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Astrocytes modulate microglial phenotype and dendritic cell-like properties

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ABSTRACT

ASTROCYTES MODULATE MICROGLIAL PHENOTYPE AND DENDRITIC CELL-LIKE PROPERTIES

**by
Nischal K. Padala**

Microglia are the resident immune cells of the CNS. In the healthy CNS, they express negligible levels of MHC II molecules as well as co-stimulatory molecules CD40, CD80 and CD86 necessary for antigen presentation to and activation of T cells. Co-stimulatory molecule expression can be induced in isolated microglia in vitro by sequential treatment with granulocyte monocyte colony-stimulating factor (GM-CSF) followed by lipopolysaccharide (LPS). Upon such treatment, they become mature dendritic cells (DCs), capable of activating T cells. However, microglia are not isolated in life, but rather exist in an environment enriched by other cells, notably astrocytes.

Therefore, to determine the effect of astrocytes on the assumption by microglia of the DC phenotype, microglia were treated with GM-CSF and LPS either in the presence or absence of astrocytes and assayed for the DC phenotypic marker CD11c and the co-stimulatory CD40, CD80, CD86 and MHC II by flow cytometry. When compared to isolated microglia, a significantly lower percentage of microglia co-cultured with astrocytes expressed these markers. Astrocytes also prevent the expression of these molecules in bone marrow-derived DCs. Neither interleukin-10, transforming growth factor-beta, nor prostaglandin E2 are responsible for the inhibition. Rather, contact with the astrocytic environment is responsible for the suppressive qualities.

Microglia cultured in the presence of astrocytes are also functionally distinct from those cultured in isolation. Microglia in association with astrocytes promote T cell

proliferation more robustly than do microglia in isolation. By contrast, a significantly higher percentage of CD4⁺ T cells stimulated with α CD3 in the presence of isolated microglia acquire an anti-inflammatory Foxp3⁺ T regulatory phenotype when compared to T cells cultured with microglia in the presence of astrocytes. This is not due to interactions between CD80/CD86 and CTLA4. The elevated presence of Foxp3⁺ T cells appears to be responsible for the lower level of T cell proliferation in the presence of isolated microglia. Finally, analysis of the supernatants from the T cell co-culture experiments reveal that astrocytic interaction(s) with microglia suppress T cell production of pro-inflammatory cytokines interferon- γ and interleukin-17. These data taken together suggest that astrocytes play a crucial role in modulating the microglial immune response in the CNS

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AND DENDRITIC CELL-LIKE PROPERTIES**

by
Nischal K. Padala

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APPROVAL PAGE

**ASTROCYTES MODULATE MICROGLIAL PHENOTYPE
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Mom, Dad, Brother and Sister

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CHAPTER 1

RATIONALE AND SPECIFIC AIMS

1.1 Rationale

Microglia – like other monocytically-derived cells – are capable of expressing a dendritic cell (DC) phenotype. When isolated in culture and properly stimulated, microglia produce major histocompatibility complex class II (MHC Class II) as well as the co-stimulatory molecules CD40, CD80 and CD86 that allow them to present antigen and activate T cells. The production of an appropriate T cell response is of vital importance to the healthy brain, and understanding the role of microglia in regulating this T cell response is crucial. During multiple sclerosis (MS) or its animal model experimental autoimmune encephalomyelitis (EAE), pro-inflammatory myelin-specific T cells invade the brain parenchyma destroying the myelin sheath. On the other hand, glial tumors suppress T cell responses. While these tumors contain resident microglia, they are immune incompetent, unable to mount an attack against the tumor. Their immaturity with respect to antigen-presentation may contribute to an environment in which anti-inflammatory Foxp3⁺ T regulatory cells are able to invade tumors and protect them against autoimmunity. The abundance of these regulatory cells is correlated with poor prognosis.

The interaction between both normal and malignant astrocytes and microglia is an essential one. Nevertheless, studies revealing the antigen-presenting capabilities of microglia almost exclusively been performed in the absence of astrocytes. Thus, how astrocytes might affect antigen presentation has been largely unknown. Data presented in

this thesis will indicate how important it is to understand this interaction for a complete understanding of microglial biology.

This thesis investigates the nature of an astrocytic/microglial cross-talk that affects the ability of microglia to assume a dendritic cell-like phenotype. Furthermore, the ability of microglia cultured in the presence or absence of astrocytes to influence CD4⁺ T cell proliferation and Foxp3 T cell differentiation is also examined. These studies address several specific aims, outlined below.

1.2 Specific Aims

Specific Aim 1. To determine whether the presence of astrocytes affects the ability of microglia to express dendritic cell (DC) markers CD11c, MHC Class II and co-stimulatory molecules CD40, CD80 and CD86.

Specific Aim 2. To determine whether differences in surface expression of these DC-specific molecules is the result of soluble or contact-mediated events.

Specific Aim 3. To examine the effects of differences in surface expression of these DC-specific molecules on T cell proliferation, Foxp3 expression and cytokine production

CHAPTER 2

INTRODUCTION AND BACKGROUND

2.1 Microglia

The CNS comprises two major cell types: neurons and glial cells. The glial cells include microglia, astrocytes and oligodendrocytes (He and Sun, 2007). Microglia are the resident immune cells of the CNS and are derived from CD45⁺ myeloid precursors. The same lineage also gives rise to bone marrow-derived dendritic cells and granulocytes (Aloisi, 2001; Barron, 1995; Chan et al., 2007; Gehrman et al., 1995; Kaur et al., 2001; Rezaie and Male, 2002). Microglia represent up to 20% of the total glial population in a healthy brain (Barron, 1995). It is generally accepted that microglial precursors migrate into the developing CNS from blood-producing centers during the mid-to-late embryonic and early postnatal periods (Kaur et al., 2001). There are two types of microglia in the CNS: perivascular and parenchymal. As their names suggest, perivascular microglia are found surrounding the vasculature of the CNS whereas parenchymal microglia are distributed throughout the brain and spinal cord (Barron, 1995; Chan et al., 2007; Kaur et al., 2001; Ling et al., 2001; Streit et al., 1988). Bone marrow chimera studies indicate that the perivascular population of microglia is slowly yet constantly replaced throughout life by bone marrow-derived precursors, whereas the parenchymal population is not (Simard and Rivest, 2004).

Microglia are among the first responders to CNS insults such as ischemia, trauma, and neurodegeneration (Aloisi, 1999; Glezer et al., 2007; Hickey, 2001; Kreutzberg, 1996; Minghetti and Levi, 1998; Nakamura, 2002; Rock et al., 2004; Streit et al., 1999; van Rossum and Hanisch, 2004). Microglia, by secreting brain-derived neurotrophic

factor (BDNF), nerve growth factor (NGF) and anti-inflammatory cytokines can influence the survival and regeneration of neural tissue after injury. On the other hand, microglial secretion of nitric oxide (NO), hydrogen peroxide (H₂O₂), and pro-inflammatory cytokines can be neurotoxic (Banati et al., 1993; Minghetti and Levi, 1998; Stoll and Jander, 1999; Wu et al., 2009). The composition of microglial products seems to be determined by the nature and potency of the activating signals.

In the normal brain microglia exhibit a ramified morphology, and, in the absence of an activation signal, exist in an “immature” phenotype (Nakamura, 2002; Ponomarev et al., 2005). Though un-activated, microglia constantly survey their microenvironment. Nimmerjahn and co-workers established that microglia *in vivo* are “highly dynamic surveillants of brain parenchyma.” They further reported that microglia appear to “sample” their neighboring parenchyma by rapidly extending and retracting their processes. In fact, this process is fast enough for the population of microglia to survey the entire CNS parenchyma within a few hours (Nimmerjahn et al., 2005).

Microglia express or can be induced to express a wide variety of cell surface receptors that can detect the consequences of almost any CNS insult as illustrated in Table 2.1. Prominent among these are receptors for phagocytosis, chemotaxis, pathogen associated molecular patterns (PAMPs) and stress-related heat shock proteins (Olson and Miller, 2004; Raivich et al., 1993; Ransohoff et al., 2007). Microglial phagocytic activity is critical for removing debris resulting from apoptotic or necrotic cells in the CNS. The timely removal of apoptotic cells is of great importance as it prevents potentially destructive inflammation in the CNS. Unlike necrosis, apoptosis is the orderly destruction

of a cell that ultimately results in the phagocytosis of the apoptotic cell by macrophages or by microglia in the CNS.

The expression of chemotactic receptors facilitates the migration of microglia to sites of injury within the CNS. Besides accumulating locally at the site of injury, chemokines released by activated microglia can further recruit infiltrating T cells, DCs, and macrophages (Cardona et al., 2008; Ransohoff et al., 2007; Rebenko-Moll et al., 2006). Befitting their role as “CNS resident immune cells,” microglia activated by PAMPs can secrete numerous immunomodulatory compounds. Upon activation microglia in vitro can assume a dendritic cell-like phenotype characterized by high expression of CD40, CD80, CD86, MHC Class II and the capacity to stimulate T cell activation (Aloisi et al., 2000; Matyszak et al., 1999; Ponomarev et al., 2005; Ponomarev et al., 2006; Ponomarev et al., 2007). Moreover, activated microglia can produce cytokines including tumor necrosis factor alpha (TNF- α), interleukin-1 (IL-1), IL-3, IL-5, IL-6, IL-10, IL-12, transforming growth factor beta (TGF- β) and the prostanoid prostaglandin E2 (PGE2) all of which can modulate the T cell response (Aloisi et al., 1997; Aloisi et al., 1999; Aloisi, 2001; Banati et al., 1993; Mizuno et al., 1994b; Mizuno et al., 1994a; Suzumura et al., 1996; Wu et al., 2009). Table 2.1 on following page.

Table 2.1 Molecular Signals and Modulators of Microglial Activation

<u>Class of Compound</u>	<u>Examples</u>
Surface structures of infectious agents	Lipopolysaccharide (LPS), bacterial cell wall proteoglycans, teichoic acid, gp41, gp120
Abnormal endogenous proteins	β -amyloid, prion protein
Complement	Complement factor C1q, C5a
Antibodies	Immunoglobulin A (IgA), IgG, IgM
Cytokines	Interleukin-1 β (IL-1 β), IL6, IL-10, IL-12, interferon-gamma (IFN γ), TGF β , TNF α
Chemokines (ligands for the chemokine receptors)	CCR3, CCR5, CXCR2, CXCR4
Neurotrophic factors	Brain-derived neuronotrophic factor (BDNF), glial cell line-derived neuronotrophic factor (GDNF)
Serum factors and proteases	Albumin, thrombin
Other proteins and peptides	Apolipoprotein E, CD40L, vasoactive intestinal peptide (VIP)
Neurotransmission-related compounds	β -adrenergic agonists, ATP, glutamate, NMDA
Ions	K ⁺ , Mn ²⁺
Other compounds	Cannabinoids, ceramide, gangliosides, melatonin, platelet-activating factor, PGE2

Source: Adapted from van Rossum, D. and Hanisch, U.-K. (2004), *Metabolic Brain Disease*, 19:399

By being immunocompetent microglia may play a role in the induction and etiology of autoimmune disorders such as multiple sclerosis (MS) (Becher et al., 2000b; Lubetzki et al., 2005; McQuaid et al., 2009; Ponomarev et al., 2005). The role of microglia in MS has come under intense scrutiny over the years, and yet the precise function of microglia during MS is not entirely clear. MS is an autoimmune disorder

characterized by the loss of the myelin sheath surrounding axons. Most of the extant knowledge about MS pathology comes from studies using the animal model of experimental autoimmune encephelomyelitis (EAE). EAE can be induced in rodents by immunization with myelin basic protein (MBP) or proteolipoprotein (PLP) or myelin oligodendrocyte glycoprotein (MOG) in complete Freund's adjuvant (McMahon et al., 2005). Although the precise cause of MS is not presently known, it is obvious that CD4⁺ T cells specific for myelin-derived antigens are responsible for the progression of MS (Adamus et al., 1996; Aggarwal et al., 2003; Bailey et al., 2007; Becher et al., 2000a). It is important to note that naïve T cells specific for myelin-derived antigens exist in most people; however, they only cause disease in a relatively small number of people. Several theories have been put forth to explain this apparent anomaly. The theory of molecular mimicry posits that antigens from pathogens such as viruses or bacteria may resemble myelin antigens closely enough that the immune system fails to differentiate between structurally similar self and non-self resulting in autoimmunity. It has been hypothesized that chronic inflammation may result in the activation of T cells of unrelated antigen specificities through a process called epitope spreading (McMahon et al., 2005).

Rodent studies show that auto-reactive T cells activated in the periphery need to be re-stimulated upon arrival in the CNS. In vitro, microglia can be prompted to assume a dendritic cell (DC) phenotype through a multi-step activation process. Exposure to the hematopoietic growth factors such as granulocyte/monocyte colony-stimulating factor (GM-CSF) can prime microglia to enter a DC differentiation pathway (Aloisi et al., 2000; Ponomarev et al., 2007; Re et al., 2002). Further activation in the form of inflammatory

signal such as interferon gamma (IFN- γ) or LPS or exposure to activated T cells can push microglia into a fully competent antigen-presenting cell (APC) phenotype (Aloisi et al., 2000; Ponomarev et al., 2007; Re et al., 2002; Shrikant and Benveniste, 1996).

The state of microglial activation appears to dictate whether they can activate a T cell or induce anergy. Matyszak and colleagues report that microglia treated with GM-CSF, IFN- γ or CD40 ligand (CD40L; CD154) alone are not mature APCs. They also demonstrate that microglia only partially activated induce anergy in T cells whereas microglia in the presence of GM-CSF and a pro-inflammatory secondary signal promote T cell activation and proliferation (Matyszak et al., 1999).

Given their competence in vitro as DCs microglia were initially thought to re-stimulate encephalitogenic T cells during MS episodes. There is conflicting evidence whether this re-stimulation is accomplished by microglia or by infiltrating DCs from the periphery. Furthermore, the re-stimulation of T cells either by DCs and/or microglia is accompanied by the failure of the usual protective immunosuppressive mechanism(s) employed by the CNS. Studies indicate that at least some part of this failure may be a consequence by dysfunctional or compromised astrocytes.

2.2 Astrocytes

Astrocytes are the most abundant glial cell type in the CNS. Originating from the neuroectoderm during development, astrocytes are absolutely essential for maintaining the homeostatic conditions in the CNS (Dong and Benveniste, 2001; He and Sun, 2007; Malipiero et al., 1990). Immunohistochemically identified by the expression of glial fibrillary acidic protein (GFAP), astrocytes provide a stable environment for neurons by preventing fluctuations in local ion concentrations and pH (He and Sun, 2007). Moreover, they clear neuronal waste and prevent excitotoxicity by removing excessive neurotransmitters such as glutamate from the synaptic cleft. Astrocytes also foster neuronal and oligodendrocyte survival by releasing BDNF, NGF and platelet-derived growth factor (PDGF) among others. In addition to these functions astrocytes maintain and regulate the blood brain barrier (BBB) (Aloisi et al., 1995; Becher et al., 2000b; Bechmann et al., 2007; Compston et al., 1997; He and Sun, 2007).

Traditionally, the CNS has been viewed as an immune privileged site, partly as a consequence of its separation from the rest of the body by the BBB (Hickey, 2001; Suter et al., 2003). Though the BBB is highly permeable to water and lipid soluble molecules and possesses carrier mechanisms for essential nutrients, it generally inhibits the free passage of solutes from systemic circulation into the CNS (Hickey, 2001). Also the CNS expresses very low levels of adhesion molecules necessary for the transit of immune cells into the CNS. Additionally, the CNS expresses copious amounts of TGF- β , a molecule that suppresses chemokine and adhesion molecule production as well as T cell proliferation (DeGroot et al., 1999; Li et al., 2006; Logan et al., 1992; Wesolowska et al., 2008). In addition to limiting the number of T cells that traffic through the CNS, the

microenvironment of the CNS seems to be detrimental to the fate of the T cells that do enter the CNS. Numerous studies show that T cells at least in the healthy CNS are quickly eliminated through apoptosis. The proposed mechanisms include the withdrawal of T cell trophic factors such as IL-2 or IL-7, encounter with T regulatory cells, the activation of TNF receptor 1 and/or CD95-CD95L death-inducing pathways (Kwidzinski et al., 2005; Meinl et al., 1994).

However, in the past few years the definition of CNS immune privilege has been refined. Evidence has steadily emerged demonstrating that during an immune response, activated T cells pass through the BBB and into the CNS irrespective of the involvement of CNS antigens in the initiation of the inflammation (Cayrol et al., 2008; Engelhardt, 2006; Ifergan et al., 2008; Lees et al., 2006). Furthermore, contrary to prior opinion, CNS derived antigens have also been shown to drain to cervical lymph nodes where they may be endocytosed by DCs and presented to T cells in the periphery. Though the CNS has an elaborate mechanism that seems to discourage T cell activity, under certain conditions T cells can and do overcome this inhibitory effect. During infections or autoimmune MS/EAE, for example, T cells proliferate and accumulate in the CNS resulting in inflammation that is detrimental to the health of the organism (Becher et al., 1999; Cayrol et al., 2008; Dittel, 2008; Engelhardt, 2006; Kebir et al., 2007; Lees et al., 2006; McQuaid et al., 2009; Meinl et al., 1994).

Until relatively recently, astrocytes were thought of only as “housekeeping” or “support cells,” and their immunological functions were somewhat overlooked. Though their capacity to activate T cells through antigen capture and presentation is controversial, there is no dispute about their capability to produce a wide variety of cytokines and

chemokines. These include the cytokines IL-1, IL-6, IL-10, IFN- α , IFN- β , GM-CSF, TGF- β , TNF- α , PGE2 and the chemokines monocyte chemoattractant protein 1 (MCP-1, CCL2), IFN- γ inducible protein 10 (IP-10), CCL2, CCL3, CCL4, CCL5, CCL20, CXCL12 (Aloisi et al., 1995; Constantinescu et al., 1996; Dong and Benveniste, 2001; Farina et al., 2007; Frei et al., 1989; He and Sun, 2007; Malipiero et al., 1990; Mizuno et al., 1994b; Mizuno et al., 1994a; Ohno et al., 1990; Sawada et al., 1989; Sawada et al., 1992). These immunomodulatory proteins not only affect T cells that find their way into the CNS directly, but they can also affect T cells indirectly by influencing the maturation and or function of infiltrating peripheral DCs and of potential CNS DCs like microglia.

There is some evidence that points to the modulatory affect of astrocytes on microglia and monocyte-derived cells. Astrocytes appear to down-regulate microglial expression of the IL-10 receptor (Ledeboer et al., 2002). Moreover, astrocyte-derived molecules down-regulate the production of IL-12 by microglia activated by IFN- γ /LPS (Aloisi et al., 1997). IL-12 suppression is mediated incompletely by soluble factors indicating that astrocytic cell membrane bound molecules may be important. Though the study did not identify the active molecules, IL-10 and PGE2 were found not to be responsible. Further evidence for the suppressive quality of astrocytes comes from a study conducted by Hailer and co-workers. Human peripheral blood-derived monocytes (PBMCs) activated with LPS in the presence of rat astrocytic supernatant expressed significantly lower levels of MHC Class II. Reduced PBMC MHC Class II expression could only be partially mimicked by the addition of exogenous IL-4, IL-10 or the receptor antagonist for IL-1 (Hailer et al., 1998). Furthermore, soluble astrocytic factors increase the expression of hemeoxygenase-1 in microglia under inflammatory conditions

(HO-1) and inducible-nitric oxide synthase (iNOS) in a TGF- β mediated process (Min et al., 2010; Vincent et al., 1997). More importantly, microglia, in the presence of astrocytes or astrocytic-conditioned medium (ACM) were markedly less active/neurotoxic even when exposed to amyloid peptide (A β), a potent activator of microglia in vitro (von Bernhardi and Eugenin, 2004). Taken together these reports clearly demonstrate that astrocytes and microglia behave quite differently when they are together than when they are alone.

2.3 Dendritic Cells

Dendritic cells (DCs) are the primary antigen presenting cells (APCs) of the immune system, and play an important role in linking the innate (antigen-nonspecific) and the adaptive (antigen-specific) arms of the immune system (Banchereau et al., 2000; Banchereau et al., 2003; Banchereau and Steinman, 1998). As such, they are essential for the activation and maturation of naïve CD4⁺ T cells into fully functional T helper cells (Th cells). Additionally, DCs are also crucial for B-cell maturation, boosting antibody production, Ig class switching and cytotoxic T cell activities (Banchereau et al., 2000; Mellman and Steinman, 2001; Palucka and Banchereau, 1999a; Steinman et al., 1999; They and Amigorena, 2001). Furthermore, they play a critical role in preventing autoimmunity by tolerizing T cells to self antigens (Blanco et al., 2008; Coquerelle and Moser, 2008; Hawiger et al., 2001; Wallet et al., 2005). Newly-generated immature dendritic cells (iDCs) from the bone marrow migrate via the bloodstream and take up residence in peripheral tissues where they are poised to respond to any pathogenic challenge (Hopken and Lipp, 2004; MartIn-Fontecha et al., 2003).

In the periphery, the iDCs constantly sample their microenvironment through a variety of processes. These include macropinocytosis, phagocytosis and endocytosis triggered by C-type lectin receptors, type I and II Fc γ receptors and various integrins that recognize apoptotic or necrotic cell death. “Self” antigens, arising as a consequence of apoptotic or necrotic cell death, and “non-self” antigens from invading pathogens are all efficiently captured and processed by iDCs (Basu et al., 2000; Manfredi et al., 2009; Maresz et al., 2007; Meloni et al., 2008; Sauter et al., 2000; Steinman et al., 2000). Though competent at capturing antigens, iDCs are poor activators of T cells since they either lack or express low levels of T cell stimulatory molecules such as major histocompatibility complex I or II (MHCs), CD40, CD80 (B7.1) and CD86 (B7.2) (Dhodapkar et al., 2001; Hawiger et al., 2001; Jin et al., 2007; Jonuleit et al., 2001; Mahnke et al., 2002; Roncarolo et al., 2001; Steinman et al., 2003).

The maturation of iDC from an antigen-capturing to an antigen-presenting phenotype capable of activating naïve T cells is a complex process. In order to finish maturation they must travel to the lymph nodes or the spleen and interact with T cells (Allavena et al., 1999; McColl, 2002). Changes in the expression of various chemokine receptors (CCRs) expressed by iDCs undergoing maturation facilitate their voyage to the secondary lymphoid organs. Chemokines such as macrophage inflammatory protein- 1 α (MIP-1 α /CCL3), MCP-1/CCL2) and the chemokine Regulated Upon Activation in Normal T cells, Expressed, Secreted (RANTES/CCL5) recruit CCR5- and CCR6-expressing iDCs to the site of inflammation (Sallusto et al., 1998).

As the iDCs mature, CCR7, the receptor for CCL19 and CCL21, is upregulated at the expense of CCR5 and CCR6 (Allavena et al., 1999; McColl, 2002; Randolph et al.,

2005). CCL19 is constitutively expressed by stromal cells and by mature DCs in the “T cell zone” of the secondary lymphoid organs. On the other hand, CCL21 is expressed mainly by cells of the lymphatic vessels. The restrictive expression of CCL19 and CCL21 in the lymphoid organs coupled with the switch in the expression of CCRs in DCs undergoing maturation ensures that only iDCs that are sufficiently activated undergo migration (Sallusto et al., 1998). Once the iDCs reach the lymphoid organs, chemokines released by the maturing DCs attract naïve T cells. Naïve T cells expressing high quantities of CD40L complete the DC activation process by binding to CD40 on the maturing DCs (McColl, 2002; Sozzani et al., 1998). Besides T cells, mature DCs already present in the lymphoid organs can provide the final maturation signal to the maturing DCs by secreting cytokines such as TNF α and IFN- γ . Upon receiving maturation signals iDCs undergo significant phenotypical and morphological changes that culminate in the generation of fully mature APC. Changes in the expression of many cell surface receptors and various intracellular processes are observed during this time. Down-regulation of antigen uptake activities while up-regulating the T cell stimulatory molecules and cytokine secretion are some of the hallmarks of iDCs undergoing maturation. Compared to iDCs, mature DCs (mDCs) express high levels of MHC Class I and II, CD40, CD80, CD86 and various cytokines and chemokines (Lutz and Schuler, 2002; Turley et al., 2000).

iDCs are set upon the path to maturation when they encounter a “danger signal.” Various endogenous and exogenous cues can serve as the danger signal (Bianchi, 2007; Lotze et al., 2007a). Endogenous signals released by necrotic or damaged cells (but not apoptotic cells) in the form of “damage associated molecular patterns” (DAMPs) like

heat shock proteins (HSP), nucleotides, or high mobility group 1 protein (HMGB1) can serve as activating signals. Human iDCs treated with HSP60, HSP70 or gp96 assume a mature dendritic cell phenotype (Bianchi, 2007; Flohe et al., 2003; Lotze et al., 2007a; Matzinger, 2002; Singh-Jasuja et al., 2000b; Singh-Jasuja et al., 2000a; Willart and Lambrecht, 2009). Likewise, HMGB1, a chromatin binding protein, passively released by necrotic cells can efficiently induce iDC maturation (Lotze et al., 2007b; Scaffidi et al., 2002). Furthermore, iDCs can respond to pro-inflammatory cytokines including TNF α , IL-1 α , and IL-4 that are produced during early stages of the immune reaction by innate immune system cells such as macrophages, neutrophils and eosinophils.

