Microglial Regulation by Astrocytes: Effect on CD40 Surface Expression and Its Consequences for Dendritic Cell Maturation

BY

GISELLES MARIA ACEVEDO

A dissertation submitted to the

Graduate School-Newark

RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Graduate Program in Biological Sciences

written under the direction of

G. Miller Jonakait

and approved by:

____________________________________________
G. Miller Jonakait

____________________________________________
Wilma Friedman

____________________________________________
Haesun Kim

____________________________________________
Doina Ganea

Newark, New Jersey

January 2013
ABSTRACT OF THE DISSERTATION

Microglial Regulation by Astrocytes: Effect on CD40 Surface Expression and Its Consequences for Dendritic Cell Maturation

Giselles Maria Acevedo

Dissertation Director:
G. Miller Jonakait

Microglia are the primary immune-responsive cells in the central nervous system. Monocytically derived, they can be isolated in culture where, if stimulated by sequential treatment with granulocyte/monocyte colony-stimulating factor (GM-CSF) and lipopolysaccharide (LPS), they will upregulate the expression of major histocompatibility complex class II and surface levels of the co-stimulatory molecules CD40, CD80 and CD86 necessary for efficient antigen presentation and T cell activation. Hence, they assume a mature dendritic cell (DC) phenotype in isolation culture. In preliminary studies we have shown that the continued presence of astrocytes stunts the assumption of the microglial DC phenotype in a contact-dependent manner. The current study was designed to examine the mechanism(s) by which astrocytes prevent DC maturation in microglia.

LPS signaling inhibitors SOCS1, SOCS3, TRIM30α and IRAK-M were rapidly and robustly upregulated by LPS treatment of co-cultured microglia. SOCS3 protein levels, however, were similar in both culture conditions while IRAK-M protein was elevated only in co-cultured cells. In IRAK-M<sup>−/−</sup> microglia CD11c levels were inhibited in both settings, but CD40 levels were only modestly affected.

Expression of IFNβ, an inducer of both CD40 as well as inhibitors SOCS1 and SOCS3, was highest in isolated microglia, but neutralization of it affected only CD11c.
GM-CSF pretreatment elicited a rapid and robust up-regulation of co-stimulatory molecules in isolated cells while subsequent LPS treatment increased expression primarily in co-cultured cells. CD40 and CD11c protein surface expression were maximal at the end of GM-CSF treatment in isolated cells while co-cultured cells awaited LPS signaling before placing protein on the cell surface. Differences in CD40 surface expression were not due to a more rapid mRNA or protein turnover. Rather, Western blots and flow cytometry showed that total CD40 protein levels in co-cultured microglia were high. These data suggested that CD40 protein was transcribed and translated but was retained intracellularly. Immunocytochemistry confirmed that CD40 was retained in the ER, but not in lysosomes or early endosomes.

In conclusion, astrocytes affect particularly GM-CSF signaling of microglia. Future studies would be designed to address the mechanism(s) by which the extracellular environment created by astrocytes regulates the intracellular trafficking within microglia.
ACKNOWLEDGMENTS

My life has always been blessed by the people that surround me. In this particular chapter of my life, Dr. Jonakait has been a role model, excellent guide, great support and an extraordinary friend. She was there through the rough times to encourage and give me that little push to continue. Today, I am humbly thanking you for everything you have done for me. You have been a key part in my career. It has been a blessing to have you as my advisor.

I am extremely thankful to Dr. Haesun Kim, Dr. Wilma Friedman, and Dr. Doina Ganea for being part of my dissertation committee. Your time and input have been incredibly valuable for the conclusion of my thesis. Thank you very much for being there throughout this process.

I am grateful to the Minority Biomedical Research Support Program (MBRS) for supporting me through my first six years in the PhD program. I also thank you for giving me the opportunity to attend different conferences.

I am appreciative for the time and effort Dr. Komisaruk and Dr. Bonder put into helping me in the transition from the MBRS program to the Biology Department which gave a teaching assistant position and supported me the remaining time in the Ph.D. program.

Thanks to the National Science Foundation for providing us with the funds for this project.

The lab techniques that I know today are thanks to Dr. Li Ni. He is the most skilled, outstanding and extraordinary bench scientist I have ever known. This would not be possible without his patience and support.

I am extremely grateful for the love and support of my past and present lab-mates: especially Lori, NK and Bhargavi. What would I have done without you?

The immunocytochemistry studies would not have been possible without the help and guidance from Dr. Altan-Bonnet, Felix and Sue.

Thanks for the support and encouragement from all the members of the LSC and Boyden: especially Kavya, Sue, Felix and Marianita.

Thanks to the folks in the animal facility for taking care of my mice.

Thanks to my whole family: especially my mom, dad, brothers, sisters, Kelbin and my beautiful daughter, Kamila.
I would like to dedicate my thesis to my uncle, Juan Acevedo (1940-1998). Without him, we would never have been able to come to the United States of America.
TABLE OF CONTENTS

Abstract ...........................................................................................................................ii
Acknowledgments ............................................................................................................iv
Dedication ..........................................................................................................................v
List of Figures and Tables .................................................................................................viii

I. Introduction and Background .......................................................................................1
   A. Dendritic cells (DCs) .................................................................................................2
   B. Co-stimulatory molecules ..........................................................................................5
      1. CD40 ......................................................................................................................5
      2. CD80 and CD86 ......................................................................................................7
   C. Microglia ....................................................................................................................7
   D. Astrocytes ................................................................................................................10
   E. Antigen presenting cells (APCs) and gliomas ............................................................11
   F. Signaling pathways and their inhibitors ......................................................................12
      1. Toll like receptor 4 (TLR4): MyD88 dependent pathway ........................................12
      2. IRAK-M: LPS signaling inhibitor ...........................................................................13
      3. TLR4: MyD88 independent pathway/IFN beta production .....................................15
      4. Suppressor of Cytokine Signaling 1 and 3 (SOCS1/3) ...........................................16

II. Rationale and Aims .......................................................................................................17

III. Materials and Methods .............................................................................................18

IV. Results ........................................................................................................................25
   Specific Aim 1 ...............................................................................................................25
      1. Endogenous inhibitors of the LPS MyD88-dependent and IFNβ signaling pathways are differentially regulated in co-cultured and isolated microglia .................................................................25
      2. SOCS3 protein levels are unchanged ....................................................................26
      3. IRAK-M regulates CD11c, but not CD40 ..............................................................27
      4. IFNβ differentially regulates CD40 and CD11c .....................................................28
   Specific Aim 2 ...............................................................................................................32
      2. Co-stimulatory molecules are differentially regulated by GM-CSF and LPS in co-cultured and isolated microglia ...................................................................................................................32
      2. Microglia express different CD40 isoforms ............................................................34
      3. LPS induces CD40 surface expression in co-cultured microglia in a time dependent manner, but did not increase its MFI ...................................................35
      4. mRNA degradation does not account for differences in co-stimulatory molecule expression ........................................................................................................................36
      5. Surface protein degradation does not account for differences in the MFI ............37
      6. Co-cultured microglia express high levels of CD40 protein, but fail to place it on their surface ...................................................................................................................37

V. Discussion ....................................................................................................................40

VI. References ..................................................................................................................50

VII. Figures and Tables .....................................................................................................70
VIII. Appendices
   Appendix A: Microglial expression of IL-35..........................91
   Appendix B: microRNAs are differentially expressed in co-cultured and
   isolated microglia...............................................................93
IX. Curriculum Vitae.................................................................97
LIST OF FIGURES AND TABLES

Figures

Figure 1. Astrocytes inhibit microglial production of a mature DC phenotype.......................................................... 71
Figure 2. T cell interaction activation with DCs.................................................... 72
Figure 3. T helper cell differentiation................................................................. 73
Figure 4. The LPS signaling pathways............................................................... 74
Figure 5. The IFNβ signaling pathway............................................................... 75
Figure 6. Co-cultured microglia express elevated levels of mRNA for endogenous LPS inhibitors............................................ 76
Figure 7. SOCS3 protein levels are similar in isolated and co-cultured microglia........................................................................................................... 77
Figure 8. IRAK-M affects CD11c surface expression........................................ 78
Figure 9. IFNβ regulates CD11c.......................................................... 79
Figure 10. QRT-PCR analysis of microglial expression of mRNA for MHC II and co-stimulatory molecules......................... 80
Figure 11. Multiple CD40 mRNA isoforms are detected in microglia............... 81
Figure 12. Protein surface expression is differentially regulated in isolated vs. co-cultured microglia.................................................. 82
Figure 13. Neither mRNA nor protein degradation rate accounts for differences in surface expression of protein at 48 hrs.......... 83
Figure 14. Multiple bands of CD40 protein are expressed in co-cultured microglia.................................................................................. 84
Figure 15. Surface vs. total CD40 expression.................................................. 85
Figure 16. CD40 cellular distribution............................................................. 86
Figure 17. Immunocytochemistry reveals CD40 sequestered in co-cultured cells in the ER......................................................... 87
Figure 18. M1 and M2 phenotypes......................................................... 88

Appendix Figures

Figure 1. Members of the IL-12 family of cytokines are differentially regulated by astrocytes.............................................................. 95
Figure 2. MicroRNAs are elevated in co-cultured microglia following LPS treatment................................................................. 96

Tables

Table 1. Primary and Secondary antibodies used................................. 89
Table 2. QRT-PCR Primers........................................................................... 90
I. Introduction and Background

Previous studies have investigated the assumption by monocytically-derived brain microglia of a dendritic cell (DC) phenotype. While lacking (or having very low) major histocompatibility complex Class II (MHC Class II) expression in vivo, microglia can be stimulated in culture to express MHC class II and co-stimulatory molecules CD40, CD80 and CD86 that are necessary for antigen presentation and T cell activation (Ford et al., 1996). We and others have shown that these molecules are up-regulated upon microglial isolation in culture (Satoh et al., 1995; Wei and Jonakait, 1999). Granulocyte/ monocyte colony-stimulating factor (GM-CSF) added into microglial cultures stimulates microglial proliferation (Lee et al., 1994), and upregulates CD11c (a DC marker), MHC class II and co-stimulatory molecules (Fischer et al., 1993; Re et al., 2002; Wei and Jonakait, 1999). These molecules can be further up-regulated by interferon-γ (IFN-γ) (DeGroot et al., 1991; Menendez et al., 1997; Nguyen and Benveniste, 2000; Williams et al., 1994), lipopolysaccharide (LPS) (Menendez et al., 1997), CD40L (CD154) (Fischer and Reichmann, 2001) and unmethylated CpG dinucleotides (Dalpke et al., 2002). As a result, isolated, stimulated microglia are effective T cell activators in vitro (Askew and Walker, 1996; Cash and Rott, 1994; Fischer and Reichmann, 2001; Frei et al., 1987; Matyszak et al., 1999b; Re et al., 2002; Williams et al., 1994).

Almost all of the experimental work done on microglial DCs has involved their isolation from other glial populations and neurons. In routine culture preparations, mixed glial cultures are prepared and carried for 10-14 days followed by the removal of loosely adherent microglial populations. Thus isolated, microglia attach firmly to tissue culture plastic and can be pharmacologically manipulated with relative ease. Studies on
microglia thus isolated, like those cited above, have been useful in establishing what microglia can do.

Microglia, however, do not exist in isolation; instead, they are surrounded by a sea of other cell types, notably astrocytes. In order to examine a possible role of astrocytes in regulating the DC phenotype, microglia were studied both in the presence and absence of astrocytes. Preliminary studies revealed that microglia co-cultured with astrocytes fail to express a fully mature DC phenotype. Measured by flow cytometry, surface levels of MHC Class II and co-stimulatory molecules are significantly lower on co-cultured microglia than on their isolated counterparts, and the mean fluorescence intensity is dramatically lower (Figure 1). In these preliminary studies, controls were done in which isolated microglia either remained in isolation or were added back to a bed of purified astrocytes and co-stimulatory molecules again measured by flow cytometry. In these reconstituted cultures, co-cultured microglia again expressed low levels of co-stimulatory molecules suggesting that it was astrocytes and not other glial or neuronal elements of the culture that were responsible for the inhibition. Moreover, it is not a soluble astrocytic molecule that inhibits the expression; microglia must be in contact with the astrocytic microenvironment in order for the inhibition to occur. The aim of the current study is to determine mechanism(s) by which astrocytes inhibit the microglial assumption of a fully mature DC phenotype. The focus will be on CD40 as representative of co-stimulatory molecule expression.

A. Dendritic cells (DCs)

DCs are monocytyically-derived immune cells in the periphery whose main function is to capture, internalize and process antigens leading to the presentation of
antigenic peptides to naïve T cells. Therefore, DCs initiate primary immune responses that subsequently lead to adaptive immunity. DCs also play a critical role in the regulation of autoimmunity. Depending on their level of maturity, they promote the differentiation of various T cell subsets that either promote or suppress autoimmunity. As a result, DCs are major regulators of immune function throughout the body.

