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Epinephrine-primed murine bone marrow-derived dendritic cells facilitate production of IL-17A and IL-4 but not IFN-γ by CD4+ T cells

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Abstract

Sympathetic activation leading to the release of epinephrine and norepinephrine, is known as an important regulatory circuit related to immune-mediated diseases. However, questions still remain on the behavior of antigen presenting cells (APC) dictated by stress-induced sympathetic neurotransmitters. The purpose of this study was to examine the fate of bone marrow derived dendritic cell (BMDC)-associated influences on resting $CD4^+$ T cell activation. We hypothesize that pre-exposure of dendritic cells (DCs) can modify the intensity of cytokine production, leading to preference in resting $CD4^+$ T cell activation. BMDCs were pretreated with epinephrine for 2 hr followed by subsequent treatment of lipopolysaccharide (LPS). Subsequently, BMDCs were cocultured with purified $CD4^+$ T cells from mouse spleen in the absence or presence of anti-CD3 stimulation in epinephrine-free media. Epinephrine pre-treatment enhanced surface expression of MHCII, CD80 and CD86. Quantitative RT-PCR showed that epinephrine pretreatment induced a significant transcriptional decrease of IL-12p40 and a significant increase of IL-12p35 and IL-23p19. In addition, β2-adrenergic-blockade was shown to reverse these effects. Epinephrine pretreatment also induced a significant decrease of IL-12p70 and a significant increase of IL-23 and IL-10 cytokine production. Importantly, these changes corresponded with increased IL-4 and IL-17A, but not IFN-γ cytokine production by $CD4^+$ T cells in a β2-adrenergic receptor-dependent manner. These results suggest that exposure to stress-derived epinephrine dictates dendritic cells to generate a dominant Th2/Th17 phenotype in the context of subsequent exposure to a pathogenic stimulus.

Keywords

epinephrine; β2 adrenergic; dendritic cells; $CD4^+$ T cells; Th17

1. Introduction

Mechanisms which foster imbalanced $CD4^+$ T cellular responses play an important role in the initiation and progression of most chronic diseases. For example, polarized Th2 cellular activation is known to exacerbate allergic asthma (Mazzarella et al., 2000, Robinson et al.,

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1992, Robinson et al., 1993). Individuals who suffer from irritable bowel disease and rheumatoid arthritis have documented elevations in Th1-mediated inflammatory responses (Bregenholt and Claesson, 1998, Cao et al., 2008, Finnegan et al., 2002, Han et al., 2005, Neurath et al., 2002, Parronchi et al., 1997, Powrie et al., 1994). Moreover, recent clinical and experimental studies have highlighted the significant role of Th17 as well as T regulatory cell activation in modulation of acute (Bai H et al., 2009, Sellge G et al., 2010) and chronic inflammatory conditions (Boden and Snapper, 2008, Costantino et al., 2008, Fletcher et al., 2009, Fujino et al., 2003, Fujiwara et al., 2007, de Kleer et al., 2004, Komiyama et al., 2006, Lawson et al., 2006, Nakae et al., 2003, Martin et al., 2004, Seiderer et al., 2008, Wakashin et al., 2008, Zhang et al., 2006).

Antigen presenting cells (APCs), which present antigen on major histocompatibility (MHC) II molecules and produce cytokines/chemokines are principal determinants of $CD4^+$ T cell-mediated inflammatory responses (Guermonprez et al., 2002). The APC capacity of dendritic cells (DC) which consists of heterogenous phenotypes (Shortman and Liu, 2002, Villadangos and Schnorrer, 2007) are capable of presenting antigen, providing co-stimulatory recognition signals and cytokine responses needed for $CD4^+$ T cell function (Guermonprez et al., 2002, Wilson and Villadangos, 2005). For example, the divergence of $CD4^+$ T cell phenotypes driven by preferences in IL-10, IL-12, IL-23 or TGF-β cytokine production by DC is believed to play a pivotal role in regulation of inflammatory disease (Antoniv and Ivashkiv, 2006, Henry et al., 2008, Lee et al., 2004, Letterio and Roberts, 1998, Murphy et al., 1994, Ogawa et al., 2008, Uemura et al., 2009). In particular, there is emerging interest in investigating the relationships between the IL-12/IL-23 axis by DC in controlling the fate of $CD4^+$ T cell responses. IL-12 represents a heterodimeric cytokine comprising of covalently linked p40 and p35 subunits (Langrish et al., 2004), and is widely known as an important regulator of Th1 cellular responses (Goriely et al., 2001). Through the co-expression of both subunits, IL-12 is biologically active in its secreted form. More recently, a novel subunit p19 that associates with the IL-12p40 subunit was discovered (Langrish et al., 2004). Similar to IL-12, IL-23 cytokine production requires both p19 and shared p40 subunit expression (Langrish et al., 2004). In contrast to IL-12, the production of IL-23 by DC is found to regulate IL-17 production by $CD4^+$ T cells (Aggarwal et al., 2003). IL-17 plays a pivotal role in the regulation of chemokines and adhesion molecules associated with the infiltration of neutrophils to inflammatory sites, through binding of its receptor on epithelial cells and endothelial cells (Laan et al., 1999, Lee et al., 2008, Ruddy et al., 2004). Thus, given the plasticity of DC to direct the fate of $CD4^+$ T cells, defining factors, which alter their function can be a preemptive target in the prevention and treatment of chronic inflammatory disease.

Perceived stress has emerged as a key causative factor in the initiation and progression of many chronic diseases including irritable bowel disease (Kiank et al., 2009, Mawdsley and Rampton, 2005, Melgar et al., 2008), rheumatoid arthritis (Cutolo and Straub, 2006, Straub and Cutolo, 2001), atherosclerosis (Hamer and Malan, 2009, Rozanski et al, 1999), and asthma (Bailey et al., 2009, Kimura et al., 2009, Turyk et al., 2008). The underlying mechanism of action is thought to be derived from stress-induced neuroendocrine regulation of cellular immune function. In response to stressful experiences (e.g. psychological, physical), activation of the central nervous system leads to neuronal activation and neuropeptide release of stress factors such as catecholamines (norepinephrine and epinephrine), corticosteroids (cortisol), parasympathetic-mediated acetylcholine activation, and others (Miller and O'Callaghan, 2002, Stojanovich and Marisavljevich, 2008). Furthermore, through nervous innervations of lymphoid tissue and/or by neuroendocrine receptor expression, immune cells receive nervous system stimuli resulting in altered function (Glaser and Kiecolt-Glaser, 2005, Yang and Glaser, 2002). Such neuroendocrine-mediated influences on immune function have since been shown to impact disease susceptibility by facilitating either hyperactivity or suppression of immune responses (Dokur et al., 2004, Glaser and Kiecolt-Glaser, 2005, Yang and Glaser, 2002).

