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Roles for the Aryl Hydrocarbon Receptor in the Immune Response to Toxoplasma Gondii

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Abstract
One of the major challenges faced by the immune system involves mounting an inflammatory response to control pathogen growth while limiting immune-mediated damage to the host. In order to achieve this balance, responding immune cells need to detect signals from the environment and react appropriately by promoting or attenuating inflammation. Cells of the immune system employ an array of sensors to respond to environmental cues, such as nuclear hormone receptors, cytokine receptors, and Toll-like receptors. The aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, provides immune cells with an additional means of detecting and responding to environmental signals to promote immunity. The work presented in this thesis examines the effects of AHR signaling during infection with the protozoan parasite Toxoplasma gondii, a medically significant pathogen that naturally infects mice. A variety of AHR ligands are produced by the host and the parasite during toxoplasmosis, which raised the question of whether AHR activity influences the immune response in this setting. Chapter 2 of this thesis describes a role for the AHR in promoting natural killer cell production of IL-10 in vitro and in vivo following infection. NK cells basally expressed the AHR and IL-12 stimulation increased AHR levels in these cells. Inhibition of the AHR led to impaired NK cell IL-10 production in vitro, and NK cells isolated from T. gondii-infected Ahr-/- mice had defective expression of IL-10. Chapter 3 demonstrates context-dependent roles for the AHR during oral and chronic toxoplasmosis. Orally infected Ahr-/- animals exhibited more severe weight loss and increased intestinal tissue pathology compared to wild-type mice, which was associated with CD4+ T cell hyperactivation. Chronically infected Ahr-/- mice developed elevated parasite burdens, but the CD4+ T cell responses in these animals were comparable to those in wild-type animals. Collectively these studies indicate that the AHR has multiple context dependent roles in the immune response to T. gondii.

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ROLES FOR THE ARYL HYDROCARBON RECEPTOR IN THE IMMUNE RESPONSE TO *TOXOPLASMA GONDII*

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ABSTRACT

ROLES FOR THE ARYL HYDROCARBON RECEPTOR IN THE IMMUNE RESPONSE TO *TOXOPLASMA GONDII*

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One of the major challenges faced by the immune system involves mounting an inflammatory response to control pathogen growth while limiting immune-mediated damage to the host. In order to achieve this balance, responding immune cells need to detect signals from the environment and react appropriately by promoting or attenuating inflammation. Cells of the immune system employ an array of sensors to respond to environmental cues, such as nuclear hormone receptors, cytokine receptors, and Toll-like receptors. The aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, provides immune cells with an additional means of detecting and responding to environmental signals to promote immunity. The work presented in this thesis examines the effects of AHR signaling during infection with the protozoan parasite *Toxoplasma gondii*, a medically significant pathogen that naturally infects mice. A variety of AHR ligands are produced by the host and the parasite during toxoplasmosis, which raised the question of whether AHR activity influences the immune response in this setting. Chapter 2 of this thesis describes a role for the AHR in promoting natural killer cell production of IL-10 *in vitro* and *in vivo* following infection. NK cells basally expressed the AHR and

IL-12 stimulation increased AHR levels in these cells. Inhibition of the AHR led to impaired NK cell IL-10 production *in vitro*, and NK cells isolated from *T. gondii*-infected *Ahr*$^{-/-}$ mice had defective expression of IL-10. Chapter 3 demonstrates context-dependent roles for the AHR during oral and chronic toxoplasmosis. Orally infected *Ahr*$^{-/-}$ animals exhibited more severe weight loss and increased intestinal tissue pathology compared to wild-type mice, which was associated with CD4^{+} T cell hyperactivation. Chronically infected *Ahr*$^{-/-}$ mice developed elevated parasite burdens, but the CD4^{+} T cell responses in these animals were comparable to those in wild-type animals. Collectively these studies indicate that the AHR has multiple context dependent roles in the immune response to *T. gondii*.

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CHAPTER 1: LITERATURE REVIEW

Introduction

One of the major challenges faced by the immune system involves mounting an inflammatory response to control pathogen growth while limiting immune-mediated damage to the host. In order to achieve this balance, responding immune cells need to detect signals from the environment and react appropriately by promoting or attenuating inflammation. Cells of the immune system employ an array of sensors to respond to environmental cues, such as nuclear hormone receptors, cytokine receptors, and Toll-like receptors. The aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, provides immune cells with an additional means of detecting and responding to environmental signals to promote immunity. The work presented in this thesis examines the effects of AHR signaling during infection with the protozoan parasite *Toxoplasma gondii*, a medically significant pathogen that naturally infects mice. A variety of AHR ligands are produced by the host and the parasite during toxoplasmosis, raising the question of whether AHR activity influences the immune response in this setting. Studies that were performed to assess the functions of AHR signaling in natural killer cells during acute *T. gondii* infection are described in Chapter 2 of this thesis. Chapter 3 presents work examining the role of the AHR in the gut following oral infection with *T. gondii,* and its impact on the chronic phase of toxoplasmosis.

Toxoplasma gondii: opportunistic pathogen and model organism

T. gondii is an intracellular apicomplexan parasite that has the ability to naturally infect any warm-blooded vertebrate through an oral route in two infective forms, the tissue cyst and the oocyst. Thus, the ingestion of undercooked meat or contaminated fruits or vegetables is a major mode of transmission to humans (Jones and Dubey, 2012). *T. gondii* is a widespread and highly successful parasite, which is reflected in infection rates as high as 70% in certain regions (Pappas et al., 2009). Human toxoplasmosis results in a spectrum of disease, ranging from asymptomatic to severe. In most immunocompetent individuals, chronic infection is generally regarded as asymptomatic (Montoya and Liesenfeld, 2004). However, the public health importance of toxoplasmosis was first recognized in the context of congenital *T. gondii* infection that occurs via maternal-fetal transmission (Wolf et al., 1939). Congenital toxoplasmosis results in birth defects such as hydrocephalus, chorioretinitis, and neurological deficits (Havelaar et al., 2007), and is estimated to affect up to 4000 births per year in the United States (Jones et al., 2001). A second group of patients that are highly susceptible to toxoplasmosis consists of individuals with immune-deficiencies including patients with AIDS, hyper-IgM syndrome, or those treated with immunosuppressive drugs (Derouin and Pelloux, 2008; Israelski et al., 1993; Leiva et al., 1998). The vulnerability of these patients highlights the importance of T cells in maintaining protective immunity to *T. gondii,* which will be discussed in more detail in the following sections. As a

consequence of these susceptible individuals, infection with *T. gondii* has been identified as a leading cause of food borne mortality in the U.S. (Mead et al., 1999).

Although toxoplasmosis is generally asymptomatic in patients without immune defects, infection with certain strains of *T. gondii* can cause severe symptoms in immunocompetent patients, resulting in ocular disease or death (Demar et al., 2007; Grigg et al., 2001). Genetic studies indicate that the structure of the *T. gondii* population in Europe and North America predominantly consists of three clonal lineages, termed type I, II, and III (Howe and Sibley, 1995). In other parts of the world, the parasite population includes these three strains as well as additional recombinant and exotic strains (Boothroyd and Grigg, 2002; Saeij et al., 2005). While type I strains are highly virulent and lethal in mice, the type II and type III strains are relatively avirulent (Boothroyd and Grigg, 2002). These strain differences are also thought to impact human toxoplasmosis, as severe ocular disease in immunocompetent patients is associated with type I strains of *T. gondii* (Boothroyd and Grigg, 2002; Grigg et al., 2001).

Although *T. gondii* can broadly infect warm-blooded vertebrates, domestic and wild cats are the definitive hosts for this parasite. Thus, *T. gondii* sexually reproduces in cats, forming highly infectious oocyts that are released into the environment (Elmore et al., 2010). Intermediate hosts, in which the parasite replicates asexually, can become infected following the ingestion of oocysts. The parasite then converts to a tachyzoite form, which replicates and disseminates throughout the host (Dubey et al., 1997). Tachyzoites can infect any nucleated host cell through the process of active invasion,

which allows the parasite to replicate in a specialized nonfusogenic parasitophorous vacuole, eventually leading to host cell lysis and the release of progeny (Sibley, 2011). In immunocompetent mice infected with type II or type III strains of *T. gondii*, the immune response leads to the control of the acute phase of parasite growth. The parasite then differentiates into a bradyzoite form that replicates slowly and is found in tissue cysts located primarily in the brain and muscle tissue (Sibley, 2011). These cysts persist chronically, and new hosts can become infected by consuming them (Sibley, 2011; Su et al., 2003). Tissue cysts can sporadically reactivate and release tachyzoites, which are controlled by the ongoing host immune response, leading to a cycle of reactivation and latency that results in life-long infection (Ferguson et al., 1989).

Several features of *T. gondii* make infections with the parasite an effective means of studying host-pathogen interactions. *T. gondii* can readily be cultured in the laboratory and is genetically tractable. Additionally, mice are a natural host for the parasite, and given the availability of tools to study murine immunity and genetically manipulated mouse strains, *T. gondii* is a useful organism to study the *in vivo* immune response to infection. The pathogenesis of toxoplasmosis, which includes an acute and chronic phase and involves parasite replication in multiple tissue sites including the brain and the intestines, facilitates the use of this parasite to investigate multiple aspects of immunity to infection. Indeed, studies with *T. gondii* have led to a number of fundamental insights on innate immune activation, the generation of Th1 responses, mechanisms of immune regulation, and mucosal immunity, which are described in the following sections.

Immune reponses to *T. gondii*

Innate immunity

Following infection, the innate immune system plays a critical role in producing the cytokine IL-12, which promotes the development of protective Th1 mediated immunity by driving the expression of IFN-γ from innate and adaptive sources (Figure 1.1). Consequently, IL-12 depletion renders mice highly susceptible to infection (Gazzinelli et al., 1994). In order to initiate anti-parasitic responses, innate immune cells need to sense the presence of *T. gondii,* and multiple studies have addressed the mechanisms involved in parasite recognition. Toll-like receptors (TLRs) were implicated in this process based on the finding that mice deficient for the TLR adaptor protein MyD88 have defects in IL-12 production during infection, develop elevated parasite burdens, and succumb to acute toxoplasmosis (Scanga et al., 2002). TLR2, 4, 7, 9, 11, and 12 have subsequently been shown to play a role in innate sensing of the parasite. TLR2 and TLR4 recognize glycosylphosphatidylinositols found on the surface of *T. gondii*, and mice deficient for both receptors develop increased parasite burdens during chronic infection (Debierre-Grockiego et al., 2007). Sensing through TLR7 and TLR9 induces responses to parasite-derived nucleic acids (Andrade et al., 2013), while TLR11 and TLR12 recognize a parasite profilin-like molecule (Koblansky et al., 2013; Yarovinsky et al., 2005). TLR11 deficient mice concordantly develop higher parasite burdens in the chronic phase of infection, whereas mice that lack TLR12 succumb to acute infection (Koblansky et al., 2013; Yarovinsky et al., 2005). Notably, there are

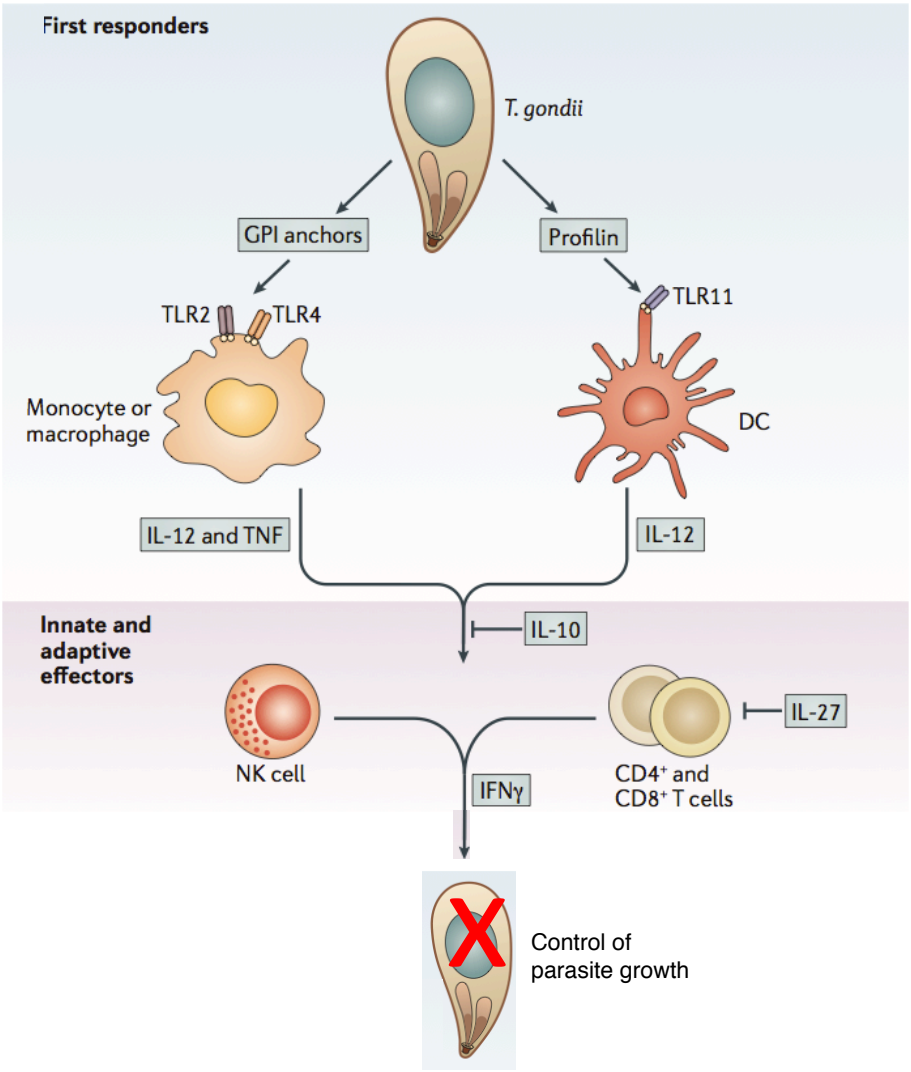

Figure adapted from Hunter, C.A., and L.D. Sibley. 2012. Modulation of innate immunity by Toxoplasma gondii virulence effectors. Nature reviews. Microbiology 10:766-778

Figure 1.1: Protective immunity to *T. gondii.* Innate recognition of *T. gondii* elicits IL-12 production by innate cell populations, which promotes IFN-γ expression by NK cells and T cells. IFN-γ signaling induces indoleamine 2,3 dioxygenase, inducible nitric oxide synthase, and p47 GTPases, which act to restrict parasite replication. IL-10 and IL-27 limit the immune response to toxoplasmosis and promote the control of immune-mediated pathology.

MyD88 independent innate mechanisms of responding to the parasite, as IL-12 production in response to *T. gondii* is not completely abrogated in MyD88 deficient cells (Scanga et al., 2002). The existence of additional innate recognition pathways was also suggested by the finding that mice lacking MyD88 are capable of generating protective immunity to rechallenge following immunization with a replication deficient vaccine strain of the parasite (Sukhumavasi et al., 2008). One MyD88 independent sensing pathway occurs through the recognition of the parasite protein cyclophilin-18 by the chemokine receptor CCR5, which promotes IL-12 production by dendritic cells (Aliberti et al., 2003).

Innate recognition of *T. gondii* elicits the production of IL-12 from neutrophils, monocytes, macrophages, and dendritic cells (Goldszmid et al., 2012; Mordue and Sibley, 2003; Scanga et al., 2002; Whitmarsh et al., 2011). Dendritic cells, and the $CD8\alpha^+$ subset of dendritic cells in particular (Reis e Sousa et al., 1997), serve as a critical source of IL-12 during toxoplasmosis, as suggested by the finding that the depletion of dendritic cells during infection leads to a profound defect in systemic IL-12 levels and mortality (Liu et al., 2006). Additionally, mice that lack expression of the transcription factor Batf3, which leads to a loss of $CD8\alpha^+$ dendritic cells, succumb to infection with *T. gondii* but are protected by treatment with recombinant IL-12 (Mashayekhi et al., 2011).

Natural killer cells, another component of the innate immune system, also respond to toxoplasmosis. NK cells from *T. gondii* infected mice have enhanced cytolytic activity against tumor cells and tachyzoites (Hauser and Tsai, 1986), and NK cells exhibit

cytotoxicity against cells that have been infected with the parasite (Persson et al., 2009). Additionally, NK cells produce IFN-γ during infection, which is induced by IL-12 signaling (Gazzinelli et al., 1993b). In mice with T cell deficiencies, this IFN-γ expression provides a limited degree of protection (Goldszmid et al., 2007; Johnson et al., 1993) and promotes the priming of $CD8^+$ T cells in CD4 deficient mice (Combe et al., 2005). Although NK cell IFN-γ is generally not thought to play a critical protective role in mice without T cell defects, some studies have indicated that NK cell depletion leads to increased mortality during the chronic phase of infection (Goldszmid et al., 2007).

Adaptive immune responses

Some of the earliest studies on immunity to *T. gondii* focused on the role of B cells (Sabin and Feldman, 1948), and humoral immune responses were found to be essential during infection. *In vitro* studies have indicated that antibodies can opsonize *T. gondii* or activate complement mediated parasite lysis (Erbe et al., 1991; Konishi and Nakao, 1992; Schreiber and Feldman, 1980; Suzuki and Tsunematsu, 1971; Vercammen et al., 1999), suggesting that these processes may be involved in controlling the parasite during infection. This role is illustrated by the phenotype of μMT mice, which lack B cells, and when challenged with *T. gondii* develop elevated parasite burdens and succumb to infection (Kang et al., 2000). The transfer of parasite-specific antibodies protects B cell deficient mice, suggesting that their susceptibility is due to defects in antibody production (Kang et al., 2000).

Although initial studies on the immune response to toxoplasmosis focused on humoral immunity, $CD4^+$ and $CD8^+$ T cell responses are also essential, and for almost 30 years the role of cell-mediated immunity has been a major area of interest in the field of immunoparasitology (Tait and Hunter, 2009). As described in the preceding section, innate cells produce IL-12 during infection, and they also present antigen. Together these stimuli promote the differentiation of IFN-γ producing effector $CD4^+$ and $CD8^+$ T cells (Aliberti et al., 2004; Szabo et al., 2003). Costimulatory molecules also contribute to this process, as indicated by the finding that signaling through CD28 and ICOS promotes T cell IFN-γ production during toxoplasmosis (Villegas et al., 2002; Wilson et al., 2006). *T. gondii* infection thereby leads to the induction of Th1 polarized $CD4^+$ T cells, which are characterized by the expression of IFN-γ and the transcription factor T-bet (Zhu et al., 2012). Concomitantly with IFN-γ expression, additional functions of $CD4^+$ T cells contribute to the establishment of protective immunity. $CD4^+$ T cells activate macrophage effector mechanisms through their expression of CD40L (Subauste and Wessendarp, 2006), and promote the generation of optimal B cell and $CD8^+$ T cell responses (Johnson and Sayles, 2002; Lutjen et al., 2006). Consistent with these roles in protective immunity, CD4 deficient mice exhibit increased susceptibility to chronic toxoplasmosis (Johnson and Sayles, 2002). Like $CD4^+$ T cells, $CD8^+$ T cells produce IFN-γ following infection (Shirahata et al., 1994) and additionally exhibit perforin-mediated cytotoxicity that promotes the control of parasite burdens (Denkers et al., 1997; Suzuki et al., 2010). Consequently, mice that are deficient for CD8 display increased mortality during chronic

infection (Denkers et al., 1997; Schaeffer et al., 2009). These findings in mouse models are consistent with the increased susceptibility of adult patients with T cell deficiencies to toxoplasmosis.

IFN-γ mediated control of parasite growth

IFN-γ plays a critical role in inducing anti-parasitic effector mechanisms during infection, and mice in which IFN-γ has been depleted are highly susceptible to *T. gondii* (Suzuki et al., 1988). One of the functions of IFN-γ is to drive the expression of inducible nitric oxide synthase (iNOS), which catalyzes the production of nitric oxide. This pathway limits *in vitro* parasite growth in macrophages and microglia (Adams et al., 1990; Chao et al., 1993; Langermans et al., 1992; Scharton-Kersten et al., 1997). Notably, IFN-γ signaling alone is not sufficient to induce optimal levels of nitric oxide production, and other stimuli, such as TNF-α, potentiate the expression of this molecule during infection (Gazzinelli et al., 1993a; Langermans et al., 1992). An additional effect of IFN-γ is to promote the expression indoleamine 2,3 dioxygenase (IDO), which catalyzes tryptophan degradation. Since *T. gondii* is auxotrophic for tryptophan (Sibley et al., 1994), IDO activity limits parasite growth in macrophages and other cell types *in vitro* (Gupta et al., 1994; Heseler et al., 2008; Murray et al., 1989; Pfefferkorn et al., 1986). Critically, IFN-γ also induces the expression of members of the p47 GTPase family. Certain p47 GTPases localize to the parasitophorous vacuole and disrupt its membrane, which can lead to parasite killing and host cell death by a mechanism that is

poorly understood (Hunn et al., 2008; Khaminets et al., 2010; Ling et al., 2006; Martens et al., 2005; Zhao et al., 2009).

In addition to their effects on parasite growth *in vitro*, iNOS, IDO, and the p47 GTPases have been implicated in resistance to *T. gondii in vivo*. Thus, mice that are deficient for iNOS do not have a defect in controlling *T. gondii* replication during the acute stage, but exhibit elevated parasite burdens and increased mortality during chronic infection (Scharton-Kersten et al., 1997). Mice treated with an IDO inhibitor similarly develop elevated parasite burdens and succumb to infection in the chronic phase (Divanovic et al., 2012). Additionally, members of the p47 GTPase family contribute to immune responses *in vivo*, as mice deficient for IGTP or LRG-47 have a major defect in their ability to control acute parasite replication (Collazo et al., 2001; Taylor et al., 2000). In contrast, mice deficient for the p47 GTPase IRG-47 succumb to infection chronically (Collazo et al., 2001). The phenotypes of these mice collectively suggest that different mediators of IFN-γ-induced resistance to *T. gondii* contribute to immunity in different stages of infection. Accordingly, the expression of iNOS and IDO appear to play an important role during chronic toxoplasmosis, while IGTP and LRG-47 are essential in the acute control of parasite growth.

Immune regulation

A Th1 immune response characterized by the production of IFN-γ has a critical role in controlling *T. gondii* infection. Importantly, this inflammatory response can also cause collateral damage to the host and needs to be appropriately regulated to prevent

immune-mediated pathology. The cytokine IL-10 is one of several factors employed by the host to limit the immune response during toxoplasmosis. IL-10 is a pleiotropic cytokine that has multiple immunosuppressive effects, including the downregulation of macrophage cytokine production, the inhibition of costimulatory molecule expression on antigen presenting cells, and the promotion of Treg suppressive activity (Chaudhry et al., 2011; Moore et al., 2001). Notably, IL-10 can also enhance NK cell IFN-γ production and promote B cell expansion (Cai et al., 1999; Moore et al., 2001), indicating that this cytokine has additional activating effects. The importance of IL-10 during toxoplasmosis was revealed in studies with IL-10 deficient mice, which have no defect in controlling parasite burdens but produce elevated levels of IL-12 and IFN-γ, develop severe tissue pathology and succumb to acute infection (Gazzinelli et al., 1996). Mice given an IL-10 receptor-blocking antibody during chronic infection also exhibit increased mortality, indicating that IL-10 also limits pathological responses chronically (Jankovic et al., 2007). Similarly, IL-10 deficient mice that have been treated with anti-parasitic drugs that allow them to survive acute toxoplasmosis develop lethal inflammation during chronic infection (Wilson et al., 2005).

IL-10 deficient mice in which $CD4^+$ T cells have been depleted are able to survive acute toxoplasmosis, indicating that these cells are responsible for the immune-mediated damage that occurs in the absence of IL-10 (Gazzinelli et al., 1996). However, $CD4^+$ T cells, and IFN-γ producing Th1 cells in particular, are also a critical source of IL-10 during toxoplasmosis (Jankovic et al., 2007). Mice in which T cells are unable to express

IL-10 consequently die of immune-mediated pathology when challenged with *T. gondii* (Roers et al., 2004). Additionally, mice deficient for both IL-10 and RAG2 that have been reconstituted with $CD4^+$ T cells that are capable of producing IL-10 survive *T. gondii* infection while mice given IL-10 deficient $CD4^+$ T cells do not (Jankovic et al., 2007). Although $CD4^+$ T cells are an essential source of IL-10 during toxoplasmosis, a number of other cell types produce this cytokine, as indicated by the finding that infected IL-10 deficient SCID mice, which lack B and T cells, exhibit improved survival compared to SCID mice (Neyer et al., 1997). These results suggest that innate sources of IL-10 can limit protective immunity during toxoplasmosis and have detrimental effects in the absence of adaptive immune responses. Subsequent studies have found that NK cells are a major early source of IL-10, which is induced by IL-12 signaling (Perona-Wright et al., 2009). Additional signals that promote IL-10 expression by NK cells are described in Chapter 2 of this thesis. NK cell IL-10 has been suggested to limit IL-12 production during infection, leading to a negative feedback loop that regulates the expression of IL-12 (Perona-Wright et al., 2009).

The cytokine IL-27 is another regulator of immune-mediated pathology following infection. Although IL-27 was initially implicated in promoting the development of Th1 responses, IL-27Rα deficient mice infected with *T. gondii* produce elevated levels of IL-12 and IFN-γ and succumb to immune-mediated pathology during toxoplasmosis (Villarino et al., 2003). The increased mortality of these mice is rescued by $CD4^+$ T cell depletion (Villarino et al., 2003). IL-27 can act directly on $CD4^+$ T cells to promote the

expression of IL-10 and inhibit IL-2 production (Stumhofer et al., 2007; Villarino et al., 2006), which is consistent with its immunoregulatory role during infection. Moreover, IL-27 has immunosuppressive effects on innate cell populations, such as dendritic cells (Mascanfroni et al., 2013).

In addition to IL-10 and IL-27, regulatory T cells have been implicated in limiting infection-induced pathological responses. Challenge with *T. gondii* leads to a dramatic reduction in Treg numbers in multiple tissues (Oldenhove et al., 2009). This Treg crash has been proposed to facilitate the initiation of a protective inflammatory response, but it is also associated with increased tissue damage during infection (Benson et al., 2012; Oldenhove et al., 2009). Thus, mice that have been treated with IL-2 complexes to increase the numbers of Tregs during toxoplasmosis exhibit decreased liver damage (Oldenhove et al., 2009). The Treg population also displays phenotypic changes following infection, such as increases in the expression of IFN-γ, IL-10, T-bet, and CXCR3 (Hall et al., 2012; Oldenhove et al., 2009). T-bet and CXCR3 promote the trafficking of Tregs to sites of inflammation (Koch et al., 2009), and are induced in these cells by IL-27 (Hall et al., 2012). IL-27 also promotes IL-10 production by Tregs, and the effects of IL-27 on these cells may contribute to the infection-induced phenotype of IL-27Rα deficient mice (Hall et al., 2012). Thus, the effects of IL-27 on IL-10 production and Tregs link all three of these immunoregulatory mediators.

In addition to cytokines, certain lipids also limit the immune response to toxoplasmosis. Lipoxin A_4, an eicosanoid that interacts with both the formyl peptide

receptor-like 1 and the aryl hydrocarbon receptor, inhibits IL-12 production by dendritic cells following stimulation with *Toxoplasma* antigen (Aliberti et al., 2002a; Machado et al., 2006). Mice deficient for 5-lipoxygenase, which is required for the synthesis of lipoxin A_4, develop increased brain inflammation and succumb to toxoplasmosis (Aliberti et al., 2002b). Additionally, glucocorticoids limit pathology during infection, as mice that lack expression of the glucocorticoid receptor in T cells exhibit signs of increased tissue damage and mortality during acute toxoplasmosis (Kugler et al., 2013). This phenotype is associated with an elevated frequency of IFN-γ-producing $CD4^+$ T cells in these glucocorticoid receptor deficient mice (Jankovic et al., 2010). Collectively these studies indicate that multiple mediators orchestrate the immunoregulatory response during toxoplasmosis, which is required to prevent lethal immune-mediated damage to the host.

Mucosal immunity

Given the natural oral route of *T. gondii* infection, the local immune response in the gut can be studied upon cyst ingestion. Following oral inoculation, cysts rupture and release parasites that infect or invade in between intestinal epithelial cells, leading to infection of the small intestinal lamina propria (Barragan et al., 2005; Dubey et al., 1997; Speer and Dubey, 1998). *T. gondii* then invades a variety of cells in this region, including macrophages, neutrophils, and T cells (Gregg et al., 2013). Infection results in localized areas of intense parasite replication in the small intestine and damage to the epithelium (Gregg et al., 2013), with the highest parasite burdens found in the distal portions of the small intestine (Dubey et al., 2012). Dissemination of *T. gondii* may occur through the

intestinal lumen (Gregg et al., 2013), or by the transport of the parasite to new sites within infected migratory cells (Coombes et al., 2013).