The ability of DCs to control T cell fate is dependent upon their maturation status, which can be manipulated in vitro (Groux et al., 2004; Jonuleit et al., 2000; Steinbrink et al., 1997; Buelens et al., 1995). In vitro immature DCs (iDCs) can be generated from bone marrow cells by growth in granulocyte/monocyte colony-stimulating factor (GM-CSF) (Sallusto and Lanzavecchia, 1994) with maturation accomplished by subsequent ligation of toll-like receptors (TLRs) by bacterial products like lipopolysaccharide (LPS; which binds TLR4 (Kaisho and Akira, 2003; Kaisho et al., 2001)), peptidoglycan (PGN, which binds TLR 2 or 6), viral RNA (TLR3), or CpG motifs (TLR9) (Agrawal et al., 2003), IL-4 (Sallusto and Lanzavecchia, 1994), TNFα (Menges et al., 2002; Inaba et al., 2000), CD40 ligation (Sender et al., 2010; Kato et al., 2001), a mixture of these (Jonuleit et al., 1997) and/or prostaglandins (Steinbrink et al., 2000; Sheibanie et al., 2007). In the process of maturation, DCs lose their ability to capture antigen (Sallusto et al., 1995), but upregulate levels of surface major histocompatibility complex class II (MHC class II) and co-stimulatory molecules such as CD40, CD80 and CD86 that are necessary for efficient antigen presentation and T cell activation (Figure 2). The up-regulated surface expression of chemokine receptors CCR7 and CXCR4 promotes migration of DCs to local lymph nodes (Scandella et al., 2002; Kellermann et al., 1999; Sallusto et al., 1998) where they act as potent stimulators of CD8^+ and/or CD4^+ T cells. Because DCs not only recognize
foreign antigens but also are capable of presenting them to naïve T cells, they are considered to be the link between innate and adaptive immunity.

DCs control the quality of the T cell response, driving naïve lymphocytes into distinct classes of effectors (Figure 3). Different DCs are characterized by specific cell surface markers as well as the cytokines that they release, and these cytokines determine the type of cell that a naïve CD4+ T helper cell (Th0) will become. These include CD4+ Th1, Th2, Th17 or regulatory T cells (T_{regs}). In mice the CD8α+ DC (lymphoid) but not the CD8α− DC subset (myeloid) produces IL-12 which is responsible for the induction of Th1 cells (a pro-inflammatory phenotype). The CD8α− DC subset (myeloid) favors the Th2 phenotype by producing IL-4 (Maldonado-Lopez and Moser, 2001; Pulendran et al., 1999). DCs differentiated in an in vitro environment rich in IL-10 and/or transforming growth factor-beta (TGF-β) induce T_{regs} due in part to the ability of these cytokines to inhibit the upregulation of MHC class II and co-stimulatory molecules (Buelens et al., 1995; Jonuleit et al., 2000; Kubach et al., 2005; Steinbrink et al., 1997; Fiorentino et al., 1991; Volpe et al., 2008). T_{regs} express CD4+CD25+ surface markers and the transcription factor Foxp3. Some of them produce IL-10 and express the negative regulator cytotoxic T lymphocyte-associated molecule 4 (CTLA-4) (Jonuleit et al., 2000). They suppress effector T cells (T_{eff}) by inhibiting their proliferation and cytokine production in an antigen non-specific manner (Jonuleit et al., 2001; Gad et al., 2004). The combination of TGF-β and IL-6 skews the T cell to a Th17 phenotype (Volpe et al., 2008). These cells are responsible for mediating autoimmunity by producing the damaging pro-inflammatory cytokine interleukin-17 (IL-17). The ability of T_{regs} to suppress Th17 cells makes them essential in ameliorating or preventing autoimmunity. Therefore, depending on the
cytokine environment, T cells adopt different phenotypes. Once a DC has directed a T
cell into a specific subset, the T cell will be able to provide the organism with long-
lasting protective immunity. In addition, DCs can also prevent autoimmunity in the host
by inhibiting the pro-inflammatory Th17 cells and/or inducing the tolerant T_{reg}
phenotype.

Immature dendritic cells (iDCs) with low surface expression of MHC class II and
co-stimulatory molecules have the ability to promote T cell anergy and/or differentiation
of T_{regs}. Thus, the ability of DCs to control T cell fates is dependent upon the maturation
status of the DCs, and that status can be manipulated in vitro. DCs differentiated in vitro
in an environment rich in IL-6, IL-10, vasoactive intestinal peptide (VIP) and/or TGF-β
have low surface expression of MHC and co-stimulatory molecules and have the capacity
to endocytose foreign proteins and/or proteins from dying cells. However, when
encountering a naïve T cell, they deliver a “suboptimal” signal, leading to anergy and
subsequent T cell death and/or the conversion of naïve T cells to T_{regs} (Delgado et al.,
1999; Buelens et al., 1995; Steinbrink et al., 1997; Dhodapkar et al., 2001; Jin et al.,
2007; Jonuleit et al., 2000; Mahnke et al., 2002). By promoting T_{reg} expression, iDCs
help to maintain self-tolerance.

B. Co-stimulatory molecules

1. CD40

CD40, a tumor necrosis factor receptor (TNFR) family member, was first
characterized in B lymphocytes. It was first described as a transmembrane protein with a
molecular weight between 30-50 kDa (Clark, 1990; Stamenkovic et al., 1989). This
surface-related molecule is composed of a typical type 1 extracellular domain which is
rich in cysteines and threonine, a transmembrane domain, and other structural homologies to the TNFR family members within the cytoplasmic domain that initiate important signaling pathways (Noelle et al., 1997; Nonoyama et al., 1993). CD40 is also expressed in other antigen-presenting cells including DCs, monocytes/macrophages, endothelial cells, epithelial cells and microglia (Grewal et al., 1996; van Kooten and Banchereau, 1996).

Only trimerized CD40 molecules are functional (Pullen et al., 1999b). Once in its trimeric aggregate in lipid rafts, CD40 can be ligated by CD154 (also known as CD40 ligand or CD40L) on CD4+ T lymphocytes. CD154 exists in both membrane and soluble forms (Noelle et al., 1997; Lane et al., 1993). The engagement of CD40 by its ligand causes the activation of tumor necrosis factor-associated (TRAF) 2 and 3 resulting in the activation of Jun N-terminal kinase and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways with subsequent activation of NF-κB (Hanissian and Geha, 1997; Leo et al., 1999; Pham et al., 2002; Pullen et al., 1999a; Tsukamoto et al., 1999). NF-κB translocation to the nucleus results in the secretion of cytokines such as IL-1, IL-2 and IL-12 and chemokines such as IL-8, MCP-1 and RANTES, molecules that increase the efficiency of antigen presentation (Pham et al., 2002; Grewal et al., 1996; van Kooten and Banchereau, 1996).

Five isoforms of CD40 RNA have been described (Tone et al., 2001). Isoform I, which codes for the surface signal transducing molecule, contains an extracellular domain with 4 cysteine repeats, a transmembrane domain and a cytoplasmic tail. Isoform II lacks both the transmembrane domain as well as the cytoplasmic tail. Isoforms III and IV have truncated cytoplasmic tails, while isoform V lacks a transmembrane domain. Isoforms I
and II are the most abundant forms in LPS-stimulated macrophage cell lines and bone marrow-derived DCs, while III, IV, and V are detectable, but at extremely low levels (Tone et al., 2001).

2. CD80 and CD86

For T cells to acquire the functions of effector cells, a second transmembrane signal is required (Geppert and Lipsky, 1986). This second signal is provided by co-stimulatory molecules, CD80 (B7.1) and CD86 (B7.2), expressed on APCs including dendritic cells and certain macrophages (Linsley and Ledbetter, 1993; June et al., 1994; Young et al., 1992; Caux et al., 1994; Hathcock et al., 1994). CD80 and CD86 share two receptors on T cells, CD28 and CTLA4, respectively. Binding of either CD80 or CD86 to CD28 results in proliferation of the T cells and production of IL-2 (Lenschow et al., 1996; Boussiotis et al., 1996). On the contrary, binding of either CD80 or CD86 to CTLA-4 appears to activate a phosphatase enzyme pathway which results in T cell anergy (Krummel and Allison, 1995; Marengere et al., 1996). Low surface expression of these molecules -- like that of CD40 -- is characteristic of immature DCs.

C. Microglia

Microglial cells are the primary immune reactive cells in the central nervous system (CNS). They form a regularly spaced network of resident glial cells that comprise between 5-15% (development) and about 20% (adult) of the total glial cells in the CNS (Lawson et al., 1990). There are at least two subsets of microglia: perivascular, which reside within the basal lamina of capillaries, and parenchymal, which are embedded in brain matter. According to bone marrow chimera experiments, a high percentage of the perivascular cells undergo continual replacement by bone marrow-derived precursors. The parenchymal microglia, on the other hand, are derived from peripheral mesodermal
(myeloid) tissue during embryonic development (Imamoto and Leblond, 1978; Ling et al., 2001; Ling and Wong, 1993; Rezaie and Male, 1999) and form a highly stable pool of CNS cells with slight turnover (Lassmann et al., 1993). Therefore, they are morphologically, immunophenotypically and functionally related to cells of the monocyte/macrophage lineage. As such, they are capable of becoming mature antigen-presenting cells (APCs).

Microglia can become activated by a variety of stimuli. Once activated, they produce a variety of cytokines, including (but not limited to) IL-1α, IL-1β, IL-3, IL-6, IL-10, IL-12, and TNFα (Ransohoff and Brown, 2012; Woodroffe et al., 1991). In addition, they can produce prostaglandins like PGE2 (Minghetti and Levi, 1995; Minghetti et al., 1998); growth factors like vascular endothelial growth factor (VEGF) (Zand et al., 2005), brain-derived neurotrophic factor (BDNF) (Trang et al., 2011), and transforming growth factor-β (TGF-β) (Wesolowska et al., 2008); chemokines like macrophage inhibitory protein (MIP)-1α (Kremlev et al., 2004; Simpson et al., 1998); enzymes like matrix metalloproteinases (MMPs); and reactive oxygen species H₂O₂ (Colton and Gilbert, 1993), NO and others (Stoll et al., 2002). Various stimulators of microglia produce various cocktails of these molecules (Carson et al., 2004). Some of these cocktails are lethal, producing reactive oxygen species, prostaglandins, and TNFα that can kill neurons (Thery et al., 1993); some can be neuroprotective, producing IL-10, TGF-β or protective growth factors (Herx et al., 2000; Werry et al., 2011).

Monocytically-derived brain microglia are capable of expressing a mature dendritic cell (DC) phenotype. While lacking (or having very low) MHC Class II expression in vivo, microglia can be stimulated in culture to express MHC class II and
co-stimulatory molecules CD40, CD80 and CD86 that are necessary for antigen presentation and T cell activation. We and others have shown that these molecules are up-regulated upon microglial isolation in culture (Satoh et al., 1995; Wei and Jonakait, 1999). Granulocyte/monocyte colony-stimulating factor (GM-CSF) added into microglial cultures stimulates microglial proliferation, (Minghetti and Levi, 1995; Liva et al., 1999; Aloisi et al., 2000; Xiao et al., 2002; Re et al., 2002) and slightly upregulates CD11c (a DC marker), MHC class II and co-stimulatory molecules (Fischer et al., 1993; Illes et al., 2005; Re et al., 2002; Satoh et al., 1995; Wei and Jonakait, 1999; Xiao et al., 2002; Aloisi et al., 2000). These molecules can be further up-regulated by interferon-γ (IFN-γ) (DeGroot et al., 1991; De Simone et al., 1995; Frei et al., 1987; Menendez et al., 1997; Nguyen and Benveniste, 2000; Williams et al., 1994; Aloisi et al., 1999; Loughlin et al., 1993), lipopolysaccharide (LPS) (Menendez et al., 1997; Wei and Jonakait, 1999), CD40L (CD154) and unmethylated CpG dinucleotides (Dalpke et al., 2002). As a result, isolated, stimulated microglia are effective T cell activators in vitro (Fischer and Reichmann, 2001; Askew and Walker, 1996; Cash and Rott, 1994; Frei et al., 1987; Matyszak et al., 1999a; Re et al., 2002; Williams et al., 1993).

Microglia are routinely studied in isolation, neglecting the fact that they normally exist in a complex glial environment made up largely of astrocytes. In recent studies from the Jonakait lab, it has been confirmed that microglia isolated from astrocytes in culture can be stimulated to express MHC class II as well as co-stimulatory molecules. However, when they remain with astrocytes, exposure to GM-CSF and LPS – treatments that would otherwise lead to the assumption of a mature DC phenotype – are ineffective and the microglia express low levels of the molecular machinery necessary for antigen
presentation. In short, they express a phenotype reminiscent of iDCs. Thus, the astrocytic environment profoundly affects the ability of microglia to become efficient APCs. Glial tumors have a similar – and more profound – suppressive effect on the microglial DC phenotype (Kostianovsky et al., 2008) rendering microglia less than useful in mounting an immune response against glioblastomas. Studies in this thesis will address the mechanism(s) by which astrocytes inhibit this phenotype.

D. Astrocytes

Astrocytes are the most abundant cells in the brain and spinal cord and are essential for brain homeostasis. Their functions include the physical structuring of the brain, biochemical support of the endothelial cells that form the blood brain barrier (Abbott et al., 2006), provision of nutrients (Kasischke et al., 2004; Pellerin, 2005), maintenance of extracellular ion balance, modulation of synaptic transmission (Mazzanti and Haydon, 2003; Ullian et al., 2004) and, importantly, the protection, repair and scarring processes of the brain and spinal cord following traumatic injury (Bush et al., 1999; Faulkner et al., 2004; Voskuhl et al., 2009).

In response to injury or disease astrocytes, like microglia, can produce cytokines such as IL-1β and growth factors such as ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), activity dependent neurotrophic factor (ADNF), or fibroblast growth factor-2 (FGF-2) (Rudge et al., 1992; Schwartz and Nishiyama, 1994; Friedman et al., 1996). However, in contrast to some microglial cytokines, most of these molecules are considered beneficial for a damaged neuronal population (though see (Volosin et al., 2008)).
Astrocytes or conditioned medium from them play a protective role by suppressing microglial activation. They inhibit microglial production of proinflammatory mediators (Aloisi et al., 1997; Siglienti et al., 2007; Kostianovsky et al., 2008; Kim et al., 2010b), promote the production of anti-inflammatory molecules (Min et al., 2006), inhibit microglial proliferation (Jones et al., 1998), and inhibit NMDA-mediated neuronal damage (Hailer et al., 2001). Moreover, data from the Jonakait lab suggest that astrocytes suppress the ability of microglia to assume a DC phenotype (see below). Studies in this thesis are designed to address the means by which astrocytes suppress the DC phenotype.

