NEUTROPHIL-DERIVED IFN-γ IN *TOXOPLASMA GONDII* INFECTION AND INNATE IMMUNITY

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DEDICATION

To all those who were at my side, before, during, and after.

NEUTROPHIL-DERIVED IFN-γ IN *TOXOPLASMA GONDII* INFECTION AND INNATE IMMUNITY

by

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NEUTROPHIL DERIVED IFN-γ:
A STORY OF *TOXOPLASMA GONDII* INFECTION AND INNATE IMMUNITY

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Interferon-gamma (IFN-γ) is a major cytokine that is critical for host resistance to a broad range of intracellular pathogens. Production of IFN-γ by Natural Killer (NK) and T cells is initiated by the recognition of pathogens through Toll-like receptors (TLRs). In an experimental model of toxoplasmosis we have identified the presence of a non-lymphoid source of IFN-γ that was particularly evident in the absence of TLR-mediated recognition of *Toxoplasma gondii*. Flow-cytometry and morphological examinations of non-NK/non-T IFN-γ-positive cells identified neutrophils as the cell type capable of producing IFN-γ. Selective elimination of neutrophils in TLR11-/- mice infected with the parasite resulted in

acute susceptibility similar to that observed in IFN-γ-deficient mice. These data show that neutrophils are a biologically significant source of IFN-γ during *T. gondii* infection. Additionally, we investigated the role of neutrophil IFN-γ in another intracellular infection, *Salmonella typhimurium,* and found that neutrophils were also IFN-γ-positive. Examination of neutrophils in different locations in a mouse model revealed that they all expressed low amounts of IFN-γ regardless of infection status. In particular, the bone marrow niche contained an IFN-γ+ population that was negative for the Ly-6G marker characteristic of mature neutrophils in peripheral tissues. Recent work defining neutrophil developmental stages by flow-cytometry allowed us to discern that precursor neutrophils at the promyelocyte stage (Ly-6G negative) were positive for IFN-γ. Furthermore, neutrophil-derived IFN-γ was prestored in granules during neutrophil lineage development although the mechanisms behind this phenomena are not yet understood. This work, combined with the recent work of other laboratories, suggests that neutrophils can have defined phenotypes and cytokine production similar to that of T cells or Innate Lymphoid cells (ILCs). These findings have broad implications for all disease states where neutrophils are the first responders to infections.

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α- – Anti (e.g. αLy-6G)

AIDS – acquired immunodeficiency syndrome

ARE - AU-rich elements

ASC – apoptosis-associated speck-like protein containing a carboxy-terminal CARD

APC – antigen presenting cell

Caspase - **c**ysteine-dependent **asp**artate-directed prote**ases**

CCR# – chemokine (C-C motif) receptor 'number' (e.g. CCR2)

CD# – Cluster of differentiation 'number' (e.g. CD4)

C/EBPα – CCAAT-enhancer-binding protein alpha

c-kit – Mast/stem cell growth factor receptor (SCFR)/proto-oncogene c-Kit/tyrosine-protein kinase Kit/CD117

CHX - cycloheximide

CMP – common myeloid progenitor

CNS- conserved non-coding sequence

CSF – cerebral spinal fluid

CXCR# – chemokine (C-X-C motif) receptor 'number' (e.g. CXCR2)

DC – Dendritic cell

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

Eomes – eomesodermin

FBS – fetal bovine serum

GFP – green fluorescent protein

GM-CSF – granulocyte macrophage colony-stimulating factor

Gr-1 – granulocyte-differentiation antigen 1

GRA# – dense granule protein ‘number’ (e.g. GRA15)

GM-CSF – granulocyte monocyte - colony stimulating factor

GMP – granulocyte-monocyte progenitor

HEPES - (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (media component)

HIV – human immunodeficiency virus

HSC – hematopoietic stem cell

IFN-‘symbol’ – Interferon (e.g. IFN-α, IFN-γ)

IFNAR - IFN-α receptor

Ig? – immunoglobulin ‘letter’ (e.g. IgG, IgM)

IL-# – interleukin ‘number’ (e.g. IL-12)

ILCs – Innate Lymphoid Cells

IRGs - immunity-related GTPases

IRI – ischemic reperfusion injury

Lin- – Lineage negative

LPS – lipopolysaccharide

LSK – lin- sca-1+ c-kit+ cells

LT-HSC – Long-Term Hematopoietic Stem Cell

Ly-6? – lymphocyte antigen 6 complex, locus ‘letter’ (e.g. Ly-6G, Ly-6C)

MCP-1 – monocyte chemoattractant protein 1

MDSC – Myeloid Derived Suppressor Cell

Me49 – a *T. gondii* type II strain

MFI – mean fluorescence intensity

MHC I – major histocompatibility complex I

MHC II – major histocompatibility complex II

miRNA - microRNA

MPP – Multi-Potent Progenitor cell

MRI – magnetic resonance imaging

mRNA – messenger ribonucleic acid

MyD88 – Myeloid differentiation factor 88

NFκB – nuclear factor κB

NK – natural killer

NLRP# – NACHT, LRR, and PYD domains-containing protein or NOD-like receptor 'number' (e.g. NLRP1)

$P2x_7$ - purinergic receptor P2X, ligand-gated ion channel 7

PBS – phosphate buffered saline

PCR – polymerase chain reaction

pDC – plasmacytoid dendritic cell

PEC – peritoneal exudate cells

PMA – phorbol myristate acetate

PMN – polymorphonuclear cell (a neutrophil)

PU.1 – PU-box binding transcription factor

QCA – Quantitative Colocalization Analysis

RAG – recombination-activating gene

RNA – ribonucleic acid

RORγt – RAR-related orphan receptor gamma t

ROS – reactive oxygen species (e.g. O^-,$ONOO^-$, H_2O_2)

RPMI - Roswell Park Memorial Institute (media)

Sca-1 – stem cell antigen-1

ST-HSC - Short-Term Hematopoietic Stem Cell

STAT# – signal transducer and activator of transcription 'number' (e.g. STAT1)

T-bet – 'T box expressed in T cells'

TE – toxoplasmic encephalitis

TGFα/β – transforming growth factor alpha or beta

Th# – T helper type 'number' (e.g. Th1)

TLR – Toll-like Receptor

TNF – Tumor Necrosis Factor

T_{reg} – T regulatory cell

UNC93B1 - Unc-93 homolog B1 [C. elegans]

YFP – Yellow Fluorescent Protein

CHAPTER I

INTRODUCTION

Toxoplasma gondii and toxoplasmosis

Discovery of T. gondii

The protozoan parasite known as *Toxoplasma gondii* was first isolated in 1908 and named by the scientists Nicolle, Manceaux, and Splendore in 1908. It was named "Toxoplasma," 'toxon' meaning arc or bow and 'plasma' meaning life due to its shape and 'gondii' a misspelling of the African rodent from which it was first isolated, *Ctenodactylis gundi* (Ferguson, 2009). It was subsequently isolated from many animal species over the next thirty years, including humans. Since the known parasite stage could be found in circulating macrophages, *Toxoplasma* was originally believed to be related to another parasite, *Leishmania*. However lack of any proof for an insect vector led to broader investigations and in 1965 a researcher isolated the parasite from domestic cat feces (Hutchison, 1965). This discovery accelerated research and just a few years later multiple research groups found that the infective material was coccidian oocytes, solving the mystery of Toxoplasma's lifecycle and allowing for its classification as a subclass (Coccidia) of the Apicomplexan protozoa (Ferguson, 2009).

T. gondii *Lifecycle*

There are many Phyla of protozoan parasites (a phylogentic relationship can been viewed in (Sibley, 2011)), but the Apicomplexans are defined by their commmon apical

complex structure of microtubules which are involved in movement and penetrating the host cell. Apicomplexans include important human pathogens such as *Toxoplasma*, *Plasmodium*, and *Cryptosporidium*. Most Apicomplexans have a complex life-cycle that involves both sexual and asexual reproduction. *Toxoplasma* oocytes result from the sexual reproduction cycle and are shed from infected feline (primary host) feces into the environment. In this form, *Toxoplasma* is protected by a highly resistant oocyte wall and can easily survive in the environment for more than a year suspended in water (Hutchison, 1965), until it is ingested by another mammal, such as livestock or a feline prey-animal. Ingested *Toxoplasma* is released from its protective layer and differentiates into the fast replicating form of the parasites - tachyzoites. Tachyzoites invade the intestinal epithelia and replicate. In non-feline hosts (secondary hosts) only asexual tachyzoites are formed whereas in a feline both the asexual tachyzoites and sexual gametocytes are produced. Tachyzoite infected cells die 24-48 hours after infection allowing tachyzoites to escape and continue the infection cycle. Tachyzoites will disseminate throughout the body as they are capable of infecting any nucleated cell type. As infection progresses, tachyzoites differentiate into bradyzoites, the slow replicating form of the parasite, and form long-lived cysts in brain and muscle tissues; cysts persist for the lifetime of the host. *Toxoplasma* tissue cysts are highly resistant to destruction by acid or trypsin but, unlike the oocytes, they can be heat killed or dessicated (Jacobs et al., 1960). Therefore if the host animal is killed and eaten by another mammal, the tissue cysts are capable of surviving digestion long enough to infect the new host. Both the cysted and oocyst *Toxoplasma* infection routes are likely important for humans, as the consumption of undercooked meats, fecal-oral contamination of unwashed produce, and

association with domestic cats are regular occurrences. It is worth noting that humans and large mammals are considered an accidental host as they are a dead-end for parasite reproduction because, unlike small mammals, most large mammals are not a typical food source for cats. Yet *Toxoplasma*'s relative lack of species and cell-type specificity, highly resistant cysts and oocysts, and wide geographical distribution (see section below) with the absence of any seasonal variations has arguably resulted in the most successful parasitic pathogen in the world.

Prevalence of T. gondii *infection in humans and our livestock*

It is estimated that 1/3 of the entire human population is infected with *Toxoplasma gondii* (Pappas et al., 2009). This estimate was made by combining data from a large number of studies that examined the seroprevelance of antibodies against *T. gondii* in pregnant human populations. In the United States, the seropositivity is estimated to be around 15%, whereas in countries that eat more undercooked meats, especially lamb (e.g. France), or have poor sanitation and contamination of their drinking water (e.g. Brazil), the incidence is greater than 60% (Jones and Dubey, 2012; Pappas et al., 2009).

Similarly to humans, it is estimated that domestic and feral cats are anywhere between 15-80% seropositive depending on region (Elmore et al., 2010). Infected felines generally only shed oocytes for one 1-2 week period during their lives (Elmore et al., 2010), so although they shed thousands of resistant oocytes, direct infection from a shedding feline is less likely than indirect infection from oocyte contamination in the environment or ingesting infected meat products. Livestock animals also exhibit a large range of infection

prevalence. Here in the United States, several studies have shown that *T. gondii* can be isolated from pigs, sheep, and deer, but not from cattle or chickens. The prevalence of infection can range greatly, from 0.3% in retail pork to 92.7% in market hogs (Jones and Dubey, 2012). Typical retail processing of pork and chicken usually includes injection of salt water and often the meat is frozen, both of which will kill encysted parasites. Cattle seem to be more resistant or less exposed to infection as the studies do not generally find infected cattle. Sheep have a high incidence of *T. gondii* infection; in the United States they have anywhere from 4-78% infection rates and in Scotland their flocks were found to be roughly 50% infected (Katzer et al., 2011). *T. gondii* infection in sheep does not cause any symptoms in adult animals, but in Europe and the UK it is estimated to be the second most common cause of fetal sheep abortions, resulting in significant economic loss (Katzer et al., 2011). In Europe and New Zealand there is a live attenuated (does not form tissue cysts) strain of *T. gondii*, S48, licensed for veterinary vaccine use but it is expensive and has the potential to revert to being pathogenic, therefore it is considered unsuitable for human use (Montoya and Liesenfeld, 2004).

T. gondii *causes toxoplasmosis*

In Immunocompetent humans, *T. gondii* causes few if any symptoms upon initial infection; the symptoms are mild and nonspecific, such as fever aches and lymph node swelling which are easily confused with the flu. An acute infection progresses to the chronic stage where *Toxoplasma* forms persistent cysts, found mainly in brain and muscle tissue, but causes no symptoms. The disease state, known as toxoplasmosis, generally occurs during the

chronic stage after the host becomes immunocompromised which allows for the encysted *T. gondii* to reactivate. Therefore, *T. gondii* usually causes disease in the very young, old, and otherwise immunocompromised due to organ transplants, chemotherapies, and AIDS. Classical toxoplasmosis seen in immunocompromised adults causes severe encephalitis with multiple organ involvement that is usually fatal if untreated. Toxoplasmosis and *T. gondii* research gained public attention in the 1970s and 1980s with the rise of the AIDS epidemic. Toxoplasmosis was one of the most common complications of AIDS in the 1980s. It was estimated that *Toxoplasma* encephalitis occurred in up to 25% of AIDS patients and was always fatal (Janssen, 1997). The antimalarial drug Pyrimethamine, in combination with Sulfadiazone, were found to be particularly effective in killing the fast replicating tachyzoites. Both Pyrimethamine and Sulfadiazone disrupt different parts of folic acid synthesis, which is needed for DNA and RNA synthesis. These drugs also have some activity in humans cells; therefore those who use these drugs long-term, especially those with HIV, consume supplemental folic acid to reduce the risk of inhibiting immune cell and platelet production, which would be counterproductive to patient survival (van der Ven et al., 1996). While the mortality rate from toxoplasmosis has greatly decreased with the administration of these drugs and anti-viral treatment for HIV, there are no drugs that can completely eliminate the encysted parasites, leaving those who are infected with the constant possibility of reactivation of *T. gondii* and potential for disease. Additionally, the rise of immunosuppressive treatments given after organ or bone marrow transplants has spurred additional screening to decrease the incidence of tissues from seropositive donors being

given to seronegative recipients and to allow for prophylactic drug treatment if a mismatch is unavoidable (Derouin and Pelloux, 2008).

The first documented case of human disease where *T. gondii* was identified as the causative agent was in 1937. It was identified in a newborn that presented with encephalitis, seizures, and choriorentinitis who later died of the illness. Researchers were able to definitely show that brain tissue lesions from the deceased newborn injected into several rodent species caused encephalitis in these animals (Wolf and Cowen, 1937). This form of toxoplasmosis, known as congenital toxoplasmosis, is a special form of this disease in which an unborn child is infected via the placenta. Fetal infection only occurs when a mother with no prior exposure to the parasite is infected during pregnancy. Congenital toxoplasmosis is diagnosed by the presence of hydrocephalus and intracranial calcifications. The prognosis for the fetus is dependent on its stage of development when first infected; the earlier the fetus is infected with *T. gondii* the more damage to their central nervous systems (encephalitis) occurs, often resulting in spontaneous abortion in the first trimester of pregnancy or varying degrees of physical and mental retardation with potential blindness if infection occurs late in pregnancy (Montoya and Liesenfeld, 2004). Congenital toxoplasmosis can occur in many other species, including sheep (as mentioned above).

Outside of the United Sates, perhaps associated with different strains of the parasite most prevalent in Brazil and other countries in South America, *T. gondii* infection can cause ocular disease – retinochoroiditis – independent of encephalitic toxoplasmosis. This ocular form of disease is unique compared to other forms of *T. gondii* infection in that it can occur in otherwise healthy adults in addition to congenital and immunodeficient patients.

Symptoms of retinochoroiditis are ocular pain, photophobia, and blurred vision or blindness. Similar to the brain, *T. gondii* forms lesions in the eye. In brain and eye infections, short term corticosteroids are often used in combination with anti-*T.gondii* drugs to control the swelling and minimize damage.

Clinically, those at risk for toxoplasmosis are diagnosed by looking for elevated *T. gondii*-specific IgM and IgG in the serum; the ratio of the affinities of these two immunoglobulins can indicate how recently the infection was acquired, which is of particular importance for pregnant females (Remington et al., 2004). However, routine screening of pregnant woman is only undertaken in a few places where the incidence of disease is high, mainly France and Austria (Montoya and Liesenfeld, 2004). Patients suspected of active disease can be diagnosed by the presence of contrast-enhanced brain lesions on an MRI, ophthalmological examination for lesions in the eye, *T.gondii*-specific IgG in their CSF fluids, or PCR of tissues for *T. gondii* DNA.

Host immunity to *T. gondii*

Adaptive immunity is essential for host immunity to T. gondii

Due to the renewed interest in *T. gondii* and AIDS associated research in the 1980s, initial research into the immune response to *T. gondii* focused on the importance of establishing immunocompromised mouse models during the chronic stage of infection. Early work in the field demonstrated that combined depletion of CD4+ and CD8+ T cells during chronic infection in mice resulted in parasite reactivation and host mortality (Gazzinelli et al., 1992). Additionally SCID mice, which lack T cells, are susceptible during the transition

from the acute to chronic stage of infection, illustrating the requirement for T cell responses to survive the infection, especially during chronic toxoplasmosis. Both of these models helped to define the importance of the adaptive immune system and T cells specifically in controlling the parasite. This data is perhaps unsurprising given what we now know about HIV patients' transition to having AIDS, which is characterized by a loss of CD4+ T cells and consequent immune system compromised status of the host.

From research on mouse models of *T. gondii* infection, we have learned that T cells, both CD4+ and CD8+ cells, seem to have an important synergistic role to play during *T. gondii* infection, because individual depletions of CD4+ or CD8+ T cells do not recapitulate the phenotype of double depletion or T cell-deficient mice (Gazzinelli et al., 1992; Suzuki and Remington, 1988). The role of CD4+ T cells during intracellular infections is the production of IFN-γ (more on IFN-γ in a following section) which is needed to prime macrophages to produce potent antimicrobial responses. CD8+ T cells have a complex role to play in control of *T. gondii* infection: they secrete IFN-γ and they can specifically kill infected cells. The balance between these two roles for CD8+ T cells is dependent on the mouse models being utilized; C57BL/6 mice, MHC I H-2^b haplotype, have the H-$2L^{null}$ allele of MHC I and are susceptible to toxoplasmic encephalitis (TE) but BALB/c mice, MHC I H-2^d haplotype, have the H-$2L^d$ allele and are genetically resistant to TE (Brown et al., 1995; Brown and McLeod, 1990; Suzuki et al., 1994). This homozygosity of MHC I alleles in inbreed mice explains why CD8+ T cells are the dominant T cell responder in BALB/c models, but are a minor player in C57BL/6 mouse models. Experimentally it has been shown by many groups that CD8+ T cell responses can be generated against epitopes of

several identified antigenic *T. gondii* proteins. Yet surprisingly the exact *T. gondii* protein used to generate a CD8+ T cells response seems to be far less important than the location of the epitope within the protein sequence processed for antigen presentation (Feliu et al., 2013), further illustrating the MHC I haplotype of the host as critical for determining the contribution of CD8+ T cells to protection from the parasite.