The adrenergic (catecholaminergic) stress response pathway is highly integrated within the immune defense system. The sympathetic nervous system innervates almost all major lymphoid tissues such as thymus, spleen, bone marrow, and regional lymph nodes (Felten et al., 1985). Adrenergic receptors are also expressed on immune cells including NK cells, T cells, and macrophages (Abrass et al., 1985, Baker and Sanders, 1997, Dokur et al., 2004, Garcia et al., 2003, Ramer-Quinn, Sanders et al., 1997) which exhibit paracrine/autocrine function, through release of catecholamines and the expression of adrenergic receptors (Flierl et al., 2007). In studies by Maestroni et al., DC were found to express $\alpha 2_A$-, $\alpha 2_C$- β1-, and β2-adrenergic receptors. Importantly, *in vivo* administration of β2-adrenergic antagonists was found to regulate the production of Th1 cytokines IL-2 and IFN-γ in lymph node after adoptive transfer of DC (Maestroni, 2000, Maestroni and Mazzola, 2003). Panina-Bordignon et al., also have shown that β-adrenergic agonists can preferentially prevent IL-12 production and promote Th2 development (Panina-Bordignon et al., 1997). More recently findings also highlight the potential impact of adrenergic stimulation on Th17 responsiveness associated with IL-23 expression in effector immune cells such as macrophages (Liu et al., 2009). Thus, the ability of DC to facilitate the activation and effector function of $CD4^+$ T cells in response to adrenergic stimulation could be a determinant in disease pathogenesis under conditions of stress.

Utilizing an experimental system of epinephrine-mediated regulation of DC activation through MHCII and co-stimulatory signaling molecule (CD80 and CD86) as well as cytokine production (IL-10, IL-12p70 and IL-23, the current study determined the fate of $CD4^+$ T cell activation under the control of DC previously influenced by adrenergic stimulation. The results from the current study provide evidence that adrenergic stimulation can enhance surface expression of MHCII, CD80 and CD86 and also preferentially augment p40, p35, and p19 heterodimeric subunit expression by DC, resulting in a preferential IL-23/IL-17 phenotype in a β2-adrenergic manner. These data provide evidence that neuroedocrine effects on APC is important in understanding stress-induced augmentation of $CD4^+$ T cell responses that may be important in defining the hidden mechanisms of stress and chronic inflammatory disease.

2. Materials and methods

2.1. Animals

Adult (6–8 weeks of age) female CD-1 mice (Harlan Sprague–Dawley, Indianapolis, Indiana) were used in all studies. Mice were maintained under specific pathogen-free conditions on a 12:12 light/dark cycle (7:00 PM to 7:00 AM). Mice were kept under optimal temperature and humidity controlled conditions and provided proper care as directed by the institutional animal care and use committee. Before bone marrow cell isolation, mice were acclimated at housing facility for 7 days to eliminate shipping stress.

2.2. Generation of bone marrow-derived dendritic cell (BMDC)

Bone marrow cells were flushed from the *femur* and *tibia* with wash media (RPMI 1640 with 1% FBS and 1% penicillin/streptomycin) using a 25-gauge needle. After removing red blood cells using ACK (ammonium-chloride-potassium) lysis method (Kruisbeek, 2001), total mononucleated cells were purified by gradient centrifugation using lympholyte M solution (Cedarlane laboratories Ltd. Hornby, ON, Canada). Cells were maintained in RPMI 1640 media containing 10% FBS and 1% penicillin/streptomycin supplemented with recombinant murine GM-CSF (10 ng/ml) (Biosource, invitrogen cytokines & signaling, Camarillo, CA) and IL-4 (10 ng/ml) (R&D systems, Inc. Minneapolis, MN). All floating cells and loosely adherent cells were removed by gentle swirling and fresh media was replaced on day 3. On day 6, half amount of fresh media was gently added to cell culture. On day 7, cells were transferred to either 6 well plates with 1×10^6 cells per well or 48 well plates with 1×10^5 cells per well for experiments. Purity of $CD11c^+$ cells was confirmed by flow cytometry (~90%).

2.3. Cell treatment and harvest

BMDC plated on 6 well- or 48 well-plates were exposed to 10^{-6} M of epinephrine (Sigma, St. Louis, MO) in the presence or absence of specified concentrations of the selective β2-adrenergic antagonist, butoxamine (Sigma) for 2 hr. After 2hr epinephrine exposure, cells were stimulated by lipopolysaccharde (LPS) (Sigma) (1 μg/ml) for an additional 3 hr. For gene expression analysis, 1 ml of TriZol reagent (Invitrogen.Co. Carlsbad, CA) was added to each well of a 6 well-plate, and cell lysates were collected for mRNA expression analysis. Similarly, BMDC cultured in 48-well plates were pre-exposed to epinephrine in the presence or absence of butoxamine for 2hr. BMDC culture supernatants were collected 24hr after LPS stimulation and stored at −80° C until analysis. For ELISA analysis, culture supernatant was collected from 48-well plates after 24 hr of stimulation.

2.4. Flow cytometry

Collected BMDC were incubated with anti-CD16/CD32 FcRII/III blocker in 4°C for 10 min. Two color immunofluorescence staining were performed using PE-labeled anti-mouse CD11c with either FITC-labeled anti-mouse CD80, FITC-labeled anti-mouse CD86 or FITC-labeled anti-mouse MHCII in 4°C for 30 min. After two time of washing, positive cells for immunostaining were identified using cytomic FC500 flow cytometry analyzer (Beckman-Coulter). Further analysis of mean fluorescence intensity and generation of histograms were performed using FlowJo analysis program (version 8.8.7, Ashland, OR). Fc blocker and antibodies were purchased from BD biosciences (BD biosciences, San Jose, CA)

2.5. Magnetic cell sorting for $CD4^+$ T cell and BMDC-$CD4^+$ T cell coculture

$CD4^+$ T cells were purified and enriched from total splenocytes using IMagnet $CD4^+$ T Lymphocyte Enrichment Set (BD PharMingen Inc., San Jose, CA) as described in procedures from the manufacturer. Briefly, after elimination of red blood cell using ACK lysis method (Kruisbeek, 2001), total splenocytes were incubated with an optimal concentration of biotin-conjugated antibody cocktail (BD PharMingen Inc.) containing anti-mouse CD8a, anti-mouse CD11b, anti-mouse CD45R/B220, anti-mouse CD49b and anti-mouse TER-119/Erythroid for 20 min on ice, followed by incubation with streptavidin-conjugated magnetic nanoparticles. Non-$CD4^+$ T cells were selected by magnetic force using BD IMagnet (BD PharMingen Inc.). Purity of enriched $CD4^+$ T cells was analyzed using flow cytometry technique (~ 93%). BMDC were plated (1×10^5 cells/well) in 48 well plates and pre-primed for 2 hr by epinephrine (10^{-6} M) in the absence or presence of butoxamine (10^{-5} M) followed by subsequent LPS stimulation (1 μg/ml) for 24 hr. 4×10^5 of purified $CD4^+$ T cells was added to each well with fresh new media after removing old media from BMDC culture to prevent direct epinephrine stimulation on T cells. To mimic T cell receptor (TCR)-independent or dependent CD4+ T cell activation, BMDC-CD4+ T cell coculture was performed in the absence [(−)CD3] or presence [(+)CD3] of anti-CD3 monoclonal antibody (2.5 μg/ml) (BD PharMingen Inc.). Culture media was collected after 96 hr incubation period and used for cytokine expression analysis.