Exogenous signals in the form of PAMPs like LPS recognized by particular “pattern recognition receptors” (PRRs) such as those belonging to the Toll-like family of receptors (TLR) may also serve to induce iDC maturation (Akira et al., 2000; Bianchi, 2007; Edwards et al., 2002; Kaisho and Akira, 2003; Kaisho and Akira, 2006; Medzhitov, 2001; Miller et al., 2005; O'Neill, 2008). TLRs are the most widely studied PRRs in mammals. At least 10 different TLRs in humans and 13 in mice have been identified (Medzhitov and Janeway, Jr., 2000b). All TLRs share a common extracellular domain rich in leucine repeats and an intracellular Toll/interleukin-1 receptor (TIR) homology domain (O'Neill, 2008). The downstream signaling pathways for TLRs are activated by the binding of the appropriate ligand as illustrated in Table 2.2.

Downstream TLR signaling can occur in one of two ways. All TLRs, with the exception of TLR 3, can activate either the MyD88 adapter dependent or independent pathway (Akira et al., 2000; Yamamoto et al., 2003). TLR 3 can only activate the MyD88-independent pathway. However, regardless of the stimulating ligand, all TLR

ligation culminates with the activation of the transcription factors NF- κ B, activating protein 1 (AP-1) and/or interferon regulatory factor 3 (IRF3).

In the MyD88 dependent pathway, MyD88 is recruited to the intracellular TIR domain by the adapter protein TIR Activating Protein (TIRAP/MAL). MyD88 in turn recruits the IL-1 Receptor Associated Kinase (IRAK) family members IRAK-4 and IRAK-1 to further the signaling event. Phosphorylated IRAK-1 associates with the TNF Receptor Associated Factor-6 (TRAF-6) and activates it through phosphorylation. At this point the signal bifurcates with one arm leading to the activation of MAP kinases and AP-1 and the other arm leading to the activation of NF- κ B by the ubiquitination and degradation of the NF- κ B inhibitor, I κ B. TLR activation of the MyD88 independent pathway requires the TIR domain-containing adapter inducing IFN- β (TRIF) protein. Intracellular TLR 3 activated by dsRNA can interact directly with TRIF. TRIF activation leads to the activation of the kinase IKK which phosphorylates the interferon regulatory factor-3 (IRF-3) and facilitates its nuclear translocation. The activation of the transcription factors NF- κ B, AP-1 and IRF-3 can lead to the maturation and up-regulation of co-stimulatory molecules and secretion of chemokines and pro-inflammatory cytokines in iDCs (Medzhitov and Janeway, Jr., 2000a; O'Neill, 2008). Table 2.2 on page 17.

Table 2.2 TLRs and Their Ligands

TLR	Ligands
TLR 1	Triacyl lipopeptides
TLR 2	Peptidoglycan
TLR 3	dsRNA
TLR 4	LPS, taxol, HSP's, envelop protein
TLR 5	Flagellin
TLR 6	Diacyl lipopeptides
TLR 7	ssRNA
TLR 8	ssRNA (only in humans)
TLR 9	CpG DNA

Source: Takeda and Akira, 2007

In the lymphoid organs, the captured and by-now-processed foreign antigen is presented by DCs expressing MHC I or II molecule to an antigen-specific naïve CD8⁺ or CD4⁺ TCR respectively (Banchereau et al., 2000; Guermonprez et al., 2002; Mellman et al., 1998; Mellman and Steinman, 2001; Pierre et al., 1997; Wallet et al., 2005). This is one of the two signals required for T cell activation. The second signal is provided by co-stimulatory molecules belonging to the B7 family (CD80, CD86) or CD40 (Avni and Rao, 2000; Brehin et al., 2008; Bretscher, 1999; Cardona et al., 2006; Chen et al., 2003; Green et al., 1995). These molecules engage their binding partners CD28/CTLA-4 and the CD40L to mediate T cell responses.

2.4 CD4⁺ T Cells

2.4.1 Activation

CD4⁺ T cells are critical mediators of the adaptive immune system. Activated T cells can recruit other members of the immune system such as B cells, basophils, and neutrophils by producing various cytokines and chemokines, to further bolster the immune response (Palucka and Banchereau, 1999b). Naïve CD4⁺ T cells responses can be polarized into at least four important categories: Th1, Th2, Th17 and T_{regs}. Of these, Th1 and Th17 are generally considered to be pro-inflammatory whereas, Th2 and T_{regs} are usually thought of as anti-inflammatory.

The proper activation of naïve T cells requires two distinct signals. Evidence for the existence of a two-signal mechanism in T cell activation was first presented by Lafferty and Woolnough. In a report on the mechanism of allograft rejection, they proposed that proper T cell activation requires an “antigenic” as well as an “inductive stimulus.” They further claimed that neither stimulus by itself was sufficient to induce T cell activation (Lafferty and Woolnough, 1977). Further studies since then have proved this to be the case (Acuto et al., 2003; Tuosto and Acuto, 1998). Sufficiently mature DCs can supply T cells with the required signals, both in vivo and in vitro. In case of naïve CD4⁺ T cells, DCs present a captured and processed antigen peptide in the context of MHC II to the TCR to provide signal one. The TCR is a multi-subunit complex composed of variable α and β chains non-covalently linked to a non-variable CD3 unit (Dietrich et al., 1996). The variable $\alpha\beta$ subunit of the TCR possesses the capacity to bind the multitude of antigens that may be encountered during the lifetime of an organism. The non-variable CD3 seems to serve primarily as a signal transducing protein since T

cells in vitro may be activated by agonist CD3 antibodies without the help of APCs or antigens (Hermans and Malissen, 1993; Wegener et al., 1992). Stimulation of the TCR leads to a complex chain of events that culminates in the activation of transcription factors including nuclear factor- κ B (NF- κ B) and nuclear factor of activated T cells (NFAT) (Masuda et al., 1998). The activation of NF- κ B and NFAT results in the transcription of various genes necessary for the further maturation of T cells.

In spite of all the changes in gene transcription and translation, signal one alone cannot elicit a proper response from T cells. Belying its name as mere “co-stimulation,” signal two is as important as the signal provided by the MHCII:antigen-peptide complex. Various B7 family members such as B7.1 (CD80), B7.2 (CD86), B7-H1/programmed death ligand-1 (PD-L1) and B7-H2 expressed by DCs can provide the secondary stimulus when they interact with their cognate receptors CD28, cytotoxic T-lymphocyte antigen-4 (CTLA-4), inducible co-stimulator (ICOS) and programmed death (PD-1) expressed on T cells.

T cells constitutively express CD28 (Gross et al., 1992). Stimulation of CD28 by either CD80 or CD86 can lead to the enhancement of IL-2 production which then acts in a paracrine/autocrine manner to promote T cell proliferation (Appleman et al., 2000; Lucas et al., 1995). Additionally, CD28 stimulation also increases the expression of the anti-apoptotic protein Bcl-x1 in an IL-2 independent manner to further bolster the ability of T cells to resist apoptosis (Boise et al., 1995). Unlike CD28, CTLA-4, PD-L1 and ICOS are rapidly upregulated upon T cell activation (Latchman et al., 2004; Teft et al., 2006). ICOS is a positive regulator of T cell function. ICOS stimulation after T cell activation appears to be important for enhancement of effector T cell functions and

cytokine secretion, especially for Th2 polarized cells (Watanabe et al., 2005). On the other hand, CTLA-4 and PD-L1 act negatively to limit the T cell response (Nishimura et al., 2001; Walunas et al., 1994).

Co-stimulation can also be provided by stimulation of the T cell CD40L by CD40-expressing DCs (Fujii et al., 2004). CD40 is a member of the TNF super family of receptors and is expressed by a variety of cells such as DCs, monocytes, B and epithelial cells (van Kooten and Banchereau, 1997). Stimulation of CD40/CD40L pathway has been shown to improve the efficiency of DCs by upregulating their expression of CD80/CD86 while simultaneously providing T cells with a secondary stimulus (Quezada et al., 2004; van Kooten and Banchereau, 1997).

Not only are DCs capable of activating T cells but they also have the capacity to modulate and fine-tune the T cell response. The strength and duration of DC signaling seems to play a role in determining the direction of the immune response, with evidence showing that a “strong” and prolonged signaling leads to type 1 inflammatory response (Th1), whereas a “weak” signal gives rise to anti-inflammatory Th2 responses (Bretscher, 1999; Green et al., 1995; Guermonprez et al., 2002). Another important factor in determining T cell fate is the cytokine milieu present during their activation. To date, four main differentiation pathways have been identified for naïve CD4⁺ T helper cells. T cells can become T helper (Th) 1, -2, -17, or T regulatory cells (T_{regs}) (Murphy and Reiner, 2002). Development of the appropriate subset of Th cells is of vital importance not only to fight off infections but also to prevent autoimmunity.

DCs at different stages of maturation or belonging to different subsets can affect the outcome of an immune response (Lutz and Schuler, 2002; Mahnke et al., 2002).

Depending on conditions, DCs can secrete many immunomodulatory chemokines and cytokines that can affect the quality of the immune response. For instance, DCs stimulated by LPS primarily secrete IL-12 and stimulate a pro-inflammatory Th1 response (Hsieh et al., 1993; Murphy et al., 1997).

2.4.2 Th1 Cells

First described more than two decades ago by Mosmann and Coffman, Th1 and Th2 cells are probably the most widely studied subsets of T helper cells (Mosmann et al., 2005). In that seminal study, two distinct clones of T cells were identified, based mainly on the production of certain “signature” cytokines. Since then, knowledge of these two closely related Th cells has expanded considerably.

Th1 is a pro-inflammatory phenotype of T cell characterized by high production of IFN- γ , lymphotoxin- α (LT- α /TNF- β), and IL-2. Naïve CD4⁺ cells can be polarized into attaining a Th1 phenotype on TCR stimulation in the presence of IL-12 (Manetti et al., 1993). DCs activated by TLR ligation or pro-inflammatory cytokines acquire the ability to produce copious amounts of IL-12. IL-12 is a heterodimeric pro-inflammatory cytokine and the founding member of the IL-12 family of cytokines which also includes IL-23, IL-27 and IL-35. An IL-12-specific p35 subunit combines with a common p40 subunit to form bioactive IL-12p70 (Trinchieri et al., 2003). DCs and other innate immune cells such as monocytes, neutrophils and natural killer (NK) cells are able to produce IL-12. Naïve T cells constitutively express the receptors for IFN- γ and IL-12R β 1, which act together with the inducible β 2 subunit to confer IL-12 sensitivity (Chua et al., 1995; Presky et al., 1996; Wu et al., 1996). IL-12 β 2 subunit expression is rapidly upregulated by T cells upon TCR stimulation in an interferon regulatory factor-1 (IRF-1)

dependent manner (Kano et al., 2008). The activation of IRF-1 is a critical step in making T cells responsive to the effects of IL-12. Abrogation of IRF-1 signaling in vivo or in vitro results in a significant skewing of the Th response in favor of Th2 cells while simultaneously reducing the number of Th1 cells significantly (Taki et al., 1997).

IL-12 acts in an autocrine manner to maintain its own expression through the activation of signal transducer and activator of transcription-4 (STAT-4) (Nishikomori et al., 2002). The activation of STAT-4 also leads to the production of IFN- γ , that acts in a paracrine/autocrine manner to activate STAT-1 and the Th1-specific transcription factor “t-bet” (Lighvani et al., 2001; Szabo et al., 2000). Moreover, IFN- γ can initiate the Th1 developmental pathway by itself in the absence of IL-12 though not as efficiently as IL-12 (Lugo-Villarino et al., 2003). The activation of STAT-1 stabilizes the expression of the IL-12R complex thereby maintaining Th1 IL-12 responsiveness over their lifetime. Interestingly, the expression of STAT-1 appears to be absolutely required to promote Th1 development only in the presence of both Th1 and Th2 polarizing cytokines. The deletion of STAT-1 in the absence of Th2 cytokines and in the presence of IL-12 does not affect the ability of CD4⁺ T cells to become Th1 cells (Afkarian et al., 2002). However, the expression of t-bet is required in all instances and naïve T cells lacking t-bet cannot efficiently differentiate into Th1 cells (Szabo et al., 2000).

Proper Th1 responses are critical for the clearance of certain intracellular pathogens such as protozoa, bacteria and fungi. IFN- γ , the main effector cytokine produced by Th1 cells, is indispensable for their action. For instance, infections by the bacterium *Listeria monocytogenes* require a robust Th1 response to be cleared (Scott et al., 1988; Sher and Coffman, 1992) Though originally implicated as the critical players in

the etiology of many autoimmune disorders, the role of Th1 cells has been significantly downgraded upon the recent discovery of Th17 cells and findings about their role in autoimmune pathology (See below).

2.4.3 Th2 Cells

Along with Th1 cells, Th2 cells were one of the two subsets of Th cells originally described by Mosmann et al. in 1986. Th2 cells are considered primarily to be anti-inflammatory in nature and are defined by their production of the cytokines IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 (Mosmann et al., 1986; Swain et al., 1988; Zhu and Paul, 2008). While mostly anti-inflammatory in nature, Th2 cells are responsible for airway allergy inflammation and are required for efficient action against certain extracellular pathogens such as helminthes and nematodes (Zhu and Paul, 2008). Furthermore, the cytokines produced by Th2 cells can promote antibody class switching by B cells (Sher et al., 1990).

Not unlike Th1 cells, the polarization of Th2 cells also depends on TCR signaling and the presence of the requisite cytokines in the immediate microenvironment. IL-4, the signature cytokine produced by Th2 cells, is also required to initiate their development from CD4⁺ naïve T cells (Swain et al., 1990). In vitro, T cells can be polarized into Th2 cells by TCR stimulation in the presence of exogenous IL-4. Moreover, Th2 cells can also be generated in the absence of exogenously added IL-4 through TCR stimulation and the simultaneous blockade of Th1 polarizing signals. Upon TCR stimulation CD4⁺ T cells produce low amounts of IL-4 which appear to be sufficient to elicit a positive feedback loop resulting in Th2 differentiation when antagonizing stimuli are neutralized (Rao and Avni, 2000). Also, as mentioned above, the strength of DC-T cell signaling

plays an important role in Th1 or Th2 polarization. Naïve T cells weakly stimulated by DCs tend to produce low quantities of IL-4 and IL-2 which act in a paracrine manner to drive Th2 polarization whereas a strong TCR stimulation leads to a Th1 phenotype.

The presence of exogenous or endogenous IL-4 when the TCR is activated leads to the induction of the Th2-specific transcription factor GATA binding protein-3 (GATA-3) in a STAT-6 dependent manner (Ho et al., 2009; Pai et al., 2004). STAT-6 is both necessary and sufficient for IL-4 signal transduction and Th2 induction (Zhu et al., 2001). Conditional knockout studies of GATA-3 provide ample evidence of the role GATA-3 plays in directing Th2 cell function. Studies using STAT-6 negative mice prove that activation of GATA-3, independent of IL-4/STAT-6, pathway is sufficient for Th2 differentiation and function (Zheng and Flavell, 1997). Although IL-4 is required for Th2 differentiation, recent evidence reveals that IL-2 induced STAT-5 may be just as important as IL-4 induced GATA-3 signaling. Neutralizing IL-2 signaling in vitro in the presence of the requisite Th2-polarizing conditions results in a near elimination of Th2 cells (Cote-Sierra et al., 2004). Furthermore, the effect of IL-2 was a result of impaired Th2 differentiation and was independent of cell survival or proliferation. The same study also reveals that IL-2 signaling is not required for the induction of Th2 differentiation; rather it is required for the continued expression of the Th2 phenotype, and it does so by maintaining the accessibility of the IL-4 gene to transcription factors. These findings have been recapitulated in vivo.

A dichotomy of sorts exists between Th1 and Th2 phenotypes. Under unambiguous polarizing conditions, induction of t-bet actively antagonizes GATA-3 and vice versa. Abrogation of either transcription factor leads to a deficiency in the generation

of the phenotype associated with that particular factor both in vivo and in vitro, even in the most favorable of polarizing stimuli. Unlike t-bet, GATA-3 is expressed at a very low level under basal conditions and seems to negatively affect t-bet transcription. The deletion of basal GATA-3 signaling promotes Th1 induction even under strong Th2-promoting and Th1-impeding stimuli (Pai et al., 2004). It appears that the basal levels of GATA-3 are required for the maintenance of an immature phenotype in naïve CD4⁺ T cells and to prevent “spontaneous” Th1 polarization.

2.4.4 Th17 Cells

Th17 cells are a relatively newly-identified subset of T cells and are so named due to their ability to efficiently produce the pro-inflammatory cytokine IL-17 (Harrington et al., 2005). IL-17 is an inflammatory cytokine with roles in inflammation and autoimmune disorders. In addition to their main effector cytokine, Th17 cells can also produce IL-17F, IL-6, TNF, IL-21 and IL-22. The development and regulation of these cells has remained obscure until the past few years. Various studies have shown that efficient Th17 polarization from naïve CD4⁺ T cells can be achieved through the combined action of TGF- β and IL-6/IL-21 and IL-23 at distinct points during the differentiation process or IL-1 β , IL-6 and IL-23 simultaneously.

Naïve CD4⁺ T cells can be induced to differentiate into Th17 cells by TGF- β and IL-6 or IL-21 (Korn et al., 2007; Veldhoen et al., 2006). Because TGF- β is primarily anti-inflammatory in nature whereas IL-6 and IL-21 are pro-inflammatory, it is surprising that cytokines with apparently contradictory characteristics can work together to induce Th17 polarization. TGF- β by itself can generate T regulatory cells (T_{regs}, see below), but the presence of IL-6/IL-21 in the cytokine milieu promotes the differentiation of Th17 cells.

There is conflicting information about the requirement of TGF- β in Th17 polarization with the majority of reports claiming that TGF- β is absolutely required for Th17 differentiation. Mice with TGF- β -unresponsive CD4⁺ T cells appear to be unable to produce Th17 cells whereas mice with CD4⁺ T cells that overexpress TGF- β have significantly more Th17 cells (Qin et al., 2009). However, a very recent study by Ghoreschi et al. reports that IL-1 β with IL-6 and IL-23 can also induce Th17 cells from naïve T cells in the absence of TGF- β (Ghoreschi et al., 2010).

IL-6 was originally thought to be as indispensable as TGF- β for Th17 induction. However, in vitro, Th17 cells can also be generated from naïve CD4⁺ T cells by TGF- β and IL-21. Although IL-21 can efficiently replace IL-6 in vitro, the scenario in vivo is peculiar. IL-6 knockout mice can produce Th17 cells only upon the depletion of naturally occurring T_{regs} (Korn et al., 2007). During pathological conditions a variety of cells including Th1, Th2, NK cells, and T follicular cells can produce IL-21 (Suto et al., 2008). It would appear that TGF- β and IL-21 produced by other immune cells coupled with the abrogation of normal anti-inflammatory controls can produce Th17 cells even in the absence of IL-6.

TGF- β and IL-6/IL-21 acting through the SMAD and STAT-3 pathways respectively lead to the induction of the Th17-specific transcription factor RAR-related orphan receptor gamma (ROR γ t) (Ivanov et al., 2006). Activation of SMAD or STAT-3 alone is not sufficient to induce fully functional ROR γ t. STAT-3 deficient mice cannot induce ROR γ t and are, therefore, incapable of generating Th17 cells (Wei et al., 2007). Interestingly, a study by Zhou and colleagues suggests that TGF- β alone may be enough to induce the expression of ROR γ t but its activity is suppressed through an unknown

post-translational mechanism and IL-6 or IL-21 through STAT-3 somehow relieve ROR γ t suppression (Xiao et al., 2008; Zhou et al., 2007). Additionally, STAT-3 leads to the upregulation of IL-21 production by Th17 cells undergoing polarization. IL-21 acts in a self-amplifying loop to increase Th17 cell numbers by promoting their survival and by actively inhibiting the differentiation of Th1 cells leading to skewing of the Th cell repertoire towards the Th17 phenotype (Wei et al., 2007). And as noted above, under certain situations in vivo, IL-21 produced by innate immune cells may substitute for IL-6.

In addition to TGF- β , IL-6 and IL-21, IL-23 stimulation of T cells undergoing Th17 differentiation is required to stabilize their phenotype and complete the maturation process (Langrish et al., 2005). IL-23 is a heterodimeric cytokine belonging to the IL-12 family of cytokines. It is composed of the novel IL-23p19 and the IL-12 shared IL-12p40 subunits (Oppmann et al., 2000). It is important to note that IL-23 is not a Th17 differentiation factor and naïve CD4⁺ T cells do not express the IL-23 receptor (McGeachy et al., 2009). Nonetheless, the activation of ROR γ t and STAT-3 leads to the expression of IL-23R on T cells stimulated with TGF- β and IL-6/IL-21 thus rendering them responsive to IL-23 (Xiao et al., 2008). IL-23 derived from innate immune cells such as DCs and macrophages acts on Th17 cells to not only maintain their phenotype but also to promote their survival. In fact, mice lacking IL-23p19 or IL-23R cannot generate Th17 cells in substantial numbers (Cua et al., 2003; Langrish et al., 2005; McGeachy et al., 2009). Furthermore, since uncontrolled inflammation is detrimental to the health of the organism, Th17 sensitivity to IL-23 may also serve to limit the duration and potency of Th17 mediated inflammation. However, Ghoreschi et al. report the existence of another pathway that is similar to the above-mentioned one in some respects

but also differs in significant ways. In their hands IL-1 β in conjunction with IL-6 and IL-23 was also able to induce Th17 differentiation independent of TGF- β . The upregulation of IL-23R through IL-6-dependent activation of STAT-3 renders differentiating T cells responsive to IL-23, which then acts in a positive feedback manner to further upregulate IL-23R and Th17 polarization (Ghoreschi et al., 2010). Although contrary to prior opinion suggesting that TGF- β and IL-6 have both been shown to be dispensable, IL-23 still appears to be required for Th17 polarization.

Th17 cells are an essential part of the adaptive immune system and their proper function is obligatory for the health of an individual. A well-regulated Th17-derived cytokine response is absolutely critical for the proper clearance of certain bacterial and fungal infections (Khader et al., 2007; Rudner et al., 2007; Ye et al., 2001). The exposure of naïve Th cells to cell components of particular pathogens seems to polarize them towards a Th17-inducing phenotype characterized by high expression of IL-23 and IL-6 among other cytokines (Infante-Duarte et al., 2000). In addition to contributing to host defense, Th17 cells have also been identified to play a critical role in the etiology of many autoimmune diseases. These include psoriasis, rheumatoid arthritis (RA), MS, inflammatory bowel disease (IBD) and asthma (Komiyama et al., 2006; Murphy et al., 2003; Zheng et al., 2007).

The discovery of Th17 cells has fundamentally changed the understanding of autoimmune disorders. Studies utilizing rodent models or MS and RA have provided many insights into the mechanism of Th17-mediated autoimmunity. These disorders were originally thought to be the result of unchecked Th1 related activities. However, support for the involvement of Th17 cells in these pathologies has been accumulating steadily.

Studies using mice deficient in the molecules required for Th1 differentiation conclusively show that EAE and collagen-induced arthritis (CIA) are Th17-dependent conditions. IL-12 receptor KO mice which are unable to induce Th1 polarization, as well as mice with IFN- γ -unresponsive T cells remain highly susceptible to EAE (Krakowski and Owens, 1996) suggesting an alternative T cell requirement. Furthermore, Gran et al. show that targeted deletion of the IL-12p40 (a subunit shared with IL-23) but not of IL-12p35 confers immunity against EAE induction in mice (Gran et al., 2002). Taken together, these revelations provide an explanation for the discrepancies regarding Th1 cells being the cause of certain autoimmune disorders. Inadvertent interference with molecules required for Th17 polarization while examining the roles of IL-12 and Th1 cells may have led to the mistaken conclusion that Th1 cells were the culprits in autoimmunity. Further evidence against Th1 cells and in favor of Th17 cells comes in the form of adoptive transfer studies. When transferred into naïve mice, myelin basic protein (MBP) specific Th17 cells induce potent EAE whereas transfer of Th1 or Th2 cells does not (Stockinger et al., 2007).

Intriguingly, the two Th17 polarization pathways described above appear to give rise to subsets of Th17 cells with very distinct phenotypes. T cells polarized in the presence of TGF- β and IL-6 seem to be non-pathogenic in relation to autoimmune diseases and have the capacity to produce anti-inflammatory IL-10 and IL-9 while suppressing pro-inflammatory IL-22 secretion (McGeachy et al., 2007). On the other hand, T cells polarized in the absence of TGF- β and in the presence of IL-1 β and IL-23 have a greater potential to cause autoimmune harm. The pathogenic subset of Th17 expresses not only ROR γ t but also the Th1 transcription factor t-bet, as illustrated by

their capacity to produce IFN- γ . Additionally, they also express markers such as IL-18R1 and CXCR3 that have been shown to be prevalent during episodes of autoimmunity (Ghoreschi et al., 2010).