E. Antigen presenting cells (APCs) and gliomas

Due to their differential functions in immunity, DCs, microglia and macrophages have become of great interest in studies related to cancer immunity. Renal cell carcinomas release IL-6 and monocyte colony stimulating factor (M-CSF) to inhibit DC maturation leading to reduced APC function (Cabillic et al., 2006). A population of mononuclear cells in the peritoneal cavity of patients with peritoneal carcinoma exhibit low levels of CD80 and CD86 antigens when compared to autologous peripheral blood mononuclear cells (PBMC) suggesting that the tumor is inhibiting their expression (Melichar et al., 1998). Myeloid DCs from cutaneous cell carcinoma do not efficiently stimulate T-cell proliferation (Bluth et al., 2009); IL-10-producing DCs prevent effective DC therapies in hepatocellular carcinomas (Chen et al., 2007); and the upregulation of IL-1 receptor associated kinase-M (IRAK-M) by a variety of tumor cells render monocytes insensitive to TLR signaling (del Fresno et al., 2005) (see below). DCs along with macrophages, microglia and T cells have the capacity to infiltrate gliomas (Hussain
et al., 2006). However, once these cells reach the gliomas, these tumors – like normal astrocytes -- suppress or promote defective differentiation of monocytic-derived DCs (Kostianovsky et al., 2008). Moreover, while CD11b/c⁺CD45⁺ glioma-infiltrating microglia/macrophages (GIMs) express substantial levels of Toll-like receptors (TLRs), they don’t become stimulated to produce the pro-inflammatory cytokines necessary to promote T-cell proliferation. In addition, despite surface major histocompatibility complex class II expression, they lack expression of the co-stimulatory molecules CD86, CD80, and CD40 which is critical for T-cell activation (Hussain et al., 2006). Thus, elucidating the mechanism(s) by which astrocytes quash the DC-like phenotype in microglia may facilitate an understanding of immune suppression by gliomas.

**F. Signaling pathways and their inhibitors**

1. Toll like receptor 4 (TLR4): MyD88 dependent pathway

Following the protocol of others, LPS is used here as a “maturing” signal for the microglial DC phenotype. In our first Specific Aim, we will examine whether endogenous inhibitors of the LPS signaling pathway may be involved in stunting the expression of MHC Class II and co-stimulatory molecules.

When activated by LPS, the TLR4 homodimer causes the assembly and activation of myeloid differentiation primary response protein 88 (MyD88) and Tirap/Mal at the cell membrane (Xu et al., 2000). The gathering of these molecules leads to the recruitment of the serine/threine Interleukin Receptor Associated Kinases (IRAK)-1 and IRAK-4. Subsequent phosphorylation of these kinases leads to their dissociation from the membrane complex allowing association with TNF Receptor Associated Factor-6 (TRAF-6). TRAF-6 then activates TGF-β-Activating Kinase (TAK-1) through a linking
adaptor molecule TAK-1 Binding protein (TAB1/2). TAK-1 in turn causes the phosphorylation and activation of IkB kinases (IKKα and IKKβ) which directly phosphorylate members of the inhibitory IkB family. This eventually enables the activation and translocation of NFκB into the nucleus. Once in the nucleus, NFκB will induce the production of pro-inflammatory proteins and, in this case, the co-stimulatory molecules necessary for antigen presentation (Qin et al., 2005; Wu et al., 2008) (Figure 4).

The LPS signaling pathway can be inhibited at various points. Data from others suggest that tolerance to LPS signaling may involve the down-regulation of IRAKs 1 and 4 (possibly through the action of ubiquitin protein isopeptide ligases known as Pellino proteins), action(s) of Suppressor Of Cytokine Signaling-1 (SOCS-1) and/or SOCS-3 (Frobose et al., 2006; Mansell et al., 2006), degradation of TAB1 and/or TAB2 by activation of E3 ubiquitin ligases like TRIpantite Motif containing 30 (TRIM30α; (Shi et al., 2008), and/or the up-regulation of the IRAK inhibitor IRAK-M (IRAK-3; (Rosati and Martin, 2002; Kobayashi et al., 2002; Nakayama et al., 2004; van't Veer et al., 2007). One means by which astrocytes could affect the microglial DC phenotype is by promoting the production of these inhibitors. This possibility will be addressed in the studies described below.

2. IRAK-M: LPS signaling inhibitor

Human IRAK-M is a signaling molecule which contains an NH2-terminal death domain and a central kinase domain similar to the domain structures of IRAK-1 and IRAK-2 (Wesche et al., 1999). While subsequent studies identified IRAK-M as an inhibitor of LPS signaling (Kobayashi et al., 2002), Wesche et al. showed that
overexpression of IRAK-M is similar to IRAK-2 in activating NF-kB. However, it is not as efficient as IRAK-1. By contrast, the same group showed that overexpression of the death domains of these three IRAK molecules are alike in being able to inhibit IL-1-induced NF-kB activation. In support of its role as a TLR-signaling activator, IRAK-M binds MyD88, but it does not possess autophosphorylation activity. Nevertheless, it can be activated by IRAK-1 to undergo autophosphorylation (Wesche et al., 1999) and release from the TLR.

Murine IRAK-M shows 71% sequence identity to the human IRAK-M and shares the same structure with the same highly conserved death and kinase domains. Murine IRAK-M acts like its human counterpart when it has to do with IL-1 signal transduction. Even though human IRAK-M has weak autophosphorylation capability (see above), murine IRAK-M has autophosphorylation activity and can induce activation of NF-kB (Rosati and Martin, 2002).

As mentioned above, IRAK-M also has inhibitory properties (Kobayashi et al., 2002). IRAK-M−/− cells show an increased cytokine production and an increased inflammatory response upon TLR/IL-1 stimulation. There is a more rapid phosphorylation and degradation of IkBα and a stronger and faster phosphorylation of JNK, p38 and ERK1/2 in IRAK-M−/− macrophages when compared to wild type. Others have identified IRAK-M as a specific negative regulator of p38 (Su et al., 2007) and as an inhibitor in the non-canonical NFκB pathway (Su et al., 2009). Elimination of IRAK-M in antigen-pulsed DCs promotes maintenance of a mature phenotype and prolongs their survival (Turnis et al., 2010).
Due to this evidence, in our studies (see below) we have used IRAK-M\(^{+/+}\) animals on the assumption that IRAK-M is acting as an LPS signaling inhibitor.

**3. TLR4: MyD88 independent pathway/IFN\(\beta\) production**

A MyD88-independent signaling pathway for LPS also exists. In studies using MyD88-deficient mice, LPS could still induce activation of NF\(\kappa\)B but with delayed kinetics when compared to wild-type cells (Kawai et al., 2001). MyD88-deficient mice also lost their ability to produce pro-inflammatory cytokines TNF\(\alpha\), IL-1\(\beta\), IL6 and IL-12p40 in response to LPS, but retained the ability to produce interferon (IFN)-inducible genes and co-stimulatory molecules. Moreover, their DCs matured in response to LPS (Kaisho et al., 2001). In the MyD88-independent pathway, LPS binds to TLR4 causing the recruitment of TRIF-Related Adaptor Molecule (TRAM) which in turn recruits the TIR domain-containing adaptor Inducing IFN\(\beta\) (TRIF) (Oshiumi et al., 2003). At this point, TRIF can interact with different proteins. It can cause NF\(\kappa\)B activation by binding to either TRAF6 or kinase Receptor-Interacting Protein 1 (RIP1; Palsson-McDermott and O'Neill, 2004). It can also associate with TRAF family member-Associated NF\(\kappa\)B activator (TANK)-Binding Kinase 1 (TBK1) and to inducible IKK (IKK-i), which in turn phosphorylates Interferon Response Factor 3 (IRF3), leading to its nuclear translocation and induction of interferon beta (IFN\(\beta\)) and co-stimulatory molecules (Yamamoto et al., 2002; Yamamoto et al., 2003b; Yamamoto et al., 2003a).

IFN\(\beta\) acts in an autocrine way ligating the IFN\(\beta\) receptor thereby activating the JAK/STAT pathway (Figure 5). Once activated, this pathway causes the up-regulation of CD40 (Qin et al., 2005). In addition, however, IFN\(\beta\) inhibits its own action by inducing the production of SOCS-1 and 3, inhibitors of STAT-1\(\alpha\) activation (Qin et al., 2006a).
Therefore, overproduction of SOCS-1, whether generated by IFNβ or by some other mechanism, would be expected to result in the inhibition of IFNβ-induced CD40 expression. The possibility that this MyD88-independent pathway is involved in the down-regulation of the DC phenotype will be examined in studies described below.

4. Suppressor of Cytokine Signaling 1 and 3 (SOCS1/3)

Suppressors of Cytokine Signaling are a family of molecules comprising eight members (SOCS1-7 and Cytokine inducible SH2-containing protein [CIS]). Each consists of a conserved box, a central SH2 domain and an N-terminus of different length and organization (see reviews by (Dimitriou et al., 2008; Palmer and Restifo, 2009). SOCS1 can be activated by LPS and is a notable inhibitor of type 1 interferons (interferon α [IFNα] and IFNβ) as well as IFNγ (see review by (Baker et al., 2009). Inhibition is largely due to the blockade of STAT1 phosphorylation by Janus kinases (Jaks). This prevents nuclear translocation (Fenner et al., 2006) and the binding of STAT1 to GAS sites on relevant promoters. By blockade of interferon signaling, SOCS1 prevents IFNγ-induced expression of MHC Class II (O'Keefe et al., 2001) and CD40 (Wesemann et al., 2002) in macrophages as well as IFNβ-induced expression of CD40 in macrophages and microglia (Qin et al., 2006a). In addition to inhibiting interferon signaling, SOCS1 can inhibit LPS signaling by inhibiting p65 phosphorylation with subsequent inhibition of NFκ-B activation (Mansell et al., 2006).

SOCS3 can be upregulated by LPS, IL-10, IFNγ, IFNβ and IL-6 and GM-CSF (Stoiber et al., 1999; Cassatella et al., 1999; Bode et al., 1999; Starr et al., 1997) but through different mechanisms. SOCS3 not only inhibits STAT1 and STAT3 phosphorylation (Braunschweig et al., 2011; Usui et al., 2004; Naka et al., 1997; Endo et
al., 1997), but also interrupts signaling through the gp130 co-receptor subunit of the IL-6 receptor (Lehmann et al., 2003) and through the phosphotyrosine 729 on the granulocyte colony-stimulating factor (G-CSF) receptor (Hortner et al., 2002).

Importantly for our studies, LPS induction of CD40 is maximal when both NFκ-B and IFNβ are activated (Qin et al., 2005). While NFκ-B directly induces CD40 expression, IFNβ works indirectly to stimulate STAT1 activation, nuclear translocation and subsequent CD40 induction. Once produced by LPS and/or IFNβ, SOCS3 inhibits LPS-induced IFNβ-induced STAT1 phosphorylation. This, in turn, prohibits the STAT1 nuclear translocation and subsequent production of CD40 (Qin et al., 2006b). This pathway is considered an important regulatory pathway for attenuating LPS signaling.

II. Rationale and Aims

Microglia cells have been studied extensively in isolated cell culture where they attach readily to tissue culture plastic and can be easily manipulated. When stimulated in this environment with viral or bacterial mimics, they express proinflammatory chemokines and cytokines, and can also be prompted to express a dendritic cell (DC) phenotype, allowing microglia to perform as professional antigen-presenting cells (APCs). Our laboratory has been interested in studying the ability of microglia to assume this DC phenotype, but most importantly how this phenotype might be affected by the presence of other cells -- primarily astrocytes. We have found that microglia, cultured in a more complex glial environment behave very differently from their isolated sisters. Microglia co-cultured with astrocytes express significantly lower levels of surface co-stimulatory molecules (CD40, CD80 and CD86) when compared to isolated microglia...
Several mechanisms can be invoked to explain this inhibition. These will be examined in 2 Specific Aims:

**Specific Aim 1.** To determine whether alterations within the MyD88-dependent and/or MyD88 independent TLR4 and/or IFNβ signaling pathways account for the differences in CD40 surface expression. Endogenous inhibitors of the MyD88-dependent and IFNβ signaling pathways such as TRIM30α, IRAK-M, SOCS1 and SOCS3, as well as the product of the MyD88-independent pathway, IFN-β, will be examined.

Specific Aim 2. To examine whether low CD40 surface expression in co-cultured microglia is due to differential regulation and/or trafficking. Experiments will focus on the total mRNA and protein expression, rate of decay and intracellular distribution.

**III. Materials and Methods**

**Reagents**

Recombinant murine GM-CSF is from PeproTech. Lipopolysaccharide (055:B5) is from Sigma-Aldrich. 4’, 6-diamidino-2-phenylindole, dilactate (DAPI, dilactate) and ProLong® Gold antifade reagent is from Invitrogen. Cycloheximide and Actinomycin D are from Sigma. ER-Tracker™ Dyes for Live-Cell Endoplasmic Reticulum Labeling from Molecular Probes (Invitrogen Detection Technologies). LysoTracker® from Molecular Probes (Invitrogen Detection Technologies). Antibodies used in this study are listed in Table 1.

**Mice**

C57BL/6, B10a mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IRAK-M−/− were described previously (Kobayashi et al., 2002). These mice on a
129/B6 background were obtained from Taconic Labs through the good offices of Richard A. Flavell (Yale University School of Medicine, New Haven, CT) and Koichi Kobayashi (Harvard Medical School, Boston, MA). All mice were housed in the Rutgers/Newark AAALAC-approved animal facility. Animals were mated on a regular basis to obtain neonatal pups for microglial cultures.