2.6. Quantitative real-time RT-PCR

Total RNA extracted from BMDC culture was used for reverse transcription adjusted to a concentration of 1 μg per reaction using MLV (Molony murine leukemia virus) reverse transcriptase (Promega, Madison, WI) as previously described (Sun et al., 2006). After cDNA synthesis, real-time PCR was performed using SYBR green techniques to evaluate mRNA expression levels of cytokine genes. Selected target and house keeping gene primer sets; IL-12p40, IL-12p35, IL-23p19, IL-10 and GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was purchased from SAbioscience Inc. (SAbiosicence, Frederick, MD). Real-time SYBR master mix was purchased from Applied Biosystem (Applied Biosystems, Foster City, CA). Thermal cycling procedure will be performed in 20 μl reaction volume using a

StepOne system (Applied Biosystems). Differences in gene expression was determined by relative quantification between cDNA templates from un-stimulated control cells and cells from each experimental group including LPS-treated positive controls. The expression of the housekeeping gene GAPDH was used as an internal control to normalize target gene expression between samples. Differences in target gene expression was calculated using the following formula: ΔΔCT = ΔCT (target gene) - ΔCT (GAPDH). The ΔΔCT value of cDNA amplification from the control group was considered the calibrator for baseline levels of mRNA expression. Data were expressed as the ratio of target gene expression of each group of various treatment subjects to the target gene expression of the control group, resulting in fold difference in target gene mRNA levels.

2.7 Enzyme-linked immunosorbent assay (ELISA)

IFN-γ, IL-4, IL-10, IL-17A, IL-12p70 and IL-23 were determined by sandwich ELISA method. All procedures were performed as described by the manufacturer. Briefly, flat-bottomed 96-well plate was coated with optimal titration of capture antibody and followed by blocking (10% FBS in PBS). After incubation of samples at 4°C for 16 hr, plates were incubated with biotin-conjugated detection antibody and streptavidin-HRP (horseradish peroxidase. After adding tetramethylbenzidine (TMB) peroxidase substrate solution (Rockland Immunochemicals, Inc. Gilbertsville, PA), the concentration of each cytokine was determined according to standard curved generated by reference concentration of cytokine at wavelength of 450 nm detected by colorimetric plate reader (Biotek Instruments Inc. Winooski, VT). ELISA antibody set and recombinant cytokine for standard were purchased from eBiosicence (eBiosciences, San Diego, CA) for IL-12p70 and IL-23 ELISA sets, BD PharMingen Inc. for IFN-γ, IL-4, IL-10 ELISA sets and IL-17A antibody pairs and R&D Systems (R&D Systems Inc. Minneapolis, MN) for recombinant IL-17A.

3. Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 4.0 (GraphPad Software, San Diego, USA). For multi-experimental group analysis, data were subjected to one-way and two-way ANOVA (analysis of variance) followed by post hoc tests (Newman-Keuls and Bonfferoni) for group differences. All data are expressed as means ± standard error of mean (SEM). The two-tailed level of significance was set at $p \leq .05$, .01 and .001 for group differences.

4. Results

4.1. Pre-exposure to epinephrine enhances surface expression of MHCII and co-stimulatory molecules (CD80 and CD86) by LPS-stimulated BMDC

We examined the expression of MHCII and co-stimulatory (CD80 and CD86) surface maturation molecules by epinephrine pre-treated BMDC following LPS stimulation. As expected, LPS stimulation significantly increased the intensity of MHCII, CD80 and CD86 as compared to un-stimulated BMDC. Epinephrine exposure prior to LPS-exposure induced a further significant enhancement of intensity of these molecules (figure 1). Interestingly, introduction of the β2 adrenergic antagonist, butoxamine during epinephrine pre-exposure attenuated MHCII and CD86 but not CD80 (supplemental figure 1).

4.2. $CD4^+$ T cell fate-determining cytokine gene expression by BMDC is predicted by exposure to epinephrine

We analyzed BMDC-associated cytokine gene expression that typically dictates Th1 and Th17 differentiation. Epinephrine-pretreated BMDC showed a significant (F=110.3; $p \leq .001$) decrease in the transcription level of the Th1 promoting heterodimer, IL-12 p40 (1160 fold) as

compared to LPS-stimulated BMDC. In contrast, transcription of the IL-12 p35 and IL-23 p19 heterodimer was significantly (F= 357.3; $p \leq .001$ and F=127.9; $p \leq .001$) increased by 18 and 25-fold by epinephrine pre-treatment, respectively (figure 2A).

β2-adrenergic receptor is known as a major receptor isotype involved in catecholamine-mediated responses in immune cells (Ramer-Quinn et al., 1997, Maestroni and Mazzola, 2003, Tan et al., 2007). We examined whether epinephrine-mediated effects of IL- 12p40, IL-12p35 and IL-23p19 heterodimer expression was impacted by β2-adrenergic receptor activation. Using a selective β2 adrenergic receptor antagonist, butoxamine, a transcriptional down-regulation of IL-12 p40 by epinephrine challenge was significantly (F=11.49; $p \leq .05$) recovered in a dose-dependent manner ($10^{-4} \sim 10^{-7}$ M). Conversely, increased transcriptional levels of IL-12p35 and IL-23p19 were significantly (F=38.24; $p \leq .05$ and F= 20.29; $p \leq .05$) reduced by butoxamine (10^{-4} M) (figure 2B).

4.3. IL-12p70, IL-23 and IL-10 cytokine production by BMDC exposed to epinephrine corresponds with preferences in p35 and p19 subunit expression

The physiological concentration ($10^{-5} \sim 10^{-7}$ M) of epinephrine has been measured in major physiological compartments including lung and blood in both human and mice (Davis IC et al., 2007, Evans JM et al., 2001, Kienbaum P et al., 1998, Klingenheben T et al., 1996, Kennedy B et al., 1990). Furthermore, studies have supported that 10^{-6} M of epinephrine was in range of physiological concentrations and has been used to measure the effect of epinephrine on cell physiology such as cytokine production (Horn NA et al., 2005, Deng J et al., 2004). Based on these findings, we determined protein expression level of IL-12p70, IL-23 and IL-10 within culture supernatants of BMDC after 2hr pre-exposure of epinephrine at various concentration ($10^{-5} \sim 10^{-8}$ M) followed by additional LPS stimulation for 24 hr. IL-12p70 was significantly (F=32.02; $p \leq .0001$) decreased by epinephrine pretreatment independent of epinephrine concentration in response to LPS stimulation as compared to LPS treatment alone. In contrast, exposure to epinephrine prior to LPS stimulation induced significant (F=7.031; $p \leq .05$) increase in IL-23 in the presence of epinephrine (10^{-6} M) as compared to LPS stimulation alone. In addition, IL-10, a Th2 promoting-cytokine was significantly (F=20.23; $p \leq .001$) increased in cultures of LPS stimulated BMDC pre-exposed to epinephrine (10^{-5} and 10^{-6} M) as compared to LPS stimulation alone (figure 3). Epinephrine treatment without LPS stimulation did not impact cytokine production (data not shown).

