Macrophage Plasticity and Manipulation of Macrophage Activation Pathways by Intracellular Parasite *Toxoplasma gondii*

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1 Abstract

Macrophages (MΦ's) play an essential role in immunity through their sensing and activation by different environmental signals. When stimulated with Interferon gamma (IFNγ) and Toll Like Receptor (TLR) ligands, MΦ's are activated to a Classical, or M1, phenotype, characterized by anti-microbial activity and production of pro-inflammatory cytokines. Stimulation with chitin, Interleukin-4 (IL-4), or IL-13 polarizes MΦ's to an alternative, wound healing phenotype, important for restoration of the extracellular matrix upon tissue damage. Here I showed that alternatively activated (AA) MΦ's can be polarized upon stimulation with IFNγ and the TLR ligand, lipopolysaccharide (LPS) to a classically activated (CA) phenotype. This conversion from an AA to CA phenotype is further shown to progress through a hybrid CA-AA period. However, CA-MΦ's are less plastic, showing no conversion to an AA phenotype upon stimulation with IL-4. I further examined the effects of *Toxoplasma gondii* infection on MΦ activation pathways. Type I ROP16 was found to be essential for the induction of an AA phenotype but not necessary for the expression of Th2 promoting chemokines. Furthermore, *T. gondii* pre-infected cells retain the ability to be polarized to an AA phenotype upon IL-4 treatment. Together, these results suggest that in contrast to the canonical classification of *T. gondii* as a Th1 pathogen, *T. gondii* infection of MΦ's puts it in a versatile position to elicit and control both a host Th1 and Th2 response.

2 Introduction

Macrophages (MΦ) comprise a heterogeneous population of cells that play a pivotal role in the control of the innate response to pathogens. While the role of MΦ's in the immune system has long been known and studied, recent advances in our understanding of MΦ biology have shown their importance extends to such processes as tissue homeostasis, wound repair, and development. Therefore, while studying MΦ biology has far-reaching effects on our understanding of immunity, so too must our focus evolve to account for the growing diversity of MΦ functions.

2.1 Macrophage Biology

A complete picture of monocyte development and heterogeneity remains incomplete, but the mononuclear phagocyte system (MPS), proposed and later refined by Van Furth et al., 1972, remains widely accepted [1]. Macrophage development begins in the bone marrow where hematopoietic stem cells develop into granulocyte/macrophage colony-forming units (GM-CFU), a common myeloid progenitor cell, in a macrophage colony-stimulating factor 1 (CSF1) dependent fashion [2, 3]. These neutrophil-MΦ cell precursors further differentiate into monoblasts and then pro-monocytes which then exit the bone marrow and enter the blood stream before differentiating into monocytes [4].

Monocytes are further divided based on chemokine receptor expression patterns and the presence of specific cell surface markers. Inflammatory monocytes are characterized by expression of high levels of the surface molecule LY6C ($Gr1^+$), high levels of the CC-chemokine receptor 2 (CCR2) and low levels of CX3C-chemokine receptor 1 (CX3CR1). These monocytes, as their name suggests, are rapidly re-

cruited to sites of inflammation in a CC-chemokine ligand 2 (CCL2) dependent manner [2, 5]. Following infection, these monocytes can readily differentiate into inflammatory dendritic cells (DC), TNFa/iNOS expressing dendritic cells (Tip-DCs), or classically activated macrophages (CA-MΦ's) [5, 6].

However, in the absence of inflammation, CCR2^{+} CX3CR1^{low} LY6C^{high} monocytes have been shown to pass through a LY6C^{mid} phenotype before returning to the bone marrow and differentiating into resident or tissue monocytes. These monocytes, characterized by low levels of CCR2 and high levels of CX3CR1, can travel along the lumen of small blood vessels in a process called patrolling and enter non-inflamed tissues. As these monocytes readily reside in tissue, upon infection, these monocytes will rapidly respond by producing different chemokines and cytokines, which in turn, help in the recruitment of inflammatory monocytes [5, 7].

2.2 The Role of Macrophages in Innate Immunity

As professional phagocytes, MΦ's express a number of different pattern-recognition receptors (PRR) used to recognize different signs of infection. These PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), Retinoid acid-inducible gene I (RIG-1) like receptors (RLRs), C-type Lectin receptors (CLRs), and a number of different scavenger receptors. These different receptor families have evolved to detect specific pathogen-associated molecular patterns (PAMPs)—conserved microbial structures—and endogenous danger signals, such as signs of apoptosis. The interaction between PRRs and their respective ligands provides key information to the MΦ's, indicating the location and type of infection, and also determines

the appropriate response needed to clear the pathogen [8, 9, 10].

The TLRs in particular, have received considerable study because of the major role these receptors play in recognition of both extracellular and intracellular signals. Of the 13 TLRs identified in mice, TLR4, which binds to Lipoplysaccharide (LPS), is of particular relevance to my work because signaling through TLR4 is necessary for classical activation of MΦ's. TLR4 signaling has been divided into two signal transduction pathways: a MyD88 dependent; and a MyD88 independent pathway [11].

The MyD88-dependent response to LPS begins with stimulation of IL-1 receptor-associated kinase-4 (IRAK-4). IRAK-4 activation leads to activation of TRAF6 (TNFa receptor-associated factor 6), which in turn, complexes with UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1 isoform A) to activate TAK1 (transforming growth factor β activated kinase 1). TAK1 activation leads to downstream activation of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) which, along with MAPK (mitogen activated protein kinase) induced AP-1, leads to expression of pro-inflammatory cytokines **(Fig. 1)** [11].

The MyD88-independent pathway also signals through the NF-κB pathway but acts through TRIF (Toll-interleukin-1 receptor containing adaptor protein) which signals through TRAF3 (TNF receptor-associated factor 3) and the transcription factor IRF3 (Interferon Regulatory Factor 3). TRIF also signals through RIP1 to activate MAPK and NF-κB pathways. Signaling through this MyD88-independent pathway leads to production of Type I interferons **(Fig. 1)** [11].

In considering the biological events that lead to inflammation, the first cells to respond at a site of injury or infection are resident macrophages and mast cells

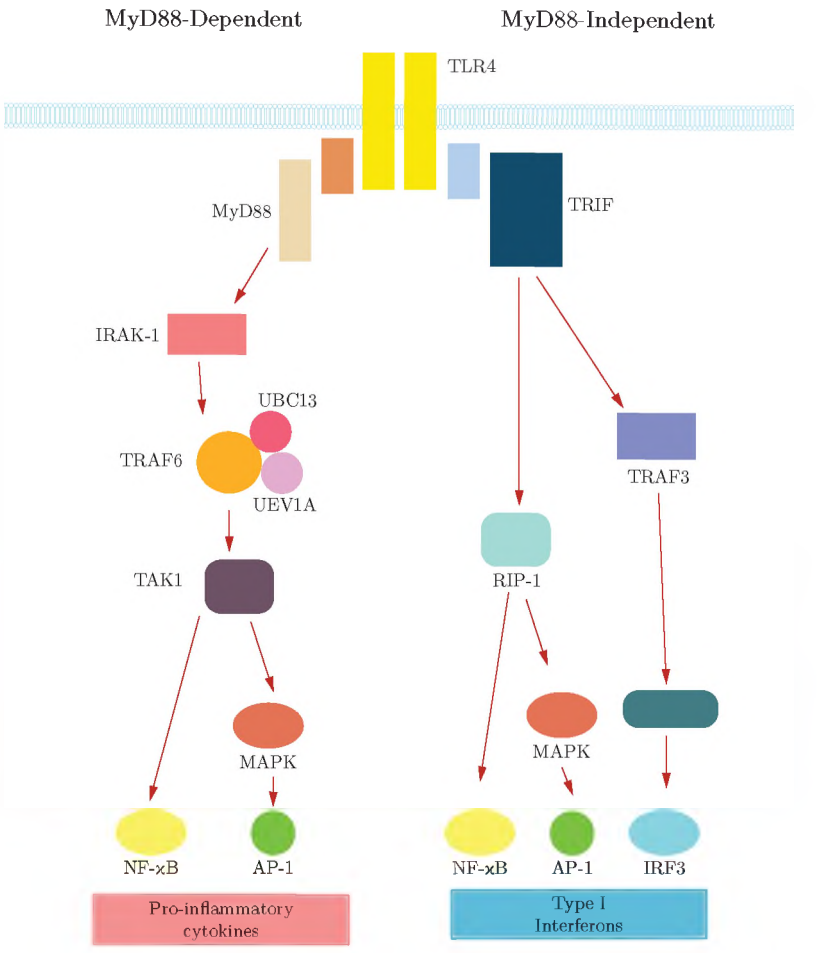


Figure 1: MyD88-dependent and MyD88-independent TLR4 signal transduction.

which, triggered by different PRR-PAMP interactions, begin the process of leukocyte recruitment through the release of chemokines, cytokines, and other products. Modification of local epithelium allows the influx of neutrophils, which extravasate into the site of infection, phagocytose invading pathogens, and release the damaging contents of their granules into the microenvironment. Additional leukocytes continue to be recruited, including monocyte-derived Tip-DCs and MΦ's, both of which act as phagocytes and as potent microbicidal agents [10].

Eventually however, inflammation must be resolved. Not only is the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) damaging to surrounding tissues, but active removal of dead neutrophils and cellular debris is necessary for the tissue to return to normal as failure to clear apoptotic cells can lead to continued aggravation of inflammation. It is here that the importance of MΦ's as janitorial cells shine. The expression of different scavenger receptors, phosphatidyl serine receptors, and complement receptors—to name a few—allows MΦ's to recognize such endogenous danger signals as heat shock proteins, extracellular ATP, histones, DNA, and other byproducts of apoptosis. Understanding of these apoptosis-cell-associated molecular patterns (ACAMPs) carries however, a whole host of other complexities; namely, that PRR engagement by ACAMPs must carry some distinguishing factor different from engagement with PAMPs. CD14 for example, which mediates transfer of LPS to TLR4 and the induction of a pro-inflammatory response has also been shown to be important for recognition of ACAMPs containing LPS-like structures [11]. However, signaling of ACAMPs by CD14, as with other ACAMPs, leads to an anti-inflammatory response. Ingestion of these molecules changes the macrophage phenotype. Several hypotheses to explain this phenomenon have been proposed including (i.) formation of CD14 with receptors other than TLR4 and (ii.) that anti-inflammatory pathways override pro-inflammatory signaling [12].

2.3 The Role of Macrophages in Adaptive Immunity

In addition to their roles in Innate Immunity, MΦ's also play an important role in activation of the Adaptive Immune system. While MΦ's readily respond to

early stimuli at the site of infection, they are also subject to signals from antigen-primed immune cells. In particular, MΦ's have been classified into different activation states in a manner, similar to the nomenclature in T-cell literature: M1 MΦ's, the classically activated innate activated MΦ's, are characterized by high levels of pro-inflammatory cytokines and microbicidal activity; M2 macrophages on the other hand, represent an umbrella of different activation states, including the alternatively activated and regulatory/deactivating MΦ's **(Table 1)**.

2.3.1 Classical Activation of Macrophages

Classically activated macrophages (CA-MΦ) are the best-documented of the different MΦ activations. Priming of MΦ's with Interferon-γ (IFNγ) followed by stimulation with Tumor Necrosis Factor (TNFa) or TLR ligands results in MΦ's vital to host defense due to their secretion of pro-inflammatory cytokines and microbial killing [2]. While an important source of early IFNγ comes from Natural Killer (NK) cells, production of IFNγ by these innate cells is not sustained. Therefore, in order for continued classical activation of MΦ's, an antigen specific Th1 response must be induced. Subsequent TLR activation can result from direct contact of primed MΦ's with TLR ligands—such as LPS—or from TNFa, produced by antigen presenting-cells (APCs) in a TLR-MyD88 dependent signaling cascade.