**Preparation of microglial cultures**

Mixed glial cultures were prepared as described previously from the cortices of neonatal mouse pups (Jonakait et al., 1996; Jonakait and Ni, 2009; Ni et al., 2007). 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 (Cell Generation, Ft. Collins, CO). 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 (350 RPM X 40 min). Floating cells were collected and plated onto uncoated 75 cm² flasks or onto glass coverslips and allowed to adhere to the substrate before being treated with GM-CSF and LPS. Microglia that remained in the mixed glial cultures were considered to be “co-cultured.”

Unless stated otherwise both mixed glial and isolated microglial cultures were treated with rmGM-CSF (PeproTech, Rocky Hill, NJ, 25 ng/ml) for 5 days and LPS (055:B5, Sigma-Aldrich, 50 ng/ml) for various periods of time noted in each experiment.
Prior to analysis, co-cultured microglia were shaken off astrocytes (350 X 30 min) so that analysis was of microglia alone, not of astrocytes. In some cases, astrocytes were analysed separately.

**Reverse-transcription Polymerase chain reaction (RT-PCR)**

mRNA was prepared from microglia harvested from co-culture or isolation culture using TRIzol® Reagent (Ambion by Life Technologies, Carlsbad, CA). cDNA was produced using 1 μg of RNA, MMLV reverse transcriptase (Promega, Madison, WI) and random hexamers. For amplification by thermocycler, forward and reverse primers for CD40 were introduced together with Taq® polymerase master mix (Promega) and PCR conditions were 5 minutes at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 51.2°C, and 1 min at 72°C. Samples (10 μl/well) were size-fractionated on a 1.8% agarose gel containing ethidium bromide (0.5 μg/ml) and bands are visualized with UV light.

**Quantitative real-time PCR (QRT-PCR)**

Cells were harvested for the determination of co-stimulatory molecules expression by quantitative real time-PCR. In these cases total microglial RNA was prepared using TRIzol® Reagent. cDNA was produced from 0.5 μg of RNA by using random hexamer and MMLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions. For QRT-PCR, cDNA was amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. The primers used for amplification were obtained from Integrated DNA Technologies, Inc. (IDT, Coralville, IA) and were designed using Primer Express® (Applied Biosystems, Foster City, CA) or PubMed.gov. All primers used are
list in Table 2. 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 was 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).

For detection of microRNA expression (see Appendix B), 5 ng of total RNA was reverse transcribed using a reaction master mix containing random hexamers, specific stem-loop microRNA primers and MuLV MultiScribe™ Reverse Transcriptase according to the manufacturer’s protocol (TaqMan® MicroRNA Reverse Transcription Kit, Applied Biosystems). For amplification, PCR conditions were 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Primers were pre-designed by Applied Biosystems using the miRBase miRNA sequence repository. The PCR products were detected using the ABI Prism 7900HT Sequence Detection System running with RQ Manager 1.2 (Applied Biosystems, Foster City, CA). Data were analyzed using DataAssist™ v1.0 Software which uses the \( C_T (\Delta \Delta C_T) \) method for determining a relative measure of gene expression. Data were expressed as arbitrary units of specific microRNA relative to a standard (miRsno202 or miRsno234).
**Flow cytometry**

Microglia were harvested from mixed glia or from isolation culture and incubated for 30 min. at 4°C in buffer containing 2% fetal bovine serum in PBS with PE-labeled hamster anti-mouse CD11c (7 µg/ml; BioLegend). After 30 min FITC-conjugated hamster anti-mouse CD40 (5 µg/ml; BD Biosciences) or FITC-conjugated CD80, CD86 or MHC Class II (10 µg/ml; BD Biosciences) were added for an additional 30 min. Non-specific binding was minimized by preincubation with FcBlock™ (BD Bioscience) used according to manufacturer’s instructions. Other cells were incubated with isotype controls (PE-labeled hamster anti-IgG [7 µg/ml; BioLegend], FITC-labeled hamster anti-IgM [5 µg/ml; BD Biosciences]), FITC-labeled mouse IgG2aκ, hamster IgG2κ, rat IgG2aκ (10 mg/ml; BD Biosciences). Cells were then washed, pelleted, and resuspended in the same buffer for flow cytometric analysis using a FACSCalibur™ flow cytometer (Becton Dickinson, Mountainview, CA). For some experiments cells were fixed and permeabilized for 30 min (Fixation/Permeabilization Concentrate and Diluent, eBiosciences). After an initial staining with antibodies against CD11c and CD40, cells were then divided into two groups, and one group was re-stained with antibodies against CD40.

Data analysis was accomplished using CellQuest® software. Gating was done by using the appropriate isotype controls as reference.

**Western blotting**

Microglia were harvested from mixed glia or from isolation culture at various time points after LPS treatment. Cells were lysed in buffer containing 10% glycerol, 1% NP40, 2.5 mM EDTA (pH 8.0), 2.5 mM EGTA (pH 8.0), 150 mM NaCl and Protease
Inhibitor Cocktail (1:100; Sigma) in a 20 mM Tris buffer (pH 7.4). Protein concentration was measured using a Bio-Rad protein assay. Protein samples (30 µg each) were size-fractionated on a 15% SDS-polyacrylamide gel, and transferred overnight onto nitrocellulose membranes. After blocking with 5% milk, the membranes were probed with antibodies against CD40 (HM40-3, 1:100), SOCS3 (1:250), or IRAK-M (1:250). All antibodies were diluted in buffer containing 5% BSA in 1X Tris-buffered saline containing 0.05% Tween 20 (TBST). After vigorous washing in TBST, membranes were probed with HRP-conjugated secondary antibodies (1:2500) in 5% milk in TBST. For HM40-3, the secondary antibody (1:1250) was in 5% BSA in TBST. Bands were detected by enhanced chemiluminescence (ECL).

**Measurement of protein degradation rate**

Isolated and co-cultured microglia were treated for 5 days with GM-CSF and then with LPS for 16 hrs at which point cycloheximide (CHX, 10 µg/ml) was added to inhibit translation. At the various time points after the addition of CHX, cells were harvested and assessed for surface protein expression of CD11c and CD40 by flow cytometry. The percent of CD11c⁺CD40⁺ cells and the MFI remaining at each time point was determined. Data were then expressed as the logarithm of that percentage. Differences between the logs at time intervals 0-4, 4-8, 8-16 and 16-24 hrs were calculated for both co-cultured and isolated microglia. Those differences were compared with an ANOVA and a post-hoc Student-Newman-Keuls test to obtain a measure of statistical significance.
**Measurement of RNA degradation rate**

Isolated and co-cultured microglia were treated for 5 days with GM-CSF and then with LPS for 3 hrs at which point actinomycin D (5 μg/ml) was added to inhibit transcription. After 30 min, 1, 2 and 4 hrs, cells were harvested and assessed for mRNA by QRT-PCR for CD40, CD80 and CD86. The percent of mRNA remaining at each time point was determined and that percentage log transformed. Differences between the logs at time intervals 0-0.5, 0.5-1, 1-2 and 2-4 hrs were calculated for both co-cultured and isolated microglia. Those differences were compared with an ANOVA and a post-hoc Student-Newman-Keuls test to obtain a measure of statistical significance.

**Immunocytochemistry**

Microglia were shaken from mixed glial cultures and seeded onto glass coverslips (isolated). Remaining microglia in the mixed glial environment (co-cultured) and the isolated microglia (on coverslips) were treated with GM-CSF (5 days) and LPS for various periods of time. After GM-CSF and LPS treatment, microglia in mixed glial cultures (co-cultured with astrocytes) were shaken off (350 RPM X 30 min) and seeded onto glass coverslips. For immunocytochemical detection of CD40 and EEA1, isolated and co-cultured microglia were rinsed with PBS and fixed with 4% paraformaldehyde. Co-cultured microglia were allowed to settle and attach to the coverslips for 20 min prior to rinsing and fixation.

Living isolated and co-cultured microglia were stained with ER Tracker™ (1 μM) or LysoTracker® from Molecular Probes (Invitrogen Detection Technologies). ERTracker™ is a compound in which red dyes are conjugated to glibenclamide which binds to the sulphophylyrea receptors of ATP-sensitive K+ channels in the ER.
LysoTracker® is a weak base linked to a fluorophore, but the nature of the weak base is proprietary. They were then fixed with 4% paraformaldehyde for 5 min, blocked and permeabilized with 5% FBS, 0.1% triton in PBS for 20 min. This was followed by incubation with CD40 antibody (HM40-3; 1:750) for 45 min. For antibodies against EEA1 (Santa Cruz Biotechnology), cells were fixed, blocked and permeabilized, and treated with the antibody (1:100) for 45-60 min at 37°C prior to inclusion of the CD40 antibody. Finally, the cells were stained with DAPI (300 nM for 20 min) then mounted in ProLong® Gold anti-fade reagent (Invitrogen). After 24 hrs, cells were examined using a Zeiss LSM 510META confocal laser scanning microscope (Carl Zeiss, USA) and analyzed with ImageJ software.

**Statistical analysis**

Relevant statistical methods are described in figure legends.

IV. Results

**Specific Aim 1. To determine whether alterations within the MyD88-dependent and/or MyD88 independent TLR4 and/or IFNβ signaling pathways account for the differences in CD40 surface expression.** Endogenous inhibitors of the MyD88-dependent and IFNβ signaling pathways such as TRIM30α, IRAK-M, SOCS1 and SOCS3, as well as the product of the MyD88-independent pathway, IFN-β, will be examined.

1.1 Endogenous inhibitors of the LPS MyD88-dependent and IFNβ signaling pathways are differentially regulated in co-cultured and isolated microglia

In all preliminary experiments LPS has been used as a maturing signal for the DC phenotype. The rapid rise of certain mRNA molecules in co-cultured microglia (see below) indicates that initial LPS signaling is intact. We speculated, however, that
endogenous LPS inhibitors might prematurely quash signaling. Data from others have suggested that tolerance to LPS signaling may involve the inhibition by IRAK-M of IRAK-1 and -4 binding, action(s) of SOCS-1 and/or SOCS-3 suppressing LPS-induced IκB phosphorylation (Frobose et al., 2006; Mansell et al., 2006; Kobayashi et al., 2002), and/or the activation of E3 ubiquitin ligases like TRIM30α (Shi et al., 2008). In order to determine whether any of these inhibitors of LPS signaling were differentially regulated, we examined the temporal expression of IRAK-M, SOCS-1/3 and TRIM30α following LPS treatment in isolated and co-cultured microglia to determine whether a premature elevation of these inhibitors might be responsible for a limited response to LPS (Figure 6). Microglia were cultured with or without astrocytes (see Materials and Methods). After a 5-day treatment with GM-CSF (25 ng/ml), cells were treated with LPS (50 ng/ml) and harvested at various time points after the beginning of LPS treatment. Cells were prepared for QRT-PCR analysis for TRIM30α (A), IRAK-M (B), SOCS1 (C) and SOCS3 (D). In these, as in all other experiments, co-cultured microglia were shaken off of astrocytes prior to analysis. Thus, data for “co-cultured” microglia represent only mRNA from these cells and do not include mRNA from astrocytes.

IRAK-M, TRIM-30α and SOCS3 were significantly higher in co-cultured microglia throughout the course of LPS treatment leading us to hypothesize that one or all of these might be involved in limiting LPS signaling.

1.2 SOCS3 protein levels are unchanged

We examined further the possibility that the elevated levels of SOCS-3 mRNA seen in the co-cultured microglia resulted in higher levels of SOCS-3 protein with concomitant inhibition of LPS signaling. However, Western blot analysis of SOCS-3
protein indicated that despite elevated message levels, protein levels were the same in both culture conditions (Figure 7). Because neither SOCS1 message levels nor SOCS-3 protein levels were different in the two culture conditions, it seemed unlikely that these were responsible for the observed differences in co-stimulatory molecule expression. Furthermore, while the increase in TRIM30α expression suggested the possibility that it played a role in inhibiting LPS signaling, no reagents (e.g., antibodies) were available to study it further. Thus, we did not study SOCS1, SOCS-3 and TRIM30α further.

1.3 IRAK-M regulates CD11c, but not CD40

The significantly higher level of IRAK-M expression in microglia cultured with astrocytes prompted us to examine whether the presence of IRAK-M in co-cultured microglia might prevent the full surface expression of the co-stimulatory molecule CD40. Two strategies were used to address this question. Western blot analysis showed that IRAK-M protein expression tracks mRNA expression. Microglia cultured with astrocytes that had shown high mRNA levels showed high levels of the translated protein as well (Figure 8A, B) suggesting that IRAK-M may be functioning as an LPS signaling inhibitor. Consistent with the low levels of mRNA in the isolated cells, there was no detectable IRAK-M protein in the isolated cells (Figure 8B). The disparity in IRAK-M expression between the isolated and the co-cultured microglia suggested that IRAK-M expression might be involved in down-regulating co-stimulatory molecule expression. In order to determine whether IRAK-M played a pivotal role in inhibiting co-stimulatory molecule expression, we used a second strategy and obtained IRAK-M knockout mice. Microglia were cultured from IRAK-M knockouts (KO) or wild-type animals in the presence or absence of astrocytes. We reasoned that if IRAK-M were essential, its
absence would lead to equivalent levels of co-stimulatory molecule expression. In these experiments we focused on CD40 as a representative of co-stimulatory molecules. These experiments were performed twice with somewhat equivocal results.