2.4.5 T-Regulatory Cells

In a healthy individual, pro- and anti-inflammatory events are kept on a tight leash. This tight control helps the immune system to respond appropriately to various pathogens and prevents autoimmunity. The job of preventing autoimmunity falls to a subset of Th cells called T regulatory cells (T_{regs}). The hypothesis that there exists an innate regulatory mechanism to keep the immune system in check and autoimmunity at bay has been around for decades. Initial evidence for this hypothesis came from studies examining the role of the thymus in the immune system. It was observed that neonatally thymectomized rodents developed a “wasting disease” similar to “host vs. graft” syndrome as a result of severe autoimmunity (Yunis et al., 1967). This broad hypothesis has been refined over the past few years and accumulating evidence has identified T_{regs} as playing the crucial role of preventing autoimmunity.

Sakaguchi et al. identified a subset of $CD4^+$ T cells expressing the IL-2 receptor α -chain, as a key mediator of autoimmunity (Sakaguchi et al., 1995). Adoptive transfer of $CD4^+$ T cells depleted of $CD25^+$ cells into nude mice was able to spontaneously produce severe autoimmunity in the host whereas transfer of $CD4^+$ cells containing $CD25^+$ T cells did not. The same group further demonstrated that neonatally thymectomized mice cannot produce $CD4^+CD25^+$ cells and therefore develop autoimmunity as a result of self-reactive T cells (Sakaguchi et al., 1996). The discovery of a new T_{reg} -specific forkhead/winged helix protein transcription factor (“Foxp3”) has eased the difficulty of

study of these cells and has led to important findings about the mechanism behind their differentiation and mode action. Brunkow et al. identified Foxp3 deficiency as the cause of autoimmunity in “scurfy” mice (Brunkow et al., 2001). Mice affected with scurfy are remarkably similar to neonatally thymectomized mice in that both conditions are characterized by excessive proliferation of CD4⁺ T cells resulting in death within weeks of birth. Significantly, a scurfy-like human disease called immune dysregulation polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) has been shown to be a consequence of mutant Foxp3 (Bennett et al., 2001). Moreover, utilizing Foxp3 KO mice, Fontenot and colleagues established that the development of CD4⁺CD25⁺ regulatory T cells originally identified by Sakaguchi et al. is contingent upon the expression and proper function of Foxp3 (Fontenot et al., 2003; Fontenot et al., 2005). Furthermore, they also showed that CD4⁺CD25⁻ cells could be endowed with suppressive functions simply by transducing them with a retroviral vector containing Foxp3.

CD4⁺CD25⁺Foxp3⁺ T cells have been classified into innately occurring natural T_{regs} (nT_{regs}), produced in a fully mature state by the thymus or inducible T_{regs} (iT_{regs}) that are generated locally in the periphery as required in response to various self and non-self antigens (Bensinger et al., 2001). Though nT_{regs} and iT_{regs} essentially perform the same function in the host and share certain traits, there are important differences between them. nT_{regs} acquire a suppressive phenotype as a consequence of strong TCR stimulation by DCs presenting self-antigen and co-stimulatory interactions between CD28 and CD80/CD86 in a thymic microenvironment (Guo et al., 2008; Jordan et al., 2001; Salomon et al., 2000). Conditional deletion of the co-stimulatory molecules significantly decreases the number of circulating nT_{regs}. Additionally, TGF-β and IL-2 have also been

implicated in nTreg development, though their involvement is controversial (Curotto de Lafaille et al., 2004; D'Cruz and Klein, 2005). Unlike nT_{regs}, TGF- β and IL-2 seem to be indispensable for the development of iTregs, both in vitro and in vivo (Guo et al., 2008). Furthermore, it is also clear that maintenance of the suppressive phenotype by nTreg_s and iTreg_s is contingent upon the availability of TGF- β and IL-2 in the immediate cytokine milieu.

Naïve CD4⁺ T cells are set on the path towards acquiring an iT_{reg} phenotype through the combined efforts of TGF- β -activated transcription factor SMAD-3 and TCR activated transcription factor NFAT. SMAD-3 and NFAT bind a Foxp3 enhancer region and drive its transcription. NFAT, in addition to binding to the Foxp3 enhancer region, also upregulates the expression of IL-2 and its receptor CD25 on T cells destined to become iT_{regs} (Wu et al., 2006). Unlike conventionally activated T cells which upregulate CD25 only transiently, iT_{regs} (and nT_{regs}) maintain their expression of CD25 throughout their life as a T_{reg}. This is an important distinction since induction and maintenance of Foxp3 requires IL-2-induced STAT-5 signaling (Wu et al., 2006). Mice deficient in IL-2 signaling as a result of deletion of the β subunit of the IL-2 receptor (IL-2R β) have a drastically reduced CD4⁺CD25⁺Foxp3⁺ number of cells both in the periphery and in the thymus resulting in severe autoimmune disorders (Burchill et al., 2007). However, this defect can be rescued through the constitutive ectopic expression of STAT-5 (Antov et al., 2003). Moreover the study by Burchill et al. also shows that STAT-5 drives T_{reg} development by binding the promoter region of the Foxp3 gene thus regulating its transcription (Yu et al., 2009). Interestingly, the effects of IL-2 but not those of the IL-2R β subunit are redundant. IL-15, a cytokine structurally similar to IL-2, can act as a

substitute in the absence of IL-2. Consequently, IL-2 KO mice do not show any deficiencies in $CD4^+CD25^+Foxp3^+$ T_{regs} whereas double IL-2 and IL-15 KO mice mimic the phenotype seen in IL-2R β KO mice (Burchill et al., 2007). Also, it appears that T_{regs} can be generated and maintained even under suboptimal IL-2/IL-15 signaling. Using a transgenic mouse model that expresses a mutant form of IL-2R β that can activate STAT-5 weakly and only transiently, Yu et al. demonstrate that T_{regs} remain prevalent and functional (Yu et al., 2009).

T_{regs} can exert their influence on the immune system in many ways. They can secrete large amounts of immunosuppressive cytokines such as TGF- β , IL-10 and IL-35 (Collison et al., 2009; Taylor et al., 2006). TGF- β and IL-10 are well known suppressors of the immune system both in vitro and in vivo. IL-35 is a newly-identified member of the IL-12 family of cytokines. Like the rest of its family members, IL-35 is a heterodimer consisting of the IL-12p35 subunit and a novel Epstein barr virus-induced-3 (EBI-3) subunit (Niedbala et al., 2007). Unlike IL-12 and IL-23, IL-35 possesses anti-inflammatory properties and has been implicated as a potential T_{reg} -mediated suppressive mechanism (Collison et al., 2007). In vitro, stimulation of T_{regs} with IL-35 under co-stimulatory conditions promotes their proliferation (Castellani et al., 2010). Furthermore, the presence of IL-35 in the cytokine milieu suppressed the differentiation of Th1 and Th17 cells. In addition, exogenous administration of IL-35 reduced the severity of CIA in vivo (Niedbala et al., 2007).

T_{regs} have also been reported to mediate immune suppression through contact-mediated inhibition of DC maturation and/or function. In vitro, co-culture of mature DCs with T_{regs} resulted in the downregulation of the co-stimulatory molecules CD80 and

CD86 expressed by the DCs (Cederbom et al., 2000). This was later found to be mediated by CTLA-4, which is expressed constitutively by T_{regs} (Takahashi et al., 2000). T_{reg} -bound CTLA-4 stimulation of mature or maturing DCs turns them into poor stimulators of T cell activation. Furthermore, CTLA-4 stimulation also plays a critical role in controlling inflammation in a mouse model of inflammatory bowel disease (Read et al., 2000). By producing the enzyme indoleamine 2,3-dioxygenase (IDO), CTLA-4-stimulated DCs can induce apoptosis in activated T cells by depriving them of tryptophan (Fallarino et al., 2006). IDO is the enzyme responsible for tryptophan catabolism and thus reduces the amount of tryptophan available to T cells in the immediate microenvironment resulting in the apoptosis of nearby activated Th cells (Lee et al., 2002a).

In addition to this, two unconventional yet equally effective mechanisms of T_{reg} -mediated control of inflammation have been proposed: Granzyme B-mediated cytotoxicity of effector T cells and T_{reg} -induced cytokine deprivation resulting in effector T cell apoptosis. T_{regs} obtained from granzyme-B deficient mice show a reduced capacity to suppress T effectors as a result of being incapable of inducing granzyme B-mediated cytotoxicity in the target T effector cells (Gondek et al., 2005). Building on this study, Cao et al. demonstrated in vivo, that T_{regs} induced by a tumor microenvironment (in a yet unknown mechanism) were incapable of suppressing the host immune response against the tumor cells if the T_{regs} were granzyme B- and perforin-deficient (Cao et al., 2007).

Death of effector T cells through cytokine deprivation is yet another suppressive mechanism available for T_{regs} . A study by Pandiyan and colleagues suggests that T_{regs} might literally “consume” more than their share of various cytokines thus depriving other Th subsets of vital cytokines required for survival and proliferation (Pandiyan et al.,

2007). Also, this mechanism seems to be functional only when the T_{regs} are in close proximity to the Th cells since separation of the two subsets with a Transwell™ apparatus abrogates T_{reg} suppression.

The dichotomous relationship between Th1 and Th2 cells seems to be recapitulated with Th17 and T_{regs} (Bettelli et al., 2006). Furthermore, a functionally antagonistic relationship also exists between the two. The decision of a naïve T cell to embark on either a T_{reg} or a Th17 polarization pathway appears to be the result of the sum of all the favorable stimuli weighed against all the opposing stimuli. In the presence of appropriate stimuli, Foxp3 and ROR γ t both have the capacity not only to antagonize each other's induction but also have the capability to reprogram an already differentiated T cell (Zhou et al., 2008). As previously stated, TGF- β in combination with IL-6 or IL-21 can drive Th17 polarization and both nT_{regs} and iT_{regs} produce TGF- β . Evidence demonstrates that IL-6 stimulation can not only inhibit the suppressive functions of a nT_{reg} but it can also reprogram the nT_{reg} into a Th17 cell capable of IL-17 production (Zheng et al., 2008). iT_{regs} do not seem to be affected in the same manner; moreover, they retain their suppressive capacity in the presence of IL-6, at least in vitro.

2.5 Maintenance of Tolerance and Prevention of Autoimmunity

Tolerance against self-antigens is a vital component of the healthy immune system. Several mechanisms seem to work in conjunction with each other to tolerize the immune system against self antigens and prevent autoimmunity. These mechanisms can be roughly classified into central and peripheral tolerance.

In the so-called “central” or “thymic” tolerance, developing T cells that react strongly to self antigens presented by thymic DCs are deleted or polarized into natural

T_{regs} (Hollander and Peterson, 2009; Jordan et al., 2001). In this first line of defense against autoimmunity, developing T cell progenitors interact with cortical thymic epithelial cells (CTEC) in the cortex of the thymus in a process that can, in the absence of further stimulus, result in apoptosis of the progenitor cell. T cell progenitors are prevented from undergoing apoptosis when they encounter and respond appropriately to co-stimulation and an MHC/self-antigen complex on DCs in the medulla of the thymus (Murphy et al., 1990; Punt et al., 1994). Only those that react with a moderate affinity to the stimulation are allowed to persist while those that react too strongly or not at all are “deleted” (Starr et al., 2003). However, the efficacy of this mechanism is limited by several factors. For instance, not all self antigens may find their way to the thymus. As a consequence, some T cells invariably escape the editing mechanism in the thymus. In addition to negatively selecting for T cells that react too strongly, the thymus also produces a phenotype of suppressive T cells called natural T_{regs} (nTregs) (Pacholczyk et al., 2002). These nTregs then migrate to the periphery where they exert their function. Curiously enough, the same mechanism that deletes self-reactive T cells also gives rise to nTregs (See below).

According to some studies, up to 25%-40% of developing T cells may escape negative selection and enter the periphery. Unchecked, these T cells have the potential to cause harm through autoimmune disorders. The job of suppressing the activity of these potentially harmful falls to nTregs or peripherally generated T_{regs} called inducible T_{regs} (iTregs). DCs play a vital role in maintaining tolerance by generating iTregs to suppress autoreactive T cells in the periphery (Banchereau et al., 2003; Coquerelle and Moser, 2008; Lutz and Schuler, 2002; Mahnke et al., 2002; Steinman et al., 2000; Wallet et al.,

2005). Moreover, DCs also terminate the T cell response at its conclusion. The activation of “co-inhibitory” CTLA-4 or PD-1 by DCs at the appropriate time can prevent wayward T cell responses and potential autoimmunity. CTLA-4 can be activated by either CD80 or CD86 whereas, PD-1 can be stimulated by PD-L1. During the course of a normal T cell response CTLA-4 and PD-1 help to inhibit T cells in part by downregulating the production of IL-2 and induction of cell cycle arrest that eventually leads to apoptosis (Fife and Bluestone, 2008; Guleria et al., 2007). However, in the absence of either CTLA-4 or PD-1, mice develop severe autoimmune/ lymphoproliferative disorders resulting in their death within a few weeks (Latchman et al., 2004; Nishimura et al., 2001; Vijayakrishnan et al., 2004).

It is hypothesized that in the “steady state” characterized by the absence of inflammation, iDCs with their high phagocytic rate and low expression of stimulatory molecules, tolerize T cells to self antigens whereas iDCs matured in response to non-self antigens and accompanying danger signals evoke an immune response (Lutz and Schuler, 2002; Steinman and Nussenzweig, 2002; Tan and O'Neill, 2005; Wallet et al., 2005).

Evidence for the role of APCs and particularly DCs in producing tolerance in the periphery was obtained relatively recently. Finkelman et al. demonstrated that APCs in addition to being immunogenic can also be tolerogenic (Finkelman et al., 1996). Mice injected with a rat monoclonal antibody (mAb) alone in the absence of other stimulatory signals induced antigen-specific T cell and B cell tolerance. This study, however, did not address the mechanism of tolerance induction or the specific type of APC responsible for it. Adler and colleagues reported that tolerizing of T cells through anergy to specific antigens requires that the antigen be processed and presented by steady state APCs to

antigen-specific T cells (Adler et al., 1998). By selectively targeting the antigen hen egg lysozyme (HEL) to a sub-population of APCs expressing the endocytic receptor DEC-205 Hawiger et al. were able to show that during steady state, DEC-205-expressing DCs captured the antigen and presented it to adoptively transferred HEL-specific T cells inducing tolerance by anergy and deletion. By contrast, the same antigen in the presence of co-stimulatory CD40L elicited a profound pro-inflammatory immune response in vivo (Hawiger et al., 2001).

iDCs along with other phagocytic cells play an essential role in clearing the body of apoptotic cells (a prime source of self antigens). Numerous studies show that DCs presenting antigens derived from apoptotic cells will induce T cell tolerization in the periphery (Basu et al., 2000; Chen et al., 2001a; Sauter et al., 2000; Steinman et al., 2000). By loading ovalbumin (OVA) into apoptotic cells and transferring them to syngeneic hosts, Liu et al. were able to induce experimental peripheral tolerance in mice. Mice thus tolerized were resistant to further immune stimulation by OVA even in complete Freund's adjuvant. The same transfer of apoptotic cells in the presence of CD40L, however, did not induce tolerance (Liu et al., 2002).

In addition to inducing tolerance directly by anergizing and deleting self responsive T cells, DCs can induce tolerance indirectly by inducing the differentiation of T_{regs} (Coquerelle and Moser, 2008; Steinman et al., 2003; Workman et al., 2009). Certain subsets of DCs present in antigen-rich environments such as the intestines, lungs and skin are especially proficient in generating iT_{regs} . For instance, DCs associated with the lamina propria of the small intestine turn naïve $CD4^+$ T cells into iT_{regs} through a retinoic acid-dependent mechanism (Manicassamy et al., 2010). Moreover, the immediate

microenvironment present while DCs are undergoing maturation also seems to play a role in their promotion of tolerogenic T cell responses. A well defined example of this is DCs matured in the presence of anti-inflammatory IL-10 (DC-10). DC-10s can induce oral allergenic tolerance in asthmatic mice by generating iT_{regs} (Huang et al., 2010).

CHAPTER 3

POTENTIAL SOLUBLE CANDIDATES

3.1 PGE2

In the studies here, astrocytes prevent the microglial assumption of a mature DC phenotype. Possible astrocyte-derived soluble molecules are proposed as possible mediators of this response. Among these are prostaglandins, IL-10 and TGF- β .

Prostaglandins are a class of small lipid molecules derived from arachidonic acid (AA) (Needleman et al., 1986). Prostaglandins have the capacity to modulate a wide variety of bodily functions including the immune system (Gualde and Harizi, 2004; Harizi and Gualde, 2002). The conversion of AA into prostaglandins begins at the cytosolic face of the cell membrane. AA cleaved by phospholipase A2 from membrane phospholipids is acted upon by prostaglandin H synthase (also called cyclooxygenase [COX]) to form the intermediate common precursor PGH2. PGH2 is in turn converted into the different prostanoids by the action of cell-specific synthases. COX is the rate-limiting enzyme in the synthesis of prostanoids (Needleman et al., 1986). There are two forms of COX. COX-1 is constitutively expressed and is responsible for basal synthesis of prostanoids within the body whereas the expression of COX-2 can be altered by a host of modulators. The expression of COX-2 and its metabolites have been demonstrated to play important roles during the course of inflammation (Boniface et al., 2009; Cheon et al., 2006; Lee et al., 2002b).

There are five different prostaglandins: PGE2, PGI2, PGD2, PGE2 α , and thromboxane (TXA2) (Needleman et al., 1986). Among these PGE2 is the most widely expressed prostanoid in the CNS under stressful conditions and is also the best

characterized (Johann et al., 2008).

The expression of PGE2 is tightly regulated by COX2. LPS, IL-1, and TNF- α can induce COX2 whereas anti-inflammatory IL-10 and IL-4 can downregulate the enzyme's expression. PGE2 has been reported to have a dual role in the immune system. Possessing both pro- and anti-inflammatory properties, PGE2 is capable of regulating DC activation, antigen presentation, migration and survival (Boniface et al., 2009; Kabashima et al., 2003; Slepko et al., 1997; Vassiliou et al., 2003). By downregulating DC-attracting/activating chemokines and cytokines and up-regulating anti-inflammatory agents, PGE2 can prevent DCs not only from migrating into an inflamed region but can also prevent and or quell the activation of DCs already in the vicinity. Conversely, by enhancing the maturation, survival and migration of certain sub-types of DCs like Langerhans cells, PGE2 can operate as a pro-inflammatory agent (Kabashima et al., 2003). For example, PGE2 can synergize with TNF- α or IL-1 β to efficiently promote the maturation of DCs. However, it can also inhibit the production of the chemokines CCL-3/4 and inflammatory cytokines such as TNF- α and IL-12 while up-regulating the expression of IL-10, an anti-inflammatory cytokine (Aloisi et al., 1999; Minghetti et al., 1998; Vassiliou et al., 2003). Moreover, PGE2 can also suppress the expression of MHC Class II in DCs (Harizi and Gualde, 2002).

The ability of astrocytes to produce PGE2 in large quantities in response to inflammatory mediators is well documented. In vitro, astrocytes treated with IL-6, TNF- α , IL-1 β or LPS upregulate the expression of PGE2 whereas treatment with IL-10 or IL-4 suppresses PGE2 production (Cheon et al., 2006; Chikuma et al., 2009; Johann et al., 2008).

3.2 Interleukin-10 (IL-10)

IL-10 was originally identified as a Th2-derived cytokine capable of inhibiting pro-inflammatory cytokine synthesis by APC-stimulated Th1 cells (Fiorentino et al., 1991). IL-10 is the “founding member” of a super family of cytokines based on their similar genomic organization, structure of receptors and signaling pathways used. The IL-10 super family includes IL-19, -20, -22, -24, -26, -28 and -29 (Conti et al., 2003). Appropriately stimulated immune and non-immune cell types including Th2, Th17, monocytes, macrophages, DCs, astrocytes, microglia, keratinocytes, epithelial cells and tumor cells can secrete IL-10 in significant quantities (Cheon et al., 2006; de Waal et al., 1991; O'Garra and Murphy, 2009; Williams et al., 1996).

Similar to other cytokines, IL-10 signaling uses the JAK/STAT pathway. The IL-10 receptor (IL-10R) is a heterodimer composed of IL-10R1 and IL-10R2 subunits. IL-10 ligation of its receptor activates the tyrosine kinases Jak1 and Tyk2, which induce the phosphorylation and activation of the downstream transcription factors STAT-1, -3 and -5 (Gonzalez et al., 2009; Moore et al., 2001; Mosser and Zhang, 2008).

IL-10 modulates Th1 activity indirectly by mediating the maturation and function of DCs IL-10 can regulate almost every aspect of DC maturation/function. It inhibits the expression of MHC Class II and the co-stimulatory molecules CD40, CD80 and CD86 on activated monocytes and isolated microglia and prevents their APC functions (Ding et al., 1993; Ding and Shevach, 1992; Fiorentino et al., 1991; Frei et al., 1994; Kim et al., 2002; O'Garra and Murphy, 2009; O'Keefe et al., 1999; Qin et al., 2006; Taylor et al., 2006; Taylor et al., 2007; Wei and Jonakait, 1999). IL-10 also has the ability to inhibit the cytokines a multitude of cytokines chemokines justifying its label as an anti-inflammatory

molecule (Bogdan et al., 1991; de Waal et al., 1991; Fiorentino et al., 1991; Harizi and Gualde, 2006; Heyen et al., 2000; Hu et al., 1999; Ledebøer et al., 2000; Wang et al., 2000). A low amount of endogenous IL-10 produced by immature monocyte-derived DCs is a potent inhibitor of DC “spontaneous” maturation (Corinti et al., 2001).

LPS treatment of isolated murine microglial cells in the presence of IL-10 leads to a reduction in the amount of CD40 expressed through the induction of the suppressor of cytokine signaling-3 (SOCS-3) gene mechanism (Qin et al., 2007). Similarly, IL-10 prevents IFN γ -induced CD40 and MHC Class II expression (O'Keefe et al., 1999; Wei and Jonakait, 1999). Furthermore, IL-10 inhibits microglial expression of both cytokines and cytokine receptors. Microglia stimulated with LPS and IL-10 show a significant decrease in the amount of TNF- α , IL-6 and IL-1 secreted in addition to reducing the expression of receptors for IL-2 and IL-6 (Ledebøer et al., 2000; Sawada et al., 1999). Importantly, mixed glial cultures express IL-10 in biologically relevant quantities (Ledebøer et al., 2002). In their hands mixed glial cultures treated with LPS show a robust time-dependent increase in the amount of IL-10 secreted, with both astrocytes and microglia contributing to this increase. Interestingly, IL-10 has very little direct effect on mature T cells since the IL-10R is downregulated in activated T cells (Fiorentino et al., 1991). However, stimulation of naïve T cells in the presence of IL-10 can result in anergy and death. It accomplishes this by interfering with the important secondary CD28-CD80/86 signaling between DCs and naïve T cells (Taylor et al., 2007). Consistent with its role in immune-suppression, IL-10 deficient mice exhibit exacerbated symptoms of EAE compared to wildtype mice (Cohen et al., 2010).

3.3 Transforming Growth Factor- β (TGF- β)

TGF- β is a pleiotropic cytokine with diverse functions, and is a potent immunoregulator. There are three members in the TGF family: TGF- β 1, -2 and -3(DeGroot et al., 1999). Upon ligating TGF- β , the TGF- β type I receptor dimerizes with the type II TGF- β receptor which in turn induces the phosphorylation of the type I receptor. The activation of the receptor leads to phosphorylation of the receptor-SMADs (R-SMAD) transcription factors. Activated R-SMADs then recruit the common-SMAD (co-SMAD) and translocate into the nucleus to initiate gene transcription (Attisano and Wrana, 2002).

TGF- β can affect T cell function directly by influencing their proliferation, differentiation and survival (Chen et al., 2001b; Dardalhon et al., 2008; Fahlen et al., 2005; Fontana et al., 1992; Ghoreschi et al., 2010; Gorelik and Flavell, 2002; Li and Flavell, 2008; Licona-Limon and Soldevila, 2007; Mangan et al., 2006; McGeachy et al., 2007; Qin et al., 2009; Veldhoen et al., 2006; Wan and Flavell, 2006; Xu et al., 2002; Zheng et al., 2008; Zhou et al., 2008). It can also effect T cells by influencing the maturation and function of DCs (Borkowski et al., 1997; Coquerelle and Moser, 2008; Dong et al., 2001; Laouar et al., 2008; Manicassamy et al., 2010; O'Keefe et al., 1999; Pazmany et al., 2000; Wei and Jonakait, 1999; Xiao et al., 2002; Xu et al., 2002; Yamazaki et al., 2007). TGF- β signaling-deficient DCs are incapable of tolerizing T cells which results in spontaneous EAE-like disease with poor prognosis (Laouar et al., 2008). However, in a few scenarios TGF- β can serve to promote the differentiation and maturation of DCs. Langerhans cells (LCs), a sub-type of DCs present in the epidermis, absolutely require TGF- β 1 for development as mice deficient in TGF- β 1 expression do not develop LCs (Borkowski et al., 1997; Caux et al., 1999).