These CA-MΦ's show enhanced microbicidal activity due to their increased production of superoxide anions ($O2^-$) and induction of iNOS (inducible nitric oxide synthase), an enzyme that catalyzes L-arginine-dependent production of microbicidal nitric oxide molecules (NO) [2]. Xia and Zweier, 1997, showed that in L-arginine depleted MΦ's, iNOS mediates production of superoxide anions which in turn, react with NO to form potent peroxynitrite species ($ONOO^-$); these potent

oxidizing species can degrade microbial cell membranes [13]. NO cytotoxicity can also occur through deamination of DNA bases, leading to widespread mutations, as well as inhibit the activity of enzymes containing iron-sulfur centers [14]. Together, these cytotoxic effects of NO render it an essential molecule for host protection against parasitic and microbial infections [14].

CA-MΦ's also upregulate production of the pro-inflammatory cytokines Interleukin-1β (IL-1), IL-6, IL-12, and IL-23. IL-12 acts in a positive feedback loop, promoting the differentiation of Th1 cells which, under the influence of IL-12, secrete increased levels of IFNγ and TNFa. CA-MΦ's have also been shown to promote differentiation of Th17 cells through secreted IL-1, IL-6, and IL-23. These Th17 cells secrete high levels of IL-17 which in turn, exacerbate the inflammatory response through recruitment of granulocyte populations [1]. CA-MΦ's have also been shown to express increased levels of Major Histocompatability Complex (MHC) Class II and B7 co-stimulatory molecules (CD86) which enhances their ability to drive a primary T-cell response [15].

Expression of markers of CA is controlled at several levels by different transcription factors, phosphorylation cascades, and transcription regulators. IFNγ signaling through the Janus-kinase (JAK) Mitogen activated protein-kinase (MAPK) signaling cascade results in phosphorylation and dimerization of the transcription factor STAT1 (signal transducer and activator of transcription 1). Phosphorylated STAT1 is then imported into the nucleus where it binds as a homodimer to gamma-activated sequences, including the promoters of Nos2 and IL-12, and induces expression. Interferon Regulatory Factor 5 (IRF5) has recently been shown to play a role in CA; IRF5 is required for maximum pro-inflammatory cytokine production and is now thought to participate with other transcription factors, such

as RelA, to induce classical gene expression while inhibiting transcription of AA markers and IL-10 [16].

The response of CA-MΦ's to pathogens has been well documented. In mice lacking IFNγ expression, an increased vulnerability to viral, bacterial, and protozoan infection is seen [2,4]. For many intracellular pathogens, impairment of these CA pathways is essential for its continued survival within its host. For example, studies of *Mycobacterium* tuberculosis-induced deactivation have implicated lipoarabinomannan (LAM), a glycolipid, in the interference of IFNγ signaling and attenuation of MΦ responses to TNFa and LPS. Similarly, work with *Leishmania spp.* has shown that attempts to activate MΦ's after infection are thwarted because infection disrupts or redirects IFNγ signaling in MΦ's [2].

Clearance of *Leishmania spp.* is dependent on induction of a robust CA-MΦ population. Whereas IFNγ treatment alone results in inefficient clearing of parasite, stimulation of MΦ's with IFNγ and TNFa before infection results in complete clearance of the parasite; similar parasite killing is observed upon IFNγ and LPS stimulation [2].

2.3.2 Alternatively Activated Macrophages

Stimulation with IL-4 or IL-13 on the other hand, polarizes MΦ's to an alternatively activated (AA) phenotype [2, 15]. IL-4 production can arise from the adaptive immune response via Th2 cells or from granulocytes of the innate immune response. AA can also occur when MΦ's come in response to contact with chitin and helminth infection [2, 3].

AA-MΦ's, or wound-healing MΦ's as they are commonly called, show markedly different expression patterns and phenotype compared to the CA-MΦ's. AA-

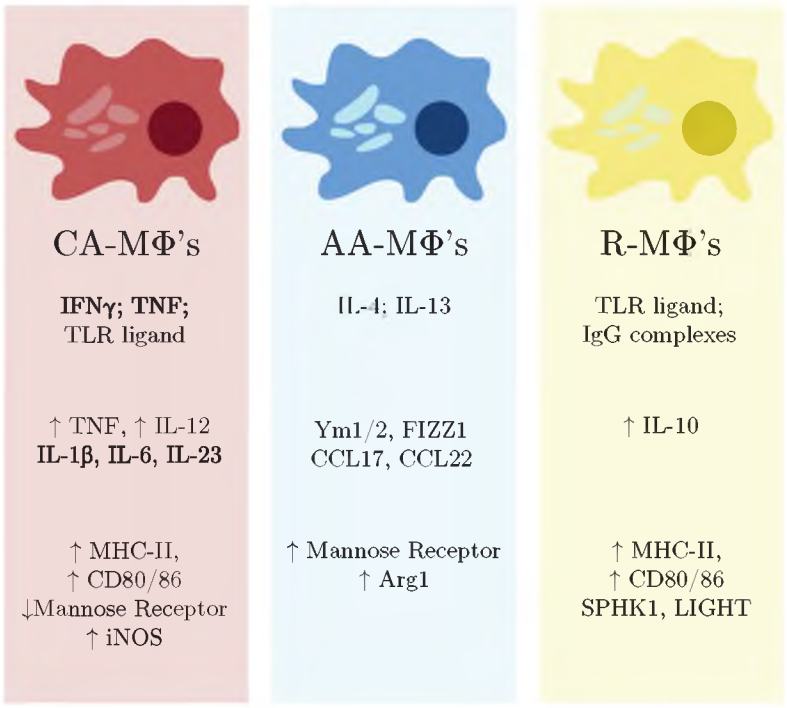
MΦ's are characterized by increased expression of Arginase 1 (Arg1), mannose receptor (CD206), CCL17 (Chemokine (C-C motif) Ligand 17), Ym1 and Ym2—two chitinase-like molecules—and FIZZ1/RELMα—found in inflammatory zone 1/resistin-like molecule alpha [2, 15, 17].

AA-MΦ's are controlled at the transcription level by several different signaling axes. IL-4 signals through the IL-4 receptor-α (IL-4Rα), which in turn signals through a JAK-STAT6, pathway and activates other signaling molecules such as phosphoinositide 3-kinase (PI3K). Whereas CA markers are until the control of STAT1, AA markers are under the control of STAT6. Expression of AA markers can also occur through the C/EBP family of transcription factors. However, whereas STAT6 mediates expression of genes such as Arg1 in response to IL-4, the cAMP-responsible element-binding-protein (CREB)-C/EBPβ axis regulates the induction of Arg1 in response to TLR stimulation. Additionally, the CREB-C/EBPβ may also play a role in the downregulation of CA activation by inducing the expression of dual specificity protein phosphatase 1 (DUSP1), which inhibits expression of pro-inflammatory genes [3].

AA-MΦ's have been shown to be necessary for protection and clearance of helminth infection. *Schistosoma mansoni* infection and subsequent egg deposition is dominated by a Th2 response while Th1 responses are downregulated. Because a robust Th2 response increases the granuloma response to parasite eggs, AA-MΦ's can be thought of as detrimental. However, infected IL4$^{-/-}$ mice fail to stimulate a Th2 response and eventually die, indicating the necessity of a Th2 response in host protection. Satoh et al., 2010, showed that the Jumonji domain containing-3 (JMJD3) histone 3 Lys27 (H3K27) demethylase is required for AA in response to helminth infection and chitin [18]. JMJD3$^{-/-}$ mice are unable to

upregulate a strong Th2 response upon infection with *Nippostrongylus brasiliensisi*, a potent Th2 inducer [18]. One such target of JMJD3 activation is the Interferon Regulatory 4 (IRF4) transcription factor promoter region; $Irf4^{-/-}$ mice are unable to upregulate AA markers after chitin stimulation. It is thought that this JMJD3 axis acts independently of IL-4 signaling and responds instead to stimulation by chitin. However, the details of chitin signaling and the PRRs associated with helminth recognition are still unknown [3, 18, 19].

While AA-MΦ's are essential for immunity against helminths, polarization of AA-MΦ's can also increase susceptibility to intracellular pathogens [2]. This may be due in part to notably different metabolism patterns of L-arginine in AA-MΦ's. Whereas in CA-MΦ's, L-arginine is rapidly catabolized by iNOS, AA-MΦ's express high levels of Arg1 which converts arginine to ornithine [2-4]. Ornithine can be acted on by ornithine amino-transferase (OAT), which leads to increased production of proline, a necessary building block of collagen. Ornithine can also be decarboxylated into putrescine by orinithine decarboxylase (ODC); putrescine, through the activity of spermine and spermidine synthase, is converted to spermine and spermidine respectively. Recently it has been shown that polyamines such as putrescine, spermine, and spermidine, may play a role in induction of certain AA-markers, including Ym1 and FIZZ1; other AA markers, like Arg1, are readily induced independently of polyamines. Additionally, polyamine-depleted MΦ populations have higher levels of pro-inflammatory gene expression but not enhanced secretion levels indicating they may play a role in maintaining an AA phenotype [4]. In the context of *L. major* infection, this sustained Arg1 activity contributes to pathology; inhibition of Arg1 is thought to decrease parasite viability by limiting the availability of polyamines [20].



	CA-MΦ's	AA-MΦ's	R-MΦ's
Activation Signal:	**IFNγ; TNF;** TLR ligand	IL-4; IL-13	TLR ligand; IgG complexes
Secretory Products:	↑ TNF, ↑ IL-12 **IL-1β, IL-6, IL-23**	Ym1/2, FIZZ1 CCL17, CCL22	↑ IL-10
Biomarkers:	↑ MHC-II, ↑ CD80/86 ↓Mannose Receptor ↑ iNOS	↑ Mannose Receptor ↑ Arg1	↑ MHC-II, ↑ CD80/86 SPHK1, LIGHT

Table 1: Characteristics and biomarkers of activated MΦ populations.

IFNγ: Interferon-gamma; TNF: Tumor Necrosis Factor; TLR: Toll-like Receptor; IL: Interleukin; MHC-II: Major Histocompatibility Complex II; iNOS: inducible Nitric Oxide Synthase; FIZZ1: found in inflammatory zone 1; CCL: CC-chemokine ligand; Arg1: Arginase-1 SPHK1: sphingosine kinase 1; LIGHT: homologous to **l**ymphotoxins, shows **i**nducible expression and competes with herpes simplex virus **g**lycoprotein D for **h**erpes virus entry mediator (HVEM) on **T**-cells

2.3.3 Regulatory Macrophages

Regulatory Macrophages (R-MΦ) are the least well characterized of the established MΦ activations. First discovered by Gerber and Mosser, 2001, these MΦ can be activated by a number of different signals. Generally, the first signal is an anti-inflammatory signal. This can come in the form of prostaglandins, adenosine, immune complexes, IL-10, or apoptotic cells; glucocorticoids for example, are secreted by adrenal cells under stress conditions and can act as a primary stimulant.

The second required signal is a TLR ligand; the combination of these two signals generates a MΦ population characterized by high IL-10 production and low IL-12 secretion [2, 15].

Before the discovery and subsequent characterization undertaken by Mosser's group, R-MΦ's have often been confused with AA-MΦ's. This is a mistake as R-MΦ's have both a unique phenotype and role in immunity. R-MΦs do not contribute to the extracellular matrix, express little to no Arg1, Ym1, and FIZZ1, and signaling in R-MΦ's does not depend on STAT6; AA-MΦ's can secrete IL-10 when stimulated by IL-4 and LPS, but only a modest increase is observed. Interestingly, these R-MΦ's also produce high levels of co-stimulatory molecules CD80 and CD86 and co-culturing experiments have been shown R- MΦ's to be the most effective, when compared with CA and AA-MΦ populations, at activating nave T-cell populations. Therefore, despite their anti-inflammatory phenotype, R-MΦ's continue to be potent APCs; furthermore, due to their production of IL-10, R-MΦ's can induce the expansion of Th2 cells, which in turn, secrete IL-4 and IL-13, amplifying the AA of MΦ's [2, 15].

There is a continued need to identify the signaling pathways that lead to R-MΦ's. While the MAPK Extracellular signal-regulated kinase (ERK) axis has been implicated, not all ERK stimulation results in IL-10 production from MΦ's. Additional characterization of R-MΦ's is also needed in order to identify distinct biomarkers. SPHK1 (sphingosine kinase-1) and LIGHT (Tumor necrosis factor ligand superfamily member 14) were recently identified by Edwards et al., 2006. How these markers complement the current anti-inflammatory profile of R-MΦ's is not completely understood but LIGHT may contribute to the effectiveness of R-MΦ mediated T-cell activation and SPHK1, to the control of pro-inflammatory

cytokine production [2, 15].