In certain mouse strains, including C57Bl/6 mice, oral infection with *T. gondii* leads to ileal inflammation that can be lethal following challenge with a high dose of cysts (Dubey et al., 2012; Dubey and Frenkel, 1973; Liesenfeld et al., 1996; McLeod et al., 1984). This ileitis is associated with tissue necrosis and the destruction of villi (Liesenfeld et al., 1996). $CD4^+$ T cell deficient mice are protected from the development of ileal inflammation, indicating that this cell population mediates intestinal pathology during toxoplasmosis (Liesenfeld et al., 1996). A number of other factors, including IFN-γ, TNF-α, nitric oxide, and IL-23 contribute to the development of infection-induced ileitis (Liesenfeld et al., 1999; Liesenfeld et al., 1996; Munoz et al., 2009). Recent studies have indicated that the intestinal microbiota, which is altered in the course of infection (Heimesaat et al., 2006; Molloy et al., 2013), also impacts disease severity. Toxoplasmosis leads to disruption of the epithelial barrier and the translocation of commensal bacteria to the liver and spleen (Hand et al., 2012). This phenomenon is associated with the generation of a commensal-specific T cell response (Hand et al., 2012). Although the role of these cells during infection is unclear, germ-free mice and animals treated with commensal-depleting antibiotics exhibit improved survival and reduced ileitis following oral challenge (Heimesaat et al., 2006), suggesting that microbiota-specific T cells may promote pathological responses. Additionally,

commensal bacteria can act as adjuvants during infection, and promote IL-12 production by dendritic cells (Benson et al., 2009).

A recently described cell population, termed the group 3 innate lymphoid cell (ILC), plays an important role in intestinal immunity and may contribute to the immune response during oral toxoplasmosis. ILCs are characterized by their lymphoid morphology and lack of rearranged antigen receptors (Spits and Cupedo, 2012). A recently proposed system for ILC nomenclature delineated three groups of these cells that are analogous to helper T cell subsets (Spits et al., 2013). Thus, group 1 ILCs, which include natural killer cells, produce IFN-γ, while group 2 ILCs produce Th2-associated cytokines such as IL-5 and IL-13. Group 3 ILCs produce IL-17 or IL-22 and are developmentally dependent on RORγt, mirroring Th17 cells (Spits et al., 2013). This category can be further subdivided into lymphoid tissue inducer cells, which are essential for the development of secondary lymphoid organs, and functionally distinct heterogeneous populations of cells designated ILC3s (Spits et al., 2013). Some of these ILC subsets exhibit plasticity, and the lineage relationships between them are unclear (Walker et al., 2013).

Group 3 ILCs have a number of effects on intestinal immunity. These cells produce IL-22 at the steady state and limit the dissemination of commensal bacteria that normally reside in Peyer's patches and mesenteric lymph nodes (Sonnenberg et al., 2012). Group 3 ILCs are also able to present antigen, but lack the expression of costimulatory molecules, suggesting that these cells act to limit T cell responses

(Hepworth et al., 2013). Accordingly, mice with a RORγt driven deletion of MHC class II exhibit increased T cell activation and intestinal inflammation (Hepworth et al., 2013). This phenotype is dependent on the presence of commensal bacteria, suggesting that ILCs restrict pathological T cell responses against the microbiota (Hepworth et al., 2013). In addition to their effects at the steady state, group 3 ILCs play a role during infection and intestinal inflammation. Thus, these cells are a critical source of protective IL-22 following challenge with *Citrobacter rodentium* (Sonnenberg et al., 2011), and produce IL-22 during infection with *Candida albicans* (De Luca et al., 2010). ILCs have also been implicated in promoting the colonic inflammation induced by *Helicobacter hepaticus* or anti-CD40 treatment in RAG deficient mice (Buonocore et al., 2010), and promote the ulcerative colitis seen in T-bet deficient RAG deficient animals (Powell et al., 2012). It remains unclear whether the functions of group 3 ILCs impact *T. gondii* infection, but data presented in Chapter 3 of this thesis suggest that there are changes in this cell population during toxoplasmosis.

The aryl hydrocarbon receptor and hypoxia inducible factor-1α

The preceding sections highlight the importance of mounting a Th1 immune response to control infection with *T. gondii* while appropriately regulating this inflammation to prevent immune-mediated pathology. Achieving this balance requires immune cells to sense environmental conditions and act accordingly to promote or limit the inflammatory response. Signaling through the aryl hydrocarbon receptor represents one mechanism that allows cells to detect environmental cues, and experiments that were

performed to evaluate how AHR activity influences immunity to *T. gondii* are presented in this thesis.

The AHR mediates cellular responses to numerous structurally diverse compounds including dietary agonists, endogenous molecules, and xenobiotic ligands that are byproducts of industrial processes. Notably, there are striking parallels in the functions of the AHR and the closely related protein hypoxia inducible factor-1α (HIF1α), which is active under low oxygen conditions and allows cells to respond to hypoxia. These similarities suggest that insights gained into the immunological roles for one pathway might shed light on functions of the other. The following sections provide an overview of the physiological roles for the AHR and HIF1α, with a focus on parallels between these two pathways.

Roles in metabolism, cancer, and vascular development

In addition to their effects on immune responses, signals through the AHR and HIF1α have parallel effects on a number of physiological processes. For example, both transcription factors have varying effects on metabolism. AHR activation promotes the metabolism of xenobiotic compounds by inducing the expression of cytochrome p450 monooxygenases as well as other enzymes (Hankinson, 1995). HIF1α induces the expression of genes involved in glycolysis, allowing cells to switch from oxidative to glycolytic metabolism, which promotes adaptation to hypoxic conditions (Seagroves et al., 2001). The AHR and HIF1α have perhaps been most extensively studied because of their roles in cancer biology. AHR activation can promote tumorigenesis via the

induction of enzymes involved in xenobiotic metabolism, which can catalyze the production of carcinogenic compounds (Bersten et al., 2013). However, in certain mouse models, AHR activity has also been shown to have a tumor suppressor role (Bersten et al., 2013). Similarly, HIF1α signaling promotes numerous aspects of carcinogenesis, including cell proliferation, changes in metabolism, and angiogenesis, and the expression of HIF1α is associated with poor prognosis in many human cancers (Bersten et al., 2013; Keith et al., 2012). However, HIF1α can also have tumor suppressor effects in certain contexts (Keith et al., 2012).

Additionally, both the AHR and HIF1α have effects on development in invertebrates and mammals. The *Drosophila* AHR homolog *spineless* contributes to leg and antennae development, as well as the establishment of the retinal mosaic that enables color vision (Duncan et al., 1998; Wernet et al., 2006). The *Drosophila* HIF1α homolog Sima promotes the branching of tracheal tubes that distribute oxygen to the tissues (Centanin et al., 2008). In mice, AHR deficiency results in vascular abnormalities including a patent ductus venosus, which connects the umbilical and portal veins to the inferior vena cava (Lahvis et al., 2000). This feature of the fetal circulatory system typically closes after birth, but adult AHR deficient ($Ahr^{-/-}$) mice retain a patent ductus venosus (Lahvis et al., 2000; Stevens et al., 2009). AHR deficient embryos also exhibit defects in liver perfusion, and additional liver abnormalities are evident in $Ahr^{-/-}$ mice, including decreased liver and hepatocyte size, fatty metamorphosis, and portal fibrosis (Fernandez-Salguero et al., 1995; Harstad et al., 2006; Lahvis et al., 2000; Schmidt et al.,

1996). *Ahr*$^{-/-}$ mice also maintain fetal vascular structures in the eye, and exhibit altered kidney vasculature, cardiac hypertrophy, and increased blood pressure (Fernandez-Salguero et al., 1997; Lahvis et al., 2000; Lund et al., 2003; Thackaberry et al., 2002). HIF1α plays a critical role in vascular development, as HIF1α deficiency leads to embryonic lethality associated with disorganized vascularization of the yolk sac and decreased cephalic vascularization (Iyer et al., 1998; Ryan et al., 1998). This function of HIF1α is analogous to the effects of *Drosophila* Sima on the development of tracheal tubes. In light of these findings it has been suggested that the AHR and HIF1α have opposing roles in vascular development; the AHR contributes to the pruning of vascular structures, which involves removing vessels as the vasculature matures, while HIF1α promotes the sprouting of new vessels (Lahvis et al., 2000). Interestingly, the developmental roles that have been described for *spineless* and Sima mirror the functions of *Drosophila* Toll, which was initially characterized for its effects on development but was subsequently shown to function in innate immunity (Lemaitre et al., 1996). Indeed, in addition to their roles in the interrelated processes of metabolism, tumorigenesis, and vascular development, the AHR and HIF1α have a number of similar effects on innate and adaptive immunity. The following sections provide an overview of the effects of AHR and HIF1α signaling on immune responses to infection, with a focus on parallels between these two pathways.

Structure and evolution of the AHR and HIF1α

The AHR and HIF1α function as heterodimers and are members of the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) superfamily of proteins, which was named after its founding members. Both transcription factors are structurally similar, with an N-terminal bHLH domain that contributes to dimerization and DNA binding, a central PAS domain that also mediates dimerization, and a C-terminal transactivation domain that activates transcription following binding to DNA (Kewley et al., 2004; Stevens et al., 2009). The PAS sequence is an evolutionarily ancient motif that has been identified in proteins expressed by *Archaea*, *Bacteria*, and *Eucarya* (Taylor and Zhulin, 1999). PAS domains are expressed in a variety of proteins, including photoreceptors, circadian clock proteins, chemoreceptors, voltage-gated ion channels, and regulators of embryonic development (Taylor and Zhulin, 1999). Proteins containing PAS sequences therefore mediate cellular responses to numerous environmental cues and developmental signals (Taylor and Zhulin, 1999).

In contrast to the expression of the PAS motif in all three domains of life, bHLH-PAS family members are found primarily in metazoans (Sebe-Pedros et al., 2011). Phylogenetic studies suggest that the AHR and HIF1α in particular arose from the duplication of an ancestral gene following the divergence of sponges but before the ancestor to Placozoa, Cnidarians, and Bilaterians (Loenarz et al., 2011; Reitzel et al., 2014). Accordingly, AHR and HIF1α homologs have not been identified in sponges, but are present in some of the simplest metazoans, including placozoans, coral, sea

anemones, and nematodes (Hampton-Smith and Peet, 2009; Loenarz et al., 2011; Reitzel et al., 2014; Rytkonen and Storz, 2011; Rytkonen et al., 2011; Taylor and McElwain, 2010). Interestingly, invertebrate AHR homologs do not bind to canonical xenobiotic AHR ligands, suggesting that this function for the AHR evolved in chordates (Hahn, 2002; Reitzel et al., 2014). Although it is unclear whether the AHR affects immunity in invertebrates, the HIF1α homolog HIF-1 affects the expression of genes involved in *Caenorhabditis elegans* host defense and mutants with increased HIF-1 activity are more susceptible to infection with *Staphylococcus aureus* (Luhachack et al., 2012). The conservation of HIF1α in a broad range of animals that occupy diverse habitats reflects the importance of oxygen sensing in the evolution of metazoans, as the maintenance of oxygen homeostasis is essential for multicellular organisms that rely on oxygen for the process of energy generation (Rytkonen et al., 2011; Semenza, 2007). Thus, the AHR and HIF1α are evolutionarily conserved transcription factors with fundamental roles in development that also contribute to immune responses.

Signaling through the AHR and HIF1α

Transcriptional activity of the AHR is induced following ligand binding (Figure 1.2). In the absence of an agonist, the AHR is localized to the cytosol in a complex with its chaperone proteins. Upon ligand binding, the AHR complex translocates into the nucleus where the AHR interacts with the AHR nuclear translocator (ARNT) to form a competent transcription factor. The AHR-ARNT heterodimer binds to dioxin responsive elements, and mediates the transcription of a number of different genes (Stevens et al.,

2009). In addition to its transcriptional effects, the AHR has E3 ubiquitin ligase activity, indicating that this transcription factor can also alter intracellular protein levels by modulating protein degradation (Ohtake et al., 2007).

Numerous structurally diverse synthetic and physiologic molecules serve as AHR ligands. Classical xenobiotic AHR agonists consist of halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons, but additional ligands with varying structures have been identified (Denison and Nagy, 2003). These molecules include the byproducts of industrial processes such as benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin), the prototypical AHR agonist. Dioxin is a potent toxin whose effects include wasting, thymic involution, liver toxicity, porphyria, and carcinogenesis (Poland and Knutson, 1982). AHR ligands are also found in cigarette smoke, vehicle exhaust, and smoke from burning wood and charcoal, suggesting that airborne molecules could be a source of AHR agonists in the lungs and skin (Kasai et al., 2006). Ligands for the AHR can also be obtained through the diet, as numerous plant compounds activate this transcription factor, making the intestines another major site of exposure to AHR agonists (Denison and Nagy, 2003). The effects of the AHR during intestinal infection are described in more detail in Chapter 3 of this thesis.

While there are numerous exogenous sources of AHR ligands, the roles for this transcription factor in development suggested that AHR agonists might also be produced endogenously. The effects of exposure to certain xenobiotic AHR ligands have been studied extensively, but less is understood about the role of endogenous compounds that

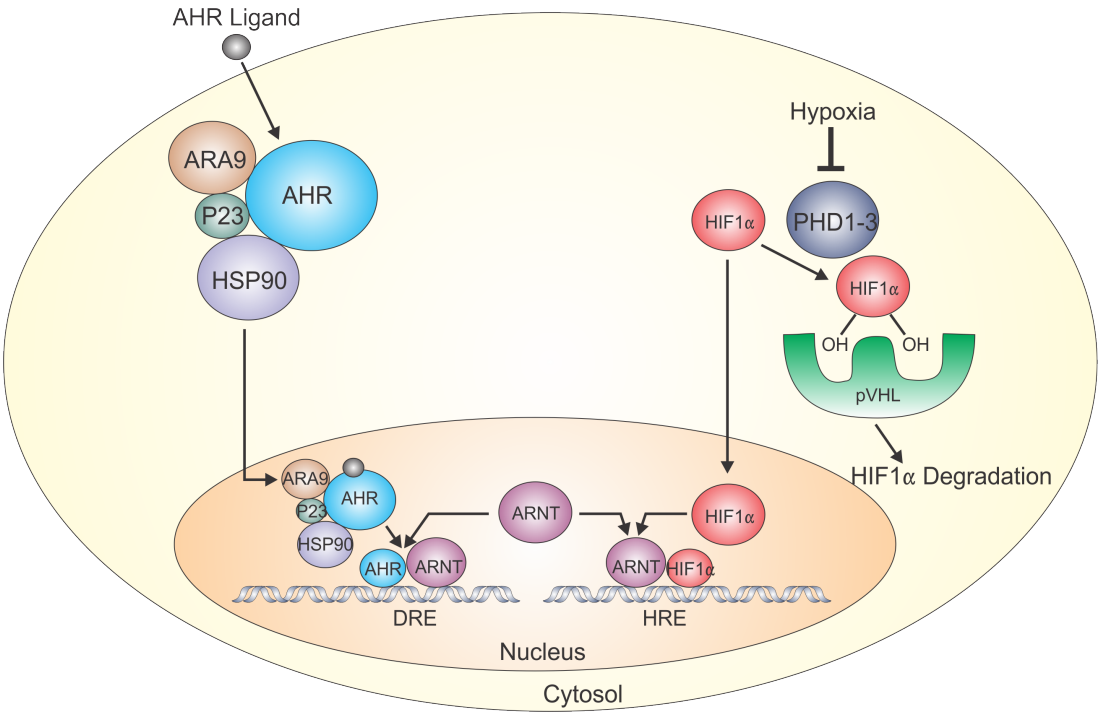

Figure created by Deborah Argento

Figure 1.2: Signaling through the AHR and HIF1α.

In its inactive form, the AHR is localized to the cytosol in a complex with the chaperone proteins ARA9, p23, and HSP90. Upon ligand binding, this complex translocates to the nucleus where the AHR heterodimerizes with ARNT, forming a competent transcription factor that binds to dioxin response elements (DREs).

Under normoxic conditions, HIF1α is hydroxylated by the PHDs, leading to its degradation by pVHL. Hypoxia inhibits the activity of the PHDs, leading to the stabilization of HIF1α and its translocation into the nucleus. HIF1α then heterodimerizes with ARNT, forming a transcription factor that binds to hypoxia response elements (HREs).

can activate the AHR. In the skin, the high affinity AHR ligand 6-formylindolo[2,3-b]carbazole (FICZ) is thought to form from tryptophan following stimulation with ultraviolet light (Fritsche et al., 2007; Rannug et al., 1995; Wincent et al., 2009). Molecules produced during the host response to infection, such as L-kynurenine, can also serve as AHR ligands. L-kynurenine is one of the products of IDO induced tryptophan degradation, and notably, this compound has been detected in the serum, brain, and other tissues following infection with *T. gondii* (Notarangelo et al., 2014; Silva et al., 2002). Additionally, microorganisms can produce AHR ligands. For example, gut resident commensal bacteria can synthesize AHR agonists (Zelante et al., 2013). Compounds that activate the AHR are also generated by *Malassezia* yeasts, and have been found in diseased human skin (Gaitanis et al., 2008; Magiatis et al., 2013; Vlachos et al., 2012). Moreover, extracts from the parasite *Toxoplasma gondii* contain peptides resembling plant lipoxygenases that may allow *T. gondii* to catalyze the production of lipoxin A4 (Bannenberg et al., 2004), an AHR ligand that can also be produced by the host (Denison and Nagy, 2003). Given the multiple sources of AHR agonists, signaling through this transcription factor may be a way of sensing environmental and dietary molecules as well as ligands produced by pathogens or the host in response to infection.

HIF1α is transcriptionally active in environments with low oxygen (Figure 1.2). Under normoxic conditions, oxygen and iron sensitive prolyl hydroxylases (PHDs) hydroxylate HIF1α, allowing it to be recognized by an E3 ubiquitin ligase, a complex that includes von-Hippel-Lindau protein (pVHL), targeting HIF1α for proteasomal

degradation (Greer et al., 2012). VHL is a tumor suppressor, and mutations in this gene cause VHL disease, which is characterized by the development of benign and malignant tumors in patients (Bader and Hsu, 2012). Under hypoxic conditions, the PHDs, which serve as oxygen sensors, are less active, allowing for the stabilization of HIF1α protein. This process leads to HIF1α translocation to the nucleus, where like the AHR it binds to ARNT and forms a competent transcription factor (Greer et al., 2012). The genes induced by HIF1α allow adaptation to hypoxic conditions by increasing erythropoiesis in part through the induction of erythropoietin, enhancing angiogenesis via the expression of genes such as vascular endothelial growth factor, increasing glucose uptake through the induction of glucose transporters, and promoting glycolysis in part by increasing the expression of glycolytic enzymes (Semenza, 2009). Notably, HIF1α can also be induced through stimulation with cytokines, growth factors, or microbial products, which is consistent with a role for this transcription factor in immune responses.

The epidermis and intestine are thought to be relatively hypoxic, suggesting that the effects of HIF1α activity may be particularly significant at these barrier sites (Karhausen et al., 2004; Rezvani et al., 2011). Importantly, inflammation is also associated with the generation of local hypoxic conditions that are thought to be caused by disruptions in blood flow as well as the metabolic demands of inflammatory cells and pathogens at sites of infection (Gale and Maxwell, 2010; Nizet and Johnson, 2009). Accordingly, challenge with a variety of pathogens leads to the formation of hypoxic regions in infected tissues (Araujo et al., 2012; Araujo et al., 2010; Grahl et al., 2011;

Heng et al., 2011; Hirota et al., 2010; Peyssonnaux et al., 2005). In order to perform their functions, immune cells need to travel through and operate in different environments with varying oxygen tensions, such as blood vessels, secondary lymphoid organs, and inflamed tissues (Gale and Maxwell, 2010). The activity of HIF1α may play a role in allowing cells to adapt to these diverse environments.

Roles for the AHR and HIF1α in innate immunity

The previous sections highlight the roles of the AHR and HIF1α in environmental sensing that can lead to changes in metabolism, which are recently developed topics of interest in the field of immunology. These transcription factors are widely expressed in immune cells including hematopoietic stem cells, T cell subsets, macrophages, dendritic cells, and NK cells, and affect multiple aspects of innate immunity. One of the functions of both the AHR and HIF1α is to modulate cytokine production. Thus, in response to LPS, AHR deficient macrophages produce decreased levels of IL-10 and elevated amounts of the cytokines IL-6, IL-12, and TNF-α, indicating that the AHR promotes IL-10 expression but limits the production of other cytokines by these cells (Kimura et al., 2009). In contrast, mice with a targeted HIF1α deletion in macrophages have impaired production of IL-12 and TNF-α following LPS injection *in vivo* (Peyssonnaux et al., 2007). Mice with HIF1α deficient macrophages consequently exhibit improved survival during LPS-induced sepsis (Peyssonnaux et al., 2007), but $Ahr^{-/-}$ mice are more susceptible to this challenge (Kimura et al., 2009). HIF1α also promotes macrophage IL-1β expression following treatment with LPS (Tannahill et al., 2013). Interestingly, LPS

stimulation promotes macrophage expression of both the AHR and HIF1α (Kimura et al., 2009; Peyssonnaux et al., 2007), raising the question of how opposing signals from these two transcription factors are integrated to ultimately determine the cytokine production response.

Signaling through the AHR and HIF1α also affects cytokine production by other innate cells. AHR deficient dendritic cells exhibit defects in IL-10 production in response to LPS (Nguyen et al., 2010). Stimulation with different AHR ligands has varying effects on dendritic cell cytokine production, with some agonists promoting the production of IL-6, IL-12, and TNF-α while certain ligands inhibit the expression of these proteins (Bankoti et al., 2010b; Benson and Shepherd, 2011; Hwang et al., 2007; Ilchmann et al., 2012; Quintana et al., 2010; Vlachos et al., 2012; Yeste et al., 2012). Although HIF1α does not impact the production of these cytokines by dendritic cells (Kohler et al., 2012), HIF1α deficient dendritic cells do exhibit defects in their ability to express type I interferons and IL-22 (Kohler et al., 2012; Wobben et al., 2013). Different AHR agonists also act to either promote or inhibit the dendritic cell expression of the costimulatory molecules CD80 and CD86 (Bankoti et al., 2010a; Bankoti et al., 2010b; Benson and Shepherd, 2011; Hauben et al., 2008; Ilchmann et al., 2012; Lee et al., 2007; Simones and Shepherd, 2011; Vlachos et al., 2012; Vogel et al., 2008; Vogel et al., 2013), and HIF1α promotes the expression of these markers (Jantsch et al., 2008; Spirig et al., 2010). These effects of the AHR and HIF1α are consistent with a role for these transcription factors in innate sensing. Although it is unclear whether HIF1α activity in dendritic cells has an

impact during infection, dioxin treatment impairs the ability of these cells to activate antigen specific $CD8^+$ T cells (Jin et al., 2010; Jin et al., 2014). The effects of AHR signaling on cytokine production by another population of innate cells, natural killer cells, are detailed in Chapter 2 of this thesis.

The AHR and HIF1α also act to promote antimicrobial effector mechanisms in innate cells. Signaling through the AHR enhances dendritic cell expression of IDO (Nguyen et al., 2010; Vogel et al., 2008). This AHR-mediated IDO expression has been implicated in the ability of dendritic cells to promote Treg differentiation (Nguyen et al., 2010; Vogel et al., 2008). However, as previously described, IDO can also deprive pathogens that are auxotrophic for tryptophan of this essential amino acid, leading to antimicrobial effects. In addition to *T. gondii*, species of *Chlamydia* and *Leishmania* are tryptophan auxotrophs (Divanovic et al., 2012; Ouellette et al., 2006). AHR activity additionally promotes the production of reactive oxygen species by macrophages following infection with *Listeria monocytogenes*, and AHR deficient macrophages are impaired in their ability to control bacterial growth. Accordingly, $Ahr^{-/-}$ mice are more susceptible to infection with *L. monocytogenes* and develop elevated bacterial burdens (Kimura et al., 2014; Shi et al., 2007). The activity of HIF1α increases macrophage phagocytosis under hypoxic conditions (Anand et al., 2007). HIF1α deficient macrophages exhibit reduced iNOS expression and NO_2^- production, which is associated with a defect in their ability to kill group A *Streptococcus* (Peyssonnaux et al., 2005). Conversely, VHL deficient macrophages, which express increased levels of HIF1α, have

enhanced killing ability (Peyssonnaux et al., 2005). HIF1α also promotes the production of antimicrobial peptides by neutrophils, and accordingly, mice with a myeloid cell specific deletion of HIF1α develop increased bacterial burdens and larger lesions following infection with group A *Streptococcus* (Peyssonnaux et al., 2005). These studies indicate that the AHR and HIF1α can promote a number of pathways in innate cell populations that act to limit pathogen growth.

In addition to its roles in inducing cytokine production and antimicrobial effector mechanisms, HIF1α has critical effects on the metabolism and survival of innate cells. Macrophages and neutrophils rely heavily on glycolysis to generate ATP, and HIF1α contributes to the glycolytic ability of these cells (Cramer et al., 2003). Thus, HIF1α deficient macrophages and neutrophils contain decreased levels of ATP even in normoxic conditions (Cramer et al., 2003). This metabolic defect is associated with impairments in macrophage migration and invasive ability as well as decreased inflammatory responses *in vivo* in mice with a myeloid cell deletion of HIF1α (Cramer et al., 2003). HIF1α also promotes neutrophil survival under hypoxia, and concordantly, neutrophils from VHL patients exhibit decreased apoptosis (Walmsley et al., 2006; Walmsley et al., 2005). It is unclear whether the metabolic effects of AHR signaling influence innate cell function. However, this transcription factor has been suggested to protect against macrophage cell death during infection by inducing expression of the apoptosis inhibitor of macrophages (Kimura et al., 2014), a secreted protein that promotes resistance to cell death (Miyazaki et al., 1999).

Effects of AHR and HIF1α signaling on adaptive immunity

Like cells of the innate immune system, adaptive immune cells need to function in diverse tissue environments to effectively respond to infection, and the AHR and HIF1α have a role in allowing these cells to sense and adapt to different settings. Both of these transcription factors are most highly expressed in the Th17 subset of $CD4^+$ T cells, and promote Th17 differentiation, IL-17 production, and IL-22 expression (Dang et al., 2011; Kimura et al., 2008; Quintana et al., 2008; Shi et al., 2011; Veldhoen et al., 2008). In spite of its effects on Th17 cells, AHR activity does not affect the expression of RORγt, the characteristic transcription factor expressed by these cells (Veldhoen et al., 2009; Veldhoen et al., 2008). In contrast, HIF1α enhances Th17 differentiation in part by promoting the transcription of RORγt (Dang et al., 2011). HIF1α may have additional effects on Th17 polarization by promoting glycolysis, as inhibiting this process also impedes Th17 development (Shi et al., 2011). The significance of AHR and HIF1α mediated effects on Th17 cells has been studied most extensively in the context of autoimmunity. Thus, *Ahr*$^{-/-}$ mice or those with a T cell specific deletion of HIF1α are less susceptible to experimental autoimmune encephalomyelitis, which is associated with decreased Th17 responses (Dang et al., 2011; Shi et al., 2011).

In addition to their proinflammatory effects on Th17 cells, the AHR and HIF1α have been implicated in the induction of regulatory T cell responses, but there are conflicting reports on the role of these transcription factors in Tregs. AHR activation by particular ligands, such as dioxin or kynurenine, promotes Treg polarization (Mezrich et

al., 2010; Quintana et al., 2008), whereas other ligands, such as FICZ, induce Th17 cells (Quintana et al., 2008). Although these findings indicate that different AHR agonists can mediate varying effects, an additional study found that FICZ and dioxin promote Th17 cells and Tregs (Kimura et al., 2008). The AHR mediates some of its effects on Tregs by binding to the FoxP3 promoter and inducing the expression of this transcription factor (Quintana et al., 2008). HIF1α has been shown to similarly promote the expression of FoxP3 and the induction of Tregs, and enhance Treg suppressive function (Ben-Shoshan et al., 2008; Clambey et al., 2012). However, contrasting studies have indicated that HIF1α inhibits Treg generation, possibly by physically interacting with FoxP3 and targeting it for degradation (Dang et al., 2011; Shi et al., 2011). The discrepancies in these findings suggest that the observed effects of the AHR and HIF1α on Tregs depend on experimental conditions.

The effects of the AHR and HIF1α on T cells influence responses to microbial challenge. Thus, AHR activation with dioxin limits the inflammatory damage caused by ocular herpes simplex virus infection (Veiga-Parga et al., 2011). This protection is associated with decreased cellular infiltration into the cornea and an elevated ratio of Tregs to effector T cells due to the increased apoptosis of effector T cells following dioxin treatment (Veiga-Parga et al., 2011). In a model of cecal ligation and puncture, T cell specific deletion of HIF1α leads to the increased production of IFN-γ by $CD8^+$ T cells, elevated expression of TNF-α and IL-6, decreased bacterial burdens, and improved survival (Thiel et al., 2007). Similarly, mice with a T cell targeted knockdown of a

HIF1α isoform display increased proinflammatory cytokine production and decreased IL-10 expression following cecal ligation and puncture, which is associated with improved survival and decreased bacterial loads (Georgiev et al., 2013). These studies indicate that the activity of the AHR and HIF1α can suppress T cell responses during infection.