Innate immunity predetermines susceptibility to T. gondii

Despite the clear importance of the adaptive response, it is components of the innate immune system that determine initial susceptibility and outcome of *T. gondii* infection. Mice deficient in Myeloid Differentiation factor 88 (MyD88), the downstream adaptor protein of most Toll-Like Receptors (TLRs) and the IL-1 receptor family, are extremely susceptible to *T. gondii* infection (Scanga et al., 2002). Pattern recognition receptors, such as TLRs, are innate immune receptors involved in recognizing conserved molecules present on bacteria, viruses, and parasites, which are not expressed by the host cells. TLRs are evolutionarily ancient, as their TIR protein domain is relatively conserved in vertebrates, invertebrates, and plants, and its members all serve various yet similar functions in pathogen recognition and resistance. The specificity of these receptors is germline encoded and therefore it requires a significant time period for selective pressure to induce evolutionary change in TLRs. This property is similar to that of the ligands that TLRs recognize which are highly conserved microbial components essential for survival of the microorganism and therefore also require a significant time period for beneficial evolutionary changes to occur. In the case of *T.*

gondii infection in mice, it was shown that MyD88-dependent responses to the parasite were primarily mediated by TLR11 (Yarovinsky et al., 2005).

TLR11/TLR12-mediated parasite recognition

TLR11 recognizes a protein component of *T. gondii* known as profilin (Plattner et al., 2008; Yarovinsky et al., 2005). Conserved in eukaryotes, profilins are known for their function in actin-filament polymerization. Profilin, much like other TLR ligands, is essential to the microorganism; inducible depletion of profilin expression in *T. gondii* results in loss of motility and host cell invasion processes that are essential for this obligate intracellular organism (Plattner et al., 2008). Specifically, it is known that *T. gondii* profilin regulates sequestration of actin filaments in the G-state (Skillman et al., 2012). Activation of TLR11 and MyD88 by *T. gondii* profilin is also required and sufficient for potent induction of IL-12 by DCs immune responses (Scanga et al., 2002; Yarovinsky et al., 2005) and this DC cell IL-12 regulates IFN-γ from NK and T cells (Hou et al., 2011; Hunter et al., 1994; Wilson et al., 2008; Yarovinsky et al., 2006). Specifically the CD8α+ DCs are the initial producers of IL-12 in response to *T. gondii* profiling *in vivo* and *in vitro*. This specificity has been explained by the discovery that IRF8 is the transcriptional factor essential for IL-12-induction downstream of TLR11 (Raetz et al., 2013b). IRF8 expression is restricted to CD8α+ DC and thus this DC subset is armed for immediate response to *T. gondii* profilin. In addition, it has been suggested that CD8α+ DC express higher amounts of TLR11 mRNA, but whether the elevated expression of TLR11 contributes to the selective responsiveness of CD8α+ DC to *T. gondii* remains to be investigated.

Other DC subsets and macrophages can acquire responsiveness to profilin, but only after priming with IFN-γ. Mechanistically it has been explain that IFN-γ triggers expression of IRF8 (initially discovered as ICSBP, interferon consensus binding protein that is induced by IFN-γ)(Kuwata et al., 2002). Thus non-CD8α+ DCs and macrophages, primed by IFN-γ, subsequently express IRF8 and acquire the ability to secrete IL-12 in response to *T. gondii* profiling. Since many TLR-driven innate immune responses in DCs activate a different transcription factor, NF-κB, it was assumed that TLR11 activated this pathway. However DCs respond to profilin by producing large amounts of IL-12, but not other proinflammatory cytokines like TNF or IL-1β. The explanation for this cytokine specificity was recently explained by the discovery that CD8α+ DCs fail to trigger NF-κB activation in response to *T. gondii* profilin stimulation (Raetz et al., 2013b).

Other Apicomplexan species also contain profilin; profilins from *Eimeria tenella*, *Theileria parva*, *Plasmodium falciparum* (malaria), and *Cryptosporiduim parvum* were examined by phylogentic and experimental approaches in comparison to *T. gondii* (Yarovinsky et al., 2005). While *C. parvum* and *E. tenella* induce IL-12 via TLR11, *P. falciparum* profilin induces little to no IL-12 (Yarovinsky et al., 2005). Additionally, replacing *T. gondii*'s profilin with malarial profilin resulted in loss of TLR11 activation in response to the parasite (Plattner et al., 2008). This represents a unique finding and perhaps a clue as to the selective pressures on TLR11. Yet, the TLR11 family is the most divergent of all vertebrate TLRs, suggesting that is may be under less purifying selective pressures than other TLRs (Roach et al., 2005).

TLR11 is part of a family of TLRs that includes TLR12 and TLR13 (Roach et al., 2005) and recent evidence points towards a role for TLR12 in *T. gondii* profilin recognition. Two groups independently observed that TLR12 is capable of forming heterodimers with TLR11, can specifically recognize *T. gondii* profilin, and is involved in the triggering the downstream IL-12 responses (Koblansky et al., 2013; Raetz et al., 2013b). This heterodimer-complex formation was suggested in earlier bioinformatics analysis (Roach et al., 2005) due to similarities observed between the TLR11 family and the TLR2 family, where TLR2 forms heterodimers with TLR1 and TLR6. It is speculated that the TLR2 family's somewhat unique heterodimer configuration allows it to attain specificity for a more diverse, but still highly conserved, repertoire of ligands (Farhat et al., 2008). It could be argued that this same idea applies to the diverse Apicomplexan profilins and TLR11. The exact importance of TLR12 beyond its role in profilin-dependent IL-12 is controversial. One group suggests that TLR12 is necessary for IL-12 and IFN-α production by plasmacytoid DCs (pDCs) in response to *T. gondii* and that IL-12 is specifically dependent on TLR12-homodimers but not TLR11 (Koblansky et al., 2013). Yet earlier reports exploring pDCs during *T. gondii* infection found that their high levels of IL-12 production and pDC maturation were entirely dependent on TLR11 (Pepper et al., 2008). This discrepancy in the TLR dependence of pDCs IL-12 needs to be clarified as does the observed dependence of IFN-α on TLR12. Although pDCs, a significant source of type I IFNs, are activated during *T. gondii* infection, the overall role of type I interferon is not well characterized. Older publications point to a possible role for type I IFNs in protection of non-hematopoietic cells from infection rather than priming of immune cells (Bogdan et al., 2004). However, in more recent experiments in C57BL/6 mice

IFN-α was not observed and IFNAR (IFN-α receptor)-deficient mice exhibited survival similar to C57BL/6 mice (Andrade et al., 2013). Furthermore, how TLR12 can trigger cytokine production in the absence of TLR11 is not clear since, in contrast to TLR11, TLR12 fails to biochemically interact with MyD88 (Raetz et al., 2013b).

Innate immunity differences (mice vs. humans)

Perhaps the strongest evidence for separate recognition systems contributing to *T. gondii* infection resistance is that TLR11 and its family members are non-functional pseudogenes or entirely absent in humans (Roach et al., 2005). Yet immunocompetent humans generally control the infection, as do TLR11-deficient mice (Benson et al., 2009; Yarovinsky et al., 2005), and both have elevated serum IFN-γ. The genetic distribution of TLR11 and TLR12 among Chordates species is evolutionarily interesting, in that the majority of animals with functional TLR11 and TLR12 tend to be small prey mammals such as rodents (Gazzinelli et al., 2014). Small prey mammals also tend to have a larger number of immunity-related GTPases (IRGs) that are implicated in parasite control in mice (Collazo et al., 2001; Liesenfeld et al., 2011; Taylor et al., 2000). However, there is only one IRG expressed in humans and it is therefore assumed to be unimportant in the human response to *T. gondii* (Howard et al., 2011). Humans and bonobos are the only sequenced primates that contain evidence of the TLR11 pseudogene and it is interesting to note that the primary host for *T.gondii*, the feline, also does not have TLR11 or TLR12 (Gazzinelli et al., 2014).

Additional MyD88-dependent signals during T. gondii *infection.*

Additional TLRs may play a role in recognition of *T. gondii*. Most TLRs utilize the adaptor molecule MyD88 and a MyD88-deficient mice has acute susceptibility to the parasite and little IL-12 production (Scanga et al., 2002) which we now know is largely due to loss of functional TLR11 and TLR12. Likewise in 3d mice, which lack functional UNC93B1, resistance to *T. gondii* is lost because all intracellular TLRs, including TLR11 and TLR12, depend on UNC93b1 for their correct endosomal localization (Melo et al., 2010; Pifer et al., 2011). The severe acute susceptibility seen in 3d mice is nearly identical to that of MyD88-deficient mice rather than the mild susceptibility observed in TLR11-deficient mice, suggesting that there are additional UNC93B1 and MyD88-dependent TLRs involved. Other intracellular TLR-deficient mice exhibit little to no phenotype during *T. gondii* infection (Benson et al., 2009; Melo et al., 2010), including TLR3/TLR7/TLR9-triple deficient mice (Andrade et al., 2013). This data illustrates the dominant effect of TLR11/TLR12 profilin recognition in generating a Th1 immune response to the parasite. Yet it has been found that TLR7 can recognize *T. gondii* RNA and TLR9 can recognize many CpG motifs in *T. gondii* DNA (Andrade et al., 2013). This likely contributes to the susceptibility of both MyD88 and 3d mice, as TLR3/7/9/11 quadruple-deficient mice have almost no IL-12 or IFN-γ in the first week of infection (Andrade et al., 2013). Nevertheless there is still an important difference between *T. gondii* infection of MyD88 and 3d mice: administering IL-12 to MyD88-deficient mice does not rescue their acute susceptibility but it does rescue the susceptibility of 3d mice (LaRosa et al., 2008; Pifer et al., 2011). This data indicates that there are even more MyD88-dependent factors necessary for survival than are currently appreciated.

MyD88 serves as the adaptor molecule for many of the IL-1 family receptors, including the receptors for IL-1α and IL-1β, IL-18, and IL-33. Signaling downstream IL-1R, IL-18R, and IL-33R leads to MyD88-dependent activation of the transcription factors NFκB and AP-1 which generally leads to pro-inflammatory responses. Of this family, IL-1β and IL-18 are unique in that they are synthesized as inactive precursor proteins in response to sensing of microbial products through TLRs or indirectly by sensing of proinflammatory TNF. Pro-IL-1β and pro-IL-18 can be cleaved by active caspase-1, a component of the larger inflammasome. The inflammasome is a multiprotein intracellular signaling complex that is part of the innate immune system and can detect microbial-derived molecular products or danger signals. The exact components of the inflammasome vary depending on the activating product but generally consist of a cytosolic sensor protein (NLR family or PYHIN family proteins), adaptor molecule ASC, and a caspase family members (usually caspase-1 and 11) (Rathinam et al., 2012). Once the inflammasome has sensed a ligand, caspase-1 is activated to cleave pro-IL-1β/pro-IL-18 into their active forms. IL-1β and IL-18 are secreted to recruit immune cells and may act in synergy with IL-12 to stimulate IFN-γ production. Active caspase-1 can also cleave other substrates to induce pyroptosis, a form of cell death in response to infection, which results in release of proinflammatory mediators to recruit additional immune cells (Franchi et al., 2012).

There is evidence in the literature that the inflammasome complex plays a role during *T. gondii* infection. Long before the inflammasome complex was known to exist, it was found that injections of IL-1β could protect mice from lethal *T. gondii* challenge (Chang et al., 1990). Following this, genetic susceptibility and resistance to the parasite was associated

with several large regions of the host genome in human and rats, one of which contains NLRP1 (also called NALP1) along many other genes (Cavailles et al., 2006; Witola et al., 2011). It was recently shown that specific knockdown of NLRP1 in rat macrophages confers resistance to pyroptosis-mediated cell death and this results in a higher parasite burden (Cirelli et al., 2014) as infected cells permit *T.gondii* replication for a greater duration before cell death. Prior to implicating NLRP1, it was known that the second signal needed for inflammasome activation and pro-IL-1β/IL-18 cleavage could be achieved through activating the $P2x_7$ receptor. $P2x_7$ receptor is an ion channel that opens in response to ATP, which acts as a danger signal when present in large quantities in the extracellular environment. Activating $P2x_7$ on *T. gondii* infected human and mouse macrophages, by adding extracellular ATP to the media, lead to more effective killing of the parasite. (Lees et al., 2010). However, $P2x_7$ receptor-deficient mice do not have an increased parasite burden or changes in pro-inflammatory cytokines, including IL-1β, rather they appeared to lose slightly more weight than control mice due to delayed IL-10 production, resulting in a prolonged inflammatory response (Miller et al., 2011). $P2x_7$ receptor has been shown in other models to be an activator of the NLRP3 inflammasome but this is just one among many diverse stimuli capable of activating NLRP3, including microbial RNA, lipopeptides, and LPS as well as particulate matter such as uric acid crystals, silica, aluminum salts, or fatty acid deposits (Franchi et al., 2012). Quite recently a group showed that mice deficient in NLRP3 or its adaptor protein ASC have decreased IL-18 and decreased survival during *T. gondii* infection (Gorfu et al., 2014). They also showed that caspase-1/11-deficient mice had a similar but slightly less severe phenotype, however in our lab caspase-1/11-deficient mice

survive infection (unpublished observations); the differential survival might be explained by a higher dose used by the other laboratory, which resulted in some C57BL/6 mouse mortality that it not normally observed.

Although NLRPs and the inflammasome complex are playing a role in host resistance to *T. gondii*, the activating ligand(s) for the NLRPs during *T. gondii* infection have yet to be elucidated. It has been hypothesized that NLRP3 activation is indirect and various intermediate adaptors may be necessary to confer specificity to such a wide range of ligands, both known and unknown (Franchi et al., 2012). Since a priming signal is required to produce pro-IL-1β/IL-18 prior to successful inflammasome cleavage of these cytokines, this raised the question as to the nature of the priming signal; in vitro experimental models typically use the TLR4 ligand LPS to prime the inflammasome response. However, *T. gondii* does not contain LPS, but it does contain other TLR ligands such as profilin, DNA, and/or RNA. Recent evidence by one laboratory suggests that at least one of these can successfully prime bone marrow-derived macrophages to secrete IL-1β, but surprisingly this is highly dependent on the strain of *T. gondii* used (Gorfu et al., 2014), suggesting perhaps that some strains are able to inhibit this response or alternatively that it isn't a *T. gondii* TLR ligand that primes production of pro-IL-1β/IL-18 but rather the indirect result of TNF production or other NFκB activator. It was suggested that *T. gondii* protein GRA15 may be responsible for these differences, as GRA15 from a type II strain has been seen to activate NFκB signaling, potentially bypassing the need for TLR stimulation for inflammasome priming (Gorfu et al., 2014; Gov et al., 2013). Yet despite this data, our lab has not been able to observe significant NFκB activation in response to type II *T. gondii* infection (naturally containing

type II GRA15) of macrophages *in vitro* or after transfection of a macrophage cell line with different TLRs prior to *in vitro* infection (unpublished observations). These data are confusing and the role of caspase-1-dependent inflammasome activation during infection is further complicated by the possibility that there is caspase-1-independent activation of IL-1β during neutrophil-predominant inflammation (Guma et al., 2009). A summary of TLR-mediated recognition and immune responses to *T. gondii* can be seen in (Fig 1-1).

Neutrophils

Basic neutrophil information

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are the most abundant phagocytic cells of the innate immune system. Their name was established by Paul Ehrlich from his observations of their multilobulated shaped nucleus and their tendency to take up neutral dyes – staining pink with hematoyxlin and eosin (H&E) stains. It was later observed by Elie Metchnikoff that neutrophil-like cells are recruited to the site of physical injury in starfish larvae and they are recruited to the site of yeast infection in Daphnia and were observed to be ingesting the yeast (Cavaillon, 2011). Much like the innate immune system itself, neutrophils are evolutionarily ancient: they can be readily identified in fish and neutrophil-like phagocytic cells can be observed in insects and slime molds (Amulic et al., 2012).

Neutrophils are a type of granulocyte - like eosinphils and basophils - they all contain storage granules that sequester molecules critical for antimicrobial responses. Upon pathogen encounter, granulocytes are capable of releasing granules that contain non-specific,

but deadly, components to control a wide range of infections. Neutrophils are historically considered to be short-lived cells with only an 8 hour circulation half-life. However, mouse neutrophils derived from inflammatory conditions and cultured *ex vivo* have a half-life closer to 16-18 hours (Fig 1-2). This extended lifespan under inflammatory conditions may be explained by inflammatory stimuli inducing cell-intrinsic anti-apoptotic factors leading to a sustained host response to infections (Pillay et al., 2013). In agreement with these observations, a group observed that naïve mouse neutrophils *in vivo* have a slightly shorter half-life of 12.5 hours (Pillay et al., 2010). Surprisingly these data may not be representative of human neutrophils which were recently observed to have a half-life of 3.8 days (Pillay et al., 2010). There is some speculation that the reason neutrophils are short-lived, before undergoing controlled apoptosis, is to prevent the accidental release of harmful granule contents (Clevers and Bevins, 2013). However, neutrophils generally need priming by cytokines (TNF, GM-CSF) or pathogen products (e.g. LPS) for full activation leading to respiratory burst and degranulation events in culture (Pillay et al., 2013). Additionally, other cell types that contain granules such as Paneth cells and pancreatic acinar cells are typically longer lived, with a turnover time of about 30 days, suggesting that the presence of granules themselves is not the only reason for the shorter half-life of neutrophils.

Neutrophil granules are classified into 4 different types: primary (azurophilic), secondary (specific), tertiary (gelatinase-positive), and secretory vesicles. The granules types were defined by differences in dye staining and their resident cargo molecules. Although many proteins are present in each granule type, there are characteristic proteins traditionally used to define each granule: myeloperoxidase (primary), lactoferrin (secondary), and

gelatinase (tertiary) (Amulic et al., 2012; Greer, 2013). Despite this classification of granules, granule proteins are thought to be made in a continuous manner during development, with the contents of each granule depending on the transcriptional program active at the time it of packaging; this granule heterogeneity has been termed 'targeting by timing of biosynthesis' (Borregaard, 2010). Granules are formed in the order mentioned with primary, secondary, and tertiary granules formed by budding from the Golgi network and lastly secretory granules are formed from the endocytosis of plasma components.