The absence of IRAK-M had a negligible effect on CD40 surface expression in both isolated and co-cultured microglia. By contrast, CD11c, the dendritic cell marker, was consistently depressed in the IRAKM KO isolated microglia (Figure 8C). In this experiment, CD11c was also severely down-regulated in the co-cultured microglia, but this was not the case in the second experiment. The IRAK-M mice proved to be difficult to mate, so we were unable to perform the experiment a third time. We tentatively concluded, however, that IRAK-M was not responsible for the down-regulation of CD40 in the co-cultured cells. If IRAK-M affected anything, it seemed to be CD11c.

1.4 IFNβ differentially regulates CD40 and CD11c

A MyD88-independent LPS signaling pathway also exists which results not only in the activation of NF-κB (see Introduction and Background) but also the activation and nuclear translocation of interferon responsive factor-3 (IRF-3). This pathway results in the elaboration of IFNβ which itself upregulates the expression of CD40 on monocytes (Marckmann et al., 2004; Wiesemann et al., 2008) and possibly on microglia (Qin et al., 2005). Similar to the data of Qin et al., LPS in both culture settings induced substantial levels of IFNβ mRNA, but isolated microglia expressed mRNA quickly (within 3 hrs) and at significantly higher levels than co-cultured microglia (Figure 9A) suggesting that IFNβ expression might be playing a role in the upregulation of CD40 expression in isolated cells. Thus, we sought to determine whether IFNβ production was having a differential effect on microglial surface CD40 expression in the two culture settings by
neutralizing its action using an antibody against it. Isolated and co-cultured microglia were thus treated for 5 days with GM-CSF as before, but during a 24-hr LPS treatment, a rat antibody against IFNβ (3.5 mg/ml) was added to some of the cultures. These were compared to cultures that did not receive the antibody. Cells were analyzed after culture by flow cytometry. If IFNβ were responsible for the disparity between the two culture settings, we expected that its neutralization would down-regulate CD40 expression in isolated microglia to the same level as that seen in co-cultured microglia. However, neutralization of IFNβ had no apparent effect on CD40 surface expression in co-cultured microglia, but had the expected (though modest) down-regulatory effect in isolated microglia (Figure 9B). More strikingly, the IFNβ antibody produced a substantial loss of CD11c expression particularly in isolated and co-cultured microglia. Thus, CD11c and CD40 were differentially regulated by IFNβ. However, the presence or absence of IFNβ could not explain the difference that we detected in the percentage of cells expressing CD40 since that difference remained intact.

**Conclusion for Specific Aim 1:** In our culture protocol (following what is routine for the maturation of peripheral dendritic cell cultures), isolated microglia and co-cultured microglia were treated for 5 days with GM-CSF and then for 2 days with LPS. Cells were routinely analyzed by flow cytometry only after the 48 hr LPS treatment, and they were analyzed as double-stained cells. That is, cells were stained for both CD11c as well as CD40 (or CD80, CD86), and the percentage of double-stained cells reported. Finding that a lower percentage of co-cultured microglia were thus double-stained, we began this aim with the erroneous assumption that some endogenous LPS signaling inhibitor must be responsible for this down-regulation. This was erroneous for two reasons: 1) LPS
signaling was intact (mRNA even for the “inhibitors” was elevated within 1-3 hrs after LPS signaling), and 2) we failed to recognize the role that CD11c played in the assessment of a mature dendritic cell phenotype. What became evident in these experiments was that the lower percentage of co-cultured cells expressing the DC phenotype was due primarily to the lower expression of CD11c, not to a lower expression of CD40. Thus, in Figure 12B, one notes that the percentage of CD40 stained cells is similar between the isolated and co-cultured microglia. What is different is the expression of CD11c. Therefore, when examining double-stained cells, it is the CD11c that is lower and brings the percentage down. This is further substantiated in experiments done for Specific Aim 2.

CD11c is an \( \alpha_\beta_2 \) integrin; it is the complement receptor 4 (CR4) for iC3b (Chen et al., 2012; Harris et al., 2000); and it binds LPS as well as osteopontin (Schack et al., 2009). As an integrin, it facilitates both adhesion and migration of leukocytes on both fibrinogen (Kukhtina et al., 2011; Georgakopoulos et al., 2008) and VCAM1 (Gower et al., 2011). From the point of view of antigen presentation, studies have shown that targeting antigen to CD11c with an agonist antibody generates a more robust response to low concentrations of antigen. Moreover, such targeting also acts as a powerful adjuvant (White et al., 2010; Wei et al., 2009). Since CD11c is necessary for the development of experimental autoimmune encephalomyelitis (EAE) – an animal model of the autoimmune disorder, multiple sclerosis – it is clear that CD11c plays a role in effective antigen presentation (Bullard et al., 2007) and that astrocytes, by limiting CD11c expression, may blunt effective antigen presentation.
We found that knocking out IRAK-M seemed to have its primary effect on CD11c, not CD40. If IRAK-M is indeed an LPS signaling inhibitor (Kobayashi et al., 2002; Wesche et al., 1999) whose upregulation results in tolerance to LPS signaling (i.e., a bacterial challenge), then its absence should *enhance* a response to subsequent LPS challenge. One would hypothesize that the absence of IRAK-M would leave the brain susceptible to an over-reactive immune response. Indeed, studies from the obesity literature have shown that the presence of damaging inflammation in adipose tissue macrophages is associated with a down-regulation of IRAK-M (Hulsmans et al., 2012). Thus, it is puzzling that the percentage of cells expressing CD11c – necessary for efficient antigen presentation – should decline. We cannot adequately explain this finding based on the role of IRAK-M as an inhibitor of LPS signaling. LPS activates SOCS1 and SOCS3, both inhibitors of CD40 expression (Qin et al., 2006a; Qin et al., 2006b; Qin et al., 2007), but a similar regulation of CD11c by SOCS has not been described. None of these facts would account for the selective effect seen on CD11c. We can speculate – on the basis of its role as an inhibitor -- that in the absence of an IRAK-M brake, a strong LPS signal preferentially activates a downstream inhibitor of CD11c expression in microglia or alters the astrocytic environment in such a way that CD11c expression is even further suppressed.

On the other hand, while many consider IRAK-M as a signaling inhibitor, other studies suggest that it, like IRAK1 and IRAK4, is an activator of LPS signaling (Wesche et al., 1999). Thus, if it is, indeed, an activator in this setting, it is easier to understand the loss of CD11c expression. Further study is needed to sort out the role of IRAK-M in this particular setting.
In our hands, the absence of IFNβ also resulted in a down-regulation of CD11c. In isolated cells, both CD40 and CD11c were down-regulated when IFNβ was neutralized. Given studies showing IFNβ upregulation of CD40 (Qin et al., 2005), this was expected. In co-cultured cells, on the other hand, only CD11c was down-regulated (Figure 9B). The action of IFNβ on DC populations is varied and complicated. It appears to be an activator of CD40 expression in macrophages, microglia and splenic DCs (Qin et al., 2005), but inhibits maturation and activation of Langerhans cells (Fujita et al., 2005). Other studies have confirmed a positive effect on DC maturation even in the face of inhibitory influences from tumor cells (Nunez et al., 2012). On the other hand, it downregulates CD11c expression on certain myeloid DC populations (Huang et al., 2005; Zang et al., 2004) including microglia. This latter effect is secondary to an IFNβ-induced upregulation of SOCS1 (Qin et al., 2006a). While we might conclude from our study that LPS-induced IFNβ had a role in the upregulation of CD40 and CD11c in isolated microglia, its function is not as clear in the more complex glial environment. We do know that astrocytes blunted its upregulation in microglia. However, why its neutralization only affected CD11c in this setting is unclear.

**Specific Aim 2. To examine whether low CD40 surface expression in co-cultured microglia is due to differential regulation and/or trafficking. Experiments will focus on the total mRNA and protein expression, rate of decay and intracellular distribution.**

2.1 Co-stimulatory molecules are differentially regulated by GM-CSF and LPS in co-cultured and isolated microglia

Because a higher percentage of isolated microglia express a mature DC phenotype, we sought to examine in more detail the temporal expression of co-stimulatory molecules by examining mRNA during the full course of GM-CSF and LPS
treatment. Isolated and co-cultured microglia were treated for various times with GM-CSF. Microglia cells from both culture conditions were examined by QRT-PCR for MHC Class II and the various co-stimulatory molecules. Co-cultured microglia were removed from astrocytes prior to analysis. Isolated microglia responded promptly to GM-CSF treatment with increases in mRNA for co-stimulatory molecules and MHC Class II (Figure 10 A-D). All were elevated by 3 or 6 hours after the beginning of treatment.

mRNA levels of these molecules in co-cultured microglia lagged behind, rarely attaining the levels seen in the isolated microglia. These data suggest that the microglial response to GM-CSF is inhibited or retarded by the presence of astrocytes. A similar time-course following LPS treatment was performed. After a 5-day treatment with GM-CSF, cells were treated for various times with LPS and collected for QRT-PCR analysis. In these experiments, co-cultured microglia, not isolated microglia responded more rapidly and with higher levels of mRNA (Figure 10 E-H). In microglia co-cultured with astrocytes mRNA levels for CD40, CD80 and CD86 increased rapidly after LPS treatment, 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 below those of co-cultured microglia. In the case of CD86 and MHC Class II, these levels were strikingly lower. Because co-cultured microglia respond promptly and robustly to LPS signaling, these data confirm that an inhibition of LPS signaling itself is not responsible for the low surface expression on co-cultured cells.
2.2 Microglia express different CD40 isoforms

The disparity between low surface expression and high mRNA levels in the LPS-treated, co-cultured microglia led to a more careful evaluation of the isoforms of mRNA that were produced. This was prompted by finding evidence in the literature of five separate isoforms of CD40 RNA (Tone et al., 2001). Isoform I, which codes for the surface signal-transducing molecule, contains an extracellular domain with 4 cysteine repeats, a transmembrane domain and a cytoplasmic tail. Isoform II lacks both the transmembrane domain as well as the cytoplasmic tail. Importantly, these authors suggest that isoform II inhibits the production of isoform I. This suggested the possibility that in our system, an upregulation of isoform II may have inhibited the expression of the signal-transducing isoform I. Three other isoforms were described that include isoforms III, IV and V. III and IV have truncated cytoplasmic tails, while V lacks a transmembrane domain. Isoforms I and II are the most abundant forms in LPS-stimulated macrophage cell lines and bone marrow-derived DCs, while III, IV, and V are detectable, but at extremely low levels (Tone et al., 2001). Using both N-terminal directed and C-terminal directed antibodies, the most abundant protein detected by Tone et al. is a 43 kDa protein presumably derived from isoform I. A smaller protein of approximately 35 kDa, presumably derived from isoform II, is detected as well, but only by the N-terminal directed antibody.

In order to examine the isoforms produced in our system, PCR was employed using the same primers utilized by these authors and by us above (Tone et al., 2001). This analysis, while not quantitative, reveals that the different isoforms are expressed in both co-cultured and isolated microglia (Figure 11). Some differences were detected. While
both expressed the higher molecular weight isoforms (probably types III, I and IV respectively), lower molecular weight forms differed. Co-cultured microglia show an isoform slightly smaller than type IV, possibly type II, at early time points following LPS treatment. This isoform is missing in the isolated cells. This is intriguing if, indeed, it is the same Type II isoform that is suggested as an inhibitor of the signal-transducing Type I. The smallest isoform (at approximately 190 base pairs), which is not described by Tone et al., is high in isolated cells following GM-CSF treatment, but declines over the 48 hr time course of LPS treatment. Without sequencing these isoforms, however, few conclusions can be drawn from the data. However, the presence of multiple isoforms of CD40 mRNA raised the possibility that there is also variability in the protein expressed by microglia in different environments. This is examined below in section 2.5.

2.3 LPS induces CD40 surface expression in co-cultured microglia in a time-dependent manner, but does not increase its MFI

The possibility existed that the disparity detected between the level of mRNA and the surface protein levels of CD40 in co-cultured microglia was because surface protein had been placed on the surface prior to our analysis at 48 hrs. In short, we missed the peak of expression. Thus, in order to determine whether CD40 protein surface expression tracked mRNA levels and to more carefully analyze temporal expression of that surface protein, we measured its expression and that of CD11c over time using flow cytometry (Figure 12). This analysis revealed that even at the end of a 5-day treatment with GM-CSF (time = 0 hrs), almost 100% of isolated microglia cells expressed CD40 and CD11c even in advance of LPS treatment (Figure 12A). Subsequent LPS treatment of the isolated microglia did not increase that percentage further (Figure 12A). However, the mean fluorescence intensity (MFI, a measure of the number of molecules/cell) increased
substantially (Figure 12C). Moreover, in the isolated microglia, most CD11c\(^+\) cells were also positive for CD40. By contrast, in co-cultured microglia a low percentage of cells express CD40, CD11c and double-positive staining at the end of GM-CSF treatment, but LPS caused those levels to gradually increase (Figure 12B). Nevertheless, CD11c expression never reached that of isolated cells; therefore, the percentage of cells expressing both markers remained low. In accord with our previous data, at the end of the 48-hr LPS treatment with LPS, the percentage of CD11c\(^+\)CD40\(^+\) cells in isolated microglia was approximately 70\% while the percentage in co-cultured microglia was closer to 50\% as indicated on the figure. Unlike their isolated counterparts, co-cultured microglia maintained constant and low MFI levels (Figure 12C).

