Signaling Cross-Talk Regulating the Expression of Arginase 1 in Murine Macrophages

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Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Biological Science

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March 25, 2010
Blacksburg, Virginia

Keywords: Arginase, Interleukin 4, IL-4, All-*trans* Retinoic Acid, ATRA, Lipopolysaccharide, LPS, Alternative, M2, Macrophage

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(ABSTRACT)

Macrophages can be activated by a variety of extracellular signals to polarize to either the M1 (inflammatory and antimicrobial) or to the M2 (wound repair and inflammation-resolution) phenotype. Expression of arginase 1 in macrophages is a key marker of the M2 phenotype. Arginase 1 expression is induced by interleukin 4 (IL-4), a cytokine secreted by Th2 helper cells. All-*trans* retinoic acid (ATRA) is a product of metabolism of dietary retinol (vitamin A). In a manner analogous to hormones, ATRA binds to nuclear receptors in cells and influences gene expression and cell physiology. ATRA is important in the resolution of inflammation systemically and on the cellular level, however it has not been linked to M2 activation or arginase 1 expression. Testing the hypothesis that ATRA can induce arginase 1 in macrophages either directly or indirectly, it was found that ATRA alone cannot cause murine bone marrow-derived macrophages (BMDM) to activate in the M2 phenotype (as indicated by arginase 1 expression), however it can dramatically potentiate induction of arginase 1 expression and activity by IL-4. This is the first observation positively linking ATRA to arginase 1.

Lipopolysaccharide (LPS), is a conserved structural component of the outer membrane of Gram negative bacteria, and a potent pyrogen. In metabolic endotoxemia, LPS concentration in the blood is slightly elevated, and over the long term this contributes to diverse inflammatory diseases such as atherosclerosis, obesity, and diabetes. LPS promotes the M1 phenotype and suppresses the M2 phenotype, but its contribution at low doses such as those found in metabolic endotoxemia are not well studied. In order to investigate mechanisms of LPS suppression at low doses, mice deficient in IRAK1 and tollip, key mediators or proinflammatory LPS signaling were used to study IL-4, ATRA, and LPS crosstalk. LPS suppression of arginase 1 is dependent on IRAK1 and tollip, but only at low doses of LPS.

This work is funded in part by a grant from the National Institutes of Health

Dedicated to my family, past, present, and future.

Acknowledgements

I thank God for the opportunity to live and to work.

I thank my lovely fiance, Miss Susan Purcell, for her support, her patience, and her love.

I thank my family for being loving, inspiring, and supportive. My father Joseph introduced me to science and engendered a sense of curiosity like his own. Dad, you are still my favorite person with whom to discuss science. My mother Kathleen taught me that value of hard work, perseverance, and of aiming high. Mom, thanks for still pushing me today. My sister Jessica has an enthusiasm and interest in my work that has never been false (MyD88). My brother Nicholas is the person who most understands my sense of humor, and always has my back. For everything I can describe but more importantly the numberless intangible joys of a wonderful family, I thank you all.

I thank my committee, Marion Ehrich, Yong Woo Lee, and Florian Schubot for their time, knowledge, and most importantly their encouragement.

Finally, I acknowledge my advisor and committee chair, Liwu Li, for his invaluable advice, guidance, and example. Daily he demonstrates a true and fundamental love for the rational pursuit of knowledge and for his students.

Michael Joseph Surace, Virginia Tech, March 25, 2010

Declaration of Work Performed

I declare that with the exception of the generation of IRAK1$^{-/-}$ and tollip$^{-/-}$ mice, all work reported herein was performed by myself.

IRAK1$^{-/-}$ mice were the kind gift of Dr. James Thomas from the University of Texas Southwestern Medical School.

Tollip$^{-/-}$ mice were the kind gift of Dr. Kimberly Burns while at the Institute of Biochemistry, University of Lausanne.

Michael Joseph Surace, Virginia Tech, March 25, 2010

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ABCA1	ATP-Binding Cassette Transporter A1
AF-1	Activating Function 1 Domain
AF-2	Activating Function 2 Domain
AKT	Activating Protein Kinase B
ALDH	Alcohol Dehyrdrogenase
AMP	Adenosine MonoPhosphate
AP1	Activator Protein 1
ATRA	All-*trans* Retinoic Acid
BLP	Bacterial Lipoprotein
BMDM	Bone Marrow-Derived Macrophage
C57BL/6	C57 black 6 mouse strain
cAMP	Cyclic AMP
CARM1	Coactivator-Associated Arginine Methyltransferase 1
CBP	CREB-binding protein
CREB	cAMP response element-binding
DBD	DNA-Binding Domain
DC	Dendritic Cell
DIC	Disseminated Intravascular Coagulation
DMEM	Dulbecco/Vogt Modified Eagle's Minimal Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOK1	Downstream of Kinase
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum
FIZZ1	Found in Inflammatory Zone 1
FoxP3	Forkhead Box Protein 3
GM-CSF	Granulocyte-Monocyte Colony-Stimulating Factor
GOI	Gene of Interest
GR	Glucocorticoid Receptor
GST	Glutathione-S Transferase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylases
HRP	Horseradish Peroxidase
IFN	Interferon
IKK	Inhibitor of Kappa B Kinase
IL	Interleukin
IL-1R	Interleukin 1 Receptor 1

IL-4	Interleukin 4
IL-4R	Interleukin 4 Receptor 1
iNOS	inducible Nitric Oxide Synthase
IRAK	Interleukin 1 Receptor Associated Kinase
IRF	Interferon Regulatory Factor
ISPF	Isonitropropiphenone
IκB	Inhibitor of Kappa B
JAK	Janus Kinases
JNK	c-Jun Terminal Kinase
KD	Kinase Dead
LBD	Ligand-Binding Domain
LBP	LPS-Binding Protein
LDL	Low-Density Lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
LXR	Liver X Receptor
M-CSF	Monocyte Colony-Stimulating Factor
MAPK	Mitogen-Activate Protein Kinase
MCF	Macrophage Chemotactic Factor
MDC	Macrophage-Derived Chemokine
MHCI/II	Major Histocompatibility Complex, Class I/II
MMR	Macrophage Mannose Receptor
MPS	Mononuclear Phagocytic System
MyD88	Myeloid differentiation primary response gene 88
NCoR	Nuclear Receptor Corepressor
NED	N-1-NapthylethylenediAmine Dihydrochloride
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2
PAGE	Polyacrylamide Gel Electrophoresis
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide 3-kinases
PI3P	Phosphatidylinositol 3-phosphate
PPAR	Peroxisome Proliferator-Activated Receptor
PRMT1	Protein Arginine Methyltransferase 1
PXR	Pregnane X Receptor
RALDH	Retinal Dehydrogenase
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element

RBP	Retinol Binding Protein
RIF	Rifampicin
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor
SDS	Sodium Dodecyl Sulfate
SMRT	Silencing Mediator for Retinoid and Thyroid-Hormone Receptors
SRC-1	Steroid Receptor Coactivator-1
STAT	Signal Transducers and Activators of Transcription
TAK	TGFβ-Activated Kinase
TBK	Tank-Binding Kinase
TBST	Trid-Buffered Saline w/ Tween
TGFβ	Transforming Growth Factor-β
TIR	Toll/ Interleukin 1 Receptor Domain
TIRAP	Toll/ Interleukin 1 Receptor 1-Associated Protein
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TNF-R	Tumor Necrosis Factor α Receptor
Tollip	Toll Interacting Protein
TRAF	TNF Receptor Associated Factor
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR Domain-Containing Adapter-Inducing Interferon-β
YM1	Chitinase 3-Like 3 Gene
γc	Gamma Common Chain

Chapter 1

Introduction

1.1 Inflammation and Innate Immunity

The inflammatory response to microbial infection is an ancient and well-conserved set of defense mechanisms. If the passive defense mechanisms of skin, mucus, saliva, sweat, and tears are eluded and a microbe finds its was into interior tissue, several mechanisms are activated to combat the foreign organism. The mechanisms present with four classic symptoms, often referred to by their Greek names; *rubor* (redness), *calor* (heat), *dolor* (pain), and *tumor* (swelling) (Nerlich and Bosch 1988). The symptoms are due to increased blood flow, extravasion of several types of leukocytes, which mediate the innate immune response, and activation of several humoral systems such as complement and clotting (Cho and Atwood 2002). Macrophages are major actors in the innate immune reponse, and they play several roles (Fujiwara and Kobayashi 2005). In one capacity, termed M1 activation, they actively seek and destroy microbes, secrete compounds that kill microbes, and educate the adaptive immune system (Classen, Lloberas et al. 2009). In another capacity, termed M2 activation, the very same cell cleans up debris from the battle, stimulates repair of the damage to the host, and suppresses remaining inflammation (Varin and Gordon 2009). If inflammation is

not resolved, then it can contribute to a wide range of diseases including atherosclerosis, asthma, diabetes, obesity, Chrons disease, and countless others (Lucas and Greaves 2001; Meyts, Hellings et al. 2006; Kilic, Ayaz et al. 2009; Devaraj, Dasu et al. 2010; Mathieu, Lemieux et al. 2010). Unresolved runaway systemic inflammation, termed septic shock, is the primary cause of death in intensive care facilities (Hunter and Doddi 2010). Once thought simply to be consumers of bacteria, macrophages have emerged as highly sensitive immune cells that are situated at the balance point between inflammation and resolution.

1.2 Macrophages

1.2.1 History

The existence of a cell-based immune system in vertebrates has been appreciated in some manner since the pioneering work of the comparative zoologist Ilya Metchnikoff in France and Russia in the late 1800s (Van 1964). His illustrated observations comprise some of the first evidence of phagocytosis, and chemotaxis by leukocytes. The term macrophage has been attributed to him. It has been a century since he received the Nobel Prize (in 1908, split with the German Paul Ehrlich, who championed a model for humoral immunity) for his work in cellular immunity (Turk 1991). In that time, our understanding of the joint humoral and cellular immune response has progressed by leaps and bounds.

The macrophage (from the Greek words makros "large" + phagein "to eat"), is unique to vertebrates, though other multicellular organisms have macrophage-like cells. The role and definition of macrophages, like many topics of research, is the subject of considerable contention. Macrophages are the terminal cell type of the mononuclear phagocytic system (MPS) (Lasser 1983). The MPS includes bone marrow progenitors, blood monocytes, and tissue macrophages, but antigen-presenting dendritic cells have recently been proposed to be a special type of macrophage and by extension a part of the MPS (Goxe, Latour et al. 1998). Monocytes are derived from bone marrow progenitors, and in turn can become resident tissue macrophages. Macrophages are defined by their ability to engage in chemotactic pursuit

of pathogens, to phagocytize said pathogens and cell debris, to process and present antigen to CD4+ and CD8+ T-cells, and to become classically (M1) or alternatively (M2) activated by exogenous ligands and by T-cell-secreted cytokines (Goerdt, Politz et al. 1999; Gordon 2003; Benoit, Desnues et al. 2008; Lolmede, Campana et al. 2009; Olefsky and Glass 2010). Markers of the monocyte/macrophage lineage include CSF-1 receptor, CD14, CD11b, F4/80 (mice)/EMR1 (human), lysozyme M, MAC-1/MAC-3 and CD68 (Khazen 2005).

Macrophages are present in most tissues, and several have specialized functions, such as microglia (brain), Kupffer cells (liver), dust cells/ alveolar macrophages (lung), osteoclasts (bone), sinusoidal lining cells (spleen), and mesangial cells (kidney). While each named type of resident macrophage is distinguished by morphology and function, all retain the characteristics of macrophages, particularly the ability to act as sentinels to detect the presence of pathogen-associated molecular patterns (PAMP) as the macrophage is the premier cell type expressing toll-like receptors (TLR), to present antigen, and to polarize to the M1 or M2 phenotype.

Differentiation of progenitor cells into naïve macrophages occurs in the context of hematopoiesis, the model for which remains unsettled. The classical theory maintains that the common progenitor cell differentiates into the myeloid lineage (cells that differentiate in the bone marrow including erythrocytes, monocytes, dendritic cells, thrombocytes, mast cells, basophil, eosinophil, neutrophil, macrophages) and lymphoid lineage (cells originally observed in the lymphatic system including natural killer cells, T-cells, B-cells) of cells. However the myeloid theory holds that the potential for lymphoid cells to differentiate into myeloid cells is maintained far longer, and that the model should not be binary but linear, with offshoots for each specific cell type. Therefore the prevailing theory is that the common progenitor cell of the bone marrow, no matter whether it is on track to differentiate to erythrocyte, T-cell, or B-cell, maintains the capacity to become a myeloid progenitor within the first 7-8 cell divisions. Differentiation of the macrophage from the myeloid progenitor cell is dependent

on two secreted cytokines; monocyte colony-stimulating factor (M-CSF) and granulocyte/ monocyte colony-stimulating factor (GM-CSF). Both contribute to macrophage differentiation through receptor-mediated activation of MAPK signaling pathways and particularly the transcription factor PU.1 (Scott, Simon et al. 1994; Grasset, Gobert-Gosse et al. 2010).

The markers of macrophage differentiation have been largely discovered and characterized quite recently. An early and commonly used marker of macrophage lineage is CD68. Originally identified as the sole target of six different antibodies that were used at the time to immunostain monocytes and macrophages, it has since been shown also to be expressed in fibroblasts (Micklem, Rigney et al. 1989; Kunz-Schughart, Weber et al. 2003). While fibroblasts were once regarded as fairly inert structural cells in healing wounds and connective tissue, it is being increasingly realized that in certain contexts, they are capable of expressing TLR, mediating inflammation, phagocytosis, and presenting antigen, behaviors once thought unique to macrophages. CD14, a pattern recognition receptor that plays a role in TLR4 recognition of LPS, was initially thought to be expressed exclusively on macrophages, but is now known to be expressed by fibroblasts and dendritic cells as well (Golenbock, Liu et al. 1993; Pugin, Heumann et al. 1994; Mahnke, Becher et al. 1997; Kitchens 2000). Among the 20+ other putative markers (Leenen, Debruijn et al. 1994), Carboxypeptidase M (Krause, Rehli et al. 1998), 7C3 (Zuckerman, Tang et al. 1987), and CD163 (Darieva, Lasunskaia et al. 2004) are more reliable. The colony-stimulating factor 1 receptor (CSF-1R) is also regarded as a reliable marker of cells of the MPS as it is required for response to M-CSF, a key inducer of macrophage differentiation.

1.2.2 Macrophage Activation

The control exerted by T-helper cells (Th1 and Th2) over macrophages is mediated by secretion of cytokines. Th1 cells induce classical activation of macrophages (M1 phenotype) and Th2 cells induce an alternative form of activation (M2 phenotype).

1.2.2.1 Classical/ M1 Activation

Although in 1905 Metchnikoff published observations of macrophage-mediated immunity to bacterial infection, it was George Mackaness in the 1960s that first determined the conditions under which macrophages were activated to kill infecting bacteria. He performed infection experiments with mice wherein he first challenged them with Listeria monocytogenes, then observed that they were resistant to infection with unrelated bacteria. He also showed that this resistance was due to the macrophage cell (and was not a humoral response) and that it correlated with the delayed-type hypersensitivity inflammatory response (Mackaness 1964). Later work by Carl Nathan identified the Th1-derived factor interferon (IFN)γ as the primary secreted activating cytokine (Nathan, Murray et al. 1983).

Classical activation of macrophages requires two independent signals. Originally identified as exogenous IFNγ and tumor necrosis factor (TNF) α (Goerdt, Politz et al. 1999). Neither IFNγ nor TNFα can induce activation in isolation, but in combination, they cause macrophages to polarize to the M1 phenotype (Goerdt, Politz et al. 1999). M1-activated macrophages are more microbicidal and tumoricidal, express and secrete the inflammatory cytokines IL-1, IL-6, and IL-23, and secrete antimicrobial products like nitric oxide, produced by the enzyme inducible nitric oxide synthase (Goerdt, Politz et al. 1999).

IFNγ is produced by T-cells. Natural killer (cytotoxic T) cells secrete IFNγ when they encounter and respond to a pathogen (Arase, Arase et al. 1996). However the primary producer of IFNγ is the Th1 cell, which secretes IFNγ following polarization to the Th1 phenotype by inflammatory cytokines or by environmental stress (Rincon, Enslen et al. 1998).

While TNFα is named for its ability to induce apoptosis in cancer cells, it is also a potent inducer of inflammation that is secreted mainly by antigen presenting cells such as macrophages or dendritic cells following detection of a PAMP which activates MyD88-dependent TLR signaling and expression/ secretion of TNFα (Bouwmeester, Bauch et al. 2004). Secreted TNFα can act in an autocrine or paracrine manner, combining with IFNγ to activate the macrophage in the M1 phenotype.

A T-cell-independent manner of classical activation has been proposed, wherein activation of IRF3 transcription factor by a TLR3 or TLR4 ligand results in expression and secretion of IFNβ, which may replace the requirement for IFNγ and act in an autocrine manner to self-activate the macrophage (Onozaki, Urawa et al. 1988).

The broad-spectrum antimicrobial activity of classically activated macrophages is not permanent, and will subside in the absence of restimulation with IFN and TNF signals. The advantage of classical activation of the macrophage is that it confers the ability to quickly mount an effective antimicrobial response. The disadvantage is that the inflammatory response is metabolically expensive as well as damaging to the surrounding host tissue. Therefore it is important to maintain strict control over this response. The counterpart to the M1 macrophages, the M2 macrophages, contributes to this control by suppressing the M1 phenotype.

1.2.2.2 Alternative/ M2 Activation

Alternative, or M2 activation of macrophages is a term that has been applied to any activation phenotype that is different from M1 or classical activation. As such, there are broad similarities between all M2 macrophages, but there are minor differences which are dependent on the differentiation factors.

All M2 macrophages share the following characteristics. They resolve inflammation by several mechanisms including macropinocytosis (engulfment of large molecules), increased expression of MHCII complex and antigen presentation, increased mannose receptor and other scavenger receptor expression, and expression of arginase 1, which suppresses iNOS activity (Datta and Lianos 1999). M2 macrophages also repair tissue damage largely by secretion of polyamines, which promote cell proliferation. M2 macrophages also express higher levels of CD36, YM1, and FIZZ1 (Gordon 2003). Alternatively activated macrophages are also characterized by reduced expression of IFNβ and the inflammatory cytokines TNFα, IL-1, IL-6, and IL-23 (Martinez, Helming et al. 2008).

The M2 phenotype of macrophages has been subdivided into M2 a, b, and c. M2a are IL-4-, IL-13-, and sometimes IL-10-polarized (Gordon 2003; Ghassabeh, De Baetselier et al. 2006). M2b are a subset that seem to be induced by TLR (LPS and Poly(I:C)) and IL-1R stimulation (Mantovani, Sica et al. 2004; El Kasmi, Qualls et al. 2008). M2c are a recently-described type that are polarized by glucocoritcoids like cortisol and dexamethasone, via the nuclear glucocorticoid receptor (GR), which heterodimerizes with the retinoid X receptor (RXR) in order to activate gene transcription using the response element 5'-GGTACAnnnTGTTCT-3 (Becker, Gloss et al. 1986; Gallardo-Soler, Gomez-Nieto et al. 2008).

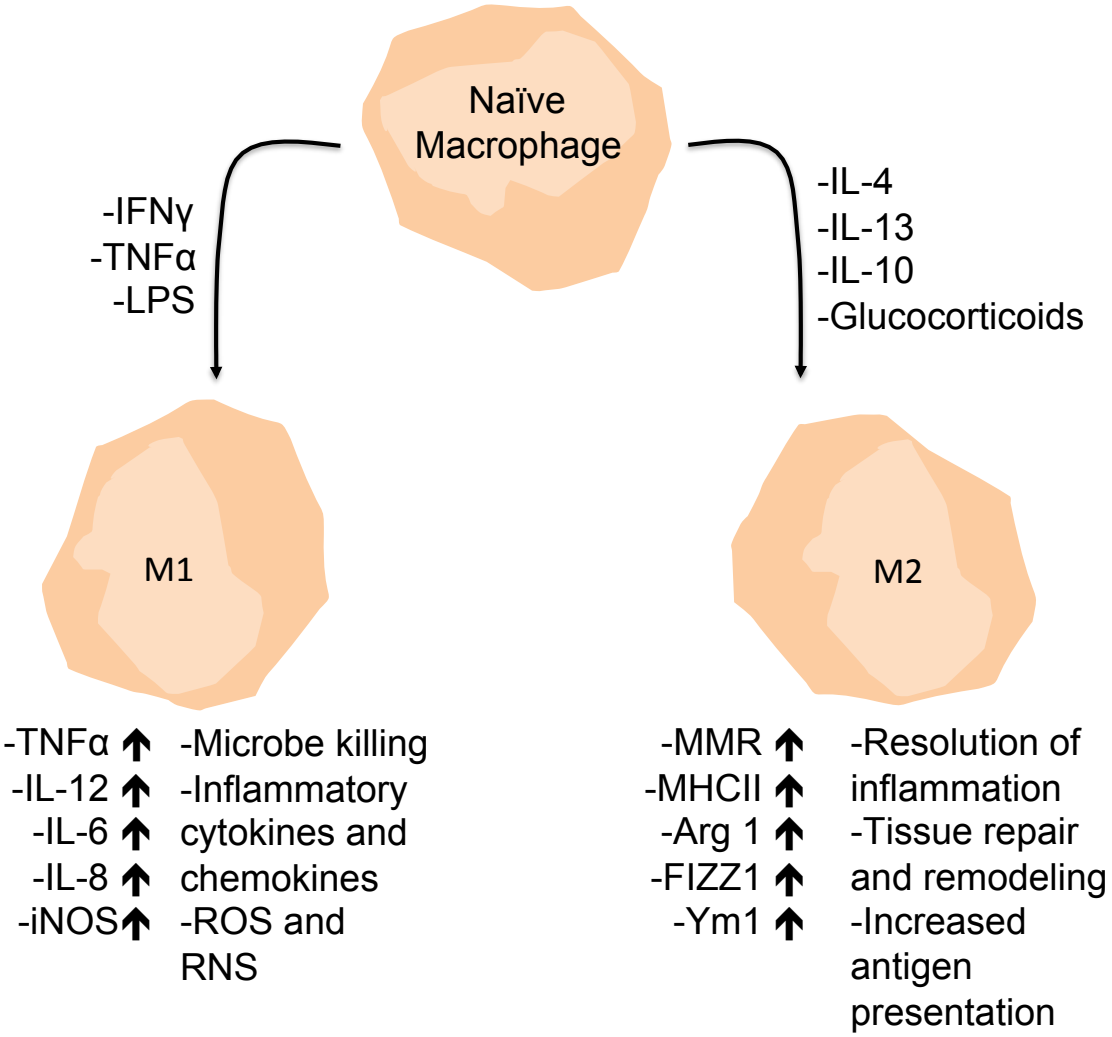
In addition to resolution of inflammation and tissue/wound repair, alternative activation of macrophages is associated with allergic inflammation and an isolative response to parasite infestation (Mabalirajan, Aich et al. 2009; Mylonas, Nair et al. 2009).

It should be noted that while under some circumstances, TLR ligands induce an M2 phenotype, LPS, Poly(I:C), and BLP suppress the polarization of macrophages to the M2a and c phenotypes by IL-4, IL-13, and glucocorticoids (Gordon 2003; Gallardo-Soler, Gomez-Nieto et al. 2008). The paradigm is that TLR ligands suppress M2 and induce M1, and IL-4, IL-13, and glucocorticoids induce M2 and suppress M1, with suppressive crosstalk between signals occurring at the level of receptor, pathway, on the chromatin, and on the mRNA. There is evidence for involvement of the nuclear receptors PPAR, LXR, GR, and RXR and for the TLR 2, 3, and 4 (Gallardo-Soler, Gomez-Nieto et al. 2008). Interestingly, recent studies have shown that LPS can induce macrophages to produce IL-4 (Mukherjee 2008). It is not only strange that IL-4 produced by macrophages, but even stranger that a Th2-assocaited cytokine is induced by LPS, which is strongly aligned with Th1 and M1 polarization. While it is incongruous, it may explain the connection between LPS and Poly(I:C) and the M2b response.

In an effort to resolve the definition of M2 macrophages, Ghassabeh et al. (Ghassabeh,

De Baetselier et al. 2006) performed *in vivo* differentiation and gene expression analysis of macrophages in response to a transplanted tumor or pathogens known to induce M2 differentiation, compared to IL-4- or IL-10-treated peritoneal macrophages *in vitro*. Some genes that were upregulated *in vivo* were not observed *in vitro*. So although the M2 phenotype is not well-defined, there is a core set of characteristic genes and behaviors (Ghassabeh, De Baetselier et al. 2006). The oldest and most representative form of M2 polarization is that induced by IL-4, thus this will be the standard of M2 differentiation in the context of this study. Both the M1 and M2 macrophage are important in both humoral and cellular immunity.

Figure 1.1: **Inducers and phenotypes of M1 and M2 polarization of macrophages.**



1.2.3 Macrophages in Humoral and Cellular Immunity

Macrophages play a part in both humoral and cellular immunity. Macrophages are one of an important class of cells called antigen-presenting cells that constantly scavenge for foreign material and cell debris using a range of receptors including TLR, mannose receptor, M160, and CD163 (Gronlund, Vitved et al. 2000; Fabriek, Dijkstra et al. 2005; Fujiwara and Kobayashi 2005; Fabriek, van Bruggen et al. 2009). Material is taken into the endosome, processed in the lysosome, and complexed with an MHCII complex for presentation to naïve T-cells, which can facilitate activation of B-cells to become plasma cells which produce and secrete antibodies. Antibodies associate in the plasma with invading organisms, and signal classical activation of complement, a system of soluble antimicrobial plasma proteins that disrupt microbial membranes (Snapper, Rosas et al. 1997). Antibodies with IgG and IgA heavy chains have opsonins, which enhance macrophage phagocytosis of the target (Neoptolemos, Wood et al. 1985). Thus macrophages are involved on the front and back end of humoral immunity.

Macrophages are just as important to cellular immunity. They can present antigen to CD4+ and CD8+ T-cells, activating them and causing them to become cytotoxic T-cells or to produce and present antibodies on their surface (Gutcher and Becher 2007). Macrophages can be classically activated themselves, as has been discussed elsewhere in this review, and along with natural killer cells can attack microbes directly by secretion of antimicrobial compounds such as nitric oxide from iNOS, matrix metalloproteinase-1, -2, -7, -9, and -12 which degrade collagen, elastin, and fibronectin (Gibbs, Shanley et al. 1999; Gibbs, Warner et al. 1999; Chizzolini, Rezzonico et al. 2000; Mueller and Pieters 2006). Macrophages also secrete chemokines and cytokines including IL-8, IP-10, MIP-1 α and β, and RANTES, which attract and activate neutrophils, dendritic cells, natural killer cells, and activated T-cells (Luster 2002). The function of the macrophage in its interactions with the other cells in cellular immunity is best understood in the context of its divergence from other leukocytes,

which all originate from the bone marrow common progenitor.

1.2.4 Macrophage Function

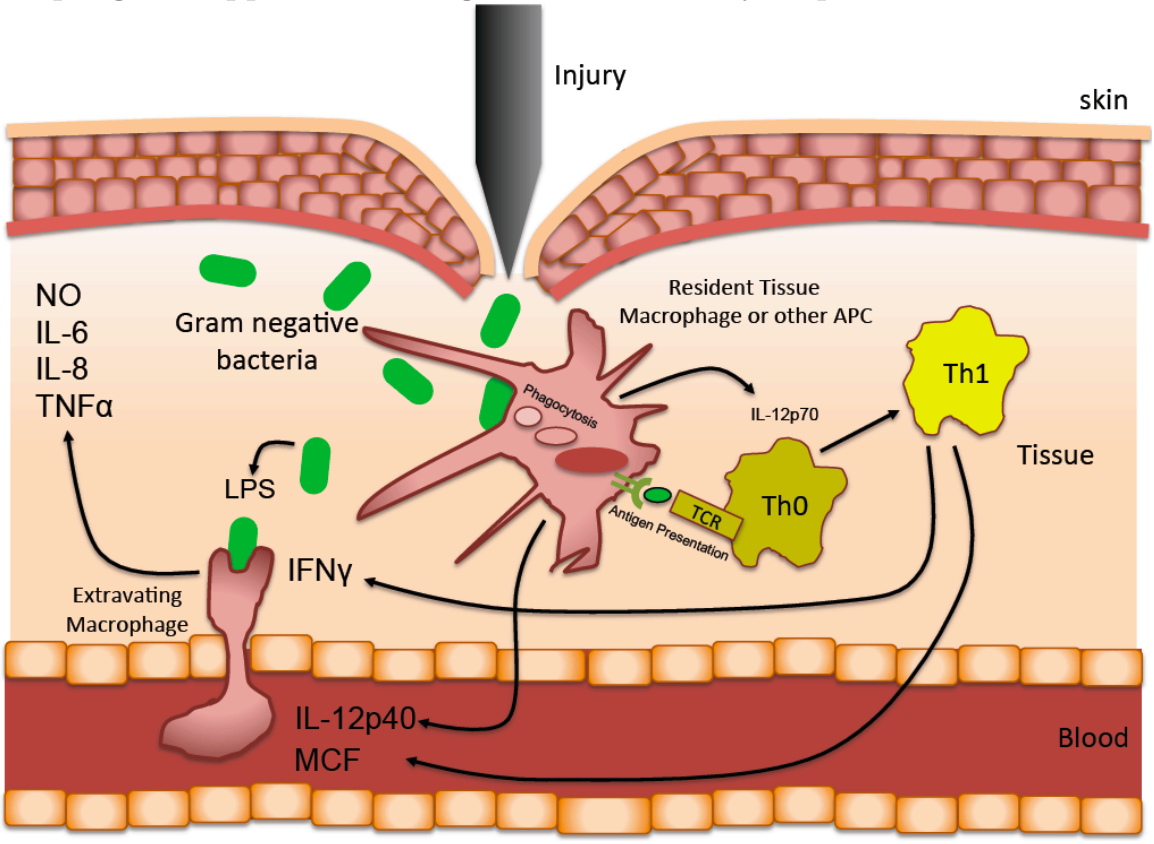
Bone marrow progenitor cells differentiate into monocytes, which are found in the circulation and act as humoral sentinels. Through the process of leukocyte extravasion, induced by chemoattractants secreted from cells that have detected pathogen or been activated by pathogen-detecting cells, monocytes leave capillaries and migrate into the damaged or infiltrated tissue (Tsukada, Kawamura et al. 1991; Jones 2000; Russell, Yan et al. 2003). Leukocyte extravasion can also occur in the absence of infection or injury, allowing for a maintenance of a population of resident tissue macrophages throughout the body.

Naïve resident macrophages constantly sample the tissue environment by phagocytosis of cell debris and other detritus through several mechanisms. Phagocytic cells such as macrophages recognize apoptotic cells using phosphatidylserine receptor and endocytic receptor protein CD91, which detect phosphatidylserine and calreticulin, molecules normally kept on the inner leaflet of the cell membrane. This recognition results in ingestion and removal of the dead cells by a process called efferocytosis (Ogden, deCathehneau et al. 2001). Recognition of opsonins such as the IgG and IgA portions of antibodies, components of the complement system; C3b, C4b, and iC3b, or mannosylated ligands by their respective receptors induces phagocytosis. Recognition of PAMP through TLR can also induce phagocytosis (Ogden, deCathehneau et al. 2001).

Antigen presentation is a key feature of macrophage function. While most cells can present antigen to CD8+ T-cells using major histocompatibility class I (MHCI) complex, professional antigen-presenting cells (APC) such as dendritic cells and macrophages utilize both MHCI and MHCII, and are capable of presenting antigen to and activating CD4+ and CD8+ naïve T-cells (McCormack, Moore et al. 1992). Phagocytized material is dismantled in the lysosome, processed, then presented on the cell surface in a complex with MHCII, integrins, and other recognition molecules necessary for successful T-cell activation. CD4+

T-cells activated by MHCII-mediated antigen presentation become helper T-cells, which exert a profound control over the cell-based and humoral-based immune response, particularly the macrophage inflammatory response (Zhu and Paul 2010).

Figure 1.2: **The role of macrophages in mediating the inflammatory response is twofold. Resident macrophages detect and engulf invading pathogens, then activate Th0 cells to polarize to Th1 phenotype by IL-12p70 and antigen presentation, as well as secrete chemoattractants such as IL-12p40. Th1 cells also secrete chemoattractants such as macrophage chemotactic factor (MCF) and IFNγ. Circulating macrophages extravate into the tissue, where they can be polarized to the M1 phenotype by IFNγ from Th1 cells and TNFα produced in response to LPS. M1 macrophages not only phagocytize, destroy, and present pathogen, but produce a range of cytokines, chemokine, and antimicrobial products. The M2 macrophage is suppressed during the inflammatory response.**



1.3 Inflammation and Innate immunity

The innate immune system is composed of specialized cells, genes, and behaviors designed to thwart infection by microbes such as bacteria, fungi, and virus. All plants and animals have some version of the innate immune system, though some are more developed than others. Following the discovery in the 1990s of the toll/interleukin 1 subfamily of receptors, so-called because they are mammalian homologues of the Toll gene found in *D. melanogaster* and all contain a toll/interleukin 1 receptor (Tireli, Ozbey et al.) domain, understanding of the innate immune system underwent a renaissance. Once thought to be a general inflammatory response, the innate immune response is now recognized not only to be an immediate response to infection, but to be finely tuned to the threat type and level, to be self-regulating, and to be necessary to educate and activate the adaptive immune system. Central to it all are the TIR domain-containing receptors.

1.3.1 Toll-like and Interleukin 1 Receptors

Because LPS is a key modulator of polarization of macrophages to the M1 phenotype, and LPS signals through toll-like receptor 4 (TLR4), a brief introduction to TLR and the signaling mechanisms used by TLR follows.

1.3.1.1 History

The toll gene in D. melanogaster was originally thought only to function in dorso-ventral patterning in the development of the fly (Hashimoto, Hudson et al. 1988). It was cloned and found to be a single transmembrane domain receptor with a series of leucine-rich repeats (LRR) in the extracellular domain, and an intracellular domain named TIR for its homology to that found in the interleukin 1 receptor (Hoffmann and Reichhart 2002). In 1996, toll was implicated in recognition of the fungus Aspergillus fumigatu and mediation of the response, secretion of the antifungal peptide drosomycin (Lemaitre, Nicolas et al. 1996). This led to the discovery of several sequence homologues of toll in almost all species of plants and animals, the first of which was TLR4 in humans (Medzhitov, Preston-Hurlburt et al. 1997).

1.3.1.2 TLR Classification

To date, there are 13 TLR in mammals, each sharing common structure components. The N-terminus is extracellular and consists of 16-28 LRR, each LRR being 20-30 residues in length. This extracellular ligand binding domain acquires a characteristic horseshoe shape that is essential for ligand binding. While each TLR ligand binding domain is similar in structure, each is capable of specifically binding a different ligand. A single membrane-spanning domain connects the extracellular domain to the intracellular TIR domain, which allows interaction with and information delivery to intracellular adaptor proteins (Janeway and Medzhitov 2002; Akira, Uematsu et al. 2006; Medzhitov 2007; Beutler 2009). The receptors function with extracellular recruitment proteins such as LPS-binding protein, hetero- and homo-dimerize, are endocytosed to signal from endosomes, and use several different adaptor proteins within the cell and thus several different signaling pathways (Su, Klein et al. 2000; Kumar, Kawai et al. 2009). The signaling pathways also interact with non-TLR pathways, thus the ultimate result is a complex web of signaling which allows for cellular responses to pathogens ranging from secretion of a specific antimicrobial peptide to broad inflammation to inception of antigen presentation and adaptive immune response.

Each TLR recognizes a different (set of) pathogen-associated molecular patterns (PAMP), well-conserved structures that are both essential and common among a given type of microbe. TLR1/2 and TLR 2/6 recognizes diacyl or triacyl lipopeptides from bacteria, mycobacteria and mycoplasma. TLR2 recognizes peptidoglycan from gram-positive bacteria. TLR4 recognizes LPS from gram-negative bacteria. TLR5 recognizes flagellin. TLR 3, 7, 8, and 9 all recognize various forms of ssRNA from virus. TLR 11 (mouse) recognizes profilin and profilin-like molecules from uropathogenic bacteria. The function of TLR 10 in humans is not known although its expression is sometimes regulated in concert with other TLR (Bourke, Bosisio et al. 2003).

Some TLR signal from the surface, some from the endocytic compartments such as the endosome and endoplasmic reticulum, and some are capable of signaling from more than one location (Kumar, Kawai et al. 2009). TLR 3,7,8, and 9 signal from endocytic compartments. These TLR recognize nucleic acids, and must be sequestered to compartments where self-nucleic acids are not present. Nucleic acid of endocytosed virus and bacteria are detected in these compartments (Barton and Kagan 2009; Kumar, Kawai et al. 2009) In deletion studies, the protein 12-membrane-spanning domain UNC-93B was found to be essential for TNFα production by intracellular TLR, suggesting a key role in intracellular TLR localization or in endosome function (Tabeta, Hoebe et al. 2006).

TLR 1, 2, 5, and 6 signal exclusively from the cell surface (Kumar, Kawai et al. 2009). TLR4 is the first TLR to be confirmed to signal both from the surface and the endosome (Barton and Kagan 2009). It is also the only TLR that can signal through both of the major TLR signaling pathways; the TIRAP/MyD88-dependent pathway and the TRAM/TRAF-dependent pathway (Barton and Kagan 2009).

1.3.2 TLR Pathways and Effects

There are two major shared pathways in TLR signaling; the toll/interleukin receptor 1-associated protein (TIRAP)/ myeloid differentiation primary response gene 88 (MyD88) pathway and the TRIF-related adaptor molecule (TRAM)/ TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway. TIRAP, MyD88, TRAM, and TRIF all contain TIR domains that are sufficient to interact with the TIR domain of TLR and IL-1R. TIRAP and TRAM are not sufficient to signal downstream, and appear to serve to recruit MyD88 or TRIF (respectively) to the receptor, but are not employed by all receptors. TIRAP associates only with membrane-bound receptors via interaction with phosphatidylinositol-4,5-phosphate lipid rafts around the receptor. TRAM can be utilized by surface-localized and endosome-localized receptors.

MyD88 mediates signals from IL-1RI, TLR 1/2, 2/6, and 4 via TIRAP and 5, 7, 8, 9,

11, and 12 sans TIRAP (Peng, Guo et al. 2005). Because interleukin 1 receptor associated kinase 1 (IRAK1) deficiency in HEK 293 cell rendered them unresponsive to IL-1, MyD88 signaling was initially thought to be mediated solely via this molecule. IRAK1 and MyD88 interact directly via the death domain (DD) present on each, upon recruitment, IRAK1 was thought to be induced to autophosphorylate at several residues and become active. Subsequent studies involving IRAK1 kinase dead (KD) mutants wherein Aspartic Acid 340 was mutated to Asparagine revealed that IRAK1 kinase activity was not required for signaling, and in fact a related kinase, IRAK4, was discovered which can both interact with MyD88 and phosphorylate IRAK1. IRAK1 kinase activity is dispensable, but its role in TLR signaling cannot be discounted, as dramatic phenotypic differences are observed between Wt and IRAK1$^{-/-}$ mice.

Two distinct families of transcription factors are induced by MyD88-dependent signaling; NF-κB and AP1. In NF-κB signaling, following phosphorylation, IRAK1 recruits TNF receptor associated factor 6 (TRAF6), which interacts with the ubiquitin-conjugating enzymes Uev1A and Ubc13. IRAK1, TRAF6, Uev1A and UBc13 dissociate from the receptor and interact with a complex composed of TAK1, TAB1, TAB2, and TAB3, activating TAK1. In turn, TAK1 phosphorylates and Uev1A and Ubc13 ubiquitinate a complex of NF-κB essential modulator (NEMO), and inhibitors of κB kinases (IKK α and β). The activated IKKs phosphorylate the inhibitors of κB (IκB α and β), which causes them to be degraded and release the NF-κB subunits p50 and p65, which hetero- and homo-dimerize with other NF-κB subunits and translate to the nucleus, where they are the major inducer or proinflammatory cytokines like IL-6 and TNFα.