Neutrophil development

Neutrophils develop from the hematopoietic stem cells (HSCs) in the bone marrow. HSC are also known as LSK cells (lin- sca-1+ c-kit+ cells) which develop into neutrophils by differentiating through several precursor stages, first the common myeloid progenitor (CMP) and then to the granulocyte-monocyte progenitor (GMP). GMPs can give raise to either the granulocyte lineages (e.g. neutrophils, basophils,) or the lineages for macrophages and monocytes. The decision between these lineages is determined by the balance between the transcription factors PU.1 and C/EBPα – higher PU.1 expression drives monocytic differentiation and higher C/EBPα expression drives neutrophil differentiation (Borregaard, 2010). Granulocyte-specific development starts with the myeloblast, which then splits into different promyelocytes depending on granuolocyte type, e.g. basophilic, eosinophilic, and neutrophilic promyelocytes. Each linage, including the neutrophil, then progresses through the following stages: promylocyte, myelocyte, metamyelocyte, and band cell to their final mature form. The developmental stages of neutrophils are typically defined by Wright-

Giemsa staining, following the progress of the nuclear segmentation into the condensed multilobulated shape seen in a mature neutrophil. Granule development can also be seen by morphological examination with the first appearance of granules thought to mark the transition of myeloblasts to promyelocytes and proceeds through the band cell stage (Greer, 2013). Immature neutrophils are maintained in the bone marrow niche by signaling through CXCR4, whose chemokine ligands produced by local bone marrow stromal cells act as retention signals. Mature segmented neutrophils, and band cells under inflammatory conditions, leave the bone marrow due to chemotactic signals received through their recently acquired receptors CXCR2, G-CSFR, and TLRs, which overcome CXCR4 signaling (Borregaard, 2010).

Neutrophils are not just granule-mediated destruction bags

Although neutrophils are primarily known as short-lived antimicrobial effector cells, in recent years our understanding of their role has been expanded greatly in several ways: 1) the discovery of Neutrophil Extracellular Trap (NET) formation 2) the appreciation of neutrophils ability to specifically activate adaptive responses and 3) the identification of Regulatory Myeloid-Derived Suppressor Cell (MDSC), a phenotype of neutrophils. NETs are a unique way in which neutrophils sacrifice themselves to trap extracellular pathogens while minimizing damage to the host. They are a combination of secreted antimicrobial granule proteins and neutrophil chromatin, which form an extracellular lattice to physically trap microbes and to concentrate the antimicrobial proteins with the trapped microbes and away from host tissues (Brinkmann et al., 2004). However, neutrophil NETS are a thought

to be a relatively rare event therefore many neutrophils contribute to pathogen resistance by other methods. Neutrophils produce cytokines that aid in the recruitment of many other cells types, including adaptive T and B cells, by the production of CCL2, CXCL9, CXCL10 and CCL20 (Mantovani et al., 2011). Neutrophils are also antigen presenting cells (APCs); similar to DCs, neutrophils can travel back to the lymph nodes to present ingested microbes or antigens to T and B cells to polarize Th1 or Th17 responses (Abadie et al., 2005; Abi Abdallah et al., 2011). Recent data that we are only just beginning to understand suggests that neutrophils can have distinct phenotypes. MDSC are a heterogeneous group of cells of granulocytic or monocytic origins that have the ability to suppress NK and T cell inflammatory responses. MDSC of granulocytic origin have been morphologically and phenotypically defined as neutrophils or immature neutrophil precursors, however there is currently not a definitive way to differentiate them from the general pool of neutrophils other than their ability to suppress T cell proliferation and cytokines (Pillay et al., 2013). Exactly how these cells arise is also not clear, but may depend on the levels of GM-CSF, IL-6, and IL-10 associated with chronic inflammation in tumor microenvironments and during chronic infections like HIV (Pillay et al., 2013). Neutrophils are just the latest example in a long line of immune cell types that we now understand to have multiple inflammatory and regulatory phenotypes, such as T cells, B cells, macrophages, and ILCs that immunologists continually struggle to define.

Neutrophils in T. gondii *infection prior to 2011*

The role of neutrophils during *T. gondii* infection has been muddled in the literature due to different interpretations of data and complications due to the tools being utilized. Original studies on the importance of neutrophils were accomplished by utilizing the antibody Gr-1 to deplete neutrophils and in these experiments exacerbation of disease was observed (Bliss et al., 2001; Sayles and Johnson, 1996). These data led to the early conclusion that neutrophils were critical for resistance to *T. gondii*. However, the Gr-1 antibody recognizes two distinctly expressed but highly related surface receptors, Ly-6C and Ly6G, which are expressed on both inflammatory monocytes and neutrophils or neutrophils alone respectively (Fleming et al., 1993). Conclusions about the importance of neutrophils drawn from experiments utilizing Gr-1 for depletion were confounded by the simultaneous depletion of both monocytes and neutrophils. The individual roles these two cell types play during *T. gondii* infection was better elucidated with the subsequent characterization of chemokine and chemokine-receptor knockout mice. CCR2 and MCP-1-deficient mice fail to recruit inflammatory monocytes and the result is a loss of parasite control and acute susceptibility to *T. gondii* infection (Robben et al., 2005). These data suggests that inflammatory monocytes are essential for parasite elimination at the site of the infection. Yet both CCR2 and MCP-1 deficient mice have unimpaired levels of IFN-γ in the serum and peritoneal lavage fluid (Robben et al., 2005). Furthermore, CD4+ T cells isolated from CCR2 and MCP-1-deficient mice produced similar amounts of IFN-γ in response to *T. gondii* antigens (Robben et al., 2005). These results indicate that IFN-γ and generation of Th1 responses to the parasite are independent of inflammatory monocytes but that inflammatory monocyte effector mechanisms for parasite elimination are indispensable for the host

protection. In contrast to CCR2 and MCP-deficient mice, CXCR2-deficient mice have impaired neutrophil recruitment during infection. CXCR2-deficient mice were found to harbor a higher parasite burden but these mice did not display an increased mortality during the acute stage of infection (Del Rio et al., 2001). It is interesting to note that unlike CCR2 and MCP-1 deficient mice, CXCR2-deficient mice did have dramatically reduced serum IFN-γ and T cell IFN-γ responses, implicating neutrophils as early players in influencing IFN-γ levels (Del Rio et al., 2001).

The recent availability of the antibody specific for Ly-6G (1A8) allowed for further insight into the roles of neutrophils during infection. In an oral infection model, WT mice treated with 1A8 mostly survived infection, whereas CCR2-deficient mice and WT mice treated with Gr-1 succumb during the acute phase (Dunay et al., 2010). The authors concluded that monocytes and not neutrophils were important for parasite control in part because administration of 1A8 had no effect on the percentage of monocyte recruitment to the site of infection. Interestingly, in a pathogen-free subcutaneous air pouch model, it has been shown that depletion of neutrophils significantly decreases the recruitment of inflammatory monocytes (Soehnlein et al., 2008). In fact, neutropenia reduces monocyte recruitment in a number of models as reviewed in (Soehnlein et al., 2009). Thus in the absence of neutrophils, there may be fewer total numbers of inflammatory monocytes, yet they could represent a similar percentage of the remaining cells in vivo as compared to the non-depleted condition. It is important to emphasize that the relative contribution of monocytes and neutrophils was largely examined in WT mice, which have an intact TLR11 recognition system responsible for IL-12-dependent NK cell IFN-γ-priming of inflammatory

monocytes to control parasite replication. Therefore, while it has become clear that inflammatory monocytes are critical for parasite elimination, the contribution of neutrophils to the inflammatory environment, including their influence on early IFN-γ levels during infection, should not be dismissed since inflammatory monocytes are only effective killers once they have been primed by IFN-γ.

Interferon-gamma (IFN-γ)

Broad importance

IFN-γ is a cytokine that is critical for coordinating protective immunity against infection with intracellular parasites and bacteria. It is produced by NK and NKT cells (Sun and Lanier, 2011) during the innate immune response and by CD4+ and CD8+ T cells (O'Garra and Robinson, 2004; Takada and Jameson, 2009) during the adaptive immune response to a variety of pathogens. IFN-γ mediates its protective effects by triggering lysosomal activation, nitric oxide production, inducing expression of effector genes such as immunity-related GTPases, and modulating metabolic activity of antigen-presenting cells including dendritic cells and macrophages (Boehm et al., 1997; MacMicking, 2012; Taylor et al., 2004). Each of these mechanisms is involved in efficient elimination of pathogens but maintaining activation of these pathways is too dangerous and metabolically costly for the host, necessitating the controlled release of IFN-γ for their activation. The importance of IFN-γ is illustrated by studies of pathogens in IFN-γ-deficient mice. IFN-γ-deficient mice are highly susceptible to a large number of bacterial and parasitic pathogens, including *Mycobacterium tuberculosis, Salmonella typhimurium, Cryptosporidium parvum,* and

Toxoplasma gondii just to name a few (Cooper et al., 1993; Jouanguy et al., 1999; Mead and You, 1998; Suzuki et al., 1988). Additionally, IFN-γ indirectly mediates protective responses by up-regulating expression of chemokines, IP-10, MCP-1 (CCL2), and MIP-1α/β (CCL3/CCL4), and adhesion molecules, ICAM-1 and VCAM-1, necessary for recruitment of immune cells to the site of infection (Schroder et al., 2004). The numerous and pleotropic effects of IFN-γ during intracellular infections has established it as the signature cytokine of Th1 immunity.

IFN-γ's specific importance to T. gondii *infection*

The importance of IFN-γ during *T. gondii* infection cannot be over emphasized: IFN-γ- or IFN-γ-receptor-deficient mice show extreme susceptibility to the parasite by day 10 post-infection (Gazzinelli et al., 1992; Scharton-Kersten et al., 1996; Suzuki et al., 1988; Yap and Sher, 1999). Depletion of cell types that make IFN-γ, such as T and NK cells, are more complicated. It was demonstrated that combined depletion of CD4+ and CD8+ T cells during chronic infection in mice resulted in parasite reactivation and host mortality (Gazzinelli et al., 1992). Individual depletions of CD4 or CD8+ T cells depends on the genotype of the mouse (as mentioned in adaptive immune to *T. gondii* section), as IFN-γ production by CD4+ T cell dominates in the C57BL/6 mouse model and CD8+T cell IFN-γ dominates in BALB/c mouse models. C57BL/6 mice lacking CD4+ T cells were found to have an extended IL-12-dependent NK cell response capable of producing adequate amounts of IFN-γ to allow for survival (Combe et al., 2005). This survival also depends on the NK cells supporting CD8+ T cell immunity to the parasite during the chronic phase of infection

(Combe et al., 2005). Depletion of CD8+ T cells alone in C57BL/6 mice does not increase mortality from *T. gondii* infection until well into the chronic phase of infection when approximately 50% of the mice dying by day 70 post infection (Gazzinelli et al., 1992). These data indicate that CD4+ T cells are sufficient for IFN-γ production in this model and that CD8+ T cells have a minor role to play in the chronic phase of infection. This idea is supported by several studies that point to CD8+ T cells cytolytic abilities as important for parasite control. Perforin-deficient mice lacking CD8+ T cell cytolytic ability survive acute *T. gondii* infection and have an unimpaired level of IFN-γ, but they have a higher cyst burden and slightly increased susceptibility at later time points of the infection (Denkers et al., 1997). Additionally, recent work supports the model of cytolytic cyst control, as transferred IFN-γ-deficient CD8+ T cells are able to greatly reduce cyst burden in a chronic reactivation model (Suzuki et al., 2010).

Early experiments into *T. gondii* infection during T cell-deficiency revealed that NK cells were also a source of IFN-γ. Splenocytes from SCID mice were able to produce significant amount of IFN-γ in response to the parasite (Denkers et al., 1993; Sher et al., 1993). Additionally, it was shown that NK cells are not directly cytotoxic for *T. gondii* infected cells but that NK cell protection is mediated by IFN-γ (Denkers et al., 1993; Hunter et al., 1994). IFN-γ from NK cells can be enhanced with the early addition of IL-12 during infection and it increases the survival of suseptibile SCID mice (Khan et al., 1994). IL-12 is both required and sufficient for triggering IFN-γ production by NK cells (Hou et al., 2011). This IL-12 is produced by DCs (via TLR11 parasite recognition) which was discussed in a prior section.

The importance of IFN-γ during *T. gondii* infection can be explained by the effector mechanisms it induces. IFN-γ is needed to prime macrophages to produce potent antimicrobial responses. IFN-γ regulates inducible nitric oxide synthase (iNOS) expression which is ultimately needed for control of the parasite, as iNOS-deficient mice succumb at 3-4 weeks post-infection (Scharton-Kersten et al., 1997). Nitric oxide interacts with diverse metabolic pathways and, as a free radical, it is toxic to bacteria and intracellular parasites. Macrophages can also use their respiratory burst to generate toxic reactive-oxygen species (ROS) in response to *T. gondii* and this response is dependent on IFN-γ (Arsenijevic et al., 2001). Additionally, IFN-γ induces indoleamine 2,3-dioxygense (IDO), an enzyme involved in tryptophan catabolism and degrades cellular tryptophan. *T. gondii* is a tryptophan auxotroph and induction of IDO-1 inhibits parasite growth in human cells (Gupta et al., 1994; Pfefferkorn, 1984). Recently it was found that inhibiting both IDO-1 and IDO-2 during *T. gondii* infection in mice also results in increased parasite burden and increased susceptibility in the chronic phase of infection (Divanovic et al., 2012). There are additional IFN-γ-induced proteins in mice known as immunity related GTPases (IRG), however this large family of proteins is not represented in the human genome (Howard et al., 2011). The importance for IFN-γ-mediated effector mechanisms are further illustrated by recent evidence that *T. gondii* actively inhibits IFN-γ signaling by inhibiting the turn-over rate of its downstream transcription factor STAT1 in human cell lines (Rosowski et al., 2014). Removal of any one of the IFN-γ-induced mechanisms to control *T. gondii* leads to higher parasite burdens in mice but acute survival is not impaired in contrast to the extreme susceptibility of IFN-γ-deficient mice.

Regulation of IFN-γ

Expression of IFN-γ is regulated at several different levels: transcription factors, chromatin and DNA modifications, and mRNA degradation. The most familiar of these in immunology involves the transcription factor regulated paradigm of Th1 IFN-γ production. The essential transcription factor associated with Th1 IFN-γ expression is T-bet, although there are many other transcription factors that can also directly bind around the IFN-γ promoter and surrounding loci (Balasubramani et al., 2010; Schoenborn and Wilson, 2007). While T-bet is essential for IFN-γ in CD4+ Th1 cells, T-bet-deficient CD8+ T cells can acquire IFN-γ production dependent on IL-12 (Szabo et al., 2002). IL-12 acts on cells via its receptor and activation of the transcription factor STAT4, which has been shown to affect IFN-γ production in both T and NK cells (Thierfelder et al., 1996). Additionally, T-bet-independent IFN-γ from CD8+ and gamma-delta (γδ) T cells was shown to require the transcription factor Eomes, which is in the same T-box transcription factor family as T-bet (Chen et al., 2007; Pearce et al., 2003). This data illustrates the two main pathways needed for IFN-γ production – the IFN-γ→IFN-γR→STAT1→T-bet pathway and the IL-12→IL-12Rβ2→STAT4/3→Eomes pathway – that have differing importance even in closely related IFN-γ producing cell types.

NK cells express low levels of IFN-γ transcript without any stimulation, whereas T cells do not, and therefore the importance of various transcription factors are different in distinct cells. NK cells can be potentially stimulated to make IFN-γ via the IL-12->IL-

12Rβ2->STAT4/3 pathway mentioned above (Thierfelder et al., 1996) and this can be enhanced by exposure to additional cytokines such as IL-18, IL-15, IL-2, TNF, or type 1 IFNs that activate STAT1, STAT4, or NF-kB (Schoenborn and Wilson, 2007). The transcription factors T-bet and Eomes are required for NK cells to maintain their fully mature state, yet knockout or temporary deletion of either or both of these transcription factors in NK cells does not affect their IFN-γ production through NKG2D stimulation, suggesting that other transcription factors or stable epigenetic factors regulate the IFN-γ loci in NK cells (Gordon et al., 2012).

The epigenetic modifications surrounding the IFN-γ loci have been closely examined due to its importance in global regulation of the immune system and more specifically, in the context of Th1 vs Th2 fate choice of CD4+ T cells. Naïve T cells (also called Th0) are thought to be poised to express IFN-γ, in that the promoter region of IFN-γ has neither repressive or permissive histone modifications, but the gene itself is methylated (Schoenborn and Wilson, 2007). Examination of the differences in chromatin modifications in the regions upstream and downstream of the IFN-γ locus during differentiation to Th1 or Th2 allowed several groups to identify multiple distal regulatory elements, or conserved noncoding sequences (CNS), present in the 100kbs surrounding the IFN-γ loci (Chang and Aune, 2007; Schoenborn et al., 2007). Some of these CNS sites identified have non-redundant roles in IFN-γ expression by Th1 effector and memory cells (Collins et al., 2012). IFN-γ is also produced by NK cells, and it has been shown that NK cells have less methylation in their IFN-γ gene, on average, and more methylation in nearby CNS regions than either Th0 or Th1 cells (Chang and Aune, 2005; Tato et al., 2004). Perhaps because of this, the CNS sites in

NK cells, in contrast to T cells, were shown to have redundant roles in IFN-γ expression (Collins et al., 2012). Additionally, gamma-delta (γδ) T cells, which are thought to be more innate-like than adaptive, also have less methylation in their IFN-γ loci (Chen et al., 2007). These patterns of chromatin modifications can help to explain the dependence, or lack thereof, of IFN-γ expression by each cell type in response to various stimuli.