Due to their anti-inflammatory phenotype, R-MΦ's may be exploited by pathogens. *Leishmania spp.* amastigotes are coated with host IgG, which engages MΦ Fc receptors for IgG (FcγR) upon entry. Similarly, African trypanasomes, through the constant shifting of their surface antigens, induce a strong antibody response and generation of immune complexes. These immune complexes too can bind to MΦ Fcγ R and produce anti-inflammatory IL-10 production [2, 15]. Therefore, there is a growing need to better understand and characterize these therapeutically relevant MΦ population.

2.3.4 Plasticity of Macrophage Activation

Unlike cells of the adaptive immune system, which undergo extensive epigenetic modifications during their differentiation, the MΦ landscape remains plastic, allowing them to respond to different environmental cues [2]. One example of this shift in phenotype is observed during long-term exposure to LPS. Endotoxin tolerance is the progressive attenuation of pro-inflammatory responses after repeated or sustained LPS stimulation. What is interesting about endotoxin tolerance is that different genes are affected in different ways. Whereas, pro-inflammatory gene expression is reduced, anti-inflammatory and anti-microbial genes continue to be readily inducible [3].

If we consider a typical inflammatory response, CA-MΦ's dominate the inflamed tissues and play an important role in the initial response and subsequent clearance of a pathogen at the site of infection. Eventually, this CA-MΦ population is replaced by AA-MΦ's so that tissue damage and wound-healing can be initiated. What is unclear however, is whether this anti-inflammatory population is the re-

sult of a shift in gene expression—namely, that these MΦ's arise from the original classically activated population—or if this population results from the subsequent migration and replacement by anti-inflammatory MΦ's from the blood. A caveat of this latter model is that migration or apoptosis of the original MΦ population must occur in order for an anti-inflammatory landscape to dominate the tissue. These two models are not mutually exclusive as monocyte populations in tissues are constantly replenished, but the growing literature on this topic suggests that MΦ's can undergo a transcriptional reprogramming at the onset of inflammation [2].

Work done by Stout et al., 2005, has often been cited as evidence of this genetic reprogramming. Stout et al. sequentially treated bone marrow derived macrophages (BMMΦ) with different cytokines and LPS in order to identify a shift in cytokine profile. Stout et al. conclude that the functional phenotype of MΦ's—that is, their pro-inflammatory or anti-inflammatory phenotype—can shift as the microenvironment is changed; pro-inflammatory MΦ's produce lower levels of pro-inflammatory cytokines IL-12, TNFa, and IL-6 upon IL-10 treatment. Similarly, IL-4 treated MΦ's can shift to become potent producers of pro-inflammatory cytokines upon LPS or IFNγ/LPS treatment [21].

There are however, shortcomings to this study. First, because of the time scale investigated, only MΦ phenotypes at the initiation and endpoint of treatment are examined. What this fails to answer, is whether this shift in phenotype is a direct from a pro to anti-inflammatory phenotype or whether MΦ's return to a state of deactivation or navet (neither pro-inflammatory or anti-inflammatory) before becoming re-polarized. Because Stout et al. only measured pro-inflammatory cytokine profiles, no information is given about the anti-inflammatory profile of

these MΦ's; it is possible that sequential treatment of MΦ populations can result in a hybrid anti-pro-inflammatory population. Similarly, Stout et al. only examined the impacts of IL-10 on attenuation of pro-inflammatory signaling. This fails to address the effects of IL-4 stimulation of pro-inflammatory MΦ populations.

I examined the changes in gene expression of CA-MΦ's following IL-4 treatment for a number of timepoints; the opposite scheme—stimulation of AA with CA polarizing IFNγ/LPS—was examined as well. My Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR) data shows that rather than return to a state of deactivation, MΦ's can display a hybrid pattern of gene expression, upregulating both CA and AA markers. Lastly, my examination of CA and AA gene expression profiles indicates that there is a differential underlying plasticity associated with classical and alternatively activated MΦ's.

2.4 *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular parasite and member of the phylum Apicomplexa that infects anywhere from a third to eighty percent of the population in select geographic locations [22, 23]. *T. gondii*'s prevalence throughout the human population is a testament to its well-studied ability to manage and balance the immune system of its host; because *Toxoplasma* is such a master manipulator of immune system, it has become a model for the study of host-pathogen interactions.

2.4.1 Life Cycle and Clinical Importance

Transmission of *Toxoplasma* begins with ingestion of cysts in undercooked meat or by accidental ingestion of food or water contaminated with oocysts from cat feces. After ingestion, *Toxoplasma* rapidly replicates and differentiates into tachyzoites within the digestive tract of the host. These tachyzoites cross the gut epithelium and disseminate throughout the host [22, 23, 24]. During this acute phase of infection, *Toxoplasma* can invade all nucleated cells and this widespread replication, dissemination, and subsequent tissue damage induces a Th1 response characterized by high levels of IL-12, TNF, and IFNγ, vital for parasite control [25] It is around this time that under pressure from the host immune system, tachyzoites transform into the inactive bradyzoite form associated with cyst-formation in the host musculature and central nervous system (CNS) [22, 23, 24].

Because humans are an intermediate host, the life cycle of *T. gondii* in humans differs from that in its definitive host, the cat. Cats become infected with *Toxoplasma* upon predation and ingestion of cysts in the musculature of their prey. In the intestinal tract of feline species, *Toxoplasma* undergoes both sexual and asexual development and oocysts are shed in feces as early as 3-10 days after ingestion. These oocysts are extremely infectious and ingestion of a single oocyst can result in infection [24].

T. gondii infection is normally innocuous, manifesting only as general malaise during the acute stage of infection. However, infection becomes a serious threat in immunocompromised hosts and in Acquired Immunodeficiency Syndrome (AIDS) patients, toxoplasmosis presents as tissue destruction and encephalitis due to rupture of cysts in the musculature and CNS [23]. While current HIV treatment

has shown success in keeping *Toxoplasma* at bay, in the underdeveloped world, infection remains a vital threat [24].

Toxoplasma can also be transferred congenitally if infection occurs in seronegative pregnant women. As with immunocompromised adults, toxoplasmosis results in severe neurological diseases. Affected fetuses show signs of hydrocephalus, chorioretinits, and intracranial calcification. In addition, infected infants will often show signs of mental retardation, convulsions, and blindness [23].

Toxoplasma research also has the potential to be applied to a number of other intracellular parasites. *Toxoplasma* has become a model for studying the host-pathogen response to other members of the Apicomplexa phylum such as *Plasmodium*, and other intracellular parasites including *Trypanasomes* and *Leishmania*; together these parasites affect and kill millions of people worldwide.

2.4.2 Manipulation of Macrophage Signaling by *Toxoplasma gondii*

In-vivo studies have shown that *T. gondii* has a preference for infecting cells of the innate immune system including MΦ's and dendritic cells. While this may seem paradoxical at first, it is now understood that *Toxoplasma* does this as a way to manipulate the host immune response into a safe reservoir for dissemination and replication [26].

Toxoplasma entry into host cells is an active process distinct from host endocytic processes. Studies have shown that *T. gondii* actively invades and that this is dependent on formation of a parasite actin-myosin motor and interactions between bridge moieties of the parasite and host cell surfaces [27]. As the parasite enters, the host membrane is dragged inward to form a specialized vacuole. In marked contrast to normal receptor-mediated endocytosis, *T. gondii* invasion

forms a paristophorous vacuole (PV) that resists acidification and fusion with host lysosomes [26, 27]. This PV is important for recruitment of host organelles, including mitochondria and the endoplasmic reticulum, which is thought to aid in utilization of host lipids and metabolites [27, 28]. This positioning allows the PV to come into close contact with the nucleus and through injection of rhoptry proteins, to manipulate host signaling pathways.

Study of these parasite proteins was made possible by identification of three parasites lineages. Type I parasites are the most lethal in mice, resulting in death before encystment. Type II and III strains on the other hand, are nonlethal and establish dormant infection. Further observations that Type I and Type III—but not Type II—infection is able to induce high levels of IL-12 led to the use of quantitative trait locus mapping and the identification of the rhoptry protein, ROP16 [25].

Type I ROP16 is a potent activator of both STAT3 and STAT6, capable of directly phosphorylating tyrosine residues. STAT3 is a member of the JAK/STAT signaling axis and its activation by *Toxoplasma* downregulates inflammatory cytokine production and serves as a means to promote parasite persistence [28]. Previous work done in the Denkers lab has shown that Type I ROP16 deleted strains (ΔROP16) deleted strains are unable to sustain STAT3 phosphorylation; this sustained STAT3 activation is restored in ΔROP16 strains complemented with Type I ROP16 (ΔROP16:1) [25].

STAT6 activation as previously discussed, is important in the upregulation of Arg1 and this has been shown to be dependent on Type I ROP16. It is thought that activation of Arg1 confers several benefits to *Toxoplasma*. First, because both iNOS and Arg1 compete for L-arginine, Arg1 induction prevents excessive produc-

tion of microbicidal NO by iNOS. Second, Arg1 induction produces polyamines which are necessary for parasite growth. And third, paradoxically, activation of STAT6 may serve as a way to limit parasite replication; *Toxoplasma* is an arginine auxotroph and is dependent on host arginine for its survival. By inducing Arg1, arginine concentrations are limited in the host cell, thus preventing excess growth and host burden by *Toxoplasma* [25].

While *Toxoplasma* infection downregulates pro-inflammatory cytokine signaling upon initial infection, eventually *T. gondii* itself will induce expression as a way to promote a protective Th1 response. Host cells infected with *T. gondii* are non-responsive to TLR signaling pathways and IFNγ. IFNγ signaling as discussed previously, is dependent on signaling through the STAT1 transcription factor. While it is still unclear how *Toxoplasma* inhibits STAT1 mediated signaling, *T. gondii* is very effective at silencing this signaling cascade, inhibiting expression from over a hundred gamma-activated sequences, including expression of Nos2. TLR signaling is also inhibited by *T. gondii* infection through manipulation of the MAPK and NFκB signaling axis. It is still unclear how *Toxoplasma* blocks late-stage MAPK activation and whether nuclear translocation of NFκB is inhibited by *Toxoplasma* infection, but LPS-induced production of TNF and IL-12 is inhibited in *T. gondii* infected cells [26].

I examined the manipulation of MΦ activation pathways by *T. gondii* using RT-qPCR, looking specifically at different markers of CA and AA; while it has been shown that Type I infection results in STAT6 activation and induction of Arg1, it is not known whether *T. gondii* infection results in an AA phenotype. Using different Type II ROP16 mutants, I show that alternative activation of MΦ's by *Toxoplasma* is dependent on Type I ROP16 and that pre-infection with *Toxoplasma* does not

prevent AA. Furthermore, I show that *T. gondii* infection upregulates production of chemokines CCL17, CCL21, and CCL24. These chemokines are important in the recruitment and activation of Th2 effector cells. Altogether, my work indicates that in contrast to the classification of *T. gondii* as a canonical Th1 pathogen, *T. gondii* also induces and promotes a Th2 response upon infection.

3 Results

IL-4 treatment polarizes MΦ's to an alternative activation phenotype. Stimulation of BM-MΦ's with IL-4 for six hours resulted in upregulation of the alternative activation markers Arg1 **(Fig. 2a)**, Ym1 **(Fig. 2b)**, and FIZZ1 **(Fig. 2c)**. Co-stimulation of BM-MΦ's with IFNγ and LPS (IFNγ/LPS) on the other hand resulted in little to no induction of these AA markers.

IFNγ/LPS treatment polarizes MΦ's to a classically activated phenotype.

Stimulation of MΦs with IFNγ/LPS for six hours increased expression of the classical activation markers Nos2 **(Fig. 3a)**, and the pro-inflammatory cytokines IL-12 **(Fig. 3b)**, TNFa **(Fig. 3c)**, and IL-6 **(Fig. 3d)**. IL-4 treatment resulted in little to no induction of these CA markers relative to untreated cells.