In MAPK activation of AP1 by TLR, activated TAK1 also can activate mitogen-activated protein kinase (MAPK) signaling cascades, resulting in activation of p38 and c-jun terminal kinase (JNK), kinases that activate members of the AP1 transcription factor family, which like NF-κB, dimerize and translocate to the nucleus, inducing expression of inflam-

matory cytokines.

Activation of the interferon regulatory factor (IRF) family of transcription factors was initially thought to be exclusively mediated by TRIF-dependent pathways. Recent observations of direct interaction between the MyD88/IRAK4/IRAK1/TRAF6 complex and IRF 1, 5, and 7, resulting in activation and translocation of these transcription factors has shown that there is crosstalk between the pathways below MyD88 and TRIF (Honda, Yanai et al. 2004; Balkhi, Fitzgerald et al. 2008; Wang, Zhang et al. 2008). There is also evidence that the MyD88-dependent pathways are somewhat abrogated in the absence of the adaptor TRAM, thought at one time only to be involved in the TRAM/TRIF pathway (Harari, Alcaide et al. 2006).

Although TRIF required TRAM in order to signal from TLR4, TRIF mediates signals from TLR 3 sans TRAM (Huang, Liu et al. 2005). However, be it through TRAM or not, TRIF activation by TLR3 or TLR4 results in activation of a common set of cell signaling proteins, resulting in activation of the interferon regulatory factors (IRF) 3 and 7, which induce expression of IFNβ, which is secreted and can act on the same cell that produced it.

1.3.3 Lipopolysaccharide

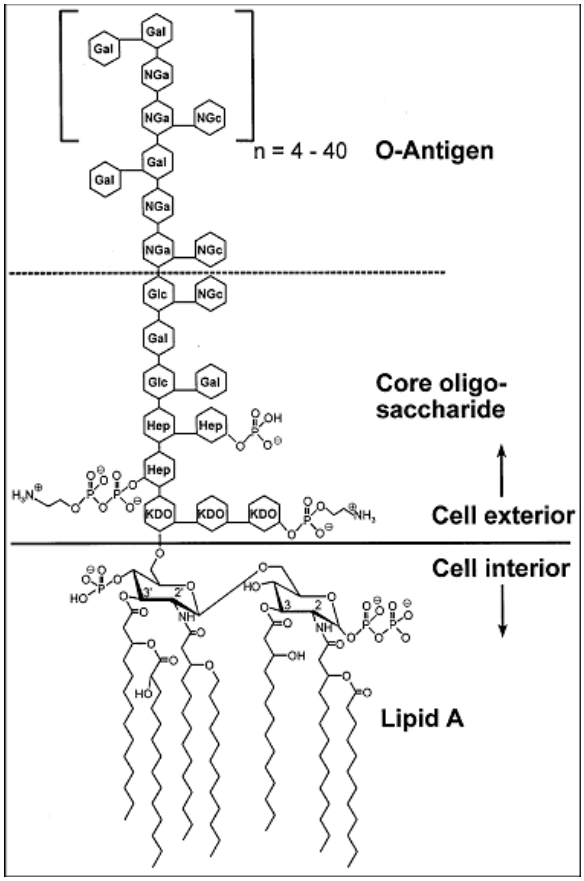
Lipopolysaccharde was discovered in the context of determination by of a non-cellular inflammation-inducing component left behind by cultures of *Eberth bacillus* (Zimmerman 1991). The name lipopolysaccharide was applied in 1943 by Shear and Turner, who developed a method for isolation of LPS and determined its bimolecular nature (Hitchcock, Leive et al. 1986).

LPS is a component of the outer membrane of Gram-negative bacteria. It is ubiquitous in the environment, and a common contaminant in pharmaceutical and experimental preparations (Probey and Pittman 1945). It is such a potent inducer of inflammation that even minimal contamination can make a drug or cell culture reagent useless. It is composed of three distinct parts (Magalhaes, Lopes et al. 2007). The O-antigen, which is a

chain of repeating subunits of 4-40 oligosaccharides of 3-8 monosaccharides each (Petsch and Anspach 2000). The second part, the core oligosaccharide, is a more conserved structure that is composed of monosaccharides covalently linked to form a hydrophilic moiety (Petsch and Anspach 2000). The core oligosaccharide is linked to the lipid A moiety, also called endotoxin, is the most conserved part of LPS and the part that is responsible for its inflammation-inducing properties (Petsch and Anspach 2000; Lin, Williams et al. 2005). Lipid A is composed of two β-1,6-linked D-glucosamines, which are covalently bound to 3-hidroxy-acyl components with 12-16 Carbon atoms on each (Ohno and Morrison 1989). These give the lipid A its hydrophobic quality, which causes it to embed in the membrane and to form large aggregates of up to 1000 KDa with other LPS when shed by dividing or dying cells (Darkow, Groth et al. 1999).

Lipid A and the core oligosaccharide are well-conserved, while the O antigen is less well conserved. Differences in the O antigen account for the different serotypes of LPS, such as *E. coli* B4 vs B8. In all serotypes, the lipid A moiety is conserved (Petsch and Anspach 2000).

Figure 1.3: **Structure of LPS from B4 serotype E. coli (Balkhi, Fitzgerald et al. 2008). Lipid A is well conserved and cytotoxic. Core oligosaccharide is less well-conserved, not toxic. O-antigen is variable, and in the case of "smooth" varieties, missing.**



1.3.4 LPS Signaling

Understanding of LPS signaling has progressed greatly over the past decade since the toll-like receptors were discovered (Chuang and Ulevitch 2000; Du, Poltorak et al. 2000; Hemmi, Takeuchi et al. 2000). There are 13 TLR in humans and mice. Individually or in heterodimers, they can detect a diverse set of pathogen-associated molecular pattern (PAMP) at the cell surface and in endocytic compartments (Barton and Kagan 2009). Ligands include LPS (TLR4), dsRNA (TLR3), bacterial lipoprotein (TLR2 + TLR1 or TLR6), flagellin (TLR5), and others (Akira 2001; Medzhitov 2001; Takeda, Kaisho et al. 2003). Each TLR elicits a highly specific cellular response, but regardless of the PAMP or receptor, much of the downstream signaling requires a common set of adaptor proteins, enzymes, and transcription factors (Zhang, Kirschning et al. 1999).

The first arm of LPS signaling is mediated by myeloid differentiation primary response gene 88 (MyD88) (Burns, Martinon et al. 1998) and is associated with ligand binding on the surface of the cell. MyD88 interacts with the intracellular domain of TLR4 via toll-interleukin 1 receptor (Tireli, Ozbey et al.) domain containing adaptor protein (TIRAP) (Henneke and Golenbock 2001; Horng, Barton et al. 2001). MyD88 binds interleukin receptor associated kinases (IRAK) 4 and 1 via their death domains, facilitating phosphorylation and activation of IRAK4, which then phosphorylated IRAK1 (Jensen, Muzio et al. 2000; Strelow, Kollewe et al. 2003). TNF receptor-associated factor-6 (TRAF6) is recruited by IRAK1 and the IRAK1/TRAF6 dimer dissociates from the receptor complex. TRAF6 recruits transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1), which binds and phosphorylates TAK-1-binding kinases (TAK) 1,2, and 3 (Dauphinee and Karsan 2006). Phosphorylated TAK3 translocates to the cytoplasm and activates inhibitors of κB kinases (IKK) α and β, which phosphorylate the inhibitor of κB (IκB)α, causing it to degrade and release NF-κB subunits for dimerization and nuclear translocation (Dauphinee and Karsan 2006).

Activation of phosphatidylinositol-3-kinase (PI3K) by MyD88-dependent signaling leads to Akt phosphorylation, and has been shown to contribute to NF-κB activation via IKK activation (Faissner, Heck et al. 2006).

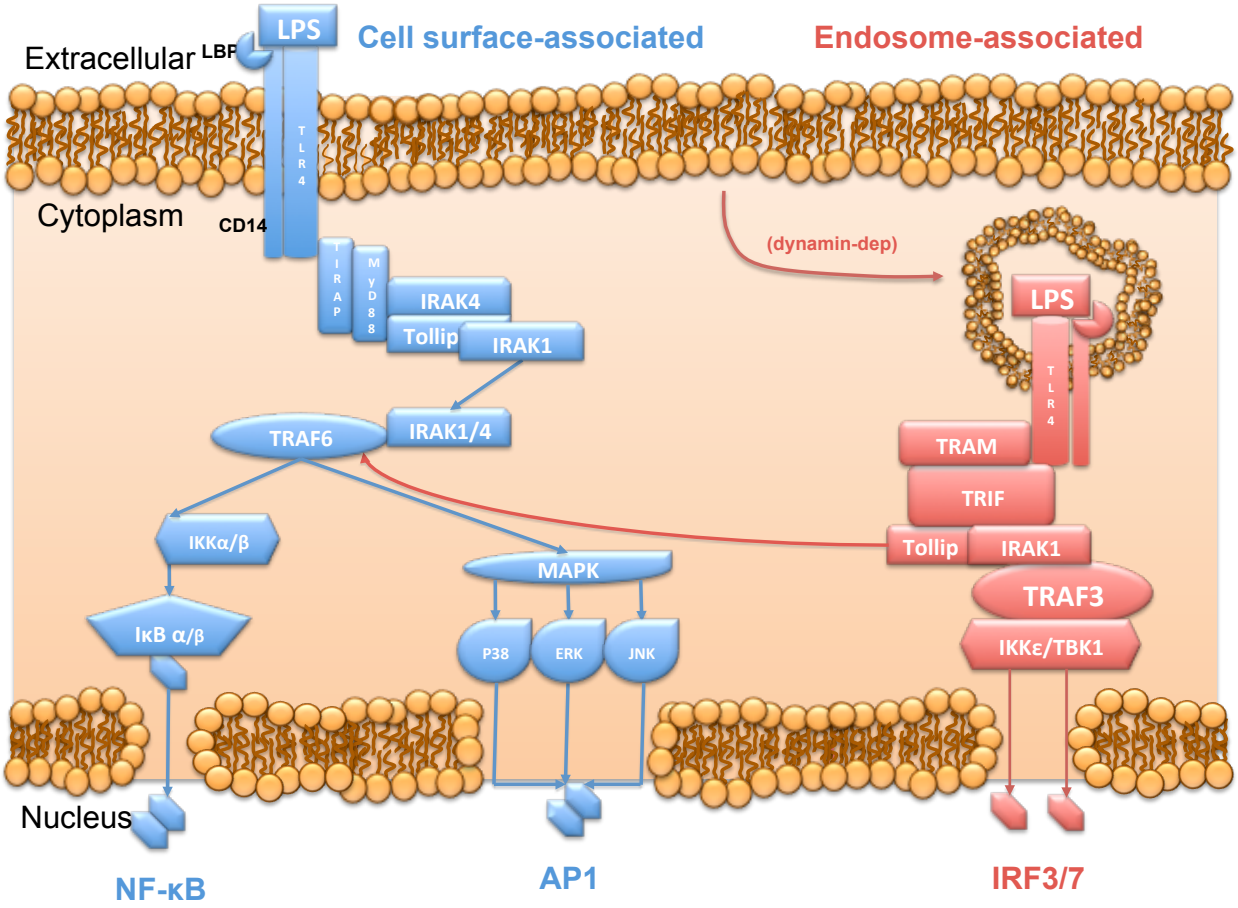
Transcription factor activator protein (AP) 1 is also activated by the MyD88/surface pathway of TLR4 signaling (Schanke, Marcuzzi et al. 1994). AP1 refers to any dimer consisting of members of the c-Fos, c-Jun, activating transcription factor (ATF) and Jun dimerization protein (JDP) families of proteins. AP1 is activated as the final phosphorylation substrate of the mitogen-activated protein kinase (MAPK) cascade (Geppert, Whitehurst et al. 1994). Several MAPK are activated by LPS (Chang, Lin et al. 1997; Jiang, Liu et al. 1999). In murine endothelial cells, TRAF6 mediates activation of c-Jun N-terminal kinase (JNK), and p38, but not extracellular signal-regulated kinase (ERK), which requires Tpl2 and coordination with the Ras signaling pathway, which can independently activate MAPK (Dauphinee and Karsan 2006).

The second arm of TLR4 signaling does not require TIRAP and MyD88, and it is associated with TLR4 signaling from the endosome, rather than the cell surface (Bjorkbacka, Fitzgerald et al. 2004). It is most recently hypothesized that the two types of signaling occur sequentially, as both can activate NF-κB, but with different kinetics (endosomal being slower) (Barton and Kagan 2009). The adaptor molecules in place of TIRAP and MyD88 are TRIF-related adaptor molecule (Sorrentino, Avellini et al.) and TIR-domain-containing adapter-inducing interferon-β (TRIF). TRAM and TRIF are recruited to the TIR domain of TLR to mediate signaling through TRAF3, which associates with TRIF, IRAK1, TANK-binding kinase (TBK) 1, IKKϵ, and interferon regulatory factors 3 and 7 (Hemmi, Takeuchi et al. 2004; Takeuchi, Hemmi et al. 2004; Oganesyan, Saha et al. 2006). IKKϵ and TBK1 are both IKK-related kinases and appear to have overlapping functions, the chief one being activation of IRF3 and IRF7 (Solis, Romieu-Mourez et al. 2007; Chau, Gioia et al. 2008). IRF3 and IRF7 translocate to the nucleus and induce expression of interferon (IFN) β. Secreted

IFNβ can bind type 1 IFN receptor and activate signaling through janus kinases (JAK) and signaling transducers and activators of transcription (Lawson, Polosukhin et al.) 1, as well as PI3K-NF-κB and p38 MAPK (Platanias 2005). Not only does the MyD88-independent arm of signaling impact players in the MyD88-dependent arm, but MyD88-dependent activation of the MAPK JNK has been shown to contribute to IRF3 activation (Solis, Romieu-Mourez et al. 2007). Although the mechanism has not been elucidated, TBK1 and IKKϵ can also activate NF-κB directly through an undefined mechanism (Sankar, Chan et al. 2006; Wietek, Cleaver et al. 2006).

Evidence suggests that the signal-transduction kinase IRAK1 is dispensable for the MyD88-depenent pathway as some IRAK1 functions overlap with those of IRAK4 (Song, Talamas et al. 2009). IRAK1 does, however appear to be required for the MyD88-independent arm of LPS signaling (Deng, Radu et al. 2003; An, Hou et al. 2008; Hou, Wang et al. 2009).

Figure 1.4: **There are two pathways (MyD88/cell surface-dependent and TRIF/endosome-dependent) and three families of transcription factors (NF-κB, AP1, and IRF) that mediate most LPS signaling. There is communication between the two pathways, and the TRIF-dependent pathway is capable of inducing NF-κB as well. The role of IRAK1 and of tollip in LPS proinflammatory signaling is still being debated, but the role in LPS suppressive signaling remains poorly-defined.**



1.4 IL-4 Signaling

In stark opposition to LPS, IL-4 is the classical and key cytokine secreted by Th2 cells that polarizes macrophages to the M2 phenotype, a short review of IL-4 and its signaling is provided herein. IL-4 is a cytokine which not only is expressed by Th2 cells, but also induces Th2 polarization. IL-4 secreted by Th2 induces M2 differentiation in macrophages. The original source of IL-4 that induces Th2 polarization is thought to be basophils (Min, Prout et al. 2004), although as the M2 polarization commonly follows M1 response, it is possible that the macrophage itself is the original source of IL-4 for Th2 polarization (Mukherjee 2008).

Soluble secreted IL-4 binds to the IL-4 receptor α (IL-4Rα), which is bound in the membrane and requires heterodimer formation with the γ common (γc) chain in order to signal properly (Letzelter, Wang et al. 1998). The γc chain also heterodimerizes with the IL-13R, although the IL-4Rα complex is somewhat responsive to IL-13 even in the absence of IL-13R anyway (Russell, Keegan et al. 1993). Intracellularly, γc helps to recruit unphosphorylated STAT6 and janus kinases (JAK) 1 and 3 (Darnell 1997). JAK 1 and JAK3 phosphorylate STAT6 at tyrosines 575, 603, and 631, after which two STAT6 homodimerize and translocate to the nucleus where they bind STAT6 response elements and induce transcription of many IL-4-inducible genes including components of MHCII, CD23, and IL-4Rα (Nelms, Keegan et al. 1999).

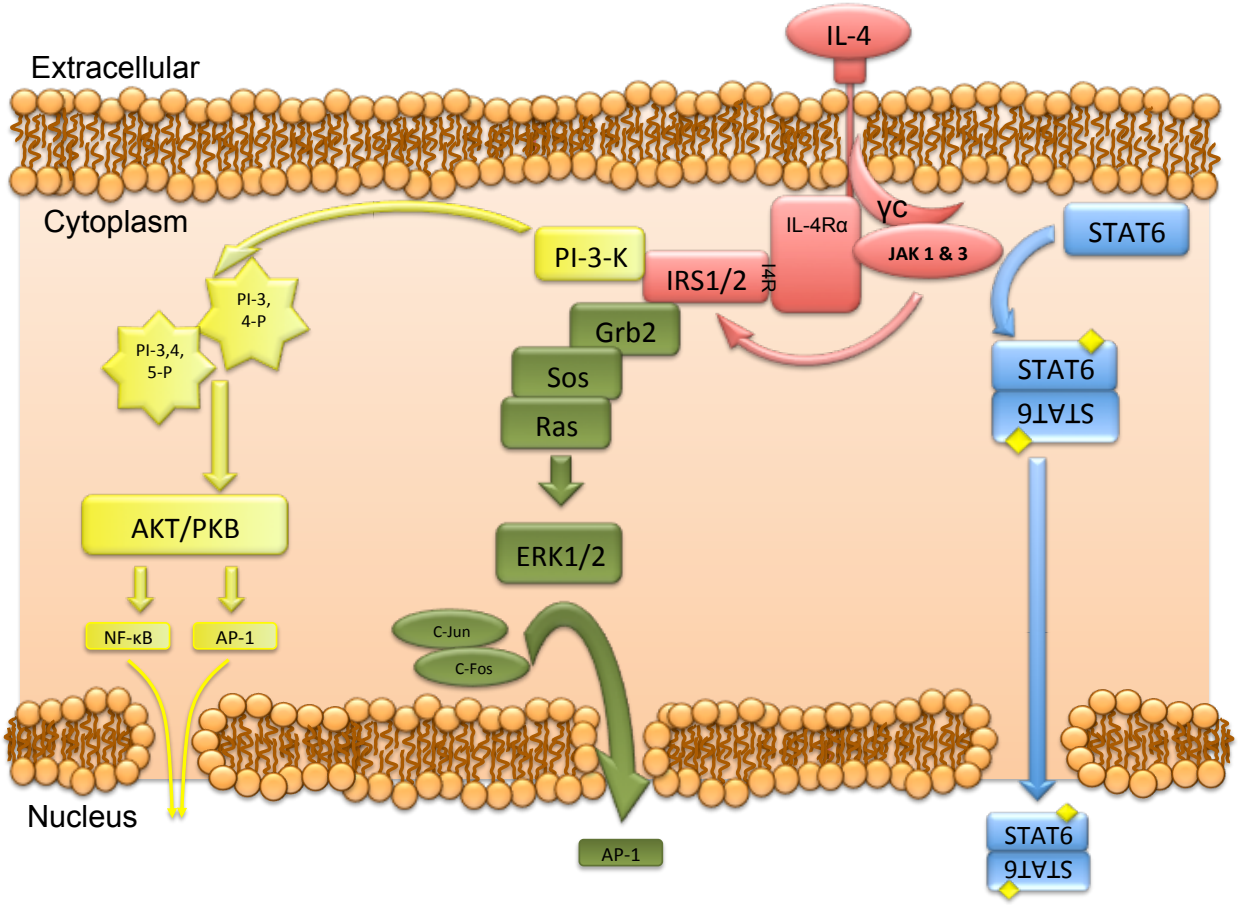
The IL-4Rα/γc chain complex also recruits insulin receptor substrates (IRS) 1 and 2 via Tyrosines 497 and 960 in the conserved interleukin 4 receptor (I4R) domain (Keegan, Nelms et al. 1994; Wang, Paul et al. 1996). IRS 1/2 is hyperphosphorylated in a JAK-dependent manner (Keegan, Nelms et al. 1994), upon which it is capable of interaction with phosphatidylinositol-3-kinase (PI-3-K) (Sun, Wang et al. 1995) and the adapter molecule, Grb-2 (Sun, Crimmins et al. 1993), through which it can activate PI-3-K and Ras/MAPK signaling pathways. Once activated, PI-3-K composed of a p85 and

p110 subunit can phosphorylate membrane lipids as well as Serine/Threonine residues on proteins. Phosphotidylinositol-(3,4,5)-triphosphate and phosphotidylinositol-(3,4)-bisphosphate are produced rapidly upon IL-4 ligand binding (Auger, Serunian et al. 1989; Stephens, Jackson et al. 1993). PI-3,4-P and PI-3,4,5-P are capable of activating protein kinase B (AKT) and protein kinase C families of proteins, which are involved in a modulating a number of cell processes, including MAPK and NF-κB activation (Nishizuka 1995) as well as TGFβ signaling though SMAD3 and SMAD4 (Franke, Kaplan et al. 1997; Franke, Kaplan et al. 1997; Remy, Montmarquette et al. 2004).

IRS 1/2 activates Ras via interaction with Sos though Grb2 (Chardin, Camonis et al. 1993; Gale, Kaplan et al. 1993). Ras-GTP activates Raf, which can phosphorylate ERK 1/2 and cause nuclear translocation and gene expression. Other ERK-like kinases such as JNK may also be activated (Denhardt 1996) ERK 1/2 activates member of the transcription factor family AP1 (Davis 1995). Mutation of the I4R domain reduced not only the IRS 1/2-dependent signaling, but the STAT6 transactivation as well, suggesting that there is some collaborative effort between either the MAPK or PI-3-K pathway (Wang, Zamorano et al. 1998). STATs 1 and 3 are phosphorylated by MAPK signaling (Wen, Zhong et al. 1995), thus ERK 1/2 phosphorylation and augmentation of the ability of STAT6 to successfully signal is likely.

Induction of arginase 1, as well as that of YM1 and other key markers of the M2 phenotype by IL-4 has been attributed to STAT6-dependent signaling (Welch, Escoubet-Lozach et al. 2002). IL-4-induced NF-κB, however, is required for successful DNA binding by STAT6 (Thieu, Nguyen et al. 2007). This introduces a candidate for interaction between IL-4-induced arginase 1 and the many receptors and signaling networks that employ members of the NF-κB family of transcription factors (Liu, Krutzik et al. 2005; Schedel, Frei et al. 2009).

Figure 1.5: **IL-4 Signaling. In the macrophage, IL-4 activates three cell signaling pathways which make it capable of seemingly converse pleiotropic effects. The STAT6 pathway is absolutely required for induction of arginase 1 by IL-4 (Nelms, Keegan et al. 1999). There is evidence in HEK293 cells for involvement of the NF-κB-inducing PI3K pathway in full activation of STAT6-dependent gene induction by IL-4 (Mikita, Campbell et al. 1996; Shen and Stavnezer 1998).**



1.5 Nuclear Receptors

Nuclear receptors are a unique family of receptors, some of which have already been implicated in modulation of important functions of macrophages. A short review of the classification, function, and immunomodulatory properties of nuclear receptors follows.

1.5.1 History and General

In pursuit of the mechanism of action of estrogen, Elwood Jensen identified the first human nuclear receptor, the estrogen receptor, in 1961 (Jensen 1962). There are now 48 known nuclear receptors in humans and hundreds in other animal species (Antebi 2006). Nuclear receptors are ligand receptors that also act as transcription factor, modifying transcription of target genes in response to endogenous hormones, metabolites of dietary origin, and endogenous metabolic intermediates such as cholesterol (Novac and Heinzel 2004).

Nuclear receptors range in mass from 40 to 55 Kilodaltons, and share a general structure composed of five domains. The N-terminal domain contains the activation function 1 (AF-1), which is ligand-independent and weak, but can augment the ligand-dependent activation function (AF-2). It may also be the site of some regulatory modification, such as phosphorylation. The second domain is the DNA binding domain (DBD). It is highly conserved and composed of two zinc fingers, oriented at right angles to one another. It recognizes and binds to well-conserved 4-6 base pair motifs in the proximal and distal (up to five Kilobases from the start codon) gene promoter. The structures for the DBD of many nuclear receptors including RAR and RXR have been solved (Bourguet, Andry et al. 2000; Bourguet, Vivat et al. 2000; Klaholz, Mitschler et al. 2000; Renaud and Moras 2000; Rochel, Wurtz et al. 2000; Kammerer, Germain et al. 2004). The DBD is followed by a short hinge region that has no catalytic or binding functions. The hinge region connects the DBD to the ligand binding domain (LBD). The LBD is the main regulatory center of the receptor. It is composed of 12 well-conserved α-helices and a β-turn (Renaud, Rochel et al. 1995; Wurtz, Bourguet et al. 1996). It contains the ligand-dependent activation function (AF-2), ligand-binding

pocket (LBP), and the main dimerization domain (Rochette-Egly 2005). It is the regulatory center of the molecule, as it is the target of coactivators, repressors, antagonists, and inverse agonists of the receptor. The LBD is followed by the C-terminal region, which is also highly conserved and has more recently been determined to be important for binding coactivators of nuclear receptors (Saatcioglu, Lopez et al. 1997).

There are four general classes of nuclear receptors, based on the ability and requirement to heterodimerize or homodimerize, as well as the cellular location before and after activation by ligand, if the ligand is known. These classes help to categorize but are by no means without exception, as will be seen in the review of the retinoic acid receptor.

Type I nuclear receptors reside in the cytosol complexed with heat shock proteins until they bind ligand, after which they dissociate from the chaperones, homodimerize, and translocate to the nucleus where they recognize and bind to the response elements, recruit histone acetyltransferase to uncoil the chromatin and other cofactors, and initiate assembly of the RNA polymerase and the transcription complex. This process is referred to as transactivation. Type I nuclear receptors include androstane receptor, estrogen receptor, glucocorticoid receptor, and progesterone receptor. The response elements are arranged as an direct or inverted pairs of a receptor-specific 4-6 bp recognition sequence separated by 1 to 5 bases (Xiao, Durand et al. 1995; Langston, Thompson et al. 1997).

Type II nuclear receptors normally reside in the nucleus, already bound to the response element but complexed with corepressors that deacetylate histones to keep the chromatin tightly bound and preclude recruitment of RNA polymerase and the transcription complex. Type II nuclear receptors work as heterodimers with another nuclear receptor, commonly the retinoid X receptor (RXR), making the RXR response element a common feature in gene promoters. Upon ligand binding, a change in conformation displaces the corepressors and provides for recruitment of coactivators and RNA polymerase complex, leading to gene transcription. Retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR),

liver X receptor (LXR), and thyroid hormone receptor (TR) are all type II nuclear receptors (Novac and Heinzel 2004).

Type III nuclear receptors homodimerize like type I, but bind to direct repeats instead of inverted repeats. Type III nuclear receptors have no known ligands (Novac and Heinzel 2004). Type IV nuclear receptors do not require dimerization, and only bind to a single half site, not a full repeat site (Novac and Heinzel 2004).

Nuclear receptors are subject to control by agonists (primary ligand), antagonists which bind directly to the receptor and preclude induction of gene expression, and inverse agonists, which can suppress even the basal level of expression induced by unliganded receptor.

Some nuclear receptors are capable not only of transactivation of gene expression, but also of interruption of other signaling pathways by transrepression (Scheinman, Gualberto et al. 1995). Transrepression was first observed in the context of glucocorticoid receptor direct protein-protein interaction with members of the AP1 and NF-κB families of transcription factors. Transrepression is responsible for many of the inflammation-suppressing capabilities of nuclear receptor ligands.

1.5.1.1 Post-transcriptional modifications

Nuclear receptors are not recycled. They are disposable transcription factors which once activated, induce or suppress gene transcription and are trafficked to the proteasome for degradation (Boudjelal, Wang et al. 2000). They are subject to phosphorylation, sumoylation, and ubiquitination. In the case of RARγ2, phosphorylation at serine 68 of the AF-1 domain by cdk7 (Bastien, Adam-Stitah et al. 2000) or serine 66 by p38 MAPK (Gianni, Bauer et al. 2002; Gianni, Kopf et al. 2002) promotes subsequent ubiquitination and recruitment of SUG-1, a component of the 26S proteasome, and eventual degradation in the proteasome. Degradation is required for successful signaling, and blockage of ubiquitination and degradation results in an attenuated signaling (Zhu, Gianni et al. 1999). Degradation does not, however, require signaling (Osburn, Shao et al. 2001). Indeed RXRα, which is

modified by a cul3-based ubiquitin ligase (Onozawa, Kondo et al. 2006) contains two proline, glutamate/aspartate, serine, and threonine (Sa, Gonzalez et al.) motifs, which are common to proteins that are subject to rapid turnover by the ubiquitin-proteasome pathway (Boudjelal, Wang et al. 2000).

The phosphorylation/ubiquitination scheme is common to all RAR, but phosphorylation at other sites influences the activity of the molecule differently (Gianni, Bauer et al. 2002). PKC phosphorylation in the DNA-binding domain suppresses nuclear export and dimer formation (Delmotte, Tahayato et al. 1999; Sun, Cheng et al. 2007). JNK phosphorylation in the hinge region or the C-terminal region suppresses degradation (both) and translation (C-term only) (Srinivas, Juroske et al. 2005).

In contrast to ubiquitination, sumoylation of RAR and other nuclear receptors results in higher stability, stronger DNA binding activity, and more consistent localization to the nucleus, although this is less well-studied (Wu, Lin et al. 2004; Hwang, Lee et al. 2009).

1.5.2 The Retinoic Acid Receptor

The retinoic acid receptor (RAR) is a type II nuclear receptor of which there are three isoforms; RARα, RARβ, and RARγ, encoded by separate genes with each gene encoding several transcription variants. Although each of the three isoforms is sufficient to activate transcription in response to ligand binding, perhaps as a homodimer (Carlberg 1993; Schrader, Wyss et al. 1993), the presence of retinoid X receptor (RXR), which also has three isoforms; RXRα, RXRβ, and RXRγ, results in higher levels of transcription induced via the common DR5 response element. The difference between the isoforms in mostly in expression patterns in different tissues. All are capable of heterodimerization with RXR and transactivation in response to ATRA.

The response element to which the RAR:RXR heterodimer binds is somewhat permissive in the specific bases as well as the spacing. The DR5 repeat is the classic example of a retinoic acid response element (RARE). It consists of an PuGTTCA motif followed by

five spacer nucleotides then a second direct repeat of the same motif. Because ATRA induces the expression of its own receptors; RAR α and β, the original identification of this response element was in the promoter of the RARβ gene, where it takes the form of 5-GGTTCACCGAAAGTTCA-3. Although the structure of the entire heterodimer complexed with DNA has not been solved, the DBD interactions of RAR:RXR heterodimers have. In DR5 RARE, the RXR DBD interacts with the 5 motif and the RAR DBD interacts with the 3 motif (Gronemeyer and Laudet 1995; Chambon 1996).

There are two natural variants of this response element. DR1 and DR2 elements maintain the same PuGTTCA motif, except separated by 1 or 2 nucleotides, respectively. DR2 can bind heterodimers of RAR:RXR the same orientation as DR5, except the DNA is bent at a sharper angle (Renaud and Moras 2000). DR1 RARE, the only known natural example of which is found in the promoter of the CRBPII gene, requires binding with reverse polarity, and also allows binding of RXR:RXR homodimers (Mangelsdorf, Umesono et al. 1991). Point mutations in the 1st and 6th base in either repeat affect transcription activity negligibly, but any point mutation in the internal bases results in a considerable reduction in transcription (Carlberg 1993).

As a type II nuclear receptor, in the absence of the ligand ATRA, RAR:RXR heterodimers were initially thought to remain bound to the chromatin at the response element and complexed with corepressors that preclude recruitment of the transcription complex. However, recent evidence suggests that shuttling of RAR between the cytoplasm and the nucleus is a viable and highly active form of regulation. In cells devoid of endogenous RAR and RXR, recombinant expressed RARγ localized to the cytoplasm. However coexpression with RXRα (and presumed heterodimerization) resulted in RARγ localization to the nucleus. P38 MAPK was implicated in regulation of RARγ location, as inhibition of P38 MAPK with the inhibitor SB 203580 resulted in RARγ movement from the cytoplasm to the nucleus (Han, Zhou et al. 2009). P38 MAPK has been implicated in phosphorylation of

serines 66 and 68 in the ligand-independent domain of RARγ (variant 2) in the degradation function, raising the question of whether RARγ can be reversibly localized to the cytoplasm for holding, or only for degradation (Gianni, Bauer et al. 2002). All together, it is clear that mechanisms controlling RARγ location are subject to control from other pathways, meaning that RAR, while classified as a type II (always localized to the nucleus) nuclear receptor, has at least one exception in RARγ, and it is not unlikely that the other isoforms are similarly controlled.

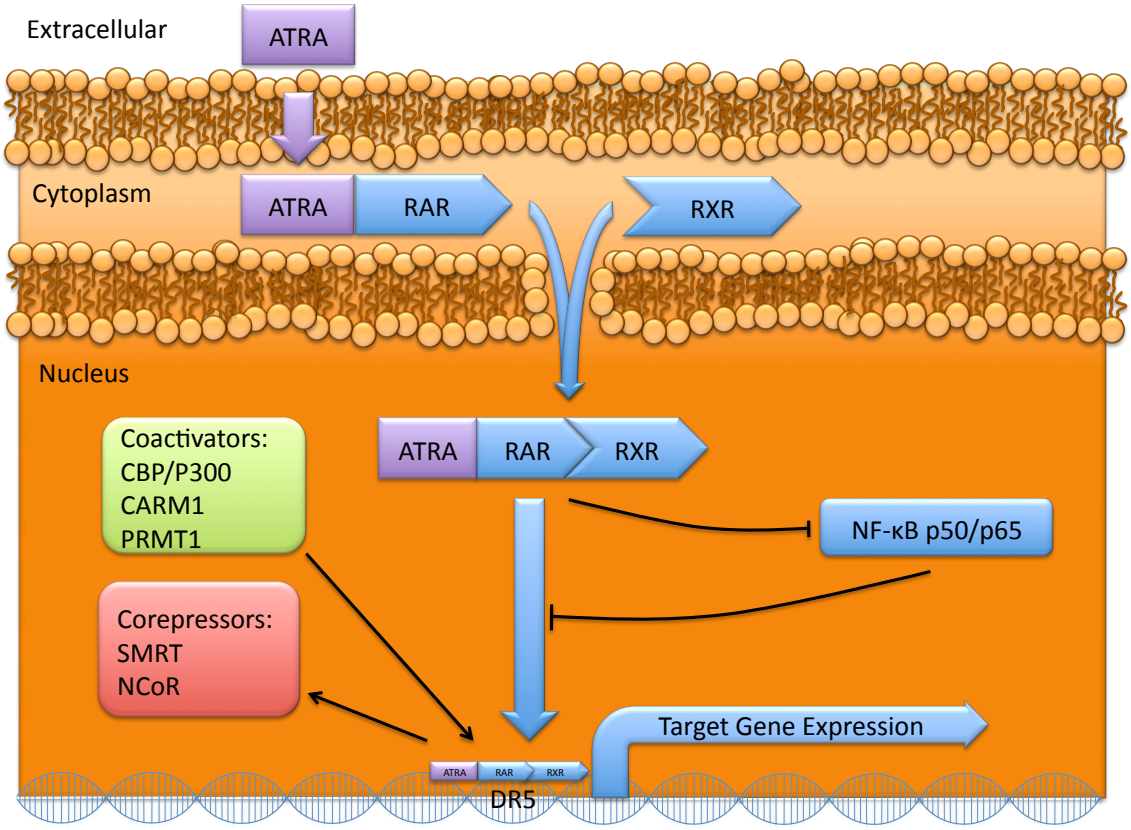
1.5.2.1 Mechanism of RAR transactivation

RAR α, β, and γ are all activated by ATRA in a heterodimer with an RXR isoform, and bind the same response elements as previously described. Prior to dimerization with the opposite partner of the heterodimer, RAR and RXR can exist in the cytoplasm and in the nucleus. This localization is regulated by phosphorylation (Taneja, Rochette-Egly et al. 1997; Bastien and Rochette-Egly 2004; Rochette-Egly 2005). After heterodimerization, RAR:RXR localizes to the nucleus and binds RARE in gene promoters. In the absence of ligand, the RAR:RXR homodimer associates with corepressors. The binding of corepressors and coactivators is mutually exclusive (Rochette-Egly 2005). There are two main corepressor complexes involved in suppression of unliganded RAR:RXR-bound RARE; the nuclear receptor corepressor (NCoR/NCoR1/RIP13) and silencing mediator for retinoid and thyroid hormone receptors (SMRT/NCoR2/TRAC) (Wen, Perissi et al. 2000; Aranda and Pascual 2001). In the absence of ligand, these complexes can bind to a hydrophobic groove in the ligand binding domain. Corepressors per se do not have any enzymatic activity, but the complexes can contain but more often recruit histone deacetylases, which deacetylate lysine residues in chromatin-associated histones, thus promoting tight winding of chromatin and opposing transcription (Jones, Sachs et al. 2001; Perissi, Aggarwal et al. 2004). Upon ligand binding, the LBD changes conformation, exposing an additional α-helix (H12), which reduces the affinity of the LBD for the corepressor complexes and promotes coactivator binding.

The coactivators associated with RAR:RXR dimers are mainly the p160 subfamily of steroid receptor coactivators (SRC). These consist of SRC-1 (NCoA-1), SRC-2 (TIF-2, GRIP-1), and SRC-3 (pCIP, ACTR, AlB1, TRAM1, RAC3) (Perissi, Staszewski et al. 1999; Glass and Rosenfeld 2000; Lefebvre, Martin et al. 2005). SCR-1,-2, and -3 recruit histone acetyltransferases such as CBP/P300 (CREB binding protein)(Dietze, Caldwell et al. 2002; Dietze, Troch et al. 2003) and histone methyltransferases such as CARM1 (coactivator-associated arginine methyltransferase 1) (Huuskonen, Fielding et al. 2004) or PRMT1 (protein arginine methyltransferase 1) (Dong, Zhang et al. 2007) that cause chromatin to become less tightly bound and consequently more likely to be transcribed (Murata, Kurokawa et al. 2001).

In addition to ligand, corepressors, and coactivators, agonists, antagonists, and inverse agonists are potent modulators of RAR and other nuclear receptors. Agonists such as the pan-RAR agonist LGD1550 (Chen, Dokmanovic et al. 2006) are ineffective in the absence of ligand, however they have been shown to be important for complete release of corepressors upon ligand binding (Germain, Iyer et al. 2002; Greschik and Moras 2003). RAR antagonists such as pan-RAR antagonist LG100815 inhibit transcriptional activation, but only in the presence of ligand. Inverse agonists, such as SR-03000000057 and SR-03000000065, identified by a Gal4 screen at the Scripps Research Institute Molecular Screening Center in as-of-yet unpublished data, selectively suppress basal unliganded transcriptional activity for RAR α, β, and γ (Rosen, H. unpublished probe report, 2009, Scripps FL).