TGF- β also prevents the maturation of isolated microglia when stimulated with LPS or inflammatory cytokines (Kim et al., 2004; Lodge and Sriram, 1996; Paglinawan et al., 2003; Suzumura et al., 1993). It inhibits the expression of co-stimulatory molecules particularly of CD40, CD80, CD86 and MHC II (Lodge and Sriram, 1996; Wei and Jonakait, 1999). It also prevents microglia from expressing pro-inflammatory agents such as IL-12, nitrous oxide (NO), RANTES and further induces the production of anti-inflammatory products (Hu et al., 1999; Paglinawan et al., 2003; Vincent et al., 1998). Importantly for these studies, TGF- β is expressed in vitro by astrocytes and microglia in mixed glial cultures and in the healthy CNS in vivo (Dhandapani et al., 2003; Hailer et al., 1998; Logan et al., 1992). Thus, it is a prime candidate to be the astrocytically-derived inhibitor of microglial activation.

3.4 Rationale for the Current Studies

This thesis investigates the nature of an astrocytic/microglial cross-talk that affects the ability of microglia to assume a DC-like phenotype. Furthermore, the ability of microglia cultured in the presence or absence of astrocytes to influence CD4⁺ T cell proliferation and Foxp3 T cell differentiation is also examined.

CHAPTER 4

MATERIALS AND METHODS

4.1 Mice

B10.A, and B6.129P-CX3CR1^{tm1Litt/J} mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the Rutgers-Newark AAALAC-approved animal care facility. In order to obtain neonates for glial cultures, males and females were placed together for 5-7 days. 16-21 days thereafter, 1-3 day old pups were taken for glial cultures (see below).

4.2 Reagents

Recombinant mouse GM-CSF, rmIL-10 and rhTGF- β 1 were purchased from Peprotech (Rocky Hill, NJ). Lipopolysaccharide (LPS; 055:B5) was from Sigma-Aldrich. Rabbit polyclonal antibodies against murine IL-10 and against all forms of TGF- β (pan-TGF β) were from R&D Systems (Minneapolis, MN). PE-conjugated Armenian hamster anti-mouse CD11c monoclonal antibody (clone N418) and IgG isotype control antibodies and the IL-17A, IFN- γ , IL-4 and IL-10 ELISA kits were from BioLegend (San Diego, CA). MatrigelTM, purified rat anti-mouse CD3, rat anti-mouse CD28, FITC-conjugated monoclonal rat anti-mouse CD40 (IgG_{2A}, κ), Armenian hamster anti-mouse CD80 (IgG₂, κ), rat anti-mouse CD86 (IgG_{2A}, κ), mouse anti-mouse MHC Class II antibodies (IgG_{2A}, κ) and the appropriate isotype control antibodies along with Mouse Fc BlockTM were obtained from BD Biosciences (Franklin Lakes, NJ). Rabbit polyclonal antibodies against CX3CR1 and FITC- and PE-labeled goat polyclonal anti-rabbit IgG were from Abcam.

TranswellTM inserts were from Corning (Lowell, MA). Anti-CD11c and anti-CD4 magnetic beads were from Miltenyi Biotec (Sunnyvale, CA). PE-conjugated rat anti-mouse/rat Foxp3 (IgG_{2A}, κ) and IgG isotype control antibodies were from eBioScience (San Diego, CA). ProstaglandinE2 is from Cayman Biologicals.

4.3 Generation of Mixed Glial Cultures

Mixed glial cultures were prepared as described previously from the cortices of neonatal (P1-P3) pups (Kreutzberg, 1995). Cortices were cleared of meninges, minced and triturated with a fire-polished Pasteur pipette and plated into poly-lysine-coated 75 cm² flasks in medium containing D-MEM/F12 (1:1), penicillin (25 U/ml), streptomycin (25 μg/ml), D-glucose (0.6%), and 10% heat-inactivated fetal bovine serum (BioAtlantic). Medium was replaced on day 3 and half the medium exchanged every 3 days thereafter. Isolated microglial cultures were generated by shaking microglia off of 12-14-day old mixed glial cultures on an orbital shaker (250 RPM X 15-20 min). Floating cells were collected and plated onto uncoated 75mm² flasks and allowed to adhere to the substrate before being treated with GM-CSF and LPS (see diagram below).

Unless stated otherwise, both mixed glial and isolated microglial cultures were treated with rmGM-CSF (25ng/ml) for 5 days and LPS (50ng/ml) for an additional 2 days to promote final maturation. A diagram of the treatment protocol on following page.

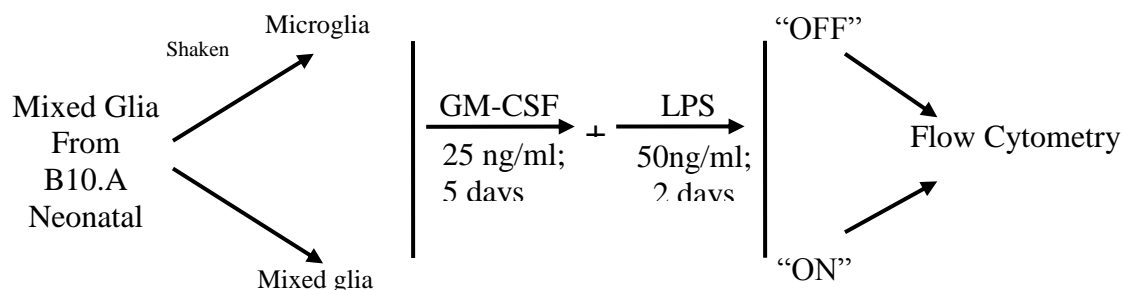


Figure 4.3 A diagram of the treatment protocol.

4.4 Generation and Purification of BM-DCs

Bone marrow (BM) cells were flushed with ice-cold RPMI medium from the femurs of 12-16 week old B10.A mice. BM cells were cultured in 100-mm Petri dishes containing 10 mL RPMI 1640 medium supplemented with 10% heat-inactivated FBS (BioAtlantic), 2 mM L-glutamine, penicillin (25 U/ml), streptomycin (25 μ g/ml), 50 μ M beta-mercaptoethanol (BME) and 15 ng/mL recombinant GM-CSF. After 3 days floating cells were harvested, pelleted and plated onto 75cm² flasks with or without enriched astrocytes (see below) in fresh RPMI medium containing GM-CSF (15 ng/ml). LPS (50 ng/ml) was added for 48 hrs on the 5th day of GM-CSF treatment. For experiments involving CD11c⁺ purified BM precursors, BM precursors were purified by CD11c immunomagnetic sorting using the AutoMACSTM system (Miltenyi Biotec). CD11c⁺ cells were plated into 75cm² flasks and treated with GM-CSF and LPS as described above. The purity of the sorted cells was determined by flow cytometric analysis (>97% for CD11c⁺ cells).

4.5 Generation of Enriched Astrocytic Cultures

Enriched astrocyte cultures were prepared from mixed glial cultures from which microglia had been shaken off. Following the removal of loosely adherent microglia, astrocyte cultures were treated for 3 days with cytosine arabinoside (AraC, 100 μ M).

They were passaged 2-3 times by light trypsinization (0.2% trypsin) followed by washing and replating into poly-lysine-coated flasks. Thus processed, they are >95% pure as defined by staining of microglial contaminants with antibodies against CD11b.

4.6 T cell Isolation and Culture

CD4⁺ T cells were isolated and purified by positive selection from spleens of B10.A mice (I-E^k) using the AutoMACS™ System. These were cultured in 48 well plates either alone or with microglia taken from the two culture settings in a microglia:T cell ratio of 1:10. Cells were plated in medium containing RPMI 1640, BME (10 μM), 10% FCS, penicillin (25 U/ml) and streptomycin (25 μg/ml) in the presence and absence of antibodies against CD3 (1.0 μg/ml, BD Biosciences). After 3 days, T cells were harvested and prepared for analysis by flow cytometry and determination of cytokines by ELISA. Mixed glia were stimulated with GM-CSF for 5 days followed by LPS for 2 days. Microglia were then shaken off and replated with CD4⁺ T cells or were reseeded with CD4⁺ T cells onto a bed of enriched astrocytes previously treated with GM-CSF and LPS.

For analysis of regulatory T cells, T cells were fixed and permeabilized with Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience) before being stained with PE-conjugated rat anti-mouse antibodies against Foxp3 (eBioscience, 0.5μg). Isotype control antibodies were handled similarly.

T cell proliferation was determined in a T cell activation assay using CD4⁺ B10.A T cells on B10.A glia that had been previously treated with GM-CSF and LPS as described above. In all cases, T cells were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, CA) prior to plating with microglia. CFSE dilution was assessed by flow cytometry.

4.7 Flow Cytometric Analysis

Microglial cells were shaken off of the mixed glial cultures or gently lifted off the isolated microglial cultures with a “cell lifter.” Cells were pelleted and re-suspended in phosphate buffered saline (PBS) containing 2% fetal bovine serum. Cells were then stained with PE-conjugated anti-CD11c and FITC-conjugated CD40, CD80, CD86 or MHC Class II or the appropriate IgG isotype control for 1 hour on ice. To minimize non-specific staining, cells were incubated with Fc Block™ (BD Bioscience) according to manufacturer’s instructions. After incubation cells were washed twice and resuspended in the same buffer before being assayed on a FACSCalibur™ flow cytometer (BD, Mountainview, CA). Data analysis was accomplished using CellQuest® software. Gating was done by using the appropriate isotype controls as reference. Non-specific staining for each experimental group was assessed through staining by isotype controls and was excluded from analysis by only counting events that were not a part of the isotype controls.

4.8 Real-time PCR

Total microglial RNA was prepared using Ultraspec™ RNA Isolation Reagent (Biotecx Laboratories, Inc., Houston, TX). cDNA was produced from 1µg of RNA by using random hexamer and MMLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instruction. The primers used for real-time PCR were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) and were designed using Primer Express (Applied Biosystem, Foster City, CA). They are listed in Table 4.1. For real-time PCR, cDNA was amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. PCR conditions

were 10 minutes at 95°C, followed by 35 cycles of 15 seconds at 95°C and 15 seconds at 60°C to amplify. After amplification, an additional cycle consisting of 95°C and 60°C (each for 15 seconds) was used for a dissociation curve to verify that the signal is generated from a single target amplicon and not from primer dimers or contaminating DNA. Serially diluted cDNA of each sample was amplified to measure the efficiencies of PCR and to draw the standard curve for each sample to calculate relative concentration of target message. The PCR products and their dissociation curves were detected using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA).

Table 4.1 Primers Used

Molecule	Forward Reverse	Sequence
CD40	F R	5'-CTG CCC AGT CGG CTT CTT CTC-3' 5'-CCT GTG TGA CAG GCT GAC AC-3'
CD80	F R	5'-CTG GGA AAA ACC CCC AGA AG-3' 5'-TGA GAA CGA TGA CGA CGA CTG-3'
CD86	F R	5'-TTA GGG AAG CAC CCA TGA TG-3' 5'-CGT CTC CAC GGA AAC AGC AT-3'
MHC CLASS II	F R	5'-CAA CAC TCT GGT CTG CTC AGT GA-3' 5'-TGT GTG GAT GAG ACC CCC A-3'

4.9 ELISAs

Supernatants were collected from T cells cultured in the presence of microglia from various culture settings. IL-17A was estimated using sandwich ELISA kits from Biolegend according to manufacturers' instructions. Both capture and biotinylated detection antibodies for measuring interferon-gamma (IFN- γ), interleukin-4 (IL-4) and IL-10 were from BDPharmingen. For IFN- γ , the capture antibody was purified monoclonal anti-mouse IFN- γ (3.5 μ g/ml); the biotinylated detection antibody was used at 1.0 μ g/ml. The detection limits of the assay were 7.8-4,000 pg/ml. For IL-4, the

capture antibody was rat anti-mouse (3 $\mu\text{g/ml}$); the detection antibody was used at 1.5 $\mu\text{g/ml}$. The detection limits of the assay were 7.8-2,000 pg/ml . For IL-10, the capture antibody was a monoclonal anti-mouse (3 $\mu\text{g/ml}$); the detection antibody was used at 1.5 $\mu\text{g/ml}$. The detection limits of the assay were 7.8-2,000 pg/ml . Optical density (OD) of standards and samples was measured using an ELISA plate reader (Packard Biosciences). Standard curves were generated for each assay and sample concentrations were obtained from the standard curve by comparing OD of samples to that of standards. Samples were assayed in triplicate and compared using an ANOVA with a post-hoc Student-Newman-Keuls test for significance at the 95% confidence level.

CHAPTER 5

RESULTS

Specific Aim 1. To determine the effect of astrocytes on the ability of microglia to express dendritic cell (DC) markers CD11c, MHC Class II and co-stimulatory molecules CD40, CD80 and CD86.

5.1 Astrocytes Modulate the Maturation of Microglial Cells into a Dendritic Cell-like Phenotype

The sequential treatment of isolated microglia with GM-CSF and LPS has been shown by us and others to induce a program of transcription that culminates in the generation of microglia with a DC-like phenotype (Aloisi et al., 2000; Ponomarev et al., 2007). However, most if not all studies to-date have dealt with microglia in isolation, and there is a dearth of research investigating microglial interactions with other cells commonly found throughout the CNS, especially astrocytes. To investigate the effect of astrocytes on the induction of a microglial DC-like phenotype, isolated microglia (labeled “OFF”) and mixed glial cultures (labeled “ON”) were sequentially treated with GM-CSF and LPS (see *Materials and Methods*) and the simultaneous expression of the DC marker CD11c and co-stimulatory molecules CD40, CD80, CD86 or MHC Class II assessed by flow cytometry.

Compared to microglia in the presence of astrocytes, a significantly higher percentage of isolated microglia expressed the DC marker CD11c ($73.4 \pm 4.2\%$ [OFF] vs. $41.9 \pm 6.5\%$ [ON]; $n=4$; $p=0.007$). Moreover, the percentage of isolated microglia

simultaneously expressing CD11c and CD40, CD80, or CD86 was also significantly higher than microglia cultured in the presence of astrocytes (Figures 5.1A and B). However, there was no significant difference in the percentage of microglia expressing CD11c and MHC Class II in the different environments (Figure 5.1B). While the percentages of cells expressing DC markers was strikingly different, the mean fluorescence index (MFI), a measure of the number of molecules per cell, shows that only CD40 differs significantly between the two culture conditions (Figure 5.1C).

The kinetics of CD11c and CD40 expression after the addition of LPS was examined to further distinguish between isolated microglia and microglia cultured on astrocytes. The percentage of co-cultured microglia expressing CD11c and CD40 increases in a time-dependent manner following the addition of LPS, while the percentage of isolated microglia expressing CD11c and CD40 remains unchanged (Figure 5.1D) suggesting that it had reached maximum levels with the treatment of GM-CSF alone.

To ensure that astrocytes were the only cells affecting the phenotype of the microglia, microglia from mixed glial cultures were purified through CD11b magnetic cell sorting and plated onto an enriched culture of astrocytes prepared as described above in *Materials and Methods*. Other microglia remained with a more heterogeneous population of mixed glia. Following treatment with GM-CSF and LPS, microglia from both settings were assessed for CD11c and CD40 expression. The percentage of CD11b⁺ enriched microglia that expressed CD11c and CD40 on enriched astrocytes is lower than microglia cultured in a more heterogeneous environment. These data, along with Figure 5.3 (see below), confirm that astrocytes, and not other constituents of the mixed glial

environment, are the primary inhibitors of microglia (Figure 5.1E). Figure 5.1 on page 56.

Figure 5.1

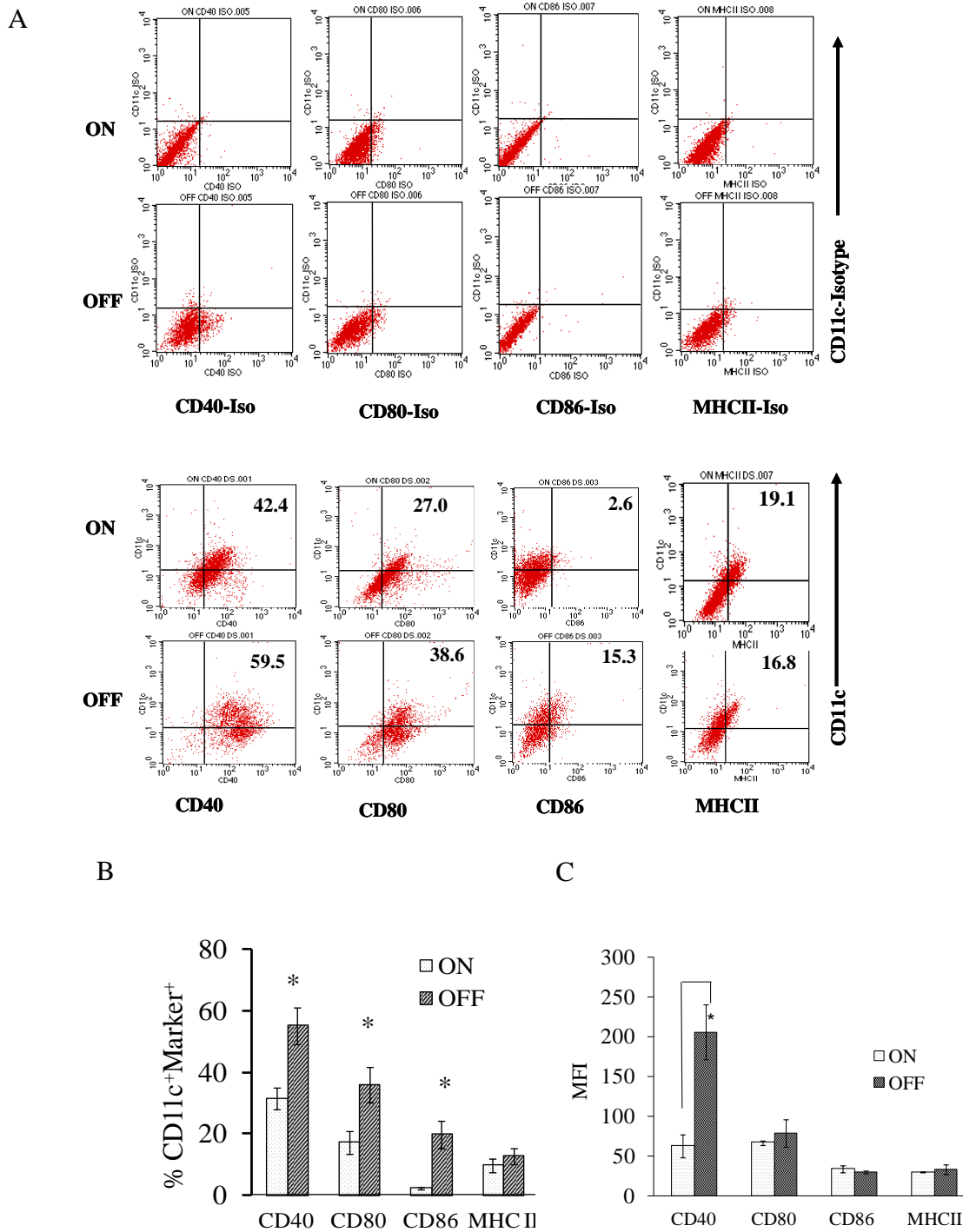
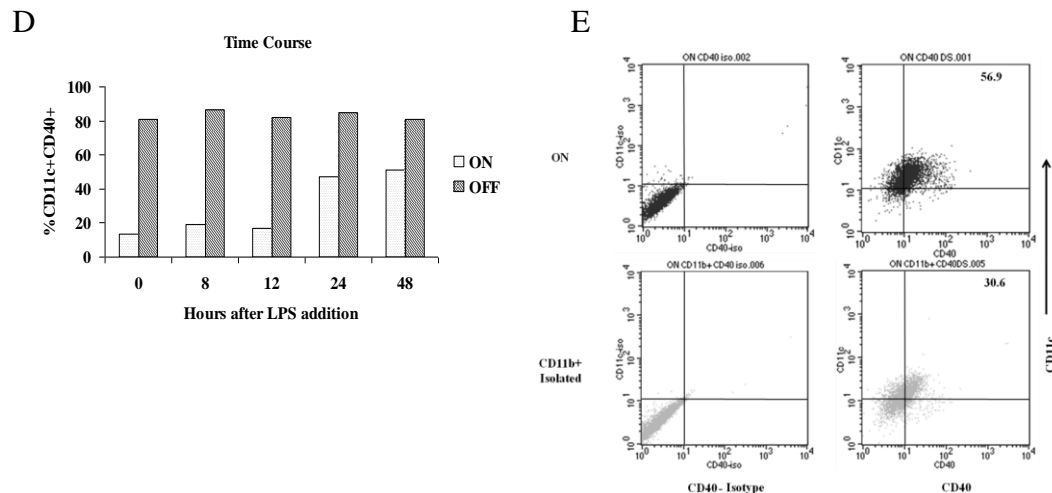


Figure 5.1 Astrocytes inhibit microglial production of a mature DC phenotype. Microglia were cultured with GM-CSF (25 ng/ml) in the presence (ON) or absence (OFF) of astrocytes for 5 days. LPS (50 ng/ml) was added for an additional two days. (Continued on page 57.)



Co-stimulatory molecule surface expression was determined by flow cytometry (A and B). Numbers in the upper right quadrants represent the percentage of CD11c⁺ cells simultaneously positive for CD40, CD80, CD86 and MHC Class II respectively.

(B) Microglial surface expression of co-stimulatory molecules in isolation is significantly higher than those on microglia cultured with astrocytes. Data are expressed as the percentage of cells expressing both CD11c and another co-stimulatory molecule \pm SEM over three individual experiments. Data from isolated and co-cultured microglia were compared by a Student's t-test. An asterisk indicates $p < 0.05$. (C) Only microglial CD40 Mean Fluorescence Intensity (MFI) differs in the presence (ON) and absence (OFF) of astrocytes. Data are expressed as the MFI \pm SEM and compared by a Student's t-test. The asterisk indicates $p < 0.05$. (D) Percentage of co-cultured (ON) microglia simultaneously expressing CD11c and CD40 increases over the course of 48 hours. Microglia were treated with GM-CSF (25 ng/ml) in the presence (ON) or absence (OFF) of astrocytes for 5 days. LPS (50 ng/ml) was added and cells were harvested at various time points after the addition of LPS and examined by flow cytometry for the expression of CD11c and CD40. The data are representative of two experiments performed. (E) Fewer CD11b⁺ enriched microglia cultured in the presence of astrocytes express CD11c and CD40 compared to microglia cultured in a more heterogeneous population. This experiment was performed once.

5.2 Microglia in the Presence of Astrocytes Express Higher Levels of Co-stimulatory Molecule mRNA

Because isolated microglia express higher surface levels of co-stimulatory molecules than their co-cultured counterparts, the temporal expression of these molecules following LPS treatment was assessed using RT-PCR (Figure 5.2A-D). In microglia co-cultured with astrocytes mRNA levels for CD40, CD80 and CD86 increased rapidly, peaking at 3 hrs and declining thereafter. By contrast, MHC Class II mRNA levels rose slowly but steadily reaching highest levels at the 12 hr time point. In the isolated microglia, CD40, CD80, CD86 and MHC Class II expression showed a similar temporal pattern. However, at all time points examined, steady-state mRNA levels in isolated microglia were significantly lower than those of co-cultured microglia. In the case of CD86 and MHC Class II, these levels were strikingly lower. Because mRNA levels rose rapidly following LPS treatment, it was clear that microglia -- even in the presence of astrocytes -- were responsive to LPS signaling.

Unexpectedly, the mRNAs for various “housekeeping” genes such as GAPDH, ARP and HTRP1 also showed significant differences between the two culture conditions (data not shown) making standardization to a housekeeping gene impossible. Figure 5.2 on page 59.