In addition to its role in acute responses, HIF1α has effects during chronic infection. In mice infected with LMCV clone 13, enhanced HIF1α activity due to a T cell specific VHL deletion leads to increased susceptibility to infection as a result of $CD8^+$ T cell induced immunopathology (Doedens et al., 2013). VHL deficient T cells exhibit resistance to exhaustion, and produce elevated levels of IFN-γ and TNF-α three weeks post-infection (Doedens et al., 2013). Additional studies are needed to determine whether the AHR has a similar role in T cell responses to chronic infection. However, during toxoplasmosis, infected *Ahr*$^{-/-}$ mice were shown to have decreased parasite burdens and increased levels of serum TNF-α (Sanchez et al., 2010). Although the role of T cells in this phenotype is unclear, these effects are consistent with a role for the AHR in limiting immune-mediated pathology in this setting. Studies that were performed to further evaluate the role of the AHR during chronic toxoplasmosis are described in Chapter 3 of this thesis.

The AHR and HIF1α also have effects on B cells, and AHR deficient chimeric mice exhibit defects in B cell development (Thurmond et al., 2000). Conversely, treatment with AHR ligands such as dioxin has suppressive effects on B cell development and inhibits antibody production (Schneider et al., 2009; Sherr and Monti, 2013; Yoshida

et al., 2012). Thus, following challenge with influenza, mice treated with dioxin produce decreased levels of virus-specific IgG, and elevated amounts of IgA (Lawrence and Vorderstrasse, 2004). HIF1α has also been implicated in B cell development, as HIF1α deficient chimeric mice display abnormalities in B cell populations and increased production of autoantibodies (Kojima et al., 2002). Additional studies are needed to assess the extent to which the effects of the AHR and HIF1α on B cells influence the development of protective immunity to infection or immunization.

Roles for the AHR and HIF1α at barrier surfaces

The sections above highlight that the AHR and HIF1α have a number of effects on the innate and adaptive immune system, suggesting important roles for these transcription factors at barrier surfaces such as the intestines and skin, which are rich in immune cell populations. Studies indicating that aging *Ahr*$^{-/-}$ mice develop colonic inflammation and rectal prolapses associated with *Helicobacter hepaticus* established that the AHR has important effects on intestinal immunity (Fernandez-Salguero et al., 1997). Subsequent work has shown that AHR activity affects the maintenance of various immune cell populations in the gut. Thus, *Ahr*$^{-/-}$ mice have a reduction in intestinal intraepithelial lymphocytes (Li et al., 2011) and a decreased population of intestinal RORγt expressing innate lymphoid cells (ILCs), as well as defects in IL-22 production by ILCs (Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012). This reduced ILC population is specific to the lamina propria, as *Ahr*$^{-/-}$ mice have normal populations of RORγt^{+} ILCs in other sites, such as the Peyer's patches (Lee et al., 2012).

The AHR dependent alterations in intestinal cell populations have consequences during infection and influence the intestinal microbiome. IL-22 elicits the production of antimicrobial peptides, and in the small intestine of *Ahr*$^{-/-}$ mice the expression of these is impaired (Qiu et al., 2012). ILCs and IL-22 promote resistance to infection with *Citrobacter rodentium*, and consequently *Ahr*$^{-/-}$ mice are highly susceptible to infection with this enteric pathogen (Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012). In addition to its effects on bacterial infection, AHR activity in the intestine impacts the populations of commensal bacteria that inhabit the gut. Although the relative composition of the bacterial flora in *Ahr*$^{-/-}$ mice is similar to wild-type controls when evaluated at the phylum level, *Ahr*$^{-/-}$ mice have elevated bacterial loads in the small intestine (Li et al., 2011; Qiu et al., 2012). *Ahr*$^{-/-}$animals also have increased intestinal levels of Th17 promoting segmented filamentous bacteria as a result of their defects in IL-22 expression (Qiu et al., 2013). This dysbiosis is associated with the increased susceptibility of *Ahr*$^{-/-}$ mice to experimental colitis (Li et al., 2011), and additional studies are needed to determine whether these altered commensal populations affect responses to infection. *Ahr*$^{-/-}$ mice have also been shown to develop cecal tumors, but germ free *Ahr*$^{-/-}$ mice are protected from tumor formation and intestinal inflammation, demonstrating that the microbiota also contributes to this phenotype (Ikuta et al., 2013).

Interestingly, mice fed a diet thought to be low in AHR agonists have reduced populations of intestinal intraepithelial lymphocytes and ILCs, indicating that the maintenance of these populations at the steady state depends on dietary AHR ligands

(Kiss et al., 2011; Li et al., 2011). Further investigation is needed to determine whether dietary ligands are also essential during infection, when compounds that activate the AHR may be produced by other sources. Additionally, vitamin A metabolism represents an underexplored pathway through which dietary compounds and AHR signaling may impact immune responses in the intestine. A vitamin A metabolite, retinoic acid, affects multiple aspects of immunity, including gut homing, Treg and Th17 differentiation, and the size of the RORγt^{+} ILC population in the gut (Spencer and Belkaid, 2012; Spencer et al., 2014). *Ahr*$^{-/-}$ mice and mice treated with dioxin exhibit perturbations in vitamin A metabolism (Andreola et al., 1997), suggesting that AHR mediated effects on this pathway could impact immune responses.

HIF1α has a number of effects on intestinal immunity and barrier function. Consistent with a role for this transcription factor in promoting the generation of Th17 cells, mice with a T cell specific deletion of HIF1α have a reduced population of Th17 cells in the colonic lamina propria and an increased frequency of Tregs (Dang et al., 2011). Concordantly, these mice develop more severe intestinal inflammation following treatment with dextran sodium sulfate (Higashiyama et al., 2012). However, in agreement with studies indicating that HIF1α promotes Treg suppressive function, RAG deficient mice are more susceptible to colitis induced by the transfer of CD45RB^{high} CD4^{+} T cells when they are given HIF1α deficient Tregs (Clambey et al., 2012), suggesting that these effects may be context dependent.

In addition to its role in T cell responses in the gut, HIF1α has a number of effects in the intestinal epithelium. Signaling through this transcription factor in epithelial cells promotes the expression of factors that enhance barrier function such as intestinal trefoil factor, ectonucleotidases, and mucin (Furuta et al., 2001; Louis et al., 2006; Synnestvedt et al., 2002). In mice with a HIF1α deficiency in the intestinal epithelium, these effects are associated with impaired intestinal barrier function and increased susceptibility to chemically induced colitis (Karhausen et al., 2004). The deletion of VHL in epithelial cells conversely protects against colitis (Karhausen et al., 2004). Interestingly, neutrophil respiratory burst activity generates hypoxic microenvironments in the intestine, inducing HIF1α signaling in intestinal epithelial cells (Campbell et al., 2014). Mice deficient for the respiratory burst develop exacerbated experimental colitis, indicating that neutrophil-induced HIF1α activity is protective in this model (Campbell et al., 2014) and raising the question of whether this pathway also plays a role during enteric infection. Notably, contrasting studies using a different model of chemically induced colitis found that epithelial VHL deficiency in the intestine increases inflammation (Shah et al., 2008).

Stimulation with intestinal bacteria can also promote the expression of epithelial HIF1α (Hartmann et al., 2008; Koury et al., 2004; Mimouna et al., 2011), and the activity of this transcription factor plays a role during intestinal infection. Thus, mice with a targeted deletion of HIF1α in the intestinal epithelium exhibit increased mortality duirng infection with *Yersinia enterocolitica* (Hartmann et al., 2008). Following exposure to *Clostridium difficile* toxin, these mice also develop increased inflammation and tissue

damage characterized by disruption of the epithelial barrier (Hirota et al., 2010). Conversely, treatment with a PHD inhibitor, which leads to increased HIF1α levels, protects against this challenge (Hirota et al., 2010).

HIF1α also has functions in the skin. Mice with a keratinocyte specific deletion of HIF1α develop more severe skin lesions following infection with Group A *Streptococcus* and elevated bacterial burdens. This phenotype is associated with decreased expression of the antimicrobial peptide cathelicidin by keratinocytes with a knockdown of HIF1α (Peyssonnaux et al., 2008). Complementary work has shown that treatment with a HIF1α agonist or a pharmacological compound that stabilizes HIF1α leads to increased resistance to *Staphylococcus aureus* (Okumura et al., 2012; Zinkernagel et al., 2008). Although it remains unclear whether AHR signaling plays a role during skin infection, the AHR has multiple effects at this barrier surface. Thus, chloracne, a condition involving the development of pustules, is characteristic of human exposure to dioxin. In studies utilizing an animal model of AHR hyperactivation, mice expressing a constitutively active form of the AHR in keratinocytes develop inflammatory skin lesions and express increased levels of antimicrobial peptides in the skin (Tauchi et al., 2005). Conversely, *Ahr*$^{-/-}$ mice have been shown to develop skin lesions as they age (Fernandez-Salguero et al., 1997), and these animals have decreased skin intraepithelial lymphocyte populations (Li et al., 2011). Taken together these studies show that the AHR and HIF1α have multiple effects on immunity in the intestines and the skin, which is consistent with

the notion that these are relatively hypoxic tissues that are also major sites of exposure to AHR ligands.

Exploitation of the AHR and HIF1α by pathogens

Although the AHR and HIF1α provide the immune system with the ability to respond to environmental conditions, these pathways can also be exploited by pathogens. The host cell metabolic state may be of particular importance to intracellular pathogens, and changes in host metabolism could limit the growth of these microbes through a variety of mechanisms. Studies describing the subversion of AHR signaling by microorganisms are limited, but treatment with AHR ligands has been shown to promote the reactivation of HIV-1 and other viruses in cell culture, indicating that AHR activity can promote viral replication (Burleson et al., 1996; Inoue et al., 2012; Murayama et al., 2002; Ohata et al., 2003). Signaling through HIF1α can have similar effects on the propagation of viruses, as HIF1α associates with the HIV-1 long terminal repeat and promotes viral gene transcription (Deshmane et al., 2009). Hypoxia also leads to the increased transcription of LCMV genes and increased LCMV protein production in a HIF1α dependent manner (Tomaskova et al., 2011). Intracellular parasites also take advantage of HIF1α activity to promote their growth. This was demonstrated by studies with *T. gondii* showing that this parasite stabilizes HIF1α by decreasing the abundance of PHD2 (Wiley et al., 2010). *T. gondii* growth is impaired *in vitro* in HIF1α deficient cells, and the parasite exhibits defects in division when grown in HIF1α deficient cells under hypoxic conditions (Spear et al., 2006). These studies also highlight the importance of

considering oxygen concentration as an experimental condition when studying the role of HIF1α, which can have more dramatic effects during hypoxia. Infection with *Leishmania donovani* also leads to increased HIF1α activity in a macrophage cell line, and HIF1α knockdown in infected cells leads to decreased intracellular *L. donovani* growth (Singh et al., 2012). Pathogens often target and inhibit cellular pathways involved in innate recognition to promote their survival. In contrast, these studies demonstrate that the AHR and HIF1α, which are employed by the host to respond to the environment during infection, can also be utilized by pathogens to enhance their survival and replication. However, not all microbes use HIF1α to their advantage, as indicated by studies showing that *Chlamydia pneumoniae* actively degrades HIF1α during later phases of infection, which is associated with host cell resistance to apoptosis (Rupp et al., 2007).

Conclusion

Signaling through the AHR allows cells to sense various environmental and endogenously produced compounds, and this transcription factor has multiple proinflammatory and immunoregulatory effects on immune responses. Notably, AHR agonists such as L-kynurenine and lipoxin A_4 are generated during infection with *T. gondii*. In order to successfully respond to challenge with this parasite, the immune system needs to establish an effective balance between inflammation that controls *T. gondii* growth and regulatory responses that limit immune-mediated pathology. Studies that were performed to evaluate how AHR activity contributes to these responses are described in the following chapters of this thesis.

CHAPTER 2: THE ARYL HYDROCARBON RECEPTOR PROMOTES IL-10 PRODUCTION BY NATURAL KILLER CELLS

Abstract

The cytokine IL-10 has an important role in limiting inflammation in many settings, including toxoplasmosis. In these studies, an IL-10 reporter mouse was used to identify the sources of this cytokine following challenge with *Toxoplasma gondii.* During infection, multiple cell types expressed the IL-10 reporter, but natural killer cells were a major early source of this cytokine. These IL-10 reporter^{+} NK cells expressed high levels of the IL-12 target genes T-bet, KLRG1, and IFN-γ, and IL-12 depletion abrogated reporter expression. However, IL-12 signaling alone was not sufficient to promote NK cell IL-10 and activation of the aryl hydrocarbon receptor (AHR) was also required for maximal IL-10 production. NK cells basally expressed the AHR, relevant chaperone proteins, and the AHR nuclear translocator (ARNT), which heterodimerizes with the AHR to form a competent transcription factor. *In vitro* studies revealed that IL-12 stimulation increased NK cell AHR levels and the AHR and ARNT were required for optimal production of IL-10. Additionally, NK cells isolated from *T. gondii*-infected *Ahr*$^{-/-}$ mice had impaired expression of IL-10, which was associated with increased resistance to this infection. Together, these data identify the AHR as a critical cofactor involved in NK cell production of IL-10.

Introduction

Toxoplasma gondii is an apicomplexan parasite that induces highly Th1 polarized immune responses characterized by the production of IL-12 and IFN-γ, which are required to control parasite growth (Gazzinelli et al., 1994; Scharton-Kersten et al., 1996). Appropriate regulation of this Th1 response is critical for surviving infection, as illustrated by reports that IL-10$^{-/-}$ mice infected with *T. gondii* control parasite burdens but succumb to immune-mediated pathology (Gazzinelli et al., 1996; Wilson et al., 2005). Although CD4^{+} T cells contribute to this pathology, these cells are also a critical source of IL-10 during toxoplasmosis. Consequently, mice in which T cells are unable to express IL-10 also develop immune-mediated tissue pathology when challenged with *T. gondii* (Roers et al., 2004). Additionally, IL-10$^{-/-}$ RAG2$^{-/-}$ mice reconstituted with CD4^{+} T cells that are capable of producing IL-10 survive *T. gondii* infection while their counterparts given IL-10$^{-/-}$ CD4^{+} T cells do not (Jankovic et al., 2007). Although these results indicate that CD4^{+} T cells are an important source of IL-10 that protects against fatal immune-mediated pathology during toxoplasmosis, a number of other cell types produce IL-10 during this infection. The biological relevance of innate sources of IL-10 was suggested by the finding that IL-10$^{-/-}$ SCID mice, which lack B and T cells, exhibit improved survival following *T. gondii* infection compared to SCID mice (Neyer et al., 1997). Recent studies have shown that NK cells can produce IL-10 and are a biologically relevant source of this cytokine during toxoplasmosis (Perona-Wright et al., 2009). NK cells are also a source of IL-10 in other murine models of infection, as NK cell IL-10

promotes increased parasite burdens during visceral leishmaniasis and limits the magnitude of the $CD8^+$ T cell response during murine cytomegalovirus infection (Lee et al., 2009; Maroof et al., 2008; Perona-Wright et al., 2009). Together, these reports indicate major biological functions for NK cell derived IL-10 in a variety of viral, bacterial, and parasitic infections.

Recent studies have identified effects of aryl hydrocarbon receptor (AHR) signaling on multiple aspects of the immune response, including IL-10 production (Apetoh et al., 2010). The AHR is a ligand-activated transcription factor that interacts with a structurally diverse array of ligands, comprising synthetic compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and endogenous molecules, which include certain tryptophan and arachidonic acid metabolites (Denison and Nagy, 2003). AHR activity was initially studied for its role in mediating tetrachlorodibenzo-p-dioxin-induced toxicity. However a number of recent studies have identified multiple effects of AHR signaling on the immune system, most notably in Th17 cells and innate lymphoid cells (Kimura et al., 2008; Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012; Quintana et al., 2008; Stockinger et al., 2009). In contrast to its effects in promoting the expression of the effector cytokines IL-22 or IL-17 in these cells, the AHR has also been shown to promote the production of IL-10. Thus, in type 1 regulatory T cells, the AHR interacts with the transcription factor c-Maf to promote IL-10 expression (Apetoh et al., 2010). *Ahr*$^{-/-}$ dendritic cells and macrophages also exhibit defects in IL-10 production (Kimura et al., 2009; Nguyen et al., 2010). Importantly, AHR activity appears to play a role in the

response to *T. gondii,* as *Ahr*$^{-/-}$ mice show increased susceptibility to this challenge, possibly due to immune-mediated pathology (Sanchez et al., 2010).

The studies presented here utilize an IL-10 reporter mouse to characterize the cell types that express IL-10 during toxoplasmosis and identified NK cells as a major source of this regulatory factor. During acute infection, NK cells that expressed the IL-10 reporter had higher levels of the IL-12 target genes IFN-γ, T-bet, and KLRG1 than reporter negative NK cells and IL-12 depletion abrogated NK cell IL-10 reporter expression. However, *in vitro* studies using NK cells suggested that IL-12 was not sufficient to induce IL-10 and that AHR activation contributed to optimal IL-10 production. NK cells basally expressed *Ahr* transcripts and these were increased following stimulation with IL-12. IL-10 production by *in vitro* expanded NK cells (lymphokine activated killer cells, or LAKs) was enhanced by augmenting AHR activity and decreased in the presence of AHR inhibitors. LAKs genetically deficient for the AHR or the AHR nuclear translocator (ARNT), which dimerizes with the AHR to form a competent transcription factor, were impaired in their ability to produce IL-10. Finally, NK cells isolated from *Ahr*$^{-/-}$ mice that had been infected with *T. gondii* exhibited defects in IL-10 expression. These data identify the AHR as a critical cofactor involved in the ability of IL-12 to promote NK cell production of IL-10, suggesting that AHR ligands can serve as signals that allow NK cells to sense and respond to their environment.

Materials and Methods

Mice and infections

Vert-X mice were provided by Dr. Christopher L. Karp (previously at the University of Cincinnati College of Medicine, Cincinnati, OH). *Ahr*$^{-/-}$ mice that had been backcrossed onto a C57BL/6J background for 21 generations were obtained from Dr. Christopher A. Bradfield (University of Wisconsin School of Medicine and Public Health, Madison, WI). Tissues from Vav-Cre *Arnt*$^{fl/fl}$ mice and control mice were provided by M. Celeste Simon (University of Pennsylvania, Philadelphia, PA). RAG1$^{-/-}$ mice and C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). CBA/J mice were purchased from the National Cancer Institute Animal Production Program (Frederick, MD). All mice were bred and housed in specific pathogen-free facilities at the University of Pennsylvania in accordance with institutional guidelines. For infections, Me49 cysts were harvested from the brains of chronically infected CBA/J mice and experimental animals were injected i.p. with 20 cysts. For IL-12 depletion, mice were injected i.p. with 1mg anti-IL-12p40 (clone C17.8) or control rat IgG (Sigma, St. Louis, MO) one day before infection and 3 days post-infection.

Cell isolation

Lymphocytes were isolated from the liver as described previously (John et al., 2009). Spleens were dissociated through a 40μm filter and red blood cells were lysed with 0.86% ammonium chloride (Sigma) in sterile water. PECs (peritoneal exudate cells) were

collected by lavaging the peritoneal cavity with 7ml PBS. Bone marrow was isolated by flushing femurs and tibias with PBS, followed by red blood cell lysis.

LAK generation

For the production of LAKs from RAG1$^{-/-}$ mice, bone marrow cells were plated at 1x10^{6} cells/ml in 10ml complete RPMI 1640 (10% heat inactivated FCS, 2mM glutamine, 10U/ml penicillin, 10μg/ml streptomycin, 1mM sodium pyruvate, 1% nonessential amino acids, 5x10^{-5}M 2-ME) and 4x10^{3}U/ml recombinant human IL-2 (proleukin, Novartis, Basel, Switzerland). Cells were fed with IL-2 on days 3 and 5 or days 4 and 6 after plating. LAKs were collected and stimulated on day 7. For the generation of LAKs from Vert-X mice, *Ahr*$^{-/-}$ mice, Vav-Cre *Arnt*$^{fl/fl}$ mice, or controls, NK cells were enriched from the bone marrow or spleen using the EasySep mouse NK cell enrichment kit (STEMCELL Technologies, Vancouver, Canada). CD3^{-} NK1.1^{+} cells were sorted on a FACSAria (BD Biosciences, San Jose, CA) and cultured for 1 week in complete RPMI with recombinant human IL-2.

Stimulation of LAKs or NK cells isolated from infected mice

LAKs were harvested and plated at a concentration of 1-2.5x10^{6} cells/ml on 96 well plates (BD Biosciences). The cells were cultured in complete RPMI (or complete IMDM where indicated). LAKs were stimulated with a concentration of 5ng/ml IL-12 (eBioscience, San Diego, CA) unless otherwise stated, 2000U/ml proleukin, 30μM CH-223191 (Calbiochem, Darmstadt, Germany), 0.625μM flavone (Sigma), 0.31μM α-napthoflavone (Sigma), 300nM FICZ (Enzo Life Sciences, Farmingdale, NY), or DMSO

(Sigma) as a vehicle control. After 48 hours, supernatants were collected and levels of IL-10 or IFN-γ were assayed by ELISA. For analysis of IL-10/GFP expression, cells were surface stained and run on a FACSCanto II (BD Biosciences). For analysis of IL-10 production from NK cells from infected mice, $DX5^+$ NK 1.1^+CD3^- cells were sorted from spleens. The purified NK cells were plated at a concentration of $2x10^6$ cells/ml on 96 well plates, stimulated for 48 hours with 50ng/ml PMA and 1μM ionomycin, and cytokine levels were measured by ELISA.

ELISAs

For IL-10 ELISAs, Immulon 4HBX plates (Thermo Fisher Scientific, Waltham, MA) were coated with anti-IL-10 (clone JES5-2A5) (BD Pharmingen San Diego, CA), blocked in 5% FBS in PBS, and loaded with samples. Biotinylated anti-IL-10 (clone JES5-16E3) was used for detection followed by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), SureBlue (KPL, Gaithersburg, MD) and TMB Stop Solution (KPL). For IFN-γ ELISAs, plates were coated with anti- IFN-γ (clone AN-18) (eBioscience) and loaded with samples. Biotinylated anti- IFN-γ (clone R4-6A2) (eBioscience) was used for detection, followed by peroxidase-conjugated streptavidin and ABTS (KPL). For IL-12p40 ELISAs, plates were coated with anti-IL-12p40 (clone C17.8), loaded with samples, followed by detection using biotinylated anti-IL-12p40 (clone C15.6) and ABTS.

RNA preparation and PCR

To evaluate the expression of the AHR and its chaperone proteins, LAKs were stimulated for 6 hours or $NK1.1^{+}DX5^{+}$ cells were sorted from the spleens of $RAG1^{-/-}$ mice. To analyze cytokine expression by NK cells from infected wild-type or *Ahr*$^{-/-}$ mice, $DX5^{+}NK1.1^{+}CD3^{-}$ cells were sorted from liver lymphocyte preparations. All sorting was performed on a FACSAria or FACSVantage SE (BD Biosciences). RNA was isolated using the RNAeasy Mini Kit (Qiagen, Valencia, CA). Samples were treated with DNAse (Promega, Madison, WI), and cDNA was generated using reagents from Invitrogen (Grand Island, NY). For RT-PCR analysis, cDNA samples were amplified with SYBR Green (Applied Biosystems, Carlsbad, CA) on the 7500 Fast Real-Time PCR System (Applied Biosystems). Primers for the AHR and β-actin were purchased from Qiagen. Primers for the AHR chaperone proteins and ARNT were generated using Primer 3 and obtained from Integrated DNA Technologies (Coralville, IA). The following sequences were used for p23 (5'-TGATTCCATGAGGCAGTTGA-3' and 5'-AATTTGTTTCCGCCTCCTTT-3'), hsp90 (5'-AAGGCAGAGGCTGACAAGAA-3' and 5'-ACAGCAGCACTGGTGTCATC-3'), Arnt (5'-TGCCTCATCTGGTACTGCTG-3' and 5'-GAACATGCTGCTCACTGGAA-3') and Ara9 (5'-GCTAGGAGTTGCCGAAACAG-3' and 5'-GAAAGTGGAACGTGGCCTTA-3'). The following sequences were used for IL-10 (5'-ACCTGCTCCACTGCCTTGCT-3' and 5'-GGTTGCCAAGCCTTATCGGA-3'), IFN-γ (5'-CTTCTTCAGCAACAGCAAGG-3' and 5'-TGAGCTCATTGAATGCTTGG-3'), and HPRT (5'-

AACTTTTATGTCCCCGGTTGA-3' and 5'-GGCTATAAGTTCTTTGCTGACCTG-3'). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

T cell polarization

$CD4^+$ T cells were sorted from the spleens and lymph nodes of C57BL/6J mice. Cells were cultured in complete IMDM for 4 days on plates coated with 1μg/ml anti-CD3 (eBioscience), with soluble 1μg/ml anti-CD28 (eBioscience). Th17 cells were cultured under previously described conditions (Stumhofer et al., 2006). Th1 cells were stimulated with 20ng/ml IL-12 and 10μg/ml anti-IL-4 (clone 11B11).

IL-12 expression in infected mice

Serum was collected 5 days post-infection and IL-12p40 levels were assayed by ELISA. Splenocytes were cultured at $1x10^6$ cells/ml in complete RPMI with or without 62μg/ml soluble Toxoplasma antigen (STAg). Supernatants were collected after 48 hours and IL-12p40 was detected by ELISA. PECs were incubated with brefeldin A (Sigma) and Golgi Stop (BD Biosciences) for 6 hours, surface stained, and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Cells were permeabilized with 0.5% saponin (Sigma) and stained for IL-12.

Antibodies and flow cytometry

For surface staining, samples were washed in flow cytometry buffer containing 1% BSA (Sigma) and 2mM EDTA (Invitrogen) in PBS, Fc blocked with 2.4G2 and normal rat IgG (Invitrogen), and stained with monoclonal antibodies. For the detection of IFN-γ

expression by NK cells, splenocytes were surface stained and stimulated with PMA and ionomycin with brefeldin A (Sigma) for 4 hours. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained for IFN-γ. For the detection of T-bet and Ki67, cells were surface stained and then stained intracellularly for T-bet and Ki67 using the FoxP3/transcription factor staining buffer set (eBioscience). For the detection of GFP in fixed cells from Vert-X mice, cells were stained intracellularly for GFP using an anti-GFP antibody (eBioscience) followed by staining with a secondary antibody (Alexa Fluor 488 conjugated AffiniPure goat anti-rabbit from Jackson ImmunoResearch). STAT phosphorylation was assayed by flow cytometry as previously described (Hall et al., 2012). NK1.1 FITC, CD8 PeCy7, CD11c PeCy7, CD4 APC, CD3 APC-eFluor 780, CD69 PerCP Cy5.5, IFN-γ PeCy7, T-bet AF647, KLRG1 APC, IL-12p40 PE and CD19 eFluor 450 were purchased from eBioscience. CD19 PerCP Cy5.5, NK1.1 Pacific Blue, and CD3 Pacific Blue were obtained from BioLegend. CD49b PE was purchased from BD Biosciences, and Ki67 AF647 was obtained from BD Pharmingen. Samples were run on a FACSCanto II and data was analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).

Parasite burdens

DNA was isolated from the PECs using the High Pure PCR Template Preparation kit (Roche, Indianapolis, IN). Parasite DNA levels were determined by RT-PCR as previously described (Silver et al., 2011).

Statistical analysis

Statistical significance was determined using paired or unpaired Student's t tests, which were performed using Prism software (GraphPad software, Inc. La Jolla, CA). For Figure 3B, 5C, 5H, 5I, 5J and 6I paired t tests were used to determine significance using pooled data from multiple experiments. In this analysis, the results from each individual experiment were paired.

Results

Characterization of cellular sources of IL-10 during toxoplasmosis

While $CD4^+$ T cells are an essential source of IL-10 during infection, studies in SCID mice have suggested that the production of IL-10 by innate cell populations promotes susceptibility to *T. gondii* (Jankovic et al., 2007; Neyer et al., 1997). To identify innate and adaptive sources of IL-10, Vert-X IL-10/GFP reporter mice, which have been used previously to assess IL-10 expression (Madan et al., 2009; Murai et al., 2009; Perona-Wright et al., 2009), were challenged with the Me49 strain of *T. gondii*. In uninfected mice, IL-10/GFP expression in the spleen was largely restricted to small populations of $CD4^+$ T cells and B cells (Figure 2.1A). Five days following infection, there was an increase in the populations of $CD8^+$ T cells and $CD4^-CD8^-$ T cells, which may be γδ T cells, that expressed the reporter (Figure 2.1B). However, within the spleen, liver and peritoneal exudate cells (PECs), a much higher percentage of NK cells were IL-10/GFP^+ at this timepoint than within any of the other cell populations analyzed (Figure 2.1B). NK cells also constituted the largest fraction of total IL-10 expressing cells in the spleen and liver, which are two of the main sites of mature NK cell localization (Huntington et al., 2007) (Figure 2.1C). These results indicated that NK cells are a major source of IL-10 during early infection, in agreement with previous findings (Perona-Wright et al., 2009).