Figure 1.6: **ATRA can signal by two major mechanisms: transactivation and transrepression. ATRA ligand binding to RAR induced heterodimerization and nuclear translocation if RAR and RXR are localized to the cytoplasm, or ATRA can bind to RAR:RXR heterodimers already on the chromatin. Conformational change induces binding of coactivators (CBP/P300, CAMR1, PRMT1) and release of corepressors (SMRT, NCoR), allowing deacetylated histones to release chromatin to allow access for RNA polymerase. RXR and RAR DNA-binding activity can be blocked by direct competition by NF-κB subunits p50 and p65.**



1.5.3 The Retinoids

Vitamin A, retinol, is an important dietary vitamin which is metabolized in the lymph, liver, fat, and target cells to yield two nuclear receptor ligands; 9-*cis* retinoic acid (9-*cis* RA) and all-*trans* retinoic acid (ATRA). Differing only in the cis/trans stereochemistry of one bond in the carbon chain, and capable of being enzymatically converted from one to the other, 9-*cis* RA and ATRA nonetheless have very distinct gene transcription profiles, which will be elaborated on later.

Vitamin A is present in the diet as retinyl esters, which upon ingestion are metabolized by pancreatic triglyceride lipase to yield retinol. Retinol is absorbed by intestinal dendritic enterocytes, re-esterified, and secreted into the lymph as chylomicrons which are incorporated into liver parenchymal cells by low density lipoprotein (LDL) receptor-mediated endocytosis (Schweigert and Raila 2002; Harrison 2005; Yokota, Takeuchi et al. 2009). Retinyl ester hydrolases in the liver again yield free retinol, which is bound to retinol binding protein (RBP). Retinol:RBP can be processed in the parenchymal cells or released into the circulation for limited storage in stellate cells (Schweigert and Raila 2002). All-*trans* retinol is metabolized to all-*trans* retinal by retinol dehydrogenases. At this point, all-*trans* retinal can be converted to the chromophore 11-*cis*-retinal by retinal isomerase or to ATRA by retinal dehydrogenases 1 and 2 (RALDH1 and RALDH2) (Wang, Bongianni et al. 2001). Isomerization of ATRA to 9-*cis* RA is not though to be spontaneous, as each has distinct *in vivo* effects (Gallardo-Soler, Gomez-Nieto et al. 2008) but the enzyme responsible has not yet been identified (Luria and Furlow 2004).

ATRA signals through RAR homodimers or RAR:RXR heterodimers, and 9-*cis* RA does not utilized RAR, binding only to RXR homodimers (Mark, Ghyselinck et al. 2006).

Retinoids are involved in regulating embryo development, sight, metabolism, and immune response. Deficiency or overabundance causes malformation of the central nervous system, and problems in limb formation and dorsal ventral patterning (Merrill, Plum et al.

2002; Loureiro, Kao et al. 2005). This is partially dues to ATRA control of Hox genes (Holland 2007). Retinal is incorporated into light-sensing cells of the retina, and photoisomerization from 11-*cis* to all-*trans* retinal changes the interaction with opsin, providing a binary system for detection and interpretation of light (Seki, Isono et al. 1998). ATRA is implicated in positive and negative regulation of hundreds of genes, several cytochrome p450-associated genes CYP1A1 and Cyp26, essential for metabolism of vitamins A and D as well as complex pharmaceuticals (Balmer and Blomhoff 2002). ATRA involvement in the innate immune system is manifold, influencing differentiation of several important cell types as well as expression of both anti- and pro-inflammatory genes.

1.5.4 Immune Effects of the Retinoic Acids

The effects of retinoic acids in the immune system are many and varied. They impact development and activity of T lymphocytes, macrophages, and dendritic cells.

1.5.4.1 In T lymphocytes

ATRA has been implicated in enhancing TGFβ-induced differentiation of naïve T-cell into T-regulatory or T-suppressor cells, which are CD4+, Forkhead Box Protein 3 (FoxP3)+, express high levels of IL-2, and express very low levels of CD127 (Liu, Putnam et al. 2006; Seddiki, Santner-Nanan et al. 2006). Through direct interaction with other immune cells, T regulatory cells are thought to suppress autoimmune responses. The mechanism by which ATRA enhances expression of the Treg-specific marker FoxP3 is not by potentiation of TGFβ signaling. Rather, via RARα, ATRA suppressed expression of cytokines IL-4, IL-21, and IFNγ in CD44hi memory and effector T-cells, a neighboring population of T-cells that suppresses TGFβ-induced Treg differentiation *in vivo* (Hill, Hall et al. 2008). Suppression of the indicated cytokines alleviated subsequent suppression of Treg differentiation in response to TGFβ.

Both ATRA and 9-*cis* RA promote the Th2 phenotype and suppress the Th1 phenotype both *in vitro* and *in vivo*, but the specific mechanism has not been established. In cultures

of primary human blood-derived CD4+ and CD8+ T-cells, treatment with either ATRA or 9-*cis* RA increased expression and secretion of the Th2 cytokines IL-4, IL-5, and IL-13 and suppressed expression of IFNγ, IL-12, and TNFα (Dawson, Collins et al. 2006). In swine infected with the parasitic nematode *Ascaris suum*, however, the story is more complex. Here markers of Th1, Th2, and Treg cells are all augmented by ATRA treatment (Tsai, Chang et al. 2008; Dawson, Solano-Aguilar et al. 2009).

1.5.4.2 In Monocytes and Macrophages

The effects of ATRA and 9-*cis* RA in monocytes and macrophages are just as profound as in T lymphocytes. While a body of information exists on phenotypes, mechanistic evidence is still lacking. In studies performed in THP-1 cells, a human blood monocyte cell line, expression of the LPS-induced Th1-associated chemokines IP-10 and pERK are suppressed by ATRA, but the Th2 chemokine MDC is also suppressed by ATRA (Tsai, Chang et al. 2008). In a study done with primary blood-derived peripheral blood mononuclear cells (PBMC), IL-8 and MCP-1 secretion was significantly increased and RANTES, IL-1β, IL-6, and G-CSF secretion were significantly decreased within 12 hours by ATRA treatment, reflecting an overall skew to the Th2/ alternative macrophage end of the binary spectrum by ATRA (Dawson, Collins et al. 2006).

In atherosclerosis, classically activated macrophages migrate from the blood into the inflamed endothelial cell layer and become foam cells, acquiring cholesterol and growing many times larger, causing partial arterial blockage that can ultimately erupt and cause a heart attack or stroke. An important part of this pathology is reduction of the activity of the ATP-binding cassette transporter A1 (ABCA1), which exports cholesterol from foam cells, thus reducing the risk of arterial blockage. Response elements for RAR and other nuclear receptors have been identified in the promoter of ABCA1, and ATRA indeed does induce its expression and may ameliorate atherosclerotic pathology over the long term (Costet, Lalanne et al. 2003; Maitra, Parks et al. 2009)

1.5.4.3 In Dendritic Cells

Dendritic cells (Manyak, Gladkova et al.) are close cousins of macrophages, and share the detection and antigen-presentation functions (Sallusto and Lanzavecchia 2002). However in the case of ATRA the effects and response are quite different. DC differentiation from monocytes is driven by GM-CSF and IL-4 and maturated with TNFα. Differentiation to DC by IL-4, as measured by DC markers with flow cytometry and functional assays, is suppressed by ATRA treatment (de Sousa-Canavez, de Oliveira Massoco et al. 2009). In contrast, ATRA cotreatment at modest concentrations (˜10 pM) augments differentiation of DC by GM-CSF to IL-12-secreting DC, capable of inducing Th1 differentiation of naïve T-cells (Mohty, Morbelli et al. 2003).

1.5.4.4 In Other Cell Types

In human acute promyelotic leukemia, ATRA is known to cause cell cycle arrest and subsequent maturation and death of leukemic cells. Not only does ATRA bind the RAR-PML fusion protein responsible for the disease, but in HL-60, a human promyelotic leukemia cell line, it enhances through response elements in the distal promoter expression of p62/DOK1 and p56/DOK2, adaptor proteins implicated in cytokine-induced inhibition of proliferation (Lamkin, Chin et al. 2006).

1.5.4.5 Target Genes

ATRA is a well-studied molecule with hundreds of putative targets. In an effort to consolidate the knowledge base, a recent review found evidence in the literature for 27 unquestioned direct targets, 105 candidates for direct control, 267 genes with unknown mechanisms of control, and 133 genes that are indirectly controlled (Balmer and Blomhoff 2002). It should be noted that all of the confirmed direct targets of ATRA are upregulated, and that RARβ and other proteins involved in metabolism or signaling are targets. Among the strong candidates however, there are many that appear at first to contradict one another but in fact demonstrate the immense influence of ATRA and the complexity of its signaling.

This belies the networked nature of signaling that is increasingly being referred to by the rather astral term "crosstalk."

1.6 Crosstalk

Crosstalk between nuclear receptors signaling pathways and toll-like receptor pathways is complex and not at all completely understood. It is in fact the object of study of several successful labs looking to exploit naturally our natural wiring for therapeutic benefit. Crosstalk should first be divided two groups; negative/ suppressive crosstalk and positive crosstalk. The term transrepression, refers to repression of a signaling pathway by mediators of another pathway without involvement of promoters or gene products. It is one mechanism of suppressive crosstalk that is widely utilized between TLR and nuclear receptor signaling pathways. It was first observed in the context of glucocorticoid receptor repression of NF-κB and AP1 (Lucibello, Slater et al. 1990). Since then it has been observed in the case of other nuclear receptors including RAR, PPAR, and LXR, inflammation-associated pathway transrepression of nuclear receptor pathways, and through mechanisms not requiring direct interaction between the repressors and their targets (Scheinman, Gualberto et al. 1995).

In one of the first descriptions of the mechanism of transrepression, Scheinman et al. showed in 1995 that GR is capable of binding NF-κB elements p50, p65, and c-Rel as well as AP1 with its zinc-finger (DNA-binding) domain (Scheinman, Gualberto et al. 1995). This interaction is induced by the GR ligand dexamethasone and suppresses NF-κB-induced gene expression. It is achieved by blocking NF-κB binding to DNA, which causes nuclear NF-κB to translocate to the cytoplasm (Scheinman, Gualberto et al. 1995).

Interestingly, in this study NF-κB/GR interaction also reduces the ability of GR to activate GR response elements on gene promoters. It was also observed that p65 is not only bound by GR, but it is also exported from the nucleus to the cytoplasm. Deletion of

most of the DBD of GR resulted in a much-reduced ability to repress p65 activation of a p65-responsive luciferase reporter in transfection assays.

In a revealing report on mechanisms in transrepression, Ogawa et al. took a closer look at the transrepression of LPS-inducible genes by GR (Ogawa, Lozach et al. 2005). They categorize LPS-induced genes that are either resistant to or subject to suppression by glucocorticoid receptor (GR) ligand dexamethasone. In response to dexamethasone, the DNA-binding domain of GR directly interacts with the Rel homology domain (RHD) of p65, competing for p65 binding with IRF3 and inhibiting p65 signaling. This transrepressive activity is completely dependent on a single residue in the DBD of GR; Lysine 471 in the second zinc finger domain. GRK471A mutant was unable to suppress p65 signaling.

Ogawa et al. also shows that transrepression of a given gene by GR is dependent on the particular TLR ligand used to induce expression (TLR3, 4, and 9 ligands used)(Ogawa, Lozach et al. 2005). Dex-activated GR was able to block p65/IRF-dependent signaling, which is used by TLR4 and 9 but not 3. They also found that PPARγ ligand and GR ligand could suppress LPS induction of iNOS in an additive manner, indicating that each nuclear receptor uses a distinct mechanism of repression. Further they found that NCor was required for PPARγ-mediated suppression but not for GR-mediated suppression, meaning that PPARγ-mediated suppression may depend on chromatin modification on the gene promoter.

Until recently, the mechanism by which nuclear receptors transrepress target genes in an NCor-dependent manner was poorly understood. Recent work by Serena Ghisletti et al. shows that NCor-dependent repression by PPARγ or LXR of LPS-induced iNOS also requires SUMO E2-conjugating enzyme Ubc9 (Ghisletti, Huang et al. 2007). LXR ligand GW3965 not only caused LXR to be recruited to the iNOS promoter, but also blocked LPS-mediated NCor removal. Ubc9 siRNA knockdown precludes LXR recruitment to iNOS promoter. They also show that SUMOylation by SUMO 2 or 3 is required for transrepression

by LXR but not transactivaton of LXR target genes.

In further clarification of the nuclear receptor-specific transrepression phenomenon, Ghisletti et al. used IL-1β. IL-1β is induced by LPS and Pam3CSK4 (TLR1/2 ligand) (Huang, Ghisletti et al. 2009). LPS-induced IL-1β is transrepressed by PPARγ and LXR. Pam3CSK4-induced IL-1β is transrepressed by PPARγ but not by LXR. It was found that NCoR clearance from the promoter correlates with nuclear receptor-specific transrepression. This data coupled with work by Ogawa et al. indicates that transrepression of the TLR pathway as well as transrepression on the target gene promoter are both very specifically regulated and restricted in terms of the inflammatory signal, the nuclear receptor signal, and the gene repressed (Ogawa, Lozach et al. 2005). This runs contrary to the generalization of signals as pro- or anti-inflammatory.

Although calreticulin, a ubiquitous Ca2+-dependent inhibitor of transcription has been shown to reduce RAR:RXR binding to RARE (Desai, Michalak et al. 1996) and to bind to and inhibit other RXR-binding nuclear receptors (Winrow, Miyata et al. 1995) and LPS signaling has been shown to lead to induction of calreticulin in silkworm (Takahashi Tadashi 2006), this is a tenuous connection at best, and it has not been explored as a mechanism of LPS suppression of nuclear receptor signaling.

Transcription factor export from the nucleus has been shown for NF-κB and IRF (Arenzana-Seisdedos, Turpin et al. 1997; Kumar, McBride et al. 2000). Though some nuclear receptors reside on the chromatin, others are known to move routinely between the cytoplasm and the nucleus. Export to the cytosol is, not surprisingly, an effective mechanism for repression of nuclear receptor signaling. While export to the cytoplasm of nuclear receptors has been reported to be dependent on activity of the DBD-binding calreticulin, the MAP kinase JNK, which is used by several TLR, has also been implicated. Itoh et al. report that JNK-dependent phosphorylation of Ser 226 of GR-GFP fusion protein resulted in nuclear export and reduced DNA binding of the nuclear receptor (Itoh, Adachi et al. 2002).

Another arena for competition is for coactivators P300 for CREB-binding protein (CBP) (Li, Pascual et al. 2000). Several studies have shown an additional mechanism of transrepression; nuclear receptor competition with TLR transcription factors such as NF-κB, AP1, and STAT1 for limited coactivators CREB binding protein (CBP) and p300. Li et al. confirmed that PPARγ transrepression of LPS-induce iNOS was dependent on its ability to bind CBP, and that a single amino acid mutation in the SRC-1 binding domain, through which PPARγ recruits CBP, ablated both transrepression and transactivation activities (Li, Pascual et al. 2000). This mechanism of transrepression not only is independent of the repressed promoter, but it does not require any association between the nuclear receptor and the TLR transcription factor.

1.6.1 LPS Signaling Suppressing RA Signaling

In this section, mechanisms by which LPS signaling through TLR4 and its downstream pathways antagonize effects of retinoic acid signaling through RAR and RXR will be reviewed. While mutual antagonism of retinoic acid and LPS has been phenotypically observed for many years, mechanistic studies have been restricted to the last decade since the discovery and characterization of TLR. Hundreds of genes have been found to be regulated by retinoic acid signaling alone, and thus the implications of interruption of RA signaling are evidently manifold (Balmer and Blomhoff 2002). Regulation of lipid storage and metabolism (Amengual, Ribot et al. 2008), drug metabolism (Zolfaghari, Cifelli et al. 2007), embryogenesis and cell differentiation (De Luca 1991; Degos 1993; Pijnappel, Folkers et al. 1998; Chen, Wang et al. 1999), the acute phase response and inflammation (Schweigert 2001; Gordon 2003), and in microbial clearance . Although much work remains to be done, here reviewed are the mechanisms that have been discovered by which LPS can effect ATRA signaling.

In 2006, Zolfaghari et al. reported that ATRA-induced Cyp26A1 and Cyp26B1, members of the cytochrome p450 family of enzymes essential for metabolism of pharmaceuticals and vitamins in the liver, were suppressed by LPS cotreatment in rat liver *in vivo* (Zolfaghari,

Cifelli et al. 2007). This correlates with hepatic and plasma decrease of retinol binding protein, a plasma carrier protein for retinol, although a mechanism is not formally proposed by the author.

A common positive-feedback mechanism is that signaling through nuclear receptors often induces expression of that receptor. This is indeed the case with RAR. RARβ has a confirmed retinoic acid receptor response element (RARE) in its promoter. Evidence is mounting for LPS repression of RXR and other nuclear receptor expression, both constitutive and nuclear receptor ligand-induced.

Some of the earliest evidence for LPS repression of nuclear receptor expression came in the form of two reports from the same San Francisco group in 2000 and 2003. In 2000, Beigneux et al. reported that LPS treatment of Syrian hamsters represses the expression of RXR α, β, and γ in hamster liver (Beigneux, Moser et al. 2000). The focus of the report was on lipid metabolism, thus several of the metrics employed were measures of PPAR and LXR signaling, but all isomers of RA require RXR as a component of the signaling complex, so RXR repression is applicable to RA signaling. Interestingly, the conclusion of the report was not that the transcription of the RXR isoforms was affected, rather that an increase in mRNA degradation was responsible for the repression. It was found that RXR proteins and mRNA were reduced by LPS in a dose-dependent manner up to 97% in the case of RXRα. However in nuclear run-on assays, wherein mRNA was produced from nuclei removed from liver cells and thus not subject to post-transcriptional modification and degradation, the RXRα repression by LPS was only 38%, and almost no repression of RXR β and γ occurred, indicating that most LPS repression of RXR is mediated by posttranscriptional mechanisms. It was also found that both isoforms of LXR (α and β) as well as all three isoforms of PPAR (α, β, and γ) were repressed by LPS. Although the pathway by which LPS mediates this repression is not elucidated in this paper, RXR, LXR, and PPAR repression by LPS is shown to correlate with reduced binding of the receptors to their respective response elements in

EMSA of hamster liver nuclear extract (Beigneux, Moser et al. 2000).

In a report by the same group in 2003, authored by Min Sun Kim (Kim, Shigenaga et al. 2004), the work was expanded into other nuclear receptors including farsenoid X receptor, thyroid receptor, constitutive androstane receptor, and pregnane X receptor. Also, suppression by IL-1 and TNF together was found to repress almost as completely as LPS alone, indicating that much of LPS repressive effect is mediated through IL-1 and TNF induction. This paper still did not define the missing mechanistic link between nuclear receptor mRNA stability and LPS signaling.

In 2006, Chow et al. broke a into a new aspect of TLR repression of nuclear receptor transcription in the context of Reyes syndrome, wherein viral infection limits the livers ability to metabolize aspirin, leading to hepatotoxicity (Chow, Castrillo et al. 2006). In a previous publication (Castrillo, Joseph et al. 2003), it was determined that IRF3, a transcription factor activated by the endosome arm of TLR4 signaling as well as TLR3 signaling, mediates TLR3/4 repression of transcriptional activity of LXR. In light of this data, the role of IRF3 was investigated in viral suppression of RXR in Reyes syndrome. Both Poly(I:C) (TLR3) and LPS (TLR4) suppress RXRα expression in mouse liver *in vivo*. The suppression was reduced by more than half in IRF3$^{-/-}$ mice. It was further found that transcriptional repressor Hes1, and not ZNF5 or ZNF202, is induced by Poly(I:C) and binds to the promoter of RXRα, recruiting HDAC1 and repressing transcription. In further work, it was shown that overexpression of Hes1 constitutively suppressed RA transcriptional activity, and Hes1 knockdown experiments reveal that Poly(I:C) is incapable of repressing RA transcriptional activity without Hes1 (Chow, Castrillo et al. 2006).

TLR can also repress retinoic acid signaling by direct intervention in the pathway. Several distinct mechanisms utilizing both arms of LPS signaling have been identified. In 2002, Li et al. characterized a unique pathway for LPS suppression of RA signaling (Li, Zimmerman et al. 2002). LPS-activated c-Jun N-terminal kinase (JNK)-dependent phosphorylation

of RXR resulted in reduced RAR:RXR transactivation of the Ntcp gene promoter. JNK is a mitogen activated protein kinase that is utilized in the MyD88-dependent arm of LPS signaling, and has been implicated in induction of inflammatory cytokines like iNOS (IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38 MAPK in a mouse macrophage cell line) and IL-6 via AP1 transcription factor (Jang, Kelley et al. 2008).

In addition to its transactivation activity via AP1, it was found that LPS- or IL-1β-activated JNK (and not MAPK p38 or ERK) is capable of repressing RAR:RXR transcriptional activity on Ntcp by direct binding to and phosphorylation of RXR. Interestingly, the natural product curcumin, an inhibitor of JNK phosphoactivation, abrogated IL-1β or LPS phosphorylation of RXR and suppression of ATRA-induced RAR:RXR transcriptional activity on Ntcp promoter.

Two years after JNK-dependent phosphorylation of RXRα was described, Zimmerman et al. published a report proposing a mechanism (Zimmerman, Thevana nther et al. 2006). In the context of hepatic acute phase response suppression of RA-induced metabolic genes, it was found that *in vivo* LPS IP injection induces JNK phosphorylation and activation in mouse livers, causing rapid (w/in 1 hour) and sustained cellular relocalization of RXRα from the nucleus to the cytoplasm. RXRα expression, it should be noted, was also reduced by LPS treatment, pointing to a multi-mechanism suppression. Interestingly, RARα expression and nuclear/cytoplasm distribution was not measurable altered in this study, indicating that RXRα, the shared binding partner, is the main target of suppression by LPS.

In 2005, a report by Srinivas et al, it is reported that activated JNK phosphorylates and causes the degradation of RARα by the ubiquitin-proteasome pathway, markedly reducing RARα transactivation activity (Srinivas, H., D. M. Juroske, et al. 2005). Although RARα degradation by the proteasome following transactivation has been known to be dependent on MAPK p38 phosphorylation, JNK-mediated degradation does not appear to require RARα transactivation in order to occur.

In a break from the JNK-mediated repression of RXRα, Gu et al. describe a distinct mechanism of RXR repression by LPS in 2006 (Gu, Ke et al. 2006). It is shown that p65, a subunit of NF-κB, is also capable of interrupting RXR signaling. In this study, pregnane X receptor (PXR), which forms a heterodimer with RXR and is activated by dexamethasone and rifampicin (RIF), is studied because PXR:RXR induce a range of genes in the cytochrome p450 family, including cyp3a4, which is a major metabolizing enzyme for prescription drugs. Primary human hepatocytes were treated with RIF 10 μM alone or with RIF and 5 μg/ml LPS. CYP3A4 mRNA was induced 35 fold by RIF and suppressed almost completely by LPS. Suppression of NF-κB by phosphorylation-incapable IκBα (SRIκBα) ablated LPS suppression of CYP3A4, so the role of NF-κB was investigated. In EMSA following *in vitro* DNA binding experiments of PXR:RXR to the consensus response element, increasing concentrations of p65, but not p50, inhibited PXR:RXR complex binding to the ER6 oligonucelotide. Interestingly, p50 and p65 together did not inhibit binding, suggesting that p50 dimerization with p65 may inhibit its repression activity. In following GST-pulldown experiments, it was found that RXRα DNA-binding domain (DBD) bound p65, whereas truncation mutants in which the DBD was removed did not bind p65. While the subcellular distribution of RXRα is not addressed here, this paper does propose a mechanism for repression of RA signaling by LPS that is distinct from JNK phosphorylation (Gu, Ke et al. 2006).

In recent work which delves deeper into the molecular mechanisms, Maitra et al. show that RARα levels in the nucleus are dramatically constitutively elevated in primary bone marrow-derived macrophages from mice deficient in IRAK1, a key signaling kinase in LPS signal transduction (Maitra, Parks et al. 2009). Furthermore, ATRA-responsive cholesterol efflux gene ABCA1 expression is higher in IRAK1$^{-/-}$ macrophages than in Wt, and LPS suppression of ABCA1 is less in IRAK1$^{-/-}$ than in Wt (Maitra, Parks et al. 2009).

In related unpublished work in the Laboratory of Innate Immunity and Inflammation

at Virginia Tech, IRAK1-dependence has been implicated in LPS suppression of arginase1, which is synergistically induced by IL-4 and ATRA cotreatment of both Wt and IRAK1$^{-/-}$ BMDM.

1.6.2 RA Signaling Suppressing LPS Signaling

Of the three naturally occurring isomers of retinoic acid, all-*trans*, 9-*cis*, and 13-*cis*, all-*trans* and 9-*cis* have the largest body of work in suppression of inflammation. In early phenotype studies, it was found in 1997 that 9-*cis* RA suppresses LPS-induced TNFα and nitric oxide (NO) production in cultured rat kupffer cells (Motomura, Sakai et al. 1997; Motomura, Sakai et al. 1997). It appears in this case to be a response primarily mediated by RXR, as RAR-specific synthetic ligands do not suppress as 9-*cis*-RA does. It was unclear at this point whether RXR:RXR ligand binding suppresses LPS signaling in the cytoplasm or in the nucleus, or whether it affects LPS suppression of RAR and RXR. It would be interesting to see the effect of 9-*cis* RA suppression of LPS signaling on LPS suppression of expression of critical hepatic metabolism genes such as the cytochrome p450 family. Do the two signals fight it out to a standoff, or does one invariably win out? Two years later, Datta et al. showed that iNOS suppression in mesangial cells is also suppressed by ATRA and 13-*cis* RA as well (Datta and Lianos 1999).

In a phenotype more relevant to human disease, it was found by Asakura et al. in 2001 that ATRA treatment effectively suppresses LPS-induced model of disseminated intravascular coagulation (DIC) in rat (Asakura, Aoshima et al. 2001). DIC is associated with sepsis and is characterized by bleeding and organ failure. In the context of treating patients with acute promyelotic leukemia with ATRA, it was observed that risk of disseminated intravascular coagulation (DIC) was decreased, even in the absence of anticoagulants. ATRA was more effective than the standard therapy, low molecular weight heparin administration, at inhibiting LPS-induced glomerular fibrin deposition, thus establishing it as an effective anticoagulant agent. It was hypothesized that the anticoagulant effect may be mediated

by induction of thrombomodulin, a cofactor of thrombin in activation of protein C in the anticoagulant pathway, and suppression of tissue factor in leukemic and endothelial cells. In an interesting result, Wang et al. reported that ATRA treatment of THP-1 monocyte cell line resulted in synergistic induction of IL-10 with LPS but antagonistic suppression of LPS-induced TNF-α and IL-12 (Wang, Allen et al. 2007). IL-10 is largely anti-inflammatory, able to block NF-κB signaling, but IL-12 and TNF-α are both inflammatory mediators. This result indicates that ATRA is upregulating an already present negative feedback loop in LPS signaling.

In an additional study using THP-1 monocytes, pretreatment with ATRA opposes LPS-induced secretion of Th1-related interferon-inducible protein 10 (IP-10) and Th2-related macrophage-derived chemokine (MDC) (Tsai, Chang et al. 2008). Realtime RT-PCR analysis indicates that transcription of IP-10 was suppressed by ATRA. Additionally, ATRA suppressed LPS-induced expression of members of the MAPK-activating pathway c-RAF; MKK1/2; ERK (Tsai, Chang et al. 2008). The authors conclude that reduced expression of members of the LPS signaling pathway is one potential mechanism by which ATRA suppresses LPS-induced Th1 and Th2 cytokines.

As in the case of LPS suppression of RA, mRNA destabilization is employed by RA to suppress LPS. In 2001, Motomura et al. reported that the mechanism by which ATRA treatment of hepatic macrophages reduced LPS-induced TNF-α was not transrepression of NF-κB or AP1, but acceleration of degradation of TNF-α mRNA was (Motomura, Ohata et al. 2001). In a similar manner, destabilization of LPS-induced NOS2 mRNA is shown to occur in response to ATRA treatment of cultured rat cardiomyocytes and microvascular endothelial cells (Grosjean, Devaux et al. 2001).

ATRA is used as a topical treatment for acne, caused by superficial infection with *Propionibacterium acnes*, which activates several TLR, including 1,2, and 4. In an effort to describe a mechanism for ATRA suppression of acne, Liu et al. reported in 2005 that TLR2

and CD14 expression is down-regulated by ATRA treatment of primary human monocytes . CD14 helps to recruit TLR ligands to TLR, including TLR4 (Liu, Krutzik et al. 2005). Liu found that downregulation of TLR expression is a mechanism distinct from transrepression for ATRA antagonism of TLR signaling (Liu, Krutzik et al. 2005).

Although examples of transrepression of LPS signaling by nuclear receptors abound, this mechanism has been noted only sparsely in the case retinoid signaling. In a 1999, Na et al. demonstrated that physical interactions between RXR and NF-κB is required for 9-*cis* RA suppression of LPS-induced IL-12 in murine macrophages (Na, Kang et al. 1999). Ligand-bound RXR associated with NF-κB subunits p50 and p65. This transrepression is dependent on the activity of the transactivation domain, as a null mutant for transactivation activity, which was incapable of binding cofactors SRC-1 and P300 was incapable of binding to and transrepressing NF-κB. Not only do RXR and NF-κB physically interact, but they also compete for cofactors p300 and SRC-1 (Na, Kang et al. 1999).

Retinoic acid is a known cell differentiating factor and is used to treat tissue tumors in addition to its widespread use in treating acute promyelotic leukemia (Degos 1993). In a 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induced tumor model, AP1 is required to mediate signals leading to neoplastic transformation. RA and other nuclear receptor ligands have long been known to suppress AP1 signaling, although the mechanism is not clear (Pfahl 1993; Fisher and Voorhees 1996; Li, Dong et al. 1996; Li, Westergaard et al. 1997). Three mechanisms have been proposed, although none is complete. Suzukawa et al. propose that retinoic acid targets AP1 dimers containing full-length AP1 subunits JunB or FOSL1 via their basic leucine zipper (bZIP) domains, as RA was unable to repress fusions containing only the transactivation domain (Suzukawa and Colburn 2002). Ramrez et al. implicate protein phosphatase 2A (PP2A) in AP1 repression. In CA-OV3 ovarian carcinoma cells, ATRA treatment suppressed AP1 transcriptional activity by 50%. This correlates with a reduction in c-Jun phosphorylation. ATRA induces PP2A, and PP2A inhibitor blocks ATRA

suppression of AP1. It is not clear whether PP2A activation is hampered or if some other mechanism is at work in the ATRA-resistant carcinoma SK-OV3 (Ramirez, Haberbusch et al. 2005). Kirchmeyer et al. found that in rat synovial fibroblast, ATRA suppressed NF-IL-6 and AP1 activity. It is proposed that NF-IL-6 and AP1 transrepression by ATRA is independent of RAR, as siRNA knockdown of each RAR isoform did not affect ATRA suppression of NF-IL-6 and AP1, although this may simply indicate redundancy in function (Kirchmeyer, Koufany et al. 2008). ATRA, but not RAR-selective agonists is capable of reducing phosphorylation of ERK1/2 but not that of JNK or p38, and the effect of ATRA on ERK1/2 and IL-6 and AP1 was recapitulated by ERK inhibitor PD-98059 (Kirchmeyer, Koufany et al. 2008).

RAR has also been shown to be constitutively bound to the AP1 response element, and to exclude the MAPK ERK and CREB-binding protein following ATRA treatment, concomitant with promoter-dependent transrepression (Benkoussa, Brand et al. 2002). This implicates a distinct mechanism of AP1 transrepression by RAR.

Whole-body NF-κB luciferase reporter mice were used to observe the effect of ATRA on NF-κB signaling *in vivo* (Austenaa, Carlsen et al. 2009). ATRA dramatically dampens LPS-induced NF-κB transcriptional activity throughout the body as well as in the human myeloblastic cell line U937. It was also found that STAT1 may play a role in ATRA-induced suppression of NF-κB as a STAT1 inhibitor partially abrogated the ATRA suppressive effect.

1.7 Rationale for this Study

High-fat diet, smoking, and excessive consumption of alcohol are contributors to a wide variety of diseases including atherosclerosis (cardiovascular disease), obesity, and diabetes. For years the reason was unknown. Recent publications implicate these behaviors, along with excessive exertion, aging, and any activity that increases permeability of the intestinal epithelium in development of endotoxemia, a condition characterized by elevation of endotoxin in the blood, which contributes to the progression of diverse inflammatory dis-

eases (Konickova, Konigova et al. 1979). Endotoxin is a pyrogenic ligand that is present in the gut due to a variety of Gram negative bacteria hosted there. Endotoxin signals through toll-like receptor 4, which activates a variety of transcription factors via surface- and endosome-associated pathways which involve Interleukin 1 receptor associated kinase 1 (IRAK1) and toll-interacting proteins (tollip) (Barton and Kagan 2009). Although the signaling is well studied, the specific mechanism by which low chronic endotoxin causes or exacerbates the aforementioned disease remains an unresolved area of intense interest for the research and medical communities. Atherosclerosis alone cost $473 billion in healthcare costs in the U.S. in 2009 and caused 831,272 deaths in 2006 . This is more deaths than are caused by cancer, accidents, and HIV combined, according to the America Heart Association.

The white blood cells, macrophages, are important players in the pathogenesis of atherosclerosis, diabetes, and many other diseases. In atherosclerosis, macrophages invade the artery wall, become foam cells, and accumulate low density lipoprotein, which eventually becomes a plaque, then a blockage, and potentially a thrombus and an embolus (Holvoet and Collen 1997). In obesity, macrophage infiltration into the adipose tissue is increased, and macrophages fuse with dead adipocytes to form a new type of fat cell called a multinucleate giant cell (Rocha and Libby 2009). In autoimmune diabetes, macrophages self-recognize the insulin-producing β cells in the pancreas (Vaarala 2008). In Macrophages can be activated into two basic phenotypes, although future research may reveal more (Classen, Lloberas et al. 2009). The M1 macrophage is the inflammatory phenotype, which can be induced by a variety of inflammatory stimulants, endotoxin among them. The M1 macrophage expresses antimicrobial and inflammatory gene products (Fujiwara and Kobayashi 2005). The M2 macrophage is the phenotype that is associated with resolution of inflammation and tissue repair (Classen, Lloberas et al. 2009). It is induced by several different anti-inflammatory stimulants, chiefly by the T-cell-derived cytokine interleukin 4 (IL-4). Expression of arginase 1 is a key marker of the M2 macrophage. Although macrophage phenotype is implicated in

these diseases, the vast majority of published data about macrophage activation uses high doses of endotoxin which exceed the low concentrations encountered in endotoxemia. As a result, the effect of low doses of endotoxin on macrophage phenotype is not known.

Furthermore, all-trans retinoic acid (ATRA), a derivative of retinol found in many vegetables, milk, cheese, butter, and particularly liver is implicated in suppression of inflammation in several specific contexts, but also systemically. ATRA treatment suppresses iNOS expression and activity (Datta, Reddy et al. 2001). ATRA alleviates airway inflammation in asthmatic rats (Fang, Jin et al. 2004), inhibits eosinophil/basophil differentiation (Leber and Denburg 1997), and downregulates TLR2 expression (Liu, Krutzik et al. 2005). Additionally, vitamin A deficiency causes diarrhea, which can indicate inflammation of the intestinal mucosa (Salazar-Lindo, Salazar et al. 1993) and is associated with increased risk of Alzheimers Disease, which has also been recently recognized to have an inflammatory component (Goodman 2006). ATRA supplementation also ameliorates ethanol-induced liver injury in rat (Pan, Dan et al. 2006). ATRA signals through the retinoic acid receptor, which is a nuclear receptor. Several closely-related nuclear receptors are involved in or capable of inducing expression of arginase 1 and M2 activation. ATRA, however, has never been linked to arginase 1 expression or M2 activation.

Hypothesis: Although the significance of ATRA in ameliorating inflammtion is clear, as stated in the introduction, the agonists that regulate the expression of arginase 1 are not well defined. I tested the hypothesis that ATRA may either directly induce or indirectly augment the induction of arginase 1. Furthermore, I hypothesized that ATRA-treated macrophages may resist LPS-mediated suppression of arginase 1 expression. Mechanistically, I tested whether deletion of several key LPS signaling molecules may further attenuate the suppressive effect of LPS on ATRA-mediated expression of arginase 1. In order to test these hypotheses, the following studies were be performed.

Specific Aim 1: To determine the role of ATRA in inducing arginase 1 expression directly or as a priming factor for other agonists.

Specific Aim 2: To characterize the impact of endotoxemia levels of endotoxin on arginase 1 expression induced by IL-4 and ATRA.

Specific Aim 3: To investigate the role of IRAK1, tollip, and endosome signaling in endotoxin suppression of arginase 1 expression in order to identify suppression targets for alleviation of symptoms of endotoxemia.

Experiments in Specific Aim 1:

1. IL-4 is a known inducer of arginase 1 and M2 activation in macrophages. In order to ensure that the IL-4 is intact and transcriptionally active and that the primary bone marrow-derived macrophages (BMDM) were capable of expressing detectable levels of enzymatically active arginase 1, BMDM were treated for 8 h with 100 pg/ml 20 ng/ml IL-4 then subjected to arginase activity assay. Positive results in the arginase activity assay were confirmed with western blot and realtime RT-PCR specifically for arginase 1.

2. Realtime RT-PCR for arginase 2 was also performed under conditions representative of all conditions tested for arginase activity in order to ensure that all arginase activity was due to arginase 1 as expected and not to arginase 2.

3. In order to test whether ATRA is sufficient to induce arginase 1 directly, murine BMDM were treated with 1 nM to 1000 nM ATRA or DMSO vehicle for 8 hours. Samples treated with ATRA were used for realtime RT-PCR, western blot, and arginase activity assay. The range of doses used initially was selected in order to encompass the wide range of basal values that exist in human plasma. Basal values of ATRA concentration in blood tend to be in the 1-300 nM range (Conley, Egorin et al. 1997). The blood concentration that causes toxicity during APL treatment is approximately 1.5-1.7 μM (Conley, Egorin et al. 1997).

4. In order to determine whether ATRA was able to indirectly augment arginase 1 expression, BMDM were treated with ATRA in the presence of 10 ng/ml IL-4. 10 ng/ml IL-4 was used as an initial concentration because that was the lowest concentration that induced arginase activity in macrophages in previous experiments.

Experiments in Specific Aim 2:

1. Because Mycobacterium bovis has been reported to induce arginase 1 expression and

activity in a manner dependent on TLR adaptor MyD88 (El Kasmi, Qualls et al. 2008), LPS was tested to ensure that it did not induce arginase 1 expression, at least in the 8 hour timecourse used in this study.

2. In order to first assess that LPS suppression of IL-4-induced arginase 1 occurred as expected, arginase activity assay was performed on samples treated with IL-4 and 1, 5, or 10 ng/ml LPS. In order to determine whether IL-4 + ATRA - induced arginase 1 was subject to suppression by LPS, samples were treated with 10 ng/ml IL-4, 100 nM ATRA, and 1, 5, or 10 ng/ml LPS, then subjected to arginase activity assay.

3. Because the suppression of arginase activity was only slightly less due to 1 ng/ml LPS than 5 ng/ml LPS, the concentration of IL-4 and ATRA was decreased by 50% from 10 ng/ml IL-4 to 5 ng/ml IL-4 and 100 nM ATRA to 50 nM ATRA in order to observe less suppression by LPS at 1 ng/ml. Wt BMDM were treated with 5 ng/ml IL-4 and 50 nM ATRA and 1, 5, or 10 ng/ml LPS then subjected to arginase activity assay.

4. In order to corroborate the previous result obtained in arginase activity assay with western blot, the same conditions were applied to BMDM except the were used to perform western blot.

Experiments in Specific Aim 3:

1. In order to investigate the role of IRAK in mediating the suppressive effect of LPS on arginase 1 expression, Wt BMDM and IRAK1$^{-/-}$ BMDM were plated equivalently and treated with 5 ng/ml IL-4, 50 nM ATRA, and 1, 5, or 10 ng/ml LPS then used for western blot.