2.4 mRNA degradation does not account for differences in co-stimulatory molecule expression

Even though the percentage of cells expressing CD40 attained high levels in the co-cultured microglia, the MFI remained low. We speculated that this could be due to a rapid turnover of the mRNA for CD40. In order to determine the rate of RNA decay, microglia grown in the two culture conditions were treated with LPS for 3 hours after GM-CSF treatment. At the end of this time, cells were treated with actinomycin D (5 \(\mu\)g/ml) and steady-state levels of mRNA were monitored by QRT-PCR over time (Figure 13A, B and C). Data are expressed as the logarithm of the percent of mRNA remaining at each time point, and the differences between the adjacent time points compared with an ANOVA to obtain a measure of statistical significance which is noted on each graph. The rate of decay of mRNA for CD40 (Figure 13A) in microglia cultured in the two different conditions was not statistically different indicating that changes in MFI could not be adequately explained on the basis of differences in the mRNA degradation rate.
Similarly, rates of decay of CD80 and CD86 were also unaffected by culture conditions (Figure 13B and C).

2.5 Surface protein degradation does not account for differences in the MFI

Because MFI in co-cultured microglia never attains that seen in isolated microglia, we sought to determine whether the loss in CD40 surface expression was due to the more rapid degradation of translated protein. Cells were cultured with their usual GM-CSF/LPS protocol, but 16 hrs after LPS addition, cultures were treated with cycloheximide (CHX, 10 μg/ml) to halt protein translation. Surface protein was assessed by flow cytometry at various time points following the addition of CHX. Data are expressed as the logarithm of the percent of surface protein remaining at each time point. Differences between individual time points were calculated, and those differences were compared with an ANOVA to obtain a measure of statistical significance which is noted on the graphs. The slope of decay of the lines indicated that CD11c, CD40 and the simultaneous expression of CD11c and CD40 surface expression was the same in both settings (Figure 13D, E and F). The lack of difference in the decay of surface expression of these molecules reveals that the observed difference is not due to the rapid degradation of CD40 or CD11c on co-cultured microglia.

2.6 Co-cultured microglia express high levels of CD40 protein, but fail to place it on their surface

Western blot analysis was performed at various time points after LPS signaling to confirm the expression of CD40 (Figure 14). Western blots performed on microglial lysates derived from isolated or co-cultured cells revealed significant quantities of protein present in the co-cultured microglia even at times when surface expression was low (Figure 14). Thus, if one compares the surface expression of CD40 at the end of GM-CSF
treatment (t = 0) as seen in Figure 12B with the protein levels detected by Western blot at the same time point, there is a significant quantity of CD40 protein that is not detected on the cell surface. The HM40-3 antibody (also used for flow cytometry and immunocytochemistry) revealed three distinct bands of protein all of which appeared to decline somewhat over the 24 hrs of LPS treatment. The highest molecular band was at approximately 43 kDa. Others have suggested that this is a glycosylated form of CD40 (Vaitaitis and Wagner, Jr., 2010). By contrast, isolated cells had low levels of protein at the end of GM-CSF treatment with only one band increasing slightly over the course of LPS treatment. These data suggested that co-cultured microglia produce significant quantities of protein, but that it remains sequestered within the cell until after LPS treatment.

In order to examine this further, cells were prepared for flow cytometry so that surface expression and total protein expression (including intracellular protein) could be compared (Figure 15). For these experiments cells were harvested after a 5-day treatment with GM-CSF or after a subsequent 24-hr treatment with LPS. They were then stained as before with antibodies against CD11c and CD40. These cells, then, revealed only surface expression. Half of these cells were then fixed and permeabilized for 30 min (Fixation/Permeabilization Concentrate and Diluent, eBiosciences) and re-stained with antibodies against CD40. These cells revealed both surface and intracellular protein levels.

Examination of total protein following permeabilization revealed that virtually 100% of both isolated and co-cultured microglia expressed CD40. Differences emerged, however, when only surface expression was analyzed. Following GM-CSF treatment
only about 36% of the co-cultured microglia showed surface expression while more than 90% had reached the cell surface in the isolated microglia. Moreover, the MFI of the protein in the isolated cells was substantially higher than their co-cultured cousins. Following 24 hrs of LPS treatment, co-cultured cells increased the percentage of cells expressing surface CD40 to approximately 72%, but the MFI remained low. These data are in accord with those in Figure 12 and suggest that most of the protein translated in the co-cultured cells is sequestered intracellularly.

Analysis by ImageJ® software of the cellular distribution of CD40 shows that isolated microglia express CD40 primarily at the cell’s edges while co-cultured microglia have a more homogeneous distribution (Figure 16). Moreover, immunocytochemistry revealed that most of it is harbored within the ER, not the early endosomes or lysosomes (Figure 17). Even after 24 hrs of treatment with LPS, CD40 still co-localizes with the ER in co-cultured microglia, while isolated microglia maintain a robust surface expression. These data suggest that astrocytes retard the placement of CD40 onto the cell surface.

**Conclusions to Specific Aim 2:** mRNA levels for co-stimulatory molecules and MHC Class II are upregulated in isolated microglia by GM-CSF while co-cultured microglia await signals from LPS in order to increase those levels. Surface protein for CD40 and CD11c have attained maximal levels following GM-CSF treatment of isolated cells while these levels don’t begin to rise in co-cultured cells until LPS treatment. The differences seen in CD40/CD11c surface expression are, therefore, not due to unresponsiveness to LPS signaling, nor are they due to differences in the turnover rates of mRNA or protein. Even when surface expression is low, co-cultured microglia express robust levels and multiple isoforms of CD40, but protein largely remains inside the cells.
Immunocytochemical analysis reveals that the CD40 protein remains in the ER in cocultured cells long after isolated cells have trafficked the protein to the surface. Taken together with previous data, these studies suggest that contact with the astrocytic microenvironment prevents microglia from responding efficiently to a GM-CSF signal, and that their response even to a strong signal like LPS is restricted.

V. Discussion

The studies in this thesis emerged from a finding by Nischal Padala that microglia isolated from their normal brain environment readily assume a mature dendritic cell (DC) phenotype with a 5-day pretreatment with GM-CSF followed by a 2-day exposure to LPS. The phenotype was complete with robust surface expression of CD11c together with co-stimulatory molecules CD40, CD80 and CD86 as well MHC Class II necessary for efficient T cell activation. By contrast, a lower percentage of microglia stimulated in the presence of astrocytes expressed mature levels of these molecules. Three other aspects of his data are worth noting: 1) enriched microglia added to a bed of enriched astrocytes mimicked the effect of microglia from mixed glia suggesting that inhibition was a property specific to the astrocytic environment; 2) bone marrow-derived DCs experienced the same down-regulation of co-stimulatory molecules when exposed to the astrocytic environment; and 3) contact with the astrocytic environment and not soluble factors was responsible for the inhibitory effect. These data raised several questions addressed in this thesis.

5.1 Co-cultured microglial response to LPS is intact

The first consideration was whether astrocytes were preventing microglia from responding to the LPS signal that followed the GM-CSF priming signal. Observations of
the co-cultured microglia had indicated that these cells were responding to GM-CSF because they proliferated as expected. But the maturing signal was LPS, and previous work by others had suggested that astrocytes limit microglial responsiveness to LPS (Aloisi et al., 1997). However, in studies designed to examine the time-course of co-stimulatory mRNA expression, co-cultured microglia responded early and robustly to LPS signaling with a significant increase in mRNA coding for CD40, CD80 and CD86. Western blotting analysis also revealed that CD40 molecule was being efficiently translated in co-cultured microglia. Therefore, failure to respond to LPS was not the reason for the immature DC phenotype.

5.2 Differential expression of CD40 isoforms in co-cultured and isolated microglia

Tone et al. (2001) have demonstrated five isoforms of CD40, and have suggested that isoform II is an endogenous inhibitor of the signaling isoform I. This study prompted us to investigate the variety of CD40 isoforms in microglia under both culture conditions. Indeed, co-cultured and isolated microglia showed slightly different patterns of expression of these isoforms. By contrast, the co-cultured cells show low levels that decline over the first 12 hrs and rise afterwards. Because these isoforms were not sequenced, we cannot be certain that they correspond to the isoforms that have been demonstrated by Tone et al. Moreover, it is unclear from the literature what are the ramifications of having these multiple isoforms. The possibility that isoform II inhibits isoform I and the possibility that isoform II is expressed only in the co-cultured cells requires additional study.
5.3 Low CD40 surface expression in co-cultured microglia is not due to a faster mRNA and/or protein degradation

Although LPS signaling was intact, we wondered whether a rapid RNA and/or protein turnover might account for low surface expression on the co-cultured cells. Neither RNA nor protein degradation studies suggested that this was the case.

5.4 miRNA 155, 146a, 146b are upregulated in co-cultured microglia

We considered the possibility that the lower surface expression might be the result of suppression of translation by microRNAs. Indeed, a recent study has identified miRNA-124 as being a key regulator of microglial quiescence (Ponomarev et al., 2011). In our experiments, co-cultured microglial expression of miRNA-155, miRNA146a and miRNA146b -- microRNAs implicated in DC expression and autoimmunity (Baltimore et al., 2008; Banchereau et al., 1995; Banchereau and Steinman, 1998) -- were substantially elevated over their isolated counterparts (see Appendix B), but Western blots (see below) suggested that inhibition of CD40 translation was not the underlying problem. Moreover, none of the co-stimulatory molecules has been identified as targets for these particular miRNAs. Without additional siRNA studies, the role of these specific miRNA molecules remains uncertain.

5.5 Astrocytes inhibit GM-CSF-induced differentiation rather than LPS-induced differentiation

The LPS signaling data were puzzling. Surface expression of co-stimulatory molecules was low, but mRNA for the co-stimulatory molecules and protein levels -- specifically those for CD40 -- were high. This prompted us to examine expression of co-stimulatory molecules prior to LPS signaling, and we turned to an investigation of expression during GM-CSF treatment. In these studies, we found that isolated microglia
responded robustly to GM-CSF treatment with an upregulation of mRNA coding for co-stimulatory molecules, while co-cultured microglia lagged behind. Analysis of surface expression (with a focus on CD40 and CD11c) revealed that the percentage of cells expressing CD11c together with CD40 on isolated microglia was maximal at the end of GM-CSF treatment, while co-cultured microglia awaited signals from LPS in order to upregulate CD11c and CD40. An analysis of surface expression over the course of LPS treatment revealed that the co-cultured microglia gradually increased the percentage of double-positive cells, but that percentage never attained the percentage seen in isolated cells. The isolated cells, on the other hand, did not increase further the percentage of double-positive cells, but unlike the co-cultured cells, the MFI, a measure of CD40 per cell, increased gradually with time of LPS treatment. Thus, these data suggest that astrocytes were primarily inhibiting GM-CSF-induced differentiation rather than LPS-induced differentiation.

Various signaling pathways for GM-CSF result in either proliferation, survival or differentiation. Jak3 is required for GM-CSF-induced differentiation of myeloid DCs. Therefore, the down-regulation of Cdk2, Cdk4, Cdk6, and Cyclin E with the concomitant upregulation of Jak3 would shift cells from a proliferative phenotype (as seen with co-cultured cells) to one capable of differentiation (as seen with isolated cells) (Mangan et al., 2006; Rane et al., 2002). We hypothesize that transcription of Jak3 is suppressed by astrocytes via a contact-mediated mechanism thus preventing GM-CSF treatment from effecting differentiation in co-cultured microglia. Investigating this hypothesis would be a future direction for this research.
What emerged, however, was that while LPS treatment raised the percentage of cells expressing CD40 in the co-cultured microglia, they never attained CD11c levels similar to those seen in the isolated cells. Therefore, it was CD11c and not CD40 that was the limiting factor. Furthermore, the MFI for CD40 was always higher in isolated when compared to co-cultured microglia. While the MFI increased in isolated microglia in a time dependent manner after LPS addition, it remained the same in co-cultured microglia. These studies did not focus heavily on CD11c regulation, but a more careful analysis of GM-CSF and/or LPS regulation of this molecule is warranted.

5.6 CD40 is retained intracellularly in co-cultured microglia

Western blots were performed on co-cultured and isolated microglia that had been treated with GM-CSF alone or at various times following LPS treatment. Surprisingly, while surface expression of CD40 was extremely low on co-cultured microglia, Western blots showed abundant protein. This raised the question of its location. A time course of total protein vs. surface protein revealed that total protein in both settings was plentiful, but protein was distributed differently in the cells. By the end of GM-CSF treatment, isolated cells had placed most of their CD40 onto the cell surface. By contrast, co-cultured cells sequestered most intracellularly. This intracellular harboring of CD40 persisted through at least 24 hrs of LPS treatment. Immunocytochemical analysis revealed that it was retained in the ER and had not reached the early endosomes on its way to the cell membrane. Staining with a Golgi marker was attempted unsuccessfully. Consequently, because electron microscopy and 3-D reconstruction of the cells were not performed, the possibility of CD40 being retained in the Golgi as well as (or instead of)
the ER cannot be discarded. However, a Z-stack analysis gave a fair indication that the subcellular localization was, indeed, the ER.

We propose a model CD40 retention that is based on that described for gp96 (Kim et al., 2010a; Han et al., 2007), a member of the heat shock protein 90 family of chaperones. Gp96 acts both within the cell, but when placed on the cell surface, can act as a receptor for TLR2/4 (Vabulas et al., 2002). Like other chaperones, gp96 contains a C-terminal KDEL sequence (Lys-Asp-Glu-Leu) that binds to a KDEL receptor on the Golgi, thus activating Src family kinases that facilitate the trafficking of proteins from the Golgi back to the ER (Semenza et al., 1990). Gp96 also forms a complex with aminoacyl-tRNA synthetase-interacting multifunctional protein 1 (AIMP1) (Han et al., 2007) which facilitates KDEL receptor binding and the retrieval of gp96 and its retention by the ER. Treatment of cells with TLR4 agonists causes the phosphorylation of AIMP1 by Jun kinase with the subsequent dissociation of the complex, the release of gp96 from the ER and its successful trafficking to the cell surface (Kim et al., 2010a). It is unclear whether CD40 contains a KDEL sequence, although Western blotting does reveal higher molecular weight isoforms in the co-cultured microglia than in isolated cells. Even if a KDEL sequence is absent, however, CD40 association with any one of a number of chaperones that do contain the KDEL sequence could form a complex with AIMP1, facilitating retention by the ER. Testing this hypothetical model might be a future direction for this work.