The amount of IFN-γ protein made is also dependent on how long the mRNA transcript is present to be translated into protein. All mRNA contains a 5' cap and a poly(A) tail that usually acts to stabilize intact mRNAs. The importance of the poly(A) tail in regulation of IFN-γ was illustrated in the original IFN-γ-YFP reporter mouse known as Yeti. The construction of these mice replaced the end of the IFN-γ transcript poly(A) with that of bovine growth hormone, which was more stable, and resulted in increased IFN-γ upon stimulation and premature death of the mice (as reported by Richard M. Locksley to Jackson Laboratory). A new IFN-γ-YFP reporter mouse was recently constructed by Dr. Locksley with the endogenous poly(A) tail (known as G.R.E.A.T. mouse) to eliminate this problem. The 3' end of the mRNA, or 3'UTR, just prior to the poly(A) tail also contains regulatory regions, known as AU-rich elements (AREs). AREs function as mediators of mRNA decay, which is mediated by different ARE-binding proteins. One such protein, tristetraprolin, has been shown to mediate the mRNA decay of many cytokines including IFN-γ (Ogilvie et al., 2009). The half-life of IFN-γ mRNA in anti-CD3-stimulated T cells in C57BL/6 mice was 23 minutes (Ogilvie et al., 2009). Regulation and half-life of IFN-γ mRNA in NK cells are likely different from T cells, as they make more IFN-γ mRNA at basel levels due to a more

open loci (mentioned above) and it has been found that stimulated NK cells seem to further stabilize their existing transcripts by productions of unknown proteins (Wilder and Yuan, 1995). More recently it has been found that miRNAs (microRNA), small non-coding RNA molecules that can regulate gene expression, can regulate many aspects of T cell fate decisions including Th1 lineage commitment. Of particular note is miR-29, a potent inhibitor of Th1 differentiation and IFN-γ expression (Steiner et al., 2011). miR-29 affects Th1 differentiation by inhibiting T-bet, Eomes, and IFN-γ transcripts, and therefore likely plays a role in regulating IFN-γ from multiple sources (Baumjohann and Ansel, 2013).

Concluding Remarks

The cytokine IFN-γ is critical for survival during *T. gondii* infection. Mice deficient in IFN-γ succumb to the acute phase of *T. gondii* infection, similar to MyD88-deficient mice. Additionally it was shown that IL-12-deficient mice are extremely susceptible to *T. gondii* and that IL-12 enhances IFN-γ production (Gazzinelli et al., 1993; Khan et al., 1994). While IL-12 is known to regulate IFN-γ, it had no protective role independent of IFN-γ-mediated pathogen resistance (Scharton-Kersten et al., 1996). These data and others established *T. gondii* as a model pathogen of Th1 immunity (Jankovic et al., 2002). Surprisingly, a lack of NK or T cells failed to recapitulate the phenotype of IFN-γ-deficiency in several infectious disease models, suggesting a possibility that there are other cellular sources of this cytokine (Denkers et al., 1993; Hess et al., 1996; Hunter et al., 1994; Mason et al., 2004).

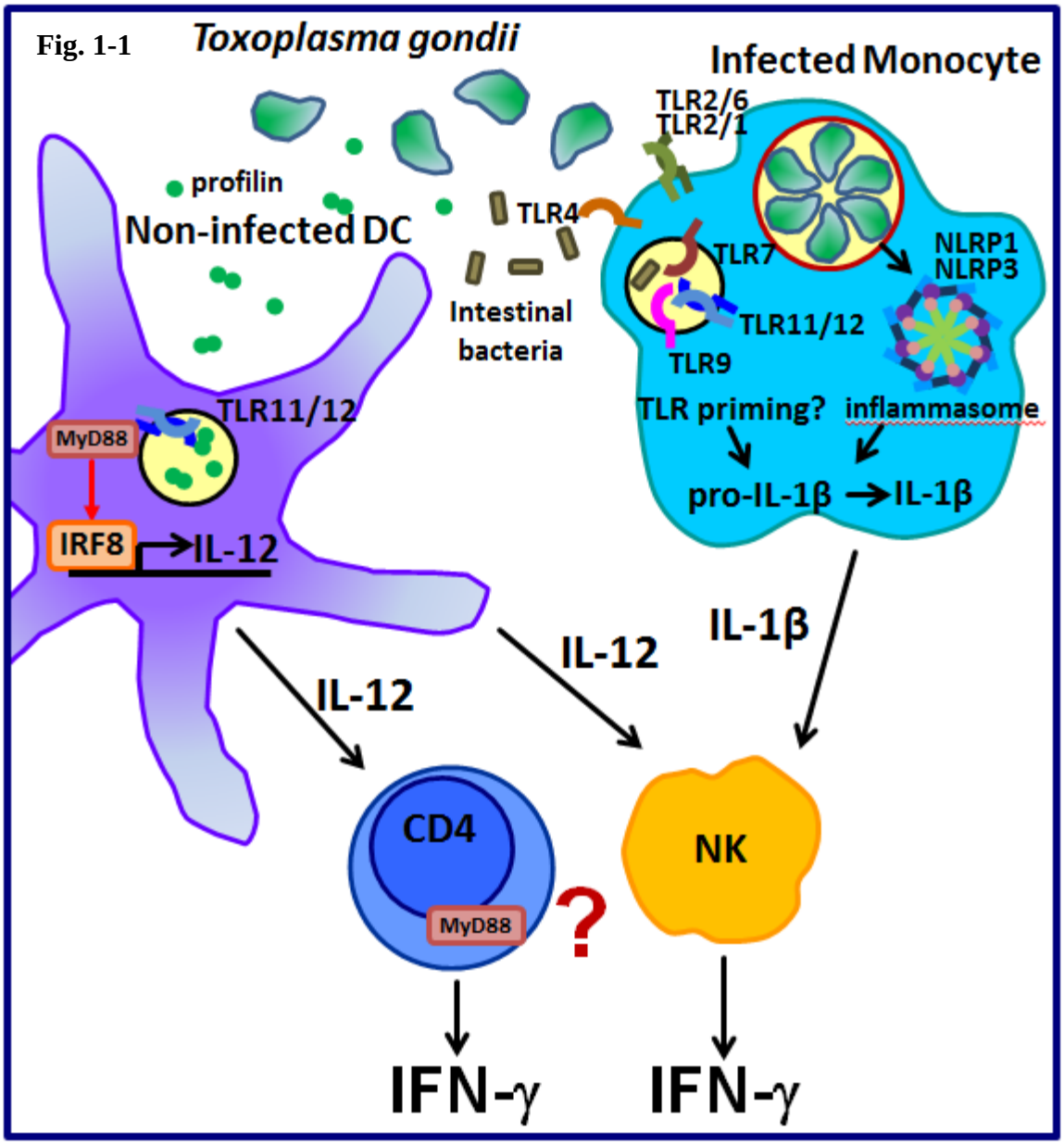


Fig. 1-1: Introduction model of TLR-mediated recognition and immune responses to *T. gondii*. When *T. gondii* infects a mouse, the endosomal TLR11/TLR12 complex recognizes a parasitic protein profilin. TLR11 via its adaptor protein MyD88 initiates downstream signaling, leading to activation of the transcription factor IRF8. This allows DCs to produce IL-12 in response to *T. gondii* infection. IL-12 mediates activation of NK cells and their IFN-γ production. Infected macrophages and monocytes may be able to respond to *T. gondii* infection through NLRP1 and NLRP3 inflammasomes. Priming by TLRs is needed to induce pro-IL-1β expression before subsequent cleavage by the inflammasome complex for maturation of IL-1β and secretion. While the specific TLRs involved in priming are not known, the process is likely initiated by two different events based on the following facts: (i) *T. gondii* itself has ligands for endosomal TLR7, TLR9, TLR11, and TLR12 and for surface-expressed TLR2 and (ii) intestinal bacteria can pass the mucosal barrier during *T. gondii*-mediated inflammation and contain ligands for TLR2, TLR4, and TLR9. IL-1β production can enhance the response of NK cells. It was recently shown that T cell IFN-γ is largely dependent on the presence of T cell-intrinsic MyD88 rather than MyD88 as seen in other cell types. Whether this MyD88 is dependent on TLRs or the IL-1 family receptors has yet to be investigated.

Fig. 1-2

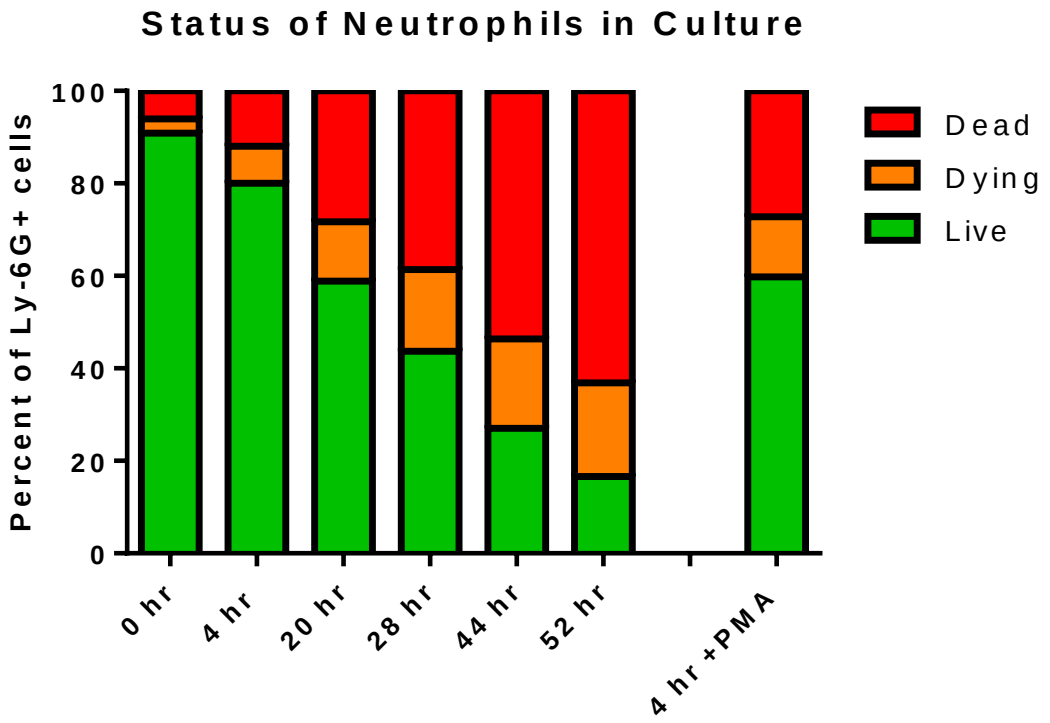


Fig. 1-2: Status of Neutrophils in Culture Media.
Mice were injected with 4% Thioglycollate for 2 hours to elicit neutrophil recruitment. PEC containing neutrophils were extract and placed in a 96 well plate in culture media at 37°C. As a positive control for neutrophil activation and death, a PMA/ionomycin condition was included. Cells were removed from culture at the times indicated, stained with anti-Ly-6G –PE to mark neutrophils, and briefly stained with 7AAD as an indicator of cell viability. Live cells (green) were not positive for 7AAD, Dying cells (orange) were lowly positive for 7AAD, indicating the start of a compromised cell membrane, and Dead cells (red) were highly positive for 7AAD.

CHAPTER II

MATERIALS AND METHODS

Mice.

C57BL/6 mice were obtained from the University of Texas Southwestern Medical Center Mouse Breeding Core Facility. RAG/IL-2R$\gamma_c^{-/-}$ mice were obtained from Taconic. TLR11$^{-/-}$, MyD88$^{-/-}$, and 3d mice have been previously described in experimental toxoplasmosis (Pifer et al., 2011; Tabeta et al., 2006; Yarovinsky et al., 2005). TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were provided by Dr. Edward Wakeland (UT Southwestern) and crossed to generate TLR2/4 doubly-deficient mice. IL-12p40-YFP reporter mice (Yet40) (Reinhardt et al., 2006) were obtained from The Jackson Laboratories and crossed to TLR11$^{-/-}$ mice to generate Yet40xTLR11$^{-/-}$ mice. IL-1R$^{-/-}$ and T-bet$^{-/-}$ mice were obtained from The Jackson Laboratories. Germ-free C57BL/6 mice were provided by Dr. Lora Hooper (UT Southwestern). All mice were maintained at an American Association of Laboratory Animal care-accredited animal facility at UTSW Medical Center. Control and experimental mice were age matched within individual experiments and maintained in the same animal room. All experiments were performed with protocols approved by the Institutional Animal Care and Use Committees of the UT Southwestern Medical Center.

Infection and Intervention.

Mice were infected i.p. with 20 brain tissue cysts of the avirulent ME49 strain of *T. gondii*, as previously described (Yarovinsky et al., 2005). In some experiments, TLR11$^{-/-}$ mice were injected i.p. with PBS vehicle control or 500µg of αIFN-γ (clone XMG1.2) on day -1 and day 6; or combinations of 200µg of αCD4 (clone GK1.5), 200µg of αCD8 (clone 2.43), or 100µg of αNK1.1 (clone PK136) twice a week. In some experiments, 200µg of αIL-12 (clone C17.8) was injected i.p on days -1 and 3 post-infection or in complementary experiments 150ng of recombinant IL-12 was injected i.p. on days 1 and 3 post-infection. In experiments to block cytokines, 4mg of IL-1R antagonist (Calbiochem) or 500µg of αTNF (clone XT22-11) was injected i.p. on days 3 and 4 post-infection. In additional experiments, TLR11-/- mice were injected i.p. with 500µg of αLy-6G (clone 1A8) on day 0 and 100µg of αLy-6G on each subsequent day. Some groups also received 1µg of recombinant IFN-γ (BioLegend) on day 0 and again every other day for the duration of the experiment. All depletion antibodies were obtained from BioXCell. *S. enterica* serovar typhimurium (SL1344) was provided by Dr. Lora Hooper (UT Southwestern). Flask cultured *S. typhimurium* in the log phase of growth was diluted in PBS to 200 CFU per mouse for i.p. infections.

***Ex Vivo* culture of cells.**

For early *in vitro* experiments with thioglycollate elicited neutrophils, TLR11$^{-/-}$ mice were injected with a 4% thioglycollate solution overnight. PEC containing neutrophils were harvested and cells were plated at a concentration of 2 million/ml in complete RPMI 1640

medium supplemented with 10% FBS, L-glutamine, penicillin, streptomycin, sodium pyruvate, nonessential amino acids, and 10μM HEPES (Life Technologies), cultured for 24 hours with or without the addition of tachyzoites in a 1:1 ratio. Supernatant from these were examined by ELISA.

For later in vitro experiments, C57BL/6 mice were infected with *T. gondii* and PECs were harvested on day 5 post-infection. In one experiment, PEC cells were incubated for 5 hours at 37°C in complete RPMI 1640 medium supplemented with 10% FBS, L-glutamine, penicillin, streptomycin, sodium pyruvate, nonessential amino acids, and 10μM HEPES (Life Technologies) with or without the addition of GolgiPlug 1μg/ml (BD Bioscience). In another experiment PECs were incubated in media with GolgiPlug and increasing concentrations of Cycloheximide (Sigma), 0.25, 0.5, 1, 2.5, 5, or 10μg/ml, with or without PMA 50ng/ml (Sigma) and ionomycin 750ng/ml stimulation (Sigma). For experiments using naïve bone marrow from C57BL/6 mice, bone marrow were isolated and single cell suspensions made complete RPMI 1640 medium supplemented as above. Cytochalasin B was added for a concentration of 10μg/ml and cells were incubated at 37°C for 10 minutes. Then there was a doubling of volume by adding media containing the indicated reagents for a final concentration of 10nM fMLP (Sigma),100ng/ml LPS (Sigma),50ng/ml PMA, or 750ng/ml ionomycin. Cells were incubated for 1 hour at 37°C, supernatants collected for ELISA and cells were stained for flow cytometry.

Quantitative Real Time-PCR.

Total cellular RNA from PEC, Ly-6G+ cells, or our defined immature neutrophils were isolated using the PureLink RNA Mini kit (Ambion) according to the manufacturers' instructions. cDNA synthesis was done using SuperScript III RT (Life Technologies). For *T. gondii* pathogen loads, total genomic DNA from PEC was isolated using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturers' instructions. PCR were done using 12.5µl of SsoFast EvaGreen Supermix (Bio Rad), 1.25µl of 10µM primers, and 1µg normalized RNA or 20ng of DNA for a total volume of 25µl. Samples were run on MyiQ and iQ5 Real-Time PCR System (Bio Rad) and data from transcripts were processed using the $\Delta\Delta C_T$ method or relative expression compared to the control gene HPRT (2^-(Ct[gene of interest] – Ct[HPRT])) and data from genomic DNA was compared to a defined copy number standard of the *T. gondii* gene B1. A list of primers used in these experiments can be found in (Table 2-1).

Flow Cytometry.

For bone marrow (BM) and peritoneal exudate cells (PEC), a single cell suspension was made in PBS plus 1% FBS and 0.5mM EDTA for immediate staining or in complete RPMI 1640 medium with supplements (see above) for cells to be incubated at 37°C prior to staining. Blood samples were directly stained in PBS plus 1% FBS and 0.5mM EDTA and then RBCs lysed with ACK lysing buffer (Life Technologies).

Cells were stained using fluorochrome- or biotinylated -conjugated antibodies according to the manufacturer's instructions. Cells stained with biotinylated antibody were

washed at least twice PBS plus 1% FBS and 0.5mM EDTA and additionally stained with a fluorochrome-conjugated streptavidin. For intracellular staining and subsequent washing, cells were permeabilized 4 hours to overnight at 4°C with the Foxp3/Transcription Factor Staining Buffer Set according to the manufacturer's instructions (eBioscience).

The following antibodies were used for surface or intracellular staining and were purchased from eBioscience unless otherwise indicated: αNK1.1 (clone PK136), αCD3ε (clone 145-2C11), αIFN-γ (BD Bioscience, clone XMG1.2), αCD11b (BD Bioscience, clone M1/70), αCD44 (clone IM7), αNeu7/4 (Abcam, clone 7/4), αGr-1 (clone RB6-8C5), αLy-6C (clone HK1.4), αLy-6G (BD Bioscience, clone 1A8), αCD45 (clone 30-F11), αCD90.2 (BD Bioscience, clone 53-2.1), αCD212 (BD Bioscience, clone 114), αCD27 (clone LG.7F9), αCD25 (BD Bioscience, clone 7D4) , αCD122 (clone TM-b1), αCD4 (BD Bioscience, clone GK1.5), αCD8α (BD Bioscience, clone 53-6.7), αB220 (clone RA3-6B2), αIgM (clone eB121-15F9), αCD19 (clone eBio1D3), αNKp46 (clone 29A1.4), αCD49b (BD Bioscience, clone DX5), αNKG2D (clone CX5), αF4/80 (clone BM8), αCD11c (BD Bioscience, clone HL3), αI-A^b (BD Bioscience, clone 25-9-17), αCD80 (BD Bioscience, clone 16-10A1), αCD86 (BD Bioscience, GL3), αLy-6G (BD Bioscience, clone 1A8), αCD34 (clone RAM34), αCD117 (clone 2B8), αCD16/32 (clone 93), αSca-1 (clone D7) , αCD19 (clone MB19-1), αF4/80 (clone BM8), αTer119 (clone Ter119), αCD11b (clone M1/70), and αGr-1 (clone RB6-8C5) and isotype IgG1κ (clone R3-34).