Figure 5.2

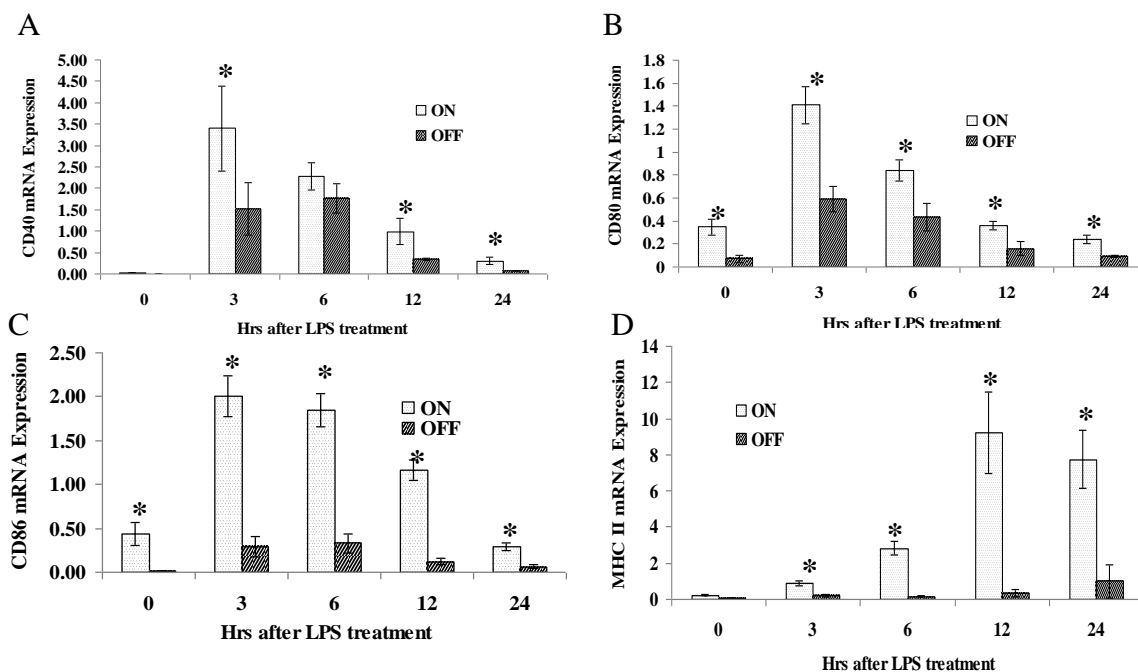


Figure 5.2 RT-PCR analysis of microglial expression of mRNA for MHC Class II and co-stimulatory molecules following a 24-hr LPS time course. Microglia in the presence of astrocytes (ON) or in their absence (OFF) were treated with GM-CSF (25ng/ml) for 5 days and LPS (50ng/ml). Microglia were harvested at various time points indicated after LPS addition and (A) CD40 (B) CD80 (C) CD86 (D) MHC class II mRNA expression assessed through real-time PCR. The data are expressed as arbitrary units \pm SEM from 3 separate experiments. Levels at each time point were compared by a Student's t-test. The asterisk indicates $p < 0.05$.

(Data are courtesy of Giselles Acevedo.)

5.3 Astrocytes Prevent the Efficient Maturation of Bone Marrow-derived DC (BM-DC) Precursor Cells into Fully Mature DCs.

BM-DCs are considered to be professional APCs of the immune system and are a vital component of the adaptive immune response. To investigate the effect of astrocytes on the maturation of BM-DC precursor cells, precursors were extracted and cultured in the presence of GM-CSF (15-20 ng/ml) for three days in isolation. After three days, floating cells were collected and either plated onto enriched astrocytic cultures or cultured alone and treated with GM-CSF for a further 5 days and LPS (50 ng/ml) for an additional 2 days to induce maturation. BM-DC precursors cultured alone expressed robust levels of co-stimulatory markers as measured by flow cytometry (Figure 5.3A and B). By contrast, BM-DCs in the presence of astrocytes expressed significantly lower levels of CD11c and CD40, CD80 or CD86. Similar to microglia, the percentage of BM-DCs simultaneously expressing CD11c and MHC Class II was the same in both conditions (Figure 5.3B).

Bone marrow cells are a heterogeneous mix of assorted precursor cells as well as cells at various stages of maturation. Thus, in order to ensure that the results observed were due solely to an interaction between BM-DCs and astrocytes alone CD11c⁺ cells were isolated through magnetic cell sorting prior to placement onto astrocytes. Before purification, 35% of the precursor cells were CD11c⁺; after purification, the percentage of CD11c⁺ cells rose to 97% (Figure 5.3C). Purified CD11c⁺ BM cells in the presence or absence of astrocytes were then treated as described above and the percentage of cells expressing CD11c and CD40 or CD80 was ascertained by flow cytometry. As observed earlier, a higher percentage of isolated BM-DCs were CD11c⁺ and CD40⁺ or CD80⁺ compared to BM-DCs in the presence astrocytes (35.4% vs. 62.2% and 19.8% vs. 54.5%) respectively (Figure 5.3D) Figure 5.3 on page 61.

Figure 5.3

A

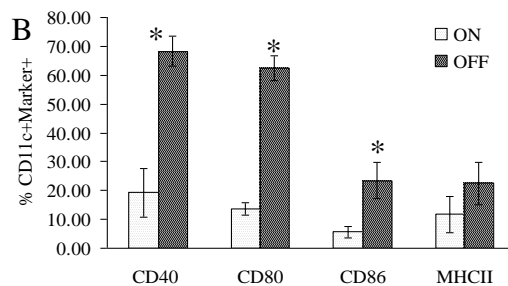
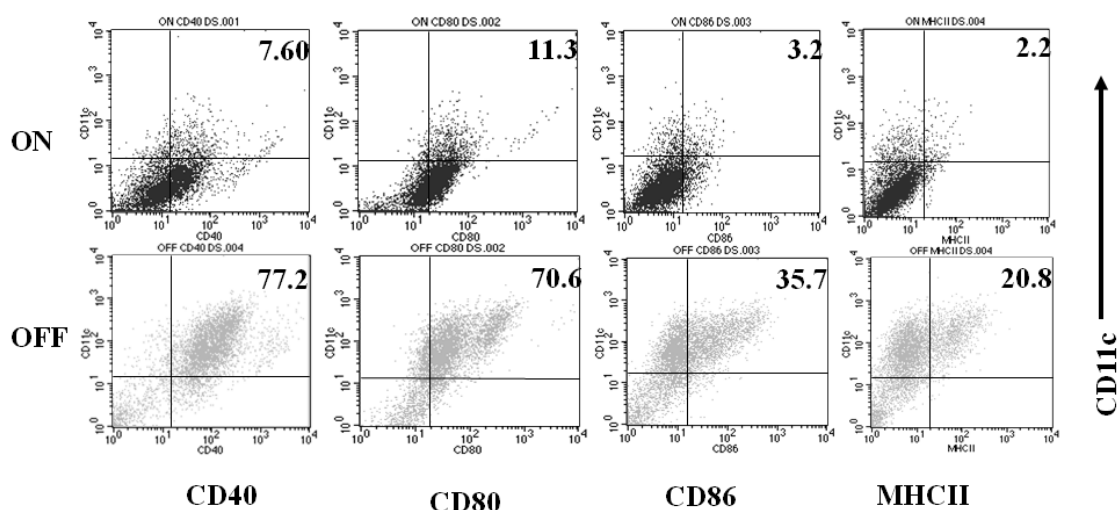
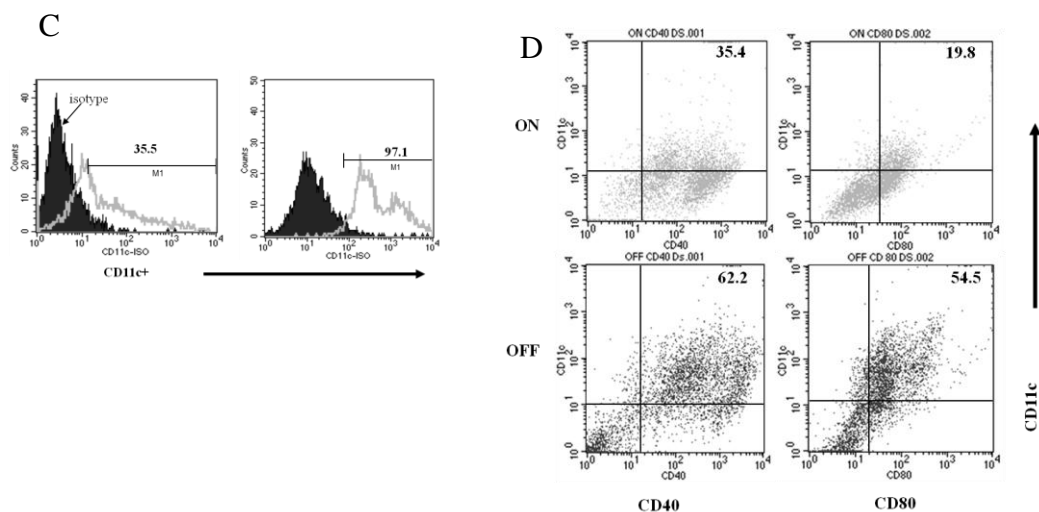


Figure 5.3 Astrocytes inhibit the expression of a mature DC phenotype in bone marrow-derived cells. BM precursors were obtained from the femurs of B10.A mice and cultured for 3 days in the presence of GM-CSF (25 ng/ml). Floating cells were collected and treated with GM-CSF (25 ng/ml) for 5 days and LPS (50 ng/ml) for an additional 2 days in the presence (ON) or absence (OFF) of astrocytes. (A) CD11c and co-stimulatory molecule surface expression was determined by flow cytometry. Numbers in the upper right quadrants represent the percentage of CD11c⁺ cells simultaneously positive for CD40, CD80, CD86 and MHC Class II respectively. (B) BM-DC surface expression of co-stimulatory molecules in isolation is significantly higher than those on BM-DCs cultured with astrocytes. Data are expressed as the percentage of cells expressing both CD11c and another co-stimulatory molecule [±] SEM over 3 individual experiments. Data from isolated and co-cultured BM-DCs were compared by a Student's t-test. An asterisk indicates $p < 0.05$. A higher percentage of purified CD11c⁺ cells cultured in the presence of astrocytes show surface expression of CD40 and CD80. (Continued on page 62.)



(C) Selection of CD11c⁺ cells by magnetic cell sorting from bone marrow cells yielded an almost pure population of cells. (D) Purified CD11c⁺ BM precursors were treated with GM-CSF (25 ng/ml) for 5 days and LPS (50 ng/ml) for two more days in the presence (ON) or absence (OFF) of astrocytes. Cells were then analyzed for CD11c and CD40 or CD80. Numbers in the upper right corner represent the percentage of cells expressing CD11c and CD40 or CD80. Fewer CD11c⁺ enriched BM precursors cultured in the presence or absence of astrocytes express CD11c and CD40 or CD11c and CD80 compared to BM precursors cultured alone. This last experiment was performed once.

5.4 Astrocytes Preserve the Expression of the Fractalkine Receptor (CX3CR1) on Microglia

Fractalkine is a 373 amino-acid chemokine that is widely expressed in the CNS (Bazan et al., 1997; Chinnery et al., 2007; Rossi et al., 1998). It is constitutively expressed by neurons whereas astrocytes can be induced to express it in the presence of pro-inflammatory stimuli (Harrison et al., 1998; Hatori et al., 2002). It can exist as a membrane bound molecule or -- when proteolytically cleaved -- as a soluble 90 kD isoform (Hatori et al., 2002). CX3CR1, the receptor for fractalkine, is expressed throughout the body by various cell types; among them are monocytes, DCs and T cells (Jung et al., 2000). Microglia are the only cell type in the CNS to express the fractalkine receptor (Hatori et al., 2002), and as a result, microglia respond to fractalkine by converging towards its source (Tarozzo et al., 2002). In addition to its chemotactic properties, fractalkine, by inhibiting microglial activation, can promote neuronal survival. In vitro, microglia activated with LPS in the presence of fractalkine down-regulate the expression of pro-inflammatory mediators detrimental to neuronal survival (Mizuno et al., 2003; Zujovic et al., 2000). While LPS-treated isolated monocytes and microglia down-regulate the expression of CX3CR1 (Boddeke et al., 1999; Ramos et al., 2010) little else is known about the regulation of the receptor on microglia.

To determine the effect of astrocytes on microglial expression of CX3CR1, mixed glial cultures were obtained from neonatal B6.129P-Cx3cr1^{tm1Litt}/J mice. In these transgenic mice, GFP has been placed under the control of the CX3CR1 promoter and is expressed instead of the fractalkine receptor in all cells derived from a monocytic lineage (Jung et al., 2000). This allows not only the identification of microglia but also allows measurement of their expression of CX3CR1^{GFP/GFP}. After treatment with GM-CSF and

LPS a significantly higher percentage of microglia in the presence of astrocytes retain their expression of CX3CR1^{GFP/GFP} compared to microglia cultured alone (Figure 5.4A) a fact confirmed by immunohistochemistry (Figure 5.4B). The mere withdrawal of microglia from an astrocytic environment is sufficient to induce the down-regulation of CX3CR1. A high percentage (~56%) of microglia 48 hours after isolation from astrocytes and in the absence of any exogenously added ligands do not express GFP whereas more than 95% of freshly isolated microglia are GFP positive (Figure 5.4C). Taken together these data show the importance of an astrocytic environment for the expression of microglial CX3CR1.

Because of the dramatic decrease in GFP expression, the experiment was repeated on wild-type C57Bl/6J animals using a antibody against CX3CR1. After 48 hrs, 53% (n=2) of isolated microglia retained cell surface expression of CX3CR1 (Not shown).
Figure 5.4 on page 65.

Figure 5.4

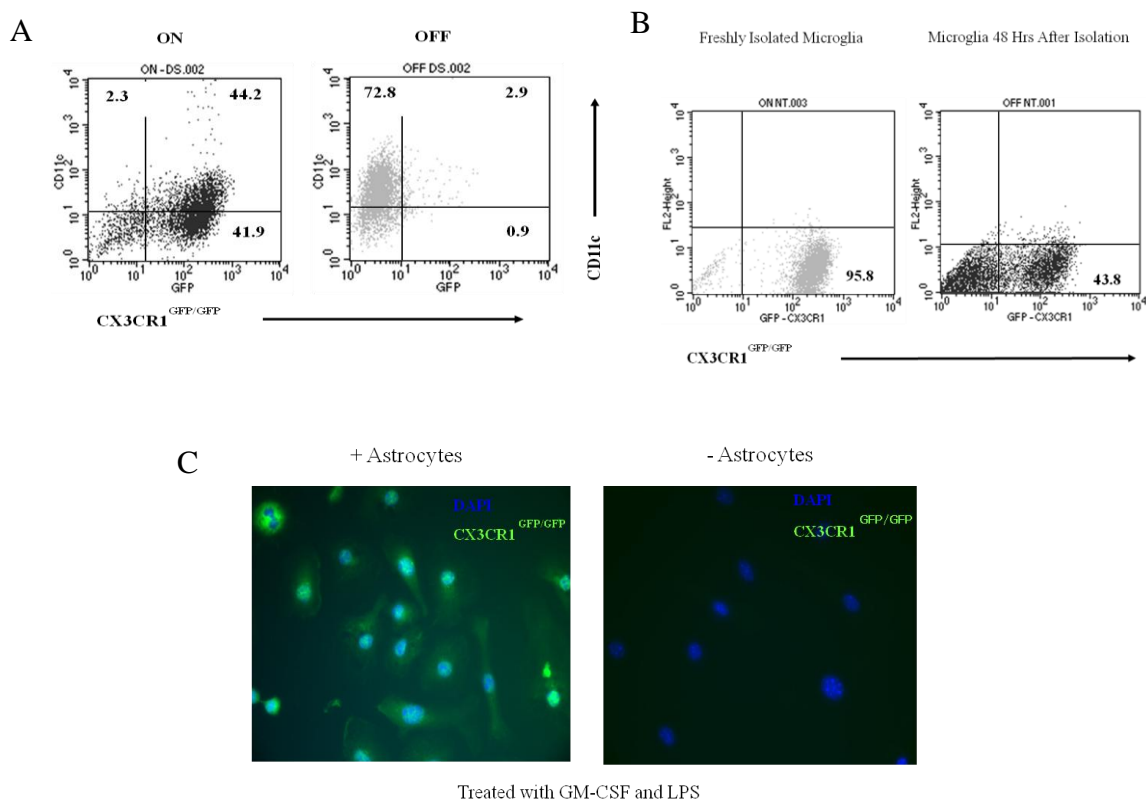


Figure 5.4 Astrocytes preserve microglial $CX3CR1^{GFP/GFP}$ expression. Mixed glial cultures were obtained from neonatal B6.129P- $CX3CR1^{tm1Lit/J}$ transgenic mice. $CX3CR1^{GFP/GFP}$ microglia were cultured with GM-CSF (25 ng/ml) in the presence (ON) or absence (OFF) of astrocytes for 5 days. LPS (50 ng/ml) was added for an additional two days. CD11c and GFP expression was determined by flow cytometry. (A) A higher percentage of microglia in the presence of astrocytes are $CX3CR1^{GFP/GFP}$ positive than microglia cultured in isolation. The figure is representative of data replicated 4 times. (B) Isolation of microglia from astrocytes leads to a decrease in $CX3CR1^{GFP/GFP}$ expression. Microglia were cultured in isolation for 48 hours in the absence of any exogenously added ligands. $CX3CR1^{GFP/GFP}$ expression was examined by flow cytometry. Numbers in the quadrants indicate the percentage of microglia expressing GFP. This experiment was performed once. (C) Removal of microglia from an astrocytic environment leads to a loss of microglial GFP expression. “ON” and “OFF” microglia were examined by fluorescence microscopy for GFP expression. Microglia were cultured in the presence or absence of astrocytes and treated with GM-CSF (25ng/ml, 5 days) and LPS (50ng/ml, 2 days). Microglia with astrocytes (+Astrocytes) or in isolation (- Astrocytes) were fixed, stained with DAPI and examined by fluorescence microscopy for GFP and DAPI.

Specific Aim 1. To determine whether differences in surface expression of these DC-specific molecules was the result of soluble or contact-mediated events.

Conclusions from Specific Aim 1:

In the presence of astrocytes:

- a significantly smaller percentage of microglia express CD11c together with co-stimulatory molecules CD40, CD80 and CD86, molecules necessary for T cell activation;
- microglial mRNA for CD40, CD80 and CD86 and MHC Class II is significantly higher than in isolated microglia;
- a significantly smaller percentage of bone marrow-derived DCs express CD11c together with co-stimulatory molecules CD40, CD80 and CD86;
-
- neither microglial nor BMDC MHC Class II surface expression differs from that seen in isolated cells;
-
- microglia maintain expression of CX3CR1^{GFP/GFP}.

Specific Aim 2: To determine whether differences in surface expression of these DC-specific molecules is the result of soluble or contact-mediated events.

5.5 Neither PGE2, IL-10 nor TGF- β Mediate Microglial Suppression

Several anti-inflammatory cytokines -- PGE2, IL-10 or TGF- β -- produced by astrocytes were examined as possible mediators of microglial suppression. Data from the Jonakait lab and from others (Levi et al., 1998) suggest that the addition of PGE2 to isolated microglial inhibits the expression of CD40 (Figure 5.5A and B). In addition, the data here and that of others (Kim et al., 2002; Qin et al., 2006) suggested that IL-10 is significantly elevated in the mixed glial cultures (Figure 5.5D). Others have pointed to TGF β as a highly anti-inflammatory molecule (Paglinawan et al., 2003; Wei and Jonakait, 1999). These three molecules were, therefore, selected as likely soluble mediators of the microglial suppression. To test the effect of these factors on isolated or mixed glial cultures, they were either exogenously supplied or their endogenous activity neutralized through specific antibodies.

In order to determine whether PGE2 qualified as a glial-derived factor responsible for suppressing co-stimulatory molecule expression, it was added to isolated microglial and mixed glial cultures in a concentration (10 μ M) shown to suppress CD40 expression in isolated cultures (Figure 5.5B). With the exception of MHC Class II, the percentage of isolated microglia expressing CD11c and the other co-stimulatory molecules was suppressed by PGE2 (Figure 5.5C). By contrast, addition of PGE2 to mixed glial cultures increased the percentage of microglia expressing CD11c and CD40, CD80 or MHC Class

II. CD86 remained unchanged (Figure 5.5C). These data suggest that PGE2 is not the mediator of microglial suppression in the presence of mixed glia.

The addition of IL-10 to isolated microglia did not lower the percentage of cells expressing CD11c and CD40 (Figure 5.5E). There was no significant difference in the percentage of CD11c⁺CD40⁺ cells even with increasing concentrations of IL-10 (assessed by an ANOVA; $p=.701$). Moreover, a neutralizing antibody against IL-10 that was used to counteract the high levels of endogenous IL-10 in the mixed glial cultures did not reverse the effect of astrocytes (Figure 5.5F). There was no significant difference in the percentage of CD11c⁺CD40⁺ cells even with increasing concentrations of the antibody (assessed by an ANOVA; $p=.786$). If IL-10 were the mediator, this antibody would be expected to raise the percentage of cells expressing CD11c and CD40 particularly in the mixed glial environment. An IgG control for the antibody had no effect on the percentage of CD11c⁺CD40⁺ microglia in either culture condition (Figure 5.5G). IL-10 failed to meet the requirement for an appropriate mediator.

Next, the effect of TGF- β was examined on both “ON” and “OFF” microglia. rhTGF- β 1 added to isolated microglia increased the percentage of microglia co-expressing CD11c and CD40 while significantly reducing that expression in a mixed glial environment (Figure 5.5H). Moreover, TGF- β 1 exerted its effect on mixed glia even at the relatively low concentration of 10 ng/ml. In order to determine whether endogenous TGF- β was responsible for the observed effect of astrocytes on inhibiting CD40, endogenous TGF- β was neutralized using a pan-anti-TGF- β antibody. This particular antibody efficiently neutralizes most of the common isoforms of TGF- β (R&D Systems). The addition of the pan-anti-TGF- β antibody to the mixed glial cultures did not enhance

the percentage of CD11c⁺CD40⁺ microglia in the mixed glial condition (Figure 5.5I). The IgG control for the antibody had no effect on the percentage of CD11c⁺CD40⁺ microglia in either culture condition (Figure 5.5J). Had TGF- β been the mediator of suppression, the addition of the antibody would have been expected to reverse the suppression. Because this did not happen, TGF- β was eliminated as a possible mediator.

Taken together, these data show that neither PGE2, IL-10 nor TGF- β is the astrocytic mediator responsible for suppressing microglial activation in a mixed glial environment. Figure 5.5 on page 70.