2. In order to investigate the role of tollip in mediating the suppressive effect of LPS on arginase 1 expression, Wt BMDM and tollip$^{-/-}$ BMDM were plated equivalently and treated with 5 ng/ml IL-4, 50 nM ATRA, and 1, 5, or 10 ng/ml LPS then used for western

blot.

3. Because tollip is associated with the endosome (Brissoni, Agostini et al. 2006) and LPS signals through TLR4 from both the cell surface and through the endosome (Barton and Kagan 2009), the role of endosomal signaling pathways in LPS suppression of ATRA-induced arginase 1 was assessed. In order to assess the role of endosome signaling, the selective dynamin inhibitor dynasore was employed. Dynamin is a GTPase necessary for dynamin-dependent endosome formation. The concentration of IL-4 and ATRA were lowered to 1 ng/ml and 50 nM, respectively. The purpose of this was that as the experiments progressed, it was realized that the IL-4 concentration could be lowered to values that better represent concentrations observed *in vivo*, and measurable levels of arginase 1 expression and activity could still be observed because of the augmentation of expression by ATRA. Wt BMDM were treated with 1 ng/ml IL-4, 50 nM ATRA, and 0 or 10 pg/ml LPS, in the presence or absence of 1 μM dynasore. Suppression was significant at the level of p less than 0.05 in the absence of dynasore, but in the presence of dynasore, the suppression became insignificant.

4. In order to better characterize the importance of endosome signaling and to corroborate the previous result found in arginase activity assay, the dynasore experiment was expanded, the concentrations altered slightly, and the samples used for western blot. Wt BMDM were treated with 1 ng/ml IL-4, 50 nM ATRA, and 0, 10, 50, 100, or 1000 pg/ml LPS in the presence or absence of 5 μM dynasore. 5 μM dynasore was used because no morphological change was observed at 1 μM dynasore and a stronger suppressive effect was desired.

Chapter 2

Materials and Methods

2.1 Transgenic Mice

Wildtype C57BL/6 mice and IRAK1$^{-/-}$ mice derived from C57BL/6 mice were used. Wildtype mice were from Charles River Laboratories, and IRAK-1/ mice with a C57BL/6 background were kindly provided by Dr. James Thomas from the University of Texas Southwestern Medical School. James Thomas et al. designed a targeting vector from the sequence of two human IRAK1 cDNA, which replaced 7 kb of the IRAK1 gene, including 3.5 kb of the 5' regulatory region, exons 18, and most of exon 9 with a neomycin resistance gene and included the HSV-tk gene in the vector sequence (Thomas 1999). The construct was moved into E1.1C ES cells by electroporation, upon which it homologously recombined with the chromosome, and was selected on the basis of G418 and ganciclovir resistance. Selected E1.1C ES cells were cultured, screened by southern blot for recombination, then injected into C57BL/6 embryo. Chimeric mice were bred with Wt C57BL/6, and the offspring which screened hetereozygous for the KO were bred with heterozygous KO siblings and offspring were screened by southern blot in order to identify IRAK1 double KO individuals (IRAK1$^{-/-}$).

Tollip$^{-/-}$ mice with a C57BL/6 background were developed and kindly provided by Dr. Kimberly Burns while at the Institute of Biochemistry, University of Lausanne (Didierlaurent, Brissoni et al. 2006). A targeting vector was developed in which the first exon of tollip was replaced with a neomycin resistance cassette flanked with an upstream noncoding region of tollip and exons 2-4 in order to facilitate homologous recombination into the genome. The construct was transformed into embryonic stem cells from 129sv mice, which were selected with neomycin then used to generate heterozygous chimeric mice. The heterozygous mice were intercrossed to produce double KO progeny, which were confirmed by PCR. Double KO 129sv mice were backcrossed 4 times with C57BL/6 mice. Although the backcrossing restores some of the C57BL/6 background, it should be considered a caveat that the and tollip$^{-/-}$ mice are not of the same background as the Wt or IRAK1$^{-/-}$ mice. The tollip$^{-/-}$ mice were developed from 129sv mice, presumably for reasons of availability of 129sv embryonic stem cells, whereas Wt and IRAK1$^{-/-}$ are both of C57BL/6 stock. There is precedence for differential expression of gene product between the two lineages as K. Reue et al. observed dramatically different levels of mRNA in the liver of the C57BL/6 and 129/J mice (Reue, Purcell-Huynh et al. 1993). While all results are corrected to yield percent change within a genotype, the difference in backgrounds should not be ignored when drawing conclusions about the contribution of tollip, particularly to baseline expression levels of mRNA and protein. Populations of Wt and IRAK1$^{-/-}$ and Tollip$^{-/-}$ mice were maintained in Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University.

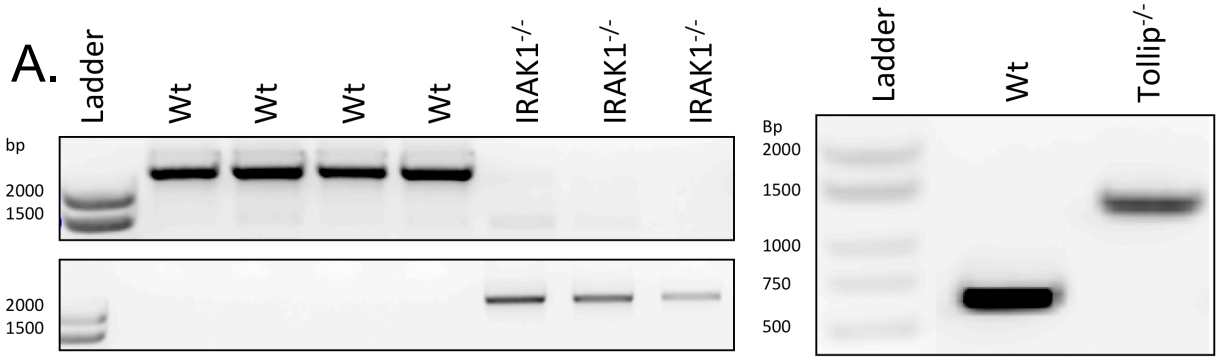
2.1.1 Genotyping

The genotype of mice used in experiments was confirmed after they were sacrificed for BMDM isolation. Tail tips were removed and frozen at $-80\,^{\circ}$C until several were collected for processing. Tail tips were placed in a 1.5 ml tube, with 300 μl of DirectPCR Lysis Reagent (Tail) (Cat # 101-T from Viagen Biotech, Inc.) with 4% proteinase K solution (500 μl 20

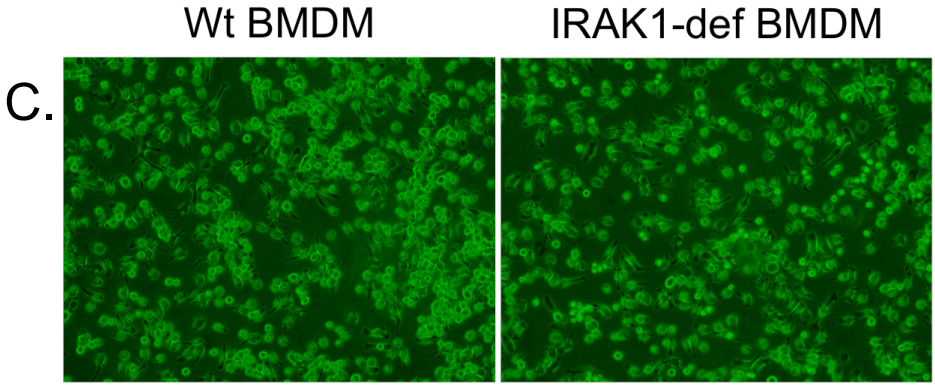
mM $CaCl_2$, 50 μl 1 M Tris, pH 8.0, 450 μl dH_2O, and 10 mg proteinase K) and incubated in a rotating incubator for 4 hours at 55 °C followed by 45 minutes in an 85 °C water bath. Tubes were centrifuged at 12000 rpm for 2 minutes, then 100 μl of supernatant collected and stored at −20 °C for later PCR.

PCR was performed using GoTaq master mix according to the protocol provided by Promega. PCR conditions were 1, 94 °C for 2 minutes, 2, 94 °C for 30 seconds, 3, 56 °C for 30 seconds, 4, 72 °C for 2 minutes (35 cycles of steps 2, 3, and 4), 5, 72 °C for 7 minutes, then hold at 4 °C. The primers used to detect Wt and KO versions of each gene are noted in figure 2.1. 10 μl of each sample was electrophoresed and imaged using a FUJI LAS-3000 gel documentation system.

Figure 2.1: **Genotyping of mice to confirm gene knockout status. A. Agarose gels of PCR product from genomic DNA of Wt and IRAK1 or tollip KO mice using gene-specific primers which will only produce product in either Wt or KO mice for specific gene. B. Gene-specific primer sequences. Common forward primers were employed when possible. C. Photographs of BMDM from Wt and IRAK-1 KO mice plated in 150 mm polystyrene plates reveal no apparent morphological differences.**



B. Primers:
Wt IRAK1 F: GCAAGCCAGAGCAGTACT
Wt IRAK1 R: GCCTCTGTAAGAGATCAG
KO IRAK1 F: GCCTCCTATCGCCTTCTT
KO IRAK1 R: GCCTCTGTAAGAGATCAG
Wt Tollip F: GGATTTGGGATTGATGAGAGGC
Wt Tollip R: ACAAGAGTGGGAGGGAAACTTC
KO Tollip F: GGATTTGGGATTGATGAGAGGC
KO Tollip R: GGAGAGGCTATTGGGCTATG



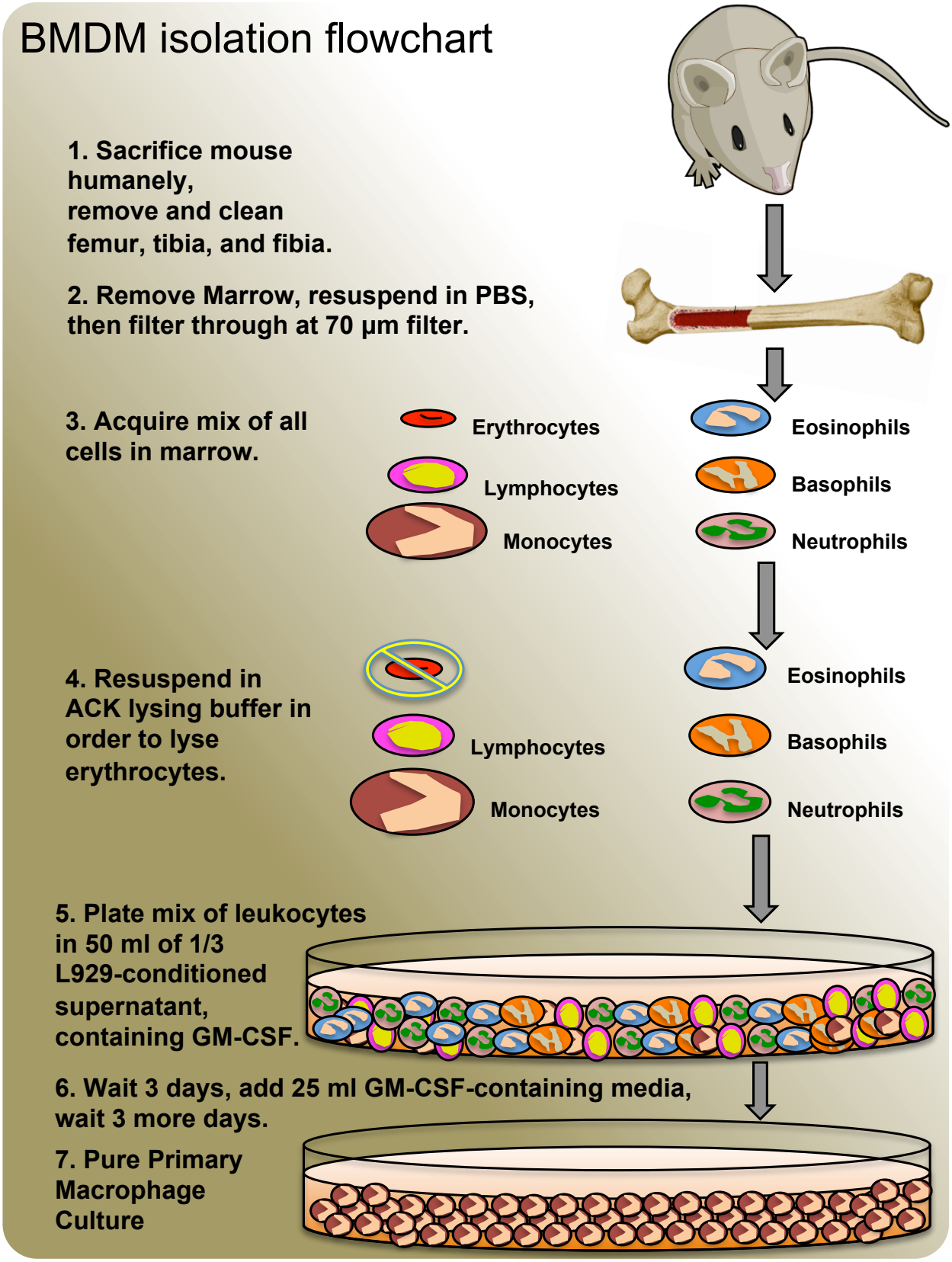
2.1.2 Isolation and Differentiation of Bone Marrow Derived Macrophages

Mice were anesthetized by inhalation of Isoflurane, USP (Abbott), and sacrificed by cervical dislocation in accordance with Virginia Tech IACUC protocol 08-193-BIOL. The mouse was sterilized with 70% EtOH, then femurs, tibia and fibia were briefly isolated in 15 ml sterile 0.01 M phosphate buffered saline (NaCl 0.138 M; KCl^- 0.0027 M; pH 7.4). Bones were cut and bone marrow isolated by repeated flushing using 20 gauge hypodermic needle with 3 ml control syringe. PBS added to 30 ml and moved to 50 ml tube, centrifuged 8 minutes, 1200 RPM, 4 °C. PBS was removed and resuspended by pipetting in 5 ml ACK lysing buffer (8,024 mg/l NH_4Cl, 1,001 mg/l $KHCO_3$, 3.722 mg/l EDTA, Na_2, $2H_2O$, Lonza) in order to lyse erythrocytes. ACK was inactivated by addition of 40 ml PBS, after which remaining cells were centrifuged under identical conditions, and resuspended in 300 ml of media composed of 2/3 fresh DMEM (containing phenol red, sodium bicarbonate, 10% heat-inactivated fetal bovine serum, 4,500 mg/L D-glucose, sodium pyruvate, L-glutamine, penicillin, streptomycin) and 1/3 L-929-conditioned DMEM (same formulation, except sterile-filtered following conditioning by a 60-90% confluent monolayer of L-929 cells for 4 days, in order to provide a supply of murine GM-CSF, which is constitutively secreted from L-929, a cell line derived from subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse) per mouse and plated on 150 mm-diameter tissue-culture treated sterile polystyrene culture plates and placed in tissue culture incubator at 37 °C, 5% CO_2, 100% humidity for 6 days. After 4 days, 25 ml of the aforementioned media mixture wass added to each plate in order to reinforce GM-CSF. On day 6, media was removed and replaced with 15 ml PBS. Plates were placed at 4 °C for 15 minutes, after which macrophages disarticulate from culture vessels with gentle washing. Buffer was collected, cells counted using a hemacytometer, centrifuged as before, then resuspended in DMEM (containing phenol red, sodium bicarbonate, 1% heat-inactivated fetal bovine serum, 4,500 mg/L D-glucose, sodium pyruvate, L-glutamine, penicillin, streptomycin) (henceforth 1% FBS DMEM) and

plated at desired concentration for experiments.

In unpublished data from the laboratory of inflammation and innate immunity at Virginia Tech, BMDM isolated in the manner described were a uniform population of CD11-expressing cells as assessed by flow cytometry.

Figure 2.2: **Murine BMDM isolation flowchart.**



2.2 Arginase Activity Assay

This arginase activity assay was based on Corralizas modification (Corraliza, Campo et al. 1994) of Schimkes method (Schimke and Doyle 1970). Following collection in PBS and counting, cells were resuspended in 1% FBS DMEM at a concentration of 1M cells/ml. 500,000 cells/well were plated in a volume of 500 μl/ well in a 24 well tissue culture treated plate (well diameter 1.02 cm) and allowed to rest overnight before further treatment. Ligands were diluted in either PBS with 0.02% BSA (by mass) as a carrier or in anhydrous dimethyl sulfoxide (DMSO) to 1000x of the desired final concentration. Treatment was done by addition of 1 μl of 1000x stock to each 1 ml of media. All samples which were not treated with ligand were treated with PBS or DMSO vehicle control. During treatment, cells were kept in tissue culture incubator.

Following the treatment period, normally 8 hours, the media was removed by aspiration and the cells briefly washed once with room temperature PBS. In order to lyse the cells without denaturing the enzymes, the PBS was removed by aspiration and replaced with 50 μl sample buffer (0.1% Triton X-100, with 5 μg pepstatin, 5 μg antipain, and 5 μg aprotinin). Cells were lysed by shaking at 37 °C, 208 RPM for 30 min. Because manganese is an essential cofactor to arginase enzyme activity, to 50 μl of lysate was added 50 μl of 10 mM $MnCl_2$ in 50 mM Tris-HCl (pH 7.8), followed by 10 minute incubation at 55 °C. 25 μl of this activated lysate was added to 25 μl of 500 mM L-arginine (pH 9.7) in a 1.5 ml tube. Tubes were incubated 1 hour at 37 °C, then 400 μl acid (1:3:9, Sulfuric Acid: Phosphoric Acid: ddH_2O) and 25 μl 9% (m/m) ISPF in EtOH were added to each tube. Tubes were boiled at 100 °C for 45 minutes, then 200 μl was moved to a 96-well polystyrene plate to determine the absorbance of 540 nm wavelength light (abs540). Standard curve; the abs540 at a given [urea] was plotted against the concentration, a linear regression was done to determine the relationship between abs540 and [urea].

2.3 iNOS Activity Assay

The inducible nitric oxide synthase (iNOS) activity assay was a kit from the Promega Corporation, product G2930. The kit consists of two reagents, a solution of sulfanilamide, which reacts with NO_2^- to yield water by removing two hydrogens and donating a nitrogen to sulfanilamide. The new species reacts with the second reagent, N-1-napthylethylenediamine dihydrochloride (NED) to yield a light-absorbing azo compound.

A concentration curve of nitrite was generated from the included 0.1M sodium nitrite standard in the media used to culture the BMDM used in the experiment. In order to test the production of nitrite, BMDM were treated for 16 hours with LPS and ATRA of varying concentrations, then 50 μl of the supernatant was isolated and centrifuged to pellet any cell debris. In a 96-well polystyrene plate, to each sample or standard, 100 μl of sulfanilamide solution and 100 μl of the NED solution were added sequentially 5 minutes apart. The absorbance of 550 nm light was measured after 15 minutes, triplicate wells were averaged and the standard deviation determined.

2.4 Western Blot

2.4.1 Lysate Preparation

Cells were plated in 6-well tissue culture-treated plates at a concentration of 1 million cells/ well, each well containing 2 ml of 1% FBS DMEM. Treatments were added as 2 μl of 1000x stock in PBS or DMSO as described. Following treatments, cells were washed 1X with PBS then incubated 30 min on ice with 1X lysis buffer (694 μM SDS, 50 mM Tris-HCl, 10% glycerol v/v, 500 μM phenylmethylsulfonyl fluoride 1 μg/ml leupeptin, 1 μg/ml pepstatin). Lysates were scraped from 6-well plates and collected in 1.5 ml centrifuge tubes, then heated at 100 °C for 10 min in order to denature protein.

Assessment of Protein Concentration: Total protein concentration was assessed by the colorimetric D_c Protein Assay (BioRad) following a 1:5 dilution with ddH_2O of each sample. A calibration curve using bovine serum albumin from 0.2 to 1.5 mg/ml was used to calculate the protein concentration.

5x Cell Lysis Buffer: 50 ml Glycerol, 10 g SDS, 25 ml ddH_2O, 25 ml 1M Tris-HCL (PH 6.8).

5X Laemmli Loading Buffer: 8 ml of 1 M Tris-HCl, pH 6.8, 2 g SDS, 10 ml glycerol, 1 ml 1% bromophenol blue, and 1.54 g DTT.

SDS-PAGE Gels: Separating gel (10%): 3.33 ml 30% polyacrylamide (29:1), 100 μl 10% SDS, 3.75 ml 1 M Tris-HCl (pH 8.8), 2.92 ml ddH_2O, 50 μl; 10% APS, and 10 μl TEMED. Stacking gel: 650 μl 30% polyacrylamide (29:1), 50 μl 10% SDS, 630 μl 1 M Tris-HCl (pH 6.8), 3.64 ml ddH_2O, 25 μl 10% APS, and 5 μl TEMED.

SDS-PAGE Running Buffer: 1 L ddH_2O, 2.9 g Tris base, 14.4 g Glycine, 1 g SDS.

Electrotransfer Buffer: 1 L ddH_2O, 2.9 g Tris Base, 14.4 g Glycine, 200 ml Methanol.

SDS-PAGE: The volume of lysate containing 15-40 μg total protein (dependent on lysate quality, antigen abundance, antibody quality, and well capacity) was calculated and combined with 1/4 of that volume of 5X Laemmli buffer, heated at 100 °C for 10 minutes to prepare for SDS-PAGE. Samples were loaded into the 10% polyacrylamide gel mounted in a Mini-PROTEAN Tetra cell (Bio Rad). Conditions were generally 80 V for 20 minutes followed by 100 V for 100 minutes. Electrophoresed gel was transferred to PVDF membrane using a trans-blot cell from Bio Rad. Following transfer, membranes were immediately blocked in 5% skim milk in autoclaved TBST (Tris-buffered saline (150 mM NaCl, 2 mM KCl, 24 mM Tris base, pH 7.4) with 0.05% Tween 20) for 30-60 minutes on a rocker at room temperature, then immunoblotted overnight at 4 °C with primary antibody diluted in 5% skim milk in TBST. Primary antibody was removed and stored at −20 °C for reuse. The membranes were washed 3-5x with TBST on a shaker at room temperature. HRP (horseradish peroxidase) -conjugated secondary antibody diluted 1:25000 in 5% skim milk in TBST and applied to membranes for 1-2 hours on rocker at room temperature. This was followed by 3-5x with TBST on a shaker at room temperature, then immunoblots were developed by using the Amersham ECL Plus chemiluminescent detection system (GE Healthcare). Photographs

and densitometrics of the band intensities were quantified using the Fujifim LAS-3000 gel and membrane documentation system and Fujifilm Multi Gauge software. Membranes were stripped of antibodies using ReBlot Plus Mild from Chemicon. In order to assess equal loading, stripped membranes were reprobed with antibodies for β-actin or GAPDH.

Antibodies: Arginase 1 (V-20) sc-18354 (Santa Cruz) goat polyclonal. β-actin sc-47778 (Santa Cruz) mouse monoclonal. GAPDH sc-25778 (Santa Cruz) rabbit monoclonal. Donkey 2 - HRP conjugated anti-goat IgG sc-2020 (Santa Cruz). Mouse 2 Anti-mouse IgG, HRP-linked Antibody (Cell Signaling). Rabbit 2 - Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling).

2.5 Realtime RT-PCR

BMDM seeded onto 12- or 6-well plates at 1 M cells/ ml in 1% FBS DMEM and allowed to rest overnight before treatment. Treatments performed in same manner as in enzyme activity assay, followed by one wash with room temperature PBS and then lysis in 1 ml Trizol reagent (Invitrogen). Trizol processed according to manufacturers protocol in order to isolate RNA pellet, which was resuspended in 30 μl RNAse-free water. The concentration and quality (regarding protein contamination) of RNA in each sample was assessed by measurement of absorbance of 260 nm and 280 nm light of 1:99 diluted samples. Samples with too little RNA or a 260/280 ratio below 1.8 were generally not used.

1.5 μg of RNA were used to produce cDNA in a 20 μl reverse transcription reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Samples were incubated 10 minutes at 26 °C, 120 minutes at 37 °C, then 5 minutes at 85 °C. cDNA were diluted 1:4 in PCR-grade ddH_2O, then stored at -20 °C until realtime RT-PCR.

Realtime RT-PCR was performed using the IQ5 realtime thermocycler and PCR detection system and Sybr Green 2x realtime master mix from Bio Rad. Samples were prepared in 25 μl reactions in a 96-well PCR plate with primers for target genes or for GAPDH and the Sybr green master mix according to the manufacturers protocol. A standard amplification

PCR program was performed for 35 cycles followed by a melt curve in order to exclude to possibility that non-specific amplification occurred.

Threshold cycles were selected automatically by analysis software, but were adjusted by the user in some cases. Triplicate samples were run in order to ensure accurate data. For a given sample, the C_t (cycle of threshold) for the gene of interest (GOI) was determined, then the Delta C_t (dC_t) was assessed by subtraction of the GOI C_t from that of the loading control (GAPDH) for that sample. The dC_t for a treatment sample was subtracted from the dC_t for the no-treatment control to yield the ddC_t. $2\hat{}ddC_t$ = fold induction of gene expression attributable to that treatment.

2.5.1 Primers for Realtime RT-PCR

GAPDH Fwd	AAC TTT GGC ATT GTG GAA GGG CTC
GAPDH Rev	TGT TGA AGT CGC AGG AGA CAA CCT
P62/DOK1 Fwd	TTT CTG CCT TGG AGA TGC TGG AGA
P62/DOK1 Rev	ACC CAA AGT CAG GAG AGT CAG CTT
Arginase 1 Fwd	ATG TTG ACG GAC TGG ACC CAT CTT
Arginase 1 Rev	TGC AAC TGC TGT GTT CAC TGT TCG
Arginase 2 Fwd	AAA TGT TCA GGA GGG TCC ACG TCT
Arginase 2 Rev	AAA TGT TCA GGA GGG TCC ACG TCT

2.6 Reagents

Mouse recombinant IL-4 (404-ML) was obtained from RnD Systems and was reconstituted in at 10 μg/ml in sterile PBS containing 0.1% bovine serum albumin, aliquoted in 1.5 ml centrifuge tubes at 5 μl/ tube and stored at $-80\,^{\circ}$C for up to 3 months. ATRA was obtained from Sigma Aldrich (R2625) in sealed amber ampules under Argon gas and resuspended in DMSO at 3 mg/ml (100 mM) and aliquoted into amber polypropylene screw-top (with rubber gasket) tubes and stored up to 6 months at $-80\,^{\circ}$C. ATRA was protected from light in order to minimize photo-isomerization between all-*trans* and *cis* isomers. LPS

was obtained from B4 serotype *E. coli* by Sigma Aldrich (Standard LPS, *E. coli* 0111:B4). Endotoxic activity of this LPS is 1-10x10^6 EU by definition. LPS was resuspended in PBS at 1 mg/ml followed by sonication in ice water bath for 4 x 30 seconds. Dynasore monohydrate, inhibitor of GTPase dynamin, was obtained from Sigma Aldrich (D7693-5MG). It was resuspended in DMSO at 100 mM and stored at $-20\,^{\circ}$C protected from light.

2.7 Calculations

2.7.1 Suppression

The percent suppression of arginase activity by LPS was calculated for realtime RT-PCR, western blot, and arginase activity assay. In each case the basal activity or expression was accounted for by subtracting the DMSO control sample value from each of the experimental sample values first. This operation yields a corrected value for each sample. The ratio between the corrected value for the sample in which arginase activity or expression was induced (by IL-4 or IL-4 + ATRA) that was not treated with a suppressive ligand (normally LPS) and the sample in which arginase activity or expression had been induced but also suppressed by LPS or in some cases another TLR ligand was calculated. This ratio was multiplied by 100 to yield the percent of the unsuppressed sample that the suppressed sample represents, then subtracted from 100 in order to yield the % suppression from the induced state. It should be noted that LPS was resuspended in PBS with 0.2% bovine serum albumin as a carrier protein. All of the material used to resuspend LPS was already present in the media in which the cells were treated, and was thus not tested separately.

2.7.2 Statistics

In all figures where error bars are illustrated, they represent the standard deviation calculated by Students 2-tailed t-test utilizing 3 independent biological replicate samples plated and treated in different wells of culture plates and processed completely independently of one another (in the case or arginase activity assay) or three independent PCR performed using the same cDNA (in the case of realtime RT-PCR).

P values, where represented, were calculated using a two-tailed t-test with 2 degrees of freedom for sets of three values for each sample. The mean and standard deviation for each group of samples was calculated, then used to calculate the t-distribution, and from that a p value. The level of significance is indicated where applicable.

Chapter 3

Results

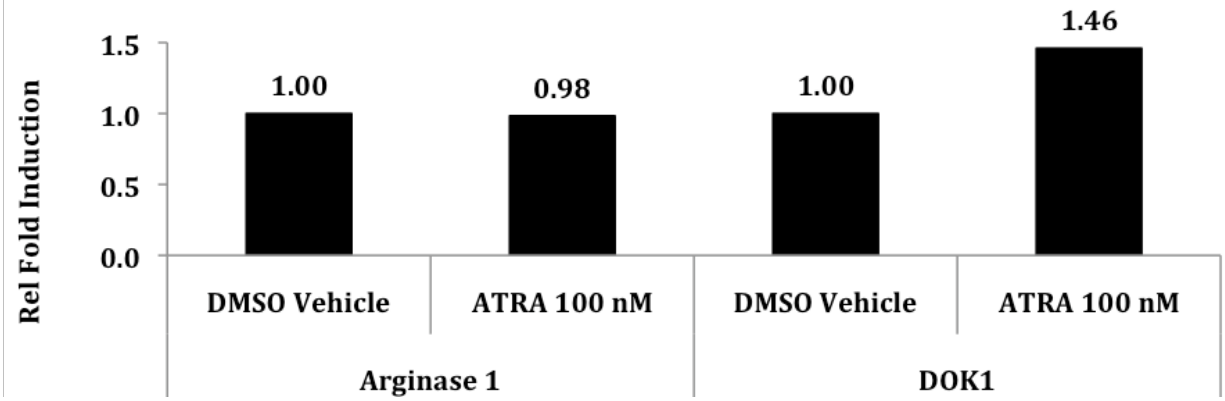
3.1 Role of ATRA in Control of Arginase 1

Previous results by Rhodes and Oliver indicate that 10^{-7} M ATRA minimally induces intracellular arginase activity in guinea pig peritoneal macrophages (Rhodes and Oliver 1980). In order to investigate whether this induction of arginase activity was indicative of an unrealized role of ATRA in M2 activation, ATRA was obtained to treat murine BMDM.

3.1.1 Validation of ATRA

Because ATRA is an intermediate metabolite that is normally derived from hydrolysis of all-*trans* retinal within the target cell, and because it is light-sensitive with respect to isomerization (the closely-related opsonin complex, which detects light in retina relies on the ability of all-*trans* retinyl ester to be photo-isomerized from trans to 13-*cis*), the ability of the ATRA to induce expression of downstream of tyrosine kinase 1 (p62/DOK1) was assessed (Lamkin, Chin et al. 2006). ATRA is also known to suppress LPS-induced iNOS activity (Datta and Lianos 1999). DOK1 mRNA was induced 1.46 fold by 8 hour treatment with 100 nM ATRA and LPS-induced iNOS activity was suppressed by 100 nM ATRA over a wide range of LPS concentrations (Figure 3.1 and 3.2).

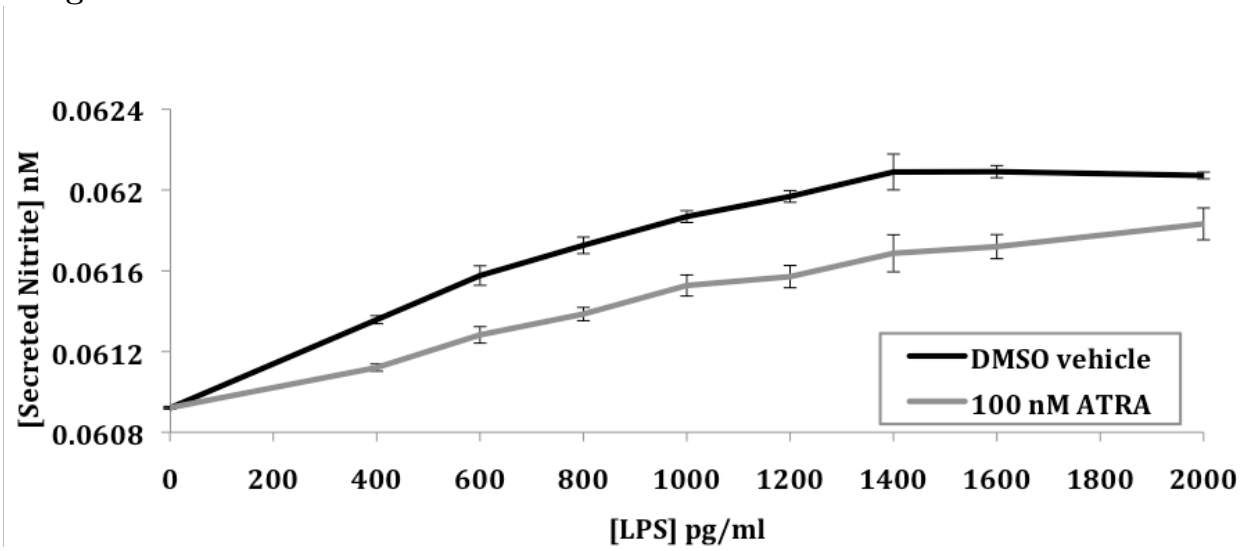
Figure 3.1: **ATRA cannot induce expression of arginase 1 alone, however at the same concentration and time course, it can induce expression of the control gene P62/DOK1 by 1.5 fold. Realtime RT-PCR. Wt BMDM were treated with DMSO vehicle or 100 nM ATRA for 4 hours, the samples were assessed for mRNA expression of arginase 1 and DOK1, with GAPDH as the housekeeping gene.**



3.1.2 Validation of IL-4 and BMDM

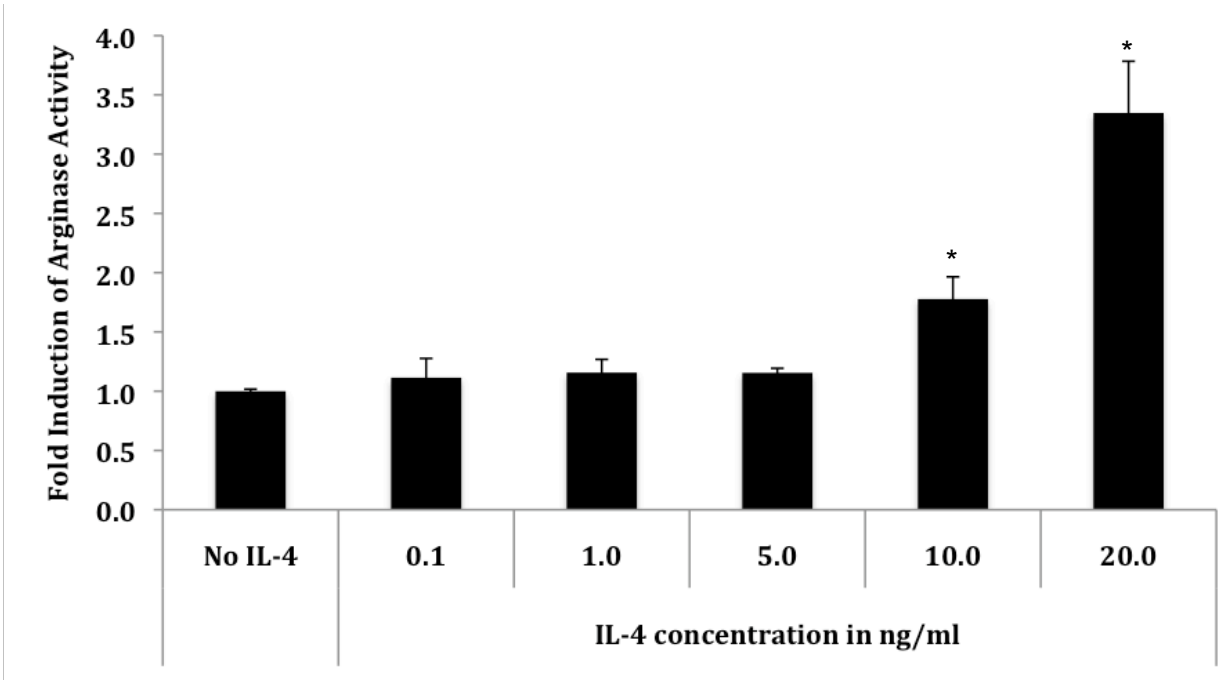
In order to assess the ability of murine bone marrow-derived macrophages to be activated to the M2 phenotype, and in order to validate the murine recombinant IL-4, arginase 1 expression and arginase activity was assessed. Following differentiation of bone marrow progenitor cells into bone marrow-derived macrophages by L-929-derived GM-CSF, the cells were replated in DMEM containing 10% FBS but no L-929 supernatant. The cells were allowed to recover overnight, then they were treated with 0.1-20 ng/ml IL-4 for 4-8 hours. 20 ng/ml IL-4 induced arginase activity by more than 3-fold. There was minimal but significant induction at 1 ng/ml and dose-dependent induction at the intervening concentrations (Figure 3.3). Realtime RT-PCR was used to assess whether transcription of arginase 1, arginase 2, both isozymes, or neither was induced by IL-4 (Figure 3.10). Only arginase 1 mRNA

Figure 3.2: **ATRA suppresses iNOS activity. ATRA suppression of LPS-induced iNOS expression is well-characterized (). This serves as an additional positive control for ATRA. iNOS Activity Assay. Wt BMDM were treated in triplicate for 16 hours with increasing concentrations of LPS in the presence or absence of 100 nM ATRA. Metabolism of Griess reagent by iNOS yields a color substrate. At all concentrations of LPS tested, 100 nM ATRA suppressed induction of iNOS activity. Error bars represent standard deviation calculated among three replicates. The difference between ATRA-treated and non-ATRA-treated is significant at the level of p less than 0.05 at all concentrations of LPS (except 0) using Students 2-tailed t-test.**



was induced. No change in expression of arginase 2 mRNA was observed in BMDM, and all changes in arginase activity were accompanied by induction of arginase 1 transcription as well as translation of a 37 KDa protein that was immunoreactive with anti-arginase 1 antibody (Figure 3.5). Induction of arginase 1 not only serves to indicate that the macrophages have been alternatively activated, but also as a marker for monocyte/macrophage lineage, as arginase 1 expression is rare in tissues other than M2 macrophages and the liver (it is sometimes called liver arginase) (Haraguchi, Takiguchi et al. 1987). Arginase 1 expression in other tissues is rare, but limited expression has been observed in erythroblasts, skeletal muscle, and human placental villi (Takiguchi, Haraguchi et al. 1988; Gordon 2003; Gerhard, Wagner et al. 2004; Ishikawa, Harada et al. 2007).