These data were acquired with FACSCalibur or FACSCanto cytometers (BD Bioscience) or, for sorted cells, with the MoFlo cytometer (Dako Cytomation) at the UTSW Flow Cytometry Core Facility and were analyzed with FlowJo software (TreeStar).

ELISA.

For PEC supernatant, the peritoneal cavity was lavaged with 5ml of ice-cold PBS containing 0.5mM EDTA. Supernatants from these and from in vitro culture experiments were collected and stored at -20°C. Blood was obtained by retro-orbital bleeding, sera were separated by centrifugation and stored at -20°C. The amount of IL-12p40 and IFN-γ concentration in the supernatants was determined by using standard sandwich ELISA kit according to the manufacturer's instructions (eBioscience).

Microscopy.

Ly-6G+ sorted PEC cells or Ly-6G+ magnetic bead sorted (Miltenyi) bone marrow cells were deposited onto glass slides using a CytoSpin 3 centrifuge (Thermo Shandon). For morphological examination, slides were stained with Giemsa according to the manufacturers' instructions (Sigma-Aldrich). Slides were examined using a light microscope with a 63X objective and attached camera.

For immunofluoresence, cells were fixed to slides with PBS buffered 4% paraformaldehyde for 15 minutes, blocked for 10 minutes in PBS with 2% BSA plus 0.2% TritonX-100 and then stained for 20 minutes in the same buffer containing antibodies of interest. Slides were washed in PBS plus 0.2% TritonX-100, stained with a secondary as per above if needed, washed again, then some slides were stained for 5 minutes with SYTO 62 nuclear stain (Life Technologies) and washed, allowed to air dry, and mounted with ProLong Gold with DAPI (Life Technologies). Images were acquired with a Leica SPE system fitted

with a Leica 633 or Leica TCS SP5 laser scanning confocal microscope objective NA 1.4 (Leica Microsystems) and data were processed with the Leica Advanced Fluorescence software. Downstream images for analysis were examined with ImageJ v1.48 (NIH, public domain), Z stacks underwent 3D blind deconvolution with AutoQuant X software (Media Cybernetics Inc), and colocalization analysis and 3D modeling done with Imaris x64 7.7.1 (www.Bitplane.com).

Antibodies used were: αIFN-γ Alexa488 (BD Bioscience, clone XMG1.2), αMyeloperoxidase (Abcam, ab9535), αLactoferrin (Abcam, ab135710), αGelatinase (Abcam, ab38898), and donkey anti-rabbit IgG Alexa568 (Invitrogen, A10042).

Statistical Analysis.

All data were analyzed with Prism (Version 5 and 6; GraphPad). These data were considered statistically significant when p values were less than 0.05 by a two-tailed t test.

Table 2-1: List of Primers used for qRT-PCR.

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
HPRT	GCCCTTGACTATAATGAGTACTTCAGG	TTCAACTTGCGCTCATCTTAGG
IFN-γ	ACTGGCAAAAGGATGGTGAC	TGAGCTCATTGAATGCTTGG
Toxo (gene: B1)	TCCCCTCTGCTGGCGAAAAGT	AGCGTTCGTGGTCAACTATCGATTG
Protinase 3	AGCTACCCATCCCCCAAG	TCGTGCCCACCTACAATCTT
Lactoferrin	GGGCAAGTGCGGTTTAGTT	CCATTGCTTTTGGAGGATTT
Gelatinase	AGCTACCCATCCCCCAAG	TGTCGGCTGTGGTTCAGTT
T-bet	CTCAGGACTAGGCGAAGGAG	CCCCGCGTCTGTTTTCTAAG
Eomes	CACAGTTCATCGCTGTGACG	AGCCGTGTACATGGAATCGTAG
ID2	CTCCAAGCTCAAGGAACTGG	ATTCAGATGCCTGCAAGGAC
STAT1	GTGGGAACGGAAGCATTTGG	ATCGTACAGCTGGTGGACCT
STAT3	GCAATACCATTGACCTGCCG	AACGTGAGCGACTCAAACTG
STAT4	CCTAGAGGAATGGCGTCAGC	CTTGATTCCACTGAGACATGCTGG

CHAPTER III

Results Part 1: TLR-independent neutrophil-derived IFN-γ is important for host resistance to intracellular pathogens.

The information in this chapter has been previously published in PNAS 2013

IFN-γ protects TLR11-/- mice from *T. gondii* infection in the absence of NK and T cells.

To investigate the mechanisms of TLR11-independent immunity to *T. gondii*, we examined the relative contribution of NK and T cells in IFN-γ-mediated resistance to the pathogen. We focused on this cytokine because treatment of TLR11-/- mice with IFN-γ-blocking antibody resulted in acute susceptibility to the infection which was similar to that observed in IFN-γ-/- mice **(Fig. 3-1A)**. Therefore, effector mechanisms responsible for host protection to *T. gondii* in the absence of TLR11 depend on IFN-γ. TLR-mediated recognition of pathogens, including *T. gondii*, regulates IFN-γ production from both NK and T cells (Scanga et al., 2002). To identify the cells contributing to IFN-γ-dependent protection in the absence of TLR11, the major innate immune sensor for *T. gondii* in mice (Yarovinsky et al., 2005), we depleted NK cells, CD4 or CD8 T cells and compared the pathogen burden and IFN-γ-/- levels during infection. No difference in serum levels of IFN-γ or pathogen burden were observed in lymphocyte-depleted mice when compared to control animals on day 5 post-infection **(Fig. 3-1B)**. Furthermore, depletion of NK cells, thought to be the early source of IFN-γ during *T. gondii* infection, did not alter the survival of TLR11-/- mice **(Fig. 3-2)**. This suggests that IFN-γ produced by NK or T cells alone is insufficient for early host resistance to the parasite. Surprisingly, even when TLR11-/- mice were simultaneously depleted of NK and T cells, they showed a significant increase in survival compared to mice

treated with IFN-γ-blocking antibody **(Fig. 3-1A)**. Analysis of NK and T cells depletion ruled out a possibility that enhanced survival of mice treated with anti-NK1.1, anti-CD4, and anti-CD8 was due to residual production of IFN-γ by those cells **(Fig. 3-2)**. Consistent with the increased survival of NK and T cell depleted TLR11-/- mice, these animals had a reduced pathogen compared to mice treated with an IFN-γ-blocking antibody **(Fig. 3-1C)**. Moreover, levels of IFN-γ in the peritoneal cavities of NK and T cell depleted mice were only slightly reduced as compared to those of control mice **(Fig. 3-1C)**. Taken together, these data suggest that there is a source of IFN-γ that is not affected by the depletion of NK and T cells in *T. gondii* infected TLR11-/- mice. Furthermore, this IFN-γ controls pathogen burden and is essential for enhanced survival of TLR11-/- mice infected with the parasite.

IFN-γ production is not limited to NK and T cells.

To identify cells capable of producing IFN-γ besides NK and T cells we used flow-cytometry to analyze viable cells expressing IFN-γ. A distinct population of IFN-γ-positive cells was observed in the peritoneal cavity 5 days after infection in WT and TLR11-/- mice **(Fig. 3-3A)**. Strikingly, substantial numbers of IFN-γ-positive cells were negative for NK1.1 and CD3ε, the prototypic surface markers expressed by all NK and T cells respectively. Additional analysis revealed that lack of TLR11-mediated recognition of the parasite resulted in a dramatic increase in frequency and total number of IFN-γ-positive cells **(Fig. 3-3A-B)**. This increase was due to a large expansion of the IFN-γ-positive CD3ε- NK1.1-negative cell population **(Fig. 3-3B)**. In addition, combined deficiencies in TLR11 and TLR2, a receptor

involved in recognition of GPI anchors expressed on the cell surface of *T. gondii* (Gazzinelli and Denkers, 2006), did not abrogate expansion of the IFN-γ-positive CD3ε- NK1.1-negative cells. Furthermore, abrogation of all intracellular TLRs including TLR11, TLR12, TLR7, and TLR9 by using 3d mice - deficient in UNC93B1 protein (Pifer et al., 2011; Tabeta et al., 2006) – resulted in IFN-γ production dominated by CD3ε- NK1.1-negative cells **(Fig 3-4)**. Thus, all TLRs known to be involved in *T. gondii* recognition (Andrade et al., 2013; Koblansky et al.; Yarovinsky et al., 2005) were not involved in induction of IFN-γ-positive CD3ε- NK1.1-negative cells. These IFN-γ-positive cells were also observed in WT mice, although at reduced frequencies **(Fig. 3-3A-B)**, since both NK and T cells contribute to IFN-γ production in response to TLR11 activation by *T. gondii* **(Fig. 3-3B and 3-2)**. The appearance of IFN-γ-positive cells lacking NK1.1, CD4, or CD8 is in agreement with the survival data of lymphocyte-depleted mice **(Fig. 3-1A)**, emphasizing the physiological importance of these cells in host defense against this parasite.

One possible explanation for the early appearance of IFN-γ-positive CD3ε- NK1.1-negative cells is that *T. gondii* infection induces the appearance of an unusual NK cell population not expressing NK1.1. Arguing against this possibility, IFN-γ-positive CD3ε-NK1.1-negative cells did not express the NK cell markers DX5, NKp46 and NKG2D, suggesting that these were not NK cells **(Fig. 3-5)**. Additionally, IFN-γ-positive CD3ε-NK1.1-negative cells did not express any other lymphoid cell lineage markers that we examined, including B220, CD19, CD4, CD8, Thy1.2 or CD27 which would identify them as B or T cells **(Fig. 3-5)**.

To formally determine whether IFN-γ-positive CD3ε- NK1.1-negative cells were of the lymphoid lineage, we utilized a genetic approach and examined the appearance of these cells in RAG2/IL-2Rγ_c-/- mice. These doubly-deficient mice do not develop T or B cells as a result of Recombination Activating Gene 2 (RAG2) inactivation and they also lack NK cells due to deficiency in IL-2 and IL-15 signaling caused by deletion of the IL-2Rγ_c gene (Cao et al., 1995; Shinkai et al., 1992). The presence of IFN-γ in these mice would indicate that expression of this cytokine is not limited to lymphoid cells, including NK and T cells. *T. gondii* infection of RAG/IL-2Rγ_c-/- mice resulted in the appearance of IFN-γ positive cells and the induction of IFN-γ transcripts at levels comparable to those observed in TLR11-/- mice **(Fig. 3-3C)**. Taken together, these data suggest that NK and T lymphocytes are not the only cell types capable of producing IFN-γ.

Neutrophils produce IFN-γ in response to *T. gondii* and *S. typhimurium* infections.

In order to define the nature of IFN-γ-positive CD3ε- NK1.1-negative cells we next examined other surface markers expressed by these cells. Expression of CD45 on IFN-γ-positive CD3ε- NK1.1-negative cells pointed to their hematopoietic origin although they were negative for the common lymphoid cell surface markers CD25, CD27, and CD122 **(Fig. 3-5)**. These IFN-γ-positive non-NK-non-T cells expressed CD11b, an integrin associated with myeloid lineage cells. They also expressed CD44, an adhesion receptor on activated cells migrating into inflammatory sites **(Fig. 3-3D)**. Nevertheless, IFN-γ-positive CD3ε-NK1.1-negative cells did not express the myeloid lineage markers CD11c, F4/80, or MHC-II

– present on DC and macrophages **(Fig. 3-5)**. Instead, IFN-γ-positive CD3ε- NK1.1-negative cells shared a surface marker present on monocytes and neutrophils, granulocyte receptor-1 (Gr-1) (Fig. 2D). Anti-Gr-1 antibody recognizes two related surface receptors, Ly-6C and Ly-6G (Fleming et al., 1993), and the precise characterization of their expression discriminates between monocytes and neutrophils. Our experiments demonstrated that IFN-γ-positive CD3ε- NK1.1-negative cells expressed intermediate levels of Ly-6C compared to monocytes and were highly positive for Ly-6G **(Fig. 3-3D)**, a surface marker highly expressed on neutrophils. The presence of the additional neutrophil-specific marker Neu7/4, also known as Ly-6B (Rosas et al., 2010) **(Fig. 3-3D)**, further suggested that IFN-γ-positive CD3ε- NK1.1-negative cells were neutrophils.

To formally examine the ability of neutrophils to produce IFN-γ, Ly-6G-positive neutrophils were purified from the peritoneal cavity of *T. gondii* infected TLR11-/- mice. Their ability to produce IFN-γ was next compared with that of NK cells isolated from the same mice **(Fig. 3-6A-C)**. We established that the majority of neutrophils stained positively for IFN-γ, strongly indicating that these cells were capable of producing this cytokine without any *in vitro* re-stimulation **(Fig. 3-6B)**. Furthermore, the presence of IFN-γ transcripts excluded a possibility that neutrophils stained positive for IFN-γ as the result of their phagocytic activity and uptake of the other IFN-γ-producing cells, such as NK and T cells **(Fig. 3-6C)**.

In the same experiments, purified Ly-6G-positive cells were morphologically characterized by Giemsa staining. They were observed to have the multi-lobed nucleus structure characteristic of neutrophils **(Fig. 3-6D)**. Purified neutrophils were stained with

anti-IFN-γ and immunofluorescent analysis revealed the presence of discrete puncta of IFN-γ present in their cytoplasm **(Fig. 3-6E)**. Additionally, TLR11-/- neutrophils in vitro produced IFN-γ in response to incubation with parasite (**Fig. 3-7**).

To determine whether IFN-γ production by neutrophils was specific for *T. gondii,* we evaluated neutrophils in response to another pathogen, *S. typhimurium*. In WT mice infected with *S. typhimurium*, neutrophils, NK and T cells all contributed to IFN-γ production. Importantly, the early IFN-γ responses against *S. typhimurium* were dominated by neutrophils in TLR2/4 doubly-deficient mice **(Fig. 3-8A-B)**, which are deficient in TLR-mediated pathogen recognition of the bacteria. Overall, analyses of IFN-γ responses to *T. gondii* and *S. typhimurium* infections revealed that neutrophils were a major cellular source of IFN-γ, especially in the absence of TLR-mediated pathogen recognition.

Neutrophil-produced IFN-γ is IL-12-independent, but is regulated by TNF and IL-1β.

IFN-γ production from NK and T cells is regulated by TLR11, UNC93B1, and TLR12-dependent induction of interleukin-12 (IL-12) (Hou et al., 2011; Koblansky et al.; Pifer et al., 2011; Scanga et al., 2002). The appearance of IFN-γ-positive neutrophils in the absence of TLR11 or UNC93B1 raised the question of whether IL-12 plays a role in triggering a neutrophil-derived IFN-γ response. We therefore analyzed the kinetics of IL-12 and IFN-γ production in WT and TLR11-deficient mice. The experiments revealed a paradoxical result in which *T. gondii* infected TLR11-/- mice showed a large increase in the number of IFN-γ-positive cells compared to WT mice **(Fig. 3-9A)**. This was in contrast to

the IL-12 response analyzed by quantification of IL-12p40-YFP expressing cells (Reinhardt et al., 2006) that was consistent with previously published data, demonstrating the dependence on TLR11-mediated parasite recognition (Yarovinsky et al., 2005). Lack of correlation between the appearance of IL-12 and IFN-γ-positive cells suggested that in contrast to NK and T cells, neutrophil-derived IFN-γ, is not regulated by IL-12. To formally test this hypothesis, TLR11-/- mice were infected with *T. gondii* and IL-12 was blocked during the course of infection using an anti-IL-12/23p40 antibody. Successful blocking of IL-12 was confirmed by examining serum levels of IL-12 and by the examination of IFN-γ production by NK cells **(Fig. 3-10A)**, which is known to be regulated by IL-12 (Hou et al., 2011). Quantification of IFN-γ- Ly-6G-positive CD3ε- NK1.1-negative cells demonstrated that administration of an anti-IL-12 antibody had no effect on the production of IFN-γ by neutrophils **(Fig. 3-9B)**. Additionally, the pathogen load of anti-IL-12 treated mice was intermediate and significantly different from that of control or anti-IFN-γ treated groups **(Fig. 3-9C).** These levels of pathogen burden are reminiscent of those seen with depletions of both NK and T cells simultaneously **(Fig 3-1C)** but are slightly elevated. As anti-IL-12 treatment has previously been shown to cause acute susceptibility (Hunter et al., 1994; Scanga et al., 2002), it may be a fine line between the parasite levels that result in acute versus intermediate susceptibly or alternatively that a role of IL-12 exists independent of eliciting IFN-γ from NK and T cells. In complementary experiments, administration of recombinant IL-12 to infected TLR11-/- mice had little effect on the number of IFN-γ-positive neutrophils **(Fig. 3-10B)**. Taken together, these results strongly suggest that IFN-γ production by neutrophils does not depend upon IL-12.

TNF and IL-1β have been implicated in the regulation of IFN-γ (Hunter et al., 1995). Therefore these cytokines were blocked during *T. gondii* infection and IFN-γ-positive neutrophils were quantified. Administration of anti-TNF significantly decreased the number of IFN-γ-positive neutrophils **(Fig. 3-9D)** without affecting early IFN-γ production by NK or T cells **(Fig. 3-10C)**. Flow cytometry experiments revealed that Ly-6C^{hi} pro-inflammatory monocytes were the major source of TNF and IL-1β during *T. gondii* infection **(Fig. 3-10D)**. Neutrophils were also capable of TNF and IL-1β production but at lower levels than was seen from monocytes, suggesting neutrophils themselves may have some minor positive feedback in this system **(Fig. 3-10D)**. Blocking responses to IL-1β utilizing a receptor antagonist revealed a decrease in IFN-γ-positive neutrophils **(Fig. 3-9D)**. IL-1R-/- mice provided a more decisive model to examine the role of IL-1β. Comparison of WT and IL-1R-/- mice exposed a significant decrease in IFN-γ-positive neutrophils in the absence of IL-1R (**Fig. 3-9E**). This observation is in agreement with the phenotype of MyD88-deficient mice that fail to induce IFN-γ from neutrophils **(Fig. 3-11)**. Together these data suggest that both TNF and IL-1β, but not IL-12 regulate IFN-γ-positive neutrophils.