4.4. Epinephrine promotes IL-4 and IL-17A, but not IFN-γ under BMDC-mediated preferential cytokine production of $CD4^+$ T cells

We first determined the level of IFN-γ, IL-4 and IL-17A cytokine production by $CD4^+$ T cell without BMDC coculture in the absence and presence of anti-CD3 stimulation to examine T cell receptor-dependent cytokine production. Anti-CD3 stimulation induced IL-4, IFN-γ and IL-17A cytokine production with IL-17A being the highest concentration detected in culture supernatants. None of these cytokines were detected in $CD4^+$ T cell culture without anti-CD3 stimulation (table 1).

For comparison, we examined the intrinsic ability of un-stimulated BMDC to induce cytokine production by $CD4^+$ T cells in absence or presence of anti-CD3 stimulation. IFN-γ was the only cytokine detected in $CD4^+$ T cell culture supernatants in the presence of un-stimulated BMDC without anti-CD3 stimulation. A further significant (F=136.2; $p \leq .001$) increase in IFN-γ cytokine production was observed in the presence of un-stimulated BMDC given CD3-mediated stimulation of $CD4^+$ T cells as compared to $CD4^+$ T cell cultures without BMDC in the presence of anti-CD3 antibody. In contrast, the presence of un-stimulated BMDC significantly (F=136.2; $p \leq .001$) attenuated IL-4 cytokine production as compared to $CD4^+$ T cell only cultures in the presence of anti-CD3 antibody. Interestingly, IL-17A cytokine

production by $CD4^+$ T cells alone or in the presence of BMDC did not significantly differ in the absence of anti-CD3 antibody. Furthermore, BMDC did not significantly alter IL-17A cytokine production by $CD4^+$ T cells in the presence of anti-CD3 antibody stimulation as compared to their $CD4^+$ T cell only counterparts (table 1). Thus, the innate ability of resting BMDC was demonstrated to facilitate a preference for IFN-γ production, but not for IL-4 or IL-17A.

We next determined $CD4^+$ T cell cytokine production mediated by epinephrine-primed BMDC. IFN-γ production by $CD4^+$ T cells in the absence or presence of anti-CD3 antibody was not significantly attenuated by epinephrine-treated BMDC. However, LPS-stimulated and epinephrine-primed LPS-stimulated BMDC showed significantly (F=53.09; $p \leq .001$) decreased IFN-γ production by anti-CD3-stimulated $CD4^+$ T cells as compared to un-stimulated and epinephrine only experimental conditions group (table 1). This response was modestly reversed in the presence of butoxamine.

In response to LPS stimulation, pre-treatment of epinephrine supported a significant (F=6.329; $p \leq .05$ and F=108.2; $p \leq .001$) increase in IL-4 production as compared to BMDC stimulated with LPS alone in the absence or presence of anti-CD3 stimulation. BMDC exposed to epinephrine with butoxamine significantly (F=6.329; $p \leq .05$) reduced IL-4 production by $CD4^+$ T cells in the absence of anti-CD3 stimulation and modestly diminished IL-4 production in the presence of anti-CD3 stimulation.

The presence of un-stimulated BMDC resulted in a modest attenuation of IL-17A production by anti-CD3 stimulated $CD4^+$ T cells as compared to IL-17A induced by T cell only culture stimulated by anti-CD3 monoclonal antibody. BMDC exposed to epinephrine only resulted in a modest increase in IL-17A by $CD4^+$ T cells in the presence of anti-CD3 stimulation as compared to $CD4^+$ T cell only culture. This effect was not observed in the absence of anti-CD3 stimulation. LPS stimulation of BMDC exposed to epinephrine resulted in a significant (F=108.6; $p \leq .001$ and F=30.50; $p \leq .05$) increase of IL-17A production by $CD4^+$ T cells in the absence of or presence of anti-CD3 stimulation. Importantly this increase in IL-17A was significantly (F=108.6; $p \leq .001$ and F=30.50; $p \leq .01$) attenuated in the presence of butoxamine within both conditions.

5. Discussion

Both anecdotal and experimental evidence support a link between stress and disease susceptibility, particularly in the case of chronic inflammatory conditions (Bailey et al., 2009, Kiank et al., 2009, Kimura et al., 2009, Mawdsley and Rampton, 2005, Melgar et al., 2008, Rozanski et al., 1999, Straub and Cutolo, 2001, Turyk et al., 2008). For years, researchers have considered this phenomenon to result from biological interactions between the central nervous and immune systems. Yet, to date the mechanisms of action that define their relationship remain unresolved.

An abundance of knowledge demonstrates the important role that $CD4^+$ T cells have in mediating various inflammatory diseases. Few reports however, distinguishes neuroendocrine-mediated DC responsiveness as a potential pathway influencing innate (Powell ND et al, 2009) and adaptive immunity (Elftman MD et al., 2010), including determination of $CD4^+$ T cell phenotypes. Thus, there is a further need to define the cellular immune mechanisms influenced by stress response factors.

The current study focused on the role of epinephrine as a major stress response factor regulating DC function. As shown in figure 1, we demonstrated that epinephrine exposure contributes to LPS-induced increase in surface CD80, CD86 and MHCII expression. Such findings demonstrate the potential relevance of epinephrine's influence during the initial stages of DC

activation and maturation independent of antigen exposure. In that epinephrine exposure enhanced MHCII as well as CD80 and CD86 co-stimulatory molecule expression suggests that epinephrine supports an elevated DC activation status. This is in contrast to the effects previous reported regarding the role of corticosteroids in which, attenuation of activation was observed by DC (de Jong et al., 1999,Vanderheyde et al., 1999). Most intriguing was the preferential unresponsivness of epinephrine-induced up-regulation of CD86 to β2-receptor blockade as compared to CD80 and MHCII (supplemental data). This finding suggests that specificity epinephrine action on DC maturation and activation can be controlled in a β2-adrenergic dependent manner. This is important in light of the reported influence that differential co-stimulatory expression by DC has on priming of T cell responses (Maroof A et al., 2009). Thus, further investigation of adrenergic receptor specificity may provide novel insight of epinephrine actions on DC maturation and co-stimulatory signaling pathways important for the cellular interactions between DC and T cells, constituting T cell activation.