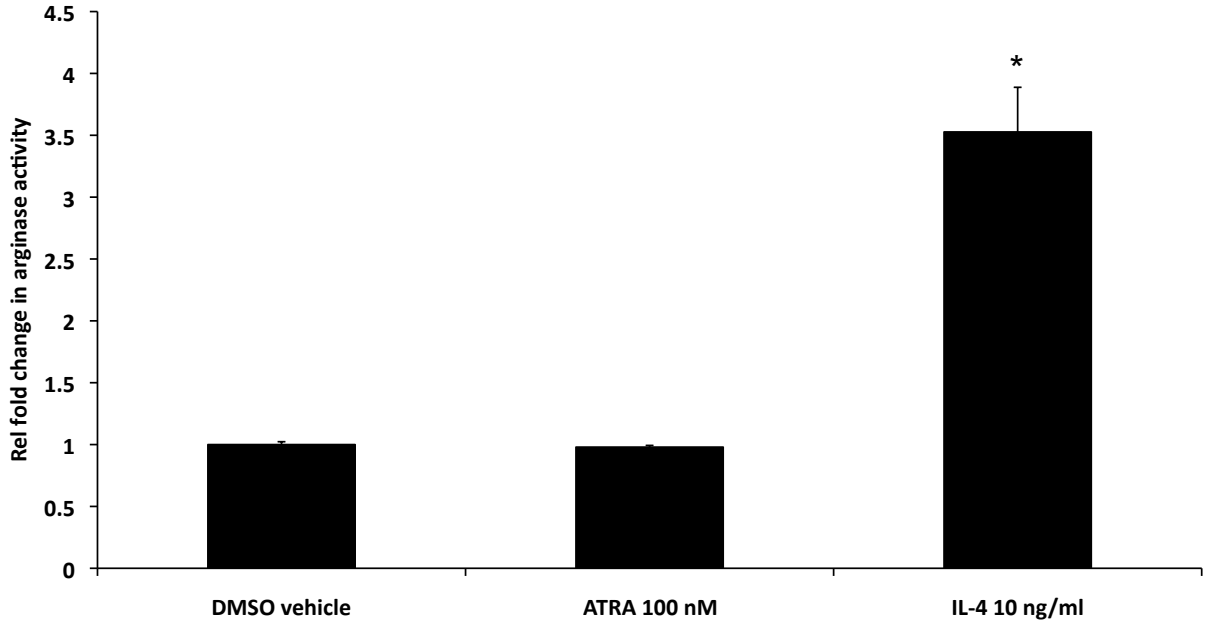
Figure 3.3: **IL-4 induced arginase activity in murine BMDM at 10 ng/ml and higher. Arginase Activity Assay. Wt BMDM were treated for 8 hours with increasing concentrations of recombinant murine IL-4, then subjected to arginase activity assay. 0.1, 1.0, and 5.0 ng/ml did not induce arginase activity significantly. 10 and 20 ng/ml induced arginase activity in a dose-dependent manner. Induction of arginase activity at 10 ng/ml and 20 ng/ml was calculated to be significant at the level p less than 0.05 using Student's t-test.**



3.1.3 ATRA Control of Arginase 1 Expression

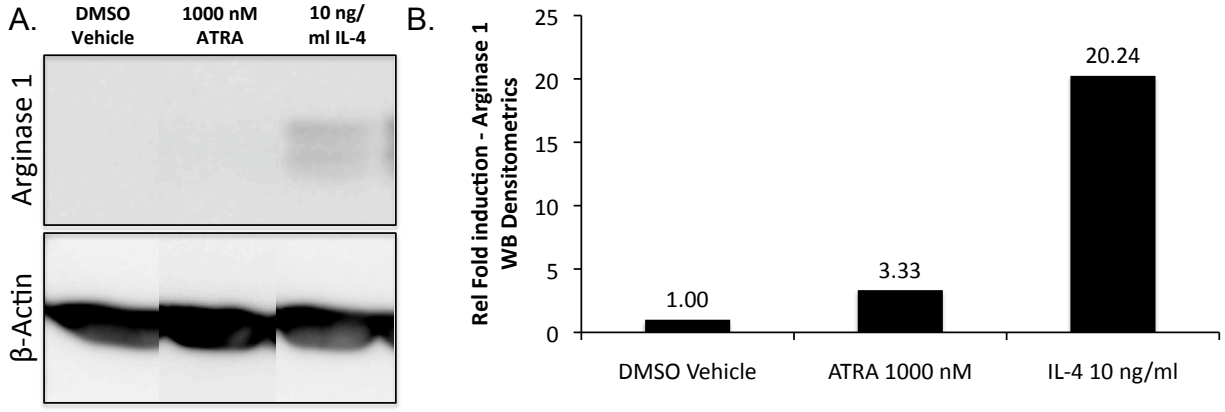
In order to determine the potential role for ATRA in M2 activation of macrophages, the effect of ATRA on expression and activity of arginase 1, a key marker of alternative activation in murine macrophages in mice, was assessed. In the arginase activity assay of Wt BMDM treated with DMSO vehicle, 100 nM ATRA, or 10 ng/ml IL-4 for 8 hours, no significant difference in activity was detected in response to ATRA, but IL-4 induced expression ˜3.5 fold over the basal level (Figure 3.4).

Figure 3.4: **100 nM ATRA does not induce arginase activity whereas 10 ng/ml IL-4 does. Arginase Activity Assay. Wt BMDM treated for 8 hours with 0.1% DMSO, 100 nM ATRA, or 10 ng/ml IL-4. No induction observed with 100 nM ATRA, 10 ng/ml IL-4 induced ˜3.5 fold, significance p less than 0.05 by Student's t-test. Error bars represent standard deviation. Arginase activity at 1000 nM ATRA was also not significant (Figure 3.9).**



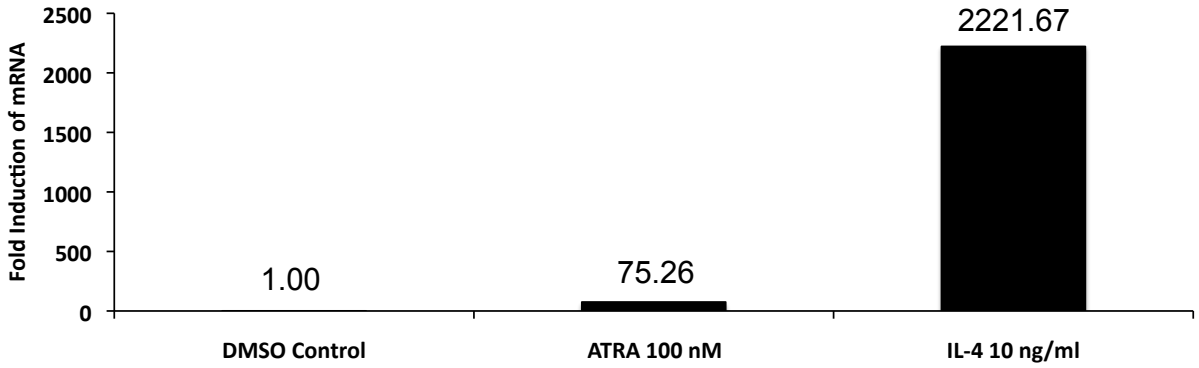
In a western blot for arginase 1 using BMDM treated for 8 hours with DMSO vehicle or with 1000 nM ATRA, the arginase 1 band in the ATRA-treated lane is not *visibly* brighter than that in the DMSO vehicle lane. Upon analysis with densitometric software, however, the band in the ATRA-treated lane is slightly darker than the control, reflecting slight induction of the expression of arginase 1 (Figure 3.5). A theory for this phenomenon is outlined in the discussion.

Figure 3.5: **Arginase 1 protein is only appreciably induced in the presence of IL-4. A. Western blot for arginase 1 of Wt BMDM treated for 8 hours with 0.1% DMSO, 1000 nM ATRA, or 10 ng/ml IL-4. Densitometric quantitation represented as columns. 3.33-fold induction of arginase 1 protein by 1000 nM ATRA is not detectable by eye. B. In order to correct for small differences in protein loading into the gel, densitometric analysis was done by calculating the ratio between absolute intensity of the arginase 1 bands and the β-Actin bands, then correcting a lane to the arbitrary value of 1.00 and all performing the same operation on all of the other values.**



This induction is calculated to be 3.33 fold that of the untreated sample, but it is questionable whether or not this induction is significant. For comparison, the induction by 10 ng/ml IL-4 was calculated to be 20.24 fold that of the DMSO vehicle. In order to corroborate this result, realtime RT-PCR of Wt BMDM was used. 100 nM ATRA induced a 75.26 fold induction of arginase 1 mRNA. 10 ng/ml IL-4, induced arginase 1 mRNA 2221.67 fold (Figure 3.6).

Figure 3.6: **Realtime RT-PCR. Expression of arginase 1 mRNA in Wt BMDM treated for 4 hours with 0.1% DMSO, 100 nM ATRA, or 10 ng/ml IL-4. Bars represent fold induction of arginase 1 mRNA. Housekeeping gene was β actin.**



Even at doses as high as 1 μM, which exceeds by 10-fold the requirement for most transactivation events and by 1000-fold the requirement for induction of arginase observed by Rhodes and Oliver (Rhodes and Oliver 1980), no significant induction of arginase activity was detected, and only minimal induction of arginase 1 protein and arginase 1 mRNA (when compared to induction by IL-4 alone) was detected.

3.2 ATRA Potentiates IL-4 Induction of Arginase 1

The potential for an alternative form of activation in macrophages (M2) was first recognized only in 1990 (Abramson and Gallin 1990), and understanding of the mechanisms by which M2 activation occurs was less compared to our understanding of classical activation. So the fact that ATRA alone was incapable of inducing the alternative activation marker arginase 1 did not by any means indicate that ATRA does not contribute to alternative activation of macrophages. Treatment of bone marrow progenitor cells that have been differentiated *in vitro* with only the cytokines from L-929 cells is not identical to the conditions under which macrophages are activated *in vivo*. IL-4 was not added to the first *in vitro* experiment, while the concentration of the cytokine IL-4 in the plasma of a healthy human is 100 pg/ml (Hohnoki, Inoue et al. 1998). The concentration of IL-4 in mouse plasma is not as well documented, but seems to be somewhat lower, around 1-20 pg/ml (Moheno, Pfleiderer et al. 2009). Also, there is no evidence that L929 fibroblasts produce IL-4, and though the concentration of bovine IL-4 in sera is not tested, the maximum concentration of sera to which the cells are exposed is 10%, so even in the event that bovine IL-4 is present and intact in heat-inactivated FBS, then it is likely present in exceedingly low concentrations. It is not present in concentrations high enough to induce arginase 1 expression in murine BMDM which respond to 1-5 ng/ml mouse recombinant IL-4. Only T-cells and basophils produce IL-4, and those only under conditions such as resolution of inflammation, wound healing, parasitic infection, and memory T-cell dependent response (Khodoun, Orekhova et al. 2004; Min, Prout et al. 2004).

Ligands that act through heterodimers of LXR:RXR or PPAR:RXR, as well as 9-*cis* RA, which works through RXR:RXR homodimers, are capable of inducing arginase 1 expression and activity in murine macrophages (Gallardo-Soler, Gomez-Nieto et al. 2008). The pathways activated and genes induced by nuclear receptor ligands, especially those that share RXR or another common heterodimer partner, frequently overlap. ATRA, which also acts via a homodimer requiring RXR, and shares the DR1 RARE with RXR:RXR heterodimers, may then have some impact on arginase 1 expression in macrophages.

A consensus on the concept of M2 activation of macrophages is being developed largely under the auspices of Dr. Siamon Gordon of Oxford University (Heinsbroek and Gordon 2009; Helming and Gordon 2009; Martinez, Helming et al. 2009). While the broad strokes of suppression of inflammatory cytokine secretion, promotion of wound healing, and resolution of inflammation are understood, the mechanisms and complete implications are not all known. Similarly, ATRA has broad anti-inflammatory properties, however, the mechanistic aspect of which are not completely understood.

One possible explanation for some of the anti-inflammatory effects of ATRA, such as suppression of iNOS, is that ATRA is suppressing M1 activation and promoting M2 activation of macrophages. So while ATRA may not be sufficient to initiate M2 activation alone, it may play a modulating role after initiation. Alternatively, ATRA may oppose some signaling that constitutively or inducibly suppresses M2 activation of macrophages.

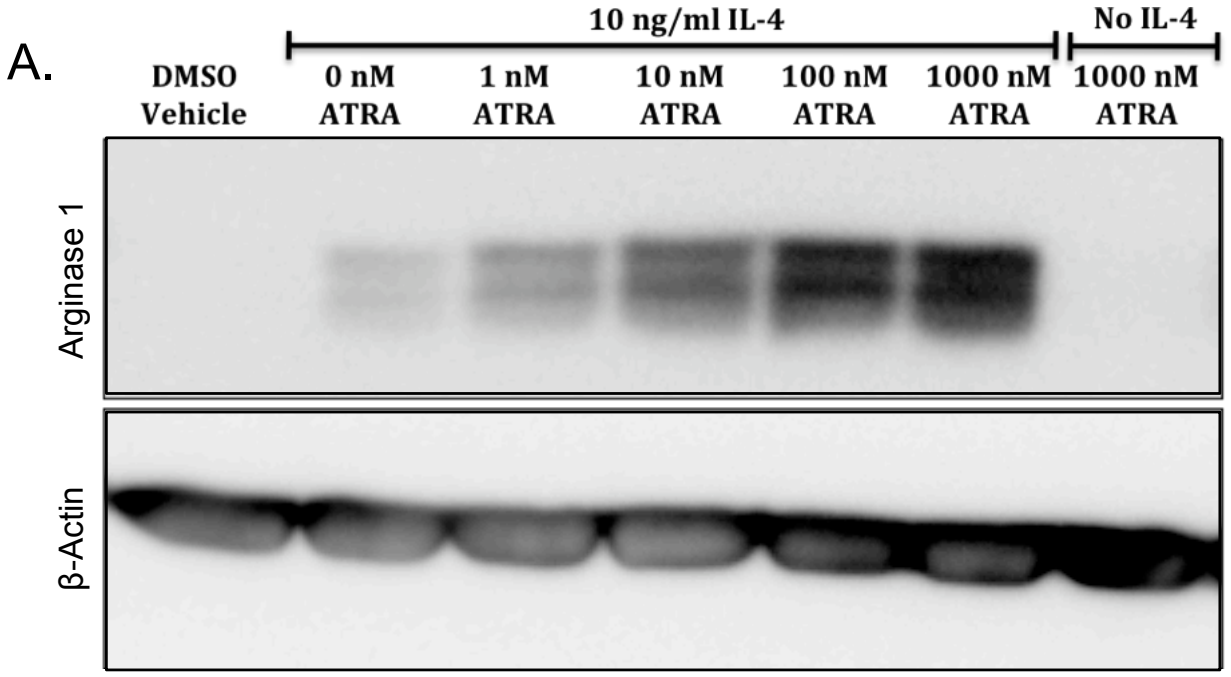
As understanding of cell signaling has progressed, the linear interpretation of signaling from receptor to adaptor to gene promoter has been replaced with a complex web of conditional positive, negative, and neutralizing interactions between receptors, adaptors, enzymes, the membrane, transcription factors, and chromatin. The conditions, cell type, history, and phase of mitosis are now regarded as much more important that a given single ligand in understanding cell responses. This is reflected in the bevy of recent publications investigating crosstalk between signaling networks (Castrillo, Joseph et al. 2003; Gallardo-Soler,

Gomez-Nieto et al. 2008) as well as in the pharmaceutical community, which has recently begun to market drugs in "polypills," wherein the combined benefit of two drugs together in fighting atherosclerosis, in the example of Caduet® (Wimett and Laustsen 2004), a single capsule combining Norvasc® (amlodipine besylate) and Lipitor® (atorvastatin calcium) , which treat high blood pressure and high LDL cholesterol, respectively (McKeage and Siddiqui 2008), is greater than the sum of the benefit of each individual drug in isolation. It behooves the researcher to consider carefully the context when investigating the effect of a particular ligand.

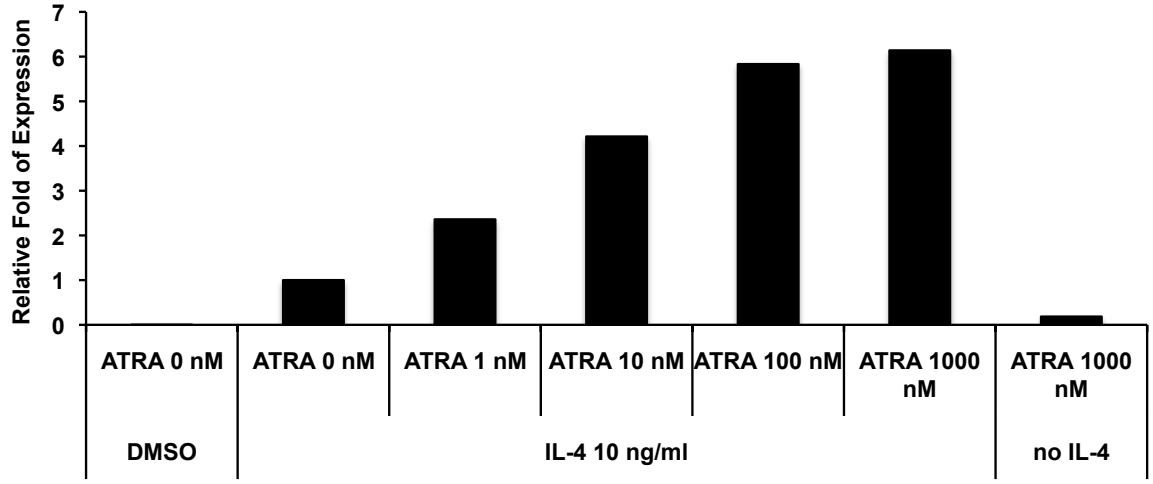
3.2.1 Impact/Effect of ATRA on Arginase 1 Expression Induced by IL-4

Basal expression of arginase 1 was scarcely detectable on the western blot in figure 3.5, which was representative of several results (Figures 3.7, 3.14, 3.16). In a western blot experiment designed to determine the effect of ATRA on arginase 1 protein expression in the presence of IL-4, treatment with 10 ng/ml IL-4 alone significantly induced expression over the basal level of expression (figure 3.7, IL-4 alone was adjusted to 1.00). Upon cotreatment with 10 ng/ml IL-4, 1-1000 nM ATRA potentiated induction of arginase 1 protein, augmenting expression 2-6 fold higher than 10 ng/ml IL-4 alone (Figure 3.7). The potentiation by 1000 nM ATRA was little more than that of 100 nM, indicating either that the capacity of the cells to express arginase 1 protein or the capacity of ATRA to potentiate induction over that of 10 ng/ml IL-4 alone is limited to a maximum of approximately 6-fold. 1000 nM ATRA in the absence of IL-4 did not induce arginase 1 expression visibly, however on densitometric analysis, there is a small increase in expression between the control and 1000 nM ATRA alone. This corroborates the small induction by ATRA alone in figure 3.5.

Figure 3.7: **All-*trans* retinoic acid potentiates induction of arginase 1 expression by IL-4 at a low dose and continuing in a dose-dependent manner. A. Western Blot. BMDM from C57BL/6 mice treated with the specified stimulants for 8 hours, then whole cell lysate harvested and subjected to western blot with anti-arginase 1 antibody, reprobed for β-actin to ensure equal loading of protein in each lane. B. As in previous figures, densitometric analysis accounts for loading and is corrected to give fold induction.**



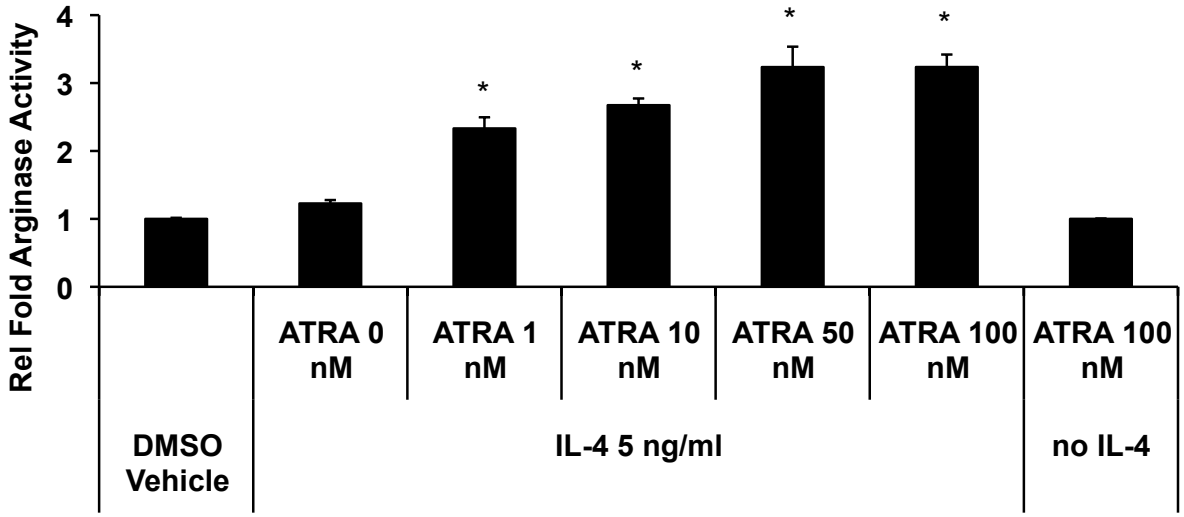
B.



Potentiation Effect of ATRA on 10 ng/ml IL-4		
IL-4 10 ng/ml	0 nM ATRA	1.00
	1 nM ATRA	2.36
	10 nM ATRA	4.21
	100 nM ATRA	5.83
	1000 nM ATRA	6.14

In light of the fact that 10 ng/ml IL-4 and 1000 nM ATRA appeared to saturate the cells ability to induce expression of arginase 1, the concentrations used in the arginase activity assay one week later were adjusted to 5 ng/ml IL-4 and 1, 10, 50, and 100 nM ATRA. Wt BMDM were treated with DMSO vehicle for the control, 5 ng/ml IL-4 alone, the indicated curve of ATRA concentrations in addition to 5 ng/ml IL-4, then a single well was treated with 100 nM ATRA and no IL-4. The cells were harvested and assessed for arginase activity (Figure 3.8). Induction of arginase activity by 5 ng/ml IL-4 is lower than induction of expression of arginase 1 by 10 ng/ml IL-4. This is likely due to the detection method, which is less sensitive as a result of natural absorbance of light in the system resulting in a higher minimal threshold of sensitivity, to the fact that enzyme activity is being measured, and not protein presence, and to the lower level of IL-4 employed. The pattern of dose-dependent ATRA potentiation of IL-4-induced arginase activity was, however similar to that observed in figure 3.7. 100 nM ATRA did not induce arginase activity.

Figure 3.8: **ATRA not only potentiates arginase 1 expression but also arginase activity. BMDM from C57BL/6 mice treated for 8 hours, then arginase enzyme activity was assessed. Error bars represent standard deviation among three independent replicates. Potentiation was significant at the level of p less than 0.05 at all concentrations of ATRA. 100 nM ATRA did not induced significant arginase activity. Fold increases were calculated from average absorbance of three replicates[1].**



Fold Induction of Arginase 1 Expression of ATRA with 10 ng/ml IL-4		
IL-4 10 ng/ml	0 nM ATRA	1.00
	1 nM ATRA	1.90
	10 nM ATRA	2.18
	50 nM ATRA	2.64
	100 nM ATRA	2.64

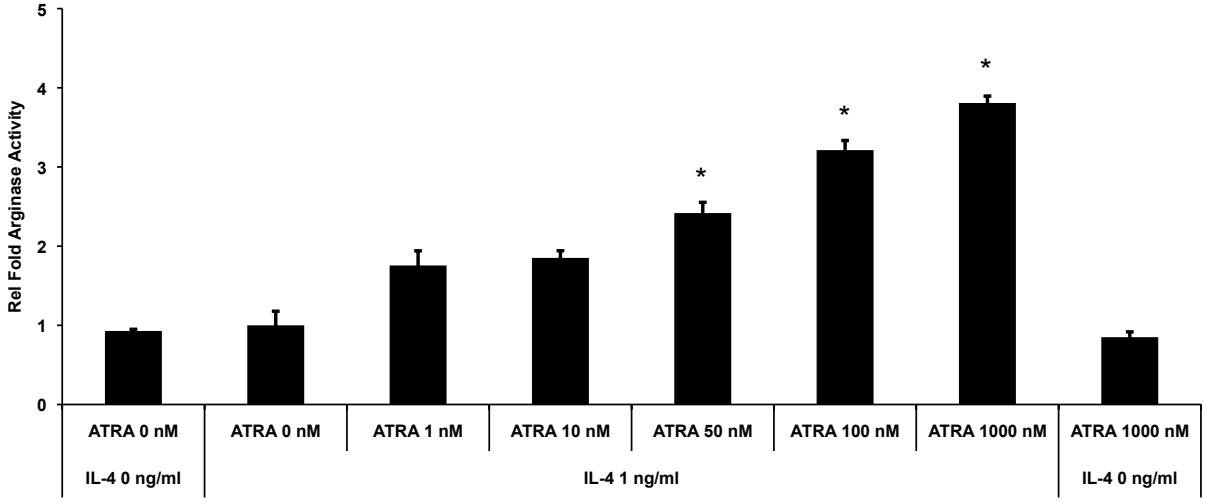
[1] Note: the method for detection of arginase activity requires measurement of absorbance of 540 nm light of 200 μl samples in a 96-well UV-clear polystyrene plate. Even if no conversion of L-arginine to urea occurs, a minimum amount of light is absorbed by the sample and plate. In addition, a minimal amount of the color substrate, isonitrosopropiophenone, is spontaneously converted to the light-absorbing form in the absence of urea, which introduces a minimum limit of detection. This amount must be set arbitrarily to 1 in order to calculate the fold induction. This may cause low levels of induced arginase activity to appear closer

to zero than is true, and may contribute to discrepancies between detection methods.

The initial IL-4 treatment was for 8 hours at 10 ng/ml because that was the lowest concentration at which IL-4 significantly induced arginase activity in initial validation experiments. Also, 1-10 ng/ml IL-4 is a concentration commonly used to suppress superoxide and IL-8 and to induce expression of arginase 1 MHCII class switching in macrophages (Cao, Wolff et al. 1989; Wirth, Kierszenbaum et al. 1989; Abramson and Gallin 1990; Standiford, Strieter et al. 1990; Gallardo-Soler, Gomez-Nieto et al. 2008). The initial concentration of ATRA used was 100 nM. In recent publications (Elias, Laurence et al. 2008; Jin, Chen et al. 2009), 10^{-6} M to 10^{-14} M ATRA has been used, 10^{-7} M used to differentiate T-regulatory (Treg) cells. In addition, the concentration of ATRA detected in blood of patients being treated with ATRA as a differentiation therapy for renal cancer were 56 +/- 37 ng/ml (˜19 nM) (BonhommeFaivre, Paule et al. 1996).

In later experiments, the IL-4 and ATRA concentrations were adjusted in light of earlier results. In some cases, IL-4 was able to induce arginase 1 expression even at 1 ng/ml (Figure 3.9). This was attributed to variability in the recombinant murine IL-4, which was available only in 10 μg aliquots, several of which were ordered throughout the course of research, as well as variability among batches of BMDM, which were isolated weekly from male mice of different age, as well as use of different lots of reagents such as FBS, the composition of which is incompletely defined. Despite these variations, the trend is consistently observed.

Figure 3.9: **Even at a dose of 1 ng/ml IL-4, which does not induce arginase activity, 1 nM ATRA can potentiate arginase activity. ATRA Potentiation Curve. In the presence of 1 ng/ml IL-4, a concentration that did not induce significant arginase activity alone, ATRA induced arginase activity in a dose-dependent manner up to 1000 nM. 1000 nM ATRA alone did not significantly induce arginase activity above vehicle control. Error bars represent standard deviation among three independent replicates. Potentiation was significant at the level of p less than 0.05 at 50nM, 100 nM, and 1000 nM ATRA. 1000 nM ATRA did not induced significant arginase activity. Fold potentiation calculated from average absorbance of three replicates.**



Fold Induction with 1 ng/ml IL-4	of Arginase Activity of ATRA	
IL-4 1 ng/ml	0 nM ATRA	1.00
	1 nM ATRA	1.76
	10 nM ATRA	1.85
	50 nM ATRA	2.42
	100 nM ATRA	3.21
	1000 nM ATRA	3.81

3.3 LPS-Mediated Suppression of Arginase 1 induced by IL-4 and by IL-4 + ATRA

3.3.1 Role of LPS in Macrophage Activation

LPS is a well-studied pyrogen and the ligand of TLR4. LPS induces expression of IFNβ via IRF3/7-dependent pathways and TNFα via NF-κB and MAPK activation of AP1 (Geppert, Whitehurst et al. 1994). These two signals are sufficient for classical activation of macrophages, although this may be less efficient than priming by IFNγ from natural killer or cytotoxic T-cells (Dalton, Pitts-Meek et al. 1993; Schoenborn and Wilson 2007). Not only is LPS a classical activator of macrophages, but in the short term it opposes alternative activation (Gordon 2003). The mechanism for this is not completely clear, but the situation is certainly complex as LPS induces expression of IL-4 in macrophages in a delayed manner (Mukherjee 2008). This lends credence to the concept that alternative activation is a macrophage state that is not in opposition to classical activation; rather it is required to follow classical activation so that inflammation does not get out of control.

LPS is always present at low concentrations in the plasma of healthy adult humans because we maintain populations of Gram-negative bacteria such as *E. coli* living in our gut (Walther and Millwood 1951). Some of the LPS that these bacteria shed is absorbed into the circulation. In the condition of metabolic endotoxemia, plasma LPS is elevated slightly to 10-50 pg/ml (Cani, Amar et al. 2007). This elevation is the key feature of metabolic endotoxemia, which results from obesity and high fat diet and can exacerbate the risk of type 1 diabetes due to chronic activation of the inflammatory response (Cani, Amar et al. 2007).

The level of LPS following Gram-negative bacterial infiltration into the blood or tissues or infection is elevated, and can cause the acute-phase response, an inflammatory response which is characterized by activation of the complement system and secretion of the inflammatory cytokines IL-1, IL-6, IL-8, inducible nitric oxide synthase (iNOS), and TNF-α

(Baumann and Gauldie 1994). These cytokines trigger neighboring cells to become inflamed as well. Interestingly, the acute phase response is also characterized by suppression of retinol binding protein, the carrier protein that chaperones retinol from the lumen of the intestine to the liver for metabolization into retinal and the retinoic acids (Stephensen and Gildengorin 2000).

Excessive plasma LPS or unresolved cytokine storm causes septic shock. Briefly, septic shock results when the cytokines released in the initial detection of infection cause too great an alarm, and too many cells produce and release the antimicrobial nitric oxide gas. Nitric oxide causes systemic vasodilation, drop in blood pressure, and hypoxia in all tissues. Persistent hypoxia causes multiple organ failure and death (Tsalis 2006).

LPS induces iNOS via NF-κB (Chan and Riches 2001; Moon, Kim et al. 2008; Yoo, Choi et al. 2008). iNOS utilizes L-arginine as a substrate for the production of nitric oxide. Furthermore it has been shown that arginase and iNOS competed for L-arginine (Johann, Barra et al. 2007). Thus LPS may not only suppresses expression of arginase 1 but also induce the expression of an enzyme that competes with arginase for substrate. It is expected that the effect of LPS on suppression of arginase activity may be greater than on arginase 1 expression alone.

Basal expression of arginase I is effectively null in resting murine BMDM. A product is detectable by realtime RT-PCR only after 33-35 cycles. A single cDNA can be amplified to detectable levels in 28-30 cycles, so allowing for some permissiveness in transcription and in primer binding, any product requiring more than 30 cycles is effectively invisible in terms of protein presence in the cell. In western blot, a very faint arginase 1 band is visible if 40-50 μg of total protein from vehicle-treated BMDM are loaded per lane, but any less and the band is not visible above background.

The relatively new field of crosstalk between TLR and nuclear receptor signaling pathways has exploded in the past decade, providing a bevy of examples wherein LPS-induced

NF-κB subunits p50 and p65 bind to and suppress RAR and RXR isoforms, preventing them from binding to response elements (Gu, Ke et al. 2006). Therefore LPS suppression of IL-4 signaling, which presumably occurs independent of ATRA/RAR/RXR and LPS suppression of RAR and RXR represent two independent routes by which LPS might potentially suppress IL-4 + ATRA-induced arginase 1 expression. Arginase activity might be suppressed via the third route of iNOS utilization and depletion of the pool of L-arginine available to arginase 1.

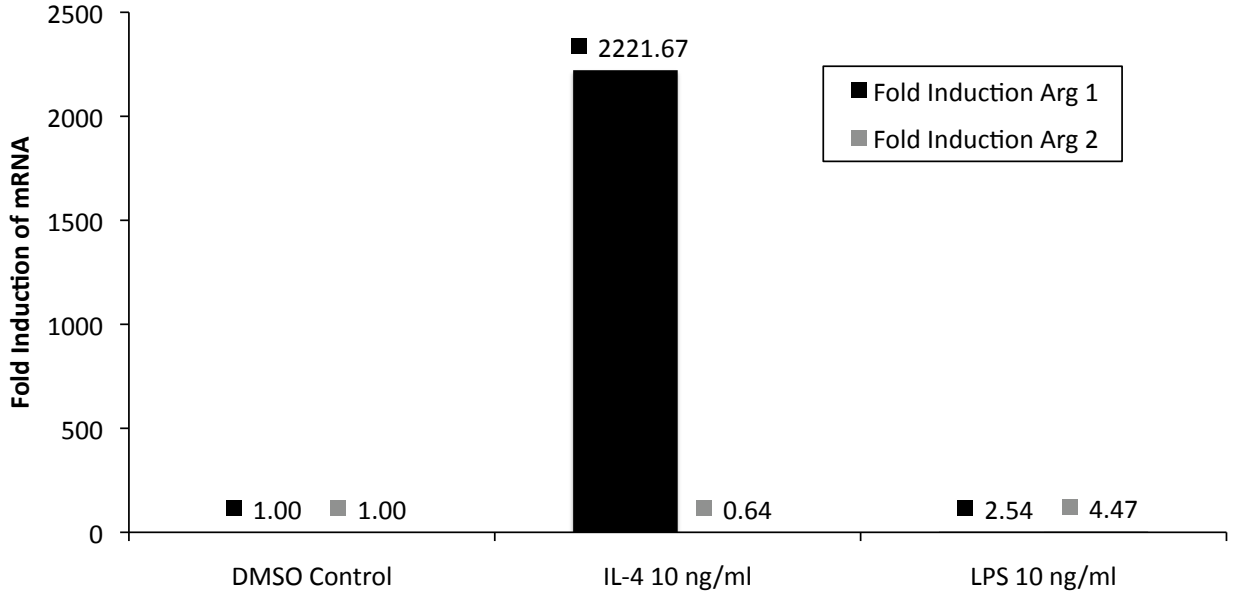
It was also hypothesized that the order of treatment would affect the ability of LPS to effectively suppress arginase expression and activity. Pretreatment with LPS was expected to suppress completely and concurrent or post-treatment was expected to partially suppress at an equivalent dose. Thus three treatment schemes were employed. The first was 60 minutes of pretreatment with LPS, followed by concurrent treatment with IL-4 and ATRA. The second was concurrent treatment with IL-4, ATRA, and LPS. In this case the addition of the treatments to each well was done as quickly as possible, but LPS was added first, then IL-4, then ATRA. In a small side experiment, the order of addition was tested, with LPS being added last, and no difference in its capacity to suppress was apparent. The third treatment scheme was treatment with IL-4 + ATRA followed by post-treatment with LPS 60 minutes later. In all cases of pre- and post-treatment with LPS, the treatment time was measured from the time at which IL-4 and ATRA treatments were administered. This time period was initially somewhat variable, from 4-16 hours, but all data shown in this document, unless otherwise noted, was derived from cells treated for 4 hours (in the case of RNA) or 8 hours (in the case of western blot or enzyme activity assay.

3.3.2 LPS Treatment Does Not Affect Basal Arginase 1 Espression and Activity

LPS treatment alone did not appear to impact arginase 1 mRNA or arginase enzyme activity. Although the level of expression and activity was effectively null in the absence of IL-4, no measurable change, positive or negative, was observed in realtime RT-PCR for

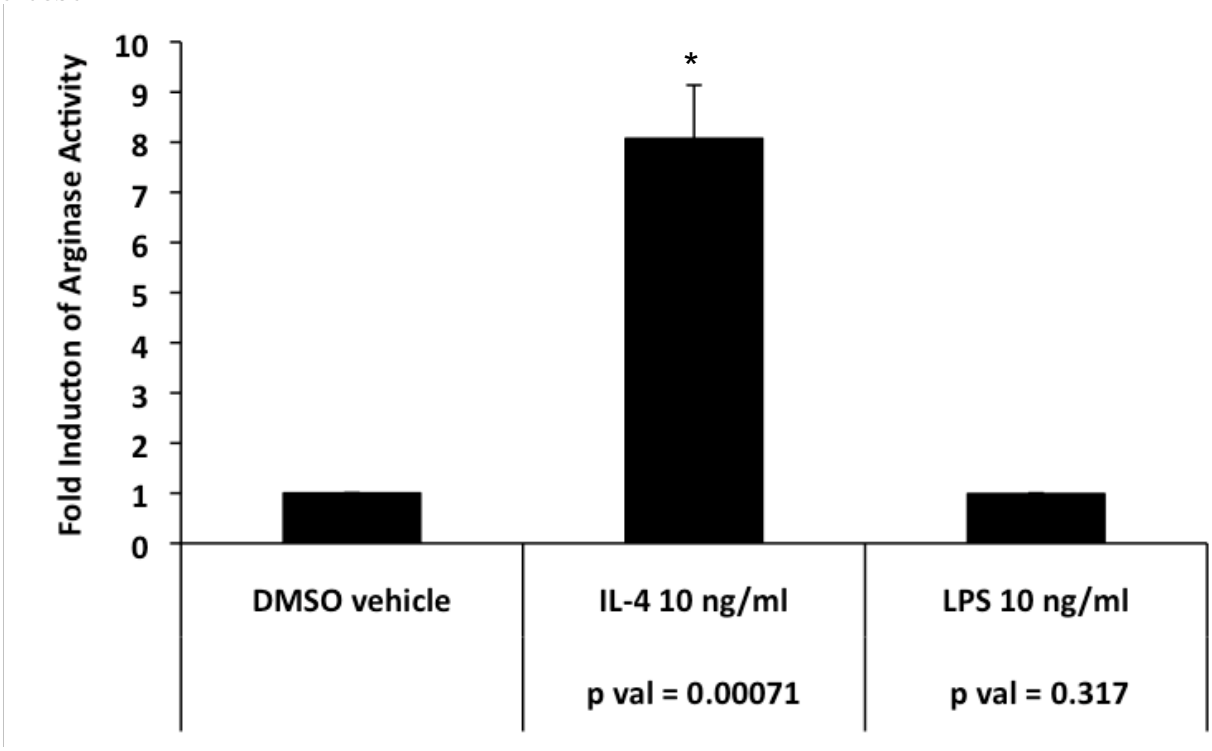
arginase 1 or in arginase activity assay (Figures 3.10 and 3.11). Western blot is not shown because DMSO-vehicle and LPS-treatment lanes contain no visible bands. This is in distinct contrast to recent results indicating that LPS is capable of inducing arginase 1 expression and activity in macrophages (El Kasmi, Qualls et al. 2008; Zhang, Baban et al. 2009).