Further characterization of NK cells in the Vert-X mice revealed that infection led to a global increase in CD11c and CD69 expression and that these markers of NK cell

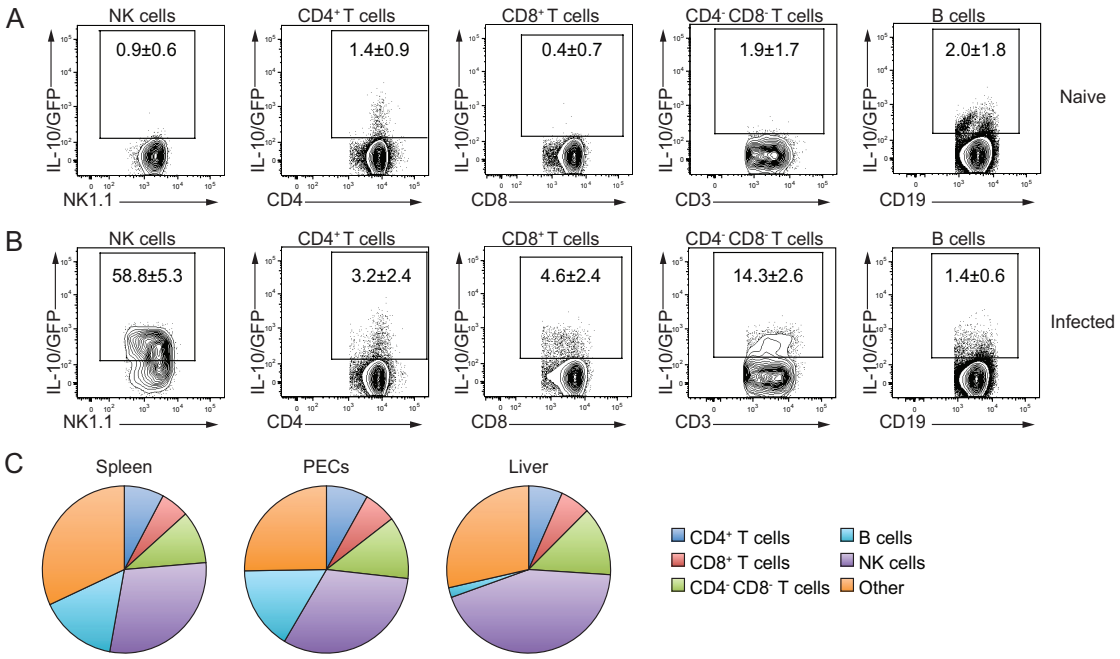

Figure 2.1: NK cells are a major source of IL-10 early during infection with *T. gondii*. IL-10/GFP expression by different cell populations in the spleens of Vert-X mice that were either uninfected **(A)** or infected with *T. gondii* five days prior **(B)** Numbers indicate the mean ± the standard deviation from 3-4 independent experiments, with a total of 6-9 mice. **(C)** The percentages of different cell types within the total IL-10/GFP$^+$ population in different tissues (spleen, PECs and liver) of infected Vert-X mice, represented as pie graphs. Average percentages were calculated from 2-3 independent experiments, with a total of 8-10 mice. Various cell types were distinguished based on the expression of surface receptors; NK cells (DX5$^+$NK1.1$^+$CD3$^-$CD19$^-$), CD4$^+$ T cells (CD3$^+$CD4$^+$), CD8$^+$ T cells (CD3$^+$CD8$^+$), CD4$^-$CD8$^-$ T cells (CD3$^+$CD4$^-$CD8$^-$), and B cells (CD19$^+$CD3$^-$).

activation were expressed similarly by IL-10/GFP$^+$ and IL-10/GFP$^-$ NK cells (Figure 2.2A) (Karlhofer and Yokoyama, 1991; Vosshenrich et al., 2007). The levels of Ki67, a marker of proliferating cells, also increased in IL-10/GFP$^+$ and IL-10/GFP$^-$ NK cells from infected mice (Figure 2.2B). However, several other markers were differentially expressed by IL-10/GFP$^+$ and IL-10/GFP$^-$ NK cells. Following PMA/ionomycin stimulation, a higher percentage of IL-10/GFP$^+$ NK cells produced IFN-γ than IL-10/GFP$^-$ NK cells (Figure 2.2B, C). Most NK cells in naïve mice stained positive for the transcription factor T-bet, but T-bet expression increased following infection (Figure 2.2B) and IL-10/GFP$^+$ NK cells expressed the highest levels of T-bet (Figure 2.2D). Both IL-10/GFP$^+$ and IL-10/GFP$^-$ NK cells from infected mice expressed high levels of KLRG1, but a significantly higher percentage of IL-10/GFP$^+$ cells were KLRG1$^+$ (Figure 2.2B, E). The marked increases in NK cell expression of CD69 and CD11c following infection indicated that both IL-10/GFP$^+$ and IL-10/GFP$^-$ NK cells became activated, but IL-10/GFP$^+$ NK cells expressed higher levels of IFN-γ, T-bet, and KLRG1, which are targets of IL-12 signaling (Joshi et al., 2007; Wilson et al., 2008).

IL-12 induces NK cell IL-10 expression

The finding that NK cells are a major source of IL-10 in early infection raised the question of which signals induce IL-10 expression by this population. Previous work has shown that IL-12 promotes NK cell IL-10 production during toxoplasmosis (Perona-Wright et al., 2009), consistent with the finding that IL-10/GFP$^+$ NK cells expressed high levels of IL-12 target genes. To examine the effects of IL-12 signaling on NK cells

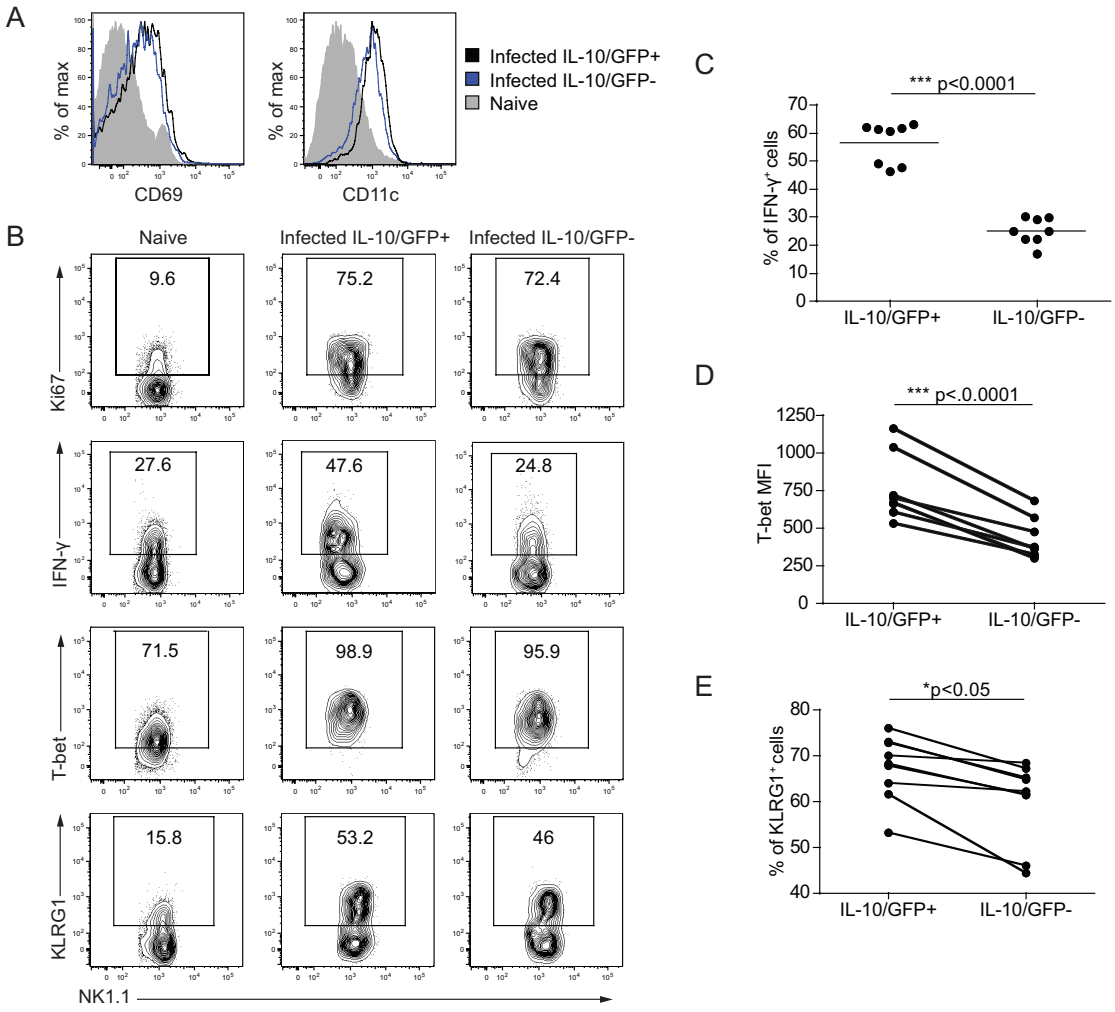

Figure 2.2: Phenotype of IL-10/GFP$^+$ and IL-10/GFP$^-$ NK cells. **(A)** Expression of CD69 and CD11c on NK cells from naïve and infected Vert-X mice. Results are representative of three independent experiments, with a total of 5 naïve and 11 infected mice. **(B)** The expression of Ki67, IFN-γ, and T-bet by total NK cells from naïve Vert-X mice and the IL-10/GFP$^+$ or IL-10/GFP$^-$ NK cells from infected mice. Cells were re-stimulated with PMA/ionomycin to evaluate IFN-γ production. Data are representative of 2-3 independent experiments with a total of 8-9 infected mice and 4-5 naïve mice. **(C)** The frequency of IL-10/GFP$^-$ and IL-10/GFP$^+$ NK cells from infected Vert-X mice that produce IFN-γ following PMA/ionomycin restimulation. Data are pooled from two experiments with a total of 8 mice. **(D)** The MFI of T-bet in IL-10/GFP$^-$ and IL-10/GFP$^+$ NK cells from infected Vert-X mice. Data are pooled from three experiments with a total of 9 mice. **(E)** Frequencies of IL-10/GFP$^-$ NK cells or IL-10/GFP$^+$ NK cells from infected Vert-X mice that were KLRG1$^+$. Data are pooled from three independent experiments with a total of 9 mice. NK cells were gated as DX5$^+$ NK 1.1$^+$CD3$^-$CD19$^-$ cells.

during infection, Vert-X mice were treated with IL-12 depleting antibodies and challenged with *T. gondii*. Depletion of IL-12 in infected Vert-X mice abrogated NK cell IL-10 reporter expression (Figure 2.3A), in agreement with previous studies (Perona-Wright et al., 2009). NK cells in infected control and anti-IL-12p40 treated mice upregulated expression of CD69 and CD11c (Figure 2.3B). However IL-12 depletion antagonized the infection-induced upregulation of KLRG1 and Ki67 (Figure 2.3C, D, E). T-bet MFIs (mean fluorescence intensities) were also reduced following IL-12 depletion (Figure 2.3C, F). These results suggested that IL-12 signaling during infection promoted NK cell IL-10 expression and proliferation. In agreement with this possibility, infected mice that had been treated with IL-12 depleting antibodies had fewer total splenic NK cells than infected controls (Figure 2.3G).

To study the factors that regulate NK cell expression of IL-10 *in vitro*, IL-2 activated lymphokine activated killer cells (LAKs) were utilized, which were generated as previously described (Cai et al., 1999). To evaluate IL-10 production by these cells, LAKs were stimulated with different combinations of cytokines for 48 hours. In the absence of stimulation, these cells did not produce detectable levels of IL-10 and stimulation with IL-2 or IL-12 alone induced little IL-10 secretion (Figure 2.4A). However, when treated with the combination of IL-2 and IL-12, LAKs produced high levels of IL-10 and IFN-γ as previously reported (Grant et al., 2008). Similar results were observed with NK cells freshly isolated from the spleens of $RAG1^{-/-}$ mice (data not shown). IL-23 and IL-27, which are members of the IL-12 family of cytokines, did not

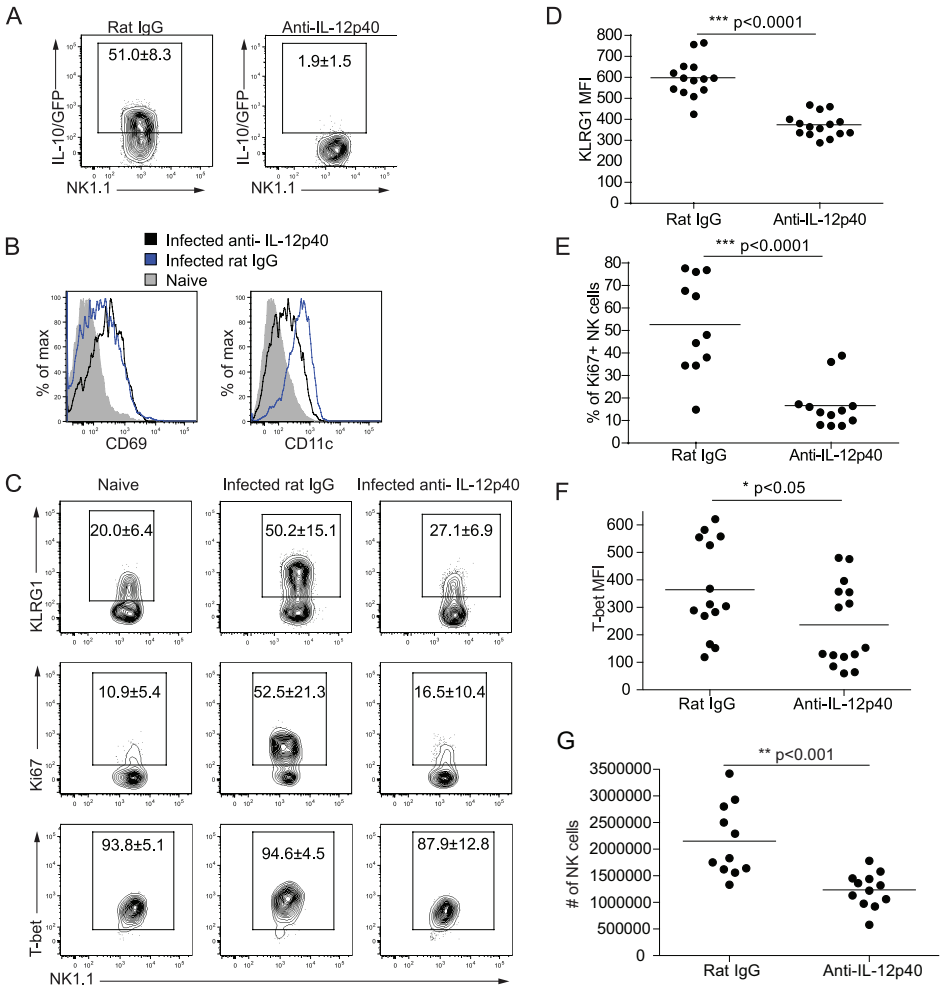

Figure 2.3: Phenotype of NK cells following IL-12 depletion during infection. Vert-X IL-10 reporter mice were infected with *T. gondii* for five days and given control rat IgG or anti-IL-12p40. NK cells were gated as $DX5^{+}$ $NK1.1^{+}CD3^{-}CD19^{-}$ cells. **(A)** IL-10/GFP expression by NK cells in the spleens of Vert-X mice that were given rat IgG or anti-IL-12p40. Numbers represent the mean ± the standard deviation from four independent experiments, with a total of 13-16 mice per group. **(B&C)** Expression of CD69, CD11c, KLRG1, Ki67 and T-bet by NK cells from naïve Vert-X mice or infected Vert-X mice that were given rat IgG or anti-IL-12p40. Numbers represent the mean ± the standard deviation from 2-4 independent experiments with a total of 5 naïve and 13-16 infected mice per group. **(D)** KLRG1 MFIs of $KLRG1^{+}$ NK cells from infected Vert-X mice given rat IgG or anti-IL-12p40. Data are pooled from 4 independent experiments. **(E)** Frequency of $Ki67^{+}$ NK cells from infected Vert-X mice given rat IgG or anti-IL-12p40. Data are pooled from 4 independent experiments. **(F)** T-bet MFIs of NK cells from infected Vert-X mice given rat IgG or anti-IL-12p40. Data are pooled from 4 independent experiments. **(G)** Number of NK cells in the spleens of infected mice. Results are pooled from 3 experiments.

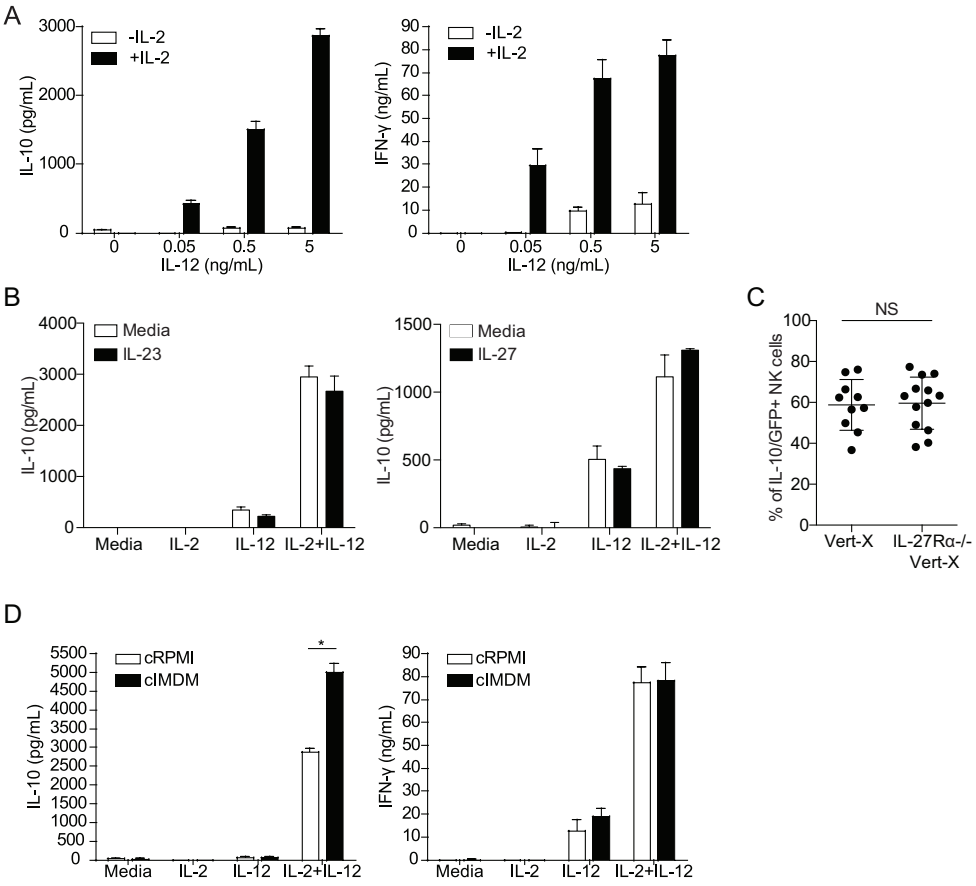

Figure 2.4: LAKs produce IL-10 when stimulated with IL-12 and IL-2. (A) IL-10 and IFN-γ production by LAKs, 48 hours after stimulation with varying concentrations of IL-12 in the presence or absence of IL-2. Results are representative of three independent experiments. **(B)** IL-10 production by LAKs 48 hours post-stimulation with the indicated cytokines. Results are representative of 2-3 independent experiments. **(C)** IL-10/GFP expression by NK cells in the spleens of mice that had been infected for 5 days with *T. gondii*. Results are pooled from 4 separate experiments. **(D)** IL-10 and IFN-γ production by LAKs that were stimulated with IL-2, IL-12 or a combination of the two cytokines in RPMI or IMDM for 48 hours. (*$p<0.05$ based on a paired Student's t test with 4 independent experiments) All error bars represent standard error.

induce IL-10 production from LAKs when used in various combinations with IL-2 or IL-12 (Figure 2.4B). Additionally, IL-27R$\alpha^{-/-}$ Vert-X mice exhibited no defect in NK cell IL-10 expression following infection with *T. gondii* (Figure 2.4C).

LAKs and NK cells express the AHR

Although these studies highlighted the critical role of IL-12 in inducing NK cell IL-10 expression, optimal cytokine production by NK cells typically requires multiple signals. Therefore, additional pathways may have contributed to IL-10 induction in these cells. One candidate was signaling by the AHR, which promotes IL-10 production by macrophages, dendritic cells, and type 1 regulatory T cells cells (Apetoh et al., 2010; Kimura et al., 2009; Nguyen et al., 2010). Indeed, LAKs that had been stimulated with IL-2 and IL-12 in the culture medium IMDM, which contains high levels of AHR ligands (Veldhoen et al., 2009), secreted significantly more IL-10 than LAKs that were cultured in RPMI (Figure 2.4D). The production of IFN-γ was not affected by the type of media used (Figure 2.4D). Since this result suggested that AHR signaling promoted IL-10 production, NK cell expression of the AHR, as well as the p23, hsp90 and ara9 chaperone proteins that associate with the AHR, were then evaluated (Figure 2.5A). LAKs basally expressed transcripts for the AHR, p23, hsp90, ara9, and ARNT (Figure 2.5A), which heterodimerizes with the AHR in the nucleus to form a competent transcription factor. While the expression of most AHR chaperone proteins and ARNT was not affected by IL-12, this treatment did result in an approximately three-fold increase in AHR mRNA within six hours (Figure 2.5A). Transcripts for the AHR, p23, hsp90, ara9 and ARNT

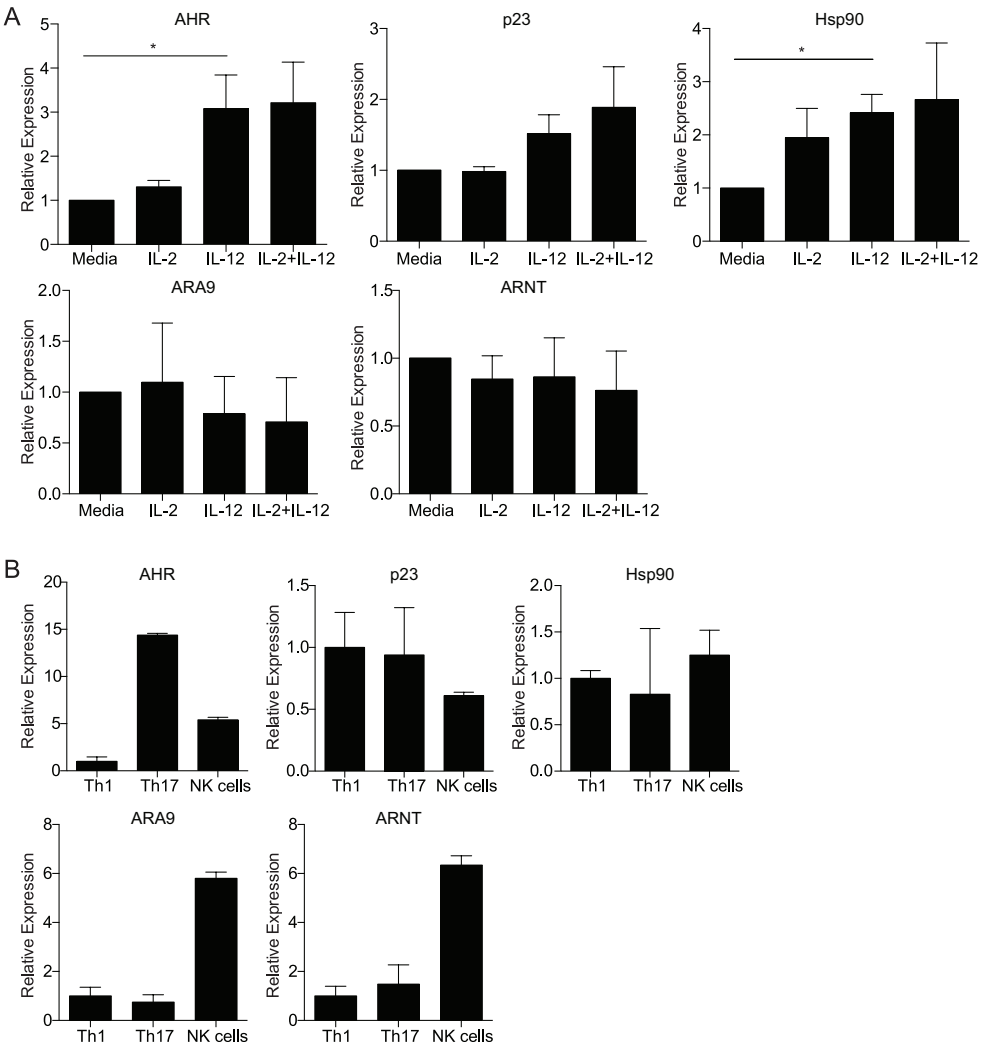

Figure 2.5: Expression of the Ahr and AHR related genes in LAKS and NK cells. (A) The expression of the Ahr and AHR related genes (p23, Hsp90, Ara9 and Arnt) in LAKs that were stimulated with IL-2, IL-12 or the combination of IL-2 and IL-12 for 6 hours. Sample transcript levels were normalized to the expression of the housekeeping gene *HPRT*, and relative levels are shown using the media sample as a calibrator. Graphs show the pooled averages from three independent experiments. (*$p<0.05$ based on a Student's t test). **(B)** Ahr and AHR related gene (p23, Hsp90, Ara9 and Arnt) expression in NK cells sorted from the spleens of $RAG1^{-/-}$ mice and *in vitro* polarized Th1 or Th17 $CD4^{+}$ T cells. Sample transcript levels were normalized to the expression of the housekeeping gene *HPRT,* and relative levels are shown calibrated to the Th1 sample. Results are representative of three similar experiments, and error bars are based on technical replicates. All error bars represent standard error.

were also detected in NK cells that had been freshly isolated from the spleens of RAG1$^{-/-}$ mice (Figure 2.5B). Since Th17 cells express high levels of *Ahr* mRNA compared to other T cell subsets, polarized Th17 and Th1 cells were included in these experiments as controls (Kimura et al., 2008; Quintana et al., 2008; Veldhoen et al., 2008). These results suggest that NK cells are able to respond to AHR signaling and that their ability to do so is enhanced by IL-12 stimulation.