Antigen presentation by DC is essential for the initiation and maintenance of adaptive $CD4^+$ T cell responses through antigen-specific recognition between MHC II - T cell receptor complexes and co-stimulatory signaling pathways. Equally important is the paracrine release of cytokines and/or chemokines by DC that direct the differentiation of $CD4^+$ T cells into Th1, Th2, T regulatory and Th17 subsets (Joffre et al., 2009, Miossec, 2008). For the purpose of understanding how neuropeptide stimulation can impact DC cytokine production, our initial studies examined the influence of exposing BMDC to epinephrine prior to lipopolysaccharide (LPS) exposure, which is known to induce co-stimulatory molecule expression and cytokine responses through toll-like receptor-4 (TLR4) signaling pathways (Higgins et al., 2003, Jing et al., 2003, Medvedev et al., 2000). Previous studies have documented the specificity of cytokine secretion by DC to direct $CD4^+$ T cell cytokine production (Joffre et al., 2009). Recently, the IL-12/IL23 axis has received significant attention regarding protection and disease pathogenesis (Lyakh L et al., 2008). DC-associated IL-12 and IL-23 cytokine production have a key role in dictating DC ability to instruct $CD4^+$ T cell phenotypes (Ross et al., 2004). IL-12 cytokine production promotes the differentiation of Th1 effector cells (Goriely et al., 2001). IL-23 production is known to induce the production of antimicrobial peptides via IL-22 (Munoz et al., 2009, Schulz et al., 2008) and maintain the Th17 cell subset (Aggarwal et al., 2003, Stritesky et al., 2008), which is an important mediator of adaptive immune responses (Fouser LA et al, 2008) as well as regulation of innate responses, including neutrophil recruitment (Laan M et al., 1999, Ruddy MJ et al., 2004) and. In the current study, we demonstrated that BMDC exposed to epinephrine followed by LPS induces a preferential reduction in IL-12p40 mRNA transcripts. In contrast, IL-23p19 and IL-12p35 mRNA expression was significantly increased in BMDC exposed to epinephrine followed by LPS stimulation (figure 2). As shown in figure 2B, selective β2-antagonist (e.g. butoxamine) demonstrated a dose-dependent impact on BMDCs p40, p35, and p19 subunit transcription, suggesting a bias for IL-23 on the basis of p19 subunit expression. This finding corresponded with ability of butoxamine to attenuate MHCII and CD80 expression (supplementary data). Thus, providing evidence of a novel mechanism where DC capacity to instruct the induction of cytokine production, particularly IL-17A is dependent on β-adrenergic stimulation.

Importantly, such preferences in gene expression were substantiated by dose- associated epinephrine dependent decreases IL-12p70 and a concomitant increase in IL-23 cytokine protein detection in culture supernatants (figure 3). Furthermore, our results demonstrated the preferential increase in IL-10 cytokine production in response to epinephrine treatment. IL-10, a key facilitator of Th2 and regulatory $CD4^+$ T cell responses is found prevalent in many chronic inflammatory disease states (Ogawa et al., 2008,Romagani, 1991,Yeatman et al., 2000). Previous studies have documented preferential Th2 responses driven by adrenergic stimulation (Maestroni, 2002,Panina-Bordignon et al., 1997,Ross et al., 2004). Together, these findings

suggested that epinephrine might direct DC cytokine phenotypes that potentially predict downstream cytokine production by $CD4^+$ T cell populations.

Based on the above studies, we predicted that preferences in cytokine production (e.g. decreased IL-12p70 and increased IL-23) by DC would subsequently lead to a preferential Th17 phenotype. First, we examined the intrinsic effect of un-stimulated BMDC to regulate $CD4^+$ T cell cytokine production. DC have been shown to promote Th1 differentiation through a preference in IL-12p70-dependent IFN-γ cytokine production by $CD4^+$ T cells (Maddur et al., 2010). Alternately, DC can also support tolerogenic $CD4^+$ T cell responses by diminishing IFN-γ production (Li et al., 2006). In comparison to cytokine production by $CD4^+$ T cells, representing a mix resting population of CD62L $CD25^{lo}$, BMDC exposed to LPS promoted marked IFN-γ cytokine production (Table 1). Secondly, consistent with previous studies (Yang et al, 2004), unstimulated BMDC elicited lower IFN-γ production. Interestingly, we were able to demonstrate that any IFN-γ produced by $CD4^+$ T cells without CD3-mediated activation was diminished by the presence of epinephrine and could be recovered by BMDC exposed to epinephrine and the β2-adrenergic receptor antagonist, butoxamine. By contrast, IL-4 production albeit modest concentrations were facilitated by epinephrine exposed BMDC and abrogated if BMDC were exposed to butoxamine. Therefore, these data demonstrated a dichotomy between IFN-γ and IL-4 in the presence of adrenergic stimulation. Most striking however, was the ability of LPS-stimulated BMDC to facilitate *de novo* IL-17A by $CD4^+$ T cells independent of CD3-stimulation at levels significantly higher than IFN-γ. Moreover, epinephrine was found to significantly increase this response that was reversed by butoxamine treatment. To our knowledge, this is the first study to test IL-17A cytokine production by a mix resting population of $CD4^+$ T cells in the absence of TCR-mediated activation in the context of LPS-associated TLR-4 DC activation. This suggests a potentially novel mechanism in which epinephrine action on DC in the presence of TLR activation constitutes a default toward IL-17A. Presumably, this result could serve as a mode of by-passing antigen-independent MCH-TCR responses for the purpose of innate immune activation. Further investigation will likely provide important insight into default actions of DC in the face of neuroendocrine stimuli.

We next examined the impact of BMDC in the presence of $CD4^+$ T cells, which were stimulated through TCR, via CD3 antibody. As expected, $CD4^+$ T cells responded to CD3 antibody stimulation and facilitated IFN-γ, IL-4 and IL-17A cytokine production (Table 1). In contrast to what was observed in the absence of CD3 stimulation, we expected that BMDC exposed to LPS would accentuate IFN-γ by CD3-stimulated $CD4^+$ T cells. Surprisingly, lower IFN-γ production was detected in the presence of LPS-stimulated BMDC as compared to $CD4^+$ T cells in the absence of DC as well as $CD4^+$ T cells in the presence of untreated BMDC, which produced significantly, the highest levels of IFN-γ. One explanation, may be the necessity for optimal signaling by DC-$CD4^+$ T cell interaction in the midst of LPS-associated maturation and activation of DC. This would be consistent with previous reports suggest functional inhibition or tolerance of T cells by matured DC (Link H et al., 2001,Zhang GX et al., 2002). Yet, despite this observation, epinephrine's effect on BMDC resulted in a similar negative impact on IFN-γ production that was similarly found reversed by β2-adrenergic receptor activity as observed under non-CD3 stimulated conditions. Thus, we concluded that epinephrine exposure of BMDC translates an inhibitory signal to $CD4^+$ T cells to produce IFN-γ that can be overcome by β2-adrenergic receptor blockade. IL-4 production by CD3-stimulated $CD4^+$ T cells was decreased in the presence of BMDC regardless of treatment. This is consistent with BMDC preference for robust IL-12p70 cytokine production corresponding with conservative IL-10 production as well as the strong IL-17A production. In support, previous studies have demonstrated DC as potent regulators of Th1 responses (Santiago-Schwarz F et al., 2001). Although BMDC exposed to epinephrine demonstrated a similar trend as compared to their non CD3-stimulated counterparts, BMDC exposed to epinephrine in the