Figure 3.10: **IL-4 induced arginase 1 but not arginase 2 mRNA, while LPS induces neither. Wt BMDM were treated for 4 hours with indicated ligand(s). In realtime RT-PCR analysis of arginase 1 and arginase 2 mRNA expression, 10 ng/ml LPS did not induce or suppress the expression of either gene. As a control, 10 ng/ml IL-4 robustly induced arginase 1 expression 2221 fold higher than basal expression. Arginase 2 mRNA was not induced by IL-4 either.**



	Fold Induction Arg 1	Fold Induction Arg 2
DMSO Control	1.00	1.00
IL-4 10 ng/ml	2221.67	0.64
LPS 10 ng/ml	2.54	4.47

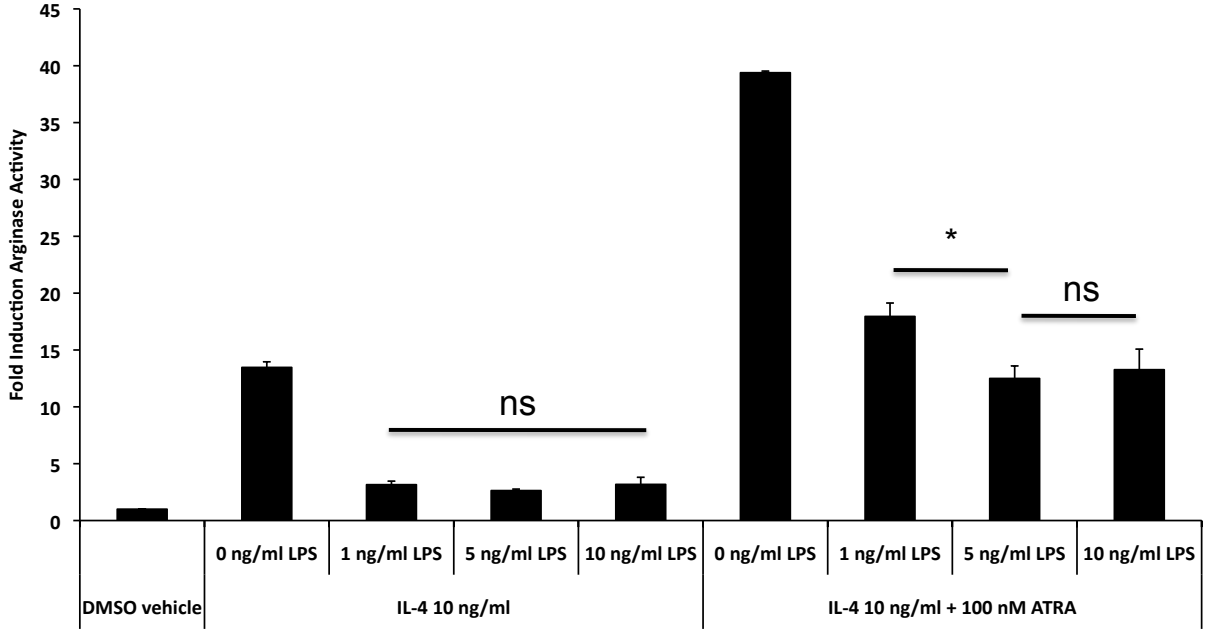
Figure 3.11: **LPS cannot induce significant arginase activity following 8 hour treatment. Arginase activity assay. Wt BMDM were plated in triplicate, treated with the specified stimulants for 8 hours, then harvested and the arginase enzyme activity was assessed. Error bars represent standard deviation of three replicates and p values were calculated from triplicate samples using Student's 2-tailed t-test.**



3.3.3 Role of LPS in Suppression of Induced Arginase 1

In order to determine the effect of LPS on IL-4-induced and IL-4 + ATRA-induced arginase activity, BMDM were treated with 10 ng/ml IL-4 or 10 ng/ml IL-4 + 100 nM ATRA and 0, 1, 5, or 10 ng/ml of LPS. All concentrations of LPS suppressed IL-4-induced arginase activity equally, but 1 ng/ml of LPS suppressed IL-4 + ATRA-induced arginase activity less than 5 or 10 ng/ml (Figure 3.12).

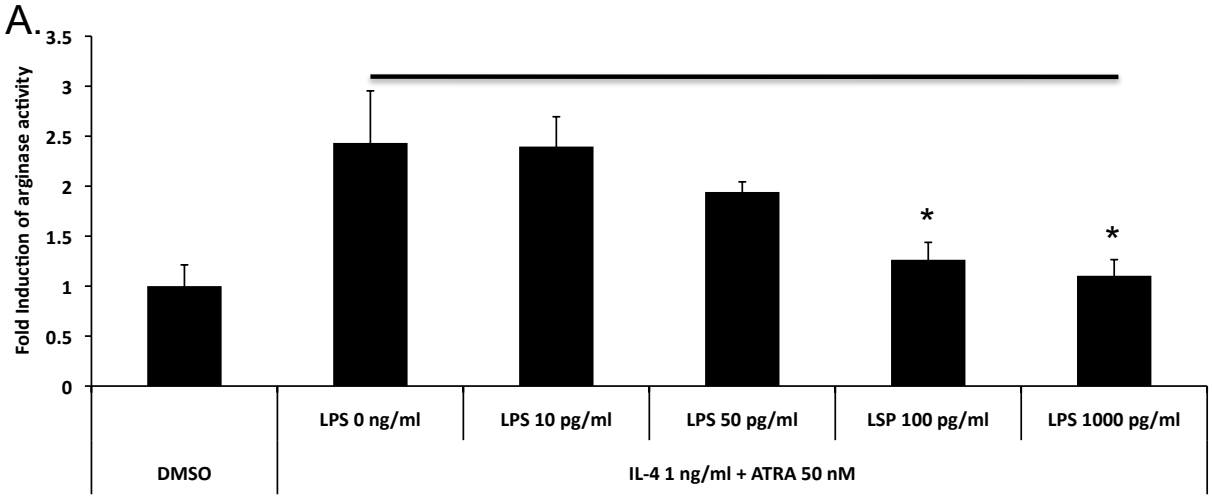
Figure 3.12: **ATRA makes BMDM more resistant to LPS suppression of arginase activity, particularly at low doses of LPS (1 ng/ml). Arginase Activity Assay. Wt BMDM were treated with DMSO vehicle, 10 ng/ml IL-4, or 10 ng/ml IL-4 + 100 nM ATRA with or without 1, 5, or 10 ng/ml LPS. Error bars represent standard deviation calculated from three biological replicates using Students 2-tailed t-test. % suppression due to LPS calculated as demonstrated in the materials and methods. The difference between 1 ng/ml LPS suppression and 5 ng/ml or 10 ng/ml LPS suppression of IL-4 + ATRA-induced arginase activity was significant at the level of p less than 0.05.**



Arginase Activity Assay - Percent Suppression of Arginase Activity Due to LPS		
[LPS] in ng/ml	IL-4 alone	IL-4 + ATRA
1	82.71	55.84
5	86.89	70.05
10	82.52	68.04

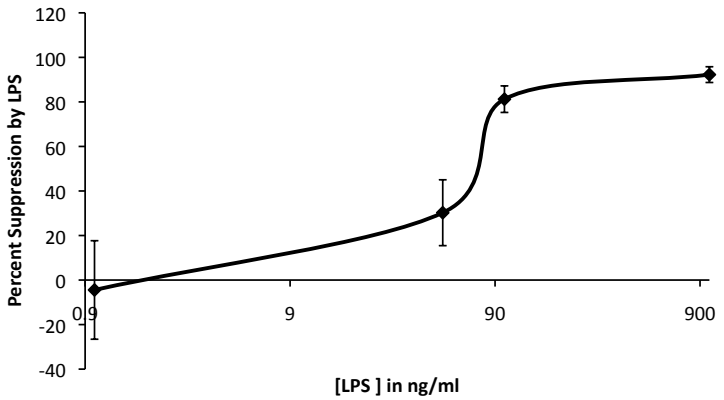
In order to use more physiologically relevant concentrations, an arginase activity assay was also performed with lower concentrations of IL-4 (1 ng/ml) and ATRA (50 nM). The LPS concentrations were also titrated lower over several experiments in an effort to identify a window of sensitivity to LPS. A similar pattern of suppression was observed (Figure 3.13).

Figure 3.13: **LPS suppression is dose-dependent. A. Arginase Activity Assay. Wt BMDM treated 8 hours with DMSO vehicle or 1 ng/ml IL-4 + 50 nM ATRA and increasing concentrations of LPS from 0 to 1000 pg/ml LPS. Percent suppression calculated as described. B. Suppression curve generated from percent suppression calculations. Error bars represent standard deviation on chart and plot. Suppression by 100 pg/ml and 1000 pg/ml was significant at the level of p less than 0.05. 50 pg/ml suppression by LPS, p=0.18.**



pg/ml LPS	% Suppression
10	2.53
50	34.24
100	81.60
1000	92.77

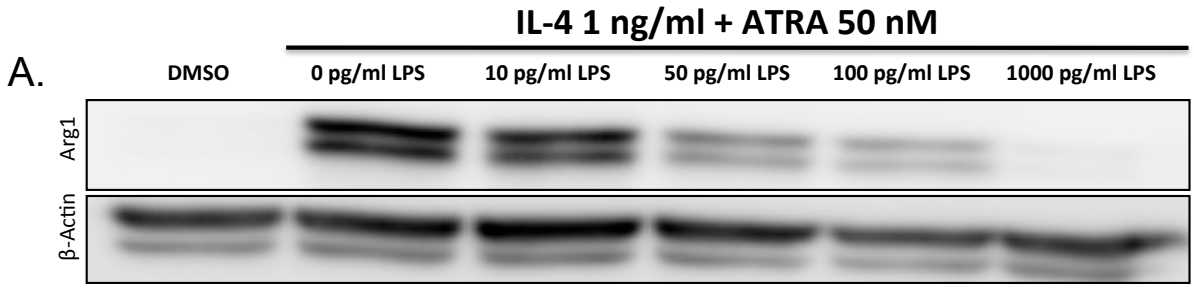
B.

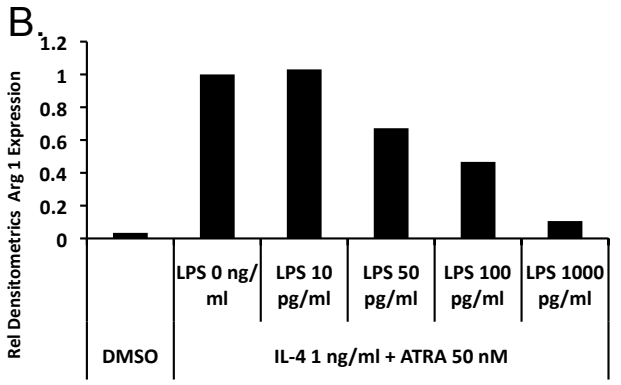


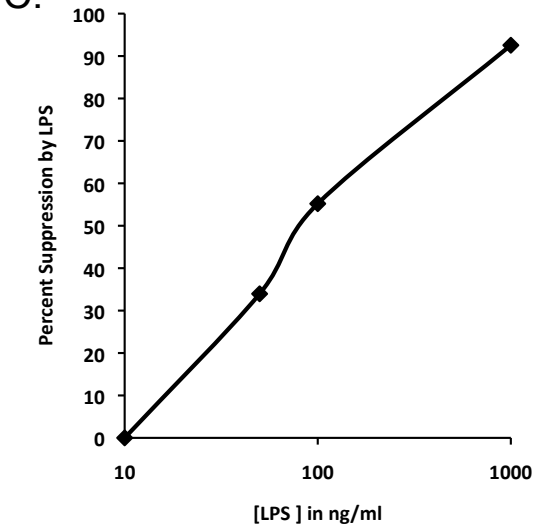
In order to determine if expression of arginase 1 was also being suppressed, western blot was performed using samples treated in the same manner as the arginase activity samples in figure 3.13 (Figure 3.14). Interestingly, arginase activity and arginase 1 expression suppression profiles match almost exactly, save higher suppression of activity than expression at 100 ng/ml LPS.

The expression of arginase 1 and arginase activity were both induced by 1 ng/ml IL-4 and 50 nM ATRA and suppressed by cotreatment with as little as 50 pg/ml LPS (Figures 3.13 and 3.14). 1 pg/ml LPS did not suppress arginase 1 expression or arginase activity. From 10 pg/ml to 1000 pg/ml, increasing concentrations of LPS suppressed arginase 1 expression in a dose-dependent manner. In most experiments, 1000 pg/ml LPS suppressed arginase activity and expression by 90 - 100%.

Figure 3.14: **LPS suppresses arginase activity at least in part by suppressing expression of arginase 1. A. Western blot. Wt BMDM were treated for 8 hours with indicated ligands. B. Densitometrics were used to calculate suppression by LPS and to generate LPS suppression curve (C.). LPS suppresses IL-4-induced and IL-4 + ATRA-induced arginase 1 mRNA, protein expression, and enzyme activity in a dose-dependent manner.**







Percent Suppression		Wt BMDM
pg/ml LPS	10	0.00
	50	33.96
	100	55.20
	1000	92.55

3.3.4 Importance of Order of LPS Treatment

It is not known if M1 or M2 activation is permanent or transient. If true polarization occurs, then LPS pretreatment would be expected to preclude the ability of macrophages to express arginase 1 in the context of alternative activation. Correspondingly, initial treatment with IL-4 and ATRA would initiate a program of alternative activation which would be insensitive to LPS suppression of arginase 1 and LPS induction of markers of classical activation such as IL-6 and TNFα. In order to assess the validity of this hypothesis, the impact of pre and post-treatment with LPS on its capacity to suppress arginase 1 expression and activity was tested. LPS treatment was performed 60 minutes prior to IL-4 + ATRA treatment for 60 minutes following IL-4 + ATRA treatment.

Pretreatment with LPS improved the efficiency of LPS suppression, achieving more suppression at each concentration of LPS than concurrent treatment or post-treatment with LPS (Figure 3.15). Pretreatment effectively shifted the suppression curve toward greater suppression while post treatment shifted the curve toward less suppression. Interestingly, neither pre- nor post-treatment resulted in complete suppression or ablation of suppression by LPS. This indicates one of two possibilities. The first is that the time course was not long enough, and that pretreatment with LPS for 1 hour is not long enough to fully engage the classical activation phenotype and that 1 hour of IL-4 + ATRA is not long enough to fully engage the alternative activation phenotype. The second is that the M1 and M2 phenotypes are not mutually exclusive or irreversible. Interestingly, arginase activity was not affected as dramatically as arginase 1 expression by pre- and post treatment with LPS. Suppression of arginase activity by 1 ng/ml LPS was not significantly alleviated by post-treatment versus pre-treatment, whereas in the western blot, post treatment with 1 ng/ml LPS was significantly less effective than pretreatment. This may be due to the fact that LPS induces iNOS in what is assumed to be a shorter time course than that required by the mechanism required to suppress arginase 1.

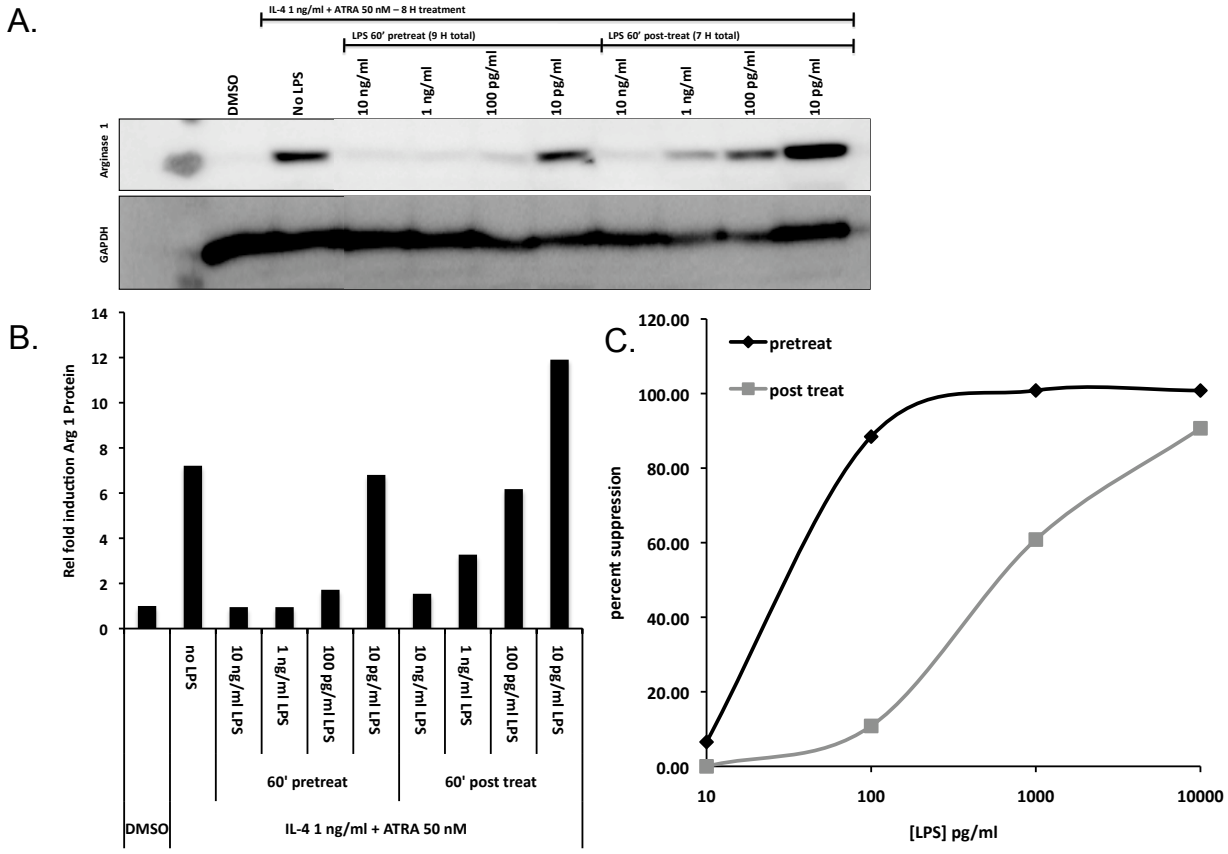
3.4 Pathways of LPS Suppression of Arginase 1

3.4.1 Role of IRAK1 in LPS Signaling

The role of IRAK1 in LPS signaling has been a hotly debated issue in innate immunity signaling. IRAK1 is utilized by tumor necrosis factor α receptor (TNF-R), interleukin 1 receptor (IL-1R) and several TLR (Lockett, Goebl et al. 2008). It is recruited to the receptor upon ligand binding, modified by polyubiquitination and hyperphosphorylation, upon which it disengages from the receptor while remaining bound to TRAF6, with which it initiates NF-κB and AP1 signaling through mechanisms described in the introduction. IRAK1 has kinase activity, but this is not required for successful activation of NF-κB (Kubo-Murai, Hazeki et al. 2008). Initially it was identified as a unique pelle-like kinase (pelle being a kinase present only in D. melanogaster and a few other arthropods for which a family of kinases including the IRAKs is named (Shiu and Bleecker 2003)) which was required for NF-κB activation in response to IL-1 and TNF in HEK293 cells (Vig, Green et al. 1999). Overexpression of IRAK1 in the keratinocyte cell line HaCaT results in augmented IL-1-induced expression of the cytokines CCL5 and CCL20 (Sanmiguel, Olaru et al. 2009). Knockdown of IRAK1 results in decreased activation of NF-κB, AP1 by IL-18 in HepG2 cells (Guo and Wu 2000). Upon the discovery of IRAK4, which phosphorylates IRAK1 and has redundant kinase activity, it was proposed that IRAK1 was redundant and unnecessary (Jiang, Johnson et al. 2003; Strelow, Kollewe et al. 2003; Song, Talamas et al. 2009). Recent work has made it clear that IRAK1 nonetheless plays a significant, if perhaps more nuanced, role in LPS signal transduction (Maitra, Parks et al. 2009; Maitra, Singh et al. 2009).

Although IRAK1 was initially only associated with the MyD88-dependent signaling from TLR4, it has also been observed in a TRAF3-dependent complex with TRIF, IKKϵ, TBK1, and IRF3 and IRF7 (Oganesyan, Saha et al. 2006). This result hints at involvement

Figure 3.15: **Early LPS suppresses arginase 1 more effectively than late LPS. A. Wt BMDM were treated 8 hours with indicated ligands. Arginase 1 induced by 1 ng/ml IL-4 + 50 nM ATRA is suppressed by LPS in a characteristic dose-dependent curve. B. Densitometric analysis of arginase 1 expression was assessed by ratio between arginase 1 band and GAPDH band intesities, then correction of unstimulated sample intensity to 1.00. C. Pretreatment with LPS shifts the suppression curve to the right, effectively increasing the % suppression/ ng LPS. Post treatment with LPS shifts the curve to the left, reducing the efficiency of suppression.**



of IRAK1 in another capacity downstream of TLR4, and puts it into the class of molecules with TRIF and TRAF6, which have been implicated as potential mediators of crosstalk between MyD88-dependent and TRIF-dependent pathways (Yamamoto, Sato et al. 2003; Hacker, Redecke et al. 2006). For this reason, the role of IRAK1 in the phenomenon of LPS suppression of IL-4 + ATRA-induced arginase 1 was investigated.

3.4.2 Role of IRAK1 in LPS Suppression of Arginase 1 Expression and Activity

The best-studied location of crosstalk between LPS and ATRA signaling is the physical interaction between p50 and p65, subunits of NF-κB, and the DNA-binding domain of RAR and RXR, the nuclear receptors through which ATRA acts (Motomura, Ohata et al. 2001; Hoo, Chan et al. 2007). As IRAK1 is critically involved in LPS activation of NF-κB, the potential for the LPS suppression of IL-4 + ATRA-induced arginase 1 to be dependent at least partly by IRAK1 was investigated.

BMDM were simultaneously harvested from Wt mice as well as mice deficient in IRAK1 in order to look for differences in the regulation of arginase 1 expression and activity in Wt and IRAK1$^{-/-}$ BMDM was tested. Cells were harvested, plated, allowed to rest, and treated for 8 hours with 1-10 ng/ml IL-4, 1-1000 nM ATRA, and 1pg/ml 10 ng/ml LPS, with pre-treatment and post treatment with LPS in some experiments.

It was found that the induction of arginase activity and of arginase 1 expression both by IL-4 alone and by IL-4 + ATRA was not affected by the absence of IRAK1. Although not always equal, induction was not consistently higher in one genotype or the other. Thus differences in basal or induced arginase induction were attributed to natural variation among the cells, minor differences in plating numbers between the genotypes from experiment to experiment, and other sources of experimental variation. In the following figures, in order to facilitate calculation of suppression efficiencies and to better visualize the effect of LPS on arginase 1 expression and arginase activity, the level of maximal induction achieved by IL-4 + ATRA has been adjusted to the arbitrary value of 1 for both genotypes.

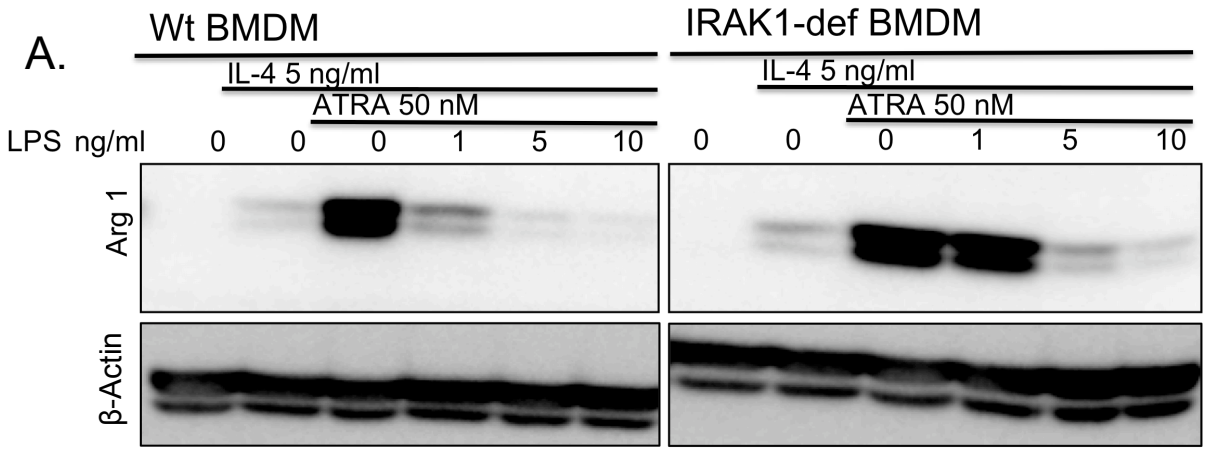
In a western blot in which high concentrations of LPS were used, the level of arginase 1 induced by 5 ng/ml IL-4 + 50 nM ATRA was suppressed from maximal induction by 80% by 1 ng/ml LPS in Wt BMDM but the same concentration of LPS only suppressed the maximal induction by IL-4 + ATRA by 20% in IRAK1$^{-/-}$ BMDM. At 5 and 10 ng/ml LPS, the difference between the genotypes was significantly smaller, with Wt at 95% and 98% and IRAK1$^{-/-}$ at 85% and 95%, respectively (Figure 3.16). These values were obtained by means of densitometric analysis of the blot photographs using the Multi Gauge software suite or with ImageJ, an open source photo analysis program. Mate samples to those used in the western blot were isolated for the arginase activity assay. (Figure 3.17). The values from the densitometric analysis or from the absorbance of 540 nm light were used to calculate the percent suppression values.

The suppression profile of LPS in arginase 1 expression and in arginase activity corroborate each other and are representative of several experiments. Both indicate that at 1 ng/ml LPS, suppression of arginase 1 and arginase activity is more efficient in terms of percent suppression/ ng/ml LPS in Wt BMDM than in BMDM lacking IRAK1. However at 5 ng/ml and 10 ng/ml, the disparity between suppression efficiencies due to genotype decreases.

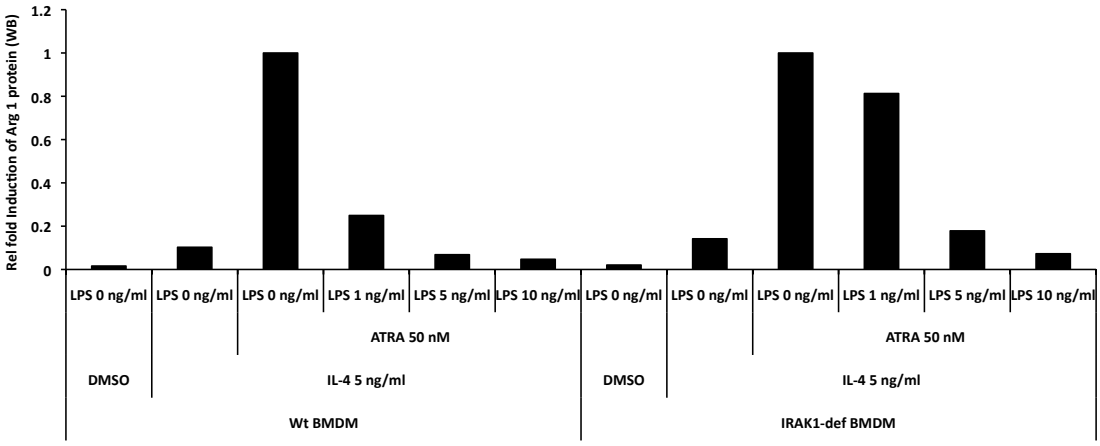
At initial appearance of an IRAK1-dependence of LPS suppression of arginase 1 and arginase activity, the concentration of LPS was titrated to lower doses in subsequent experiments. IL-4 and ATRA concentrations were also reduced in an effort to better observe window of sensitivity within which LPS suppression of arginase 1 is dependent on IRAK1.

Figure 3.16: **Suppression of arginase 1 protein expression is dependent on IRAK1 at low doses of LPS. A. Wt and IRAK1$^{-/-}$ BMDM were treated with DMSO vehicle, 5 ng/ml IL-4 alone, or IL-4 + 50 nM ATRA + 0, 1, 5, or 10 ng/ml LPS. B. Densitometric analysis was used to calculated by ratio between arginase 1 and β-Actin bands, and the control was corrected to 1.00. C. Calculated percent suppression from maximal induction is calculated from the densitometric result and represented in the table and in the line graph.**

A.

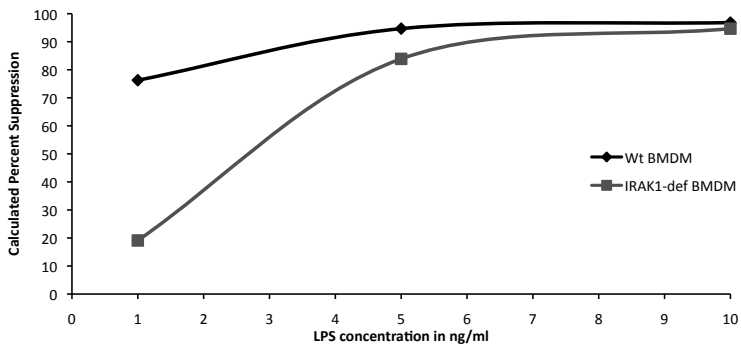


B.



C.

Calculated Percent Suppression of Arginase 1 Expression due to LPS from Densitometrics		
	Wt BMDM	**IRAK1-def BMDM**
LPS 1 ng/ml	**76.27**	**19.11**
LPS 5 ng/ml	**94.68**	**83.89**
LPS 10 ng/ml	**96.83**	**94.68**



3.4.3 Role of Tollip in LPS Suppression of Arginase 1 Expression and Activity

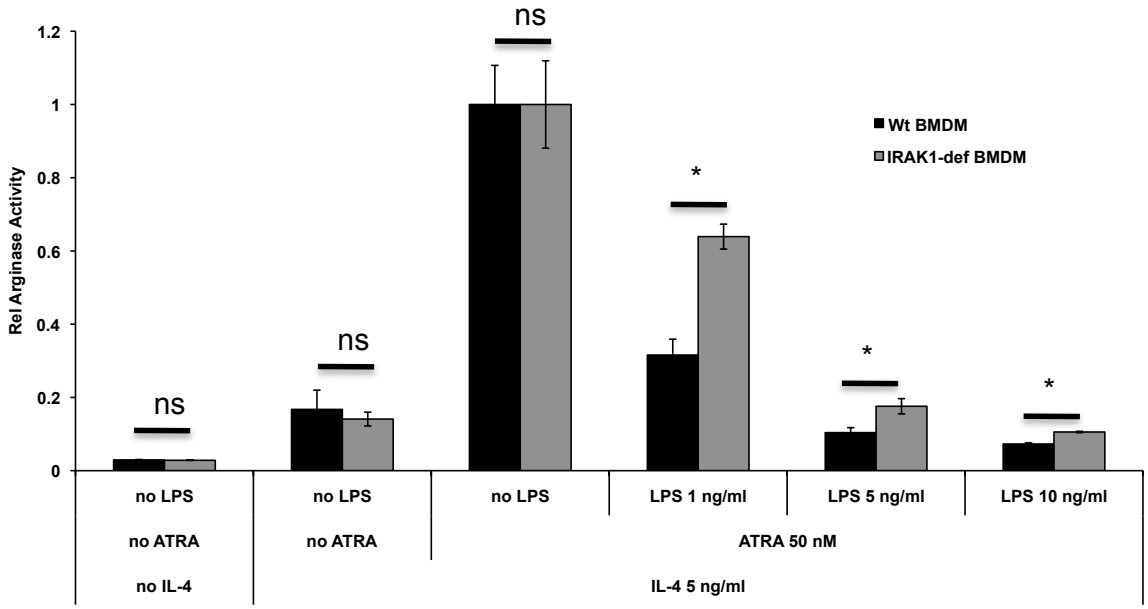
Toll-interacting protein (tollip), discovered by Dr. Kimberly Burns in 2000 (Burns, Clatworthy et al. 2000), has a C-terminal CUE domain, which interacts with ubiquitinated substrates, a C2 domain, which interacts with phosphatidylinositol-3-phosphate and targets the protein to the cell membrane, and an N-terminal Tom1-binding domain (Burns, Clatworthy et al. 2000; Shih, Prag et al. 2003; Yamakami, Yoshimori et al. 2003; Ryzhakov and Randow 2007). More importantly, tollip interacts with IRAK1 as part of the receptor kinase complex, and although initially it was found to suppress LPS-induced NF-κB when overexpressed *in vivo*, the absence of tollip in mice abrogated induction of IL-6 and TNFα by IL-1β and LPS, firmly implicating tollip as a positive regulator of inflammatory signaling and a requirement for full inflammatory response (Zhang and Ghosh 2002; Didierlaurent, Brissoni et al. 2006).

While the exact mechanism is still not known, generally tollip recognizes ubiquitinated substrate and shepherds proteins to the Tom1 complex of the endosome for degradation (Yamakami, Yoshimori et al. 2003). Its involvement in ubiquitination, sumoylation, and intracellular trafficking of proteins was discovered by its requirement for successful ubiquitination and degradation of the IL-1R (Brissoni, Agostini et al. 2006). Location, partner binding, and degradation of RAR has been shown to be influence by its sumoylation status as well as by the ubiquitin/proteasome pathway (Zhu, Gianni et al. 1999; Wu, Lin et al. 2004; Maitra, Parks et al. 2009). It is not known if tollip interacts directly or indirectly with RAR, but this may provide an additional point of contact for tollip in regulation of arginase 1.

Tollip$^{-/-}$ BMDM were employed in order to determine if tollip has a role in modulating the LPS signal that suppresses arginase 1 in BMDM. Upon treatment with IL-4 alone, arginase 1 and arginase activity are modestly induced in tollip$^{-/-}$ BMDM. ATRA alone has no measurable effect on arginase 1 or arginase activity in these BMDM, however ATRA does

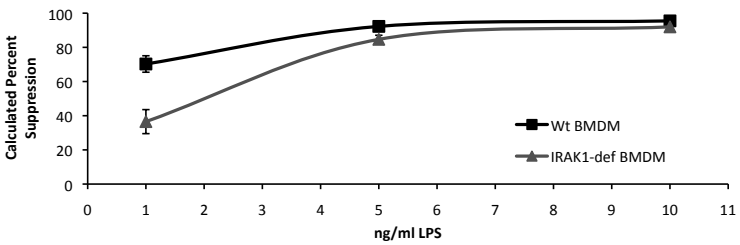
Figure 3.17: **Dependence on IRAK1 of LPS suppression of arginase 1 protein expression is reflected in arginase activity. A. Wt and IRAK1$^{-/-}$ BMDM were plated and treated in triplicate for 8 hours with DMSO vehicle, 5 ng/ml IL-4 alone, or IL-4 + 50 nM ATRA and 0, 1, 5, or 10 ng/ml LPS. B. Percent suppression from maximal induction was calculated for each concentration of LPS for each genotype, represented in the table and in the line graph. Error bars represent standard deviation in the bar graph and the suppression plot. The difference in suppression was significant at the level of p less than 0.05 at all concentrations of LPS, but was greater at the low dose of LPS representing endotoxemia.**

A.



B.

Calculated Percent Suppression of Arginase Activity due to LPS		
	Wt BMDM	IRAK1-def BMDM
LPS 1 ng/ml	70.54	36.65
LPS 5 ng/ml	92.36	84.83
LPS 10 ng/ml	95.49	91.96



potentiate IL-4 induction of arginase 1 and arginase activity to greater degree than in Wt or in IRAK1$^{-/-}$ BMDM. This result was not obtained in every experiment, but was a consistent phenomena. Tollip$^{-/-}$ mice exhibit a phenotype of smaller body weight, thinner bones and more and smaller BMDM than Wt and IRAK1$^{-/-}$ mice. Despite this variation, the cells responded in a manner similar to Wt and IRAK$^{-/-}$ BMDM, and no special alternations such as loading of less total protein in western blots, use of a different number of cells, or dilution of samples prior to measurement were necessary.

Alternatively, the phenomena of higher induction in the absence of tollip suggests a role for tollip in constitutive suppression of arginase 1, even under no stimulation by LPS.

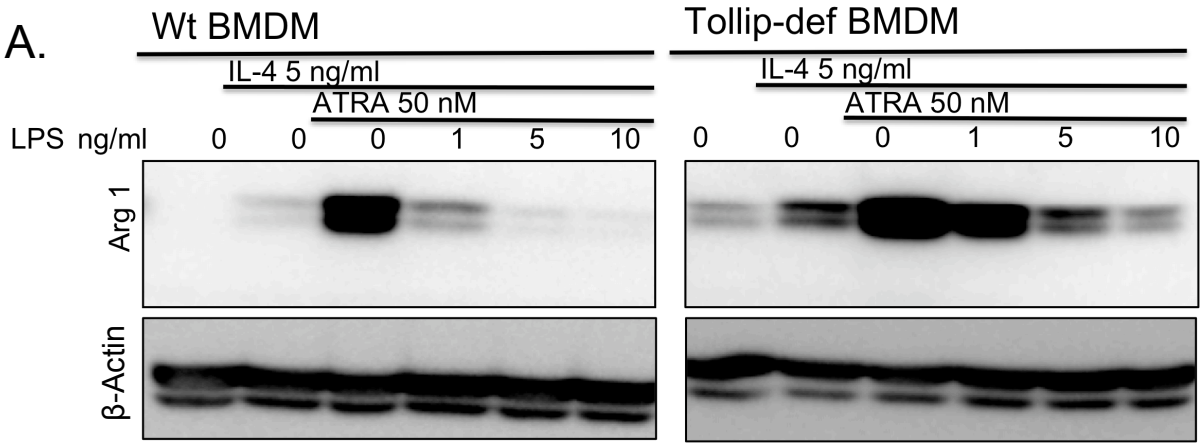
In order to determine if tollip is involved in mediating suppression of arginase 1, Wt and tollip$^{-/-}$ BMDM were treated for 8 hours with 5 ng/ml IL-4 + 50 nM ATRA and increasing concentrations of LPS. Lysates were harvested and used for western blot (Figure 3.18). Identically-treated samples were harvested in triplicate for the arginase activity assay (Figure 3.19).

The LPS suppression profile in the western blot for tollip$^{-/-}$ BMDM compared to Wt BMDM is similar to the comparison between IRAK1$^{-/-}$ versus Wt BMDM. At lower concentrations of LPS, the suppression of arginase 1 is less efficient in tollip$^{-/-}$ BMDM than in Wt BMDM. At 1 ng/ml LPS, arginase 1 expression is suppressed 76% in Wt BMDM but only 25% in tollip$^{-/-}$ BMDM. At 5 ng/ml LPS, arginase 1 expression is suppressed 95% in Wt and 75% in tollip$^{-/-}$, and at 10 ng/ml, both are effectively completely suppressed.

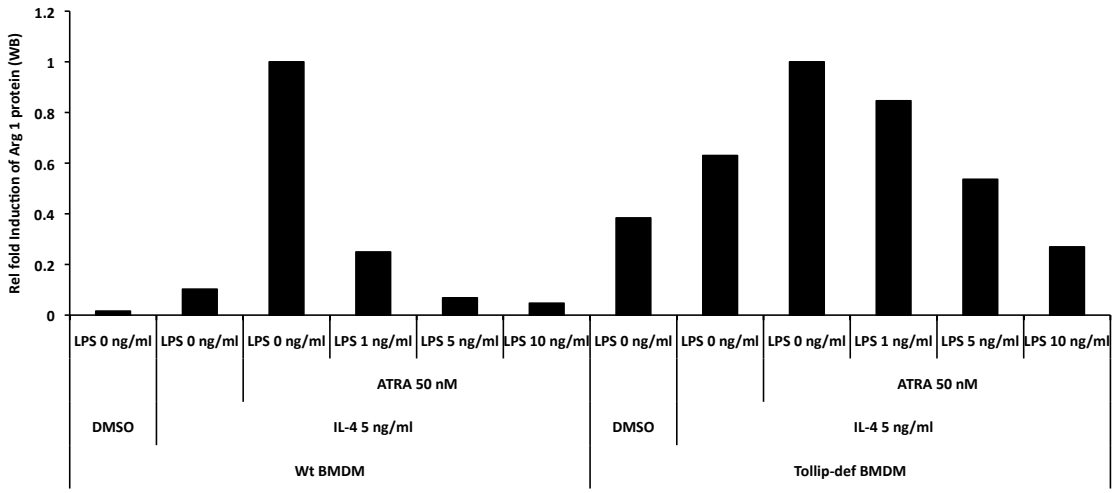
The arginase activity suppression profile for tollip$^{-/-}$ BMDM versus Wt BMDM does not corroborate the western blot. Although suppression of arginase activity is less efficient in tollip$^{-/-}$ BMDM than in Wt BMDM, the difference is smaller than expected at 1 ng/ml LPS and larger than expected at 10 ng/ml LPS.

Figure 3.18: **Overall expression of arginase 1 protein is constitutively suppressed by tollip, and suppression of arginase 1 protein expression is dependent on tollip at low doses of LPS. A. Wt and tollip$^{-/-}$ BMDM were treated with DMSO vehicle, 5 ng/ml IL-4 alone, or IL-4 + 50 nM ATRA + 0, 1, 5, or 10 ng/ml LPS. B. Densitometric analysis was used to calculated by ratio between arginase 1 and β-actin bands, and the control was corrected to 1.00. C. Calculated percent suppression from maximal induction is derived calculated from the densitometry result and represented in the table and in the line graph.**

A.