Effect of AHR signaling on IL-10 and IFN-γ production by LAKs

To determine the effects of AHR signaling on NK cell cytokine production, LAKs were stimulated with AHR inhibitors in the presence of IL-2 and IL-12 and supernatants were assayed for IL-10 and IFN-γ. The effects of these inhibitors on LAK IFN-γ expression were variable; two of the inhibitors, flavone and α-napthoflavone, had no significant effect on IFN-γ production, while treatment with CH-223191 led to decreased IFN-γ secretion (Figure 2.6A). No difference in survival was seen between LAKs treated with DMSO as a vehicle control or cells stimulated with CH-223191 (data not shown). IFN-γ transcript expression also decreased following stimulation with CH-223191 (Figure 2.6B). To further investigate the effects of AHR signaling on NK cells, LAKs were stimulated *in vitro* with the high-affinity AHR ligand 6-formylindolo[2,3-b]carbazole (FICZ), which can form intracellularly from tryptophan following exposure to ultraviolet light (Fritsche et al., 2007; Rannug et al., 1995). LAKs stimulated with FICZ in the presence of IL-2 and IL-12 produced similar levels of IFN-γ as LAKs treated

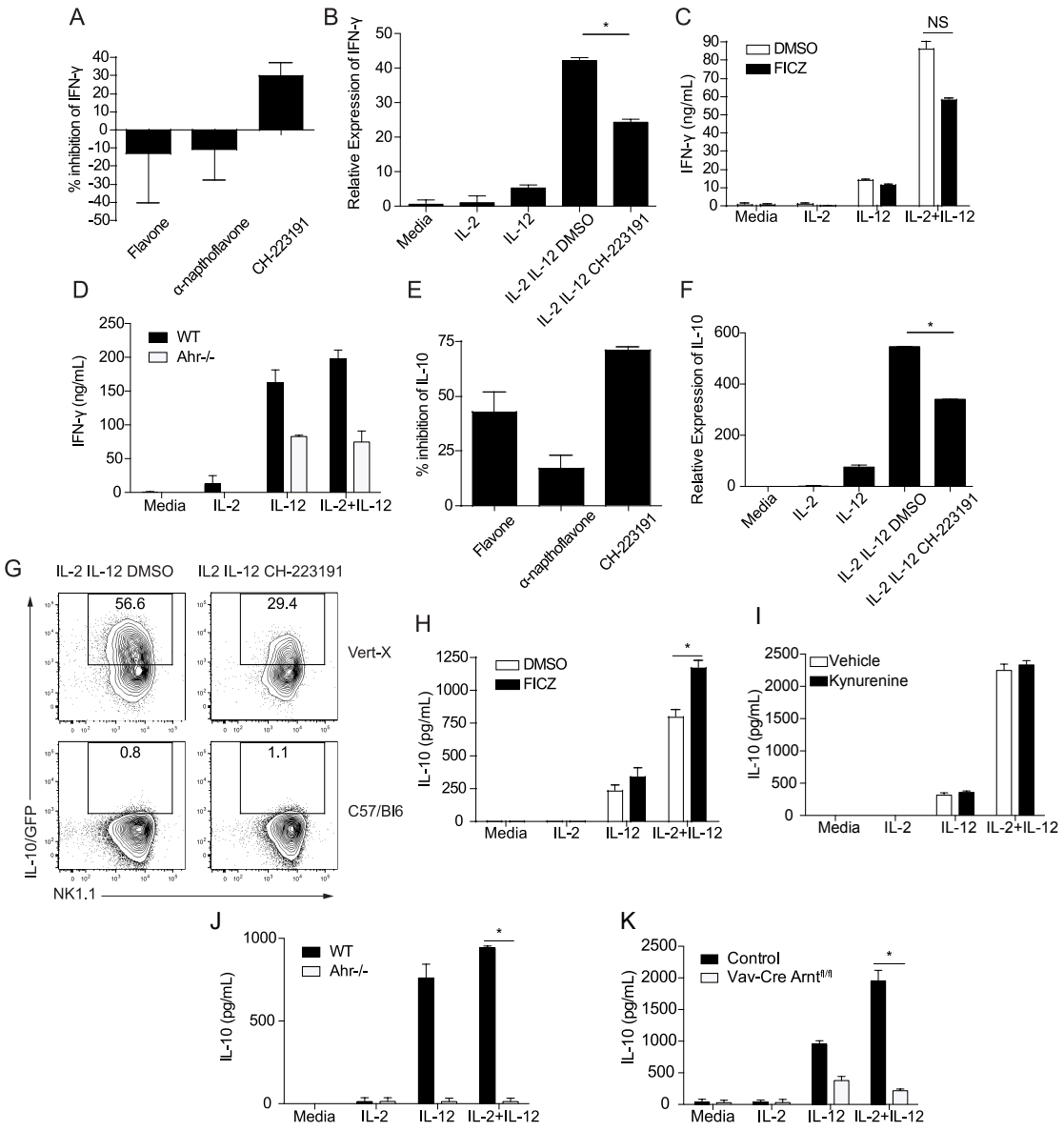

Figure 2.6: AHR signaling promotes IL-10 production by LAKs. (A, E) Percent inhibition by AHR inhibitors (flavone, α–napthoflavone and CH-223191) of IFN-γ and IL-10 production by LAKs that were stimulated with IL-2 and IL-12 compared to vehicle control (DMSO) treated cells. The data show the average inhibition from at least 3 independent experiments.

(B, F) IFN-γ and IL-10 transcript expression by LAKs stimulated for 6 hours or 27 hours respectively. Sample transcript levels were normalized based on *HPRT* expression, and calibrated to the IL-2 treated sample. Results are representative of three similar experiments, and error bars are based on technical replicates (*$p<0.05$ based on a paired Student's t test). **(C, H, I)** Cytokine production by LAKs stimulated with IL-2 and IL-12 in the presence or absence of AHR ligands for 48 hours (*$p<0.05$ based on a paired Student's t test with 5 separate experiments). **(D, J)** IFN-γ or IL-10 production by LAKs generated from *Ahr*$^{-/-}$ or wild-type mice, 48 hours after stimulation with IL-2 and IL-12 (*$p<0.05$ based on a paired Student's t test with 3 independent experiments). **(G)** IL-10/GFP expression by LAKs generated from Vert-X mice 48 hours post-stimulation. LAKs from C57BL/6J mice were used as controls to gate for IL-10/GFP^{+} cells. Representative plots from 3 independent experiments are shown. **(K)** IL-10 production by LAKs generated from a Vav-Cre *Arnt*$^{fl/fl}$ mouse or control 48 hours post-stimulation. (*$p<0.05$ based on pooled data from two separate experiments)

with the vehicle control (Figure 2.6C). Since possible off-target effects with the use of pharmacological agents as AHR agonists or antagonists were a concern, LAKs were generated from mice genetically deficient for the AHR and wild-type controls (Figure 2.6D). Following stimulation with IL-2 and IL-12, *Ahr*$^{-/-}$ LAKs secreted less IFN-γ than LAKs from wild-type mice. This reduction was statistically significant ($p<0.02$) when comparing the pooled percentages of *Ahr*$^{-/-}$ LAK IL-10 production relative to wild-type LAKs from three separate experiments. No survival differences were observed between wild-type and *Ahr*$^{-/-}$ LAKs (data not shown). Together, the results from these studies suggest a role for AHR signaling in the production of IFN-γ by LAKs.

The effects of AHR signaling on IL-10 production by LAKs were also evaluated. All three of the AHR inhibitors significantly reduced LAK IL-10 secretion in response to IL-2 and IL-12 (Figure 2.6E). Flavone and α-napthoflavone have been shown to act as AHR agonists at high concentrations (Kim et al., 2006) and their effects on IL-10 expression were concentration dependent, with maximal inhibition of IL-10 seen when LAKs were treated with intermediate concentrations of these inhibitors (data not shown). Similarly, stimulation with CH-223191, the most effective AHR inhibitor used in these studies (Kim et al., 2006), also led to a decrease in the level of IL-10 mRNA expressed by LAKs (Figure 2.6F). This was corroborated by using LAKs generated from Vert-X mice, which expressed reduced levels of the IL-10 reporter following treatment with CH-223191 compared to cells treated with the vehicle control (Figure 2.6G). While stimulation with the AHR agonist FICZ was not sufficient to induce detectable secretion

of IL-10, FICZ treatment led to an increase in the amount of IL-10 expressed by LAKs in the presence of IL-2 and IL-12 (Figure 2.6H). Thus, augmenting AHR activity in culture promoted cytokine-mediated NK cell production of IL-10. However, stimulation with the AHR ligand L-kynurenine did not lead to significant differences in IL-10 production (Figure 2.6I), suggesting that various AHR ligands have different effects, in agreement with previous studies (Quintana et al., 2008). In order to evaluate LAK IL-10 secretion in the complete absence of AHR activity, LAKs were generated from *Ahr*$^{-/-}$ mice or wild-type controls. When treated with IL-2 and IL-12, *Ahr*$^{-/-}$ LAKs exhibited significant defects in IL-10 production (Figure 2.6J). To determine whether ARNT activity also contributed to NK cell IL-10 expression, LAKs were generated from Vav-Cre *Arnt*$^{fl/fl}$ mice, in which ARNT is conditionally deleted in hematopoietic cells. Following stimulation with IL-2 and IL-12, ARNT deficient LAKs exhibited impaired IL-10 production (Figure 2.6K), suggesting that the effects of the AHR on NK cell IL-10 expression were dependent on its interaction with ARNT. Collectively, these studies indicated that AHR activity is required for optimal IL-10 expression by LAKs in response to stimulation with IL-2 and IL-12.

Possible explanations for the contribution of the AHR to NK cell production of IL-10 included reduced IL-12 receptor expression or defective IL-12 signaling in the absence of AHR activity. Consistent with this possibility, previous work has indicated that the AHR can modulate STAT activation in macrophages and T cells (Kimura et al., 2009; Kimura et al., 2008). To evaluate a role for the AHR in IL-12 signaling,

splenocytes from wild-type or *Ahr*$^{-/-}$ mice were stimulated *in vitro* with IL-12 and STAT4 phosphorylation was examined (Figure 2.7). NK cells from wild-type and *Ahr*$^{-/-}$ mice had similar STAT4 phosphorylation responses following IL-12 treatment, indicating that proximal IL-12 receptor signaling was intact in the absence of AHR activity, and defects in this pathway were not responsible for the decreased IL-10 production seen in *Ahr*$^{-/-}$ NK cells.

Role of the AHR in the innate response to *T. gondii*

To determine the role of the AHR on NK cell responses *in vivo*, *Ahr*$^{-/-}$ mice were challenged with *T. gondii* and their innate immune responses were evaluated. Analysis of IL-12 production revealed that infection led to comparable serum levels of IL-12 in wild-type and *Ahr*$^{-/-}$ mice (Figure 2.8A). Dendritic cells from these mice also expressed similar levels of IL-12 (Figure 2.8B, C), and splenocytes isolated from infected wild-type and *Ahr*$^{-/-}$ mice produced comparable levels of IL-12 upon stimulation with soluble Toxoplasma antigen (STAg) (Figure 2.8D), indicating that infected *Ahr*$^{-/-}$ mice had no early defect in IL-12 production. Infected *Ahr*$^{-/-}$ and wild-type mice also had similar numbers of NK cells (Figure 2.8E). These populations expressed comparable levels of CD69, CD11c, T-bet, KLRG1, and Ki67 (Figure 2.8F, G). However NK cells isolated from *Ahr*$^{-/-}$ mice had marked reductions in their levels of IL-10 mRNA compared to cells from wild-type mice (Figure 2.8H). *Ahr*$^{-/-}$ NK cells isolated from infected mice were also impaired in their ability to secrete IL-10 following stimulation with PMA/ionomycin *ex vivo* (Figure 2.8I). However, no differences were seen in IFN-γ production by wild-type

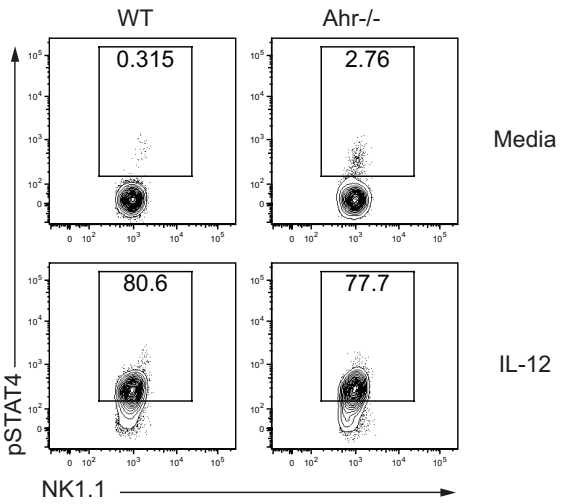

Figure 2.7: No defect in STAT4 phosphorylation in *Ahr*$^{-/-}$ NK cells following stimulation with IL-12. Splenocytes from wild-type or *Ahr*$^{-/-}$ mice were stimulated with IL-12 for 2.5 hours and pSTAT4 was assayed by flow cytometry. Plots are gated on NK cells (NK1.1^{+}TCRβ^{-}). Results are representative of two independent experiments.

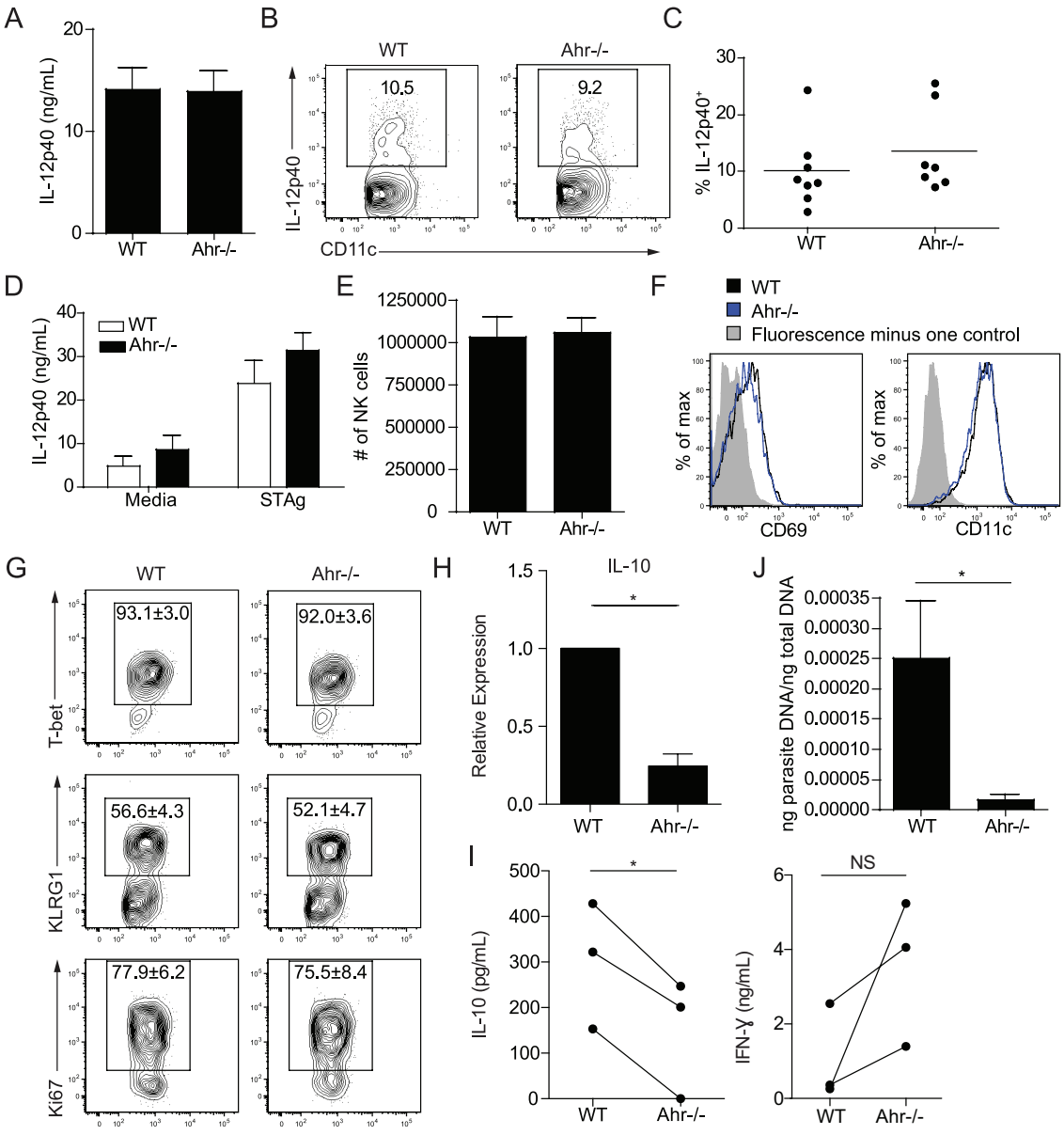

Figure 2.8: NK cells from *Ahr*$^{-/-}$ mice express decreased levels of IL-10. Wild-type or *Ahr*$^{-/-}$ mice were infected for five days with *T. gondii.* **(A)** IL-12p40 levels in the serum of WT or *Ahr*$^{-/-}$ mice. Pooled data from 3 independent experiments are shown. **(B, C)** IL-12p40 production by dendritic cells in the PECs (peritoneal exudate cells) following a 6 hour incubation with brefeldin A and Golgi Stop. Dendritic cells were gated as CD3^{-}CD19^{-}NK1.1^{-}CD11c^{+} cells. Data are pooled from three independent experiments.

(D) IL-12p40 secretion by splenocytes from infected mice 48 hours post-incubation in media alone or STAg. Pooled data from two experiments are shown. **(E)** Total numbers of NK cells in the spleens of infected WT or *Ahr*$^{-/-}$ mice. Data are pooled from three independent experiments with a total of 10 mice per group. NK cells were gated as DX5^{+} NK 1.1^{+}CD3^{-}CD19^{-} cells. **(F)** CD69 and CD11c expression by splenic NK cells from infected wild-type or *Ahr*$^{-/-}$ mice. Representative plots from three independent experiments are shown. **(G)** Expression of T-bet, KLRG1, and Ki67 by splenic NK cells from infected wild-type or *Ahr*$^{-/-}$ mice. Numbers represent the mean ± the standard deviation from three separate experiments with a total of 10 mice per group. **(H)** IL-10 transcript expression by NK cells sorted from the livers of infected wild-type or *Ahr*$^{-/-}$ mice. Data are pooled from two separate experiments. (*p<.05 from a Student's t test). **(I)** Cytokine secretion by NK cells sorted from the spleens of infected wild-type or *Ahr-/-* mice and stimulated *ex vivo* with PMA and ionomycin for 48 hours. Results from three independent experiments are shown. (*p<.05 based on a paired Student's t test). **(J)** Levels of parasite DNA in the PECs five days post-infection. (*p<0.05 from 3 independent experiments with a total of 7-9 mice per grou-p)

or *Ahr*$^{-/-}$ mice under these conditions (Figure 2.8I). While IL-10 plays a critical role in limiting infection-induced immunopathology, IL-10 signaling can also promote increased pathogen burdens by attenuating inflammatory responses and suppressing antimicrobial activity. Accordingly, infected *Ahr*$^{-/-}$ mice had decreased parasite burdens during the acute phase of infection (Figure 2.8J). Collectively these results indicate that although *T. gondii* infection induced AHR-independent activation of NK cells, optimal NK cell expression of IL-10 was dependent on AHR activity.

Discussion

Multiple studies have identified effects of AHR signaling on cells of the immune system, including Th17 cells, regulatory T cells, γδ T cells, innate lymphoid cells, dendritic cells, and macrophages (Kimura et al., 2009; Kimura et al., 2008; Kiss et al., 2011; Lee et al., 2012; Martin et al., 2009; Nguyen et al., 2010; Qiu et al., 2012; Quintana et al., 2008; Quintana et al., 2010; Veldhoen et al., 2008). In many of these instances, AHR activity affects cytokine production, but AHR signaling can also affect the survival or maintenance of certain immune cell populations (Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012). The studies presented here establish that AHR signaling also influences NK cell production of IL-10. NK cells basally expressed *Ahr* transcripts, and *Ahr* expression in LAKs increased following stimulation with IL-12. AHR signaling in turn promoted NK cell IL-10 production *in vitro* and contributed to their IL-12 dependent production of IL-10 during infection with *T. gondii*. Although previous work has suggested that NK cell IL-10 limits IL-12 production during experimental toxoplasmosis (Perona-Wright et al., 2009), in the studies presented here $Ahr^{-/-}$ and wild-type mice had similar levels of IL-12p40. Importantly $Ahr^{-/-}$ mice also had lower parasite burdens than wild-type mice, which likely impacted their IL-12 levels.

While this chapter has focused on the impact of the AHR on NK cell production of IL-10, its effects on IFN-γ expression were variable. Treatment with one of the three AHR inhibitors used in these studies led to decreased NK cell IFN-γ production, and $Ahr^{-/-}$ LAKs were impaired in their ability to express IFN-γ. In contrast, no defect in IFN-γ

production was seen in NK cells isolated from infected *Ahr*$^{-/-}$ mice following restimulation *ex vivo*, but this result could potentially be due to the effects of treatment with PMA/ionomycin, potent stimuli that may override a requirement for AHR signaling. Notably, a recent study has shown that the AHR promotes NK cell cytolytic activity and IFN-γ production, and that NK cells with deficient AHR activity are impaired in their ability to control tumor growth (Shin et al., 2013). Collectively these studies indicate that the AHR has a number of important effects on NK cell function in settings of infection and cancer. Thus AHR expression likely contributes to the ability of NK cells to sense environmental signals. NK cells express a number of receptors that allow them to detect external cues, including cytokine receptors and activating and inhibitory receptors (Yokoyama et al., 2004). AHR activity may provide these cells with an additional means of responding to the environment, in this case, by promoting the production of IL-10. NK cells have been shown to produce IL-10 following infection with *Y. pestis*, *Listeria monocytogenes,* and murine cytomegalovirus (Lee et al., 2009; Perona-Wright et al., 2009). Additionally, during visceral leishmaniasis, NK cell IL-10 promotes increased parasite burdens (Maroof et al., 2008). Indeed, in the studies presented here, the defect in NK cell IL-10 expression in *Ahr*$^{-/-}$ mice correlated with decreased levels of *T. gondii* at five days post-infection. Thus, it seems likely that AHR signaling affects NK cell function during challenge with other pathogens and thereby affects the outcome of infection.

The finding that only a subset of NK cells expressed IL-10/GFP at five days post-infection raises the question of what accounted for the differential expression of this cytokine. These subsets may reflect variability in AHR expression or access to AHR ligands. Alternatively, subsets may be distinguished by differences in cytokine responsiveness. NK cells exhibit variable expression of IL-12Rβ2, and those cells expressing higher levels of IL-12Rβ2 could be more prone to producing IL-10 in response to IL-12 stimulation (Chakir et al., 2000). Interestingly, IL-10/GFP$^+$ NK cells also expressed high levels of T-bet and KLRG1, mirroring the phenotype of short-lived effector CD8$^+$ T cells, which express higher levels of T-bet and KLRG1 than the CD8$^+$ T cells that give rise to long term memory (Joshi et al., 2007). Several reports have indicated that NK cells can also form memory populations (Cooper et al., 2009; O'Leary et al., 2006; Paust et al., 2010; Sun et al., 2009), and the expression of T-bet and KLRG1 may distinguish short-lived and long-lived populations of NK cells, paralleling the expression of these markers in different populations of CD8$^+$ T cells.

This report also raises the question of what the sources of AHR ligands are that affect NK cell function. AHR ligands are present in cell culture medium, which is consistent with the finding that LAKs stimulated in IMDM, which contains relatively high levels of AHR ligands, produced the highest levels of IL-10. *In vivo*, AHR ligands may be obtained through the diet, as a number of plant compounds are thought to activate the AHR. Diets low in AHR ligands have been shown to affect immune activity in the gut (Kiss et al., 2011; Li et al., 2011), but dietary AHR ligands may have more systemic

effects on the immune response. Alternatively, AHR ligands may be produced by NK cells themselves or by other host cells in response to infection. An additional possibility is that AHR ligands are produced directly by the parasite during infection. *T. gondii* can catalyze the production of lipoxin A4, a putative AHR ligand that can also be produced by host cells (Bannenberg et al., 2004). Another potential source of parasite-derived ligands is based on the shikimate pathway, which is a biochemical pathway found in *T. gondii* that provides precursors for the synthesis of a number of aromatic compounds (Roberts et al., 2002), which may serve as AHR ligands. The shikimate pathway is active in a number of pathogens, including other apicomplexans such as *Plasmodium falciparum* and bacteria such as *Mycobacterium tuberculosis* (Roberts et al., 2002). The generation of potential AHR ligands by *T. gondii* raises the possibility that the AHR may act as a sensor that can detect pathogen-derived metabolic products during infection. Indeed, it has been suggested that the innate immune system can distinguish between live and dead microbes through the detection of viability-associated pathogen associated molecular patterns, such as bacterial mRNA (Sander et al., 2011). By acting through the AHR, parasite-derived metabolites could potentially function as another class of viability-associated pathogen associated molecular patterns.

Attribution

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CHAPTER 3: DIFFERENTIAL EFFECTS OF ARYL HYDROCARBON RECEPTOR SIGNALING DURING ORAL AND CHRONIC TOXOPLASMOSIS

Abstract

The aryl hydrocarbon receptor is a ligand-activated transcription factor that binds to multiple xenobiotic and endogenously produced compounds and has multiple effects on the immune response. Recent reports have highlighted roles for the AHR in intestinal immunity, but less is known about its functions during enteric infection. Thus, experiments were performed to determine the effects of AHR signaling during oral infection with the parasite *Toxoplasma gondii*. Compared to wild-type mice, orally infected *Ahr*$^{-/-}$ animals exhibited more severe weight loss and increased intestinal tissue pathology, which was associated with CD4^{+} T cell hyperactivation. In order to determine whether AHR signaling also had effects on T cell activation during chronic toxoplasmosis when the parasite is primarily localized to the central nervous system, *Ahr*$^{-/-}$ mice were infected intraperitoneally with *T. gondii*. AHR deficient animals had elevated parasite burdens during chronic infection. In contrast to the T cell hyperactivation seen following acute oral infection, CD4^{+} T cell responses in chronically infected *Ahr*$^{-/-}$ mice were comparable to those in wild-type animals. Together, these results demonstrate distinct roles for the AHR in tissue specific responses during toxoplasmosis.

Introduction

A number of recent studies have shown that signaling through the AHR can influence the development of both proinflammatory and regulatory immune responses (Lawrence and Vorderstrasse, 2013; Stevens et al., 2009). The AHR is a ligand activated transcription factor that binds to various structurally diverse agonists including endogenous compounds such as certain tryptophan metabolites and synthetic molecules exemplified by 2,3,7,8-tetrachlorodibenzo-p-dioxin (Denison and Nagy, 2003). AHR ligands derived from plants can also be obtained through the diet (Denison and Nagy, 2003), making the intestines a major site of exposure to AHR agonists. Signaling through this transcription factor has multiple effects on intestinal immune responses. For example, the AHR contributes to the maintenance of intraepithelial lymphocytes and group 3 innate lymphoid cells (ILCs) in the small intestine lamina propria, and promotes IL-22 production by ILCs (Kiss et al., 2011; Lee et al., 2012; Li et al., 2011; Qiu et al., 2012). AHR activity is also required for the development of isolated lymphoid follicles and cryptopatches in the intestine (Kiss et al., 2011; Lee et al., 2012). Moreover, *Ahr*$^{-/-}$ mice have elevated levels of intestinal segmented filamentous bacteria that promote Th17 cells, indicating that AHR signaling influences commensal populations in the gut (Qiu et al., 2013).

In addition to its effects on intestinal immunity, the AHR influences other aspects of the immune response. AHR deficiency leads to decreased susceptibility to central nervous system inflammation in the experimental autoimmune encephalomyelitis model,

which is associated with decreased Th17 responses (Quintana et al., 2008; Veldhoen et al., 2008). The AHR also has contrasting immunoregulatory roles, and enhances IL-10 production by a number of cell types, including macrophages, dendritic cells, and natural killer cells (Kimura et al., 2009; Nguyen et al., 2010; Wagage et al., 2014). Similarly, in type 1 regulatory T cells, the AHR contributes to the ability of IL-27 to induce IL-10 production (Apetoh et al., 2010). Taken together, these studies indicate that AHR signaling influences the development of immunoregulatory and proinflammatory immune responses in the intestine as well as other locations, but its role during infection is not well understood.

Establishing an effective balance between inflammatory responses that control pathogen growth and immune-regulation that prevents pathology is essential during infection with *Toxoplasma gondii.* This intracellular parasite induces highly Th1 polarized immune responses typified by IL-12 and IFN-γ expression. These cytokines are critical for controlling parasite replication, as mice deficient for IL-12 or IFN-γ develop elevated parasite burdens and rapidly succumb to infection (Gazzinelli et al., 1994; Scharton-Kersten et al., 1996). While the development of an inflammatory Th1 immune response is essential for controlling parasite growth, this response must be appropriately regulated to limit immune-mediated pathology to the host. The importance of immune regulation during toxoplasmosis has been highlighted by studies in mice deficient for either IL-10 or IL-27 signaling (Gazzinelli et al., 1996; Villarino et al., 2003). Following infection, IL-$10^{-/-}$ or IL-$27R\alpha^{-/-}$ mice have no defects in controlling parasite burdens but

nonetheless develop severe tissue damage and die acutely. This pathology is mediated by $CD4^+$ T cells, as IL-$10^{-/-}$ or IL-27R$\alpha^{-/-}$ mice survive acute infection following the depletion of this population (Gazzinelli et al., 1996; Villarino et al., 2003). Therefore, establishing an effective balance between inflammation that controls pathogen growth and a regulatory response that prevents excessive immune-mediated pathology is critical during toxoplasmosis and is a common feature of many infections (Couper et al., 2008).

Interestingly, AHR ligands such as L-kynurenine and lipoxin A4 are generated during *T. gondii* infection (Bannenberg et al., 2004; Silva et al., 2002), and previously published work has suggested that the AHR acts to limit inflammation following intraperitoneal (i.p.) challenge with the parasite (Sanchez et al., 2010). Similarly, as described in Chapter 2, *Ahr*$^{-/-}$ mice have reduced parasite burdens during acute i.p. infection. Given the multiple roles for the AHR in intestinal immunity, experiments were performed to determine the effects of AHR signaling in the gut during toxoplasmosis. Compared to wild-type mice, orally infected *Ahr*$^{-/-}$ animals exhibited more severe weight loss and increased intestinal tissue pathology. This phenotype was associated with $CD4^+$ T cell hyperactivation and increased IFN-γ production, as well as a decreased population of group 3 ILCs in the lamina propria.