Neutrophils protect against *T. gondii* in the absence of TLR11.

Neutrophils participate in host response to *T. gondii* but play a limited role in survival of WT mice infected with the parasite (Denkers et al., 2004; Dunay et al., 2010; Egan et al., 2008). Instead, IFN-γ from NK and T cells predetermined susceptibility vs. resistance to the parasite (Hou et al., 2011; LaRosa et al., 2008; Mason et al., 2004). The dominance of neutrophils in IFN-γ production observed in *T. gondii* infected TLR11-/- mice prompted us to

investigate their physiological importance. Depletion of neutrophils using the highly specific anti-Ly-6G antibody resulted in early mortality of TLR11-/- mice with kinetics nearly indistinguishable from those observed in IFN-γ-/- mice **(Fig. 3-12A)**. This was in contrast to neutrophil-sufficient TLR11-/- mice which can control the parasite during the acute phase of the infection **(Fig. 3-1A)** and demonstrate enhanced susceptibility to *T. gondii* only during the chronic phase of the infection (25). Importantly, the selective depletion of neutrophils in *T. gondii* infected TLR11-deficient mice did not affect the levels of IFN-γ produced by NK, CD4, or CD8 T cells **(Fig**. **3-12B and 3-13)**, yet lymphoid cell IFN-γ production was insufficient to control the parasite in the absence of neutrophils **(Fig. 3-12A)**. Treatment of neutrophil-depleted TLR11-/- mice with recombinant IFN-γ (rIFN-γ) enhanced host resistance during the acute stage of the parasitic infection **(Fig. 3-12A)**. Overall, these results revealed that neutrophils are crucial effector cells for IFN-γ-mediated host resistance to the parasite in the absence of TLR11-dependent activation of NK and T cells.

Discussion of the Results: Part 1

It is generally accepted that activation of TLRs on DCs is a crucial event for triggering IL-12-dependent IFN-γ production by NK and T cells. The currently proposed models of TLR-mediated immune responses implies that lack of TLRs, IL-12, or NK and T cells would completely abrogate IFN-γ production and activation of inflammatory cells at the site of infection; hence, infection would be more severe. The significance of IFN-γ is exemplified by studies performed in IFN-γ-/- mice which are extremely susceptible to a broad range of pathogens, yet this susceptibility is not recapitulated in mice lacking NK or T cells. These results have raised the question of the cellular origin of this cytokine in the

initial stages of the innate immune response. In this regard, we observed that neutrophils are the major IFN-γ-producing cells during acute responses to both *T. gondii* and *S. typhimurium*. The presence of IFN-γ-producing cells in *T. gondii*-infected mice lacking T and NK cells due to deficiency in RAG and IL-2Rγ_c has formally proven the existence of non-NK/non-T IFN-γ-producing cells. In wild type mice, neutrophils produce IFN-γ; however IFN-γ-positive neutrophils were especially evident in the absence of TLR11 or UNC93B1. We also established that IFN-γ production by neutrophils is IL-12-independent, but is regulated by TNF and IL-1. These results indicated the existence of an IFN-γ pathway for controlling intracellular pathogens that is independent of TLR-recognition, as well as NK and T cells, but is dependent on MyD88 via IL-1R. Based on what we know about neutrophils, their large numbers and rapid deployment to the site of infection should provide an important means of very early, robust, and rapid elimination of pathogens.

NK cells are a well characterized source of IFN-γ that restricts *T. gondii* replication during the first week of acute infection (Denkers et al., 1993; Hunter et al., 1994). This conclusion stems from the extended survival of lymphocyte-deficient mice during parasite infection in comparison to IFN-γ-/- mice and WT mice treated with IFN-γ-blocking antibody (Suzuki et al., 1988). In addition to NK cells, CD4 T cells participate in IFN-γ production as early as 5 days after infection. The contribution of CD4 T cells to the initial IFN-γ-mediated control of the parasite is particularly evident in mice lacking the common cytokine receptor γ-chain (γc) gene which leads to the defective development of NK and T cells (Scharton-Kersten et al., 1998). Nevertheless, selective depletion of either NK cells or T lymphocytes does not diminish the resistance during the acute stages of infection, suggesting the

redundancy of these cell types in mediating IFN-γ-dependent host resistance (Scharton-Kersten et al., 1998). Paradoxically, the combined deficiency in NK and T cells does not abrogate IFN-γ triggered by *T. gondii* infection (Hunter et al., 1994). As in *T. gondii* infection, *Salmonella*-induced IFN-γ expression is only partially dependent upon NK and T cells (Mittrucker and Kaufmann, 2000). Taken together, these results suggest that in addition to NK and T cells there are innate immune cells, likely of myeloid origin, producing IFN-γ in response to microbial infections. Furthermore, neutrophils producing IFN-γ were identified in *Salmonella*, *Listeria*, and *Nocardia* infected mice (Ellis and Beaman, 2002; Kirby et al., 2002; Yin and Ferguson, 2009), but this possibility is not well accepted and is usually interpreted as a result of incomplete depletion of IFN-γ-producing NK or T cells during analysis of myeloid cells (Laouar et al., 2005; Schleicher et al., 2005). This scenario is particularly applicable to the description of IFN-γ production by DCs and macrophages, since these cells can form tight functional contacts with NK cells (Coombes et al., 2012; Lucas et al., 2007; Makarenkova et al., 2005). In addition, the presence of IFN-γ protein in antigen-presenting cells may result from the phagocytic activities of DCs and macrophages rather than cytokine production. Overall, the concept of myeloid-derived IFN-γ is still the subject of debate, in part because of the failure to identify cytokines regulating IFN-γ production in myeloid cells that are distinct from those involved in IFN-γ-production by NK and T cells (Bogdan and Schleicher, 2006). Our experimental infections of RAG2/IL-2Rγ_c-/- mice with *T. gondii* provided definitive evidence for the ability of neutrophils to produce protective IFN-γ. This conclusion was further supported by identification of TNF and IL-1 as selective

regulators of IL-12-independent IFN-γ production by neutrophils required for host resistance to *T. gondii* in the absence of TLR11.

TLR11 is a major innate immune sensor for *T. gondii* that is responsible for IL-12-dependent activation of NK and T cells. Nevertheless, in contrast to IFN-γ-/- mice, those lacking TLR11 are relatively resistant to the parasite (Yarovinsky et al., 2005). It was recently suggested that TLR12-dependent activation of NK cells in the absence of TLR11 explains the enhanced survival of TLR11-/- mice when compared with MyD88-/- or IFN-γ-/- mice (Koblansky et al.). Nevertheless, depletion of NK cells in *T. gondii* infected TLR11-/- mice did not affect the survival of these mice, strongly suggesting that NK cells play little or no significant role in TLR11-independent IFN-γ-dependent host resistance to *T. gondii*. Furthermore TLR11, as well as TLR12 inactivation, or combined deficiencies in TLR11, TLR12, TLR7, and TLR9 signaling pathways caused by mutation in UNC93B1, abolished IL-12 production *in vivo* and *in vitro*, but had a minor effect on the levels of IFN-γ observed in *T. gondii* infected mice (Pifer et al., 2011; Yarovinsky et al., 2005). This unexpected disconnect between TLR-mediated pathogen recognition and IFN-γ-dependent host resistance is not unique to *T. gondii*; it has been observed with other intracellular pathogens and is frequently interpreted as a functional redundancy among TLRs in the activation of NK or T cells. This seems to be supported by the phenotypes of MyD88-/- mice that are highly susceptible to both *T. gondii* and *S. typhimurium* (Scanga et al., 2002; Weiss et al., 2004). In addition to TLRs, IL-1R-mediated activation of MyD88 is indispensable for host resistance to intracellular pathogens (Hitziger et al., 2005) although IL-1R regulation of IFN-γ production is not completely understood, since IL-1R contributes to but is not absolutely

essential for the IL-12 and IFN-γ production by DCs and NK cells respectively (Benson et al., 2009; Hunter et al., 1995). IL-1R or caspase-1 deficiency during *T. gondii* infection has no effect on IFN-γ production by T cells (Benson et al., 2009; Raetz et al., 2013a). IL-12-independent functions of MyD88 are also supported by the inability of recombinant IL-12 to rescue *T. gondii*-infected MyD88-/- mice (Hou et al., 2011). While this treatment protects DC-specific MyD88-deficient mice as well as 3d mice with impaired functions in all endosomal TLRs (Tabeta et al., 2006), exogenous IL-12 fails to protect complete MyD88-deficient mice from *T. gondii* (Hou et al., 2011; Pifer et al., 2011). In this study we established that IL-1R-mediated MyD88 activation cooperates with TNF to regulate IL-12-independent IFN-γ.

Our results suggest that neutrophils are the early cellular source of IL-12-independent IFN-γ. The proposed model suggests that IL-1β and TNF produced by resident macrophages and circulating pro-inflammatory monocytes at the site of the infection regulate IFN-γ production by neutrophils. While neutrophil IFN-γ alone is not sufficient for complete host protection, it significantly reduces the pathogen loads and extends the survival of mice, probably because the response is rapid and robust. The importance of this myeloid-derived IFN-γ is supported by several clinical observations that human neutrophils produce IFN-γ (Ethuin et al., 2004; Yeaman et al., 1998). This is particularly relevant to human toxoplasmosis, since humans lack functional TLR11 and TLR12 (Roach et al., 2005), and the host resistance to the parasite is mediated by unknown mechanisms responsible for IFN-γ-mediated parasite killing. Taking into account the dominance of neutrophils among all immune cells, it is highly possible that in addition to direct pathogen elimination, IFN-γ

produced by neutrophils may have broad immunoregulatory effects on establishing type I immunity by suppressing IL-4 secretion by CD4 T cells (Wurtz et al., 2004) and influencing the isotypes of immunoglobulin secreted by B cells during the adaptive immune response (Coffman and Carty, 1986; Vitetta et al., 1985). Our results have unveiled a new arm in TLR-independent innate immunity and provide an explanation for IFN-γ-dependent host resistance to intracellular pathogens in the absence of NK and T cells.

Fig. 3-1

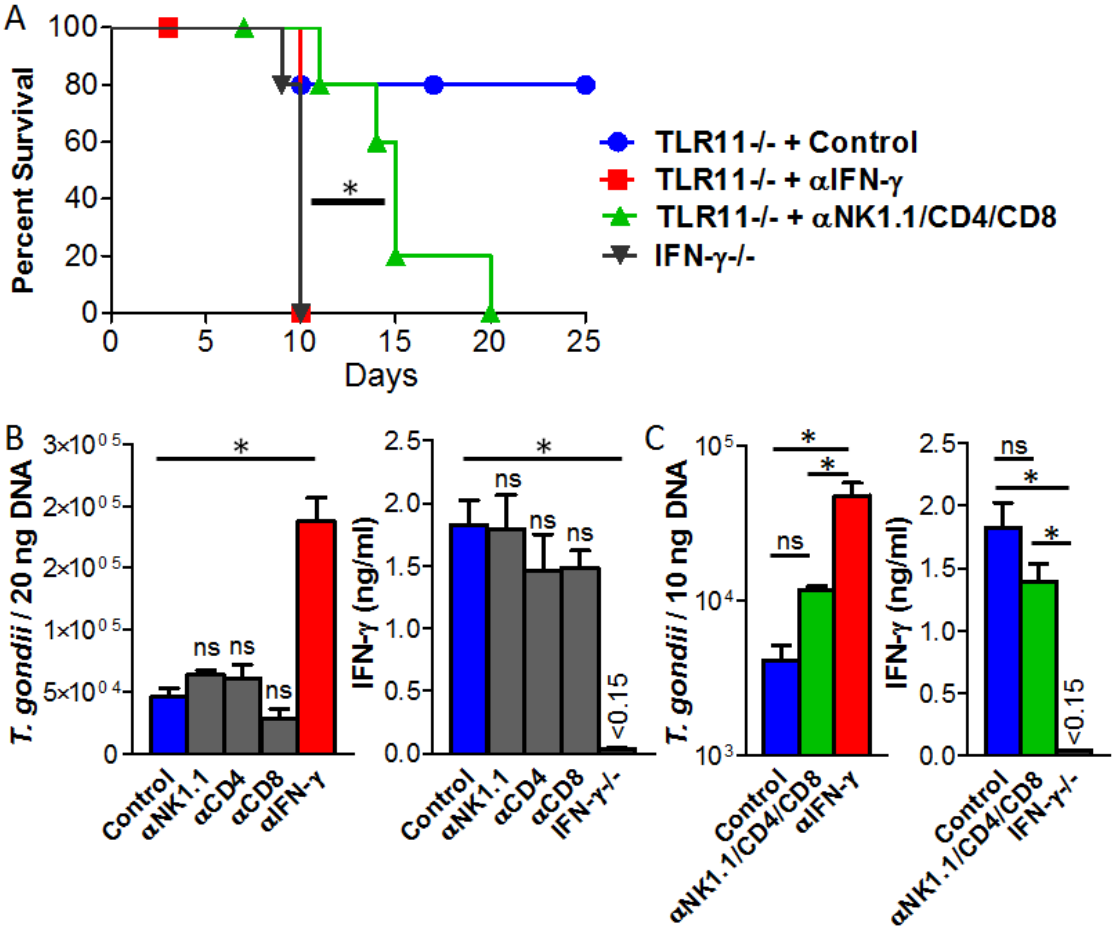


Fig. 3-1: Survival of TLR11-deficient mice during acute toxoplasmosis depends on IFN-γ but not NK or T cells.

TLR11-/- mice were infected intraperitoneally (i.p.) with 20 cysts of the ME49 strain of *T. gondii* and (A) were additionally treated with IFN-γ blocking antibody (red), αNK1.1, αCD4 and αCD8 antibodies to simultaneously deplete NK and T cells (green), or vehicle control (blue). The survival of the TLR11-/- mice was compared to similarly infected IFN-γ-/- mice (black). $p \leq 0.009$. (B) Infected TLR11-/- mice were treated with IFN-γ-blocking antibody (red), or αNK1.1, αCD4, or αCD8 antibodies to individually deplete NK and CD4 or CD8 T cells respectively, or vehicle control (blue). *T. gondii* load and IFN-γ production in the peritoneal cavity of infected mice were analyzed 5 days after infection by quantitative RT-PCR and ELISA respectively. (C) *T. gondii* load and IFN-γ production in the peritoneal cavity of infected mice were analyzed on day 5 post-infection by quantitative RT-PCR and ELISA respectively. The data shown are one of three experiments, each involving 5 mice per group, 'ns' is not significant, and error bars shown are the means ± s.e.m.

Fig. 3-2

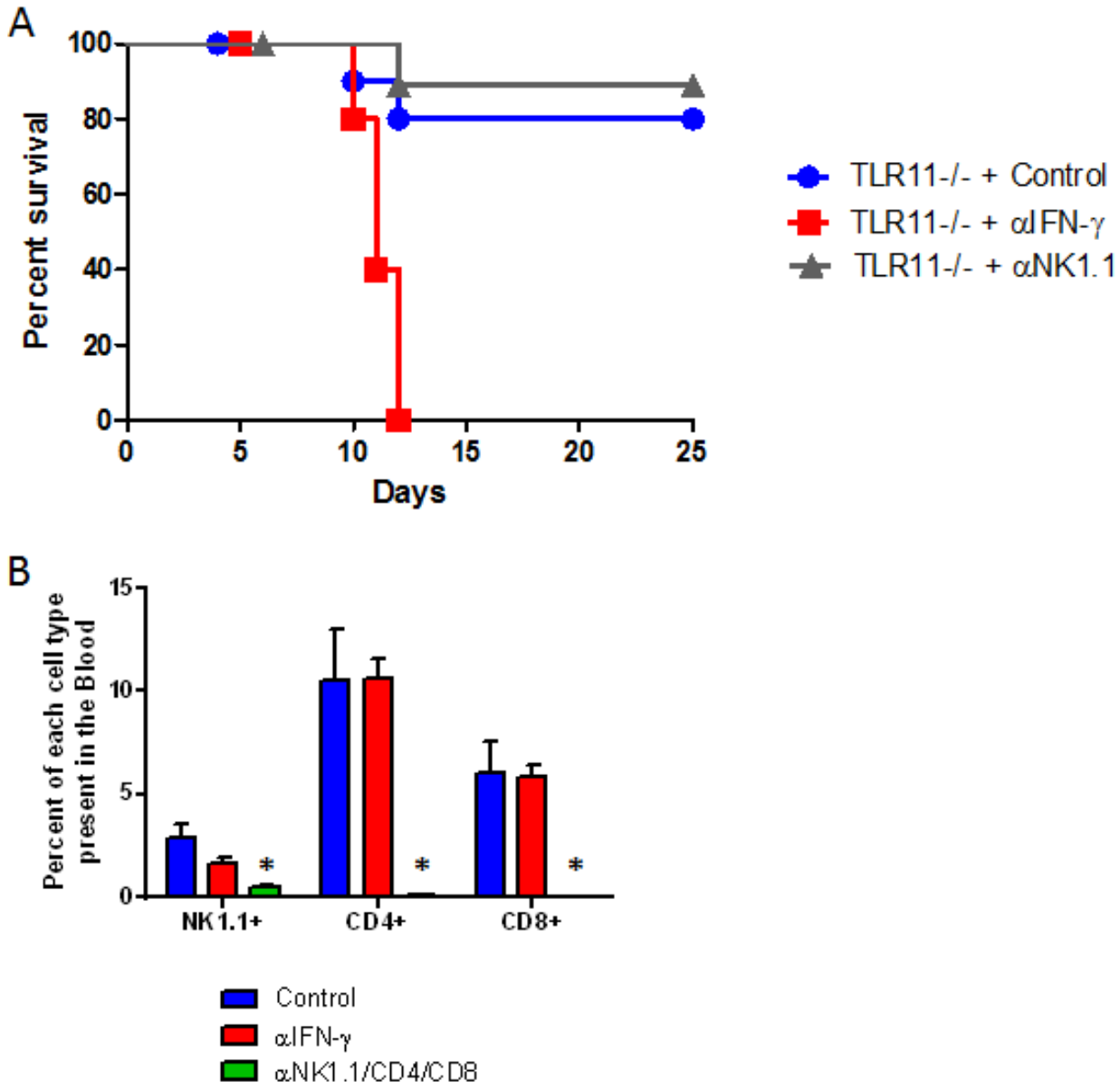


Fig. 3-2: Simultaneous depletion of NK and T cells was effective in *T. gondii*-infected TLR11−/− mice.
TLR11−/− mice were infected intraperitoneally (i.p.) with 20 cysts of the ME49 strain of T. gondii and were additionally treated with IFN-γ blocking antibody (red), αNK1.1 antibody (gray), or vehicle control (blue) and monitored for survival (A). (B) TLR11−/− mice were infected i.p. with 20 cysts of the ME49 strain of T. gondii and were additionally treated with IFN-γ–blocking (red), αNK1.1, αCD4, and αCD8 antibodies to simultaneously deplete NK and T cells (green), or vehicle control (blue). The efficacy of depletions during survival data in (Fig. 3-1A) as assessed by the relative (percentage) quantification of NKs and T cells present in the blood. The data shown are one of three experiments each involving three to five mice per group, and error bars shown are the means ± SEM and $P \leq 0.05$.