context of LPS stimulation enhanced IL-4 compared to LPS only condition. Interestingly, no significant influence of β2-adrenergic receptor antagonist was observed. Together, these studies demonstrated for the first time that epinephrine pre-exposure of BMDC can preferentially induce the differentiation of Th17 phenotype as evidence of robust IL-17A cytokine production by $CD4^+$ T cells Hence, priming BMDC, in the presence of epinephrine in the presence of LPS, served as a catalyst for induction of IL-17A cytokine production.

Earlier studies support the sympathetic nervous system as an active participant in regulation of proinflammatory conditions (Elenkov et al., 2000, Szelenyi and Vizi, 2007). However, the specific immune cell-types influenced remain unresolved. $CD4^+$ T cells have become a major focus of chronic inflammatory disease, especially the impact of the recently defined Th17 subset (Fujino et al., 2003, Nakae et al., 2003, Seiderer et al., 2008, Taleb et al., 2009, Wakashin et al., 2008, Zhang et al., 2006). Whether Th17 cells play an instrumental role in neuroendocrine-associated inflammatory disease remains undefined. In the current study, we focused on DC response as a catalyst for cytokine production by a heterogenous population of resting $CD4^+$ T cells. Recently, Seiffert et al., showed that epidermal langerhans cells as well as DC-like cell lines respond to epinephrine through β–adrenergic stimulation, resulting in impaired IFN-γ-associated contact hypersensitivity responses (Seiffert et al., 2002). In a report related to our current study, Maestroni demonstrated that BMDCs exposed to epinephrine influenced IL-12p40 cytokine production and Th1 development (Maestroni, 2002, Maestroni and Mazzola, 2003). Unique to this study however, is the findings that not only does epinephrine exposure decrease IL-12 activity (IL-12p40 mRNA and IL-12p70 cytokine production), but also results in a preferential increase in IL-23 response through induction of IL-23p19 and a reduction in IL-12p40 mRNA expression that corresponded with elevations in IL-23 cytokine production. The ability of epinephrine to influence $CD4^+$ T cell cytokine production in this study had both similarities and differences compared with previous *in vitro* studies. Maestroni et al. (Maestroni, 2002 Maestroni and Mazzola, 2003) and others (Panina-Bordignon et al., 1997) demonstrated that adrenergic stimulation modulates $CD4^+$ T cell responses. Specifically, Maestroni et al. highlighted the ability of norepinephrine exposure to decrease IFN-γ cytokine production in a mixed DC-$CD4^+$ T cell culture system. However, in contrast to other reports, we examined the exclusive impact of epinephrine solely on BMDC cultures and its subsequent ability to elicit $CD4^+$ T cell cytokine production in an epinephrine-free co-culture system. Also, in contrast to our current findings, Goyarts et al., demonstrated a decrease in IL-12p40 and IL-23 cytokine production by human cord blood $CD34^+$ precursor cells (Goyarts et al., 2008). Such discrepancies may be explained by differences between human and murine progenitors as well as *in vitro* culture conditions for the generation and maintenance of human verses murine DC (Goyarts et al., 2008, Mayordomo et al., 1997, Siena et al., 1995, Wells et al., 2005). Furthermore, although the use of BMDC highlights the significant role of epinephrine as a modifier of DCs functioning, an important point to consider is the impact of epinephrine upon its exposure to a more physiologically relevant phenotype. In this regard, further study using DC subtypes from the spleen or lymph nodes for example, will be valuable in defining the underlying mechanisms of stress-induced epinephrine effects on DC function as well as immunoregulation utilizing physiological models.

In summary, DC function represented by the type of cytokines and chemokines they secrete is critical for programming the type and quality of $CD4^+$ T cell responses. Importantly, because inflammatory conditions mediated by $CD4^+$ T cells require DC regulation, understanding the mode of action will likely benefit the development of therapies which control chronic disease. In recent years, the IL-17 cytokine family has become the center of attention in regulation of inflammatory disease states including arthritis, colitis, asthma and cardiovascular disease (Fujino et al., 2003, Nakae et al., 2003, Seiderer et al., 2008, Seiderer et al., 2006, Taleb et al., 2009, Wakashin et al., 2008, Zhang et al.). Likewise, the role that stress plays in exacerbated inflammatory conditions is believed to be pivotal in disease management. Here, we examine

the relationship between adrenergic nervous system activation on DC as a determinant in the fate of T cell cytokine production representing Th1, Th2 and Th17 phenotypes. Moreover, the consequence of adrenergic influences, DC may also contribute to innate immune responses, particularly through antigen-independent *de novo* Th17 pathway. As shown in our hypothetical model, our findings suggest that adrenergic stimulation can affect inflammatory conditions by favoring Th17 differentiation through augmentation of DC cytokine functioning (figure 4). Importantly, we hypothesize that differential pathways can be driven by epinephrine through selectivity of β2-adrenergic responses that can result in $CD4^+$ T cell cytokine production by non-classical DC-$CD4^+$ T cell interaction. On the basis of these findings, future understanding of the type of adrenergic receptor expression by DC that alter their function will provide important insight toward understanding their role in orchestration of $CD4^+$ T cell-mediated inflammatory conditions, considering stress as a catalyst.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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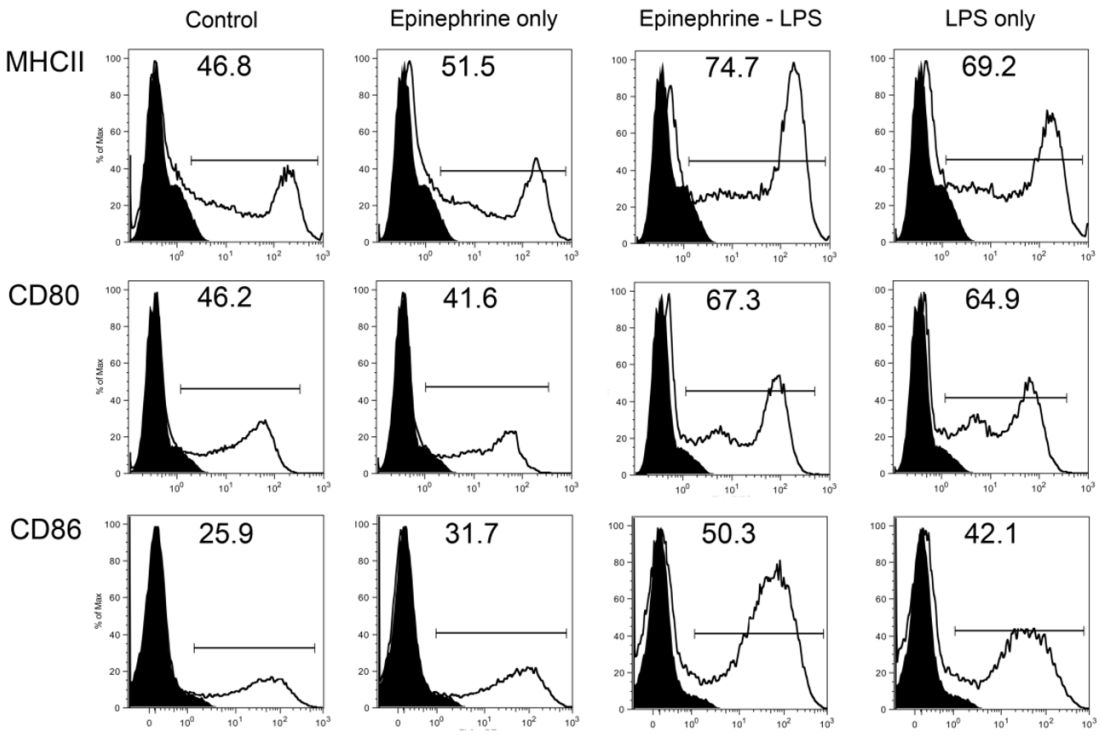