Figure 5.5

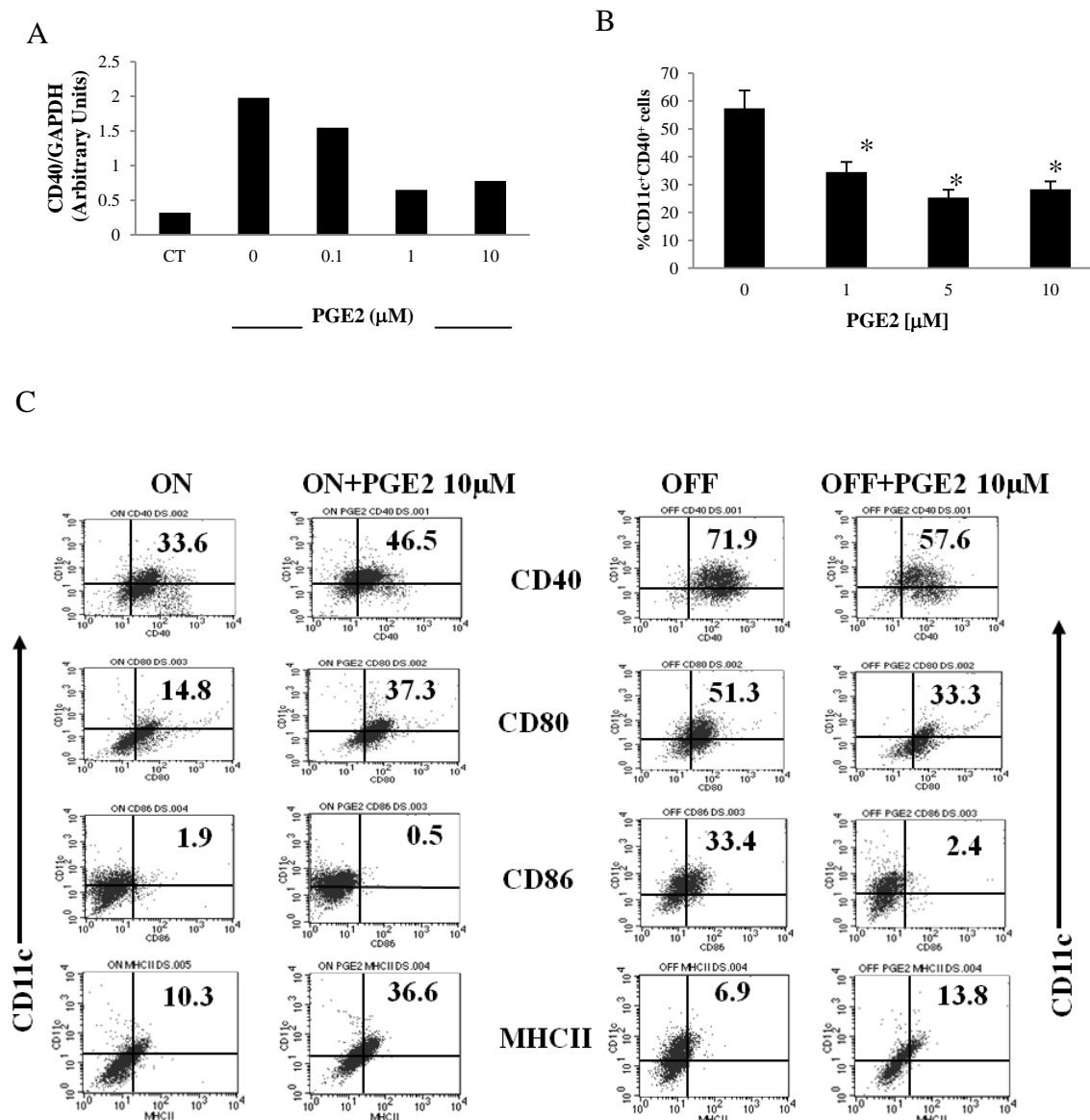
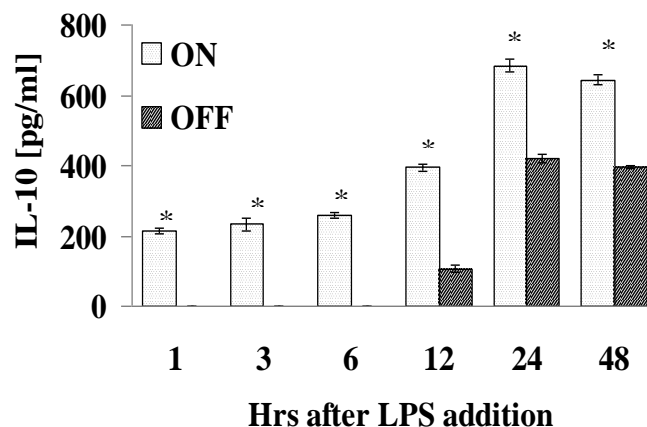


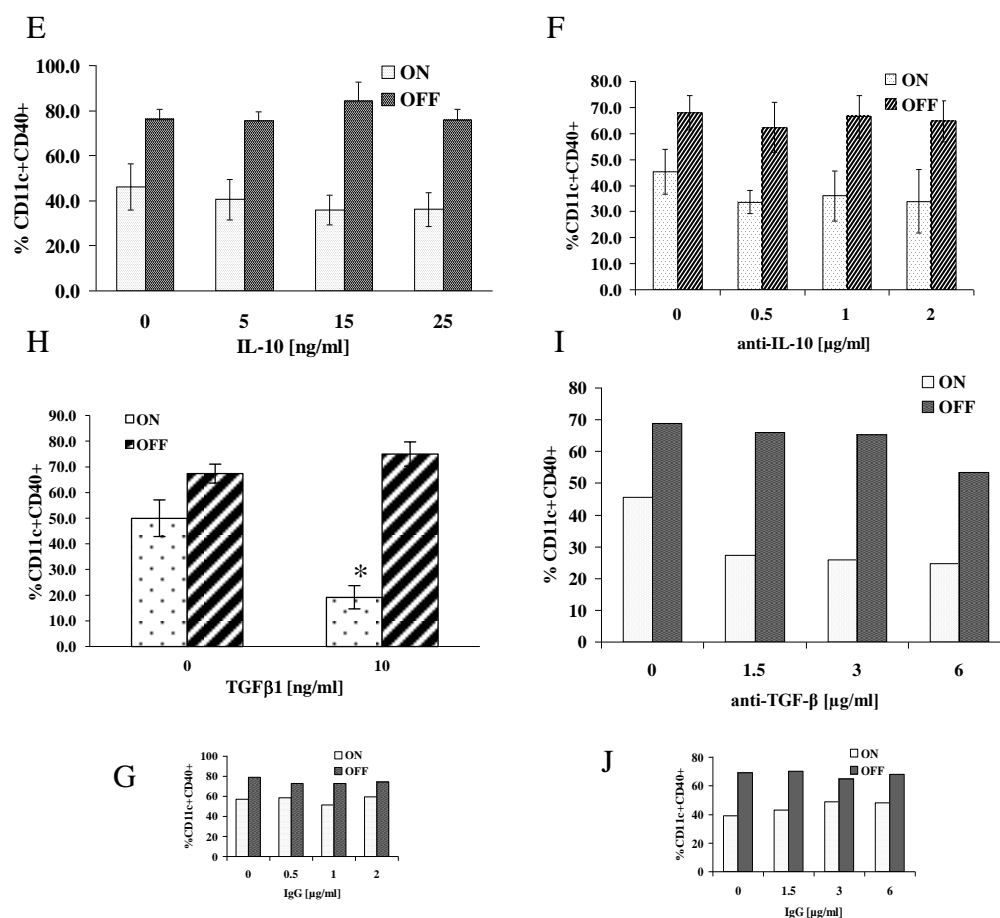
Figure 5.5 PGE2 treatment downregulates CD40 mRNA expression in isolated microglia. (A) Isolated microglia were treated with LPS and increasing doses of PGE2 and the expression of CD40 mRNA determined by RT-PCR. (B) The percentage of isolated microglia expressing CD40 and CD11c is significantly reduced in the presence of PGE2. Isolated microglia were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). PGE2 was added in increasing doses before and 24 hrs after the addition of LPS. Microglia were collected and the expression of CD11c and CD40 determined by flow cytometry. Data are expressed as %CD11c⁺CD40⁺ cells \pm SEM (n=3 or more). An asterisk represents a difference with PGE2 untreated control (*p<.05). (C) PGE2 is not the mediator of astrocytic action. ON and OFF microglia were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). PGE2 (10μM) was added before and 24 hrs after the addition of LPS. Microglia were collected and the expression of CD11c and CD40 or CD80 or CD86 or MHC II determined by flow cytometry.

(Continued on page 71.)

D



(D) Mixed glial cultures produce higher amounts of IL-10. Mixed glial and isolated microglial cultures were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days) and supernatants collected at the indicated times after the addition of LPS. The concentrations of IL-10 were determined by an ELISA. IL-10 is not the mediator of astrocytic action. ON and OFF microglia were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). Data are expressed as pg/ml \pm SEM from triplicate samples. Asterisk indicates a significant difference of "ON" vs. "OFF" at each time point ($p < 0.05$). One of two experiments is shown.
(Continued on page 72.)



(E) IL-10 was added at varying doses before and 24 hrs after the addition of LPS to isolated and mixed glial cultures. CD11c and CD40 were determined by flow cytometry. An ANOVA showed the addition of IL-10 did not significantly alter the percentage of cells expressing CD11c and CD40 in isolated microglia ($p=0.701$) (F) Neutralizing antibodies for IL-10 were added at varying doses before and 24 hrs after the addition of LPS to isolated and mixed glial cultures. CD11c and CD40 were determined by flow cytometry. An ANOVA showed that the addition of the antibodies did not significantly alter the percentage of cells expressing CD11c and CD40 in co-cultured microglia ($p=0.786$). (G) A control IgG control had no effect. (H) Microglia in both culture settings were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). TGF- β was added at 10 ng/ml before and 24 hrs after the addition of LPS to isolated and mixed glial cultures. Microglia were collected and the expression of CD11c and CD40 determined by flow cytometry. Data are expressed as percentage of CD11c⁺CD40⁺ cells \pm SEM. An asterisk represents a difference from TGF- β -untreated co-cultured (ON) microglia ($p=0.02$, $n=3$). (I) Microglia in both culture settings were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). Pan-antibodies against all forms of TGF β were added at varying doses before and 24 hrs after the addition of LPS to isolated and mixed glial cultures. Microglia were collected and the expression of CD11c and CD40 determined by flow cytometry. This experiment was performed once. (J) A control IgG control had no effect.

5.6 Repression of Microglial Activation and Maintenance of Microglial CX3CR1 Requires Continued Contact with an Astrocytic Environment.

In order to determine whether soluble astrocytic molecule(s) were responsible for the repression of microglial activation and maintenance of CX3CR1, microglia were cultured over astrocytes in a Transwell™ chamber. This design allows the two cell types to share any soluble factors while preventing the two cell types from coming into contact. Mixed glial cultures were plated onto 6 well plates and isolated microglia onto Transwell™ inserts (0.4 μm pore size) before being treated with GM-CSF and LPS. Microglia thus separated from astrocytes but in contact with soluble factors coming from them failed to mimic the repressed DC-like phenotype typical of “ON” microglia. Instead, “transwell” microglia closely resembled their isolated counterparts (Figure 5.6A).

Similarly, microglial maintenance of CX3CR1 also requires physical contact with astrocytes since microglia in the Transwell™ inserts lose their CX3CR1^{GFP/GFP} expression after being treated with GM-CSF and LPS (Figure 5.6B). To further clarify the mode of astrocytic action, CX3CR1^{GFP/GFP} microglia were cultured in isolation, in the presence of GM-CSF. After 5 days, microglia were trypsinized and plated onto a confluent bed of enriched wild-type astrocytes and cultured for a further 2 days before being assessed for CX3CR1^{GFP/GFP} expression. Thus treated, ~37% of microglia recover their expression of CX3CR1^{GFP/GFP} when compared to microglia grown in isolation (Figure 5.6C). Taken together these data effectively eliminate most soluble factors as the astrocyte-derived mediators of microglial activity and suggest that contact with the astrocytic environment is essential. Figure 5.6 on page 74.

Figure 5.6

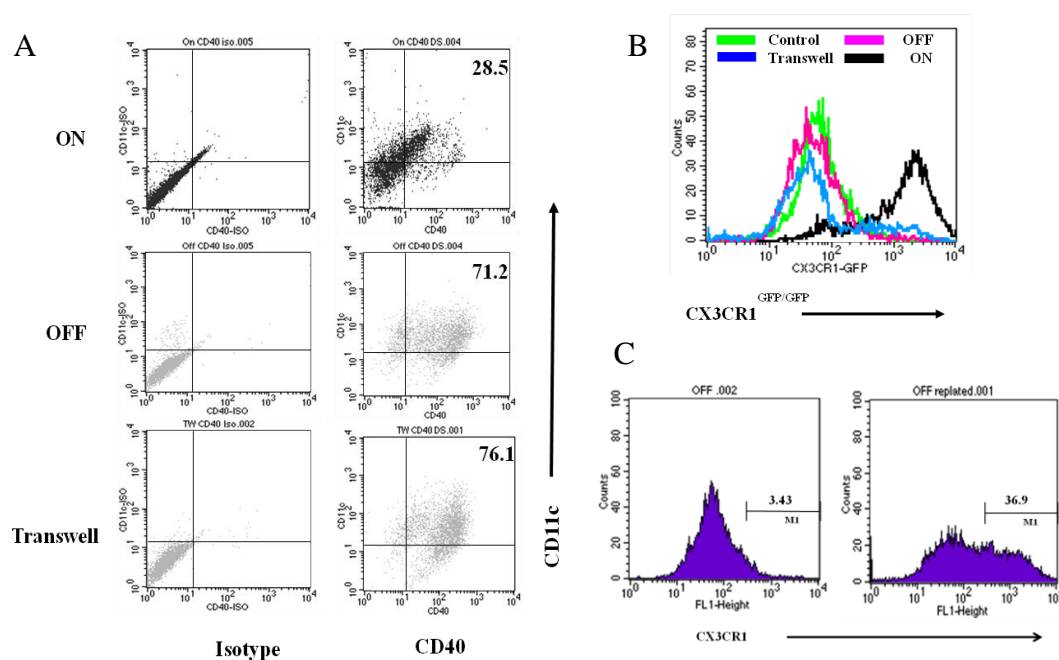


Figure 5.6 Contact with the astrocytic environment is necessary for astrocytes to modulate microglial DC maturation. (A) B10.A microglia in the presence of astrocytes (ON) or absence of astrocyte (OFF) and microglia separated from mixed glial cultures with a Transwell™ apparatus (Transwell) were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). Surface expression of CD11c and CD40 were assayed by flow cytometry. Representative data from one of two experiments are shown. (B) Cell contact with the astrocytic environment is required for CX3CR1^{GFP/GFP} microglia to retain GFP expression. CX3CR1^{GFP/GFP} microglia cultured with astrocytes (ON, black), without astrocytes (OFF, pink) or separated from mixed glial cultures with a Transwell™ apparatus (Transwell, blue) were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days) and assessed for GFP expression by flow cytometry. Control (green line) represents wild-type cells used to compensate for auto-fluorescence. (C) CX3CR1^{GFP/GFP} microglia recover their GFP expression after being replated onto astrocytes. CX3CR1^{GFP/GFP} microglia were isolated from astrocytes and cultured with GM-CSF (25ng/ml) for 5 days. Microglia were then harvested by gentle trypsinization and plated onto a bed of wild-type enriched astrocytes, cultured for an additional 2 days and GFP expression measured by flow cytometry. Numbers above the histogram represent the percentage of GFP-expressing microglia. This experiment was performed once.

5.7 Astrocytic Extracellular Matrix Molecules may Play a Role in Suppressing Microglial Expression of Co-stimulatory Molecules.

Astrocytes express a wide variety of molecules which are involved in forming the extracellular matrix (ECM). To further clarify the nature of the astrocytic membrane-bound molecule, microglia were cultured in the presence of MatrigelTM, a heterogeneous mixture of various extracellular matrix proteins, primarily laminin and collagen IV (BD Biosciences). It can be induced to form a 3D gel on plastic and has been shown by various studies to significantly affect cell behavior (Kleinman and Martin, 2005). To examine the effect of MatrigelTM on microglial activation, isolated microglia were treated with GM-CSF and LPS in flasks coated with MatrigelTM. The expression of CD11c and CD40 was assessed after treatment with GM-CSF and LPS. Compared to microglia cultured in isolation, there is only a minor difference in the percentage of microglia expressing CD11c and CD40 in the presence of MatrigelTM (Figure 5.7A). However, when compared to isolated microglia the amount of surface CD40 (as assessed by MFI) expressed per cell is lower in microglia cultured in the presence of MatrigelTM (Figure 5.7A).

Microglia derived from CX3CR1^{GFP/GFP} mixed glial cultures were similarly treated in the presence of MatrigelTM. Isolated CX3CR1^{GFP/GFP} microglia retain their expression of GFP to a large extent when cultured on MatrigelTM (Figure 5.7B). These data suggest that in addition to astrocytic membrane-bound molecules, the ECM secreted by astrocytes may also play a role in mediating the suppression of microglial activation.

Figure 5.7 on page 76.

Figure 5.7

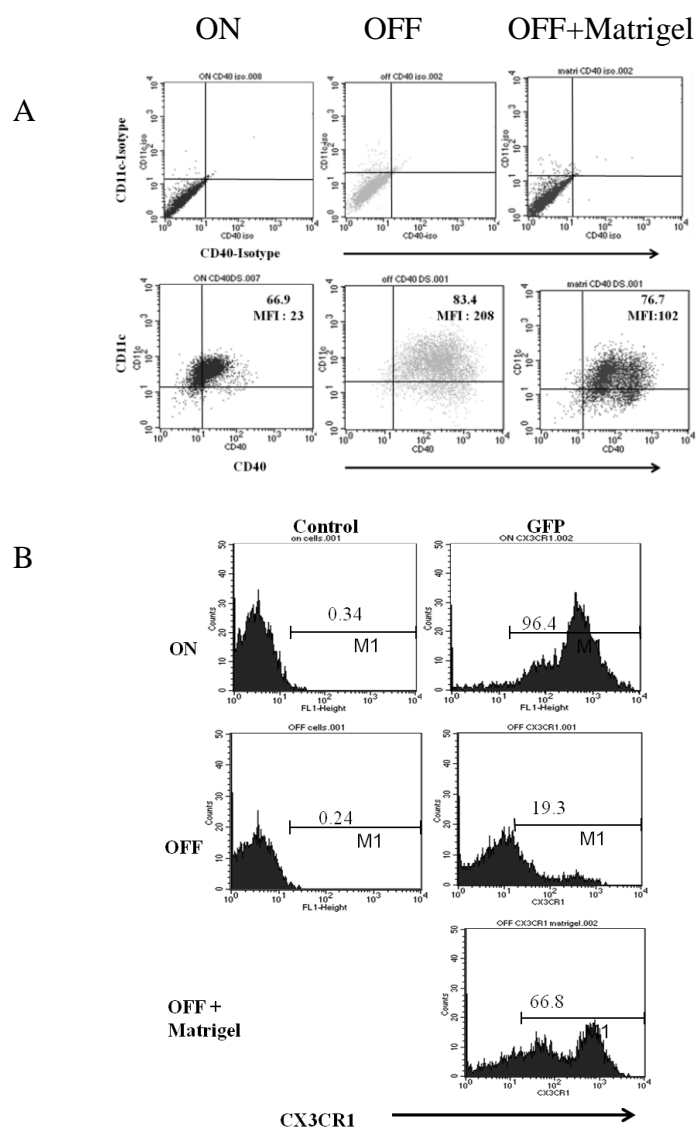


Figure 5.7 MatrigelTM partially mimics the effect of astrocytes on microglia. (A) B10.A microglia in the presence of astrocytes (ON), isolated from astrocytes (OFF) and in the presence of MatrigelTM (OFF+Matrigel) were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days) and assessed for the expression of CD11c and CD40 by flow cytometry. Numbers in the quadrants represent the percentage of CD11c⁺ and CD40⁺ microglia. MFI is mean fluorescence intensity. This experiment was performed once. (B) A higher percentage of isolated CX3CR1^{GFP/GFP} microglia retain GFP expression in the presence of MatrigelTM. Microglia derived from CX3CR1^{GFP/GFP} mice were treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days) in the presence (ON) or absence of astrocytes (OFF) or on MatrigelTM (OFF+Matrigel). Microglial GFP was assessed at the end of the treatment by flow cytometry. Numbers above the histogram indicate the percentage of GFP⁺ microglia.

Specific Aim 2. To determine whether differences in surface expression of these DC-specific molecules is the result of soluble or contact-mediated events.

Conclusions from Specific Aim 2:

- Neither PGE₂, IL-10 nor TGF β is responsible for the astrocytic effect on microglial DC phenotype.
- Contact with the astrocytic environment is required for astrocytic inhibition of the microglial DC phenotype and CX3CR1 retention.

Specific Aim 3: To examine the effects of differences in surface expression of these DC-specific molecules on T cell proliferation, Foxp3 expression and cytokine production.

5.8 A Mixed Glial Environment is More Conducive for T cell Activation and Proliferation

The data so far reveal that microglia and BM-DCs in the presence of astrocytes are not as efficient as either cell type alone in upregulating the DC marker CD11c or the co-stimulatory molecules CD40, CD80, and CD86 required for T cell activation and stimulation. To functionally differentiate between mixed and isolated microglial cultures, their ability to promote CD4⁺ T cell proliferation was ascertained. As described earlier, T cells require a primary signal provided by the T cell receptor and a co-stimulatory signal for proper activation. Here, the T cell receptor is stimulated through the addition of an exogenous CD3 antibody whereas the secondary signal is provided by the microglia with or without astrocytes or by the addition of exogenous CD28 antibody (α CD28). The optimal concentration of stimulatory CD3 antibody was determined to be 1 μ g/ml (Figure 5.8A). Due to the presence of a confluent bed of astrocytes, plate-bound anti-CD3 antibody could not be used (as is standard) to stimulate the T cells. Therefore, potential differences in T cell proliferation stimulated by plate-bound and soluble anti-CD3 antibody were assessed (Figure 5.8B). No differences were found, allowing the use of soluble anti-CD3.

CD4⁺ T cells were isolated by magnetic cell sorting from the spleens of B10.A mice and cultured with isolated microglia, microglia in the presence of astrocytes or astrocytes alone. All were stimulated for 3 days with CD3 antibody (1 μ g/ml) and T cell

proliferation determined by CFSE dilution. T cells responded robustly to the anti-CD3 stimulus (Figure 5.8C). However, when the T cells were stimulated in the presence of astrocytes, their proliferation was significantly suppressed. Moreover, in the presence of microglia together with astrocytes, that suppression was enhanced. In the presence of microglia alone, T cell proliferation was further reduced. This disparity between the effect of co-cultured microglia and isolated microglia on T cell proliferation was statistically significant. These results were puzzling given the hypothesis that higher levels of co-stimulatory molecules should promote T cell activation. In order to determine whether secondary signaling was, in fact, leading to a disparity in T proliferation, a secondary stimulus was provided exogenously by the inclusion of CD28 antibody which is expected to ligate T cell CD28 overriding the need for CD80 and CD86. Inclusion of this agonist antibody abrogated the suppressive effect of astrocytes. While microglia, unlike astrocytes, still suppressed T cell proliferation, the differences in microglial environments became inconsequential (Figure 5.8C). This would suggest that the difference in the expression of co-stimulatory molecules on microglia in the two environments was, indeed, a factor, but it did not explain why lower levels of co-stimulatory molecules (on co-cultured microglia) lead to increased T cell proliferation.

This prompted an examination of other possible reasons for this finding. Figure 5.8 on page 80.

Figure 5.8

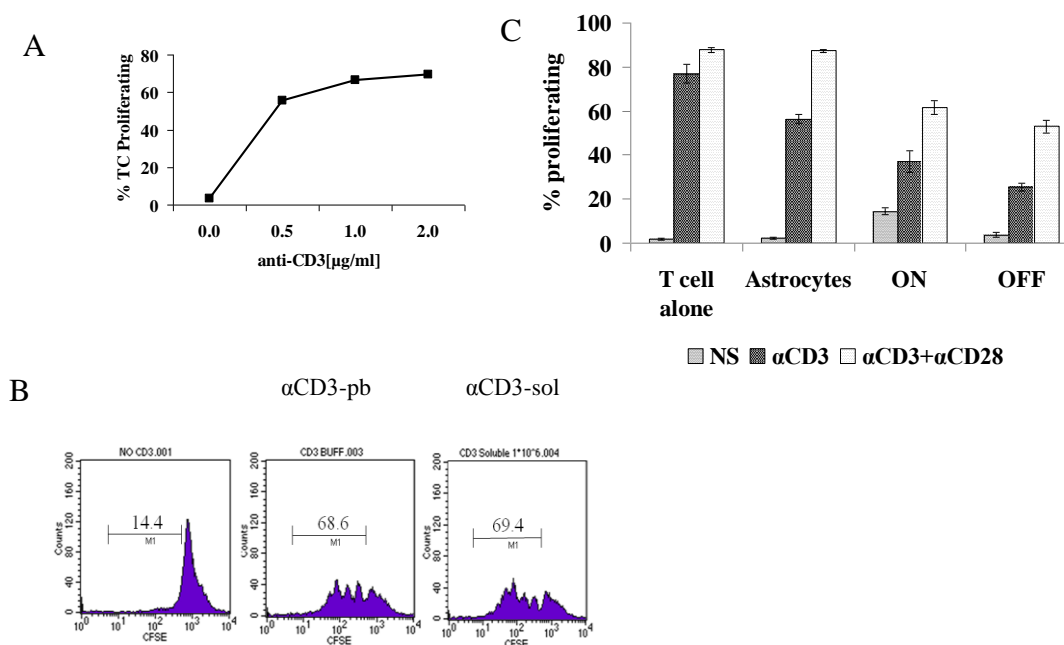


Figure 5.8 Glia affect T cell proliferation. (A) To determine the optimal concentration of the agonist anti-CD3, CD4^+ T cells were simulated with various concentrations of soluble CD3 antibody and proliferation assessed by CFSE dilution. CD4^+ T cells were magnetically isolated from the spleens of B10.A mice. 1×10^6 CD4^+ T cells were loaded with CFSE and cultured with increasing concentrations of CD3 antibody (0.0 - 2.0 $\mu\text{g/ml}$) for 3 days. T cell proliferation was optimal at 1 $\mu\text{g/ml}$. (B) Both plate-bound (pb) and soluble (sol) anti-CD3 stimulation leads to efficient CD4^+ T cell proliferation. CD4^+ T cells were obtained as before and 1×10^6 CD4^+ T cells were loaded with CFSE, cultured without exogenous stimulation (No αCD3) or with anti-CD3 (1.0 $\mu\text{g/ml}$) in a plate-bound (αCD3 -pb) or soluble (αCD3 -sol) format. Proliferation was measured after 3 days by assessing CFSE dilution through flow cytometry. Numbers above the histogram indicate the percentage of proliferating T cells. There was no difference in proliferation, allowing the use of soluble αCD3 in subsequent studies. (C) In the presence of αCD3 alone, both astrocytes and microglia impede T cell proliferation. 0.5×10^6 CD4^+ T cells were loaded with CFSE and cultured alone (T cell alone), in the presence of astrocytes alone (astrocytes), with 50,000 isolated microglia (OFF) or with 50,000 microglia in the presence of astrocytes (ON). Astrocytes, isolated microglia and microglia in the presence of astrocytes were all previously treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). T cells were stimulated by soluble anti-CD3 (1.0 $\mu\text{g/ml}$) alone or anti-CD3 (1.0 $\mu\text{g/ml}$) together with anti-CD28 (0.5 $\mu\text{g/ml}$). T cell proliferation was assessed by CFSE dilution through flow cytometry after 3 days. Data from three independent experiments are expressed as the percentage of T cell proliferation \pm SEM and compared by an ANOVA with a post-hoc Student-Newman-Keul's test for significance at the 95% confidence level. All groups were significantly different from each other in all conditions except T cells \pm astrocytes treated with αCD3 and αCD28 as well as T cells in the presence of microglia \pm astrocytes treated with αCD3 and αCD28 . Comparison of T cell proliferation in the presence of ON and OFF microglia has been performed 9 times with similar results.

