, and MCP-3 were produced in significant amount following treatment of DCs with extracellular Tax. The role of MCP-1 and MCP-3 has been implicated in patients with various neuroinflammatory diseases including multiple sclerosis and HIV dementia, where

maintenance of chronic inflammation was attributed to significant expression of these chemokines in the CSF of affected individuals [40]. It is then possible that production of these chemokines by Tax-stimulated DCs may also contribute to the neurologic damage observed in HAM/TSP.

We additionally questioned whether a transient exposure to Tax was sufficient to induce the production of cytokines using two representative cytokines, IL-12 and TNF- α . The secretion of these cytokines was completely abolished following removal of Tax from the culture media, suggesting that a continuous presence of Tax is essential to maintain the levels of these cytokines. Having identified the key proinflammatory cytokines relevant to HTLV-1-associated neurological disorders, we proceeded to examine the role of the NF- κ B signaling pathway in this process and studies were performed to determine whether the inhibition of the NF- κ B pathway interfered with Tax-mediated cytokine induction. The results demonstrated that pre-incubation of cells with NF- κ B inhibitors completely abrogated cytokine secretion indicating the involvement of NF- κ B signaling in the absence of cell death. However, the involvement of other signaling pathways known to activate DCs, such as calcium and protein kinase signaling, cannot be ruled out and is under current investigation.

Taken together, these results indicate that Tax can activate DCs and the involvement of NF- κ B in this process leads to the secretion of proinflammatory cytokines. This provides a mechanism of Tax-mediated DC activation that may be initiated from the membrane by toll-like receptors. The involvement of NF- κ B in this process may provide an autocrine loop for the continuous release of cytokines to maintain an intensive immune response observed during HTLV-1 infection. Based on the biological activities attributed to cytokines including a role in inflammation and immune response induction a comprehension of their mechanism of action will prove essential to identify molecular targets for the treatment of neuroinflammatory diseases.

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A

Alb Cy 3/5	Eotaxin	Biot-Alb
GM-CSF	IFN- γ	IL-10
IL-12p70	IL-2	IL-4
IL-5	IL-6	IL-7
IL-8	IP-10	MCP-1
MCP-3	TNF- α	TNF- β
Neg. Ctrl.	Ctrl.	Neg. Ctrl.

B

Cytokine	Description	Secreted Amounts (pg/ml) normalized		Fold-changes over mock	
		LPS	Tax	LPS	Tax
Eotaxin	C-C chemokine ligand 11	561	692	7.0	8.4
GM-CSF	Colony stimulating factor 2	0	1442	1.0	0.9
IFN- γ	Interferon gamma	496	1462	1.4	2.0
IL-2	Interleukin 2	337	117	1.8	1.5
IL-4	Interleukin 4	19	25	1.0	1.0
IL-5	Interleukin 5	0	0	0.0	0.0
IL-6	Interleukin 6	0	0	0.0	0.0
IL-7	Interleukin 7	4694	0	1.6	0.8
IL-8	CXC Chemokine ligand 8	0	0	0.0	0.0
IL-10	Interleukin 10	0	2233	0.5	1.8
IL-12	Interleukin 12	3575	126	2.5	2.0
IP-10	Interferon-inducible-protein-10	0	0	0.0	0.0
MCP-1	Monocyte chemotactic protein 1	5	98	1.1	3.5
MCP-3	Monocyte chemotactic protein 3	0	1799	0.5	5.1
TNF- α	Tumor necrosis factor α	10	7839	1.2	53
TNF- β	Tumor necrosis factor β	0	2814	1.0	2.0

Fig 1.

HTLV-1 Tax induces the secretion cytokines from primary human MDDCs. MDDCs were exposed to mock fluid (negative control), LPS (positive control) or Tax (1 μ g/ml) for 24 hr. Cell supernatants, harvested 24 hours after treatment, were analyzed for cytokine production using a human cytokine protein array as described in Materials and Methods. A: A grid layout of the antibodies as spotted on the slide. B: Normalized amounts (pg/ml) and fold-changes of cytokines secreted by Tax- or LPS-stimulated MDDCs.

Fig 2.

Validating Tax-induced cytokine expression profile. The expression of significantly expressed (≥ 2.0 -fold) cytokines was further validated by exposing MDDCs to mock fluid, LPS or two doses of Tax (0.1 and 1 μ g/ml) at two time periods (24 and 48 hours). Cell supernatants were analyzed using the cytokine protein array as described in Materials and Methods and it was found that Tax induced the expression of significantly expressed cytokines in a dose- and time-dependent manner.

Fig 3.

A transient exposure to Tax is not sufficient to induce cytokine secretion from MDDCs. MDDCs were either untreated or treated with Tax (1 $\mu\text{g/ml}$) for 4, 8, or 24 hours. Cells were then washed extensively with PBS to remove Tax and supplied with fresh medium without Tax. Supernatants were collected after 1, 4, and 24 hours from each data point and analyzed by ELISA. The results are represented by mean values in $\text{pg/ml} \pm$ standard deviation of duplicate samples analyzed in the ELISA. In case of both IL-12 and TNF- α , the presence of Tax was essential for sustained cytokine production.

Fig 4.

Role of NF- κ B in Tax-mediated induction of cytokines. MDDCs were either directly treated with mock fluid or Tax or pre-incubated (30 minutes) with NF- κ B inhibitors PDTC (10 μ M) or TLCK (10 μ M) followed by treatment with Tax (1 μ g/ml). At the end of incubation period (24 hours post-treatment), supernatants were collected and analyzed by ELISA. The results are represented by mean values in pg/ml $\pm$ standard deviation of duplicate samples analyzed in the ELISA. Pre-incubation of MDDCs with PDTC or TLCK completely blocked the Tax-mediated induction of cytokines indicating a role for NF- κ B signaling in DC activation by Tax.