In order to determine whether AHR signaling also had effects on T cell activation during chronic toxoplasmosis, when the parasite is primarily localized to the central nervous system, *Ahr*$^{-/-}$ mice were infected i.p. with *T. gondii*. Unexpectedly, chronically infected *Ahr*$^{-/-}$ mice had modestly increased parasite burdens in the brain, but no defects

in $CD4^+$ T cell responses were identified. Thus, additional studies were performed to evaluate anti-parasitic activity in the absence of AHR signaling. As noted in Chapter 1, the AHR has been implicated in promoting macrophage antimicrobial mechanisms, but no defects were observed in the ability of *Ahr*$^{-/-}$ macrophages to control parasite growth *in vitro*. Collectively these results indicate that following acute intestinal toxoplasmosis the AHR limits the $CD4^+$ T cell response, but this transcription factor does not have a similar role during the chronic phase of infection.

Materials and Methods

Mice and infections

Ahr$^{-/-}$ mice that had been backcrossed onto a C57/Bl6 background for 21 generations were obtained from Dr. Christopher A. Bradfield (University of Wisconsin School of Medicine and Public Health, Madison, WI). C57/Bl6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). CBA/J mice were purchased from the National Cancer Institute Animal Production Program (Frederick, MD). All mice were bred and housed in specific pathogen-free facilities at the University of Pennsylvania in accordance with institutional guidelines. For infections, Me49 cysts were harvested from the brains of chronically infected CBA/J mice and experimental animals were injected i.p. with 20 cysts or orally gavaged with 20 cysts. For the analysis of parasite burdens, DNA was isolated from tissues using the High Pure PCR Template Preparation kit (Roche, Indianapolis, IN). Parasite DNA levels were determined by RT-PCR as previously described (Silver et al., 2011).

Cell isolation

Spleens, lymph nodes and Peyer's patches were dissociated through a 40μm filter. Red blood cells in the spleens were lysed with 0.86% ammonium chloride (Sigma) in sterile water. Mononuclear cells were isolated from the brain and lamina propria lymphocytes were isolated from the small intestine as previously described (Hall et al., 2012; Wilson et al., 2005). Briefly, for cell isolation from the brain, mice were perfused with cold PBS. Brain tissue was passed through an 18-gauge needle and incubated with

collagenase/dispase (Roche Diagonstics, Indianapolis, IN) and DNAse (Sigma). The tissue was dissociated through a 70μm filter, resuspended in 60% isotonic Percoll (Amersham Bioscience, Uppsala, Sweden), and overlaid with 30% Percoll. Mononuclear cells were isolated from the interphase layer. For cell isolation from the gut, small intestines were cut longitudinally and the contents were washed in RPMI. Epithelial cells were stripped with 5mM EDTA and 1mM DTT in RPMI, and the tissue was digested with 0.16U/mL Liberase TL (Roche) and 0.05% DNAse. The suspension was passed through a 70μm filter, and then passed through a 40μm filter.

ELISAs

Cells were cultured at 1-2.5x10^6 cells/ml in complete RPMI with or without 62μg/ml soluble Toxoplasma antigen (STAg). Supernatants were collected after 48 hours and cytokines were detected by ELISA. For IL-10 ELISAs, Immulon 4HBX plates (Thermo Fisher Scientific, Waltham, MA) were coated with anti-IL-10 (clone JES5-2A5) (BD Pharmingen San Diego, CA), blocked in 5% FBS in PBS, and loaded with samples. Biotinylated anti-IL-10 (clone JES5-16E3) was used for detection followed by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), SureBlue (KPL, Gaithersburg, MD) and TMB Stop Solution (KPL). For IFN-γ ELISAs, plates were coated with anti- IFN-γ (clone AN-18) (eBioscience) and loaded with samples. Biotinylated anti- IFN-γ (clone R4-6A2) (eBioscience) was used for detection, followed by peroxidase-conjugated streptavidin and ABTS (KPL). For IL-12p40 ELISAs, plates were coated with anti-IL-12p40 (clone C17.8), followed by

detection using biotinylated anti-IL-12p40 (clone C15.6). Parasite specific antibody levels in the serum were assayed as previously described (Glatman Zaretsky et al., 2012).

RNA preparation and PCR

RNA from cells was isolated using the RNAeasy Mini Kit (Qiagen, Valencia, CA). RNA was isolated from tissues using Trizol (Invitrogen Grand Island, NY) according to the manufacturer's protocol. Samples were treated with DNAse (Promega, Madison, WI), and cDNA was generated using reagents from Invitrogen. For RT-PCR analysis, cDNA samples were amplified with SYBR Green (Applied Biosystems, Carlsbad, CA) on the 7500 Fast Real-Time PCR System (Applied Biosystems). Relative gene expression was calculated using the $2^{-\Delta\Delta_{CT}}$ method (Schmittgen and Livak, 2008).

Flow cytometry

For intracellular cytokine staining, cells were incubated with brefeldin A (Sigma) and PMA and ionomycin for 4 hours, surface stained, and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Cells were then permeabilized with 0.5% saponin (Sigma) and stained for cytokines. For surface staining, samples were washed in flow cytometry buffer containing 1% BSA (Sigma) and 2mM EDTA (Invitrogen) in PBS, Fc blocked with 2.4G2 and normal rat IgG (Invitrogen), and stained with monoclonal antibodies. For the detection of T-bet, FoxP3 and Ki67, cells were surface stained and then stained intracellularly for these markers using the FoxP3/transcription factor staining buffer set (eBioscience). Samples were run on a FACSCanto II or LSRFortessa and data was analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).

In vitro macrophage infections

Bone marrow derived macrophages were generated as previously described (Whitmarsh et al., 2011). Cells were harvested and plated at a concentration of $1x10^6$ cells/mL in polypropylene tubes, stimulated with IFN-γ and TNF-α for 5 hours, and infected with *T. gondii* tachyzoites. One hour later the cells were spun at a low speed and washed to remove extracellular parasites. 24 hours post-infection, cytospins of the macrophages were stained using the Hema 3 stain set (Fisher Scientific, Kalamazoo, MI) and parasites were counted.

Statistical analysis

Statistical significance was determined using unpaired Student's t tests, which were performed using Prism software (GraphPad software, Inc. La Jolla, CA). P values of less than 0.05 were considered significant. Error bars in all graphs represent the standard deviation.

Results

Increased pathology and T cell activation in orally infected *Ahr*$^{-/-}$ mice

The AHR has been shown to affect multiple aspects of intestinal immune responses, raising the question of whether this transcription factor influences immunity to a parasitic infection of the gut. To investigate the effects of the AHR during intestinal infection, wild-type and *Ahr*$^{-/-}$ mice were challenged orally with a low dose of *T. gondii* cysts and weight loss was monitored over time to determine disease progression. In wild-type mice, this dose induces modest tissue pathology and weight loss (Figure 3.1A). In contrast, infected *Ahr*$^{-/-}$ mice lost more weight than their wild-type counterparts (Figure 3.1A). H&E staining of intestinal tissue sections from *Ahr*$^{-/-}$ mice revealed increased tissue pathology (Figure 3.1B). These animals also had modestly elevated parasite burdens in the terminal ileum (Figure 3.1C).

Since CD4^{+} T cells contribute to immune-mediated pathology during oral toxoplasmosis (Liesenfeld et al., 1996), studies were performed to determine whether an excessive T cell response might be responsible for the increased pathology seen in the absence of AHR signaling. Infected *Ahr*$^{-/-}$ mice had increased numbers of CD4^{+} T cells in the lamina propria (Figure 3.2A), but not in other sites such as the spleen. T cell activation was also evaluated in the spleen and mesenteric lymph nodes following infection. CD4^{+} T cells in infected *Ahr*$^{-/-}$ mice expressed higher levels of the Th1 associated transcription factor T-bet, as well as CXCR3, a T-bet target gene (Figure 3.2B and data not shown) (Koch et al., 2009; Lord et al., 2005). The expression of Ki67, a

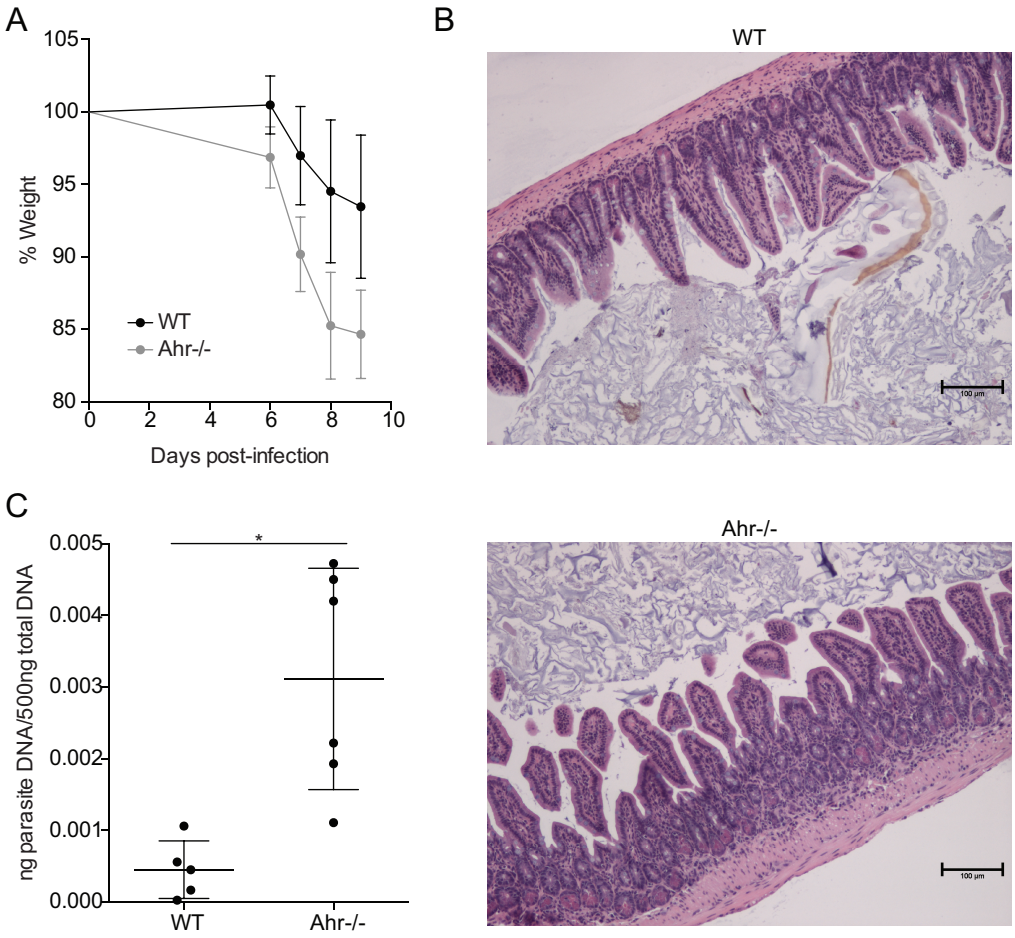

Figure 3.1: Increased susceptibility of *Ahr*$^{-/-}$ mice to oral infection with *T. gondii*. *Ahr*$^{-/-}$ mice or wild-type controls were infected orally with 20 Me49 cysts for 9 days. **(A)** Weight loss was monitored at various days post-infection. Data are pooled from 2 experiments with 6-8 mice per group. **(B)** H&E staining of small intestinal tissue sections. **(C)** Parasite burdens in the terminal ileum of infected mice was measured by RT-PCR. Results are pooled from 2 experiments.

marker of proliferating cells, was also elevated in $CD4^+$ T cells in *Ahr*$^{-/-}$ mice, suggesting that an increased proportion of these cells was undergoing proliferation (Figure 3.2B). However, the expression of T-bet, CXCR3, and Ki67 by $CD4^+$ T cells was comparable in naïve wild-type and *Ahr*$^{-/-}$ mice (data not shown). Additionally, an elevated percentage of $CD4^+$ T cells in infected *Ahr*$^{-/-}$ mice expressed high levels of CD11a, a component of LFA-1 that is associated with antigen-experienced T cells (Figure 3.2C) (Doll et al., 2013).

Consistent with the increased activation phenotype of *Ahr*$^{-/-}$ $CD4^+$ T cells, these cells produced elevated levels of IFN-γ following stimulation with PMA/ionomycin (Figure 3.3A). However, IFN-γ secretion by wild-type and *Ahr*$^{-/-}$ cells in response to soluble Toxoplasma antigen (STAg) was similar (Figure 3.3B). These results raised the possibility that the excessive T cell activation seen in *Ahr*$^{-/-}$ mice was not due to parasite-specific T cells but rather cells that were recognizing other antigens available during infection. Indeed, previous work has shown that toxoplasmosis leads to disruption of the intestinal barrier and the induction of microbiota-specific T cell responses (Hand et al., 2012). This finding suggests that one explanation for the phenotype in *Ahr*$^{-/-}$ mice is a dysregulated response against commensal bacteria. Although additional experiments are needed to evaluate this possibility (see Discussion), when taken together these results indicated that the increased susceptibility of *Ahr*$^{-/-}$ mice to oral toxoplasmosis was due to pathology mediated by a dysregulated immune response that was associated with increased parasite burdens.

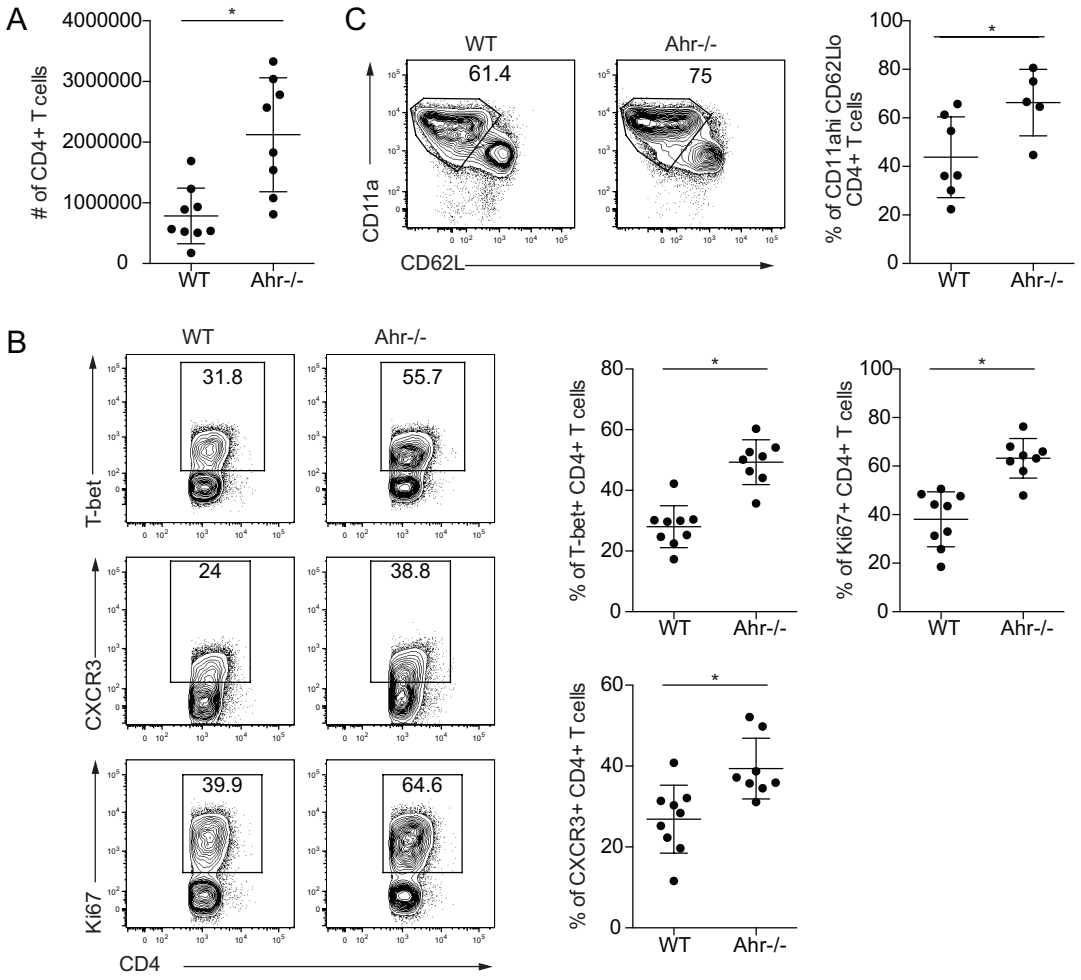

Figure 3.2: Increased T cell activation in *Ahr*$^{-/-}$ mice following oral infection. *Ahr*$^{-/-}$ mice or wild-type controls were infected orally with 20 Me49 cysts for 9 days. **(A)** Numbers of CD4^{+} T cells isolated from the lamina propria of infected mice. Data are pooled from 3 experiments. Plots are gated on FoxP3^{-}CD3^{+}CD4^{+} cells. **(B)** Expression of T-bet, CXCR3, and Ki67 by CD4^{+} T cells in the mesenteric lymph nodes of infected mice. Results are pooled from 3 separate experiments. **(C)** Percentage of CD62L^{lo}CD11a^{hi} CD4^{+} T cells in mesenteric lymph nodes. Data are pooled from 2 separate experiments. Plots are gated on CD3^{+}CD4^{+} cells.

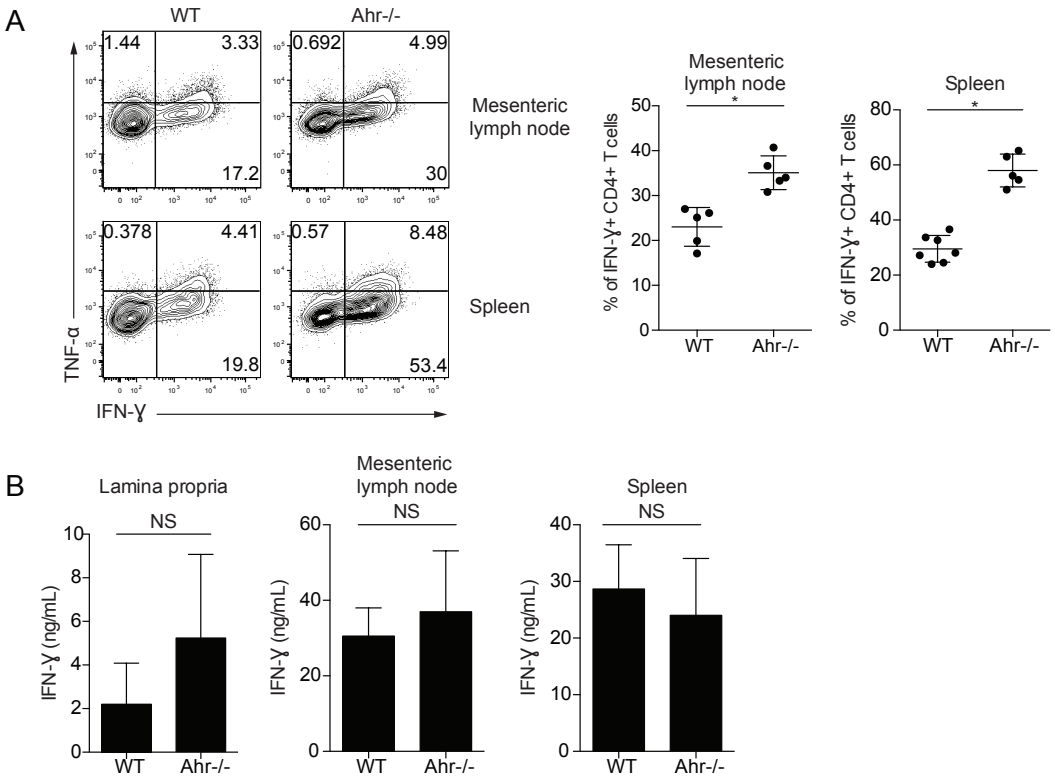

Figure 3.3: IFN-γ expression in *Ahr*$^{-/-}$ mice following oral infection.
Ahr$^{-/-}$ mice or wild-type controls were infected orally with 20 Me49 cysts for 9 days. CD4^{+} T cells were gated as CD3^{+}CD4^{+} cells. **(A)** Cytokine production by CD4^{+} T cells following stimulation with PMA/ionomycin. Data are pooled from 2 experiments. **(B)** IFN-γ secretion by cells from the lamina propria, mesenteric lymph nodes, and spleens of infected mice following restimulation with STAg. Results are pooled from 2 separate experiments.

AHR signaling can influence Treg development (Kimura et al., 2008; Quintana et al., 2008), suggesting that a defective Treg response might have promoted the excessive T cell activation seen in *Ahr*$^{-/-}$ mice. However, Treg frequencies were similar in infected wild-type and *Ahr*$^{-/-}$ mice (Figure 3.4A). Previous work has indicated that the expression of T-bet and CXCR3 promote Treg function by allowing these cells to migrate to sites of inflammation (Hall et al., 2012; Koch et al., 2009). Analysis of the Treg phenotype during infection indicated no defects in the expression of these markers in *Ahr*$^{-/-}$ animals (Figure 3.4B). Additionally, although the AHR has been shown to promote IL-10 production in a variety of cell types (Apetoh et al., 2010; Kimura et al., 2009; Nguyen et al., 2010; Wagage et al., 2014), no major defect in IL-10 production following STAg restimulation was observed in orally infected *Ahr*$^{-/-}$ mice (Figure 3.5). Collectively these results suggested that the increased susceptibility of *Ahr*$^{-/-}$ mice to oral toxoplasmosis was due to tissue damage mediated by hyperactivated CD4^{+} T cells, indicating that signaling through the AHR acted to limit T cell activation during infection.

Group 3 ILC responses during oral toxoplasmosis

A prominent phenotype in *Ahr*$^{-/-}$ mice is their lack of lamina propria group 3 ILCs (Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012), raising the question of whether these cells were influencing the pathology seen in *Ahr*$^{-/-}$ mice during oral infection with *T. gondii*. Since a role for group 3 ILCs during toxoplasmosis has not been described, experiments were performed to evaluate the phenotype of these cells following infection. In these studies, oral challenge with *T. gondii* led to a decrease in RORγt^{+} ILC frequency

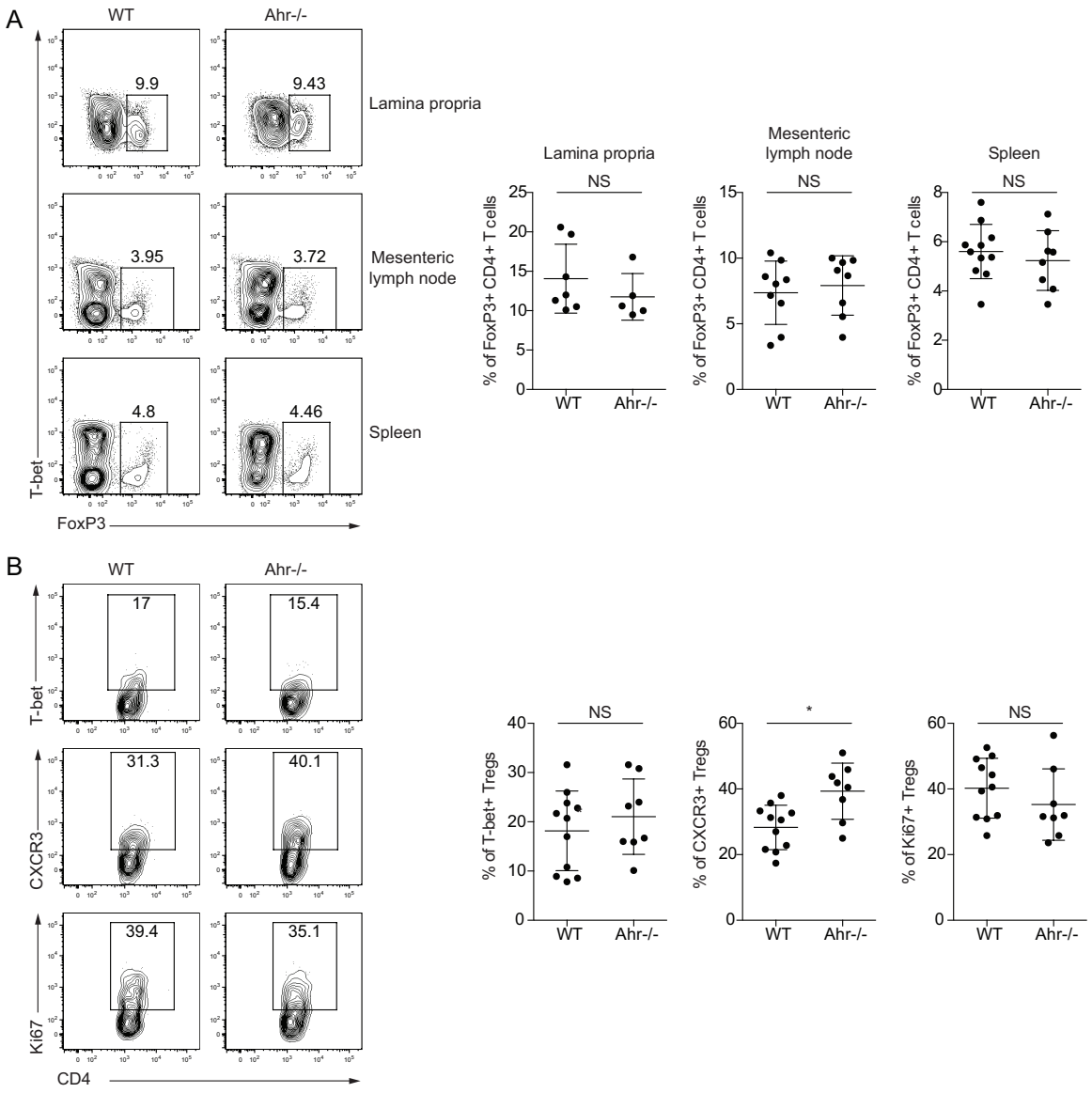

Figure 3.4: Treg phenotype in orally infected *Ahr*$^{-/-}$ mice. *Ahr*$^{-/-}$ mice or wild-type controls were infected orally with 20 Me49 cysts for 9 days. In the mesenteric lymph node and spleen Tregs were gated as CD3^{+}CD4^{+}FoxP3^{+} cells. In the lamina propria, Tregs were gated as live CD45^{+}CD3^{+}CD4^{+}FoxP3^{+} cells. Results are pooled from 2-3 separate experiments. **(A)** Frequency of Tregs in the indicated tissues of wild-type or *Ahr*$^{-/-}$ mice. **(B)** Expression of T-bet, CXCR3, and Ki67 on Tregs in the spleens of wild-type or *Ahr*$^{-/-}$ mice.

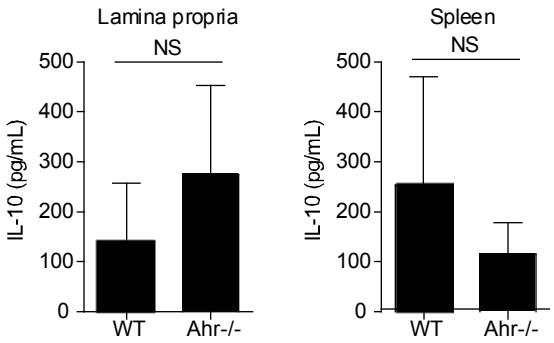

Figure 3.5: IL-10 expression by cells from orally infected *Ahr*$^{-/-}$ mice. *Ahr*$^{-/-}$ mice or wild-type controls were infected orally with 20 Me49 cysts for 9 days. IL-10 secretion by cells isolated from the lamina propria or the spleen following restimulation with STAg. Results are pooled from 2 separate experiments with a total of 5-7 mice per group.

and number in the lamina propria (Figure 3.6A, B). However, the expression of Ki67 increased, suggesting that an increased frequency of these cells was undergoing proliferation (Figure 3.6C). Group 3 ILCs in infected mice also expressed reduced levels of RORγt (Figure 3.6D). Previous work has indicated that group 3 ILCs are capable of presenting antigen, but lack the expression of costimulatory molecules, and consequently act to promote tolerance to commensal antigens (Hepworth et al., 2013). Analysis of the expression of MHC class II and the costimulatory molecule CD80 on group 3 ILCs indicated that these markers were expressed by an increased frequency of RORγt^{+} ILCs during infection, suggesting that these cells may be able to activate adaptive immune responses in this setting (Figure 3.6E). In infected *Ahr*$^{-/-}$ mice, RORγt^{+} ILCs were absent in the lamina propria but were present in the Peyer's patches at similar frequencies as in wild-type animals, consistent with previous descriptions of naïve *Ahr*$^{-/-}$ mice (Figure 3.7A) (Lee et al., 2012). Preliminary studies also indicated that these cells produced IL-22 in infected wild-type mice, while *Ahr*$^{-/-}$ RORγt^{+} ILCs exhibited defects in IL-22 expression (Figure 3.7B). Thus, the group 3 ILC defects that have been observed in naïve *Ahr*$^{-/-}$ mice were also evident in these animals during toxoplasmosis.