Plasticity of CA-MΦ's

To examine if CA-MΦ's could be converted to AA-MΦ's with IL-4 treatment, MΦ's were co-stimulated with IFNγ and LPS for six hours before incubation with IL-4 for different timepoints. Medium pre-treated cells upregulated Arg1 **(Fig. 4a)**, Ym1 **(Fig. 4b)**, and FIZZ1 **(Fig. 4c)** in a time-dependent matter when stimulated with IL-4.

IL-4 treatment of CA-MΦ's also increased Arg1 expression **(Fig. 4a)**. However, because TLR signaling can also result in upregulation of Arg1, plasticity was further examined in the context of Ym1 and FIZZ1 induction [29].

Pre-treatment of MΦ's with IFNγ and LPS downregulates Ym1 as shown by the decreased Ym1 expression relative to medium treated control cells at the 0

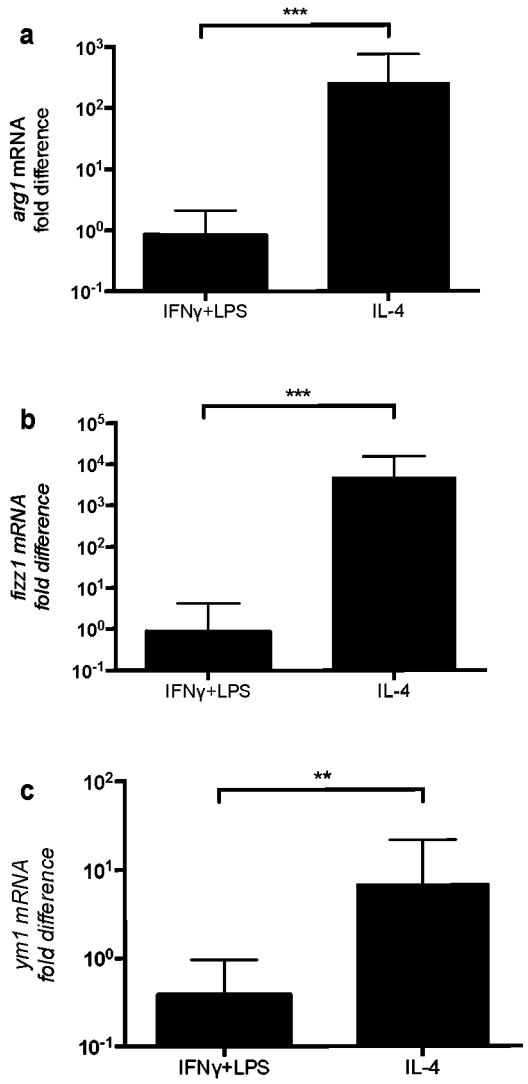


Figure 2: IL-4 treatment alternatively activates MΦs.

Quantitative PCR analysis showing expression of **(a)** Arg1, **(b)** Ym1, and **(c)** FIZZ1 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from bone marrow derived macrophages treated with IFNγ (100ng/mL) and LPS (100ng/mL) or IL-4 (10ng/mL) for 6 hours. * $P<0.05$; **$P<0.01$; ***$P<0.001$ (two-tailed Students t-test). Results are representative of four **(a)** and five **(b,c)** independent experiments; data are represented as mean $\pm$ SEM.

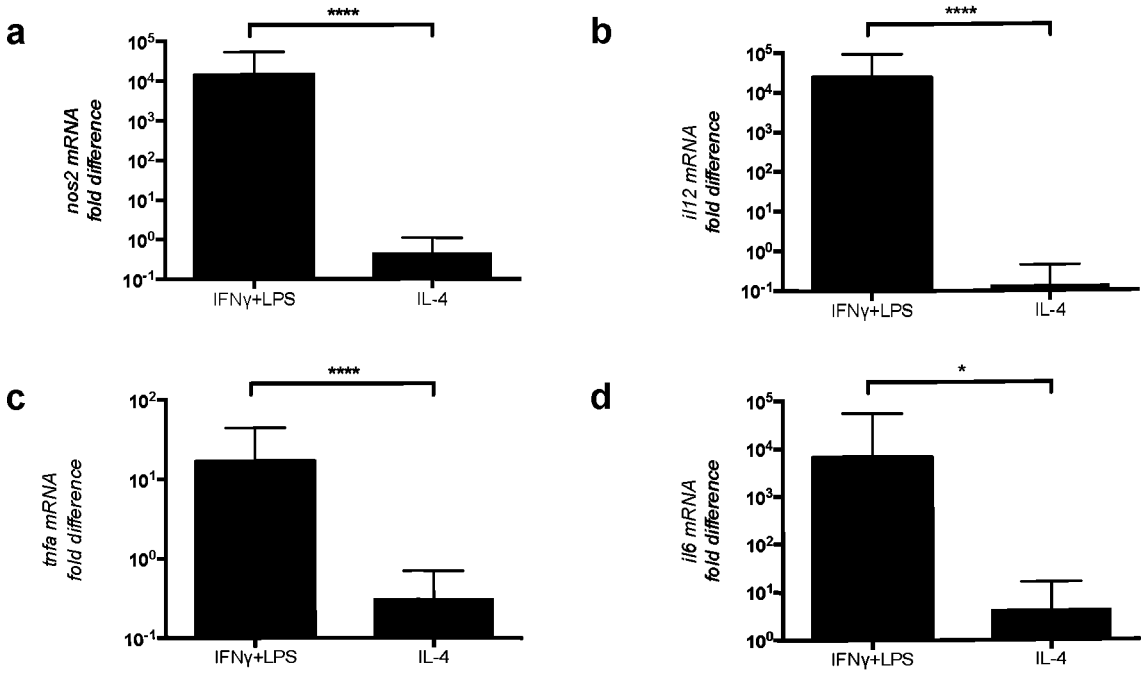


Figure 3: IFNγ/LPS treatment classically activates MΦ's.

Quantitative PCR analysis showing expression of **(a)** Nos2, **(b)** IL-12, **(c)** TNFa, and **(d)** IL-6 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BM-MΦ's treated with IFNγ (100ng/mL) and LPS (100ng/mL) or IL-4 (10ng/mL) for 6 hours. * $P<0.05$; **$P<0.01$; ***$P<0.001$ (two-tailed Students t-test). Results are representative of five **(a)**, four **(b,c)**, and three **(d)** independent experiments; data are represented as mean $\pm$ SEM.

time point **(Fig. 4b)**. While there is a slight increase in Ym1 expression with IL-4 treatment, the fold increase plateaus around one; this indicates that Ym1 expression levels however around that of the control population. Therefore, while IL-4 treatment may abrogate the inhibitory effects of IFNγ and LPS pre-treatment, it is unable to upregulate production of Ym1.

IFNγ and LPS treatment also downregulates FIZZ1 expression. While the pre-Classically activated MΦ's increase FIZZ1 expression within a six hour window of IL-4 treatment, inhibition relative to medium pre-treated cells is still observed **(Fig. 4c)**. This increase in FIZZ1 expression however, is not sustained. Between 6 hours and 18 hours post-IL-4 treatment, FIZZ1 expression decreases to levels comparable to the control population, indicating that IL-4 treatment is unable to induce expression of AA markers. These data suggest that IL-4 treatment is unable to skew CA-MΦ's to an AA phenotype and that IFNγ/LPS pre-treatment inhibits IL-4 signaling over an extended period of time.

However, it is possible that IL-4 treatment attenuates the CA phenotype, converting CA-MΦ's to an un-stimulated state. To address this possibility, the effects of IL-4 treatment on the expression of the CA markers Nos2 and IL-12 were examined **(Fig. 5)**. Nos2 expression is sustained in the CA population in the presence of IL-4, even slightly increasing between 6 to 18 hours of IL-4 treatment. IL-12 levels also show little change, slightly increasing with IL-4 stimulation. Whereas Nos2 expression is sustained in the absence of IFNγ and LPS, IL-12 expression may be attenuated; this is evidenced by the lower levels of IL-12 expression in the CA-MΦ population compared to those observed in **Fig. 3b** and **Fig. 6c.**. Nonetheless, IL-4 treatment does not decrease expression of the CA markers Nos2 and IL-12, indicating that the CA phenotype is largely maintained (i.) in the

absence of continued IFNγ and LPS stimulation and (ii.) in the presence of IL-4.

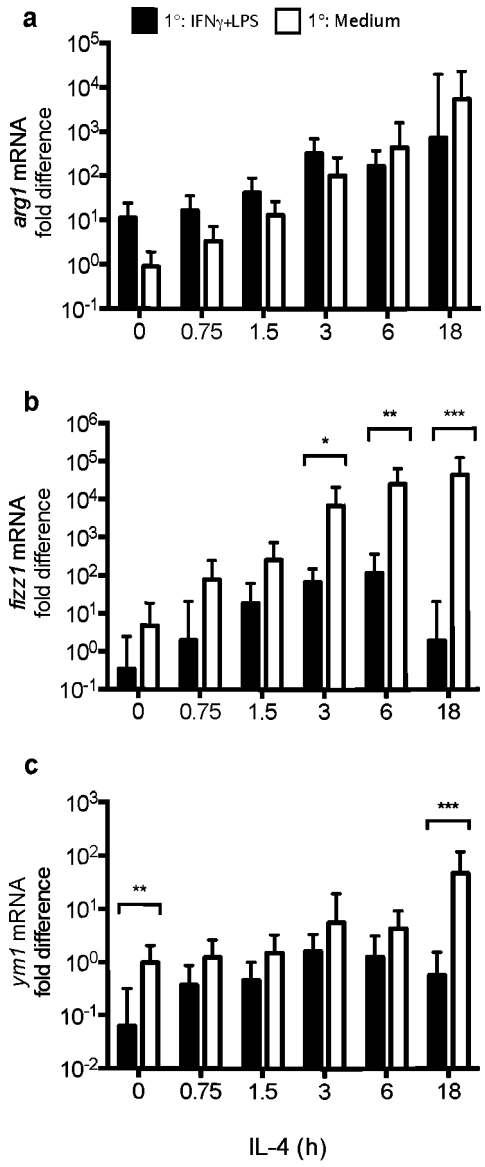


Figure 4: CA-MΦ's are not polarized to an AA phenotype upon IL-4 treatment.

Quantitative PCR analysis showing expression of **(a)** Arg1, **(b)** Ym1, and **c)** FIZZ1 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BMMΦs. MΦs are pre-treated with IFNγ (100ng/mL) and LPS (100ng/mL) or medium for 6 hours before a second stimulation with IL-4 (10ng/mL). * $P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ (2-way ANOVA with Sidak Multiple Comparisons correction). Data are representative of 2-5 independent experiments; data are represented as mean ± SEM.

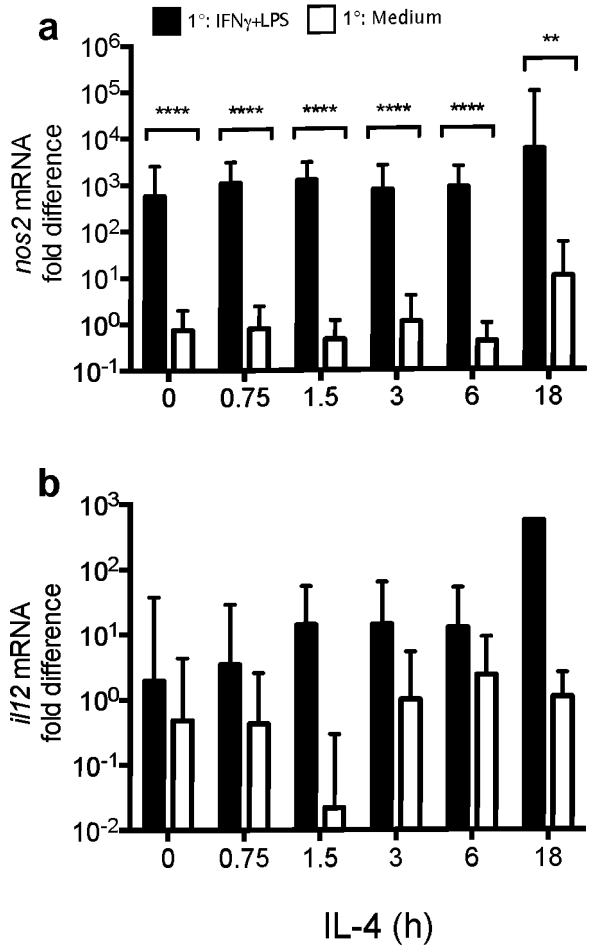


Figure 5: CA-MΦ's maintain CA phenotype upon IL-4 treatment.