5.7 Astrocytes and the M2 phenotype

Recent studies have identified differences in macrophage phenotypes: the classically activated M1 and the so-called “alternatively activated” M2 (Verreck et al.,
M1 macrophages are characterized by a pro-inflammatory phenotype. Stimulated by IFNγ, tumor necrosis factor-alpha (TNFα) or LPS, these cells express high levels of IL-12, IL-23, TNFα and inducible nitric oxide synthase and are considered a major cause of autoimmune disease, host defense to infection, and, in the case of spinal cord injury, neurotoxicity (Gordon and Martinez, 2010; Kigerl et al., 2009). Although we have not specifically investigated phenotypic characteristics of microglia in our culture settings, isolated microglia can easily be considered M1-like cells. M2 macrophages, on the other hand, are stimulated by IL-4 and are characterized by the production of anti-inflammatory molecules like IL-10 as well as high levels of arginase as well as the mannose receptor (Stein et al., 1992). These cells dampen inflammation, promote angiogenesis and lead to the resolution of inflammation. In unreported ELISA data from our lab, LPS-treated microglia cultured in the presence of astrocytes upregulate IL-10 when compared to isolated microglia (data not shown). Thus, co-cultured microglia -- i.e., cells in a more normal brain environment -- could be considered M2-like. Consistent with our findings, others have suggested that in the normal brain microglia exist in an M2 status (Ponomarev et al., 2007), but the mechanism(s) promoting this phenotype remain unclear.

Establishing these mechanism(s), however, is essential. Tumors are notoriously adept at suppressing or evading anti-tumor immunity by encouraging macrophage polarization to the M2 phenotype (Sica et al., 2006; Soares-Schanoski et al., 2012) or suppressing or promoting defective differentiation of monocytic-derived DCs (Almand et al., 2000; Liu et al., 2009; Wang et al., 2006). Renal cell carcinomas, e.g., release IL-6 and monocyte colony-stimulating factor (M-CSF) to inhibit DC maturation leading to
poor antigen-presenting function (Menetrier-Caux et al., 2001); myeloid DCs derived from cutaneous squamous carcinoma are notably poor antigen presenters (Bluth et al., 2009); excessive DC production of IL-10 prevents effective DC therapies in hepatocellular carcinomas (Chen et al., 2007); and a variety of tumor cells up-regulate the TLR4 suppressor IRAK-M (del Fresno et al., 2005).

Thus, while one appreciates that astrocytes play an important role in quelling microglial inflammation, there is a dark side to the ability of astrocytes to quash microglial DC function and maintain an M2-like status. Microglia with low or absent MHC Class I and II and co-stimulatory molecule expression reside among malignant astrocytes, notably the aggressive glioblastoma multiforme (GBM), a WHO grade IV astrocyte tumor (Hussain et al., 2006; Sinn et al., 2007; Tran et al., 1998). Possibly because of the immature status of these tumor-associated microglial DCs (Kostianovsky et al., 2008; Dibra et al., 2009), regulatory-type T cells accumulate in gliomas with concomitant suppression of anti-tumor activity (Matsui et al., 2009; Badie et al., 2002; Anderson et al., 2007; Grauer et al., 2007; Akasaki et al., 2004; Kennedy et al., 2009; Hussain et al., 2006; Jacobs et al., 2008). Most tumor cells fail to produce GM-CSF in vivo (Menetrier-Caux et al., 2001), thus limiting the molecules needed for DC maturation. Instead, they and tumor-associated microglia express TGFβ that inhibits DC maturation and promotes T_{reg} expression (Constam et al., 1992; Grauer et al., 2007; Wesolowska et al., 2008; Shinozaki et al., 2009), immunosuppressive IL-10 (Wagner et al., 1999; Hishii et al., 1995; Nitta et al., 1994; Huetter et al., 1995), and the tumor mitogen IL-6 (Li et al., 2010; Brantley and Benveniste, 2008; Loeffler et al., 2005; Tchirkov et al., 2001). Because of tumor production of IL-6 and IL-10, STAT3, the
signaling molecule for both IL-10 and IL-6, have been examined for anti-tumor efficacy (Zhang et al., 2009). Their natural inhibitors, SOCS 1 and SOCS3, are hypermethylated in GBM (Martini et al., 2008) leading to a loss of expression. The picture is not simple, however, since overexpression of SOCS3 correlates with enhanced glioblastoma growth (Brantley et al., 2008). In short, glioblastoma survival and growth are secured by production of molecules designed to promote tumor growth, inhibit antigen presentation by resident microglia and promote the accumulation of T\textsubscript{regs}.

Effects of glioblastoma on microglial assumption of a DC phenotype have not been studied in great detail. Consistent with our own findings with normal astrocytes, Kostianovsky et al. have recently found that the molecular machinery for antigen presentation by microglia is truncated by glioblastoma (Kostianovsky et al., 2008). What is becoming clear is that manipulation of the tumor environment may be able to shift tumor-associated macrophages/microglia to an anti-tumor phenotype (Kitoh et al., 2011).

On the other hand, in multiple sclerosis (MS), a neurodegerative disease characterized by demyelination of axons, microglia become activated. If normal astrocytes are effective inducers of the M2 phenotype, what changes must occur in the brain to allow for the conversion to an M1 phenotype? One can speculate that in early stages of MS, microglia exist in the M2 status. However, the massive infiltration of activated dendritic cells and macrophages from the periphery that precede CNS demyelination may change that phenotype to the M1. One means by which this could occur is due to the release of metalloproteinases (MMPs) such as MMP9 molecules by invading cells (Kouwenhoven et al., 2002). This could in turn degrade the extracellular matrix (ECM) that keeps the communication network between microglia and astrocytes.
In this instance, the microglial-astrocytic contact-dependent communication is disrupted. If astrocytes are, indeed, responsible for providing signals for the maintenance of the M2 phenotype, such signaling may be disturbed, allowing for microglial activation. Therefore, depending on the physiologic conditions microglia encounter they may exert either neurotoxic or neuroprotective responses.

The importance of the ECM in maintaining microglial quiescence has, perhaps, been underestimated. Our data would suggest that this aspect of microglial-astrocytic cross-talk should be given a more prominent research focus.


Stamenkovic I, Clark EA, Seed B 1989. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J. 8: 1403-1410.


Figures and Tables
Figure 1. Astrocytes inhibit microglial production of a mature DC phenotype. Microglia were cultured with GM-CSF (25 ng/ml) in the presence (Co-cultured; ON) or absence (Isolated; OFF) of astrocytes for 5 days. LPS (50 ng/ml) was added for an additional two days. Co-stimulatory molecule surface expression was determined by flow cytometry (A). 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 ± SEM over 3 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 CD40 Mean Fluorescence Intensity (MFI) differs in the presence (Co-cultured) and absence (Isolated) of astrocytes. Data are expressed as the MFI ± SEM and compared by a Student’s t-test. The asterisk indicates p< 0.01. Data courtesy of Nischal Padala.
Figure 2. T cell interaction activation with DCs requires Signal 1 (MHC II binding to the T cell receptor [TCR]) as well as co-stimulatory molecule binding. B7-1 (CD80) and B7-2 (CD86) are ligands for CD28 and CTLA4, activators and inhibitors of T cell activation respectively. Adapted from Donovan and Finn, 1999.
Figure 3. **T helper cell differentiation into different T cell effectors** (see text). From Tato, et al., 2006.
Figure 4. The LPS signaling pathways include MyD88-dependent and MyD88-independent pathways (see text). Adapted from http://en.wikipedia.org/wiki/IRF3.
Figure 5. The IFNβ signaling pathway involves JAK/STAT and the nuclear translocation of interferon response factor (IRF; see text). Adapted from http://www.invivoegen.com/hek-blue-ifn-ab
Figure 6. Co-cultured microglia express elevated levels of mRNA for endogenous LPS inhibitors. Microglia were cultured with or without astrocytes (see Materials and Methods). After a 5-day treatment with GM-CSF (25 ng/ml), cells were treated with LPS (50 ng/ml) and harvested at various time points after the beginning of LPS treatment. Cells were prepared for QRT-PCR analysis for TRIM 30α (A), IRAK-M (B), SOCS1 (C) and SOCS3 (D). Data are expressed as the mean ± SEM of arbitrary mRNA units from 3 independent experiments. Data from isolated and co-cultured microglia were compared at each time point using a Student’s t test. A single asterisk indicates a significant difference at the 99% confidence level; double asterisk indicates a significant difference at the 95% confidence level. Data comparisons using a two-way ANOVA yielded identical results.
Figure 7. SOCS3 protein levels are similar in isolated and co-cultured microglia. Cells were cultured in the two conditions (see Materials and Methods) and harvested at the times indicated following LPS treatment. Western blotting was performed using a rabbit antibody against mouse SOCS3 (1:250). Because actin levels change in the two culture conditions, the loading controls were blots stained with Coomassie Blue. One representative experiment of 4 is shown.
Figure 8. IRAK-M affects CD11c surface expression. Microglia from WT animals were co-cultured with astrocytes or in isolation. They were treated with GM-CSF for 5 days prior to LPS treatment. At various time points following LPS treatment cells were assessed by RT-PCR for IRAK-M mRNA (A). Data are expressed as the mean ± SEM of arbitrary mRNA units from 3 independent experiments. Data from isolated and co-cultured microglia were compared at each time point using a Student’s t-test and were found to be different from each other at the 95% confidence level. Data comparisons using a two-way ANOVA yielded identical results. (B) Western blots confirmed the presence of IRAK-M protein in the co-cultured microglia. As in Figure 7, loading controls were blots stained with Coomassie Blue. Data are representative of 4 experiments performed. In (C) cells from WT and IRAK-M KO animals were assessed by flow cytometry using antibodies against CD40 and CD11c. Data are expressed as the percentage of cells expressing either CD40 or CD11c or the two together. Data are from one experiment of two performed.
Figure 9. IFNβ regulates CD11c. (A) Co-cultured and isolated microglia were harvested after the beginning of LPS treatment at the times indicated and assessed for mRNA expression by RT-PCR. Data are expressed as the mean ± SEM of arbitrary RNA units from 10 measurements from 6 independent experiments. Data at each time point were compared using a Student’s t test for statistical significance at the 95% confidence level. An asterisk indicates p<0.0009. (B) Co-cultured and isolated microglia were cultured with or without a neutralizing rat antibody against IFNβ (3.5 μg/ml) during a 24-hr LPS treatment. Cells were harvested and stained with antibodies against CD11c and CD40 and the percentage of cells expressing either CD40 or CD11c or the two together were determined by flow cytometry. This experiment was performed twice with similar results.
Figure 10. QRT-PCR analysis of microglial expression of mRNA for MHC Class II and co-stimulatory molecules following a 48-hr treatment with GM-CSF (A-D) and a 24 hr treatment with LPS (E-H). Microglia cultured with (co-cultured) or without (isolated) astrocytes were harvested at various time points indicated after GM-CSF addition and (A) CD40 (B) CD80 (C) CD86 (D) MHC class II mRNA expression assessed through quantitative real-time PCR. In E-H, microglia were harvested at various time points after the addition of LPS. The data are expressed as arbitrary units ± SEM from 3 separate experiments. Levels at each time point were compared by a Student’s t-test. The asterisk indicates \( p < 0.01 \). A two-way ANOVA yielded identical results.
Figure 11. Multiple CD40 mRNA isoforms are detected in microglia.
Co-cultured or isolated microglia were treated with GM-CSF for 5 days. They were then treated with LPS and harvested at various time points (hrs) after LPS treatment. cDNA was prepared from extracted mRNA and run on a 1.8% acrylamide gel. The gel was probed with CD40 primers designed to recognize all mouse isoforms between exon 5 and exon 9 as described by Tone et al. 2001. Positive controls detected the expected presence of IRAK-M, IP10, and CD40. For the positive controls, primers were designed specifically to amplify fragments of 150 bps.
Figure 12. Protein surface expression is differentially regulated in isolated vs. co-cultured microglia. Isolated microglia (A) and co-cultured microglia (B) were cultured for 5 days with GM-CSF and then treated with LPS. At the various time points indicated after LPS treatment, cells were harvested, stained for CD11c and CD40 and assayed by flow cytometry. In accordance with our previous data, at the end of the 48-hr LPS treatment, the percentage of CD11c+CD40+ cells in isolated microglia was 72.4% while the percentage in co-cultured microglia was 52.9% as indicated on the figure. (C) Mean fluorescence intensity of CD11c+CD40+ cells also increased with the length of LPS treatment. These data are representative of 3 independent experiments.
Figure 13. Neither mRNA nor protein degradation rate accounts for differences in surface expression of protein at 48 hrs. For assessment of mRNA degradation, isolated microglia or co-cultured microglia were treated for 5 days with GM-CSF and then with LPS for 3 hrs at which point actinomycin D (5 μg/ml) was added to inhibit transcription. At the various time points indicated, cells were harvested and assessed for steady-state levels of CD40 (A), CD80 (B) and CD86 (C) mRNA. Data are expressed as the logarithm of the percent of mRNA remaining at each time point, and the differences between the adjacent time points compared with an ANOVA to obtain a measure of statistical significance. P values obtained are noted on the graphs. Data shown are representative of 3 experiments performed. For assessment of protein degradation rates, isolated microglia or co-cultured microglia were treated for 5 days with GM-CSF and then with LPS for 16 hrs at which point cycloheximide (10 μg/ml) was added to inhibit translation. At the various time points indicated, cells were harvested and assessed for surface protein expression by flow cytometry for CD40 (E), CD11c (F) and the simultaneous expression of both (G). Data are expressed as the logarithm of the percent of surface protein remaining at each time point. Differences between individual time points were calculated, and those differences were compared with an ANOVA to obtain a measure of statistical significance. P values obtained are noted on the graphs. Data shown are representative of 3 experiments performed.
<table>
<thead>
<tr>
<th>GM-CSF 5 days</th>
<th>LPS</th>
<th>0</th>
<th>8</th>
<th>16</th>
<th>24</th>
<th>LPS</th>
<th>0</th>
<th>8</th>
<th>16</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>43 kDa</td>
<td>CD40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Co-cultured       Isolated