Figure 1.

Epinephrine enhances LPS-stimulated BMDC maturation. Percentage of positive cell populations for MHCII and co-stimulatory (CD80 and CD86) molecule was determined by flow cytometry after 24 hr of LPS stimulation in either epinephrine-pretreated or non-treated group. Cell populations in black area indicate isotype control. Numbers on each histogram indicate the percentage of positive cell population for each molecule from representative data of experimental groups (n=3).

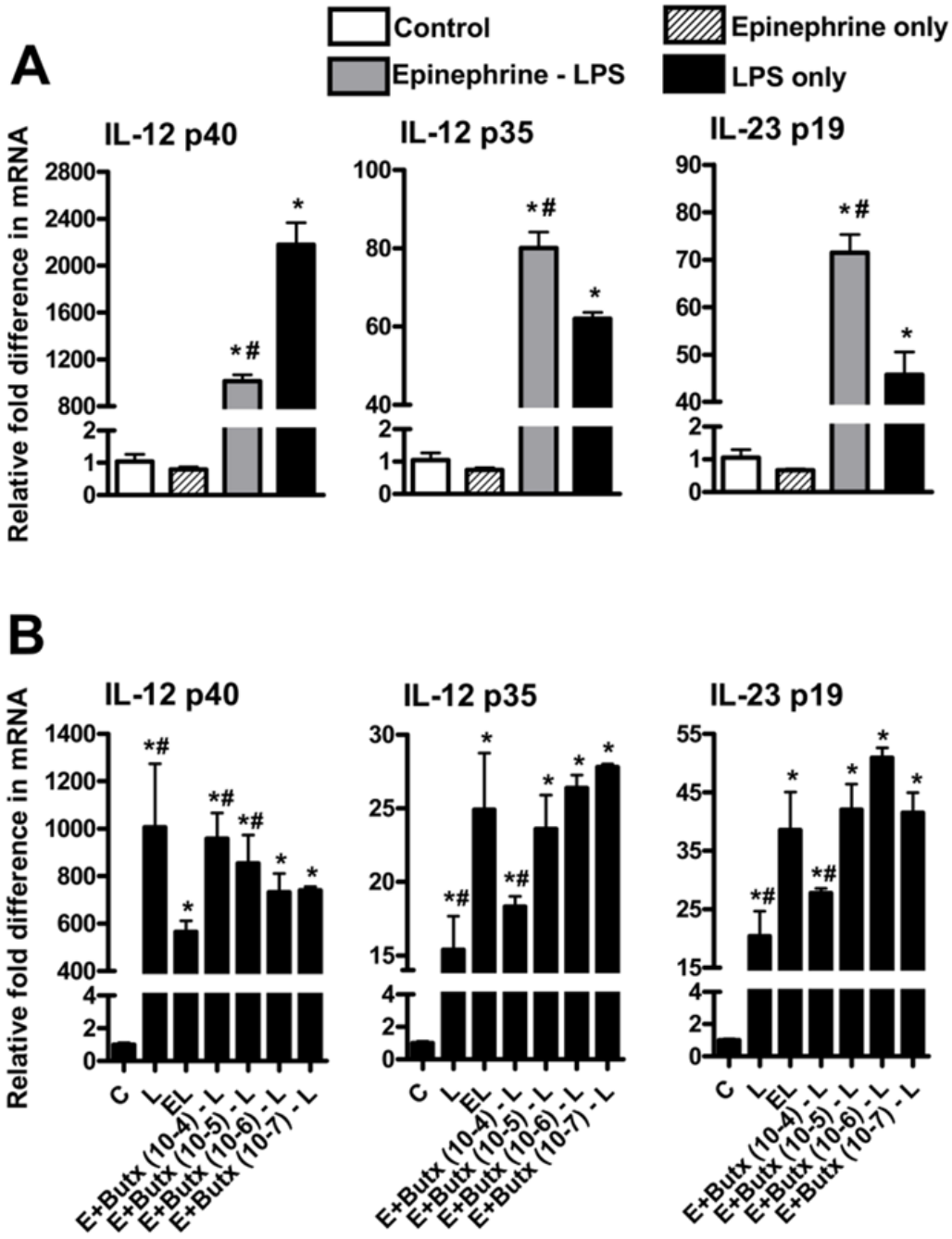

Figure 2.

$CD4^+$ T cell fate-determining cytokine expression by BMDCs is predicted by exposure to epinephrine. Total RNA was extracted from BMDCs. mRNA transcription of IL-12p40, IL-12p35 and IL-23p19 was analyzed by quantitative real-time RT-PCR technique. Relative fold difference of mRNA transcription in epinephrine only group, epinephrine-LPS treated (EL) group and LPS only group was compared to un-stimulated control BMDC group as 1. Asterisk (*) indicates significant group differences in control/epinephrine only vs other groups and sharp (#) indicates significant differences in epinephrine pre-treated (EL) vs LPS only group (A). BMDCs were also treated by epinephrine (10^{-6} M) with or without various concentration ($10^{-4} \sim 10^{-7}$ M) of butoxamine for 2 hr followed by LPS (1 μg/ml) challenge

for additional 3 hr. All data represent mean (N=3) ± standard error. Asterisk (*) indicates significant difference in control vs other groups and sharp (#) indicates significant differences in EL vs butoxamine treated and LPS only group. To present group differences, one-way ANOVA was used followed by Student-Newman posthoc test.