Quantitative PCR analysis showing expression of **(a)** Nos2 and **(b)** IL-12 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BM-MΦ's. Φ's are pre-treated with IFNγ (100ng/mL) and LPS (100ng/mL) or Medium for 6 hours before a second stimulation with IL-4 (10ng/mL). * P<0.05 ; **P<0.01; ***P<0.001; ****P<0.0001 (2-way ANOVA with Sidak Multiple Comparisons correction). IFNγ/LPS pre-treated results are representative of 2-3 experiments for **(a)**; and three **(0-6h)** and one **(18h)** for **(b)**. Medium pre-treated results are representative of 2-5 independent experiments; data are represented as mean ± SEM.

Plasticity of AA-MΦ's

To examine if AA-MΦ's can be converted to CA-MΦ's in the presence of IFNγ and LPS co-stimulation, MΦ's were incubated with IL-4 or medium for 6 hours before stimulation with IFNγ and LPS for different timepoints. Medium pre-treated cells upregulated both Nos2 and IL-12 in a time-dependent manner **(Fig. 6)**. This is mirrored by the increase in Nos2 and IL-12 expression of the IL-4 pre-treated, AA-MΦ population **(Fig. 6a)**. Notably, IL-4 pre-treated MΦ's were more responsive to IFNγ/LPS treatment, expressing higher levels of Nos2 at 3, 6 and 18 hours post IL-4 removal relative to medium pre-treated MΦ's **(Fig. 6a)**. Expression of IL-12 was also upregulated in the IL-4 pre-treated population in a time-dependent manner with sustained, high levels of expression even after 18 hours of IFNγ/LPS treatment **(Fig. 6b)**. These results indicate that AA-MΦ's can readily upregulate CA markers in response to IFNγ/LPS stimulation and adopt a CA phenotype.

I next examined whether MΦ's stimulated with IFNγ/LPS continued to express high levels of AA markers, which would indicate the possibility of a hybrid AA-CA MΦ. Alternatively, IFNγ/LPS treatment could abrogate the expression of AA markers, skewing the AA-MΦ's to a CA phenotype. Within 6 hours of IFNγ/LPS treatment, Arg1 **(Fig. 7a)**, Ym1 **(Fig. 7b)**, and FIZZ1 **(Fig. 7c)** continue to be expressed by AA-MΦ's. However, between 6 and 18 hours of treatment, expression of these AA markers is decreased. Notably, both Ym1 and FIZZ1 expression levels decrease to levels comparable to those of the medium treated control **(Fig. 7b, 7c)**. Arg1 expression, though attenuated, continues to be at levels higher than the medium control. This however is paralleled by the medium pre-treated population and can be attributed, in part, to TLR signaling. However, another possibility, as I discuss later **(Fig. 11)**, is that this decrease in AA marker expression is not

due to IFNγ/LPS treatment but rather, to the removal of IL-4 signaling. This would indicate that expression of Ym1, FIZZ1, and Arg1 (to a lesser extent) by AA-MΦ's relies on continuous IL-4 signaling

Nonetheless, AA-MΦ's can be readily skewed to a CA phenotype after 18 hours of IFNγ/LPS stimulation and this transition occurs without a return to an unstimulated state; in the presence of IFNγ/LPS, AA-MΦ's begin to upregulate expression of CA markers as early as 45 minutes **(Fig.5)**. Coupled with the sustained expression of AA markers during this time, these results suggest that the transition from an AA to CA phenotype is marked by a hybrid CA-AA MΦ expressing markers of both CA and AA.

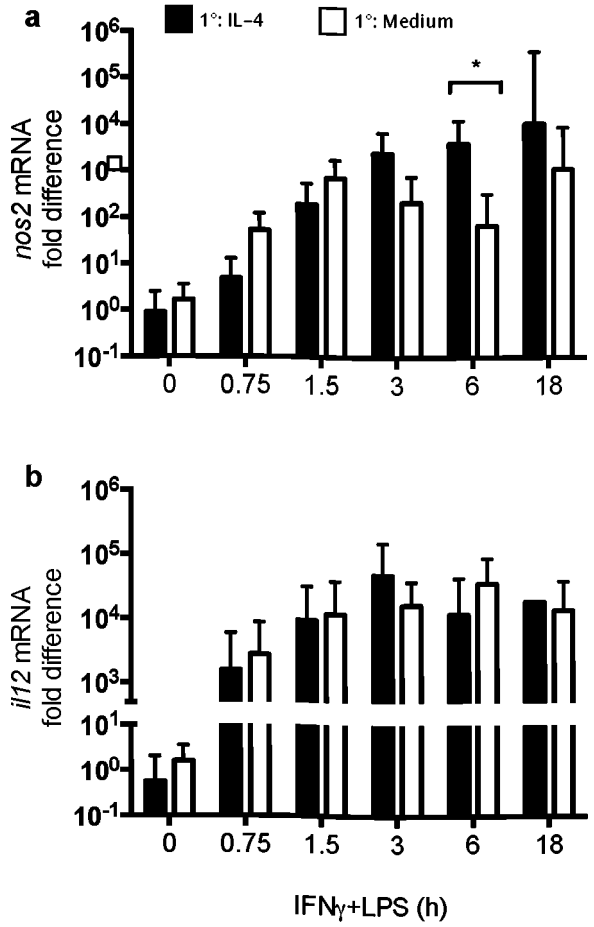


Figure 6: AA-MΦ's are polarized to a CA phenotype upon IFNγ/LPS treatment.

Quantitative PCR analysis showing expression of (a) Nos2 and (b) IL-12 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BM-MΦ's. MΦ's are pre-treated with IL-4 (10ng/mL) or medium for 6 hours before a second stimulation with IFNγ (100ng/mL) and LPS (100ng/mL). * $P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ (2-way ANOVA with Sidak Multiple Comparisons correction). IL-4 pre-treated results are representative of 2-3 independent experiments for **(a)**; two **(0-6h)** and one **(18h)** for **(b)**. Medium pre-treated results are representative of 2-5 independent experiments; data are represented as mean ± SEM.

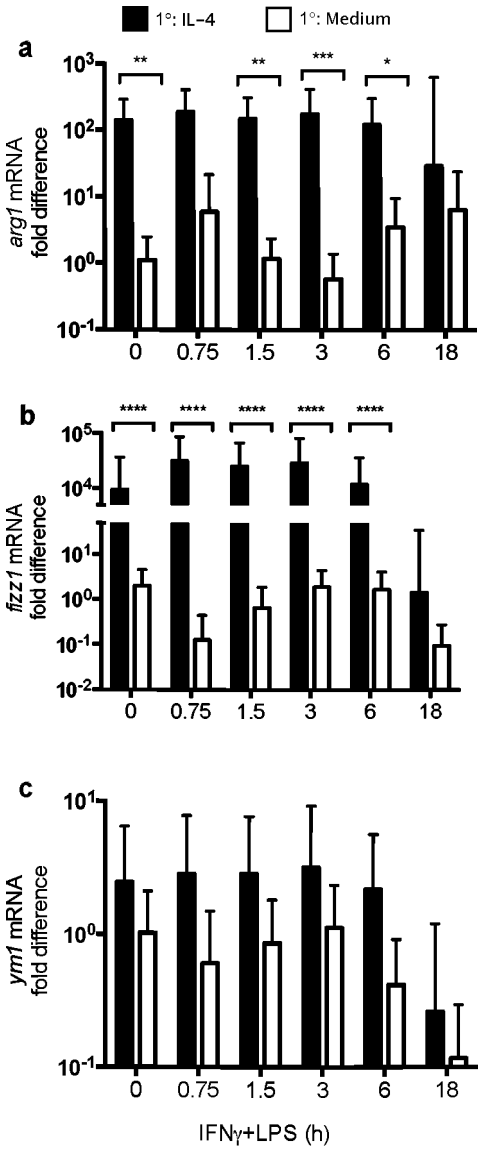


Figure 7: AA-MΦs do not maintain AA phenotype upon IFNγ/LPS treatment

Quantitative PCR analysis showing expression of **(a)** Arg1, **(b)** Ym1, and **(c)** FIZZ1 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BMMΦs. MΦs are pre-treated with IL-4 (10ng/mL) or medium for 6 hours before a second stimulation with IFNγ (100ng/mL) and LPS (100ng/mL). * $P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ (2-way ANOVA with Sidak Multiple Comparisons correction); data are representative of 3-5 independent experiments. Data are represented as mean ± SEM.

Type I, but not Type II, *T. gondii* infection polarizes MΦs to an AA phenotype.

Previous work in the Denkers lab has shown that Type I ROP16 is necessary for induction of Arg1 in MΦ's [25]. Because high levels of Arg1 expression are one of the hallmarks of AA, I wanted to investigate whether other markers of AA are also upregulated, indicating that *T. gondii* infection skews MΦ's to an AA phenotype.

MΦs were infected with either RH—wild type Type I strain—or Pru—a Type II strain—in a 3:1 ratio of parasites to MΦ for 0, 3, 6, and 18 hours. As expected, RH infection resulted in increasing and sustained Arg1 expression **(Fig. 8a)**. Similar increases in two other hallmarks of AA—Ym1 **(Fig. 8b)** and FIZZ1 **(Fig. 7c)**—suggest that RH infection skews MΦs to an alternative phenotype.

While Pru infection resulted in slight increases in Arg1 expression 6 hours post-infection this increase was not sustained. Arg1 expression levels after 18 hours of infection decreased to levels comparable with those of control medium population **(Fig.8)**. Similarly, induction of Ym1 and FIZZ1 is neither sustained nor shows clear increases upon Pru infection. 18 hours post Pru-infection, both Ym1 and FIZZ1 levels remain comparable to those of the control population. Therefore, while RH is able to induce expression of the AA markers Arg1, Ym1, and FIZZ1 and polarize MΦs to an AA phenotype, Pru infection does not. These results showed that Type I ROP16 is necessary for the induction of Arg1, Ym1, and FIZZ1.

Although Type II *T. gondii* strains have previously been reported to induce a CA phenotype [30], in my hands both Pru and RH infection result in modest increases in Nos2 **(Fig. 9a)** and IL-12 expression **(Fig. 9b)**; neither RH nor Pru infection induced expression of TNFa. In contrast to previous work, RH infection

results in slightly higher, albeit not significant, levels of Nos2 and IL-12 expression 18 hours post-infection. Notably, MΦ's infected with RH also express high levels of AA markers while MΦ's infected with Pru do not **(Fig. 8)**. This would suggest again, the possibility that MΦs can adopt a hybrid phenotype where both CA and AA markers are expressed.

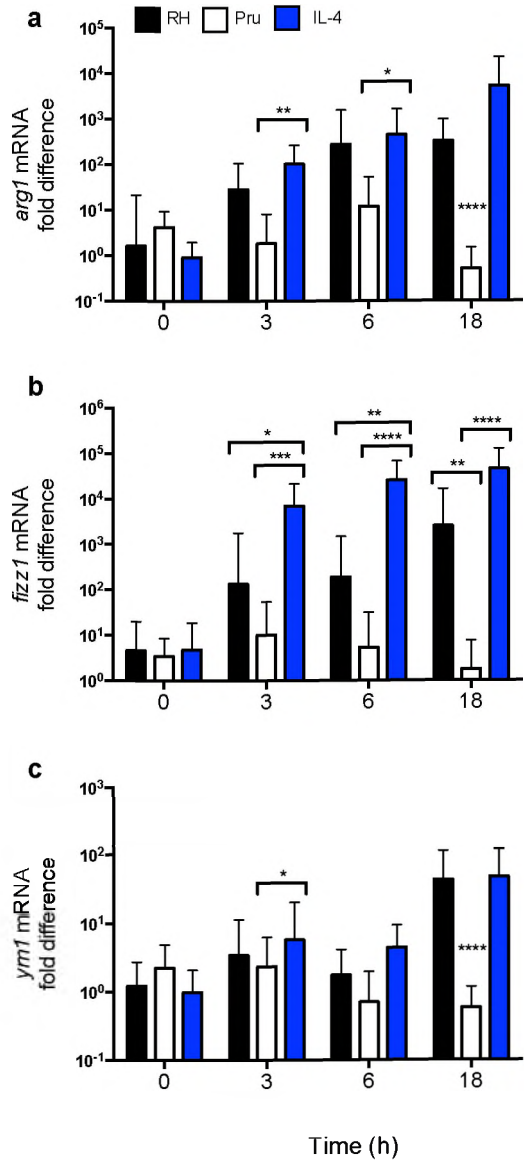


Figure 8: Type I, but not Type II, *T. gondii* infection polarizes MΦ's to an AA phenotype.