Fig. 3-3

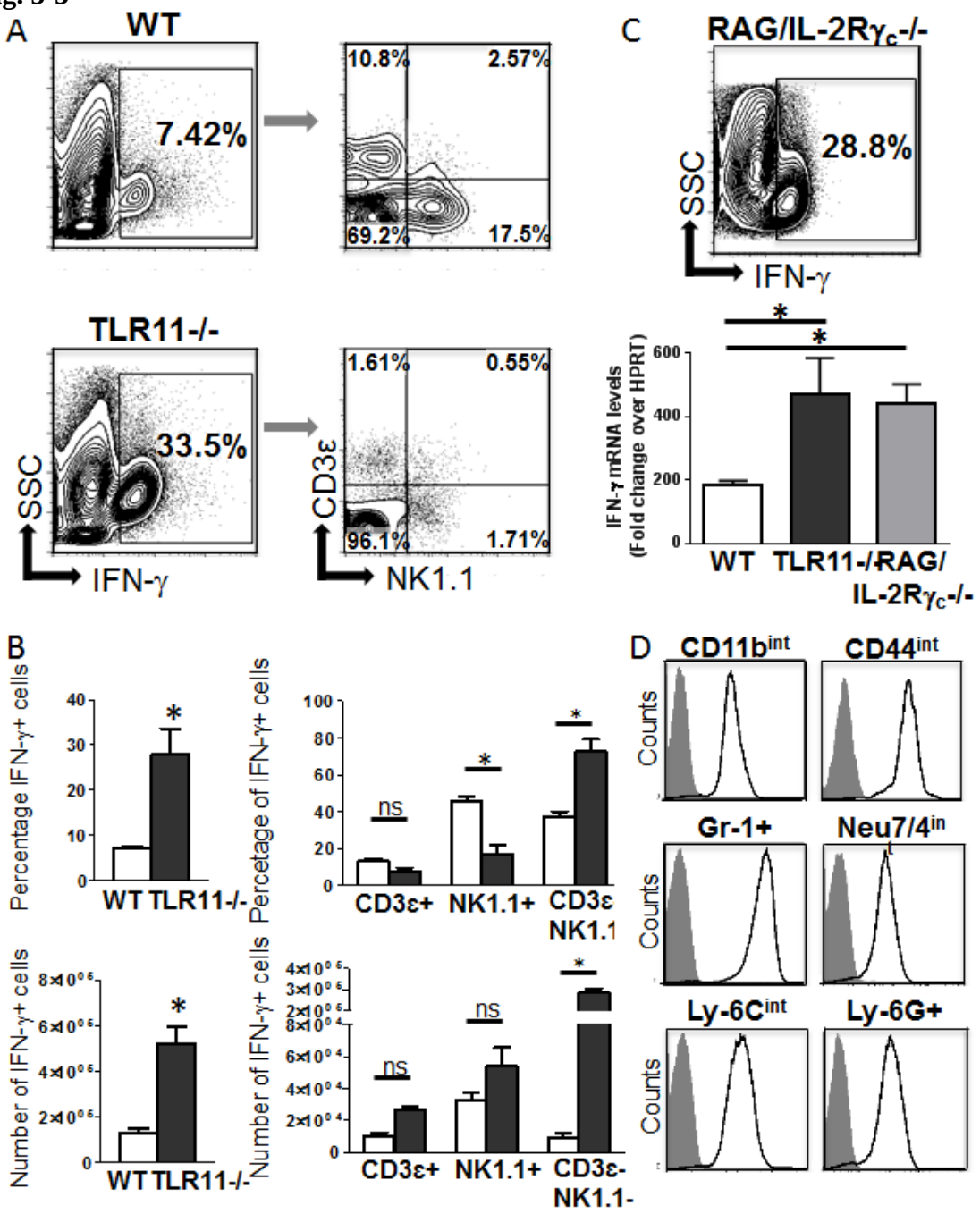


Fig. 3-3: IFN-γ production by non-NK non-T cells in *T. gondii* infected mice.
(A) WT and TLR11-/- mice were infected i.p. with *T. gondii* and IFN-γ-positive cells were identified in the peritoneal cavity of infected mice by gating on live IFN-γ+ cells on day 5 post-infection. IFN-γ-positive cells were further differentiated by plotting NK1.1 vs. CD3ε as markers for NK and T cells respectively. (B) Relative (top) and absolute quantification (bottom) of total IFN-γ-positive cells (left) and additionally broken down by cell types (right) in WT (white) and TLR11-/- (black) mice. (C) IFN-γ-positive cells were identified in the peritoneal cavity of infected RAG/IL-2Rγ_c-/- mice by gating on live IFN-γ-positive cells (top) and total IFN-γ expression in infected WT, TLR11-/- and RAG/IL-2Rγ_c-/- was measured on day 5 post-infection by RT-PCR. $p \leq 0.04$. (D) Cell surface markers expressed by IFN-γ-positive CD3ε- NK1.1-negative cells isolated from *T. gondii* infected TLR11-/- mice (open histograms) compared to appropriate isotype controls (filled histograms). The data shown are one of four experiments each involving 3-5 mice per group. Error bars shown are the means ± s.e.m.

Fig. 3-4

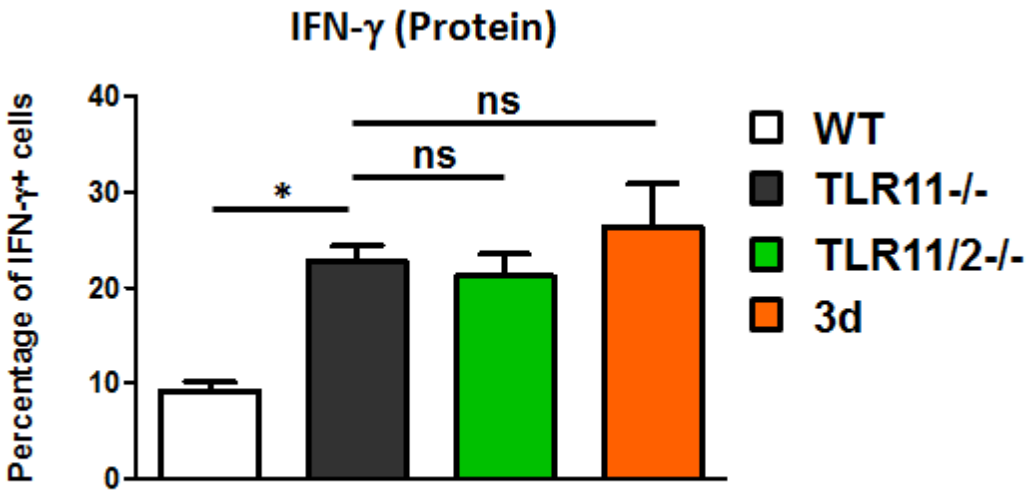


Fig. 3-4: TLR2−/− and 3d−/− mice have IFN-γ+ CD3e− NK1.1− cells.
WT, TLR11−/−, TLR11/2−/−, and 3d mice were infected i.p. with 20 cysts of the ME49 strain *T. gondii*. The relative (percent) quantification of total IFN-γ+ cells were analyzed on day 5 after infection. The data shown are one of three experiments each involving three to five mice per group, and the error bars shown are the means ± SEM and *P ≤ 0.001. ns, not significant.

Fig. 3-5

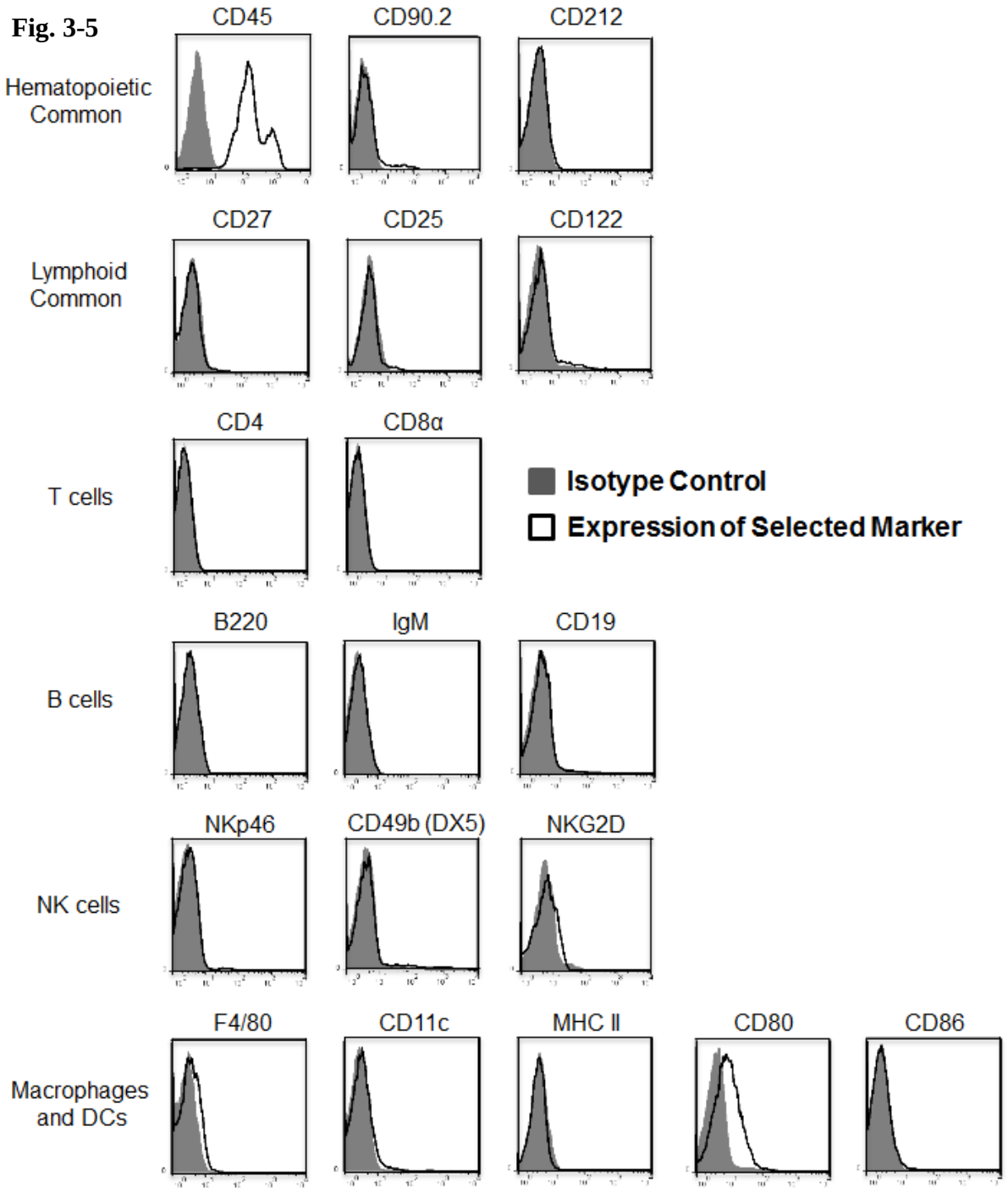


Fig. 3-5: Surface marker expression of IFN-γ+ CD3e− NK1.1− cells.
TLR11−/− mice were infected i.p. with 20 cysts of the ME49 strain of *T. gondii*. Cell surface markers expressed by IFN-γ+ CD3e− NK1.1− cells (open histograms) were compared with appropriate isotype controls (filled histograms) in the peritoneal cavity on day 5 after infection. The data shown are one representative sample of five performed experiments.

Fig. 3-6

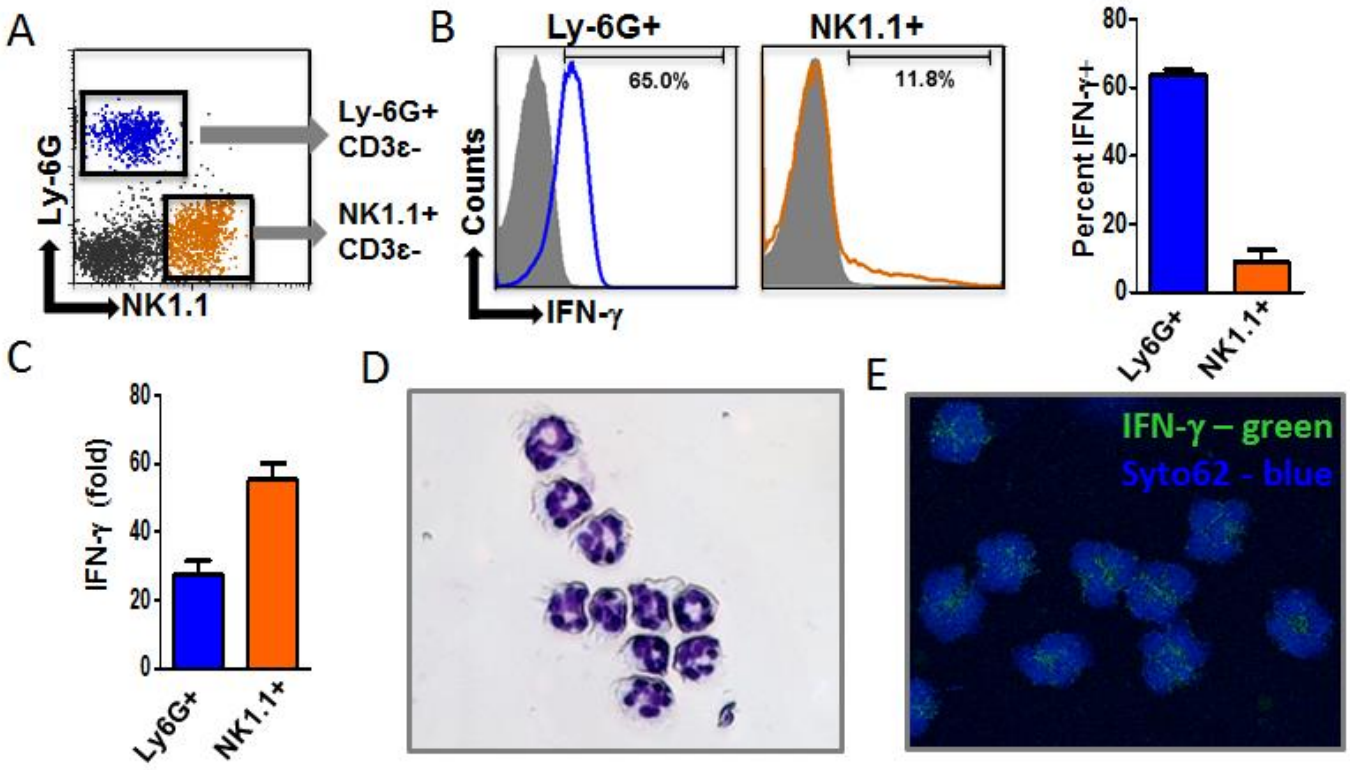


Fig. 3-6: Ly-6G-positive neutrophils produce IFN-γ.
TLR11-/- mice were infected i.p. with *T. gondii* and on day 5 (A) cells were sort-purified as shown, isolating Ly-6G+ CD3ε- and NK1.1+ CD3ε- cells from PEC. (B) Ly-6G+ cells (left) and NK1.1+ cells (middle) were defined by histogram expression of IFN-γ (open histograms) compared to its appropriate isotype control (filled histogram) and percent of IFN-γ quantified in a bar graph (right) (C) Sorted Ly-6G-positive cells contain IFN-γ transcript as quantified by fold change over bulk naïve PEC by qRT-PCR. (D) Sort-purified Ly-6G+NK1.1- CD3ε- PEC were analyzed morphologically by Giemsa staining. (E) Purified Ly-6G-positive cells were analyzed for IFN-γ protein by staining with αIFN-γ antibody and analyzed by confocal microscopy. The data shown are one of three independent experiments and error bars shown are the means ± s.e.m.

Fig. 3-7

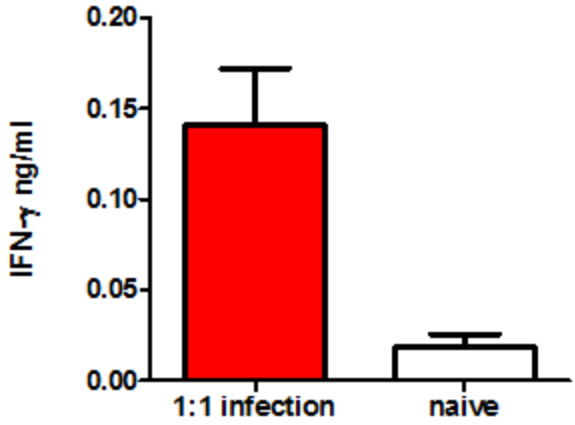


Fig. 3-7: Neutrophils produce IFN-γ in response to *T. gondii* in vitro.
Thioglycollate-elicited neutrophils from TLR11−/− mice were incubated with (red) or without (white) tachyzoites in a 1:1 ratio, and supernatant was analyzed by ELISA 24 h later. The data shown are representative of two performed experiments.