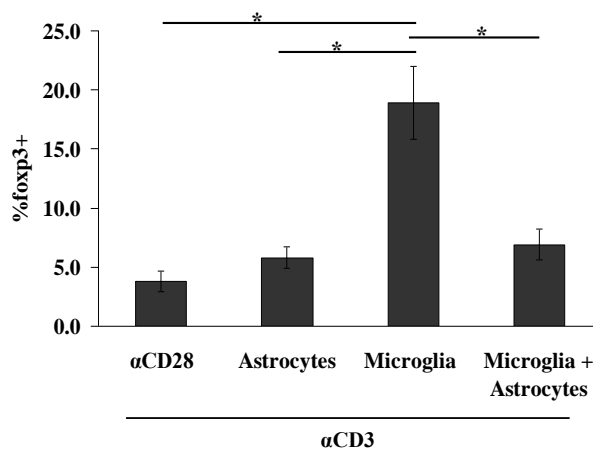
5.9 Isolated Microglia Promote the Differentiation of Foxp3⁺ T regulatory Cells

Because the presence of Foxp3⁺ T_{regs} can suppress T cell proliferation, the presence of Foxp3⁺ cells in various culture environments was determined. In a syngeneic experimental set-up, CD4⁺ T cells obtained from B10.A mice were cultured with enriched astrocytes alone, microglia in the presence of astrocytes and microglia alone. As described above, T cells were stimulated at the TCR by soluble anti-CD3. After three days in culture, T cells were collected and stained for the T_{reg} transcription factor Foxp3. There was no difference in the expression of Foxp3 among groups of T cells cultured alone, with addition stimulation by α CD28, with astrocytes alone, or with microglia and astrocytes (Figure 5.9A). Only T cells plated together with isolated microglia showed a significant increase in the percentage of T cells expressing Foxp3.

In order to determine the role of CD28 in promoting Foxp3 expression, a secondary signal was provided by soluble anti-CD28 (0.5 μ g/ml). With this treatment, the percentage of Foxp3⁺ T cells was significantly reduced in the presence of isolated microglia (Figure 5.9B). These data suggest that co-stimulatory signals provided by isolated microglial CD80 and CD86 are weak or could be stimulating an inhibitory molecule on T cells, such as CTLA-4. This prompted an investigation of a possible role of CTLA-4 in promoting T_{regs} in an isolated microglial environment. Figure 5.9 on page 82.

Figure 5.9

A



B

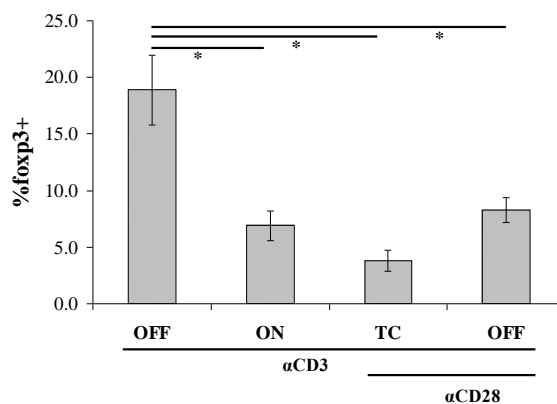


Figure 5.9 Isolated microglia promote the generation of Foxp3⁺ T cells. (A) CD4⁺ T cells were magnetically isolated from the spleens of B10.A mice. 0.5 X 10⁶ CD4⁺ T cells were cultured alone (first bar), in the presence of astrocytes alone (Astrocytes), with 50,000 isolated microglia (OFF) or with 50,000 microglia in the presence of astrocytes (ON). Astrocytes, isolated microglia and microglia in the presence of astrocytes were all previously treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). T cells were stimulated by either soluble anti-CD3 antibody (1.0 μ g/ml) alone or anti-CD3 antibody (1.0 μ g/ml) and anti-CD28 antibody (0.5 μ g/ml). After 3 days T cells were harvested, fixed/permeabilized and intracellularly stained for Foxp3. Foxp3 expression was assessed by flow cytometry. (B) Foxp3 expression was also assessed in CD4⁺ T cells stimulated with α CD3 (1.0 μ g/ml) and α CD28 (0.5 μ g/ml) in the presence of astrocytes, isolated microglia and in mixed glia. Data in both A and B are expressed as the average percentage of Foxp3⁺ T cells \pm SEM of 3 independent experiments and compared by an ANOVA with a post-hoc Student-Newman-Keuls test for significance at the 95% confidence level. An asterisk indicates $p < 0.05$ when compared to T cells cultured with isolated microglia in the presence of α CD3 alone.

5.10 CD80/CD86 Stimulation of Cytotoxic T-lymphocyte Antigen-4 (CTLA-4) is not Responsible for the Increased Percentage of Foxp3⁺ T_{regs} in the Isolation Cultures

CTLA-4 is an important negative regulator of T cells and is significantly upregulated upon T cell activation. By binding CTLA-4, CD80/CD86 can help rein in the immune response in part through the generation of T_{regs}. To determine whether CD80/CD86 stimulation of CTLA-4 was responsible for the induction of Foxp3⁺ T_{regs} in the isolated microglial cultures, varying doses of CTLA-4 neutralizing antibody were added to the cultures and Foxp3 expression assessed. Addition of increasing doses of neutralizing CTLA-4 antibodies or its IgG isotype did not affect the percentage of Foxp3⁺ T cells produced in the ON or OFF cultures (Figure 5.10). Even high doses of CTLA-4 neutralizing antibody failed to inhibit the induction of Foxp3⁺ cells, indicating that CD80/CD86 stimulation of CTLA-4 stimulation is not responsible for Foxp3 induction.

Figure 5.10 on page 84.

Figure 5.10

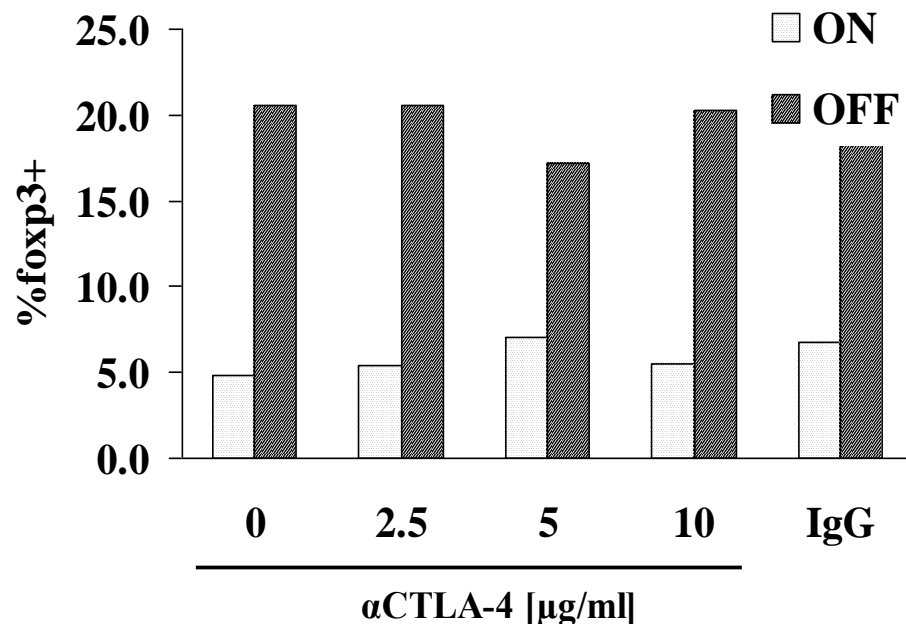


Figure 5.10 Foxp3 induction is not affected by CTLA-4 inhibition. CD4⁺ T cells were magnetically isolated from the spleens of B10.A mice. 0.5×10^6 CD4⁺ T cells were cultured with 50,000 isolated microglia (OFF) or with 50,000 microglia in the presence of astrocytes (ON). The microglia had been previously treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). T cells were stimulated by soluble anti-CD3 (1.0 μ g/ml) in the presence of increasing amounts of a neutralizing anti-CTLA4 (0.0 – 10 μ g/ml) or its IgG control (5 μ g/ml). After 3 days T cells were harvested, fixed/permeabilized and intracellularly stained for Foxp3. Foxp3 expression was assessed by flow cytometry. Data were assessed by regression analysis which rejected the hypothesis that there was a dose-response ($r = 0.3493$). A Student's t-test determined that none of the CTLA-4 antibody treatments differed from the 0 antibody control or from the IgG control ($p = .878$).

5.11 Foxp3⁺ T-regulatory Cells in the Isolated Microglial Cultures Suppress T cell Proliferation

Foxp3⁺ T_{regs} can affect the immune response by limiting the proliferation of other T-helper cell subtypes. The data show that T cell proliferation in the isolated microglial cultures -- which contain a high percentage of T_{regs} -- is significantly lower than T cell proliferation in the ON cultures. To test the effect of Foxp3⁺ T_{regs} depletion on T cell proliferation, CD4⁺ T_{regs} were depleted of CD25⁺ cells through magnetic cell sorting and cultured alone, with isolated astrocytes, microglia alone or with microglia in the presence of astrocytes. T cells were stimulated with αCD3 (1μg/ml) with or without αCD28 (0.5μg/ml) and proliferation assessed after 3 days. The differences in proliferation between T cells cultured in the presence of ON and OFF microglia disappears in the absence of CD25⁺ T cells suggesting that the presence of high numbers of CD25⁺ T_{regs} negatively affects T cell proliferation in the isolated microglial cultures. Figure 5.11 on page 86.

Figure 5.11

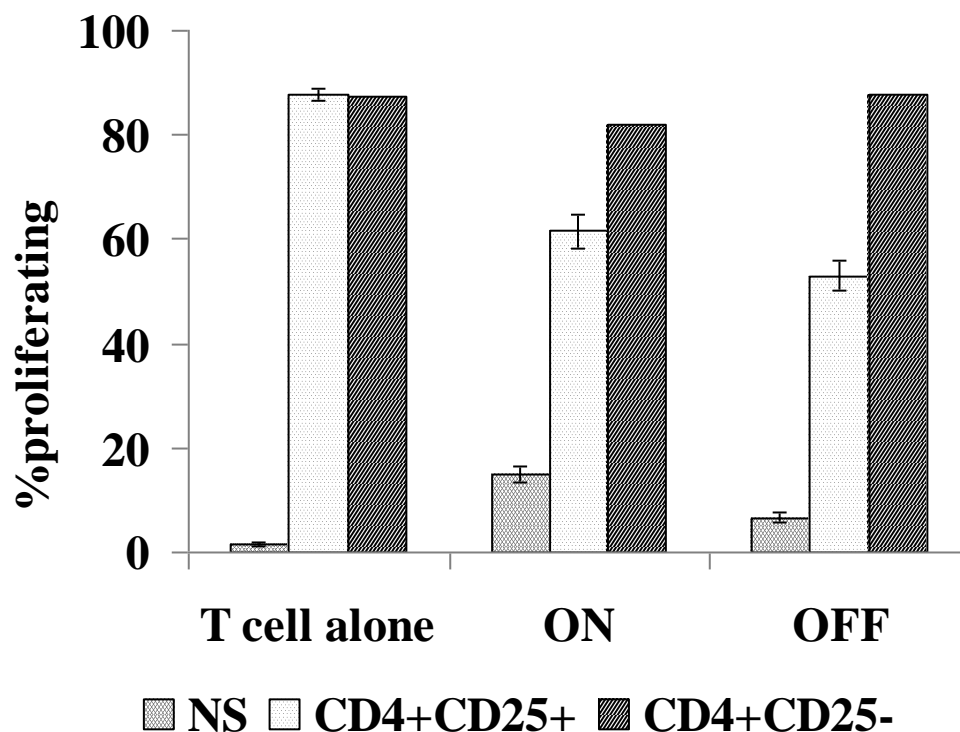


Figure 5.11 Depletion of CD25⁺ cells rescues CD4 T cell proliferation. CD4⁺ T cells were isolated and depleted of CD25⁺ T cells through magnetic cell sorting. 0.5×10^6 CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells were loaded with CFSE (5 μ M) and cultured alone, with 50,000 isolated microglia (OFF) or with 50,000 microglia in the presence of astrocytes (ON). The microglia had been previously treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). T cells were stimulated by soluble anti-CD3 antibody (1.0 μ g/ml) and anti-CD28 antibody (0.5 μ g/ml). After 3 days T cells were harvested, and proliferation was assessed by CFSE dilution. (n=1)

5.12 A Mixed Glial Environment is Less Conducive for Cytokine Production by CD4⁺ T cells

T cell-derived cytokines are important mediators of the immune response. To test the effect of various culture conditions on the production of IFN- γ , IL-17A, IL-10 and IL-4 by T cells, supernatants were harvested from T cells activated with α CD3 in the presence of isolated microglia, microglia in the presence of astrocytes and astrocytes alone. ELISAs reveal that T cells cultured in a mixed glial environment produce significantly lower quantities of these cytokines compared to T cells cultured alone or in the presence of isolated microglia (Figure 5.12A-D). Figure 5.12 on page 88.

Figure 5.12

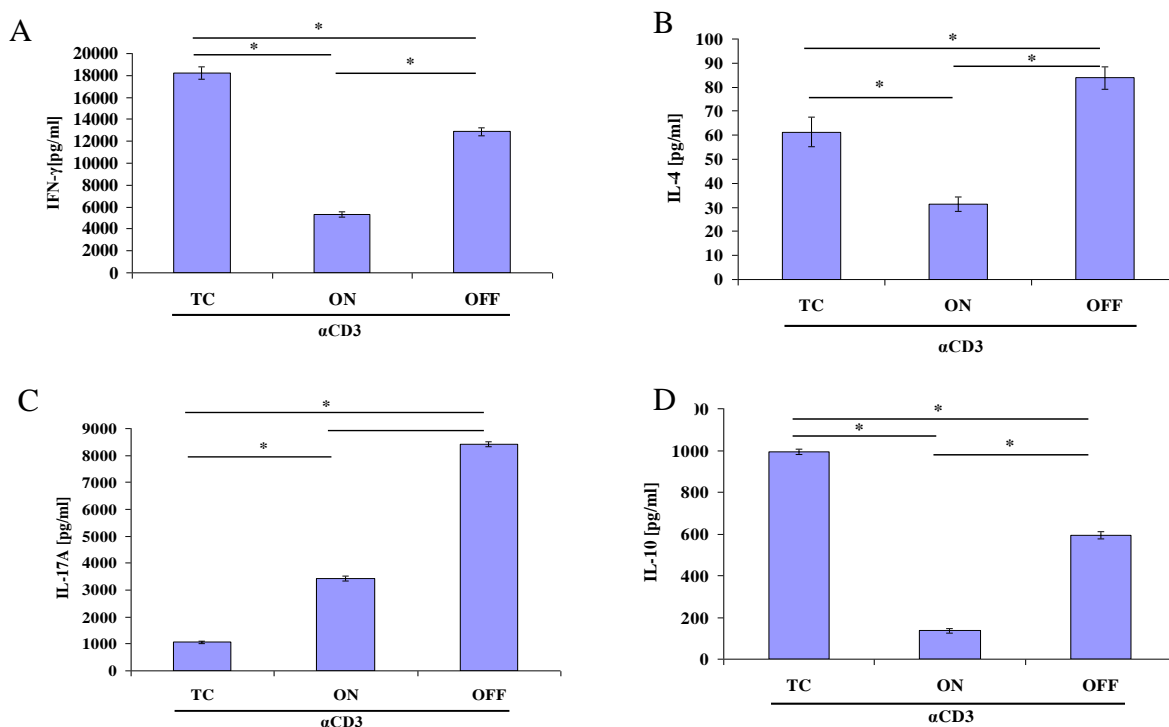


Figure 5.12 A mixed glial environment suppresses cytokine production. $CD4^+$ T cells were isolated from the spleens of B10.A mice through magnetic cell sorting. 0.5×10^6 $CD4^+$ T cells were cultured alone, with 50,000 isolated microglia, 50,000 microglia in the presence of astrocytes or with astrocytes alone. Astrocytes, isolated microglia and microglia in the presence of astrocytes were all previously treated with GM-CSF (25 ng/ml, 5 days) and LPS (50 ng/ml, 2 days). T cells were stimulated by soluble anti-CD3 antibody (1.0 μ g/ml). After 3 days supernatants were harvested and assessed for (A) IFN- γ , (B) IL-4, (C) IL-17A and (D) IL-10 using a sandwich ELISA. The data represent triplicates within a single assay and were compared by an ANOVA with a post-hoc Student-Newman-Keuls test for significance at the 95% confidence level. Asterisks reveal differences between groups. The assay has been done twice with similar results. Data courtesy of Divya Sagar.

Specific Aim 3: To examine the effects of differences in surface expression of these DC-specific molecules on T cell proliferation, Foxp3 expression and cytokine production.

Conclusions from specific aim 3:

- Glia impede T cell proliferation
- Isolated microglia promote generation of Foxp3⁺ T cells. CD80/86 interaction with CTLA-4 is not the cause of the increased Foxp3⁺ T cell generation.
- T cells cultured with microglia in the presence of astrocytes produce significantly less IFN- γ and IL-17

CHAPTER 6

DISCUSSION

There are many studies investigating the immune properties of microglia. However, a majority of these studies have been conducted on isolated microglia in the absence of other cell types – notably astrocytes. Astrocytes play a very important role in maintaining CNS homeostasis. In addition to maintaining the blood-brain barrier, they also play a vital role in preserving metabolic and biochemical homeostasis. It is not surprising, therefore, that they also have a strategic importance in regulating the brain's immune response. One of the ways in which they accomplish this is by assuring that pro-inflammatory mediators arising from the periphery or from within the CNS are kept under tight control. The ability of astrocytes to suppress immune reactions in the CNS is one reason why the brain has been labeled “immune privileged.”

Microglia are considered to be the resident immune cells of the CNS and are derived from the same myeloid lineage that also gives rise to a subset of professional antigen presenting cells (APCs) of the immune system (Barron, 1995; Chan et al., 2007). Microglia themselves are able to become APCs, expressing both MCH Class II as well as co-stimulatory molecules necessary for efficient antigen presentation and T cell activation. However, microglia do not express these molecules in the healthy CNS (Becher et al., 2000b). These studies have found that astrocytes provide a brake on the expression of these molecules, and this thesis investigates the mechanism(s) by which astrocytes perform this essential homeostatic function. Astrocytic regulation of monocyte-derived cell behavior has been investigated by others. Both soluble and

astrocytic-bound molecules have been found to exert their influence on microglial and monocyte activation. For instance, astrocyte-derived molecules down-regulate the production of IL-12 by microglia activated by IFN- γ /LPS (Aloisi et al., 1997). Though this effect on IL-12 production can occur in the presence of astrocyte conditioned medium, the effect is more potent when microglia are in contact with the astrocytes. In the presence of astrocytes or astrocytic conditioned medium, microglia are markedly less neurotoxic even when exposed to beta-amyloid peptide, a potent activator of isolated microglia in vitro (von Bernhardi and Eugenin, 2004), and recent bioinformatics studies have highlighted the importance of the microenvironment in regulating microglial expression of various molecules (Schmid et al., 2009). The results indicate that under inflammatory conditions, astrocytes temper the maturation of microglia into functional DCs.

The experiments conducted here show that microglia treated with the DC maturation factors GM-CSF and LPS in the presence of astrocytes assume an altered phenotype compared to microglia cultured in isolation. Microglia cultured with astrocytes are characterized by the reduced expression of the DC marker CD11c and T cell co-stimulatory molecules. A significantly greater proportion of the isolated microglia express CD11c together with co-stimulatory molecules CD40, CD80 or CD86 than microglia cultured in the presence of astrocytes. Though there are significant differences in the pattern of T cell co-stimulatory molecule expression between the two culture conditions, the expression of MHC class II, a molecule essential for antigen presentation to T cells, is unaffected by the presence of astrocytes.

To assure that astrocytes were the primary glial components responsible for this phenomenon, CD11b⁺ microglia (isolated by magnetic cell sorting) were plated onto an enriched bed of confluent astrocytes. When co-cultured in this more homogeneous glial environment, microglia still exhibited low surface expression of CD40, thereby confirming that the active glial component of co-cultures was, indeed, astrocytes.

To elicit an adaptive immune response, DCs routinely capture, process and present antigens to the appropriate T cells using the MHC class II complex. Engagement of the CD3/TCR complex by the MHC class II molecule containing an antigenic peptide delivers the primary activation signal to a T cell. Since the data show no significant differences in the expression of microglial MHC Class II between the two culture conditions, one might be tempted to disregard it as an inconsequential defining factor. However, it is important to note that the data only examined the surface expression of MHC Class II and not total cellular expression. In addition to the T cell stimulatory functions – which occur at the cell membrane – endosomal MHC Class II has also been implicated in intracellular signaling events that are distinct from its classical functions. A study by Liu and colleagues shows that endosomal MHC Class II molecules – unlike their membrane-bound counterparts – can promote TLR-triggered activation/maturation of DCs and macrophages by functioning as a scaffolding molecule (Liu et al., 2011). By recruiting various downstream members of the TLR signaling pathway and the kinases required to activate them (notably the tyrosine kinase Btk), endosomal MHC class II substantially enhances the DC response to various TLR ligands. Thus, the production of various pro-inflammatory cytokines is significantly attenuated in the absence of endosomal MHC Class II. Interestingly, their data show that intracellular MHC Class II

needs to form a complex with Btk through endosomal CD40 which then interacts with other factors to induce efficient TLR signaling.

Even though isolated microglia express more surface CD40 than do microglia cultured with astrocytes, microglia cultured with astrocytes express significantly more total CD40 protein as characterized by western blot (Giselles Acevedo, personal communication). Moreover, several different isoforms of CD40 can be visualized from whole cell lysates derived from microglia cultured in the presence of astrocytes while lysates obtained from isolated microglia reveal only a single CD40 isoform. Immunocytochemical data also reveal intracellular depots of both CD40 and CD86 (Jonakait, unpublished observations; Cruz, unpublished observation).

Alternative mRNA splicing can generate at least five distinct CD40 isoforms (types – I to V) (Tone et al., 2001). Of these five isoforms, only the type-I isoform is able to induce intracellular signaling from the cell surface. Though they lack an endodomain required for signaling and are thus incapable of traditional signal transduction, types II-IV are not without function. Type-II-IV isoforms may inhibit CD40 signaling by either acting in a dominant-negative fashion at the cell surface (type-III and -IV) or by preventing the trafficking of the type-I isoform to the cell surface (type-II). Another important intracellular interaction is that described above in which intracellular MHC Class II molecules form a complex with CD40 and Btk in endosomes, and intracellular CD40 mediates the interaction between MHC Class II and Btk (Liu et al., 2011) thereby regulating TLR signaling. This potentially important intracellular interaction, however, has not been demonstrated in microglia.

Microglia respond to LPS by increasing co-stimulatory surface expression, and intracellular MHC class II may play a role in facilitating that signaling (Liu et al., 2011). It is reasonable to consider the possibility that the presence of various non-signaling isoforms of CD40 in microglia cultured with astrocytes may interfere with TLR signaling that is mediated by intracellular MHC class II.

RT-PCR analysis of mRNA of MHC class II and the co-stimulatory molecules shows that microglia in both conditions upregulate mRNA expression following LPS treatment. In all cases, mRNA expression in microglia cultured with astrocytes is significantly higher compared to isolated microglia. Analysis of mRNA expression was complicated by the lack of suitable internal controls since “housekeeping” genes tested (GAPDH, HPRT1 and SDHA) also differed between the two culture conditions (data not shown), but repetitive assays were consistent in yielding this phenomenon. This was unexpected and seems inconsistent with the analysis of surface protein. The apparent inconsistency between message and protein data suggest that the surface expression of co-stimulatory molecules might be post-transcriptionally and/or post-translationally regulated. Indeed, recent studies have shown that expression of a brain-specific microRNA (miRNA-124) controls activation of peripheral macrophages and microglia (Ponomarev et al., 2011). Subsequent studies from the Jonakait laboratory have shown that other miRNAs implicated in controlling macrophage activation are elevated in co-cultured microglia (Acevedo, personal communication) (Baltimore et al., 2008; Boldin et al., 2011; O'Connell et al., 2010). While these data are preliminary, they suggest the possibility that astrocytes regulate translation of co-stimulatory molecules by regulating microRNAs.

Other means of regulating surface expression of proteins include alternative mRNA splicing and/or trafficking. Recent data from the Jonakait lab have shown that CD40 exists in higher quantities and in multiple intracellular isoforms that are only present in microglia cultured with astrocytes (Acevedo, personal communication). It is thus reasonable to suggest that alternative splicing has created isoforms that lack the signal sequence that targets proteins to the membrane. While data are lacking, it is possible to hypothesize that CD80 and CD86 are regulated similarly.