Quantitative PCR analysis showing expression of **(a)** Arg1, **(b)** Ym1, **(c)** FIZZ1 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BM-MΦ's. MΦ's are infected at a 3:1 ratio of parasites to MΦ or treated with IL-4 (10ng/mL) in a timecourse. *P<0.05 ; **P<0.01; ***P<0.001; ****P<0.0001 (2-way ANOVA with Sidak Multiple Comparisons correction). Data represent 3-5 independent experiments; data are represented as mean ± SEM.

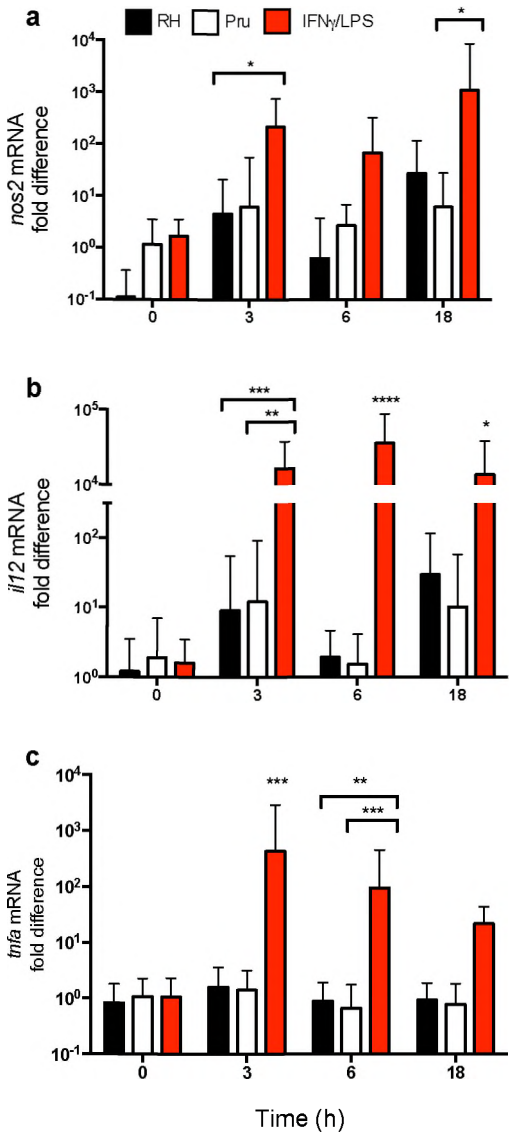


Figure 9: Type I and Type II *T. gondii* infection results in low levels of CA marker expression.

Quantitative PCR analysis showing expression of **a)** Nos2, **(b)** IL-12, **(c)** TNFa mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BM-MΦ's. MΦ's are infected at a 3:1 ratio of parasites to MΦ or treated with IFNγ (100ng/mL) and LPS (100ng/mL) in a timecourse. *P<0.05 ; **P<0.01; ***P<0.001; ****P<0.0001 (2-way ANOVA with Sidak Multiple Comparisons correction). Data represent 2-6 independent experiments; data are represented as mean ± SEM.

***T. gondii* infection induces expression of the chemokines CCL17, CCL21, and CCL24.**

Another hallmark of AA is the expression of the selective chemokine CCL17 [2, 31];. CCL17 or TARC (thymus and activation-regulated chemokine) is a T cell-directed CC chemokine that along with other chemokines, including CCL21 and CCL24, plays an important role in promoting a Th2 response [32]. CCL21, or SLC (secondary lymphoid organ), functions to recruit APCs and T-cells together to promote T-cell activation [33]. CCL24 (eotaxin-2), originally identified as a chemotactic factor for eosinophils, is a potent recruiter of other components of the innate immune system including neutrophils, basophils, and macrophages [34].

To examine the effect of *Toxoplasma* infection on induction of these chemotactic factors, MΦ's were infected in a 3:1 ratio of parasites to MΦ for six hours and expression levels were compared to IL-4 treated MΦ's. As shown in **Fig. 10a**, RH and Type II ΔROP16 parasites complemented with Type I ROP16 (Δ16 2:1) induced expression of CCL17 to levels comparable with IL-4 treatment. Pru, Type II ROP16 complemented knockouts (Δ16 2:2), and ROP16 knockouts (Δ16) instead induced significantly lower levels of CCL17 **(Fig. 10a)**. RH infection also induced significantly higher levels of CCL21 and CCL24 relative to Pru, Δ16 2:2, and Δ16 parasites **(Fig. 10b, 10c)**. Given these observations, it was clear that while Type I ROP16 induced higher levels of CCL17, CCL21, and CCL24 expression upon infection, induction was not entirely dependent on ROP16; infection with Δ16 parasites resulted in low levels of expression.

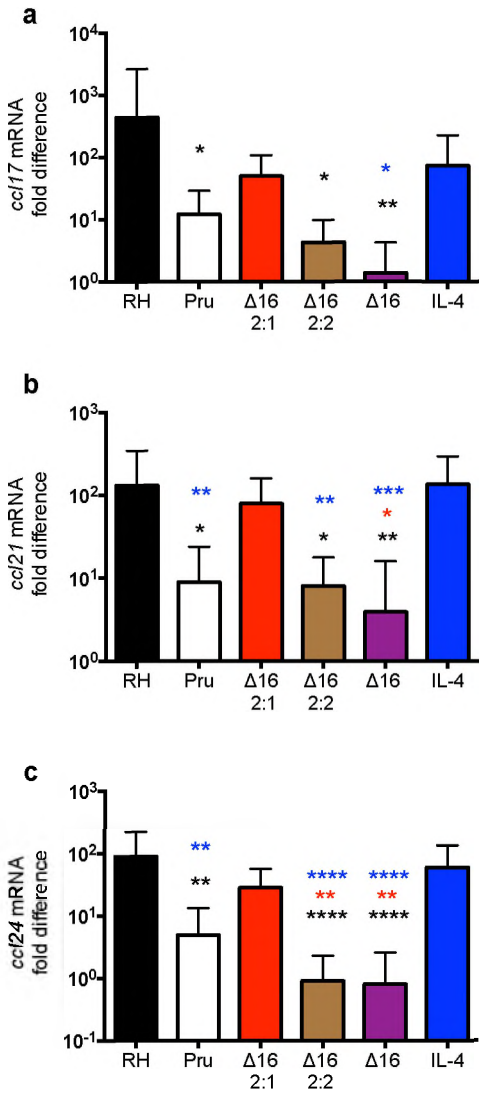


Figure 10: Induction of chemokines CCL17, CCL21, and CCL24 is enhanced by Type I ROP16.

Quantitative PCR analysis showing expression of **(a)** CCL17, **(b)** CCL21, **(c)** CCL24 mRNAs (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BM-MΦ's. MΦ's are infected at a 3:1 ratio of parasites to MΦ or treated with IL-4 (10ng/mL) for six hours. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (1-way ANOVA with Tukeys Multiple Corrections Test); Black *s denote significance vs. RH infection; Red *s denote significance relative to Δ16 2:1 infection; Blue *s denote significance vs. IL-4 treatment. Data shown represent 2-5 independent experiments; data are represented as mean ± SEM.

Effects of T. gondii pre-infection on AA polarization.

To examine whether *T. gondii* pre-infection attenuates or enhances AA polarization with IL-4 treatment, MΦ's were infected in a 3:1 ratio of parasites to MΦ for six hours before stimulation with IFNγ/LPS, IL-4, or medium alone for an additional 18 hours. Expression of the AA markers Arg1, Ym1, and FIZZ1 were assessed relative to IFNγ/LPS, IL-4, and medium pre-treatment as controls.

Beginning with **Fig. 11c**, we can see that RH and Δ16 2:1 induce sustained expression of Arg1 up to 24 hours after the initiation of infection. Additionally, both Pru and Δ16 2:2 are able to induce and sustain Arg1 induction—albeit at a lower level—and that Δ16 and IFNγ/LPS pre-treated populations also express low levels of Arg1. The induction of Arg1 with Pru, Δ16 2:2, and Δ16 pre-infection as well as with IFNγ/LPS pre-treatment is somewhat expected because TLR ligands can induce expression of Arg1 [29]. It is interesting to note that the IL-4 pre-treated population does not sustain Arg1 expression when IL-4 stimulation is removed; namely, that despite pre-treatment with IL-4 for 6 hours, during the subsequent 18 hours in medium, Arg1 expression is lost.

This same reduction in AA marker expression is seen in **Fig. 11f** where the IL-4 pre-treated population was unable to sustain Ym1 expression in the absence of IL-4 stimulation. Both RH and Δ16 2:1 however, maintained expression of Ym1 while Pru, Δ16 2:2, and Δ16 pre-infection did not. These results coincide with those depicted in **Fig. 8** where Pru infection was unable to induce AA marker expression. In **Fig. 11i** however, *T. gondii* pre-infection and IL-4 pre-treatment both conferred sustained levels of FIZZ1 expression. As with the other AA markers, both RH and Δ16 2:1 pre-infection induced higher levels of FIZZ1 expression consistent with the role of Type I ROP16 in activation of STAT6 and

polarization of MΦ's to an AA phenotype. Pru, Δ16 2:2, and Δ16 pre-infection also induced expression of FIZZ1 24 hours after the initiation of infection, again at a lower level compared to RH and Δ16 2:1 **(Fig. 11i)**. Notably, FIZZ1 expression did not require continued IL-4 stimulation, as evidenced by the sustained high levels of FIZZ1 expression with IL-4 pre-treatment **(Fig. 11i)**.

T. gondii pre-infection, regardless of strain, did not inhibit AA polarization upon IL-4 stimulation **(Fig. 11b, 11e, 11h)**. In **Fig. 11b**, we can see that in fact, *Toxoplasma* pre-infected populations showed higher expression of Arg1 and Ym1 compared to the IL-4 and medium pre-treated. Pre-treatment of MΦ's with *Toxoplasma* also resulted in high induction of FIZZ1 upon IL-4 secondary treatment **(Fig. 11c)**. Secondary IFNγ/LPS however, was able to attenuate expression of the AA markers Arg1 **(Fig. 11a)**, Ym1 **(Fig. 11b)**, and FIZZ1 **(Fig. 11c)**. We can see that secondary IFNγ/LPS treatment led to several fold lower expression of Arg1 in the RH pre-treated population (Fig. 11a) compared to its respective population in **Fig. 11c**. This decrease in AA marker expression is also seen in **Fig. 11d** and **Fig. 11g** in the expression of Ym1 and FIZZ1 respectively. Expression of these AA markers is similarly decreased in the Type II pre-infection populations **(Fig. 11a, 11d, 11g)**. These results indicate that while IL-4 was able to polarize *T. gondii* pre-infected MΦ's to an AA phenotype, IFNγ/LPS secondary treatment also downregulated the expression of AA markers, attenuating Type I ROP16-mediated alternative activation.