**Figure 14. Multiple isoforms of CD40 protein are expressed in co-cultured microglia.** Western blots of CD40 were prepared at various time points following LPS stimulation. Total cell lysates were probed with the HM40-3 anti-CD40 antibody. Total CD40 protein was substantially higher and showed multiple isoforms at all time points in the co-cultured microglia even though flow cytometry indicates lower surface expression (cf. Figure 1). Because actin levels change in the various culture settings (data not shown), loading controls were prepared from the same samples and stained with Coomassie Blue. Western blots have been performed 4 times with identical results.
**Figure 15. Surface vs. total CD40 expression.** (A) Isolated and co-cultured microglia were prepared for flow cytometry following GM-CSF treatment for 5 days and 24 hrs LPS treatment. Cells were stained with and without a permeabilizing agent to determine total CD40 protein vs. surface protein. A representative experiment of 3 performed is shown. (B) Data from (A) are depicted in graphic form.
**Figure 16. CD40 cellular distribution.** CD40 immunoreactivity in isolated (A) and co-cultured (C) microglia reveal variability in cellular localization 24 hrs after LPS treatment. Z-stacks were performed on both cell types to determine cellular localization (data not shown). Image J analysis reveals that isolated microglia (A) express CD40 at the cell surface (B) while co-cultured microglia (D) retain it intracellularly, clustered primarily around the cell nucleus (C).
Figure 17. Immunocytochemistry reveals CD40 sequestered in co-cultured cells in the ER. Isolated and co-cultured microglia were prepared as described in the text. After 24 hrs of LPS treatment, cells were prepared for simultaneous expression of CD40 and ER using ER Tracker™ (A), CD40 and lysosomes using LysoTracker® (B) or CD40 and early endosomes using an antibody against EEA1 (C). Only ER Tracker™ showed substantial overlap with CD40 expression in co-cultured microglia.
Table 1. Primary and Secondary Antibodies Used

<table>
<thead>
<tr>
<th>Source</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Probes/Invitrogen</td>
<td>Alexa Fluor® 488 donkey anti-goat IgG</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor® 488 goat anti-rabbit IgG</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor® 594 chicken anti-rabbit IgG</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor® 594 chicken anti-rat IgG</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor® 594 donkey anti-mouse IgG</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor® 594 donkey anti-sheep IgG</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>EEA1 (h-300) SC-33585</td>
</tr>
<tr>
<td></td>
<td>Rabbit polyclonal anti-human CD40 (C-20: sc975)</td>
</tr>
<tr>
<td></td>
<td>Goat polyclonal anti-mouse CD40 (T-20: sc1731)</td>
</tr>
<tr>
<td></td>
<td>HRP-conjugated donkey anti-goat IgG</td>
</tr>
<tr>
<td>BD Biosciences</td>
<td>Purified mouse anti-EEA1 clone 14/EEA1 (against human tissue)</td>
</tr>
<tr>
<td></td>
<td>FITC-conjugated Armenian hamster anti-mouse CD40 (HM40-3)</td>
</tr>
<tr>
<td></td>
<td>Unlabeled Armenian hamster Anti-Mouse CD40 (HM40-3)</td>
</tr>
<tr>
<td></td>
<td>FITC-labeled hamster IgGM_{\lambda,1} (isotype control)</td>
</tr>
<tr>
<td>BioLegend (San Diego, CA)</td>
<td>Purified anti-mouse CD11b (clone M170)</td>
</tr>
<tr>
<td></td>
<td>PE-conjugated Armenian hamster anti-mouse CD11c (clone N418)</td>
</tr>
<tr>
<td></td>
<td>PE-labeled hamster IgG (isotype control)</td>
</tr>
<tr>
<td>AnaSpec (Fremont, CA)</td>
<td>Rabbit anti-mouse SOCS3 (IN) cat. #55410</td>
</tr>
<tr>
<td>Millipore</td>
<td>Rabbit polyclonal anti-mouse IRAK-M</td>
</tr>
<tr>
<td>Abcam</td>
<td>Rat monoclonal anti-mouse interferon β^+ (clone 7F-D3)</td>
</tr>
<tr>
<td></td>
<td>HRP-conjugated goat anti-rabbit IgG</td>
</tr>
<tr>
<td>Cell Signaling</td>
<td>HRP-conjugated goat anti-rabbit IgG</td>
</tr>
<tr>
<td>Brockwood Biomedical (Birmingham, AL)</td>
<td>HRP-conjugated rabbit anti-hamster IgG</td>
</tr>
</tbody>
</table>
Table 2. QRT-PCR primers used.

**Co-stimulatory molecules**

CD40:
- 5’ CTG CCC AGT CGG CTT CTT CTC 3’ F
- 5’ CCT GTG TGA CAG GCT GAC AC 3’ R

CD80:
- 5’ CTG GGA AAA ACC CCC AGA AG 3’ F
- 5’ TGA CAA CCA TGA CGA CGA CTG 3’ R

CD86:
- 5’ TTA CGG AAG CAC CCA TGA TG 3’ F
- 5’ CGT CTC CAC GGA AAC AGC AT 3’ R

MHC II:
- 5’ CAA CAC TCT GGT CTG CTC AGT GA 3’ F
- 5’ TGT GTG GAT GAG ACC CCC A 3’ R

**LPS Signaling inhibitors**

TRIM-30 ALPHA:
- 5’ TCG TGT CCG AGT TTT CCT GG 3’ F
- 5’ GGA AGG CAG GGT CAT AGA ACC 3’ R

IRAK-M:
- 5’ TAC TCC TGT TCC GTC ACC CC 3’ F
- 5’ AAG CGT CCC GTT GCT CAT AT 3’ R

SOCS1:
- 5’ GCG CGC TCC TGG ACG CCT GCG GC 3’ F
- 5’ CCG CAC GCG GCG CTG GCG CAG C 3’ R

SOCS3:
- 5’ GCT CCA AAA GCG AGT ACC AGC 3’ F
- 5’ AGT AGA ATC CGC TCT CCT GCA G 3’ R

**MyD88-independent pathway**

IFN-β:
- 5’ CAG CTG AAT GGA AAG ATC AAC CT 3’ F
- 5’ TGG ATG GCA AAG GCA GTG TA 3’ R

IFN-β1:
- 5’ CTG GAG CAG CTG AAT GGA AAG 3’ F
- 5’ CTT GAA GTC CGC CCT GTA GGT 3’
Appendices
Appendix A – Microglial expression of IL-35

Although the issue of T cells and immune tolerance is not addressed here, an underlying subject of the thesis is the immunosuppressive nature of the healthy brain. How does the brain prevent proinflammatory destruction and what happens to allow proinflammation to take over as it does in multiple sclerosis or in the recent outbreak of fungal meningitis? A newly identified member of the IL-12 family of cytokines – IL-35 – has attracted some attention as a mediator of regulatory T cell (T<sub>reg</sub>) suppression of inflammatory T cells (Collison and Vignali, 2008; Collison et al., 2007). The IL-12 family of cytokines comprises dimers derived from combinations of subunits p19, IL-12α (p35), p40, IL-27α, IL-27β (p28) and Epstein Barr induced factor 3 (Ebi3) (see Appendix Table 1). IL-12, IL-23, IL-27 activate similar members of the JAK/STAT signaling pathways as a result of homology in their receptor components (Vignali and Kuchroo, 2012).

<table>
<thead>
<tr>
<th>α chain</th>
<th>β chain</th>
<th>Active Cytokine</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12α (p35)</td>
<td>p40</td>
<td>IL-12p70</td>
<td>Generally pro-inflammatory</td>
</tr>
<tr>
<td>IL12α (p35)</td>
<td>Ebi3</td>
<td>IL-35</td>
<td>A product of T&lt;sub&gt;reg&lt;/sub&gt;; acts to suppress T&lt;sub&gt;H1&lt;/sub&gt; cell proliferation; induces iTr35 cells</td>
</tr>
<tr>
<td>IL-27α (p28)</td>
<td>Ebi3</td>
<td>IL-27</td>
<td>Promotes differentiation of T&lt;sub&gt;H1&lt;/sub&gt; cells and Tr1 cells</td>
</tr>
<tr>
<td>p19</td>
<td>p40</td>
<td>IL-23</td>
<td>Promotes differentiation of T&lt;sub&gt;H17&lt;/sub&gt; cells</td>
</tr>
</tbody>
</table>

Studies by others had suggested that astrocytes inhibit microglial expression of IL-12 (Aloisi et al., 1997). We sought to replicate these studies. In preliminary studies we analyzed the expression of Ebi3, IL12α and IL-27 to determine whether LPS treatment of
microglia in the presence or absence of astrocytes showed differential regulation of these molecules (Appendix Figure 1).

IL-27α (p28) was elevated in microglia derived from both settings, though it seemed to attain a much higher value after 6 hrs in the isolated cells. IL-12a (p35) was also dramatically up-regulated at 6 hrs in isolated cells followed by a rapid decline. Co-cultured cells showed a gradual increase in message beginning at 6 hrs. What was most striking, however, was the fact that Ebi3 levels were elevated in co-cultured microglia as early as 3 hrs, attaining maximal elevation by 6 hrs. Levels of Ebi3 in co-cultured cells was approximately 4 times higher than in isolated cells. Even without GM-CSF or LPS treatment, co-cultured microglia expressed higher levels of Ebi3 mRNA than their isolated cousins.

An ELISA is not yet available for IL-35, so we were unable to ascertain whether the elevation of p35 together with the elevation of Ebi3 yielded the suppressive cytokine IL-35. If it does, this would be the first report of IL-35’s presence in the brain. Until now it has been reported that T_{reg}s are the sole producers of this cytokine.
Appendix B – microRNAs are differentially expressed in co-cultured and isolated microglia

Recent studies have implicated a variety of microRNAs in DC expression and autoimmunity (Baltimore et al., 2008; Boldin et al., 2011; O'Connell et al., 2010; Chen et al., 2011). We examined some of these -- miRNA155, miRNA146a and miRNA146b – in variously cultured microglia following LPS stimulation (Appendix Figure 2). All 3 were elevated, and in all three cases, co-cultured microglia showed the most robust up-regulation. Of interest was the dramatic rise in miR155 in co-cultured microglia during the 48 hrs of LPS stimulation. Not all targets of these microRNAs have been identified. As of now, co-stimulatory molecules, however, are not among them. Their elevation in the co-cultured microglia, however, was intriguing.
Appendix Figure 1. Members of the IL-12 family of cytokines are differentially regulated by astrocytes. Microglia were cultured in the presence (co-cultured) or absence (Isolated) of astrocytes and treated for 5 days with GM-CSF. Treatment with LPS was begun and cells collected at various time points indicated for RT-PCR analysis of p35 (A), p28 (B) and Ebi3 (C). Data shown are representative of 3 experiments performed.
Appendix Figure 2. MicroRNAs are elevated in co-cultured microglia following LPS treatment. Isolated and co-cultured microglia were cultured with GM-CSF for 5 days, followed by LPS. At various time points after LPS treatment, cells were collected and assessed by RT-PCR for levels of miR155 (A), miR146a (B), and miR146b (C). Data are expressed as a fold change over amounts of microRNA in the co-cultured microglia at 0 time (A) or after 1 hr of LPS treatment (B,C).
Curriculum Vitae
Giselles Maria Acevedo
Born on December 14, 1979, Santo Domingo, Dominican Republic

Education

Ph.D. in Cell and Molecular Biology 01/2013
State University of New Jersey/Rutgers University-Newark

M.S. in Cell and Molecular Biology 05/2007
State University of New Jersey/Rutgers University-Newark

B.S. in Biology 12/2003
State University of New York at Binghamton, Binghamton University

Seward Park High School 06/1999

Professional Experience

Graduate Assistant 01/2005-09/2012
Department of Biological Sciences, Rutgers University, Newark New Jersey

• Characterized the dendritic cell phenotype in co-cultured versus isolated microglia. This involved a study of the cellular distribution of co-stimulatory molecules, especially CD40.

Teaching Assistant 01/2012-06/2012
Department of Biological Sciences, Rutgers University, Newark New Jersey

• 21:120:452 Lab in Cellular and Molecular Biology: Molecular Biotechniques
• 26:120:538 Topics in Molecular Genetics

Undergraduate Research Experience Trainee 06/2003-08/2003
Wadsworth Center, SUNY Albany

• Identified APPL1 as a potential binding partner for human follicle stimulating hormone receptor (FSHR) using a yeast two-hybrid screening system.

Work Study 01/2000-05/2003
SUNY Binghamton, Binghamton, NY

• Set up teaching labs including arrangement of spectrophotometer, microscope and reagents ready on bench, cleaning glassware, and preparing reagents

Publications

1. Acevedo G, Padala NK, Ni L, Jonakait GM. Astrocytes inhibit expression of CD40 and CD11c on microglia membranes through a contact-mediated process. Submitted