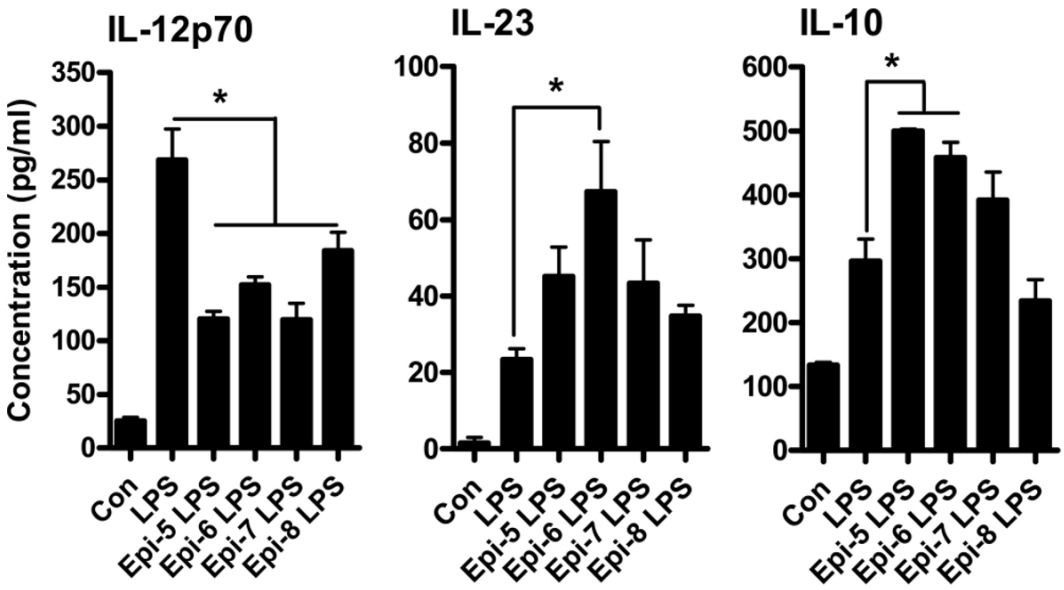

Figure 3.

IL-12p70, IL-23 and IL-10 cytokine production by BMDCs exposed to epinephrine corresponds with preferences in p35 and p19 subunit expression. BMDCs were treated by various concentration of epinephrine (10^{-5}~10^{-8} M) for 2 hr followed by LPS (1 μg/ml) challenge for additional 24 hr. Representative protein expression of IL-12p70, IL-23 and IL-10 from 4 independent experiments was determined from culture media using ELISA technique. All data represent mean (N=4) ± standard error. One-way ANOVA (analysis of variance) was used to determine group differences followed by Student-Newman posthoc test. Asterisk (*) indicates significant difference between compared groups from LPS-stimulated positive control.

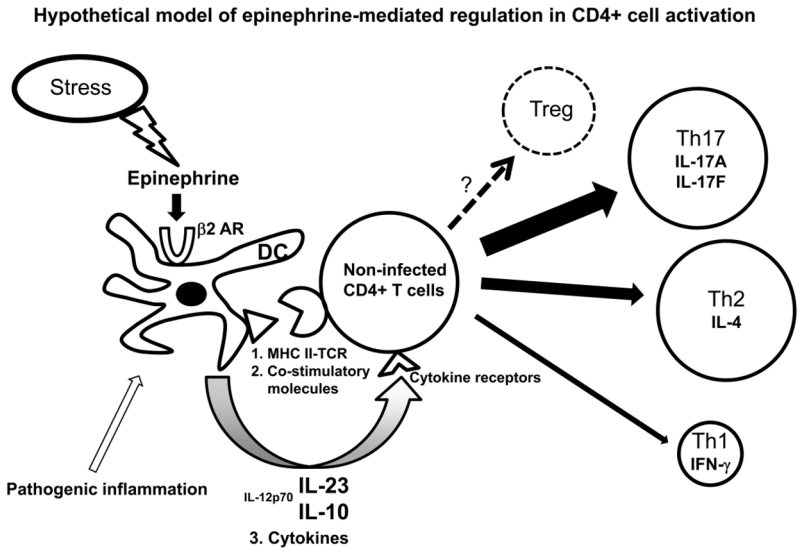

Figure 4.

Hypothetical model of epinephrine-mediated regulation of dendritic cell function to generate adaptive immunity. Diagram represents our hypothetical model that stress-induced epinephrine impacts dendritic cells through β2-adrenergic receptor to generate dominant IL-23 and IL-10 production in response to pathogenic activation, and as a consequence, drive $CD4^+$ T cell-mediated adaptive immunity into Th2/Th17 type.

Table 1

Epinephrine supports IL-4 and IL-17A production, but not IFN-γ by $CD4^+$ T cells with β2 adrenergic receptor-dependent manner. Purified $CD4^+$ T cells were cocultured with epinephrine (with or without butoxamine (10^{-5} M))-pretreated BMDCs in epinephrine-free new culture media for 96 hr with or without anti-CD3 monoclonal antibody (2.5 μg/ml). Protein expression of IFN-γ, IL-4 and IL-17A was determined from culture media collected each condition including control T cell only culture. Data has been selected as representative set from 3 independent experiements.

		(−) CD3			(+) CD3		
		IFN-γ	**IL-4**	**IL-17A**	**IFN-γ**	**IL-4**	**IL-17A**
BMDC-$CD4^+$ T cell coculture	No stimulation	272.85±91.07	N.D	N.D	2000.60±8.45	148.28±8.21	1496.03±66.99
	Epinephrine only	159.99±49.24	N.D	N.D	1768.02±80.44	103.67±7.32	2138.72±280.52
	Epinephrine - LPS	354.64±142.28	49.39±13.13 *a*	4008.65±231.84 *a*	768.83±41.80 *a*	426.43±31.53 *a*	4681.40±137.78 *a*
	Epinephrine+Butoxamine - LPS	659.13±99.75	23.75±9.60 *a b*	1352.06±212.47 *a b*	902.79±36.12 *a*	414.08±12.11 *a*	3203.10±90.25 *a b*
	LPS only	420.39±189.32	17.94±3.44 *a b*	2222.65±137.58 *a b*	760.59±34.50 *a*	245.08±23.20 *a b*	3978.60±237.94 *a b*
$CD4^+$ T cell only		N.D	N.D	N.D	826.43±20.54 *c*	841.38±26.54 *c*	1831.43±125.07

All data represent mean (n=3) ± standard error. One-way ANOVA (analysis of variance) was used to determine group differences followed by Student-Newman posthoc test.

a indicates significant difference ($p \leq 0.05$) between control/epinephrine-only group and either EL or LPS-only group.

b indicates significant difference ($p \leq 0.05$) between EL and LPS-only group.

c indicates significant difference ($p \leq 0.05$) between T cell only culture and control group.

N.D: no detective cytokine concentration.