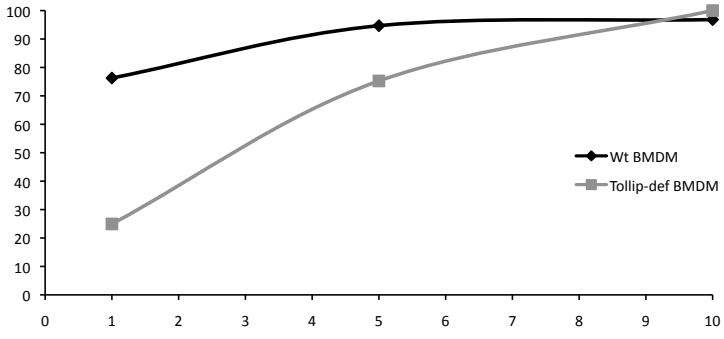


B.



C.

Calculated Percent Suppression of Arginase 1 Expression due to LPS from Densitometrics		
	Wt BMDM	**Tollip-def BMDM**
LPS 1 ng/ml	**76.27**	**24.94**
LPS 5 ng/ml	**94.68**	**75.25**
LPS 10 ng/ml	**96.83**	**100.00**



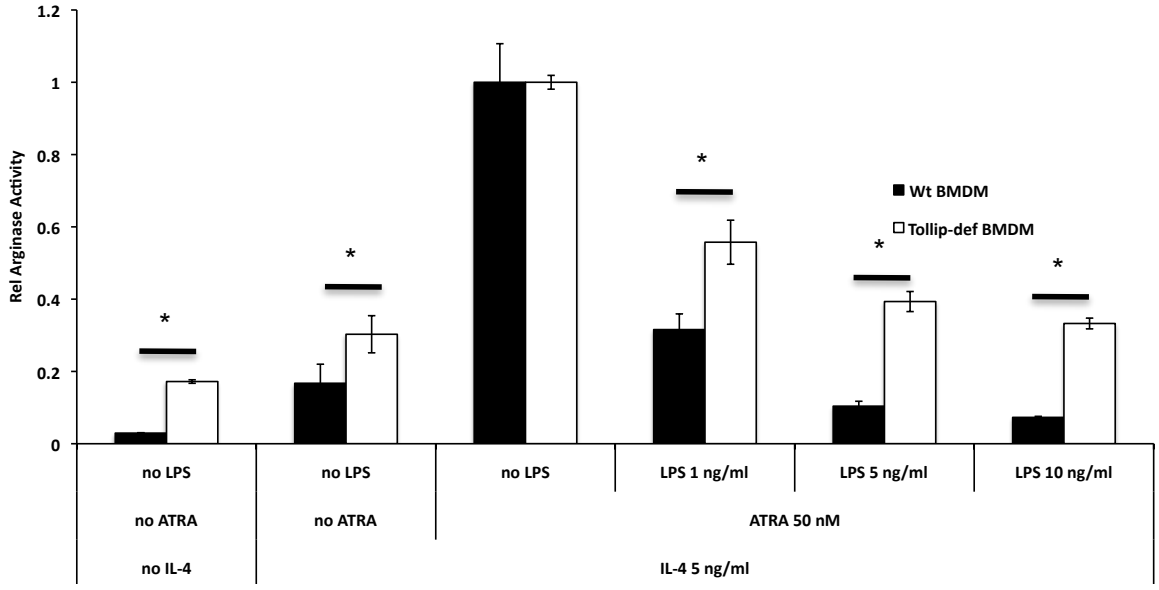
3.4.4 Subcellular Location of TLR4 in LPS Suppression of Arginase 1

Recent results indicate that while TLR 3, 7, 8, and 9 signal exclusively from intracellular endosome/lysosome compartments in order to keep them from recognizing self-nucleic acids, TLR4 is capable of signaling both from the cell surface as well as from the endosome (Barton and Kagan 2009). The current theory is that TLR4 can sequentially engage both the MyD88-dependent signaling pathway from the surface and the TRIF-dependent pathway from the endosome due to restrictive distribution of the key adaptors throughout the cell, resulting in early and late signaling events. Inhibition of the GTPase dynamin by the selective inhibitor dynasore prevents endocytosis of the receptor complex, preventing TRIF-dependent signals but allowing MyD88-dependent signals (Husebye, Halaas et al. 2006; Thompson and McNiven 2006; Kirchhausen, Macia et al. 2008).

In order to the importance of TLR4 signaling from the endosome is necessary for LPS suppression of arginase 1, and by implication whether the suppression is limited to the MyD88-dependent pathway or if it requires the TRIF-dependent pathway as well, the inhibitor dynasore was employed. In initial experiments, 80 μM dynasore, as used by Massol, R.H., et al. (Macia, Ehrlich et al. 2006) in BSC1 (African green monkey kidney cell line) resulted in dramatic morphological change and reduced expression of GAPDH as well as arginase 1 in the BMDM, so the concentration was reduced to 5 μM, which was not toxic to the cells, but was still capable of disrupting LPS signaling. Dynasore administered by dissolving in DMSO at a concentration of 5 nM, 1000x the final concentration, then adding 1μl per ml to the plated cells. DMSO vehicle was used in samples that did not receive dynasore ([DMSO] never exceeding 0.1% of culture media). Dynasore pretreatment for 30 minutes was followed by simultaneous treatment with IL-4, ATRA, and LPS for an additional 8 hours. Dynasore treatment did not affect IL-4 or IL-4 + ATRA induction of arginase 1 expression and arginase activity in BMDM. It did, however, completely ablate LPS suppression of arginase 1 expression at 10, 50, and 100 pg/ml LPS. Interestingly, 1000 pg/ml LPS was

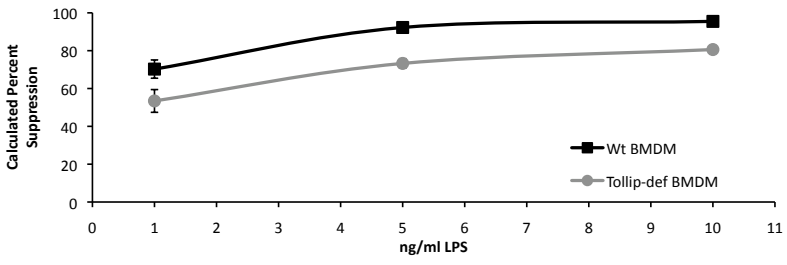
Figure 3.19: **The impact of tollip on arginase 1 expression, both in constitutive suppression and LPS-mediated suppression, is reflected in arginase activity, except more dependence at higher doses of LPS was observed. A. Wt and Tollip$^{-/-}$ BMDM were plated and treated in triplicate for 8 hours with DMSO vehicle, 5 ng/ml IL-4 alone, or IL-4 + 50 nM ATRA and 0, 1, 5, or 10 ng/ml LPS. B. Percent suppression from maximal induction was calculated for each concentration of LPS for each genotype, represented in the table and in the line graph. Error bars represent standard deviation in the bar graph and the suppression plot. The difference in suppression was significant at the level of p less than 0.05 at all concentrations of LPS, but was greater at the low dose of LPS representing endotoxemia. The difference in constitutive and induced (IL-4 alone) arginase activity was also significant at the level of p less than 0.05. the difference between maximal induction cannot be assessed with corrected data.**

A.



B.

Calculated Percent Suppression of Arginase Activity due to LPS		
	Wt BMDM	Tollip-def BMDM
LPS 1 ng/ml	70.54	53.49
LPS 5 ng/ml	92.36	73.25
LPS 10 ng/ml	95.49	80.59



still capable of suppressing arginase 1 nearly to basal levels of expression (Figures 3.20 and 3.21). LPS suppression of arginase 1 expression of 6%, 42%, 59%, and 95% by 10 pg/ml, 50 pg/ml, 100 pg/ml, and 1000 pg/ml of LPS (respectively) were reduced to -2%, -10%, -14%, and 85% (respectively).

When the same experiment was performed using IRAK1$^{-/-}$ BMDM, only minimal alleviation of the suppression by LPS was observed (Figures 3.22 and 3.23). LPS suppression of arginase 1 expression of 23%, 49%, 60%, and 93% by 10 pg/ml, 50 pg/ml, 100 pg/ml, and 1000 pg/ml of LPS (respectively) were moderately reduced to 23%, 39%, 43%, and 90% (respectively). This indicates first that LPS suppression of arginase 1 is at least partly dependent on the activity of dynamin. This, however, may or may not be because of activation of pathways from TLR4 that require endocytosis. Second, the alleviation of suppression occurs to a small degree in IRAK1$^{-/-}$ BMDM, which implies that the alleviation of LPS suppression by dynasore is not restricted to the same mechanism(s) at work in alleviation of suppression in IRAK1$^{-/-}$ BMDM as compared to Wt BMDM. Dynasore-dependent alleviation of LPS suppression of arginase 1 expression occurs at least in part independently of IRAK1-dependent signaling. So although IRAK1-deficiency and inhibition of dynamin-dependent endosome formation have similar effects on arginase 1 expression, the mechanisms by which they achieve this are likely different.

Figure 3.20: **Inhibition of the small GTPase dynamin abrogates LPS suppression of arginase 1 protein expression at low doses of LPS in Wt BMDM. Wt BMDM were treated 8 hours with DMSO vehicle or 1 ng/ml IL-4 + 50 nM ATRA + 0, 10, 50, 100, or 1000 pg/ml LPS in the presence or absence of 5 μM dynasore. Membranes were stripped and reprobed for β-actin to assess equal loading.**

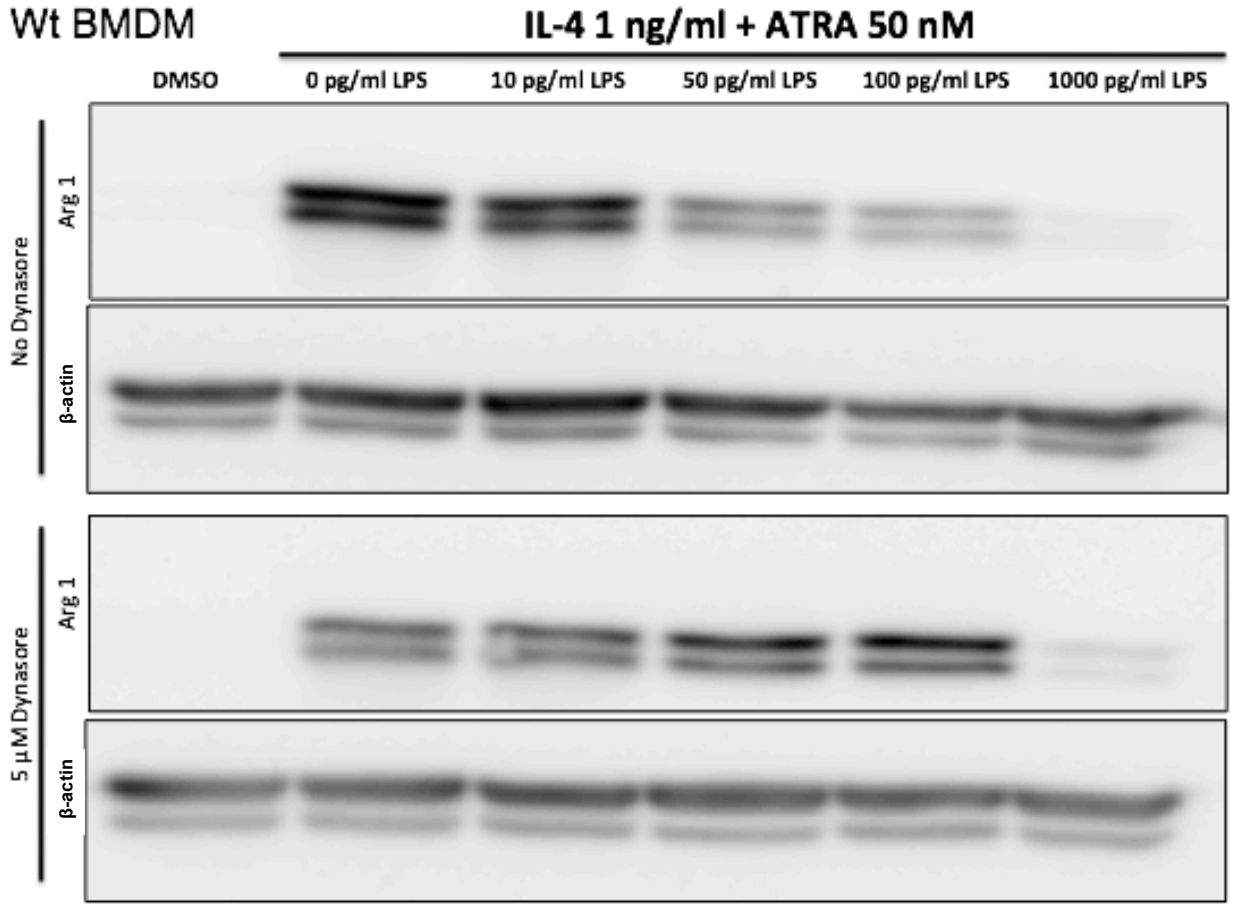
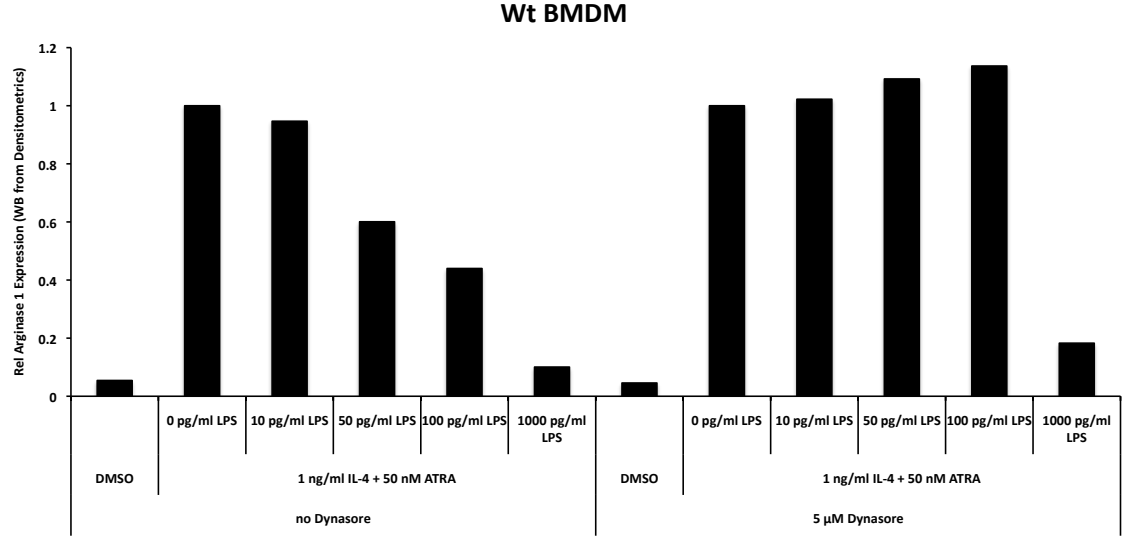


Figure 3.21: **Densitometrics and suppression calculations from figure 3.20 western blot. A. Band intensity for all arginase 1 bands in figure 3.20 were assessed and corrected for β-actin loading by ratio. B. Percent suppression was calculated and plotted against [LPS]. Statistics are not available as densitometric analysis is a semiquantitative method that yields only single values.**

A.



B.

Wt BMDM, % suppression of IL-4 + ATRA-indueced arginase 1 expression by LPS in the presence or absence of dynasore.		
	no dynasore	5 µM dynasore
10 pg/ml LPS	5.63	-2.35
50 pg/ml LPS	42.23	-9.67
100 pg/ml LPS	59.24	-14.33
1000 pg/ml LPS	95.11	85.64

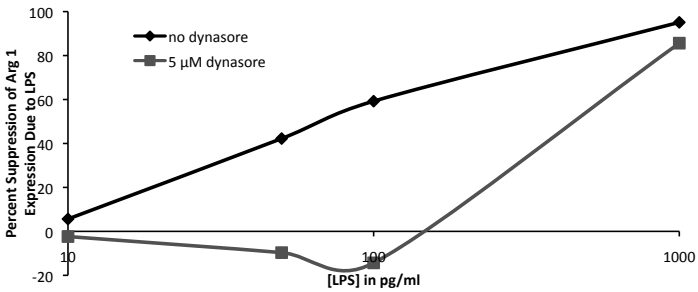


Figure 3.22: **In IRAK1$^{-/-}$ BMDM, treatment with dynasore does not inhibit LPS signaling in the same manner as in Wt BMDM. IRAK1$^{-/-}$ BMDM were treated 8 hours with DMSO vehicle or 1 ng/ml IL-4 + 50 nM ATRA + 0, 10, 50, 100, or 1000 pg/ml LPS in the presence or absence of 5 μM dynasore. Membranes were stripped and reprobed for β-actin to assess equal loading.**

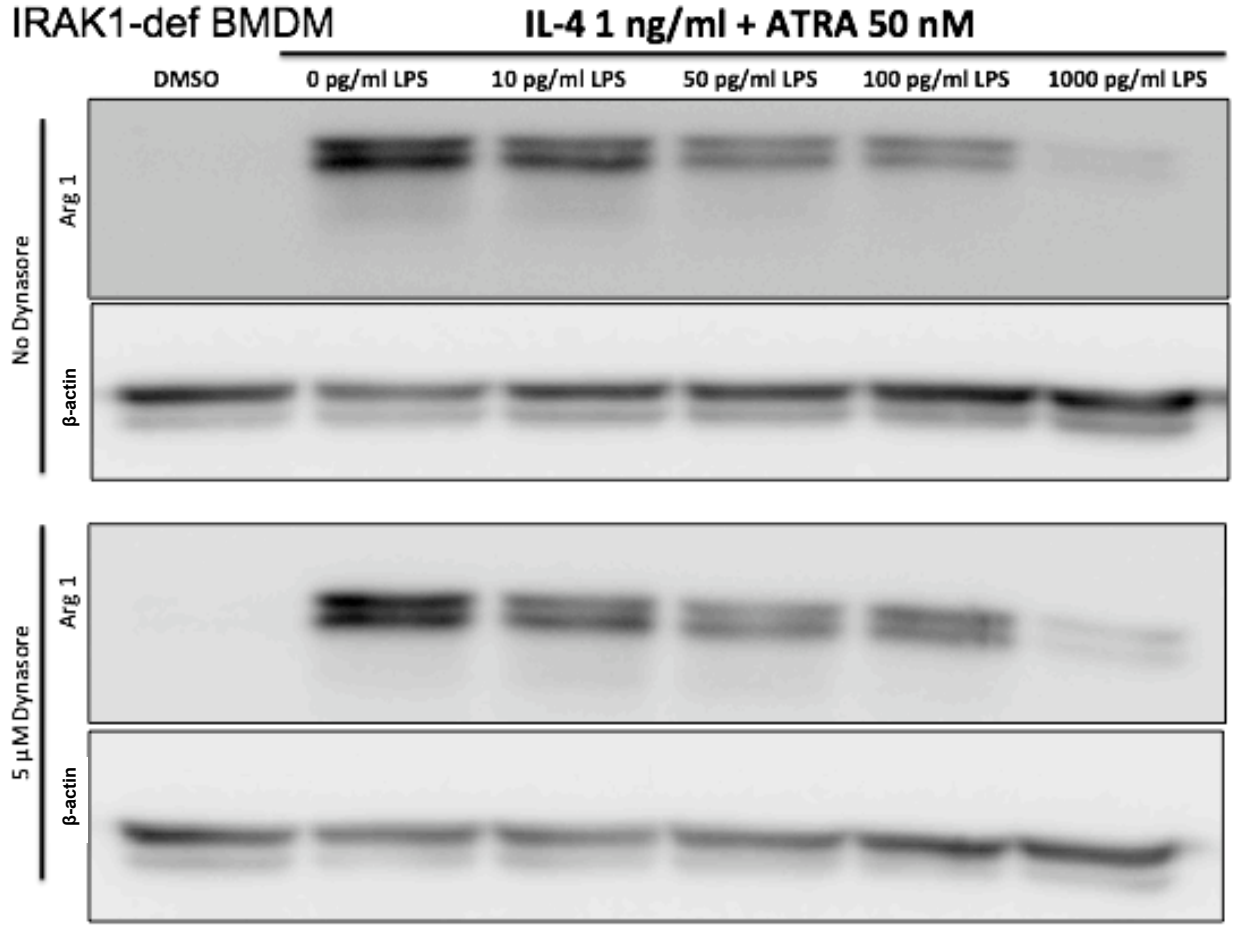
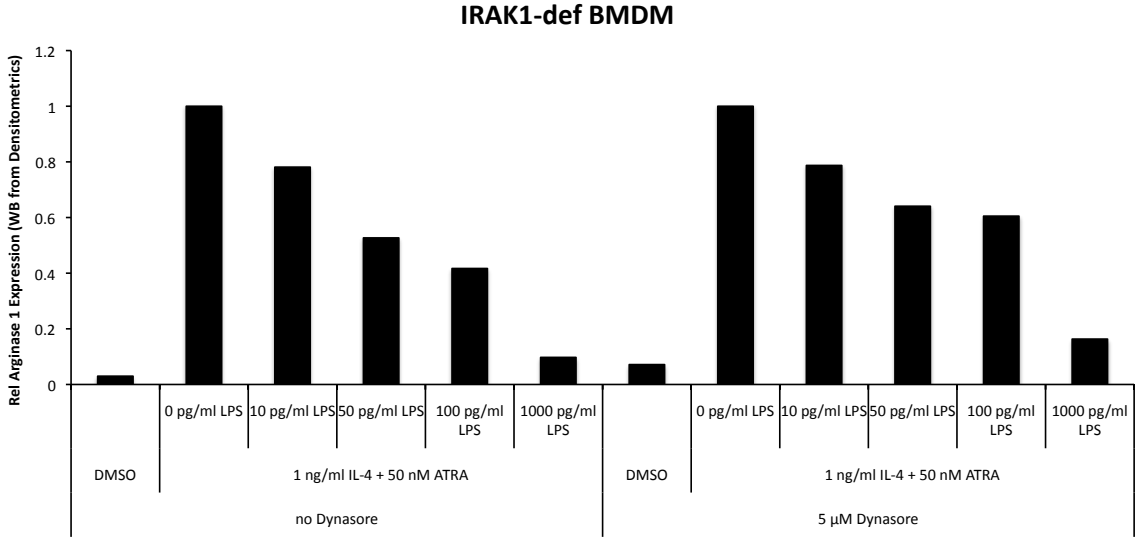


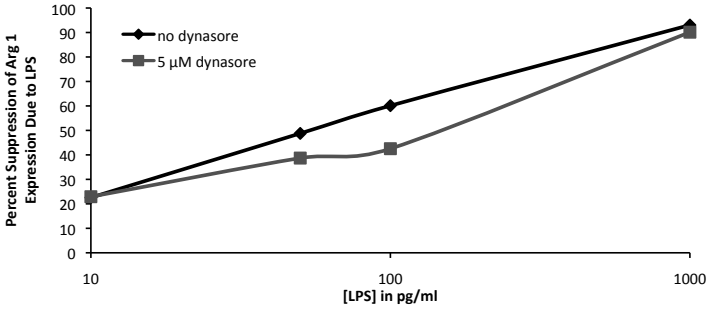
Figure 3.23: **Densitometrics and suppression calculations from figure 3.22 western blot. A. Band intensity for all arginase 1 bands in figure 3.22 were assessed and corrected for β-actin loading. B. Percent suppression was calculated and plotted against [LPS]. Statistics are not available as densitometric analysis is a semiquantitative method that yields only single values.**

A.



B.

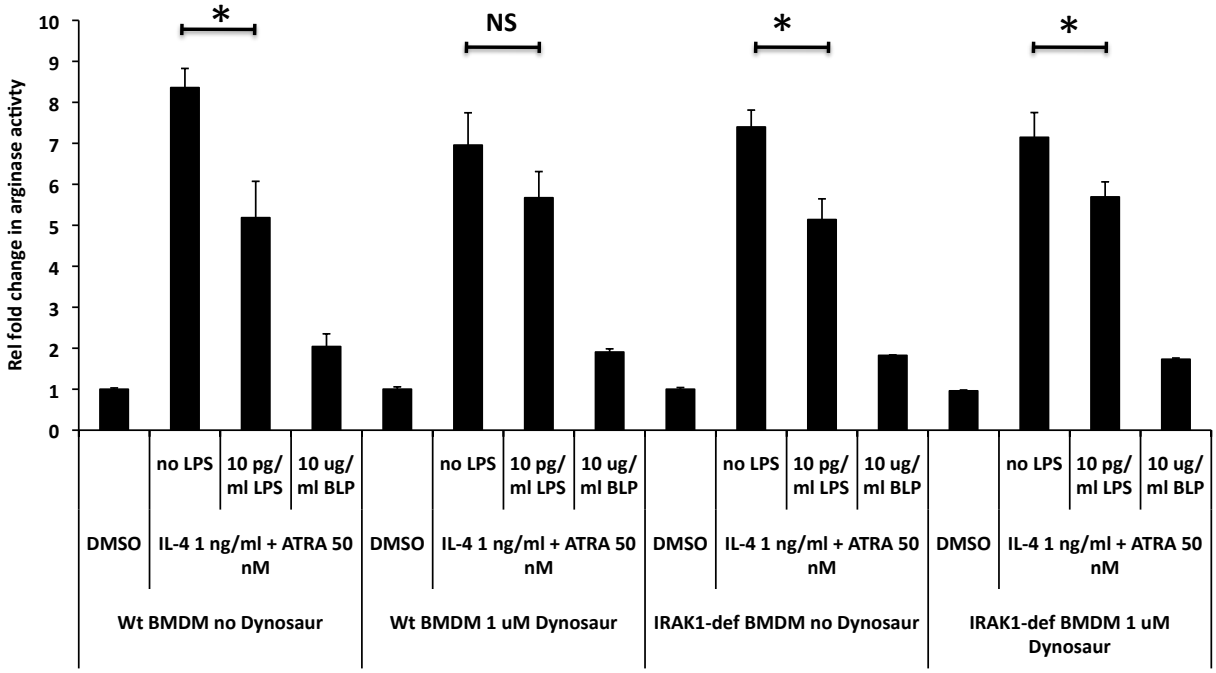
IRAK1-def BMDM, % suppression of IL-4 + ATRA-indueced arginase 1 expression by LPS in the presence or absence of dynasore.		
	no dynasore	5 µM dynasore
10 pg/ml LPS	22.55	22.92
50 pg/ml LPS	48.77	38.70
100 pg/ml LPS	60.11	42.53
1000 pg/ml LPS	93.04	90.12



For several reasons, not least of which was the need to observe the dynasore effect in a statistically measurable way, arginase activity assays were performed in order to confirm the result observed in the western blot for the effect of dynasore on arginase 1 expression. The concentration of dynasore used in the western blot was not suitable for the arginase activity assay because it was found that the arginase activity was nearly completely suppressed even by concentrations of dynasore as low as 5 μM. 1 μM dynasore, although 15 times lower than the IC50 (15 μM) determined by Massol et al, was sufficient to observe alleviation of suppression of arginase activity. In Wt and IRAK1$^{-/-}$ BMDM, arginase activity was induced by 1 ng/ml IL-4 + 50 nM ATRA and suppressed by 10 pg/ml LPS in the presence or absence of 1 μM dynasore. In the absence of dynasore, 10 pg/ml LPS suppressed arginase activity by 43% and 35% in Wt and IRAK1$^{-/-}$ BMDM, respectively. Both of these suppressions were significant at the level of p ¡ 0.05. Following 30 minutes of 1 μM dynasore pretreatment, the percent suppression was reduced to 22% and 23% in Wt and IRAK1$^{-/-}$ BMDM, respectively. This suppression was significant at p ¡ 0.05 in IRAK1$^{-/-}$ BMDM, but not significant at that level in Wt BMDM (Figure 3.24).

Bacterial lipoprotein (BLP) is the ligand for TLR2. Unlike TLR4, it is incapable of signaling through an endosome-dependent pathway. Like TLR4, it activated NF-κB and AP1 in a MyD88-dependent manner. Since BLP does not signal through endosomal pathways, suppression of arginase 1 by BLP can be used as a positive control for the study of the effect of inhibition of endosome formation on TLR4 signaling effects.

Figure 3.24: **Dynasore pretreatment alleviates LPS suppression of IL-4 + ATRA-induced arginase activity. Wt and IRAK1$^{-/-}$ BMDM were pretreated for 30 minutes with DMSO vehicle or with 1 μM dynasore, inhibitor of the GTPase dynamin, which is required for TLR4 signaling from the endosome. DMSO vehicle or 1 ng/ml IL-4 + 50 nM ATRA and 0 pg/ml or 10 pg/ml LPS or 10 μ/ml BLP (bacterial lipoprotein).**



10 pg/ml LPS in:	percent suppression
Wt BMDM no dynasore	43.12
Wt BMDM 1 µM dynasore	21.57
IRAK1-def BMDM no dynasore	35.30
IRAK1-def BMDM 1 µM dynasore	23.66

* p<0.05

Chapter 4

Discussion and Conclusions

4.1 All-*trans* Retinoic Acid Does Not Induce Arginase 1.

Although ATRA was found to be insufficient to induce expression of arginase 1 on its own, the hypothesis that ATRA may indirectly augment expression of arginase 1 was proven true. In cotreatment with 1, 5 or 10 ng/ml IL-4, ATRA significantly induced expression and activity of arginase 1 significantly above that of IL-4 alone in wildtype, IRAK1$^{-/-}$, and tollip$^{-/-}$ bone marrow-derived macrophages. This represents the first evidence that ATRA influences expression of arginase 1. As arginase 1 is expressed only in the liver and in the M2 macrophage, This may also be taken as the first evidence that ATRA is involved in macrophage activation to the M2 phenotype.

This evidence also marks ATRA and the nuclear receptors RAR and RXR as potential therapeutic targets in endotoxemia and diseases of inflammation. Of particular interest is the potential for combining ATRA with existing therapies for atherosclerosis. As ATRA has already been tested and approved for use in oral and intravenous use, it will be interesting to see if ATRA-induced arginase 1 will help to resolve or prevent the development of atherosclerotic plaques, which are well known to be dependent to a large degree recruitment

and conversion of M1 macrophages to foam cells, and subsequent accumulation of low-density lipoproteins inside the macrophage. If ATRA-dependent arginase 1 is truly indicative of the M2 phenotype, then this may indeed be the case. Other markers and behaviors of the M2 and M1 macrophage, such as expression of MHCII, MMR, FIZZ1, and YM1 (M2) and IL-6, IL-8, TNFα, and iNOS will have to be assessed in the ATRA-primed macrophage.

In using ATRA, it was important first to confirm that the compound was transcriptionally active. A known positive control for ATRA is that it induces downstream of kinase 1 (p62DOK1), a scaffolding protein involved in cell cycle regulation (Lamkin, Chin et al. 2006). ATRA is also known to attenuate the activity of the enzyme iNOS (Datta, Reddy et al. 2001). RNA from BMDM treated with DMSO vehicle or 100 nM ATRA were used for realtime RT-PCR to look for induction of arginase 1 and of DOK1. Arginase 1 was not induced while DOK1 was induced 1.5 fold (Figure 3.1). A Griess reagent assay, the classical method described by Peter Griess in 1858 of measuring production of nitrite species (Viinikka 1996), was performed on samples treated with increasing amounts of LPS, a potent pyrogen that induces iNOS, in the presence or absence of 100 nM ATRA. The samples treated with LPS and ATRA exhibited much less production of nitric oxide species than the matched samples treated with LPS alone and no ATRA (Figure 3.2). These two results taken together confirmed that the ATRA was transcriptionally active.

Arginase 1 is a key marker of alternative activation in macrophages that is induced by IL-4, IL-13, IL-10, LPS (with delayed kinetics), 9-*cis*-RA, and glucocorticoids in the several types of M2 macrophage. It is not significantly induced in the M1 macrophage or in other tissues save the liver, where it is constitutively expressed and performs a vital function in the urea cycle. ATRA is a metabolic derivative of vitamin A, which has the same structure as 9-*cis* RA, save that it is an isomer and has a cis bond in place of a trans bond in the tetraenoic acid portion. ATRA is implicated in a broad range of anti-inflammatory effects, including alleviation of *Propionibacterium acnes*-induced matrix remodeling and suppression

of TLR expression and signaling (Liu, Krutzik et al. 2005; Jalian, Liu et al. 2008). ATRA has not, however, been implicated in any way in modulation of arginase 1 expression or connected in any manner to macrophage activation in the M1 or M2 phenotypes.

The only evidence in the literature of induction of arginase in any capacity was published by Rhodes and Oliver in 1980 (Rhodes and Oliver 1980). Guinea pig peritoneal macrophages were treated with 10^{-6} M, 10^{-7} M, 10^{-8} M, or 10^{-9} M ATRA for 24 hours, after which arginase activity was assessed with an assay similar to the one employed in this study. Although induction of arginase activity was observed, it is not possible to assess the significance because the result does not show statistics, only the mean of 4 independent experiments. In order to address the claim that ATRA alone can induce arginase activity, similar experiments were performed at the outset of this study. Even at a concentrations of 10^{-7} M and 10^{-6} M, ATRA alone was not capable of significantly inducing arginase activity in several experiments wherein triplicate samples were independently treated and processed, then averaged and assessed for statistical significance (Figures 3.4, 3.8, and 3.9). This does not corroborate the result obtained by Rhodes and Oliver, but does not refute it for the least reason that the experiments are performed in different species and in macrophages of different origins.

In the arguably more sensitive assays of western blot and realtime RT-PCR, induction of arginase 1 protein and mRNA was observed in several experiments (Figures 3.5 and 3.6). This induction is very low; approximately 3.33 fold, especially in comparison to induction by any concentration of IL-4 alone; approximately 20.24 fold. In the case of the western blot, the induction is not visible in the blot photograph. However in the case of the realtime RT-PCR, the change in the cycle number is from 31.43 to 25.57. Investigators generally classify any product that requires 30 or more cycles to be effectively absent or zero because this approached the lower limit of detection of the method. However, although both values are near to the lower limit of detection, a difference of five cycles is difficult to discount.

This induction was approximately 75.26 fold, whereas 10 ng/ml IL-4 induced a 2221.67 fold induction. It is thus clear that arginase 1 mRNA is minimally induced from undetectable to scarcely detectable levels in Wt murine BMDM by treatment with 100 nM ATRA for 8 hours, that arginase 1 protein is also induced in a similar manner, but that these inductions do not translate to measurably increased arginase activity.

In light of the IL-4-potentiating capacity of ATRA described herein, arginase 1 induction by high levels of ATRA may be attributable to the possible presence of low levels of the cytokine IL-4 in the media in which the cells were plated and treated. The media used was 10% fetal bovine serum, an undefined gross preparation of serum. Inquiries to Gibco, the supplier of FBS, revealed that while the FBS is heat-inactivated and certified to be free of endotoxin, the concentration of IL-4 is not assessed. In normal human blood, IL-4 is present at a concentrations of approximately 100 pg/ml in healthy volunteers and as high as 1400 pg/ml in multiple sclerosis patients (Hohnoki, Inoue et al. 1998). Assuming similar levels of IL-4 in bovine serum allows for as much as 10 pg/ml IL-4 in the culture media. While this is not enough to induce arginase 1 expression alone (1 ng/ml was incapable alone (Figure 3.9)), it may have provided a signal that could be induced to detectable levels by high concentrations of ATRA in the same manner observed in later experiments.

It would be possible to test this theory by observation of the effects of ATRA on arginase 1 expression in BMDM cultured in defined media that is completely devoid of IL-4, or at a minimum transferred to serum-free media 12 or 24 hours prior to treatment.

4.2 Arginase II is not Induced by IL-4, ATRA, or LPS in BMDM

There are two known isoforms of arginase in both mice and humans (Satoh, Yoshida et al. 1967; Porembska 1973; Reddi, Knox et al. 1975). In both species, arginase 1 and arginase 2 are not only encoded by different genes on different chromosomes, but have different patterns of expression. Both act as a trimer of identical subunits and require manganese ions as a cofactor in order to hydrolyze L-arginine to yield urea and ornithine. Arginase 1

is the primary form implicated in the urea cycle, which occurs in the liver and is essential for conversion of amino groups from amino acids into urea, a form that can be eliminated from the body in urine. The only other circumstances under which arginase 1 is significantly induced is M2 activation of macrophages, although some expression has been observed in neutrophils during wound healing (Debats, Wolfs et al. 2009). Arginase 2 is located in the mitochondria of the cell and expressed extrahepatically, particularly in the kidney (Debats, Wolfs et al. 2009), but has also been observed in endothelial cells, keratinocytes, fibroblasts, macrophages and neutrophils (Debats, Wolfs et al. 2009).

No other enzyme is known to be capable of hydrolysis of L-arginine to yield urea, which is the measured entity in the arginase activity assay. Inducible nitric oxide synthase utilizes L-arginine as a substrate to produce nitric oxide, but it cannot produce urea. In order to measure expression of both arginase 1 and arginase 2, were induced in murine BMDM in response to the ligands used in this study, Wt BMDM were treated with DMSO vehicle, 10 ng/ml IL-4 alone, 100 nM ATRA, or 10 ng/ml IL-4 + 100 nM ATRA, and the same four treatments with 10 ng/ml LPS added, then the mRNA isolated and reverse transcribed into cDNA for realtime RT-PCR. Specific primers for arginase 1 as well as arginase 2 were designed using the primer design tools on the integrated DNA technology website and used in realtime RT-PCR as described in the methods section. Expression but not induction of arginase 2 was detected. Arginase 1 was induced and suppressed in the same manner as induction and suppression of arginase activity observed in other experiments. The truncated figure 3.10 is a selection of data from the larger experiment mentioned above. The full result is available in the appendix in figure A.1. The data indicate that all arginase activity measured in BMDM is due to arginase 1 and not to arginase 2.

4.3 ATRA Potentiates IL-4 Induction of Arginase 1

Arginase 1 can be induced by IL-4, IL-10, IL-13, glucocorticoids, 9-*cis* RA, and TLR ligands (with delayed kinetics) in macrophages. Despite the strong connection between 9-*cis*

RA and glucocorticoids with induction of arginase 1, strong enough in fact to warrant a separate class of M2 macrophage (M2c), as well as the broad anti-inflammatory effects of ATRA, ATRA has not been linked to M2 activation of macrophages in any capacity.

First, the BMDM were treated alone with increasing concentrations of IL-4, ranging from 100 pg/ml to 20 ng/ml in order to determine indeed that they had the capacity to express arginase 1 in response to IL-4 treatment. 100 pg/ml was selected as a starting concentration because of the previously mentioned Hohnoki publication (Hohnoki, Inoue et al. 1998) that identifies this as a minimal concentration in the plasma of healthy human volunteers. Information on basal concentration of mouse IL-4 is still unavailable, but was reported recently to be undetectable by ELISA with a 3.2 pg/ml lower limit of detection (Moheno, Pfleiderer et al. 2009), indicating a much lower basal secretion in mice than in humans. Although other publications reported basal levels in mice of at least 8-20 pg/ml (Niwa, Urtz et al. 2010)[2].

Initial concentration curves were performed using the arginase activity assay for three reasons. The first is that the arginase activity assay is the most physiologically relevant and stringent assay for M2 differentiation. Induction of mRNA or protein expression does not necessarily result in higher enzyme activity, without which the M2 phenotype is not fully acquired. Conversely, a change in enzyme activity normally occurs in response to a change in transcription or translation. Secondly, and less importantly, the arginase activity assay requires much fewer cells than western blot or realtime RT-PCR. As the number of BMDM that can be isolated from one mouse is a limiting factor, this not only makes it easier to attain enough cells to set up triplicate sibling wells to attain standard deviation data, but it allows for more conditions (different concentrations, treatments, time courses, etc.) to be screened in early experiments. Third, the arginase activity assay is less expensive in terms of pennies per sample as well as time investment (it can be completed in one day) than western

[2]Data from author correspondence.

blot and realtime RT-PCR.