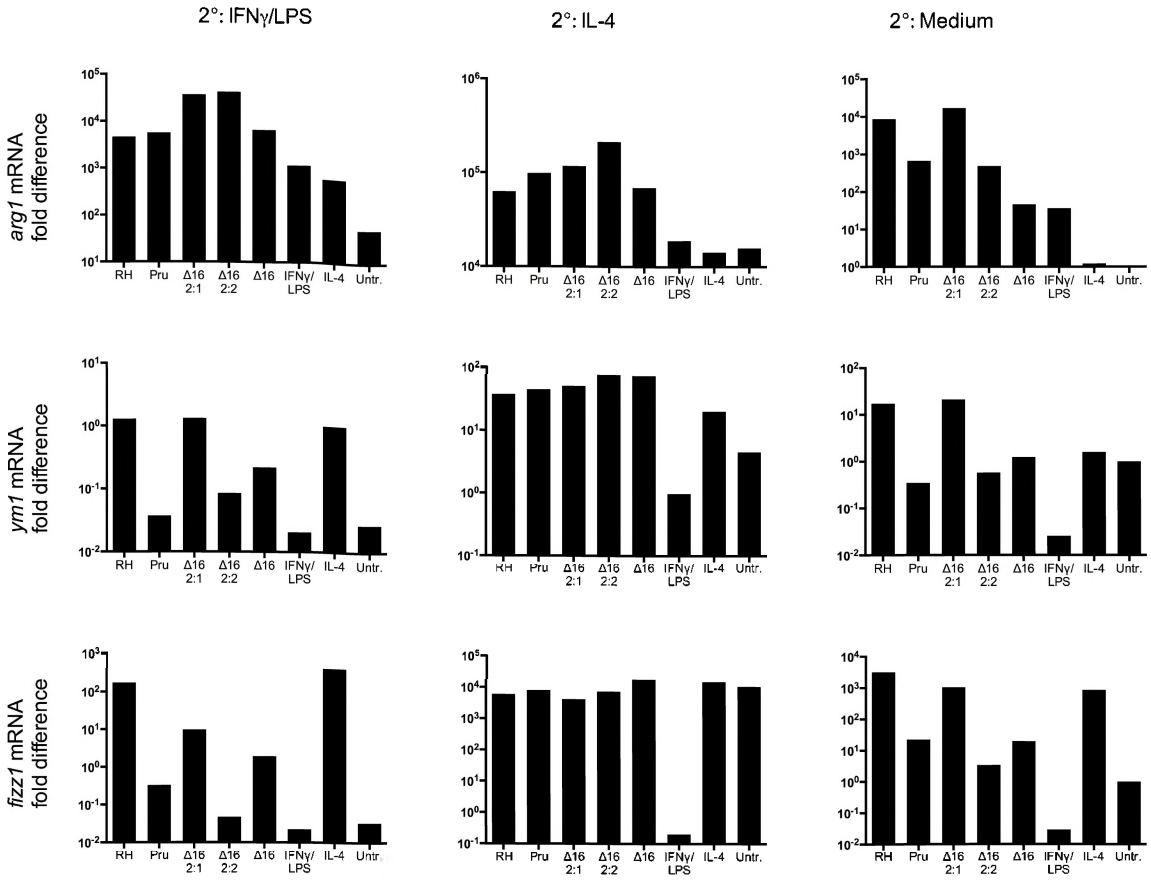


Figure 11: *T. gondii* pre-infection does not prevent AA of MΦs.

Quantitative PCR analysis showing expression of **(a-c)** Arg1, **(d-f)** Ym1, and **(g-i)** FIZZ1 mRNAS (relative to Actin mRNA and medium-treated cells) in total RNA extracted from BM-MΦ's. MΦ's are infected in a 3:1 parasite to MΦ ratio or treated with IFNγ (100ng/mL) and LPS (100ng/mL), IL-4 (10ng/mL), or medium for 6 hours before treatment with IFNγ/LPS, IL-4, or medium for an additional 18 hours. Data are represented as means of one experiment.

Effects of *T. gondii* pre-infection on CA polarization.

MΦ's from the same experiment **(Fig. 12)** were further examined for the effects of *T. gondii* pre-infection on CA polarization **(Fig. 12)**. Without any secondary cytokine treatment, *T. gondii* induced expression of Nos2 **(Fig. 12c)** and IL-12 **(Fig. 12i)** but not TNFa **(Fig. 12i)**. Markedly, RH pre-infection induced lower levels of Nos2 **(Fig. 12c)** and IL-12 **(Fig. 12i)** relative to pre-infection with the Type II strains.

Relative to secondary treatment with medium, expression of Nos2 **(Fig. 12a)** and IL-12 **(Fig. 12g)** were upregulated by several fold in *T. gondii* pre-infected MΦ's. TNFa expression was also upregulated with IFNγ/LPS treatment although at levels lower than that of IL-4 pre-treated MΦ's **(Fig. 12d)**. RH and Δ16 2:1 pre-infection attenuated expression of Nos2 **(Fig. 12a)** and TNFa **(Fig. 12g)** relative to IL-4 pre-treated cells. Therefore, while MΦ's alternatively activated by IL-4 stimulation readily express high levels of CA markers following IFNγ/LPS treatment, expression of CA markers is attenuated by infection with parasites expressing Type I ROP16.

Pre-infection with Pru and Δ16 parasites showed attenuation of Nos2 **(Fig. 12a)** and TNFa **(Fig. 12d)** expression relative to IL-4 pre-treated cells **(Fig. 12d)**. However, these populations also expressed comparatively higher levels of IL-12 relative to the IL-4 pre-treated population**(Fig. 12g)**. This is further confounded by the high expression of Nos2 by Δ16 2:2 pre-infected MΦ's **(Fig. 12a)**. Together these results implicate virulence factors distinct from ROP16 in the interference of IFNγ/LPS signaling.

IL-4 secondary treatment had little effect on the expression of CA markers by *T. gondii* pre-infection, paralleling the IFNγ/LPS pre-treated population **(Fig.**

12b, 12e, 12h); expression of Nos2, TNFa, and IL-12 were at levels comparable to those of secondary medium treated populations **(Fig. 12c, 12f, 12i)**.

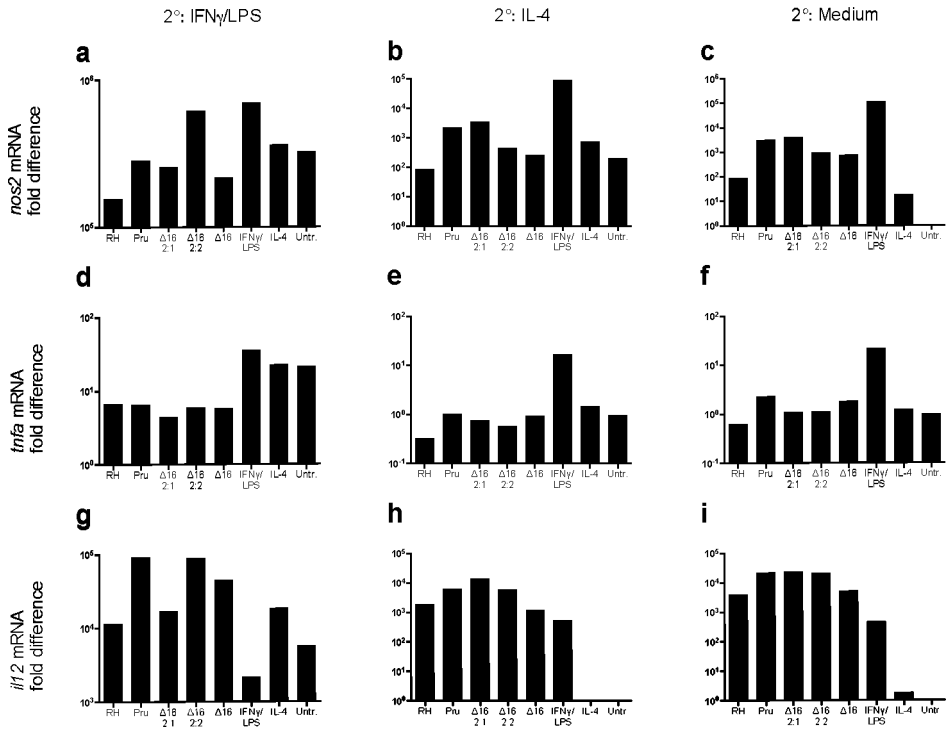


Figure 12: Pre-infection with Type I ROP16 expressing *T. gondii* attenuates CA polarization.

Quantitative PCR analysis showing expression of (a-c) Arg1, (d-f) Ym1, and (g-i) FIZZ1 mRNAS (relative to Actin mRNA and medium treated cells) in total RNA extracted from BM-MΦ's. MΦ's are infected in a 3:1 parasite to MΦ ratio or treated with IFNγ (100ng/mL) and LPS (100ng/mL), IL-4 (10ng/mL), or medium for 6 hours before treatment with IFNγ/LPS, IL-4, or medium for an additional 18 hours. Data are represented as means of one experiment.

4 Discussion

MΦ's play a pivotal role in both innate and adaptive immunity. As professional phagocytes, MΦ's play the essential role of clearing infection and activating the adaptive immune response through their APC functions. Furthermore, MΦ's are able to detect and respond to changes in their microenvironment through the expression of diverse cell surface receptors. Therefore, while the ability to mount a tailored immune response is a hallmark of the adaptive immune response, MΦ's too possess a remarkable adaptability through their ability to reshape their genetic landscape in response to different signals.

I examined the plasticity of activated MΦ's through analysis of gene expression changes in response to different polarizing stimuli. Expression changes do not necessarily reflect the activity of the gene product; nor do they address factors such as degradation rates of mRNA transcripts or of protein stability. However, expression of a gene does show how the activity of transcription factors, of genetic remodeling, and of other factors can influence expression of genes in response to different signals.

Previous work has established antagonism between the IL-4-STAT6 axis, which drives AA, and the IFNγ/STAT 1 and NF-κB signaling axes [3]. This antagonism is due in part to the Transactivator Domain (TAD) of STAT6, which binds to and sequesters shared transcriptional activators from STAT1-activated sequences [35]. Recent work has also begun to shed light on the role of SOCS (Suppressor of Cytokine Signaling) proteins, though their role in determining MΦ activation is still controversial because Type I and Type II Interferons, IL-4, and IL-13 have all been reported to induce expression of SOCS-1 [36, 37]. To further complicate

this model, SOCS-1 has been reported to antagonize MΦ responses to IFNγ [37] and IL-4 [36]. One possible explanation for this apparent paradox is that while SOCS-1 can be upregulated in both classical and alternative activation, what may determine MΦ phenotype is ultimately the ratio between SOCS-1 and SOCS-3: a high ratio of SOCS-1 to SOCS-3 promotes a AA phenotype by activation of PI3K; a low ratio of SOCS-1 to SOCS-3, on the other hand, promotes a CA phenotype through inhibition of PI3K [38]. While this is may present an appealing explanation, further work needs to be done to further establish this proposed model [38].

Here I have shown that BM-MΦ's activated to a CA phenotype by stimulation with IFNγ/LPS are unable to (i.) sustain increased expression of AA markers and (ii.) continue to express high levels of CA markers when treated with the AA polarizing cytokine IL-4. On the other hand, BM-MΦ's activated to an AA phenotype by stimulation with IL-4 readily (i.) upregulate and sustain expression of CA markers while (ii.) downregulating expression of AA markers when stimulated with IFNγ/LPS. My findings suggest that AA-MΦ are more plastic and can be readily converted to a CA phenotype. On the other hand, CA-MΦ's are less plastic and cannot be converted to an AA phenotype upon IL-4 treatment. This difference in plasticity is not readily explained by current understandings of STAT1-STAT6 antagonism and interactions.

While it is tempting to attribute this conversion entirely to stimulation with the CA-polarizing IFNγ/LPS, another possible explanation is that continuous IL-4 stimulation is needed to maintain an AA phenotype and STAT6 activation. Therefore, while IFNγ/LPS treatment is able to induce expression of CA markers, the decrease in expression of AA markers could be an *in vitro* artifact. Additionally,

the inertness of CA-MΦ's when stimulated with IL-4 may also be due to positive autocrine and paracrine feedback loops from secreted pro-inflammatory cytokines.

Nonetheless, my findings show that this conversion from an AA to CA phenotype happens through an intermediate transition state where both CA and AA signaling pathways are activated. In other words, rather than proceed through a deactivated phenotype, AA-MΦ's undergo a hybrid CA-AA phenotype before conversion into CA-MΦ's. It is also possible that due to my experimental methods, my RT-qPCR results are not reporting a hybrid phenotype, but rather, the gene expression profile of a mixed MΦ population. This is certainly possible and further studies using flow cytometry, which would allow the phenotype of individual cells to be examined, should be undertaken in the future, However, because observation of this hybrid phenotype occurs within a short 6 hour window, distinguishing MΦ phenotypes based on protein biomarkers may be difficult.