A role for the AHR in chronic infection

In order to determine whether AHR activity also influenced the response to chronic toxoplasmosis, wild-type and *Ahr*$^{-/-}$ mice were challenged i.p. with *T. gondii*, allowing the mice to progress to chronic infection. This route of infection also minimized

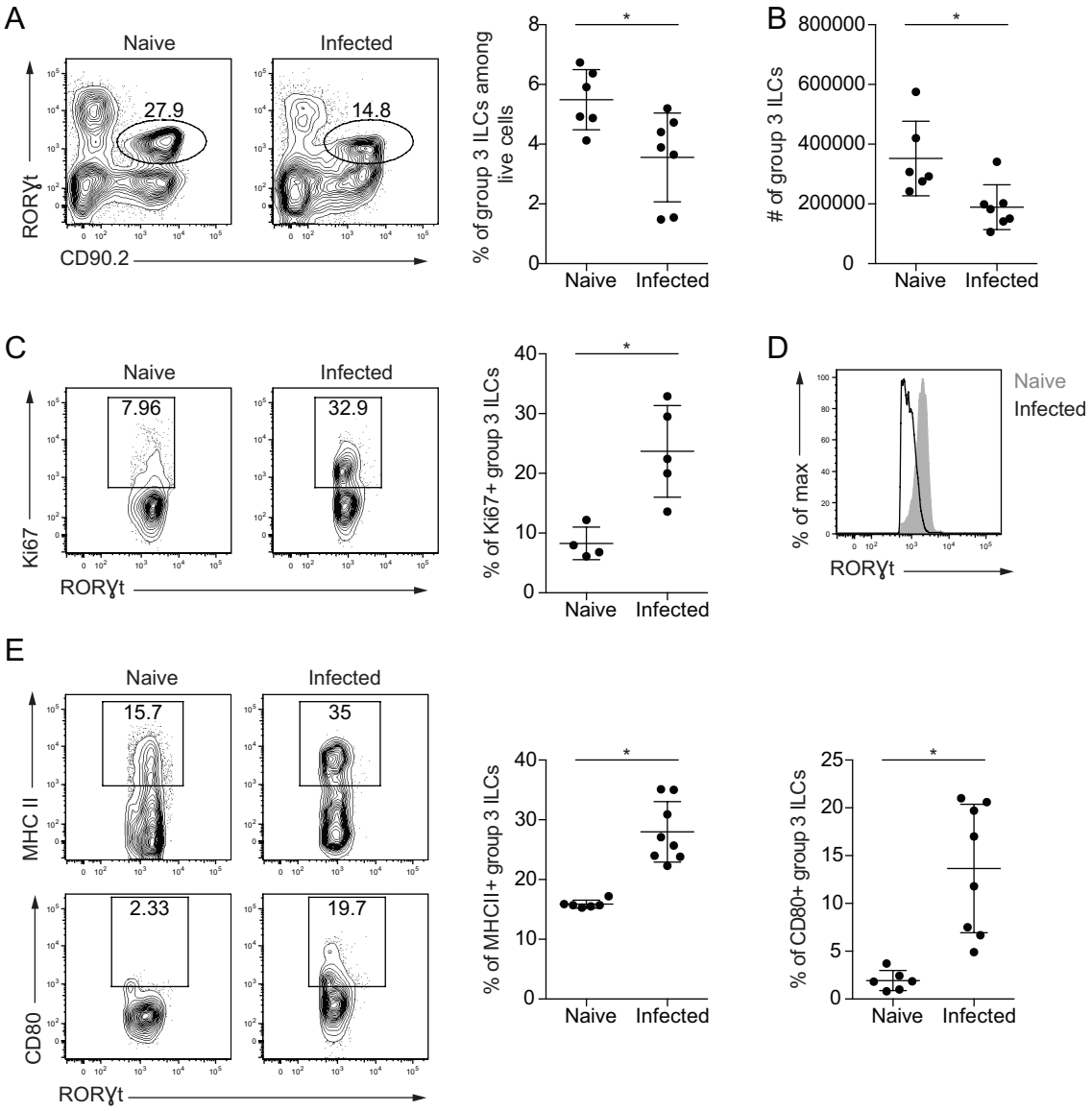

Figure 3.6: Phenotype of group 3 ILCs following infection. Wild-type mice were infected orally with 20 Me49 cysts for 9 days, and cells were isolated from the lamina propria of infected mice or naïve controls. Group 3 ILCs were gated as live NK1.1$^-$CD3$^-$CD5$^-$CD11c$^-$B220$^-$CD90.2$^+$RORγt$^+$ cells. Results are pooled from 2-3 separate experiments. **(A)** Frequency of group 3 ILCs in the lamina propria. The plots on the left are gated on live NK1.1$^-$CD3$^-$CD5$^-$CD11c$^-$B220$^-$ cells. **(B)** Number of group 3 ILCs isolated from the lamina propria. **(C)** Ki67 expression by group 3 ILCs. **(D)** RORγt expression by group 3 ILCs. **(E)** MHC II and CD80 expression by group 3 ILCs.

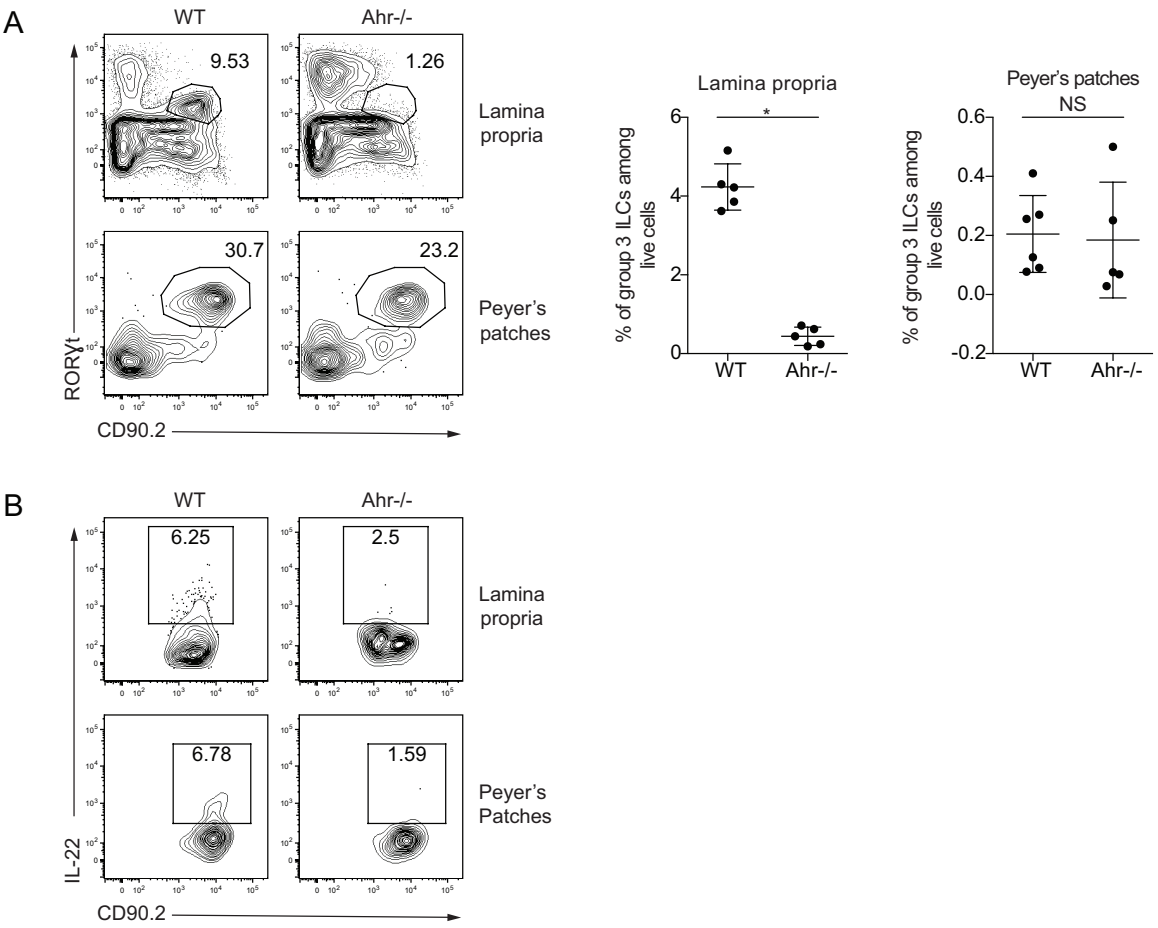

Figure 3.7: Group 3 ILCs in infected wild-type and *Ahr*$^{-/-}$ mice. Wild-type or *Ahr*$^{-/-}$ mice were infected orally with 20 Me49 cysts for 9 days. Group 3 ILCs were gated as live NK1.1^{-}CD3^{-}CD5^{-}CD11c^{-}B220^{-}CD90.2^{+}RORγt^{+} cells. Results are pooled from 2-3 separate experiments. **(A)** Frequency of group 3 ILCs in the lamina propria and Peyer's patches. The plots on the left are gated on live NK1.1^{-}CD3^{-}CD5^{-} CD11c^{-}B220^{-} cells. Results are pooled from two independent experiments. **(B)** IL-22 production by group 3 ILCs following restimulation with PMA/ionomycin. Results are based on one experiment with 2-3 mice per group. In the lamina propria, the frequency of group 3 ILCs in the IL-22^{+} gate ranged from approximately 4-9% for wild-type mice and was less than 3% for *Ahr*$^{-/-}$ mice. In the Peyer's patches, the frequency of group 3 ILCs in the IL-22^{+} gate ranged from approximately 4-8% for wild-type mice and was less than 2% for *Ahr*$^{-/-}$ mice.

potential effects of the intestinal microbiota and gut derived AHR ligands. As described in Chapter 2, i.p. infected *Ahr*$^{-/-}$ mice had reduced peritoneal parasite levels at 5 days post-infection. However, by 41 days post-challenge, *Ahr*$^{-/-}$ mice developed elevated brain parasite burdens, as measured by their levels of parasite DNA (Figure 3.8A). Brain tissue sections from *Ahr*$^{-/-}$ mice also revealed increased numbers of parasite cysts (Figure 3.8B). These results indicated that *Ahr*$^{-/-}$ mice had a reduced ability to control parasite replication in the brain, suggesting that the AHR was acting to promote effector mechanisms in this setting.

Given the T cell hyperactivation seen in orally infected *Ahr*$^{-/-}$ mice, the phenotype of CD4^{+} T cells in i.p. infected animals was also evaluated. Preliminary studies indicated that the prominent CD4^{+} T cell hyperactivation seen 9 days following oral infection was absent in *Ahr*$^{-/-}$ mice that were infected i.p. for 9 days (Figure 3.9). Similarly, the analysis of CD4^{+} T cells during chronic infection in the brains and spleens of *Ahr*$^{-/-}$ mice indicated that these cells expressed comparable levels of T-bet, CXCR3, and Ki67 to wild-type controls (Figure 3.10 and data not shown). Additionally, no differences in Treg frequency or phenotype were observed between chronically infected wild-type and *Ahr*$^{-/-}$ animals (Figure 3.11).

In order to evaluate effector cytokine production during chronic infection, mRNA was isolated from brain tissue. RT-PCR analysis demonstrated that *Ahr*$^{-/-}$ mice expressed similar levels of IL-12 and IFN-γ transcripts during chronic toxoplasmosis (Figure 3.12A). Following stimulation with PMA/ionomycin, CD4^{+} T cells isolated from the

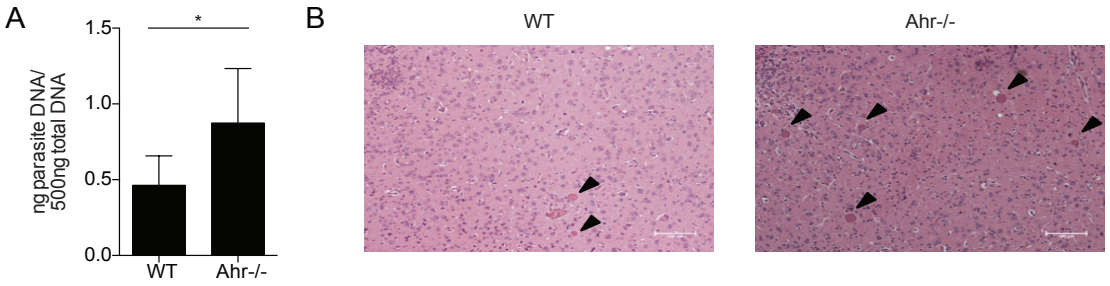

Figure 3.8: Elevated parasite burdens in *Ahr*$^{-/-}$ mice during chronic toxoplasmosis. Wild-type or *Ahr*$^{-/-}$ mice were infected i.p. with 20 Me49 cysts for 41 days. **(A)** Parasite burdens in the brains of chronically infected mice were assayed by RT-PCR. The graph shows the pooled average from 4 separate experiments with a total of 9-11 mice per group. **(B)** H&E staining of brain sections from infected mice shows parasite cysts.

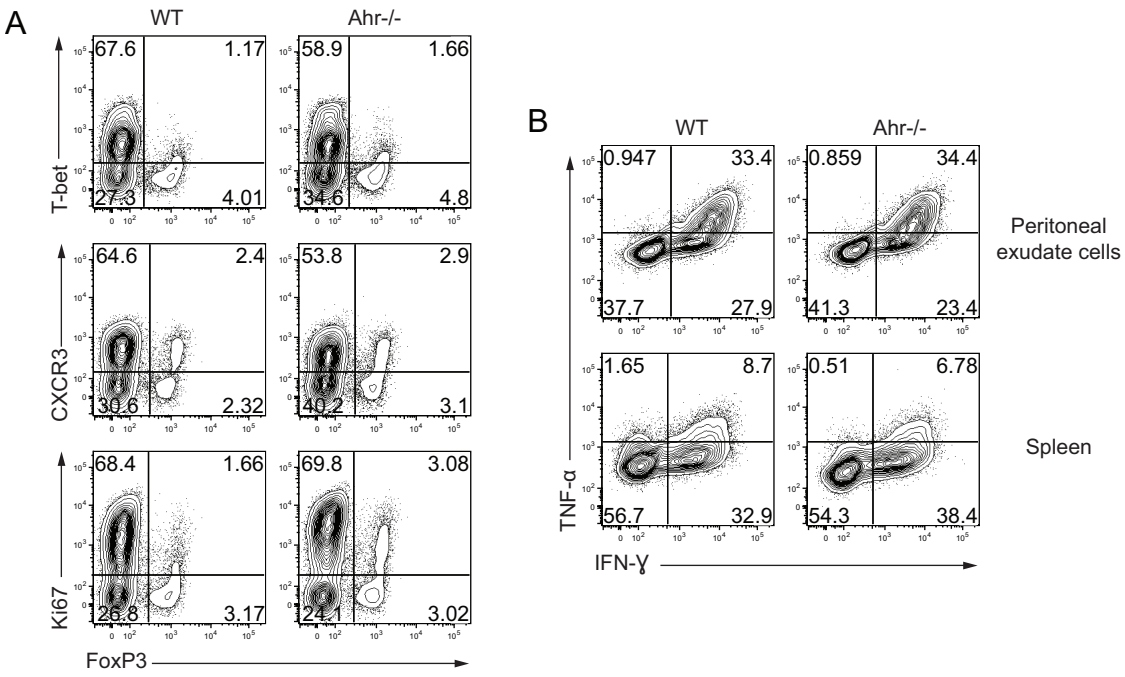

Figure 3.9: Similar T cell activation in *Ahr*$^{-/-}$ mice following i.p infection. *Ahr*$^{-/-}$ mice or wild-type controls were infected i.p. with 20 Me49 cysts for 9 days. Results are based on 1 experiment with 2-4 mice per group. CD4^{+} T cells were gated as CD3^{+}CD4^{+} cells. **(A)** Expression of T-bet, CXCR3, and Ki67 by CD4^{+} T cells in the spleen. FoxP3^{-}CD4^{+} T cells in wild-type mice were approximately 56-71% T-bet^{+}, 59-69% CXCR3^{+}, and 69-76% Ki67^{+}. In *Ahr*$^{-/-}$ mice, FoxP3^{-}CD4^{+} T cells were approximately 67-71% T-bet^{+}, 64-70% CXCR3^{+}, and 68-77% Ki67^{+}. **(B)** TNF-α and IFN-γ production by CD4^{+} T cells in peritoneal exudate cells and spleens of infected mice following restimulation with PMA and ionomycin. In the spleens of wild-type mice, 20-40% of CD4^{+} T cells stained IFN-γ^{+}, while 35-40% of *Ahr*$^{-/-}$ CD4^{+} T cells stained IFN-γ^{+}.

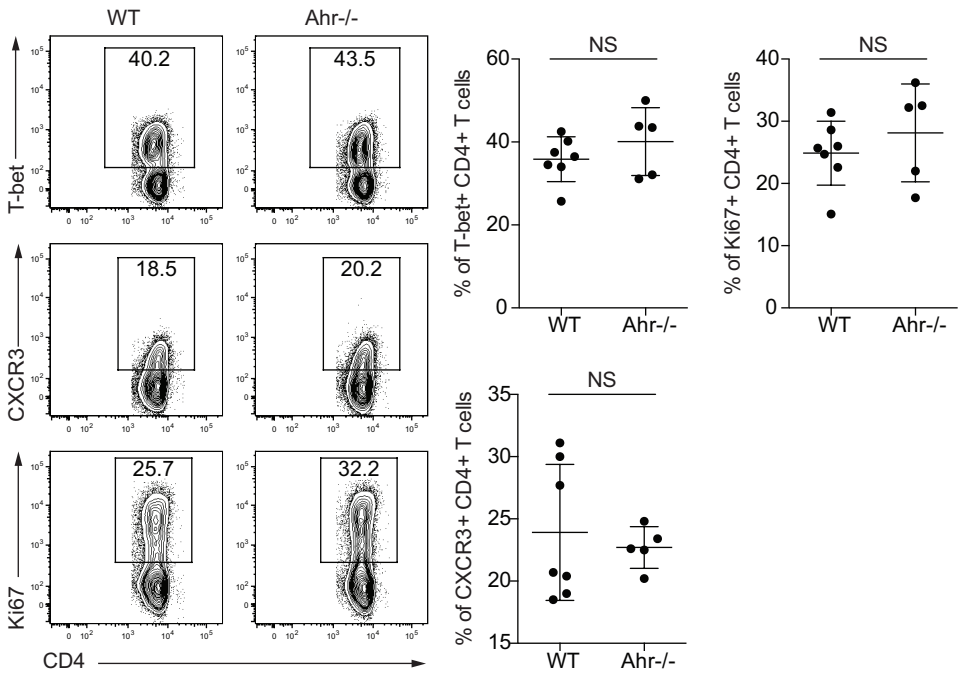

Figure 3.10: T cell activation in chronically infected *Ahr*$^{-/-}$ mice. Wild-type or *Ahr*$^{-/-}$ mice were infected i.p. with 20 Me49 cysts for 41 days. Expression of T-bet, CXCR3, and Ki67 by CD4^{+} T cells in the spleen. Plots are gated on FoxP3^{-} CD4^{+}CD3^{+} cells. Results are pooled from 2 independent experiments.

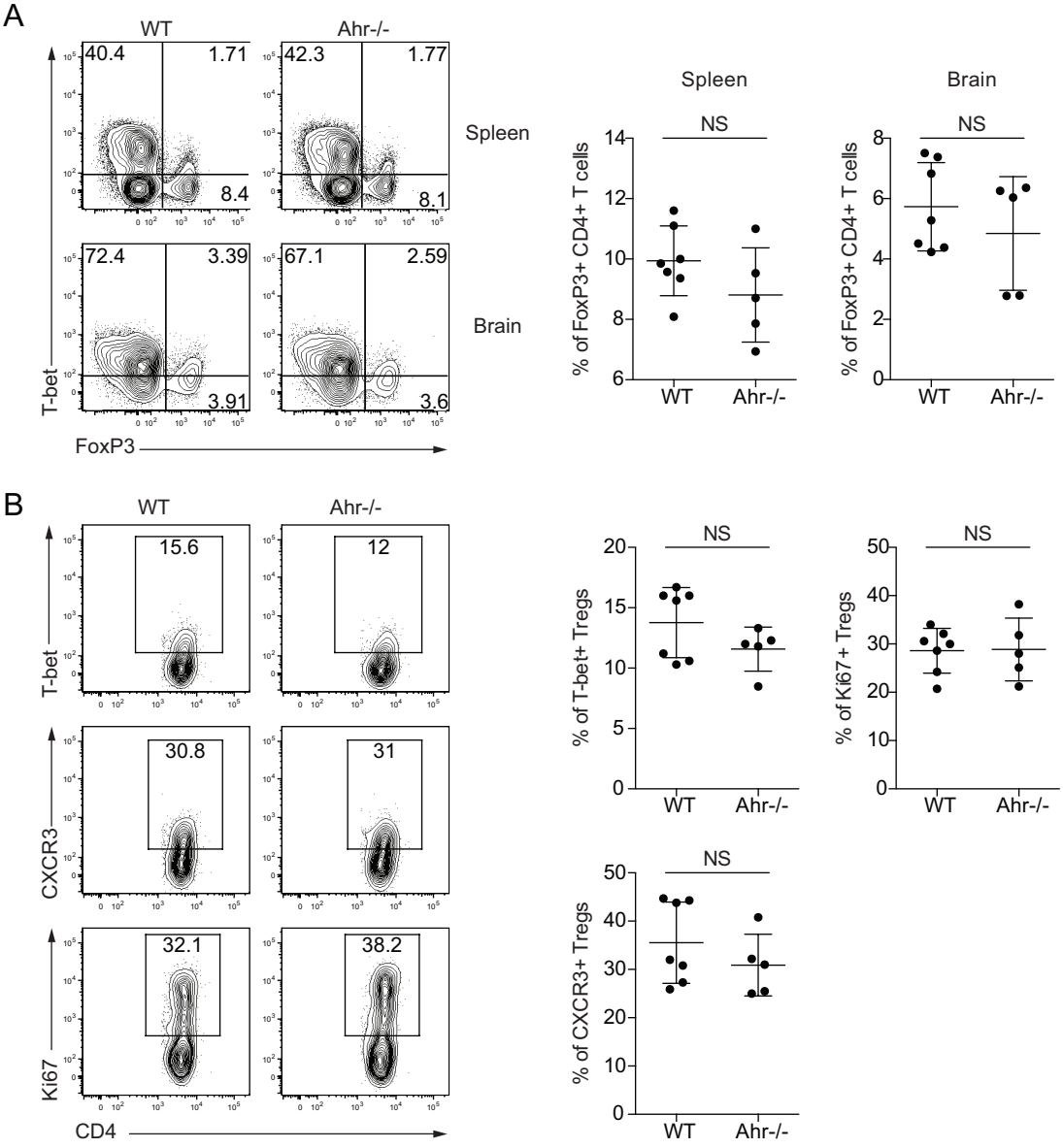

Figure 3.11: Treg phenotype during chronic infection. *Ahr*$^{-/-}$ mice or wild-type controls were infected orally with 20 Me49 cysts for 41 days. Tregs were gated as CD3^{+}CD4^{+}FoxP3^{+} cells. Results are pooled from 2 separate experiments. **(A)** Frequency of Tregs in the indicated tissues of wild-type or *Ahr*$^{-/-}$ mice. **(B)** Expression of T-bet, CXCR3, and Ki67 on Tregs in the spleens of wild-type or *Ahr*$^{-/-}$ mice.

brains of chronically infected *Ahr*$^{-/-}$ mice produced similar levels of IFN-γ as wild-type controls (Figure 3.12B). Additionally, brain mononuclear cells from infected *Ahr*$^{-/-}$ and wild-type mice secreted similar amounts of IL-12p40 and IFN-γ when stimulated with STAg (Figure 3.12C). Together, these data revealed that the prevalent T cell hyperactivation seen following acute oral infection in *Ahr*$^{-/-}$ mice was absent following i.p. challenge. Additionally, although *Ahr*$^{-/-}$ mice were impaired in their ability to control parasite burdens chronically, no defect in Th1 responses was apparent in these animals.

Other effector mechanisms in *Ahr*$^{-/-}$ mice

In an attempt to understand the basis for the increased parasite burdens in chronically infected *Ahr*$^{-/-}$ mice, additional effector mechanisms involved in controlling *T. gondii* growth were analyzed. Since humoral immunity plays an essential role in controlling parasite burdens (Kang et al., 2000), the production of *T. gondii*-specific antibodies in wild-type and *Ahr*$^{-/-}$ mice was assayed. No difference was observed in the circulating levels of parasite-specific antibodies in *Ahr*$^{-/-}$ mice (Figure 3.13A). Additionally, the activation of innate cell populations, such as microglia, was intact in the brains of chronically infected *Ahr*$^{-/-}$ mice (Figure 3.13C). A possible alternative explanation for the increased parasite burdens seen in *Ahr*$^{-/-}$ mice was a cell intrinsic defect in controlling parasite growth. Indeed, previous work has shown that the AHR promotes the expression of indoleamine 2,3, dioxygenase in dendritic cells (Nguyen et al., 2010). This enzyme catalyzes tryptophan degradation, thereby limiting the growth of *T. gondii,* a tryptophan auxotroph (Divanovic et al., 2012; Pfefferkorn et al., 1986). In

order to determine whether *Ahr*$^{-/-}$ cells had a defect in controlling parasite replication in response to cytokine stimulation, bone marrow-derived macrophages from wild-type or *Ahr*$^{-/-}$ mice were stimulated *in vitro* with IFN-γ and TNF-α. The cells were infected with *T. gondii* tachyzoites, and twenty four hours later, intracellular parasite numbers were determined (Figure 3.13D). Wild-type and *Ahr*$^{-/-}$ macrophages had similar rates of infection when left unstimulated or when treated with IFN-γ and TNF-α, indicating that macrophages had no defect in controlling parasite growth in the absence of AHR signaling. In agreement with these findings, the levels of transcripts in the central nervous system for indoleamine 2,3, dioxygenase and IGTP, which are induced by IFN-γ and promote the control of parasite growth, were similar between wild-type and *Ahr*$^{-/-}$ mice (Figure 3.13D). Thus, although chronically infected *Ahr*$^{-/-}$ mice had elevated parasite burdens, no defect was apparent in their Th1 immune response or in the cell intrinsic ability of *Ahr*$^{-/-}$ macrophages to limit parasite replication.

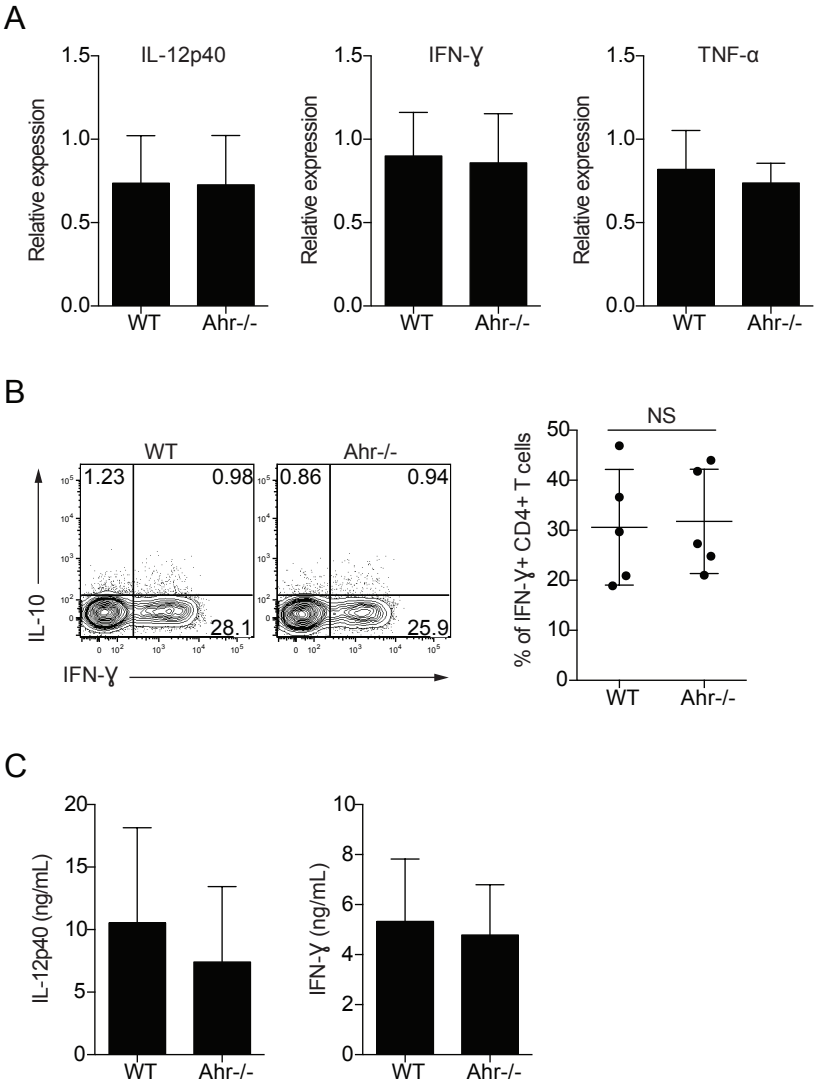

Figure 3.12: Cytokine expression in *Ahr*$^{-/-}$ mice during chronic infection.
Wild-type or *Ahr*$^{-/-}$ mice were infected i.p. with 20 Me49 cysts for 41 days. **(A)** The mRNA levels of the indicated cytokines in the brains of chronically infected mice were assayed by RT-PCR. Results are representative of two experiments. **(B)** Isolated brain mononuclear cells were stimulated with PMA/ionomycin for 4 hours and stained for cytokine production. Plots are gated on $CD3^+CD4^+$ cells. Data are pooled from 2 experiments. **(C)** Isolated brain mononuclear cells were restimulated with STAg for 48 hours. Supernatants were collected, and cytokine production was assayed by ELISA. Data are pooled from 3 separate experiments.