In the dose curve for mouse recombinant IL-4, it was found that arginase activity was not measurably induced by less than 10 pg/ml IL-4. For this reason, early experiments utilize 10 ng/ml IL-4. This concentration was titrated lower, along with those of ATRA, and LPS in subsequent experiments because induction of arginase 1 by ATRA was so dramatic that the concentrations were reduced in an effort to achieve the best window for observation of modulation of arginase 1 expression and activity.

In order to confirm that the dramatic potentiation of arginase activity was indeed due to arginase 1, and that arginase 2 was not being induced under these new circumstances, follow-up experiments were performed in realtime RT-PCR (Figure 3.10) and in western blot (Figure 3.5). It was confirmed that arginase 1 mRNA and protein was induced by 10 ng/ml IL-4, and that arginase 2 mRNA was not induced. As induction of arginase 1 is a key marker of M2 activation in macrophages and is not detected in M1 or unpolarized macrophages, these results were interpreted as a form of M2 activation analogous to M2c, which is induced by ligands of the glucocorticoid receptor (Figure 3.3).

9-*cis* RA, which differs from ATRA only in the stereochemistry of one bond and signals using RXR, just like ATRA, has been shown to be able to induce arginase 1 in macrophages alone (Ott and Lachance 1979, Gallardo-Soler, Gomez-Nieto et al. 2008). Furthermore, peroxisome proliferator-activated receptors γ and δ (which also utilize RXR as a binding partner) are fundamentally implicated in arginase 1 modulation, and have even been shown to be required for IL-4-mediated induction of arginase 1 (Gallardo-Soler, Gomez-Nieto et al. 2008). This indicates that the modulation of arginase 1 expression is not a story just about IL-4 signaling being mediated by STAT6. This information coupled with the fact that ATRA is a broad anti-inflammatory signaling molecule about which much remains unknown made ATRA modulation of M2 differentiation an interesting possibility.

Although ATRA was incapable of inducing arginase activity alone, it was hypothesized

that ATRA may only exert an effect in the presence of a factor that could initiate M2 differentiation. Thus 100 nM ATRA was administered in the presence of 10 ng/ml IL-4 in an arginase activity assay. A dramatic potentiation of IL-4-induced arginase activity was observed (Figure 3.8). Upon titration of ATRA down to 1 nM, the potentiation phenomenon was found to be preserved and to be dose-dependent on [ATRA]. These observations were confirmed in western blot and in subsequent arginase activity assays (Figures 3.7 and 3.9).

The arginase activity assay uses isonitropropiphenone (ISPF) as a colorimetric reagent. In initial assays, the induction of arginase activity was so high that an artificial maximum measurement limit caused by depletion of the ISPF, was encountered. It was decided that since the potentiation was dose-dependent on ATRA, that IL-4 and ATRA concentrations would be reduced in further experiments in order to limit the enzyme activity to reliably measurable levels.

ATRA modulation of arginase activity is significant at 1 nM, which is very low dose in the context of the doses used to treat acute promyelotic leukemia (in the 1-2 μM range) (Conley, Egorin et al. 1997). In the presence of all concentrations of IL-4 tested, even as low as 1 ng/ml, which is too low to induce arginase activity alone, concentrations as low as 1 nM ATRA potentiate the induction of arginase activity (Figure 3.9). This is corroborated by western blot (Figure 3.14).

This data firmly implicates ATRA in positive modulation of arginase 1 expression at the mRNA and protein levels as well as in enzyme activity, but only in the presence of IL-4. The fact that IL-4 is required suggests that ATRA works in some positive way on IL-4 signaling, in some negative way on and IL-4 suppressing pathway, or in both ways. There are two pieces of data that this should be considered in light of.

The first is that PPAR γ and δ were recently shown to be required for IL-4 induction of arginase 1, and that IL-4 has been shown to induce production of PPAR ligands in macrophages (Huang, Welch et al. 1999). PPAR γ and δ are both nuclear receptors of

the same class as RAR, the receptor through which ATRA works. Both PPAR and RAR heterodimerize with RXR. 9-*cis* RA works primarily through a homodimer of two RXR, and 9-*cis* RA can induce arginase 1 as well. Recently, it was also shown that IL-4 induces dendritic cells to express ALDH1A (alcohol dehydrogenase 1A), an enzyme that helps to metabolize retinol into ATRA (Yokota, Takeuchi et al. 2009). All this points to IL-4 induction of a Th2 response generally and M2 differentiation specifically through the use of several related nuclear receptor pathways.

The second piece of information is that the NF-κB member P100 is induced by IL-4 signaling, and that this activity is required for STAT6 DNA binding induced by IL-4 (Thieu, Nguyen et al. 2007). ATRA has been implicated in suppression of NF-κB family members p50 and p65 via transactivation, wherein nuclear receptor ligand binding induces the DNA-binding domain of RXR and other nuclear receptors to interact with an unknown region of NF-κB subunits, thus precluding them from translocating and binding to target promoters. The potential for ATRA to modulate NF-κB subunit activity is a potential route of ingress into an aspect of IL-4 signaling that is important for arginase 1 induction.

It is also possible that ATRA is augmenting IL-4-induction of arginase 1 expression by blocking some constitutive source of suppression. Ligand-activated RXR can transrepress p50 and p65 through direct binding (Na, Kang et al. 1999) and ATRA blocks NF-κB transcriptional activity (Austenaa, Carlsen et al. 2009). If the suppression of IL-4-induced arginase 1 by LPS is mediated by NF-κB, then constitutive activation of NF-κB, which has been observed in cells of the monocyte lineage (Frankenberger, Pforte et al. 1994), may suppress IL-4 induced arginase 1 in the absence of any additional NF-κB-inducing ligand. In this case, ATRA activation of RAR or RXR to transrepress constitutive NF-κB activity may block a source of constitutive suppression of IL-4.

4.4 IL-4 + ATRA - Induced Arginase 1 is Suppressed by LPS

The hypothesis that ATRA-treated macrophages are more resistant to LPS suppres-

sion of arginase 1 expression was also proved. The calculated percent suppression by 1, 5, and 10 ng/ml LPS was found to be smaller in IL-4 + ATRA treated macrophages than in macrophages treated with IL-4 alone.

LPS, the potent inflammatory TLR4 ligand that is shed from Gram negative bacteria, promotes M1 activation and suppresses M2 activation in the short term. There is evidence to support a role of LPS as well as other TLR ligands in activation of arginase 1 and other markers of M2 activation, but this is largely thought to be a secondary or tertiary effect because the time course is longer than what can be thought of as direct M2 differentiation. Although arginase suppresses the antimicrobial activity of iNOS by utilizing the same substrate, Induction of arginase in a delayed manner following detection of a pathogen-associated molecular pattern is not antithetical to antimicrobial mission of TLR. In fact it is quite appropriate, as arginase activity is associated with wound repair and resolution of inflammation, which should occur following the antimicrobial response. This supports the view of M2 macrophages as mopping up after clearance of microbes.

Because LPS is implicated in induction of arginase 1, initial experiments were performed to ensure that LPS did not induce arginase 1 in the time course in which the effect of ATRA was to be studied. Indeed, treatment of BMDM alone with LPS did not induce arginase activity (Figure 3.11) or arginase 1 mRNA (Figure 3.10). Interestingly, in two separate experiments, very slight induction of arginase activity was observed in Wt BMDM treated with 10 ng/ml LPS for 12 hours, but never at 8 hours (Figure A.2).

In order to determine whether the potentiation of arginase 1 expression by ATRA was subject to the same suppression as arginase 1 expressed in response to IL-4 alone, samples were cotreated with IL-4 and LPS or with IL-4, ATRA, and LPS. LPS suppressed arginase activity induced by both IL-4 alone and by IL-4 + ATRA, though IL-4 alone was suppressed more effectively than IL-4 + ATRA (Figure 3.12).

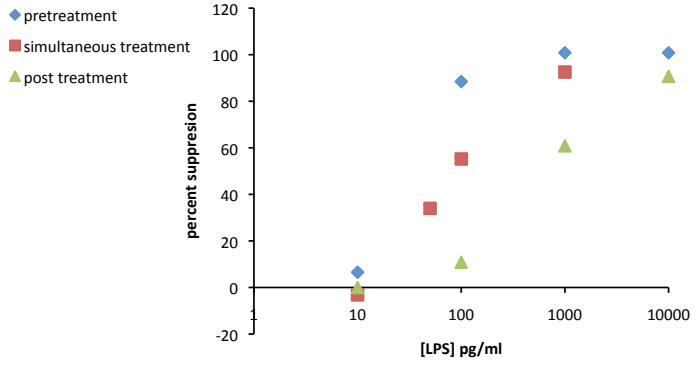
LPS suppression was found to be dose-dependent in the case of IL-4 + ATRA, but even

the low dose of 1 ng/ml LPS suppressed IL-4-induced arginase activity as efficiently as 10 ng/ml LPS. LPS suppression of arginase 1 protein was observed by western blot to mirror the pattern of suppression of arginase activity (figures 3.13 and 3.14).

Interestingly, 60 minute pretreatment with LPS strengthened its ability to suppress arginase 1 expression to the point that 100 pg/ml LPS almost completely precluded the induction of arginase 1 protein by 1 ng/ml IL-4 and 50 nM ATRA. Even on pretreatment though, 10 pg/ml LPS was insufficient to suppress arginase 1 expression (Figure 3.15). Treatment with LPS 60 minutes after administration of IL-4 and ATRA made LPS less capable of suppressing arginase 1 protein expression. Reproduced below is the suppression profile matched for LPS concentration from figures 3.14 and 3.15. It is clear that the suppression curve is shifted to the right by post treatment and to the left by pretreatment.

Figure 4.1: **Data from densitometric analysis of western blots. Calculated percent suppression of arginase 1 protein expression induced by 1 ng/ml IL-4 and 50 nM ATRA due to 1 hour pretreatment, simultaneous treatment, or 1 hour post treatment with indicated concentrations of LPS.**

	percent suppression by LPS		
[LPS] pg/ml	pretreatment	simultaneous treatment	post treatment
10	6.53	-3.18	0
50	-	33.96	-
100	88.45	55.2	10.84
1000	100.83	92.55	60.84
10000	100.81	-	90.69



LPS suppression of IL-4 + ATRA induced arginase 1 expression exhibits dose-dependence as well as a dependence on the order in which the ligands are encountered by the cells. The effect of pretreatment and post treatment was incremental and not fundamental. This lends

credence to the theory that M1 and M2 are not states of absolute polarization, rather they are poles of a continuous spectrum of activation, upon which macrophage activation may be tuned to an appropriate response, and perhaps retuned if the conditions change.

4.5 LPS Suppression of Arginase 1 is Mediated by IRAK1

Interleukin receptor associated kinase 1 (IRAK1) is a key kinase in TLR4 and several other TLR signaling pathways. Its function was once thought to be dispensable, but recent work has indicated that its role may be that of a nuanced modulator rather than a kinase that is absolutely necessary or not at all required for a given signaling pathway. For that reason, the conditions under which a role is attributed to it are critically important. An observed dependence may be easily lost if one variable is slightly altered.

Because IRAK1 has been implicated strongly in modulating induction of NF-κB in response to LPS - TLR4 signaling, and NF-κB subunits p50 and p65 have been implicated in blocking RXR binding to retinoic acid response elements on chromatin (RXR binding presumably being important for induction of arginase 1 by ATRA, 9-*cis* RA, and PPAR ligands), a potential role for IRAK1 in mediating the suppressive effect of LPS on IL-4 + ATRA induced arginase 1 was investigated. Initial experiments were performed in arginase activity assay and confirmed in western blot. Figures 3.16 and 3.17 are representative of results obtained in several experiments. The characteristic suppression curve was easily reproduced at concentrations of LPS in the range of 1 to 10 ng/ml, however when the concentration of LPS was reduced to the pg/ml range, the suppression curves became less reliable and reproducible. This may be the result of the hydrophobic nature of LPS. It is notoriously difficult to solubilize, requiring carrier protein and sonication in many cases. The concentrations of LPS used in these studies are lower by an order of magnitude or more than those used in most studies in which LPS signaling is the subject. The extremely low concentrations may have introduced a new propensity for LPS to be attracted to itself in solution, which may have resulted in an inability to dilute stocks and treat samples accurately. A possible solu-

tion to this suspected problem is to increase the concentration of the carrier protein bovine serum albumin in the solutions as the LPS is diluted. The result of this difficulty is that the LPS suppression curves do not cover the full range of suppression (from 0% to 100%) for Wt and IRAK1$^{-/-}$ BMDM. This is a key target for improvement in this study.

Despite the limitations of the experimental data, it is clear that IRAK1 plays a positive role in LPS suppression of arginase 1 expression in the presence of LPS, but does not effect induction by IL-4 and ATRA, and is thus not involved in constitutive suppression in the absence of LPS.

4.6 LPS Suppression of Arginase 1 is Mediated by Tollip

Tollip, discovered in 2000 by Dr. Kimberly Burns as a member of the IL-1R signaling pathway that was involved in recruitment of IRAK1 to the IL-1R through direct interaction with IRAK1 (Burns, Clatworthy et al. 2000). Initial function studies indicated a negative regulation role, as overexpression inhibited IL-1R-mediated activation of NF-κB (Palsson-McDermott and O'Neill 2004), however later deletion studies have shown tollip to be required for induction of IL-6 and TNFα by IL-1β and LPS, implicating tollip as a positive regulator of inflammatory signaling (Zhang and Ghosh 2002; Didierlaurent, Brissoni et al. 2006). In response to LPS, tollip protein expression is induced in the human monocyte line THP-1 and human primary blood mononuclear cells (PBMC)(Li, Hu et al. 2004). In the same study, tollip was observed to bind to phosphatidylinositol-3-phosphate (PI3P) and phosphatidylinositol-3,4,5-phosphophate (PI3,4,5P) via its C2 domain (Li, Hu et al. 2004). PI3P and PI3,4,5P are phospholipids that are synthesized in and preferentially localize to the early endosome (Gillooly, Morrow et al. 2000; Petiot, Faure et al. 2003). They can recruit proteins to the cell surface in order to act as signaling adaptors or scaffolding for complexes. They can also act as cell signaling molecules, as described in IL-4 signaling in the introduction (Gillooly, Morrow et al. 2000).

As IL-6 and TNFα are induced in a tollip-dependent and an NF-κB-dependent manner

by LPS, and NF-κB subunits have been implicated in suppression of RAR and RXR, potential involvement of tollip in LPS suppression of IL-4+ ATRA-induced arginase 1 expression was investigated.

Wt and tollip$^{-/-}$ BMDM were treated with 10 ng/ml IL-4 and 100 nM ATRA, then cotreated with 1, 5, or 10 ng/ml LPS for 8 hours. In both western blot (Figure 3.18) and in arginase activity assay (Figure 3.19), less suppression was seen for each concentration. In the western blot, the suppression curve reveals a concentration dependence, where tollip is required at 1 ng/ml but not at 10 ng/ml. The characteristic curve showing more suppression in Wt than tollip$^{-/-}$ at 1 ng/ml was not observed in arginase activity. Rather a general decrease in suppression efficiency was observed at all concentrations of LPS.

The data from the IRAK1$^{-/-}$ and tollip$^{-/-}$ studies indicates two important things. Neither the absence of IRAK1 nor the absence of tollip completely abrogated suppression by LPS. This indicates that LPS suppresses expression of arginase 1 by two or more collateral pathways. Suppression in the knockout BMDM was less efficient only at low doses of LPS. This fits the model that the pathway(s) that are dependent on IRAK1 and tollip are engaged in response to a low dose of LPS, and that the IRAK1 and tollip-independent pathway is engaged only in response to a high dose of LPS. The downstream signaling of TLR4 lends itself to this interpretation, as it engages two distinct signaling pathways that involve different sets of adaptor proteins and are engaged differentially (Barton and Kagan 2009).

TLR4 is unique among TLR in that it is capable of activating two distinct signaling pathways, dependent on two different sets of adaptor molecules. Though recent evidence has demonstrated potential for communication between members of the two pathways, NF-κB activation by the cell surface-associated MyD88-dependent pathway has been shown to be dependent on IRAK1 and on tollip while NF-κB activation by the endosome-associated TRIF-dependent pathway does not explicitly require IRAK1 and tollip.

The dependence of LPS suppression on tollip and IRAK1 is dose-dependent. At 10 ng/ml LPS, the suppression was not genotype-dependent. Wt, IRAK1$^{-/-}$, and tollip$^{-/-}$ BMDM all demonstrate the similar suppression of 94% - 100%. At 1 ng/ml LPS, suppression in Wt BMDM was 76%, but in IRAK1$^{-/-}$ and tollip$^{-/-}$ BMDM, it was 19% and 25%, respectively.

The signaling pathways downstream of TLR4 are transiently and sequentially activated, the MyD88-dependent before the TRIF-dependent (Barton and Kagan 2009). In explanation of the observed results, it is possible that a low concentration of LPS activates the MyD88-dependent pathway but not the TRIF-dependent pathway. This would result in a dependence on IRAK1 and tollip that is observed only at low doses of LPS and not at high doses. In order to test this theory, dynasore, a GTPase inhibitor that targets dynamin and blocks endocytosis, was employed to block LPS signaling via the endosome-associated TRAM/TRIF-dependent pathway.

4.7 LPS Suppression of Arginase 1 is Mediated by Endosome Signaling

The results obtained from studies employing the dynamin-specific endosome inhibitor dyansore indicate that the suppression of arginase 1 observed in Wt BMDM is at least partially dependent on a pathway that requires endosome formation. When dynasore is applied, no suppression is observed in response to any concentration of LPS except 1000 pg/ml. This indicates that in this particular experiment, at 10 pg/ml, 50 ng/ml, and 100 pg/ml LPS, the suppression was completely dependent on the ability of the cells to signal from the endosome, and that at 1000 pg/ml, endosome-dependent signaling was not of any import.

In a matched experiment, wherein IRAK1$^{-/-}$ BMDM were used, dynasore did not change the suppression curve as dramatically. In fact the suppression curves are almost identical. This result is confusing in several aspects. The first is that IRAK1 is involved in mediating the MyD88-dependent arm of TLR4 signaling, which requires TIRAP recruitment to the receptor complex. TIRAP binds PI4,5P, which is restricted to the cell membrane and

not associated with the endosome (Barton and Kagan 2009). While IRAK1 has also been observed in a complex with TRIF, TBK1, TRAF3, and IKKϵ and is thus thought to be involved in the endosome-associated TRIF-dependent pathway (Hemmi, Takeuchi et al. 2004; Takeuchi, Hemmi et al. 2004; Oganesyan, Saha et al. 2006), the role of IRAK1 and tollip in the MyD88-dependent pathway is much more established. The data from the knockout studies alone lends itself to an interpretation in which IRAK1 and tollip are involved only in one pathway (MyD88/ surface), and that the other (TRIF/ endosomal) is intact but only activated at high concentrations of LPS. The data from the dynasore experiments, however, is difficult to integrate into this model. The alleviation of LPS suppression in Wt poses no issue, as it can be interpreted as the complete blocking of the TRIF/ endosomal pathway, with only the MyD88/ surface pathway remaining intact. The data from the IRAK1$^{-/-}$ BMDM is incongruous, as both the MyD88/ surface pathway and the endosomal pathway are presumably blocked, yet suppression still occurs.

In order to account for the fact that suppression by LPS in IRAK1$^{-/-}$ BMDM was unaffected by dynasore, the model becomes slightly more complex. IRAK1 and potentially tollip are involved in both in the MyD88/ surface pathway and in the TRIF/ endosome pathway, and both are abrogated in the absence of IRAK1 and potentially tollip, but that IRAK1 at least is not absolutely required for the MyD88-dependent pathway to suppress arginase 1 expression. This is supported by data that the kinase activities of IRAK1 and IRAK4 are redundant for induction of inflammatory cytokines via NF-κB in mice (Song, Talamas et al. 2009).

It is important to remember that the dynasore suppression profile (Figures 3.20 and 3.21) cannot be compared directly the suppression profile characterized (Figures 3.16 - 3.19) because the concentrations of IL-4, ATRA, and LPS are different. While it would be ideal for every experiment to be comparable in this respect, the genotype-dependent suppression was not achievable with the lower set of concentrations, and the dynasore result was not

observed when the higher concentration set was used. It can be observed that at the low concentrations of IL-4 and ATRA used in the dynasore experiments, the genotype dependence between Wt and IRAK1$^{-/-}$ BMDM is not observed. In fact, at this concentration, the suppression curves between the two genotypes match almost exactly.

4.8 Conclusions

Although ATRA was found to be insufficient to induce expression of arginase 1 on its own, the hypothesis that ATRA would indirectly augment expression of arginase 1 was proven true. In cotreatment with 1, 5 or 10 ng/ml IL-4, ATRA significantly induced expression and activity of arginase 1 significantly above that of IL-4 alone in wildtype, IRAK1$^{-/-}$, and tollip$^{-/-}$ bone marrow-derived macrophages. This represents the first evidence that ATRA influences expression of arginase 1. Because arginase 1 is expressed only in the liver and in the M2 macrophage, this may also be taken as the first evidence that ATRA is involved in macrophage activation to the M2 phenotype.

This evidence also marks ATRA and the nuclear receptors RAR and RXR as potential therapeutic targets in endotoxemia and diseases of inflammation. Of particular interest is the potential for combining ATRA with existing therapies for atherosclerosis. As ATRA has already been tested and approved for use in oral and intravenous use, it will be interesting to see if ATRA-induced arginase 1 will help to resolve or prevent the development of atherosclerotic plaques, which are well known to be dependent to a large degree recruitment and conversion of M1 macrophages to foam cells, and subsequent accumulation of low-density lipoproteins inside the macrophage. If ATRA-dependent arginase 1 is truly indicative of the M2 phenotype, then this may indeed be the case. Other markers and behaviors of the M2 and M1 macrophage, such as expression of MHCII, MMR, FIZZ1, and YM1 (M2) and IL-6, IL-8, TNFα, and iNOS will have to be assessed in the ATRA-primed macrophage.

The hypothesis that ATRA-treated macrophages are more resistant to suppression of arginase 1 expression, particularly by low doses of LPS such as those observed in individ-

uals suffering from metabolic endotoxemia was also proven true. The calculated percent suppression by 1, 5, and 10 ng/ml LPS was found to be smaller in IL-4 + ATRA treated macrophages than in macrophages treated with IL-4 alone. Although suppression at high and low concentrations was significantly alleviated by ATRA, suppression at the lower dose was much more sensitive (Figure 3.1.2). Furthermore, in all experiments, a trend of sensitivity at low doses of LPS but not at high doses of LPS was consistently observed. The hypothesis that deletion of key signaling molecules in the LPS signaling pathway may further attenuate the suppressive effect of low-dose LPS on ATRA-induced arginase 1 was also proven true. In several experiments using different batches of cells and different sets of ligands, the suppression of arginase 1 expression in BMDM deficient in IRAK1 or in tollip, especially by the low dose of LPS, was significantly less than that observed in wildtype BMDM. This result marks members of LPS signaling pathways as a target for suppression in inflammatory conditions, particularly conditions such as metabolic endotoxemia, where low concentrations of LPS exert a profound effect on such a wide variety of inflammatory diseases.

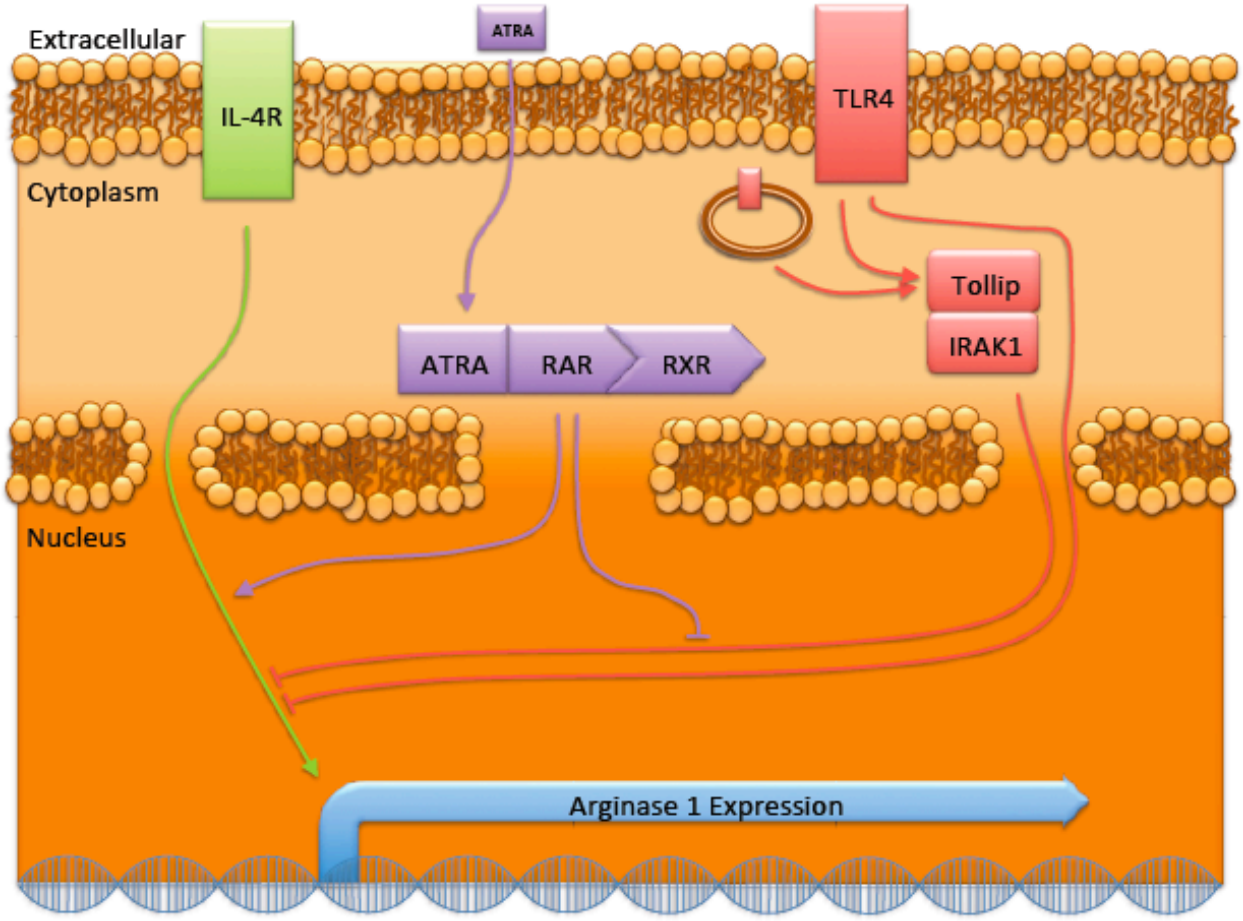
Deletion of IRAK1 did not affect induction of arginase 1 by IL-4 and ATRA. The maximum level of induction was comparable between IRAK1$^{-/-}$ and Wt BMDM. Interestingly, the induction of arginase in tollip$^{-/-}$ BMDM was greater than that observed in Wt or IRAK1$^{-/-}$ BMDM. Deletion of tollip appears to have alleviated some constitutive suppressing signal on arginase 1 expression in which IRAK1 is not involved. This introduces a new level of complexity, as up to this point, the role of tollip in LPS signaling has been considered to be largely that of a recruiter of IRAK1 to the MyD88 signaling complex.

It should be considered that tollip interacts strongly with phosphatisylinositol-3-phosphate (Li, Hu et al. 2004), which is used by IL-4 receptor as a signaling molecule to activate AKT which leads to NF-κB translocation that is required for complete induction of arginase 1 by IL-4 in HEK-293 cells (Shen and Stavnezer 1998). Deletion of tollip may result in more unbound PI3P being available in the cell for IL-4 signaling, subsequently augmenting induction

of arginase 1 expression.

Because tollip associates with the endosome (Brissoni, Agostini et al. 2006) and was found to be important in mediation of LPS suppression of arginase 1, we tested whether endosomal signaling by LPS was required for LPS suppression of arginase 1 expression. Intriguingly, suppression of dynamin-dependent endosome formation with the selective inhibitor dynasore ablated suppression of arginase 1 expression at all doses of LPS lower than 1000 pg/ml. This alleviation was even greater than that observed in the case of IRAK1 or tollip deficiency, indicating a critical role of endosome-dependent signaling in the signaling induced by low doses of LPS. The fact that almost complete suppression of arginase 1 expression was achieved at 1000 pg/ml even in the presence of dynasore indicates that higher concentrations of LPS are able to signal through an unaffected (perhaps the surface- and MyD88-associated) pathway.

Figure 4.2: **Model for signaling interactions among IL-4, ATRA, and LPS in the control of arginase 1 expression. IL-4 is the classical inducer of arginase 1 expression. ATRA potentiates the induction of arginase 1 by IL-4, but has little to no effect in the absence of IL-4. Not known if ATRA augments IL-4 signal or blocks suppression by constitutive TLR signaling. LPS suppresses induced arginase 1, not basal arginase 1, in a manner dependent on IRAK1, tollip, and on endosome formation.**



4.9 Future Studies

This study lends itself to expansion in several interesting directions. The intriguing involvement of ATRA in induction of arginase 1 is an exciting clue to a mechanism by which ATRA may exert a profound and systemic inflammation resolving influence. The effect of ATRA with and without IL-4 on other markers of the M2 macrophage, particularly MHCII and mannose receptor induction, as well as suppression of inflammatory cytokines, chemokines, and antimicrobial mediators that mark the M1 macrophage (IL-6, IL-8, TNFα, Etc.), would be particularly interesting, in that if ATRA potentiates the effect of IL-4 in several markers of M2 differentiation, it may represent a form of M2 differentiation distinct from M2a, b, or c, and not just modulation of expression of an inflammation-resolving gene. It also may assign more importance to dietary retinol and its derivatives in augmenting the inflammation-resolving behavior of the M2 macrophage following infection. Of equal interest is the mechanism by which ATRA is able to potentiate IL-4-induced arginase 1. There are several potential retinoic acid response elements (RARE) in the promoter of the arginase 1 gene. Before investigating the potential for secondary effectors, the direct impact of ATRA on the promoter of arginase 1 should be investigated. Does RAR:RXR heterodimer coprecipitate with the arginase 1 promoter in BMDM? If so, then can ATRA induce transcriptional activity in a luciferase construct assay for the arginase 1 promoter in the absence of IL-4 or serum-containing media? Also, does ATRA potentiation of IL-4-induced arginase 1 effect extend to other M2 inducers such as IL-10, IL-13, or glucocorticoids? Does ATRA suppression of iNOS benefit in a more-than-additive manner from cotreatment with IL-4?

The other aspect that deserved more work is the mechanism of suppression. The data points to LPS concentration-conditional use of both the surface and endosome-associated pathways of TLR4. The fact that both pathways induce NF-κB in addition to the several examples of NF-κB-mediated suppression of nuclear receptors immediately identifies this transcription factor as a key suspect for mediation of the suppressive effect on arginase 1.

A key experiment would be to determine the dependence of LPS suppression of arginase 1 on IKKϵ by use of a selective inhibitor. IKKϵ is responsible for activation of NF-κB activation by TRIF-dependent signaling from TLR4.

It would also be wise to test for involvement on the MAC1 receptor in suppression of arginase 1 expression. MAC1 was recently found to sense LPS and initiate signaling that results in production of superoxide (Pei, Pang et al. 2007).

Other TLR ligands such as BLP and Poly(I:C) can suppress arginase 1. A simple experiment would be to test whether or not IL-1β and TNFα, which both also induce NF-κB, can suppress arginase 1 in the same manner. If indeed they can, then inhibitor or siRNA studies wherein the various NF-κB subunits are selectively blocked or knocked down might serve to identify which, if any are responsible.

Because of the importance of ATRA in modulating arginase 1 expression and activity in macrophages, the fact that in treatment of acute promyelotic leukemia, very high concentrations on the level of 1-2 μM are used for several days or weeks, and the fact that APL targets granulocytes, not macrophages, it would be interesting to take peripheral blood from APL patients before and after ATRA therapy and test the monocytes for M1 and M2 markers using flow cytometry to see if the population is skewed to the M2 phenotype by the ATRA treatment.

In a similar direction, high-fat diet mice are prone to atherosclerosis. It would be revealing first to establish that metabolic endotoxemia is present, by testing the endotoxic units in the blood of HDF mice, but then to look at M1 markers on the macrophages and the effect of ATRA on expression of those markers. This would allow the researcher to quickly determine not only if the benefits of ATRA in inflammation extend to atherosclerosis, but also to determine if a connection could be drawn between any observed benefit and macrophage activation state.

4.9.1 Potential for therapeutic applications.

In addition to urea, arginase 1 produces polyamines, a class of molecules that is strongly associated with promotion of cell proliferation and with wound healing. Arginase 1 also suppresses iNOS activity, which while killing microbes, does damage to surrounding host tissue and causes vasodilation, which is part of what makes septic shock so deadly. In cases of excessive inflammation or sepsis, dramatic activation of arginase 1 in macrophages, particularly in the short-term, may be an effective strategy for suppressing production of nitric oxide.

If the impact of ATRA on arginase 1 expression translates to verifiable M2 activation, then the possibilities for the use of ATRA in combination with other anti-inflammatory treatments may increase efficacy of those treatments. One example is in treatment of atherosclerosis, wherein inflammation in the artery wall causes macrophages to take up residence and take in triglycerides, and over the course of years becoming foam cells (van Eijk, Aust et al. 2010). In this case, several risk factors inflammation and high fat diet contribute to the disease state, so reduction of triglycerides in the blood coupled with therapies that push macrophages from the M1 end to the M2 end of the spectrum may prove to be beneficial.

It is also worth noting that since ATRA is used to treat acute promyelotic leukemia, many different doses and treatment courses have been attempted, from which weve learned a great deal. Although at 1.7 μM and above, ATRA is toxic (Conley, Egorin et al. 1997), the effects are not deadly, and can be ameliorated simply by abstaining from treatment for as little as 8 hours (Fenaux, Chomienne et al. 2001; Ruiz-Arguelles, Morales-Toquero et al. 2005). Not only is it safe, but ATRA is a natural compound that is easily obtained from a diet rich in vegetables or in a purified cream, injection, or capsule (Molin and Ruzicka 2009; Wang, Liu et al. 2009).

Appendices

Figure A.1: **Arginase 1 is significantly induced by IL-4 and ATRA and suppressed by LPS, but arginase 2 is not induced by IL-4, ATRA, or combined treatment. Realtime RT-PCR. Wt BMDM were treated for 4 hours before harvest in Trizol reagent. Expression of arginase 1 and arginase 2 relative to GAPDH. 10 ng/ml IL-4 and 10 ng/ml IL-4 + 100 nM ATRA both induced arginase 1 but not arginase 2. Arginase 1 was suppressed by LPS. This data is from a single representative experiment without replicates or statistical calculations.**

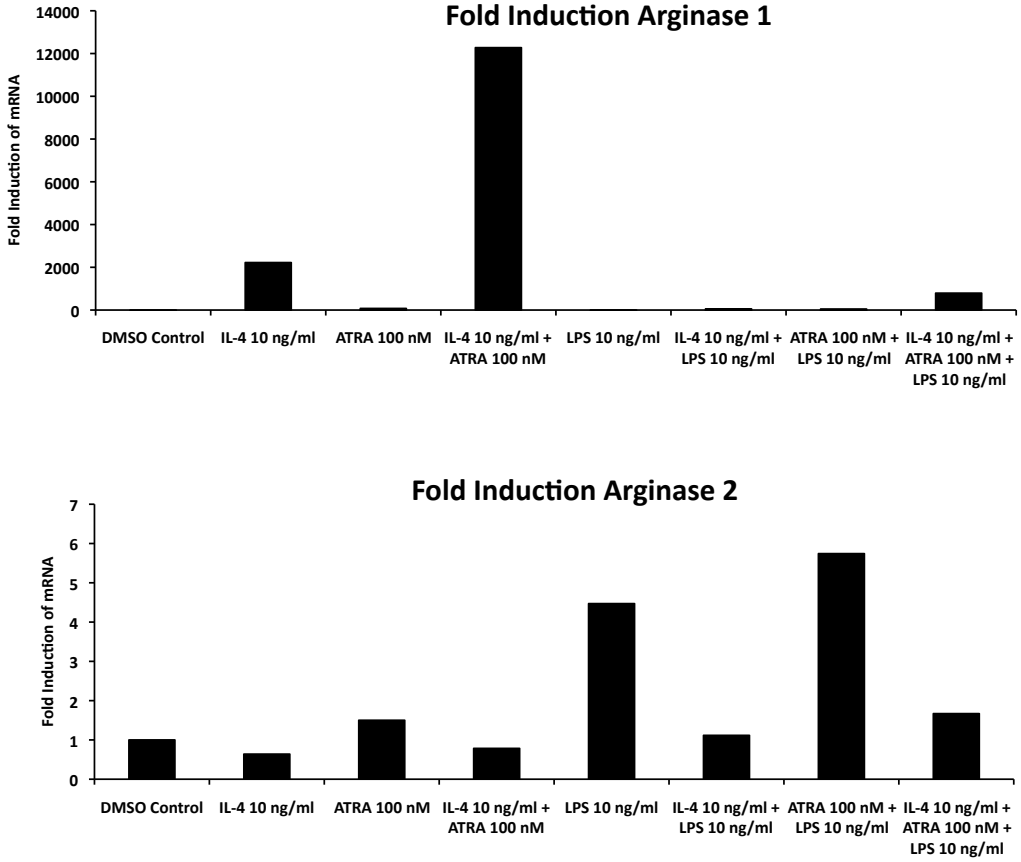
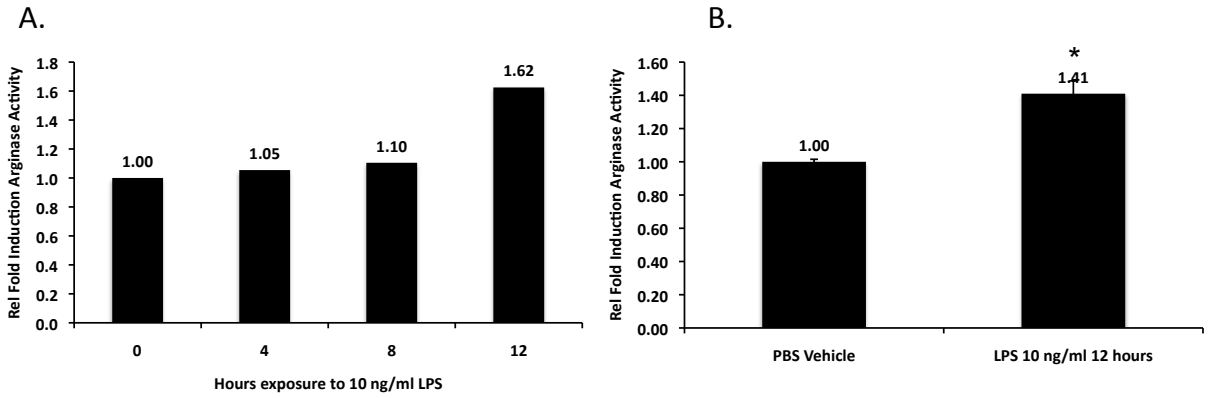


Figure A.2: **LPS induces arginase activity on a longer time course. Arginase Activity Assay. Two separate experiments performed with the same Wt BMDM. A. Time course for arginase activity in response to 10 ng/ml LPS indicated slight induction between 8 and 12 hours. B. Vehicle control and 12 hour time point only in triplicate. Error bars represent standard deviation. In B, the induction is significant at p=0.0018< 0.05.**



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