Still, I believe my findings support the gradient of MΦ activations proposed by Mosser [2]. Rather than think of activated MΦ's as distinct, mutually exclusive populations at opposite ends of a linear scale, it is more apt to designate a spectrum of MΦ activation whereby MΦ's are not restricted to expressing exclusively CA or AA markers **(Fig. 13)**. This new approach to MΦ activation addresses issues associated with classifying MΦ's into activation states based on their expression of activation markers. For example, in my hands IFNγ/LPS stimulated Arg1 expression, a hallmark AA marker after 12 hours of stimulation **(Fig. 4a)**, but also high levels of Nos2 **(Fig. 5a)** and IL-12 **(Fig. 5b)**—hallmark CA markers. It would be difficult to designate this population as solely classically activated or alternatively activated.

This model of MΦ activation is also more conducive to our understanding of

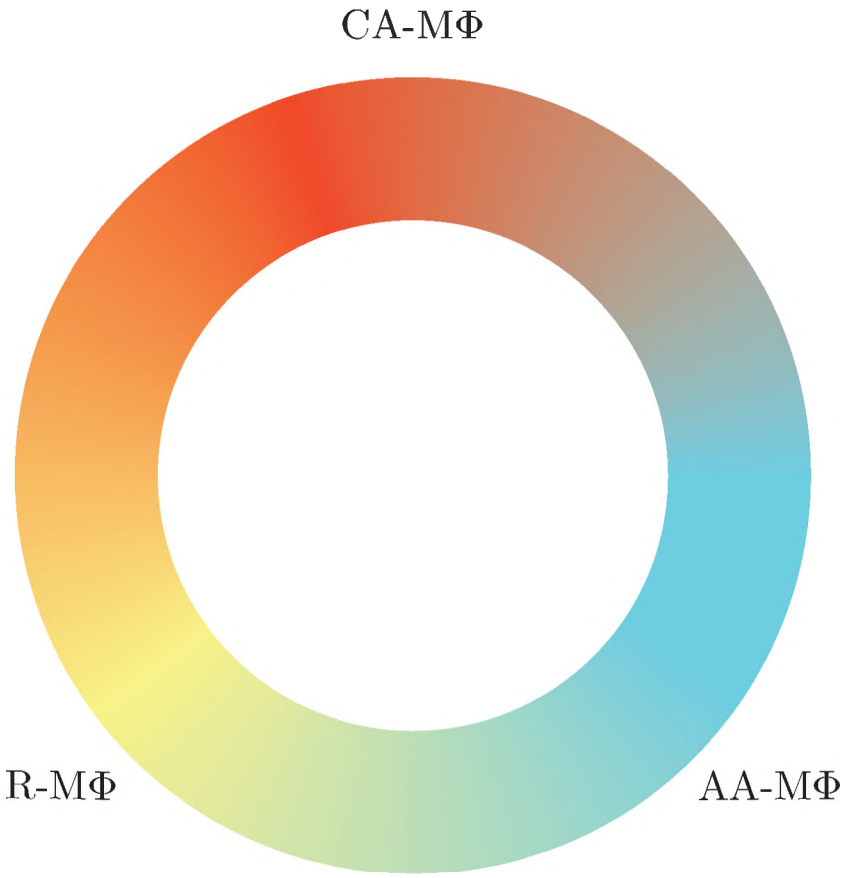


Figure 13: The Spectrum of MΦ Activation.

the role of MΦ's in the body. Because MΦ's are subject to numerous environmental signals at any given time, it is unlikely that MΦ's are purely one activation phenotype versus another. Tumor associated macrophages (TAMΦ), for example, display characteristics of both regulatory and alternatively activated macrophages while MΦ's associated with obesity patients have characteristics of both CA and AA-MΦ's [2].

I further showed that *T. gondii* infection resulted in induction of Arg1, Ym1, and FIZZ1 expression, characteristic of AA-MΦ's, and that this polarization was dependent on expression of Type I ROP16 **(Fig. 8)**. Yet, these infected MΦ's

also expressed low levels of the CA markers Nos2, IL-12, and TNFa **(Fig. 9)**. Therefore, rather than characterize *Toxoplasma* as solely a Th1-inducing pathogen, the parasite may more accurately be balancing a Th2 response, protective to the parasite, with a Th1 response, protective for the host. Therefore, while seemingly paradoxical, by infecting MΦ's, *Toxoplasma* situates itself in a uniquely versatile position where manipulation of the host immune system can readily be achieved. Indeed, parasite infection induced expression of the chemokine CCL17, important for the recruitment of Th2 cells. Infection also induced production of CCL24, which is a potent eosinophil chemotactic factor. Secretion of these two chemotactic factors amplify the Th2 response because eosinophils are important providers of IL-4 in the body. While I showed that expression of the hallmark AA markers Arg1, Ym1, and FIZZ1 **(Fig. 8)** was dependent on Type I ROP16, induction of these chemokines was not. This may indicate a conserved mechanism for inducing a Th2 response, independent of ROP16.

Preliminary data also suggest that *T. gondii* infection does not prevent AA of MΦ's by IL-4 stimulation; in fact, both Type I and Type II pre-infected MΦ's showed increased expression of AA markers following IL-4 treatment. Notably, IL-4 treatment was not able to downregulate T. gondii induced Nos2, TNFa, or IL-12 expression **(Fig. 12b, 12e, 12h)**. This parallels the unresponsiveness of CA-MΦ's to IL-4 stimulation **(Fig. 4)**. Furthermore, my preliminary data shows that IFNγ/LPS co-stimulation upregulated expression of CA markers, although at lower levels compared to uninfected MΦ's. This contradicts the current literature citing the inhibition of IFNγ and TLR signaling by *Toxoplasma* infection. One hypothesis is that simultaneous stimulation with IFNγ and LPS is able to synergistically override the ability of *Toxoplasma* to interfere with pro-inflammatory signaling.

However, another possibility for this observed increase in CA marker expression is that upregulation may be reflective of un-infected MΦ's.

Overall, my work lends insight into plasticity of CA and AA MΦ's. Whereas previous work has shown the deactivating effects of IL-10 on pro-inflammatory cytokine production, my work shows that IL-4 stimulation is not able to deactivate CA-MΦ's and skew them to an AA phenotype. On the other hand, AA-MΦ's are readily converted to a CA phenotype and possess a hybrid AA-CA gene expression profile during their transition.

By further understanding the molecular basis of MΦ plasticity, we can begin to better understand how these activated MΦ populations play a role in disease [39]. For example, CA-MΦ's are generally associated with early stages of cancer, possibly due to their production of ROS and RNS which can lead to DNA damage [2]. However, as tumor growth proceeds, the MΦ phenotypes become more similar to those of R-MΦ's and these TAMΦ's secrete factors that modify the tumor microenvironment [2, 40]. Furthermore, studies have shown that MΦ's can vary in phenotype depending on their spatial location [39]. Understanding of how these complex groups of MΦ activations arise in the tumor microenvironment will further our understanding of cancer progression and holds therapeutic potential.

I further showed that *T. gondii* infection alternatively activates MΦ's and is able to induce expression of Th2 promoting chemokines. While expression of these chemotactic factors occurs in Type II ROP16 parasites, it is enhanced by Type I ROP16-expressing parasites. Furthermore, I have shown preliminary data that indicates *T. gondii* does not interfere with with AA polarization. These results indicate that rather than characterize *Toxoplasma* as a Th1 pathogen, it may be more accurate to classify it as a Th2 parasite as well. *T. gondii*'s preference for

MΦ invasion may therefore be a way for the parasite to display extreme versatility in balancing the host Th1 and Th2 response. In light of the proposed spectrum of MΦ activations, so too must our understanding and classification of pathogens into polarized extremes change to meet this complexity.

5 Materials and Methods

5.1 Mice

Female C57BL/6 mice, 6-8 weeks old, were purchased from Taconic Farms (Germantown, NY) and housed under pathogen-free conditions in the Cornell University College of Veterinary Medicine animal facility, an accredited institution by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

5.2 Parasites

Tachyzoites were maintained by twice weekly passage on human foreskin fibroblast monolayers in DMEM (Life Technologies, Gaithersburg, MD) 1% heat-inactivated bovine growth serum (HyClone, Logan, UT), 100 U/mL Penicillin (Life Technologies) and 0.1 U/mL Streptomycin (Life Technologies).

5.3 Bone marrow-derived MΦ preparation

Bone Marrow MΦ's were flushed from femur and tibia and cultured for 5-6 days in DMEM, 9% heat-inactivated bovine growth serum (HyClone, Logan, UT), 100 U/mL Penicillin (Life Technologies), 0.1 U/mL Streptomycin (Life Technologies), 30% supernatant from L929 cells as a source of M-CSF. After 5-6 days of culture, non-adherent cells were removed, and adherent monolayers were washed and resuspended in PBS before plating in DMEM with 1% heat-inactivated bovine growth serum, 100 U/mL Penicillin (Life Technologies) and 0.1 U/mL Streptomycin.

5.4 Macrophage Activation and Cell Culture

MΦ's were plated in the presence of 100ng/mL IFNγ (Peprotech) and 100ng/mL LPS (*S. minnesota*, ultrapure, List Biological Laboratories, Campbell, CA for classical activation and IL-4 (Peprotech) at 10ng/mL. Infection was accomplished by addition of tachyzoites to MΦ cultures in a 3:1 ratio of parasites to MΦ's. Plates were briefly centrifuged (600-1200 x *g* for 2 minutes to synchronize infection. In experiments with multiple treatments, plates were washed two times with PBS before secondary cytokine stimulation or parasites were added.

5.5 Reverse Transcriptase Quantitative Polymerase Chain Reaction

Total RNA was prepared from cell cultures using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek) with on-column RNAse free DNAse treatment (Omega Bio-Tek). RNA was converted to cDNA using qScript cDNA Supermix kits (Quanta Biosciences, Gaithersburg, MD). Quantitative PCR was performed and normalized to the expression of housekeeping gene *Actin* using SYBR green chemistry (Quanta Biosciences, Gaithersburg, MD) and ABI 7500 fast machine (Life Technologies Corporation, Carlsbad, CA). Expression relative to untreated cells was assessed using the ΔΔCt method.

5.6 Primer Sequences

Actin-Forward: TGGAATCCTGTGGCATCCATGAAAC

Actin-Reverse: TAAAACGCAGCTCAGTAACAGTCCG

Arg1-Forward: AAGAATGGAAGAGTCAGTGTGG

Arg1-Reverse: GGGAGTGTTGATGTCAGTGTG

CCL17-Forward:TACCATGAGGTCACTTCAGATGC

CCL17-Reverse: GCACTCTCGGCCTACATTGG

CCL21-Forward:GTGATGGAGGGGGTCAGGA

CCL21-Reverse: GGGATGGGACAGCCTAAACT

CCL24-Forward:ATTCTGTGACCATCCCCTCAT

CCL24-Reverse: TGTATGTGCCTCTGAACCCAC

FIZZ1-Forward: CCTCCACTGTAACGAAGACTCTC

FIZZ1-Reverse: GCAAAGCCACAAGCACACC

IL-12p40-Forward: GGAAGCACGGCAGCAGAATA

IL-12p40-Reverse: AACTTGAGGGAGAAGTAGGAATGG

IL-6-Forward: GAGGATACCACTCCCAACAGACC

IL-6-Reverse: AAGTGCATCATCGTTGTTCATACA

Nos2-Forward: AAGCTGATGGTCAAGATCCAG

Nos2-Reverse: CCACCTCCAGTAGCATGTTG

TNFa-Forward: CATCTTCTCAAAATTCGAGTGACAA

TNFa-Reverse: TGGGAGTAGACAAGGTACAACCC

Ym1-Forward: ATCTATGCCTTTGCTGGAATGC

Ym1-Reverse: TGAATGAATATCTGACGGTTCTGAG

5.7 Statistics

Statistical analysis was performed as indicated using GraphPad Prism.

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