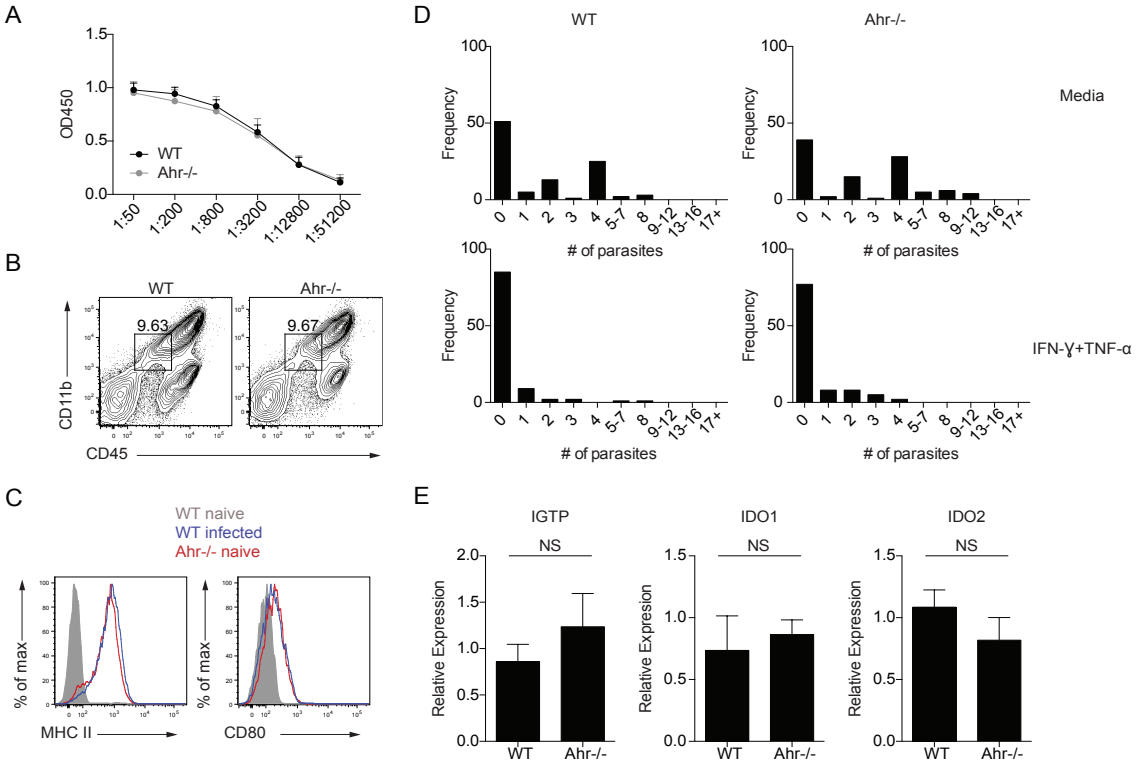

Figure 3.13: Antibody production and innate cell activation in *Ahr*$^{-/-}$ mice. **(A)** Levels of parasite-specific IgG2c in the serum of wild-type or *Ahr*$^{-/-}$ mice that were infected i.p. with 20 Me49 cysts for 41 days. Results are pooled from three experiments. **(B)** Gating for microglia in brain mononuclear cells that were isolated from mice that had been infected i.p. for 41 days. Plots are gated on CD3^{-}CD19^{-} NK1.1^{-}cells. Results are based on three independent experiments. **(C)** MHC II and CD80 expression on microglia 41 days following i.p. infection. Plots are gated as shown in figure 3.13B. Results are representative of 3 experiments. **(D)** Bone marrow-derived macrophages were stimulated with IFN-γ and TNF-α and infected with *T. gondii* tachyzoites. The cells were stained and parasites were counted 24 hours post-infection. Results are representative of 5 experiments. **(E)** The mRNA levels of IGTP and IDO in the brains of chronically infected mice were assayed by RT-PCR. Results are representative of two experiments.

Discussion

Collectively these studies indicate that AHR signaling has different roles during acute oral toxoplasmosis and chronic infection. Following oral challenge with *T. gondii*, the absence of AHR activity led to increased pathology and $CD4^+$ T cell hyperactivation, raising the question of how this transcription factor limits T cell responses in this setting. Although the AHR plays a role in T cell IL-10 production and Treg development, which constrain the immune response during toxoplasmosis, no deficiencies in these pathways were observed in *Ahr*$^{-/-}$ mice. The group 3 ILC defects seen in infected *Ahr*$^{-/-}$ mice raised the possibility that this phenotype could contribute to the pathology observed in AHR deficient animals. In naïve mice, group 3 ILCs and IL-22 signaling limit the dissemination of commensal bacteria (Sonnenberg et al., 2012), suggesting that these cells may act in a similar capacity during toxoplasmosis. Intestinal *T. gondii* infection in wild-type mice leads to the translocation of gut resident commensal bacteria to other sites and the generation of microbiota specific T cell responses (Benson et al., 2009; Hand et al., 2012). These findings raised the possibility that the *Ahr*$^{-/-}$ defect in group 3 ILCs may lead to increased bacterial translocation during infection and an elevated immune response against the microbiota. This model could be tested by treating *Ahr*$^{-/-}$ mice with antibiotics during toxoplasmosis to deplete commensal bacteria and evaluating whether this treatment attenuates the T cell hyperactivation seen in these animals. Additional studies with a tetramer that detects T cells specific for an epitope expressed by a group of commensal bacteria could determine whether there are increased microbiota-specific T

cell responses in *Ahr*$^{-/-}$ mice. Alternatively, other cell types or pathways may contribute to the T cell hyperactivation seen in these animals. For example, inflammatory monocytes were recently shown to express IL-10 and prostaglandin E_2 and limit pathology during infection (Grainger et al., 2013), and the loss of the AHR may lead to reduced production of these immunoregulatory mediators in monocytes.

Orally infected *Ahr*$^{-/-}$ mice had modestly elevated parasite burdens, which could also contribute to the T cell hyperactivation seen in these animals. Increased levels of *T. gondii* were also observed in the brains of chronically infected *Ahr*$^{-/-}$ mice. This phenotype differs from a previous finding indicating that AHR deficient animals exhibit decreased parasite burdens but increased mortality following i.p. infection (Sanchez et al., 2010). One factor that might contribute to this discrepancy is the timepoint post-infection at which parasite burdens were assayed. The previously published work described reduced parasite levels in *Ahr*$^{-/-}$ mice at 25 days post-challenge, and in agreement with this, the studies discussed in Chapter 2 identified decreased parasite burdens in these animals during early *T. gondii* infection. However, in multiple experiments, *Ahr*$^{-/-}$ mice had increased parasite levels in the brain 41 days post-challenge. These results suggest that the relative *T. gondii* burdens in *Ahr*$^{-/-}$ mice increase over time, eventually leading to increased parasite levels compared to wild-type animals. As discussed in Chapter 2, AHR activity in NK cells may contribute to the decreased parasite burdens seen in *Ahr*$^{-/-}$ mice during acute infection, and NK cell numbers and activity decline later in toxoplasmosis (Neyer et al., 1997). Alternatively, this increase in

parasite levels may be due to differences in the environment of the central nervous system compared to the primary site of infection during acute i.p. challenge. Previous work has also shown that *Ahr*$^{-/-}$ mice generated in different laboratories can have different phenotypes following infection (Lawrence and Vorderstrasse, 2013), which may explain the variability in these findings. Some of the phenotypic differences in strains of *Ahr*$^{-/-}$ mice have been attributed to the extent to which these mice were backcrossed prior to analysis (Esser, 2009). The *Ahr*$^{-/-}$ mice that were used in these studies had been backcrossed onto a C57/Bl6 background for 21 generations (Harstad et al., 2006).

No differences were seen in chronic Th1 responses or cell-intrinsic control of *T. gondii* growth in the absence of AHR signaling, but *Ahr*$^{-/-}$ mice developed elevated parasite burdens, raising the question of why these animals have a defect in limiting parasite replication. One possibility is that the AHR has cell-type specific effects that were not identified in these studies. For example, AHR signaling may control parasite replication in neural cells in the central nervous system during chronic infection but not in the macrophages that were used for *in vitro* analysis. Alternatively, functions of the AHR in nonhematopoietic cells may impact the outcome of infection, which could be addressed through the use of chimeric approaches. For example, the vascular defects in *Ahr*$^{-/-}$ mice could lead to altered dissemination of *T. gondii* in the brain or the metabolic changes induced by AHR activity may influence parasite growth.

Together, the use of different routes of *T. gondii* infection highlight context-dependent roles for the AHR during infection. The effects of the microbiota during

intestinal infection offer one possible explanation for the different phenotypes seen in orally and i.p. infected *Ahr*$^{-/-}$ mice. Alternatively, given findings indicating that different AHR ligands can have distinct effects, these varying outcomes may be a reflection of the different sources of AHR ligands available in discrete sites of infection. AHR agonists found in the diet or produced by commensal bacteria are prevalent in the gut, whereas compounds produced by the host such as L-kynurenine may be more important in the brain. Gaining an understanding of how AHR signaling mediates context-dependent effects could facilitate targeting this pathway therapeutically and provide insight into how exposure to environmental or infection-induced AHR agonists impacts health.

Attribution

Gretchen Harms Pritchard, Lucas A. Dawson, and Arielle Glatman Zaretsky contributed to the work presented in this chapter.

CHAPTER 4: DISCUSSION

Increasing appreciation of AHR-mediated effects on immunity

Some of the earliest reports that suggested a role for the AHR in the immune system were toxicological studies examining the effects of exposure to synthetic AHR agonists such as dioxin. Dioxin treatment has multiple effects on immunity, including thymic involution, the inhibition of delayed hypersensitivity responses, and the suppression of antibody production (Buu-Hoi et al., 1972; Faith et al., 1978; Tucker et al., 1986). Concordantly, exposure to low doses of dioxin was found to increase murine susceptibility to challenge with *Salmonella* and *Plasmodium yoelli* (Thigpen et al., 1975; Tucker et al., 1986). These studies need to be interpreted carefully due to the systemic toxic effects of dioxin, which make identifying the specific mechanisms for dioxin-mediated effects on the immune system challenging. Relatively recent studies revealing a role for the AHR in Th17 cells and Tregs led to increased interest in evaluating the effects of this transcription factor on immunity (Kimura et al., 2008; Quintana et al., 2008; Veldhoen et al., 2008). As described in Chapter 1, subsequent work has identified multiple functions for the AHR in the immune system, particularly in the intestine.

The experiments presented here contribute to this body of work by describing context-dependent effects of the AHR following oral and i.p. infection with *T. gondii* and a role for this transcription factor in NK cell production of IL-10. When taken together with other reports demonstrating that AHR activity promotes IL-10 production by

macrophages, dendritic cells, and T cells (Apetoh et al., 2010; Kimura et al., 2009; Nguyen et al., 2010), these studies indicate that the AHR is a conserved regulator of IL-10 expression by innate and adaptive cells. Importantly, the effects of the AHR on IL-10 production are context dependent, as suggested by the intact STAg-induced expression of IL-10 in cells from orally infected *Ahr*$^{-/-}$ mice (Chapter 3). Additional implications of this work and questions that it raises are discussed in the following sections.

Challenges in using *Ahr*$^{-/-}$ mice

One of the challenges in using *Ahr*$^{-/-}$ mice to study immunity is caused by the widespread phenotypic changes in these animals, which make identifying the precise mechanisms behind any infection-induced effects difficult. The altered liver morphology, vascular defects, and changes in commensal populations in *Ahr*$^{-/-}$ mice could have effects on outcomes of infection in addition to the broad roles for the AHR in multiple aspects of immunity. These studies are further complicated by the finding that AHR deficient mice generated in different laboratories can have markedly different phenotypes following infection (Lawrence and Vorderstrasse, 2013). As described in Chapter 3, some of these effects may be due to the extent to which different strains of *Ahr*$^{-/-}$ mice had been backcrossed (Esser, 2009). Further studies done in parallel with multiple strains of AHR deficient animals could clarify the basis for this observed phenotypic variability. *Ahr*$^{-/-}$ mice additionally have reproductive defects and decreased fertility, which need to be taken into account when breeding these animals (Baba et al., 2005). However, mice with floxed alleles of the AHR have been developed, and provide a useful tool for studying the

effects of AHR deficiency on specific cell types *in vivo*. Alternatively, studies in *Ahr*$^{-/-}$ mice could be complemented by experiments in which animals are administered AHR ligands or inhibitors (Denison and Nagy, 2003; Zhao et al., 2010), but many of these compounds are not soluble in chemicals that can readily be administered to mice. Although there are a number of challenges to working with fully AHR deficient mice, one advantage to this approach is that it provides a means to investigate the effects of a complete absence of AHR signaling in every cell type.

Sources of AHR ligands

The finding that the AHR affects immune responses to toxoplasmosis raises the question of what the relevant sources of ligands are in this setting. This consideration is important, as different AHR agonists can have disparate effects. For example, as described in Chapter 1, FICZ induces Th17 cells whereas dioxin promotes Treg development (Quintana et al., 2008). Similarly, the studies in this thesis found that FICZ promotes IL-10 production from NK cells but kynurenine does not (Chapter 2). These results raise the question of how various AHR agonists mediate different responses. The rate at which AHR ligands are metabolized could explain some of their differential effects; FICZ is metabolized rapidly but dioxin persists, which may contribute to its toxic consequences (Bergander et al., 2004; Neal, 1985).

As detailed in Chapter 1, possible sources of AHR ligands during toxoplasmosis include the diet, commensal bacteria, the host immune response, or *T. gondii*. The prevalence of various AHR agonists in different sites of infection may explain some of

the disparate roles for this transcription factor during oral and chronic toxoplasmosis. Thus, dietary and microbiota-derived ligands may be particularly important during oral infection while high levels of the host-derived AHR agonist kynurenine are found in the brain during the chronic phase (Notarangelo et al., 2014). A potential role for dietary AHR ligands during toxoplasmosis could be tested by feeding infected mice diets that are low in AHR agonists, or conversely, by supplementing the diet with high-affinity AHR ligands. Recent insights into the roles for the diet and commensal bacteria as sources of AHR agonists that impact immunity (Kiss et al., 2011; Li et al., 2011; Zelante et al., 2013) contribute to a growing appreciation of how food intake and the microbiota can influence the immune system. For example, dietary nutrients such as vitamin A have a number of effects on immune responses (Spencer and Belkaid, 2012), and the production of butyrate by commensal bacteria promotes the generation of Tregs (Arpaia et al., 2013; Furusawa et al., 2013). Sensing through the AHR provides an additional means through which the immune system can respond to dietary and microbiota-derived compounds. Importantly, the diet directly affects the composition of the intestinal microflora as different groups of bacteria compete for nutrients (Honda and Littman, 2012). Dietary compounds might also shape the intestinal microbiota indirectly by activating the AHR, which influences IL-22 production, group 3 innate lymphoid cells, and the expression of antimicrobial peptides (Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012; Veldhoen et al., 2008).

As discussed in Chapter 2, the production of AHR ligands by pathogens such as *T. gondii* raises the possibility that the AHR might also act as a sensor of pathogen-derived metabolites. The immune system has been proposed to respond differently to living and dead microorganisms due to the detection of molecules such as bacterial mRNA, which serve as viability associated pathogen associated molecular patterns (vita-PAMPs) (Sander et al., 2011). By acting through the AHR, metabolites produced by pathogens could potentially act as vita-PAMPs. Although previous work has shown that some of the consequences of AHR signaling are consistent with a role in innate sensing (see Chapter 1), no such effects of this transcription factor were observed following *T. gondii* infection. For example, *Ahr*$^{-/-}$ mice had no defect in IL-12 production by dendritic cells during acute toxoplasmosis (Chapter 2), and microglial activation in the chronic stage was comparable between wild-type and AHR deficient animals (Chapter 3). Additionally, although stimulation with the AHR agonist FICZ promoted NK cell IL-10 production *in vitro*, treatment with STAg had no effect on NK cell IL-10 (data not shown). Although these results suggest that *T. gondii* is not a major source of AHR agonists following infection, the effects of parasite-derived ligands during toxoplasmosis remain unclear. A possible role for these compounds could be further evaluated by generating genetically manipulated parasites that are unable to catalyze the production of the AHR ligand lipoxin A_4, but this not the only potential pathway through which *T. gondii* might create AHR agonists.

Parallels between the AHR and HIF1α

As detailed in Chapter 1, increasing attention is being paid to the widespread and context dependent roles for the AHR and HIF1α in multiple aspects of innate and adaptive immunity. The parallels in the immunological functions of these transcription factors suggest that gaining an understanding of the roles of one pathway might also shed light on contributions of the other. For example, the effects of AHR signaling on group 3 innate lymphoid cells and the intestinal microbiota raises the question of whether HIF1α might also influence these populations. Similarly, HIF1α has multiple effects in intestinal epithelial cells that promote barrier function, suggesting that the AHR may play a comparable role. The studies in this thesis describing a contribution of the AHR to NK cell IL-10 expression raise the question of whether HIF1α also affects cytokine production by these cells. Additionally, the effects of AHR signaling during oral and chronic toxoplasmosis suggest that HIF1α might affect responses to challenge with *T. gondii*. This is a particularly interesting question, given the finding that parasite growth is impaired in HIF1α deficient cells (Spear et al., 2006). Thus, while HIF1α might contribute to the immune response to toxoplasmosis, signaling through this transcription factor also promotes parasite replication. Although many studies have investigated the effects of AHR and HIF1α signaling in isolation, work that examines the effects of these transcription factors in parallel could provide valuable insight into how they function during immune responses to infection.

The striking parallels in the effects of the AHR and HIF1α on immune responses may be coincidental, but also raise the possibility that low oxygen and the presence of AHR ligands are interpreted by the immune system as being indicators of similar challenges that warrant similar responses. What might account for these parallels? One possibility is that AHR and HIF1α signaling may play an especially important role at barrier surfaces such as the skin and intestines, which are likely major sites of exposure to AHR ligands and are thought to be relatively hypoxic. In agreement with this notion, the work presented here found that *Ahr*$^{-/-}$ mice exhibited more dramatic phenotypic differences following oral infection with *T. gondii* than after i.p. challenge. Additionally, the AHR and HIF1α have been shown to promote responses such as the production of antimicrobial peptides and the generation of Th17 cells, which are critical at barrier surfaces (Berger et al., 2013; Dang et al., 2011; Kimura et al., 2008; Qiu et al., 2012; Quintana et al., 2008; Shi et al., 2011; Veldhoen et al., 2008).

Sources of IL-10 during *T. gondii* infection

Studies described in Chapter 2 of this thesis using Vert-X IL-10 reporter mice identified multiple cellular sources of IL-10 following infection, in agreement with previous work (Perona-Wright et al., 2009). These results raise the question of whether IL-10 production from varying cell types has different functional effects during toxoplasmosis. Indeed, $CD4^+$ T cells are a critical source of IL-10 following infection with *T. gondii* (Jankovic et al., 2007; Roers et al., 2004), indicating that IL-10 expression by other cell types is not sufficient to prevent lethal immune-mediated pathology. The

finding that IL-10$^{-/-}$ RAG2$^{-/-}$ mice reconstituted with IL-10 sufficient but not IL-10$^{-/-}$ CD4^{+} T cells survive *T. gondii* infection implies that innate sources of IL-10 are not essential during toxoplasmosis (Jankovic et al., 2007). The requirement for CD4^{+} T cell IL-10 may be due to the amount of IL-10 produced by these cells, the time post-infection at which IL-10 is produced by CD4^{+} T cells, or the specific microenvironment in which IL-10 is secreted by these cells. Interestingly, IL-10 is produced by Th1 cells that also express IFN-γ (Jankovic et al., 2007). The work presented in Chapter 2 indicated that NK cells expressing the IL-10 reporter during infection similarly produce high levels of IFN-γ. These two cytokines have a number of functions that directly antagonize each other, raising the question of whether Th1 cells or NK cells simultaneously release IL-10 and IFN-γ *in vivo* and what effects these cytokines mediate when acting in concert.

Although innate sources of IL-10 may not be essential during toxoplasmosis, IL-10 deficient SCID mice are more resistant to infection than SCID mice (Neyer et al., 1997), indicating that innate IL-10 expression does have functional consequences. The finding that NK cells are a major early source of this cytokine raises the question of what effects NK cell IL-10 mediates. The production of IL-10 by NK cells has been suggested to limit the CD8^{+} T cell response following murine cytomegalovirus infection (Lee et al., 2009) and NK cell IL-10 promotes increased parasite burdens during visceral leishmaniasis (Maroof et al., 2008). Similarly, in the studies in Chapter 2, the NK cell IL-10 defect in *Ahr*$^{-/-}$ mice was associated with reduced parasite burdens. NK cell depletion studies during toxoplasmosis have suggested that IL-10 expression by these cells limits

IL-12 production (Perona-Wright et al., 2009), but NK cell depletion could also influence IL-12 levels by altering parasite burdens. Thus, although NK cells are a major early source of IL-10, it remains unclear how their production of this cytokine affects the immune response to toxoplasmosis. These effects could be further elucidated through the use of mice with an NK cell specific deletion of IL-10. However, the specificity of NK cell targeted Cre expression is a concern, as the commonly used NK cell marker NK1.1 is expressed by a variety of cell types including NK T cells and some activated $CD8^+$ T cells (Wang et al., 2013). NKp46 driven Cre mice that have been developed (Eckelhart et al., 2011) may offer more specificity, but NKp46 is also expressed on other cell types, including a subset of group 3 ILCs.

Although IL-10 production plays a critical role in limiting pathology during acute and chronic toxoplasmosis (Gazzinelli et al., 1996; Jankovic et al., 2007; Wilson et al., 2005), the cell populations that IL-10 needs to act on to mediate its protective effects are unclear. IL-10 was initially characterized as a factor produced by Th2 cells that inhibited Th1 cytokine production (Moore et al., 2001). Subsequent studies indicated that IL-10 mediated consequences on T cell cytokine production were the indirect result of the effects of IL-10 on antigen presenting cells such as macrophages and dendritic cells (Moore et al., 2001). More recently, IL-10 signaling has been shown to affect regulatory T cells (Chaudhry et al., 2011; Murai et al., 2009), and it is unclear whether this function of IL-10 influences the phenotype of $IL\text{-}10^{-/-}$ mice during toxoplasmosis. The importance

of different populations of IL-10 responding cells during infection could be evaluated through the use of mice with cell type specific deletions of the IL-10 receptor.

Possible functions of NK cell subsets

In addition to producing IL-10, the studies described in Chapter 2 found that NK cells upregulated the expression of T-bet and KLRG1 during infection, raising the question of how these molecules affect NK cell function. KLRG1 binds to E-cadherins and limits NK cell cytolytic activity (Ito et al., 2006). Thus, the population of NK cells emerging during infection that produces IL-10 and expresses high levels of KLRG1 may have a less cytotoxic and more immunoregulatory role.

As noted in Chapter 2, the subset of NK cells expressing high levels of T-bet and KLRG1 mirrors the phenotype of short lived effector $CD8^+$ T cells. Studies describing NK cell memory responses raise the question of whether these cells form populations of short lived effectors and memory precursors like $CD8^+$ T cells (Cooper et al., 2009; O'Leary et al., 2006; Paust et al., 2010). Additionally, the signals that regulate the generation of NK cell memory are not well understood, but the activity of IL-12 contributes to this process (Sun et al., 2012). The results presented in Chapter 2 indicate that AHR activity promotes some of the IL-12-mediated effects on NK cells, and it is unclear whether this transcription factor also affects the establishment of NK cell memory populations.

Potential roles for group 3 ILCs during toxoplasmosis

The studies described in Chapter 3 found that the group 3 ILC population also exhibits phenotypic changes following infection. Interestingly, challenge with *T. gondii* led to a reduction in the frequency and number of RORγt$^+$ ILCs in the lamina propria as well as decreased RORγt expression by these cells. Previous work has found that a subset of group 3 ILCs can downregulate RORγt expression and develop the ability to produce IFN-γ, a process that is promoted by IL-12 signals (Vonarbourg et al., 2010). A similar phenomenon may occur in response to the IL-12 that is expressed during oral toxoplasmosis. Notably, the downregulation of group 3 ILC RORγt is associated with the proliferation of these cells (Vonarbourg et al., 2010), and an increased frequency of group 3 ILCs expressed Ki67 following challenge with *T. gondii* (Chapter 3). RORγt GFP reporter mice could be utilized to assess the fate of RORγt$^+$ ILCs during toxoplasmosis by transferring congenically marked group 3 ILCs from these mice into infected animals.

The observed decrease in the lamina propria RORγt$^+$ ILC population during toxoplasmosis raises the question of whether the infection-induced changes in these cells have functional consequences. Given the role of group 3 ILCs in promoting the anatomical containment of commensal bacteria (Sonnenberg et al., 2012), a reduction in this cell population could contribute to the barrier dysfunction and microbial translocation that has been observed during toxoplasmosis (Benson et al., 2009; Hand et al., 2012). Since group 3 ILCs promote tolerance to commensal bacteria (Hepworth et al., 2013), decreases in this cell population might facilitate the induction of immune

responses to infection. This effect would be analogous to the suggestion that the Treg crash seen during *T. gondii* infection promotes the generation of protective immunity by diminishing the immunosuppressive effects of Tregs (Benson et al., 2012). However, an increased frequency of RORγt$^+$ ILCs expressed MHC II and CD80 during toxoplasmosis, suggesting that these cells might promote the development of adaptive immune responses following infection. The functional effects of group 3 ILCs are challenging to assess *in vivo*, because these cells are characterized by the absence of numerous of surface markers, which makes them difficult to deplete or target through genetic approaches. However, strategies to deplete these cells in RAG deficient chimeric mice have been developed (Sonnenberg et al., 2012), providing a means to interrogate the effects of RORγt$^+$ ILCs during toxoplasmosis. Additionally, *T. gondii* infection of mice with an RORγt driven deletion of the AHR (Kiss et al., 2011) could address whether group 3 ILCs in the lamina propria contribute to the immune response following challenge.

Epidemiological and therapeutic implications of the effects of the AHR on immunity

Given the presence of numerous compounds that activate the AHR in environmental pollution, findings that the AHR influences immune responses to *T. gondii* as well as a number of other infections (see Chapter 1) suggest that the effects of AHR signaling on the immune system may have epidemiological consequences. The effects of the AHR in promoting Th17 generation and the development of experimental autoimmune encephalomyelitis led to the suggestion that signaling through this transcription factor might contribute to the increasing rates of autoimmunity seen in

highly industrialized countries (Veldhoen et al., 2008). Notably, in addition to its role in autoimmune disease, AHR activity has a number of effects on immune responses to infection, suggesting that AHR signaling in response to changing exposure to environmental ligands may impact human health. Indeed, epidemiologic studies indicate that elevated exposure to pollutants that activate the AHR is associated with increased incidences of ear infections, chicken pox, and respiratory infections in children (Weisglas-Kuperus et al., 2000; Weisglas-Kuperus et al., 2004; Yu et al., 1998).

Findings describing multiple effects of the AHR on immune responses have also led to interest in targeting this pathway therapeutically. Although transcription factors are generally regarded as poor drug targets, the ligand dependent nuclear hormone receptors, which include immunologically relevant proteins such as retinoic acid receptors and peroxisome proliferator-activated receptors, are considered "druggable" (Blumberg and Evans, 1998; Shi, 2007). The AHR is structurally distinct from nuclear hormone receptors, but signaling through the AHR is analogous to nuclear hormone receptor function in that ligand binding induces transcriptional activity. The availability of various compounds that modulate AHR activity suggests that it may be possible to manipulate this pathway for therapeutic benefit (Denison and Nagy, 2003; Zhao et al., 2010). Studies in which AHR ligands have been delivered in nanoparticles (Quintana et al., 2010) indicate that it may be possible to alter AHR activity in specific cell types for therapeutic purposes. However, gaining an understanding of the context dependent effects of AHR signaling is essential in effectively targeting this transcription factor.

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