Though microglia are the resident immune cells of the CNS, numerous studies utilizing various rodent models for EAE, as well as CNS-specific viral, bacterial and parasitic diseases clearly demonstrate that various subsets of peripheral DCs and T cells enter the CNS in response to these challenges (Brehin et al., 2008; Fischer et al., 2000; Kostulas et al., 2002; Pashenkov et al., 2002). Moreover, MHC class II⁺ peripheral DCs have been identified in the choroid plexus, meninges and cerebrospinal fluid (Chinnery et al., 2010; Serot et al., 1997). The ability of astrocytes to express immunomodulatory capacity against peripherally derived DCs similar to that seen with microglia was determined. Consistent with the data of others (Hailer et al., 2001) the data show that astrocytes significantly suppress DC maturation of heterogeneous peripheral bone marrow-derived precursors. When CD11c⁺ bone marrow-derived cells are purified by magnetic cell sorting and cultured on a bed of enriched astrocytes, there is a profound reduction in the percentage of cells that express CD40 and CD80 compared to cells cultured in isolation suggesting that it is astrocytes and not other hematopoietic cell types nor other glial components that are responsible for the down-regulation.

In addition to suppressing the co-stimulatory molecules and MHC class II, astrocytes also prevent the downregulation of the chemokine receptor CX3CR1 in microglia in response to GM-CSF and LPS treatment. Data show that the mere removal of microglia from an astrocytic environment is enough to cause the downregulation of CX3CR1. The ability of astrocytes to prevent the loss of this receptor is important in the control of microglial-mediated neural cell death. CX3CR1 has been shown to be neuroprotective in various CNS infectious diseases and neurodegeneration models (Corona et al., 2010; Jaworski et al., 2011; Suzuki et al., 2011). For instance, Cardona and colleagues utilizing these same transgenic mice and various clinically relevant models of neurodegeneration show that microglial cells unresponsive to fractalkine are significantly more neurotoxic (Cardona et al., 2006). Similarly, Cipriani et al. report that CX3CL1 by acting at the microglial CX3CR1 receptor protects against damage caused by microglial activation in a permanent middle cerebral artery occlusion (pMCAO) model (Cipriani et al., 2011).

Given the data obtained from the transgenic CX3CR1^{GFP/GFP} mice, the expression of endogenous CX3CR1 receptor in wildtype microglia was assessed. A flow cytometric analysis of the CX3CR1 receptor in WT C57BL6/J isolated microglia treated with GM-CSF and LPS revealed that approximately 50% of the microglia still retained the receptor (data not shown). This was surprising since none of the transgenic microglia retain their GFP expression in the absence of astrocytes. Moreover, since GFP is under the direct control of the CX3CR1 promoter, one would expect the presence or absence of GFP in the transgenic microglia to correlate directly with the presence or absence of the CX3CR1 in the wild-type microglia.

Though the discrepancies between the transgenic and wild-type data are admittedly troubling, the transgenic data might prove to be useful and may yet shed light on how CX3CR1 expression is regulated by astrocytes. For instance, data reveal that a large portion of isolated GFP-microglia retain their GFP expression when cultured with GM-CSF and LPS in the presence of MatrigelTM even in the absence of astrocytes. Furthermore, a sizeable percentage of isolated GFP microglia devoid of GFP expression *regain* GFP expression after being placed into an astrocytic environment indicating that contact with the astrocytic environment has initiated *de novo* transcription and translation of the CX3CR1^{GFP/GFP} in the transgenic microglia. These data suggest that extrinsic cues provided by the astrocytic environment may somehow affect the transcription, translation and/or the stability of the CX3CR1.

Taken together, the above data demonstrate that an astrocytic environment not only prevents the efficient maturation of microglia and BM precursors into efficient DCs but it may also enhance neuroprotection by preserving microglial expression of the fractalkine receptor.

Since astrocytes are capable of secreting a wide variety of pro- and anti-inflammatory immune cytokines, the effects of endogenous and exogenous PGE2, IL-10 and TGF- β on microglial maturation in the presence or absence of astrocytes was investigated. As described above (see Background), numerous studies have shown these cytokines to broadly suppress isolated microglial activation in response to inflammatory stimuli. In agreement with other studies, addition of PGE2 suppressed the expression of co-stimulatory molecules in isolated microglia. Intriguingly, PGE2 had the opposite effect when microglia were in the presence of astrocytes. This result, however, was not

totally unexpected; others have also shown that PGE2 can be pro-inflammatory under certain conditions (Sheibanie et al., 2007).

Analysis of IL-10 expression through an ELISA revealed that the production of IL-10 in both culture conditions increased in a time-dependent manner. However, the amount of IL-10 in the mixed glial culture was significantly higher when compared to that in the isolated microglial cultures. Furthermore, unlike isolated microglial cultures, IL-10 was present consistently throughout the entire duration of LPS treatment in the mixed glial cultures. Given the elevated concentrations of endogenous IL-10, it is perhaps not surprising that the addition of exogenous IL-10 had no discernible effect. However, given the anti-inflammatory role of IL-10 on microglial activation, neutralizing IL-10 was expected to reverse the suppression of microglial activation. ELISA data showed that the addition of the antibody successfully neutralized IL-10 (data not shown). Even so, it had no significant effect in either culture condition ruling out IL-10 as the mediator of glial inhibition of the DC phenotype.

In addition to PGE2 and IL-10, the role of TGF- β 1 was tested. TGF- β 1 has been shown by others to mediate astrocytic neuroprotection (Dhandapani et al., 2003), to down-regulate activation of invading macrophages (Hailer et al., 1998) and microglia (Kim et al., 2004; Ledebor et al., 2000; Suzumura et al., 1993), and promote an immature DC-like phenotype in isolated microglia (Xiao et al., 2002). The addition of exogenous TGF- β 1 to mixed glial cultures significantly reduced the number of CD11c⁺CD40⁺ microglia, suggesting a possible role in mediating the DC inhibition. In order to determine whether endogenous TGF- β was indeed responsible, the endogenous molecule was neutralized by a pan-TGF- β antibody capable of countering the effects of

all three TGF- β isoforms. However, the addition of the antibody also decreased the percentage of CD11c⁺CD40⁺ microglia in mixed glial cultures. The apparent contradiction that both the addition and the neutralization of TGF- β had the same action might be explained by the fact that the antibody neutralized all three isoforms of TGF- β which might have contradictory effects. The consequences of adding exogenous TGF- β 2 and 3 was not pursued.

Because the three mediators examined did not appear to be the relevant candidates, a TranswellTM system was employed to examine the possibility that other astrocyte-derived soluble molecules were involved. CX3CR1^{GFP/GFP}-expressing microglia were used in initial studies because the down-regulation of CX3CR1 in some conditions is inhibited by soluble factors such as IL-10 and IFN- γ (Ramos et al., 2010). Separation of microglia from a confluent bed of astrocytes failed to maintain GFP expression. The expression of CX3CR1 was maintained only when microglia were in direct contact with astrocytes thereby eliminating most soluble factors as mediators.

Similar results for CD40 were obtained when isolated microglia were cultured in the TranswellTM setting. The percentage of cells expressing CD11c and CD40 is the same in microglia grown apart from astrocytes and those cultured in a TranswellTM system. These data conclusively show the need for contact between microglia and astrocytes in order for the latter to inhibit the maturation of the former.

These data, however, do not eliminate all soluble factors as potential astrocyte-derived mediators. One such factor that warrants further investigation is thrombospondin (TSP). Though TSP is a soluble factor, it can function as a “bridging” molecule capable of binding receptors on two different cells in very close proximity

(Brown and Frazier, 2001; Gao et al., 1996). TSP exerts its functions over distances that are much smaller than a Transwell™ apparatus allows. The anti-inflammatory properties of TSP are well characterized. Treatment of DCs with TSP-1 reduces the expression of molecules required for T cell stimulation (Doyen et al., 2003; Mittal et al., 2010). TSP-1 also decreases the production of inflammatory cytokines by PBMCs (Demeure et al., 2000). Furthermore, TSP-1 ligation of CD47 on T cells induces their differentiation into Foxp3⁺ T_{regs} in response to inflammatory signals (Grimbert et al., 2006). And importantly, astrocytes produce copious amounts of TSP-1 (Lanz et al., 2010). TSP has some unique properties that make it a candidate for consideration as an astrocytically-derived molecule that can affect microglial function. TSP-1 has a heparin binding domain (HBD) at its N-terminus and a C-terminal cell binding domain (CBD) (Krispin et al., 2006). These two domains can affect cells differently based on the specific receptors expressed by the cells. For instance, TSP-1 HBD when bound to DCs induces a phagocytic state whereas CBD binding induced a tolerizing phenotype (Tabib et al., 2009). Future studies should consider TSP as a possible astrocytic mediator of DC inhibition.

Other molecules within the astrocytic environment may be important. ECM molecules such as fibronectin and laminin secreted by astrocytes may negatively affect microglial maturation. Components of the ECM are known to suppress the induction of microglial iNOS/NO in response to LPS stimulation (Kim et al., 2006). Furthermore, in the presence of laminin or fibronectin, human DCs tend to maintain a “more immature” phenotype characterized by high endocytic capability and low T cell stimulatory

molecules (Garcia-Nieto et al., 2010). However, these same DCs were capable of efficient T cell stimulation upon their removal from the inhibitory ECM proteins.

The data presented here show that microglia are being similarly affected. Microglia treated with GM-CSF and LPS on MatrigelTM reveal a percentage of cells expressing CD11c and CD40 that is similar to that of isolated microglia. However, the MFI of CD40 is lower, reminiscent of the effect provided by an astrocytic environment. Similarly, CX3CR1^{GFP/GFP} microglia retain their GFP expression to a large extent in the presence of MatrigelTM. These data add support to the hypothesis that astrocyte-derived ECM proteins affect microglial maturation.

Though microglia cultured on MatrigelTM seem to mimic the phenotype of microglia grown in the presence of astrocytes, the data show that the components of MatrigelTM are not entirely sufficient to replicate the action of astrocytes. This implies that there might be other astrocytic factor(s) that also play a role in modulating microglial behavior. These factors could be molecules like thrombospondin that require membrane associated proteins for their effect and/or astrocytic membrane-bound molecules that microglia are required to touch and/or ECM constituents.

The phenotype expressed by DCs significantly affects their capacity to stimulate the activation and proliferation of T cells. Owing to their immature DC phenotype, microglia in the presence of astrocytes might be expected to be inefficient T cell stimulators. To test this hypothesis, T cells were cultured with microglia alone, astrocytes alone or microglia in the presence of astrocytes. CD4⁺ T cells were stimulated in an antigen non-specific manner by an exogenously supplied anti-CD3 antibody

whereas microglial CD40, CD80 or CD86 stimulating their appropriate cognate ligand would theoretically provide the T cells with the secondary signal.

Compared to T cells cultured alone, the data show that a glial environment significantly impedes T cell proliferation. Though microglia in the presence of astrocytes were more effective in stimulating T cell proliferation than isolated microglia, T cell proliferation was significantly lower in both culture conditions when compared to T cells cultured alone or in the presence of an enriched astrocytic culture.

A different result is obtained if an exogenous stimulator for signal two is provided. The inclusion of an agonist CD28 antibody together with anti CD3 eliminates the differences between the proliferation of T cells cultured alone and with astrocytes alone. Moreover, the difference between the proliferation of T cells cultured with isolated and co-cultured microglia disappears. What remains, however, is the suppression of proliferation whenever microglia are present. Since T cells cultured in these conditions do not proliferate as much as those cultured alone or cultured with astrocytes alone, this suggests that microglia irrespective of their environment suppress T cell proliferation.

The inhibitory effect of astrocytes on T cell proliferation in the presence of anti-CD3 is unexplained, but could be the result of an unknown ligand from astrocytes interacting with inhibitory molecules on the T cell surface. The addition of activated microglia to the astrocytic environment is even less conducive to T cell proliferation. However, because isolated microglia (with their full complement of co-stimulatory molecules) are less effective than co-cultured microglia (with their immature complement of co-stimulatory molecules) in promoting T cell proliferation, molecules other than the ones investigated here may be important players. So, e.g., the rapid upregulation of

CTLA-4 following T cell activation (Walunas et al., 1994) may allow for its ligands CD80 and CD86 to have an inhibitory effect on T cell activation and cell cycle progression. Moreover, CTLA-4 has additional binding partners that include B7-H2 which can bind CD28, thereby activating T cells. With an upregulation of CTLA-4, however, B7-H2 is prevented from activating CD28. B7-H2 expression on astrocytes and microglia has not been examined.

PD-L1 and PD-L2, present on astrocytes and microglia, are upregulated by pro-inflammatory signals (Pittet et al., 2011). Ligation of PD-1 on T cells inhibits PI3 kinase activity and lowers T cell proliferation (Sandner et al., 2005). Moreover, PD-1 mediated inhibition correlates with the strength of TCR ligation (Freeman et al., 2000). While both plate-bound and soluble anti-CD3 were equally efficacious in promoting T cell proliferation, there is no information on that strength relative to an *in vivo* stimulation. Importantly, PD-1 inhibition can be overcome by strong stimulation of CD28 (Freeman et al., 2000). If PD-L1 or PD-L2 were expressed on either microglia or astrocytes, this could account for their suppressive effects on T cell proliferation and explain why such suppression was overcome by the addition of anti-CD28.

An additional candidate for T cell inhibition is B7-H3, a newly-identified member of the B7 family of co-stimulatory molecules. A B7-H3 agonist fusion protein inhibits murine T cell proliferation generated by anti-CD3 (Leitner et al., 2009; Prasad et al., 2004; Suh et al., 2003). The expression of B7-H3 has not been studied in microglia or astrocytes.

An important future direction of these studies would be an investigation of these inhibitory co-stimulatory molecules on isolated and co-cultured microglia to determine whether they may be players in the inhibition of T cell proliferation observed here.

The ability of the two culture conditions to induce Foxp3⁺ T cells was assessed. Foxp3⁺ T cells are a subtype of T-helper cells that exhibit anti-inflammatory properties and have the capacity to downregulate the immune response. Studies show that Foxp3⁺ T cell generation can be affected by the strength and timing of the primary and secondary signaling provided by DCs. Since microglia in the presence of astrocytes express lower amounts of the co-stimulatory CD40, CD80 and CD86 molecules one should expect a higher percentage of Foxp3⁺ T cells in these cultures. However, the data show that the opposite is true, i.e., a significantly higher percentage of T cells harvested from isolated microglial cultures are Foxp3 positive compared to those isolated from the mixed glial environment. Two possible explanations can be invoked to account for this discrepancy. The higher percentage of Foxp3⁺ cells in the isolated microglial cultures could be the result of proliferation of endogenous Foxp3⁺ cells or it could be due to the *de novo* induction of Foxp3 in naïve CD4⁺ cells.

The CD4⁺ T cells used in the experiments were obtained through magnetic cell sorting from the spleens of B10.A mice. Without further purification steps, isolating T cells in this manner results in a heterogeneous mix of various CD4⁺ T helper subtypes including naïve (Th0), Th1, Th2, Th17 and Foxp3⁺ T_{regs}. Data from the Jonakait lab (not shown) and others indicate that Foxp3⁺ cells may comprise 5-10% of the CD4⁺ T cell population in the spleen. The increase to approximately 20% in the presence of isolated

microglia could result from preferential proliferation of this endogenous population of Foxp3⁺ T cells or from the de novo induction of Foxp3 in Th0 cells.

While the here experiments did not distinguish between these possibilities, others have shown that a strong CD28 signal even in the presence of a weak TCR signal promotes proliferation of Foxp3⁺ T_{regs} (Hombach et al., 2007). Similarly, such disparity in signaling also promotes de novo expression of Foxp3 (Gabrysova et al., 2011; Molinero et al., 2011). In the experiments here, T cells were stimulated with soluble anti-CD3 antibody. However, anti-CD3 antibody was used in a soluble form instead of the more conventional plate-bound antibody. A plate-bound antibody is deemed to provide T cells with a “stronger” primary signal than a soluble antibody. One reason for this is that the plate-bound antibody is more likely to cross-link the various signaling proteins than an antibody in solution. A weak primary signal coupled with the relatively stronger secondary signal provided by the isolated microglia may explain the higher percentage of Foxp3⁺ cells there. Given the disparity of the signaling strengths provided here, it is likely that the increased presence of T_{regs} is due to both induction and proliferation.

The addition of exogenous anti-CD28 did not change the percentage of Foxp3⁺ cells in T cells cultured alone or in the presence of co-cultured microglia. However, it did lower significantly the percentage of Fox3-expressing cells in the presence of isolated microglia. This may be due to an increased proliferation of other T cell types relative to T_{regs}. This would tend to lower the percentage of any single population. On the other hand, one report shows that the addition of soluble anti-CD28 in a pro-inflammatory environment lowers the percentage of Foxp3-expressing cells (Bouguermouh et al., 2009). The isolated microglial environment, having been stimulated with LPS, is

assuredly pro-inflammatory (see Figure 5.12). These data together confirm 1) the importance of the strength and timing of co-stimulatory signals and 2) the importance of astrocytes in regulating the microglia response.

An experiment using an antigen-dependent T cell stimulation protocol is an important future direction. In such an experiment microglia would have to phagocytose, process and present an antigen to a CD4⁺ T cell in an MHC class II context. Since this experiment would examine all the important functional aspects of a microglial-DC, it would provide a more complete picture of how isolated microglial behavior differs from microglia in an astrocytic environment.

Another consideration for Foxp3 induction is the effect of negative stimulation by CD80/86 of CTLA-4. By inducing apoptosis or anti-inflammatory properties in the target cells, negative stimulation of T cells by CD80 and CD86 can terminate the immune response at the appropriate time. Studies show that CD80 and CD86 bind CTLA-4 preferentially and with much greater affinity than CD28. Moreover, CD80/86 binding of CTLA-4 tends to elicit anti-inflammatory responses (Vandenborre et al., 1999; Walunas et al., 1994; Wells et al., 2001). One way this is accomplished is through the production of Foxp3⁺ T_{regs}. CD80/CD86 stimulation of CTLA-4 under certain conditions can lead to the induction of Foxp3 in T cells (Pletinckx et al., 2011). Since a significantly greater percentage of isolated microglia express CD80 and CD86, it was reasonable to hypothesize that CD80/CD86 stimulation of CTLA-4 might be the reason for the increased number of Foxp3⁺ T cells. A neutralizing antibody against CTLA-4 was used to test this hypothesis. Blocking CTLA-4 interaction with CD80/86 was expected to reduce the observed percentage of Foxp3⁺ cells. However, Foxp3⁺ T cell generation remained

unaffected even in the presence of the neutralizing antibody suggesting that CD80/86 stimulation of CTLA-4 is probably not the cause of the increase in Foxp3 cell numbers. Furthermore, the elimination of CTLA-4 as a mechanism for Foxp3 generation lends more support to the suggestion that weak TCR signaling coupled with strong co-stimulatory signaling might be the cause of Foxp3 induction in the isolated microglial cultures.

In addition to suppressing T cell proliferation, the stimuli arising from the glial environment also seem to inhibit the efficient secretion of various cytokines by T cells. The pro- or anti-inflammatory activity of T cells is determined in part by the type of cytokines they produce. An analysis of the supernatants by ELISAs reveals that a mixed glial environment hinders the secretion of cytokines by the T cells whereas, isolated microglia promote both pro- and anti-inflammatory cytokine secretion by T cells. The production of pro-inflammatory IFN- γ , IL-17A and anti-inflammatory IL-10 and IL-4 is significantly reduced in T cells cultured in a mixed glial environment. Though a greater percentage of T cells cultured with isolated microglia assume a Foxp3⁺ T_{reg} phenotype, these cultures tended to produce significantly more IL-17A and IFN- γ two pro-inflammatory cytokines implicated in auto-immune disorders.

The cytokine milieu generated in the various culture conditions is likely due to the variability of the co-stimulatory signaling. The engagement of co-stimulatory molecules on DCs by their cognate ligands on naïve or differentiated T cells can modify the phenotype of the DCs themselves. For instance, the engagement of CD40 by CD40L (on T cells) is sufficient to induce the maturation of immature DCs in certain instances (Fujii et al., 2004). Moreover, following TLR signaling, CD40L-stimulated human DCs

significantly increase their production of IL-23, a cytokine essential for Th17 differentiation (Sender et al., 2010) revealing the importance of the crosstalk between DCs and T cells. The differentiation of Th17 cells also requires IL-6 and TGF- β . High levels of IL-6 were observed in both culture conditions, and it increases over time (data not shown). The presence or absence of TGF- β in the culture system was not assessed but presumably the TGF- β already present in serum is enough to drive Th17 generation. In light of this study and data showing the very high expression of surface CD40 in isolated microglia and the detection of high quantities of IL-17 in the supernatants, it is quite feasible that the mechanism put forth by Sender et al. is also at work in this study.

The production of an appropriate T cell response is of vital importance to the healthy brain, and the data indicate that an astrocytic environment plays an important role in maintaining brain homeostasis. This level of immunosuppression is generally thought to be beneficial. However, the interaction between becomes detrimental when that immunosuppression occurs within tumors. Tumors including gliomas tend to induce an anti-inflammatory environment as well as the formation of Foxp3⁺ T_{regs} which compromise the immune response and contribute to the immune evasion of the tumor and poor prognosis (Hussain et al., 2006; Wei et al., 2010) While they contain resident microglia and even peripheral DCs, these are characterized by low expression of MHC class II, CD40 and CD86 and are unable to mount an appropriate immune response. Their immaturity with respect to antigen-presentation may contribute to an environment in which anti-inflammatory Foxp3⁺ T_{regs} are generated (Kostianovsky et al., 2008). Indeed the conversion to a more mature phenotype markedly reduces the immunosuppressive qualities of the cancer cells (Wei et al., 2010). Even more effective is the intra-tumoral

introduction of mature DCs pulsed with tumor antigens (Pellegatta et al., 2010). A recent paper of interest suggests that the introduction of gliomas into the CNS does not elicit an immune response, whereas implantation into the periphery does suggesting the profound immunosuppression elicited in a brain microenvironment (Biollaz et al., 2009).

Likewise, it appears that the depletion of endogenous Foxp3⁺ T_{reg} may also provide protection against gliomas by unleashing the latent potential of the hitherto suppressed immune system (Maes et al., 2009). The same study shows that the prognosis gets even better when T_{regs} ablation is coupled with the introduction of mature DCs pulsed with tumor antigens. This suggests that maximal immune response can only be achieved in the absence of T_{reg} mediated suppression as well as the presence of DCs in a proper state of maturation.

The data in this thesis show that microglia in the presence of astrocytes are characterized by their low expression of CD80, CD86 and MHC class II as well as their reduced capacity to stimulate T cell activation and proliferation when compared to T cells cultured alone. It appears that the mechanisms that prevent an overblown T cell response in the healthy CNS are co-opted and exaggerated by tumor cells to their own advantage. Understanding the mechanism(s) through which this occurs in the healthy CNS may shed further light on how tumors evade immune surveillance.

In contrast to their detrimental role in the pathogenesis of gliomas, activation of microglia and CNS DCs has been shown to exert a neuroprotective effect. Spinal cord injury (SCI) studies indicate that the presence of activated microglia and/or mononuclear cells at the site of injury can be directly correlated with morphological and functional recovery (Mikami et al., 2004). The vaccination of immature DCs loaded with myelin

basic protein either directly into the site of the wound or in the peritoneum after the SCI lead to significant improvements in recovery (Hauben et al., 2003; Liu et al., 2009). Moreover, this recovery is contingent on the presence of mature T cells since the depletion T cells results in negligible improvement after SCI. In addition to recruiting T cells, activated DCs and microglia aided recovery by reducing the formation of glial scars that impede neuroregeneration (Hauben et al., 2003).

Glial scars comprise a heterogeneous mixture of cells including microglia, reactive astrocytes and CNS invading DCs. They produce high quantities of extracellular matrix molecules including chondroitin sulfate proteoglycans (CSPG) and laminin (Kato-Semba et al., 1995). The data here and that of others suggest that ECM molecules or their degradation products have an anti-inflammatory effect on microglia (Ebert et al., 2008; Rolls et al., 2004). While protecting against neuronal death, this down-regulation can also prevent the elimination of the glial scar either by microglial phagocytosis or T cell invasion.

Conclusions

The data presented here and that of others suggest that microglia are tightly controlled by an astrocytic environment that prevents when possible the over activation of microglia. This is neuroprotective in some instances where pro-inflammatory events could cause neuronal cell death or promote autoimmunity. But this inhibitory effect has a darker side. Microglial inflammation can be beneficial both in recovery from traumatic injury and is essential for an effective response against brain tumors. Understanding the mechanism(s) that regulate microglia in both the healthy and the diseased or injured brain are essential

for proper therapeutic intervention. The data point to the complex astrocytic microenvironment as being an important focus for this understanding.

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