Fig. 3-8

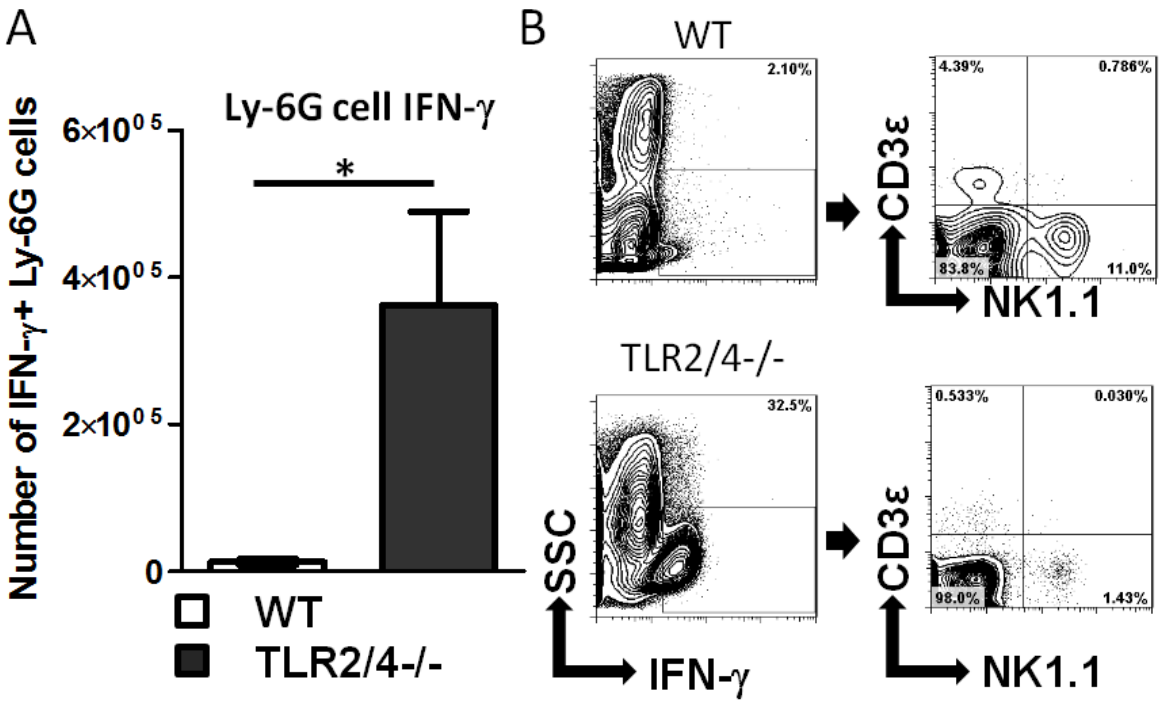


Fig. 3-8: Salmonella infection elicits IFN-γ+ neutrophils.
WT and TLR2/4−/− mice were infected i.p. with 200 cfu of S. typhimurium, and PEC cells were analyzed on day 5. (A) Absolute quantification of IFN-γ+ Ly-6G+ cells present in the peritoneal cavity of WT (white) and TLR2/4−/− (black) mice. (B) Representative flow cytometry data of IFN-γ+ cells in the peritoneal cavity of infected WT and TLR2/4−/− mice. Data were defined by gating on live IFN-γ+ cells on day 5 after infection. IFN-γ+ cells were further differentiated by plotting NK1.1 vs. CD3e as markers for NK and T cells, respectively. The data one of two separate experiments and error bars shown are the means ± SEM and $P \leq 0.03$.

Fig. 3-9

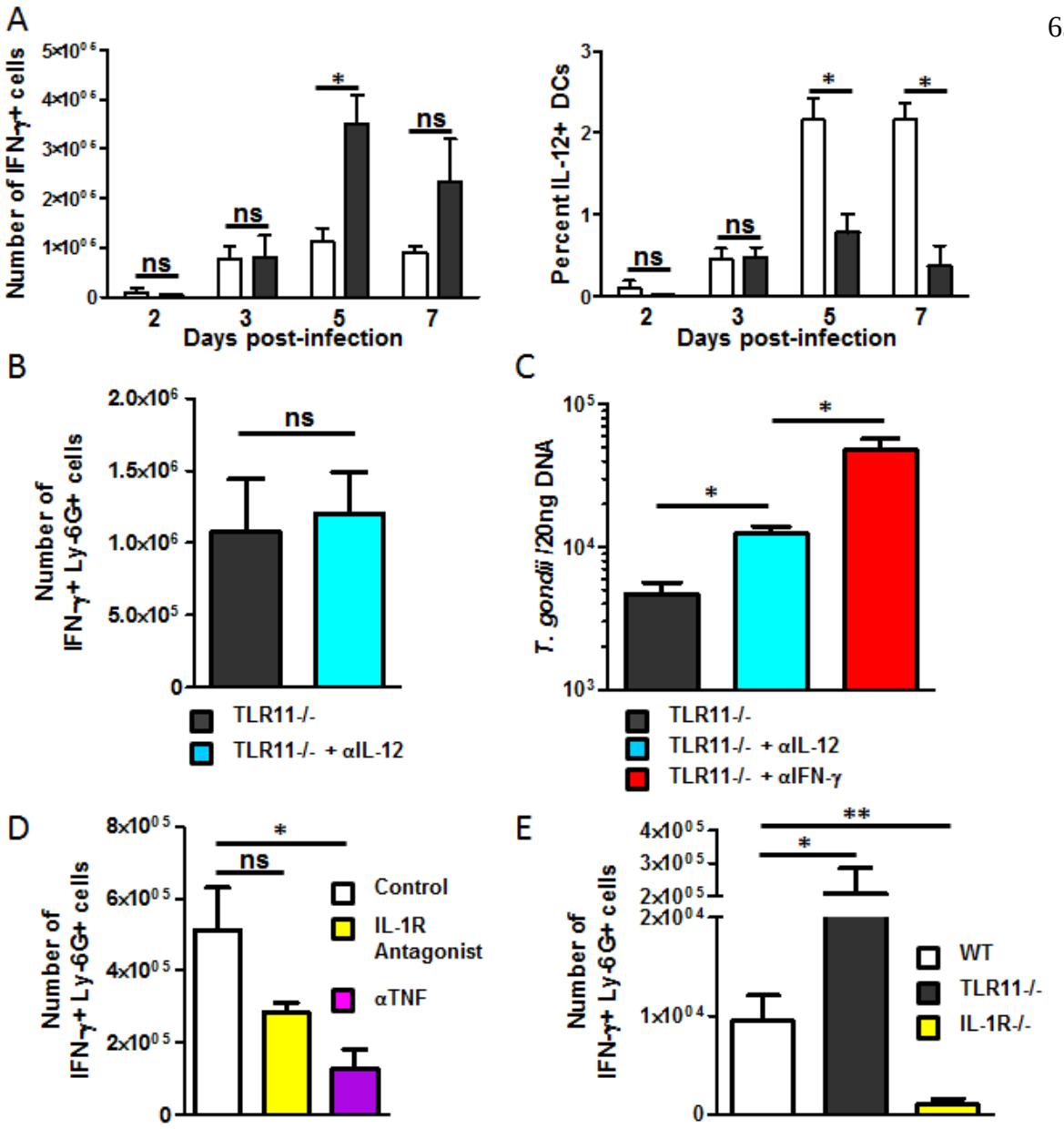


Fig. 3-9: Neutrophils produce IFN-γ in an IL-12-independent, TNF- and IL-1β-dependent manner. Mice were infected i.p. with 20 cysts of the ME49 strain *T. gondii*. (A) PEC were analyzed by flow cytometry on day 2, 3, 5 and 7. Total IFN-γ+ cells were examined by absolute quantification in WT (white) and TLR11-/- mice (black). IL-12 in PEC CD11c+ DCs was examined by relative quantification of IL-12p40 expression from IL-12-reporter mice (Yet40) (white) and Yet40xTLR11-/- mice (black). (B) Absolute quantification of IFN-γ+ Ly-6G+ cells day 5 post-infection from TLR11-/- mice that received control (black) or αIL-12 antibody (light blue). (C) *T. gondii* load in the peritoneal cavity of infected TLR11-/- mice that received control (black), αIL-12 antibody (light blue), or αIFN-γ (red) were analyzed on day 5 post-infection by quantitative RT-PCR. $p \leq 0.004$. (D) Absolute quantification of IFN-γ+ Ly-6G+ cells from infected TLR11-/- mice that received control (white) or IL-1R antagonist (yellow) or αTNF antibody (purple). $p \leq 0.05$. (E) WT (white), TLR11-/- (black), and IL-1R-/- mice (yellow), were infected and the analyzed by absolute quantification of IFN-γ+ Ly-6G+ cells day 5 post-infection. *$p \leq 0.05$ and **$p \leq 0.002$. The data shown are one of three independent experiments with 3-7 mice per group, and error bars shown are the means ± s.e.m.

Fig. 3-10

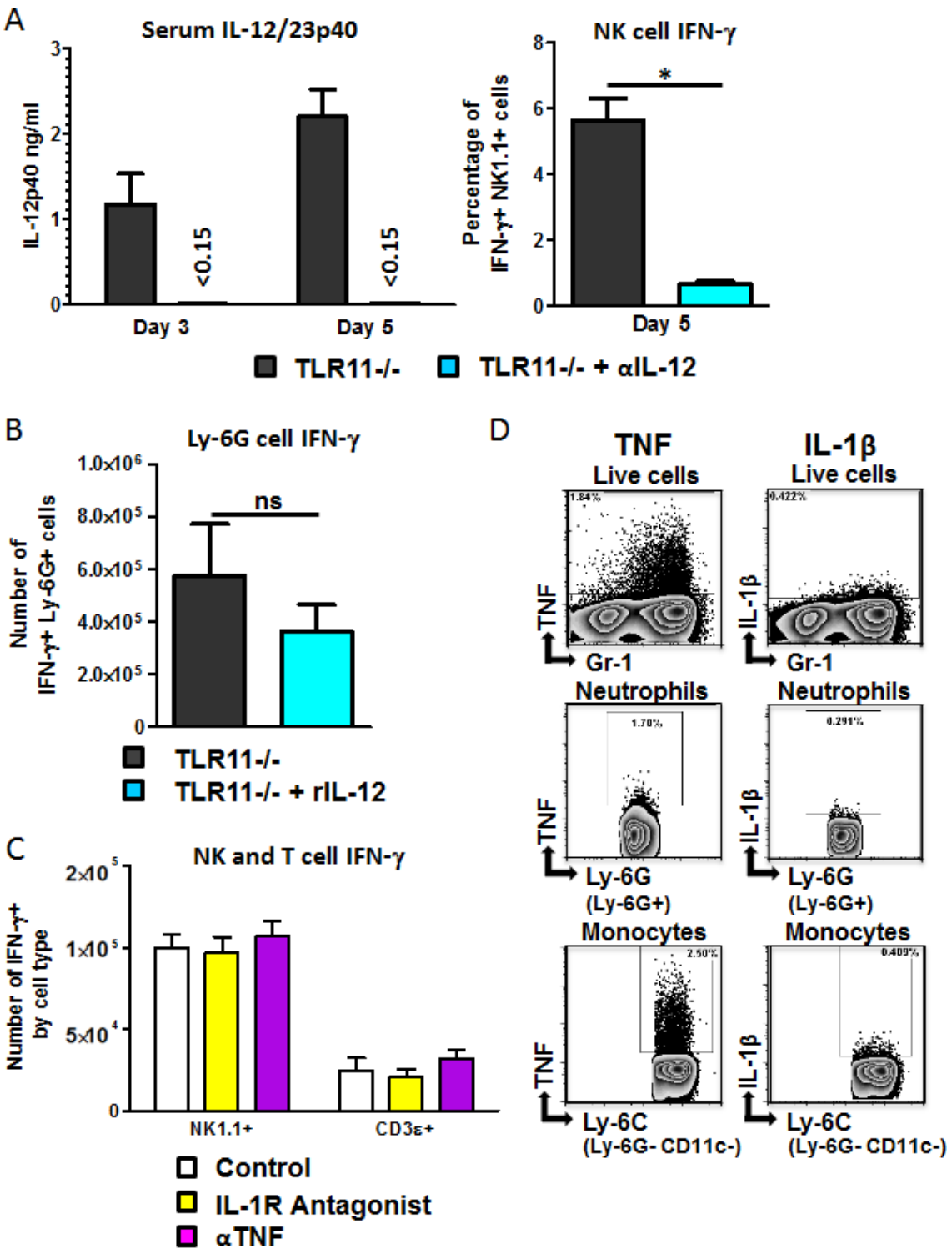


Fig. 3-10: IL-12 does not affect IFN-γ+ neutrophils and TNF and IL-1β do not affect IFN-γ+ NK and T cells. (A) TLR11−/− mice were administered anti–IL-12 (light blue) or control (black) antibody and assessed on day 5 after infection as in Fig. 5B. Serum levels of IL-12p40 were assessed by ELISA. Additionally, the relative (percentage) quantification of IFN-γ+ NK1.1+ cells present was analyzed by flow cytometry. (B) TLR11−/− mice were administered recombinant IL-12 (light blue) or control (black) and assessed on day 5 after infection. The absolute quantification of IFN-γ+ Ly-6G+ cells was analyzed by flow cytometry. (C) TLR11−/− mice were administered IL-1R antagonist (yellow), αTNF antibody (purple), or vehicle control (white), and PEC were analyzed by flow cytometry on day 5. Absolute quantification of IFN-γ+ NK1.1+ and IFN-γ+ CD3e+ cells present from data seen in Fig. 5C. (D) Infected TLR11−/− mice PEC were analyzed by flow cytometry on day 5. The data shown were gated on live CD11c-Ly-6G-Ly6C+ cells - macrophages. The data shown are one of three independent experiments with three to seven mice per group. ns, not significant. Error bars shown are the means ± SEM and $P \leq 0.001$.

Fig. 3-11

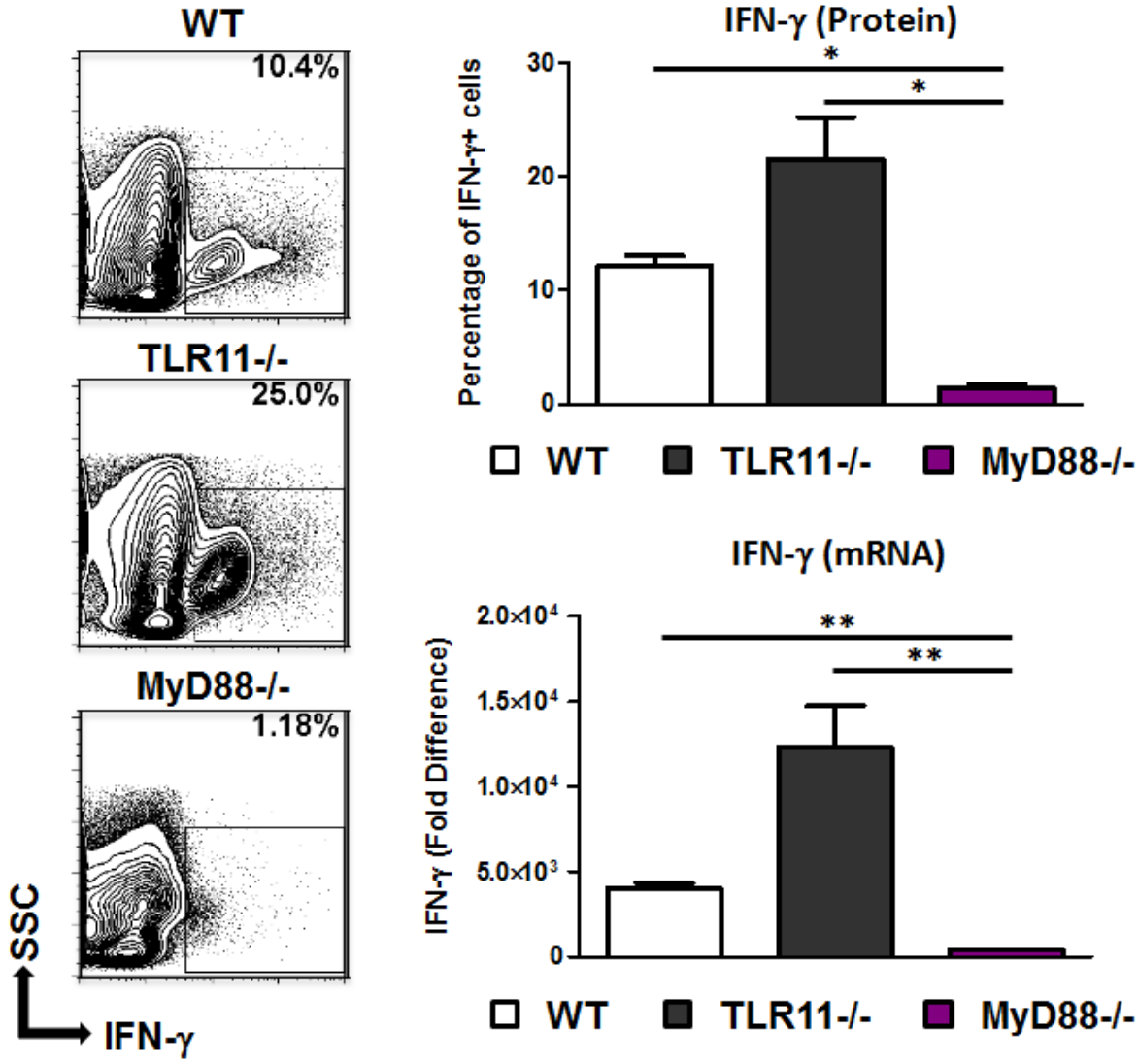


Fig. 3-11: MyD88−/− mice do not have IFN-γ+ neutrophils.
WT, TLR11−/−, and MyD88−/− mice were infected i.p. with 20 cysts of the ME49 strain *T. gondii* and IFN-γ+ cells were identified in peritoneal cavity of infected mice by gating on live IFN-γ+ cells on day 5 after infection (Left). The relative (percent) quantification of total IFN-γ+ cells (Upper) and IFN-γ transcript (Lower) for WT (white), TLR11−/− (dark gray or black), and MyD88−/− (purple) mice were as quantified by flow cytometry and RT-PCR, respectively. The data shown are one of three independent experiments with three mice per group. The error bars shown are the means ± SEM and *P ≤ 0.01, **P ≤ 0.0001.

Fig. 3-12

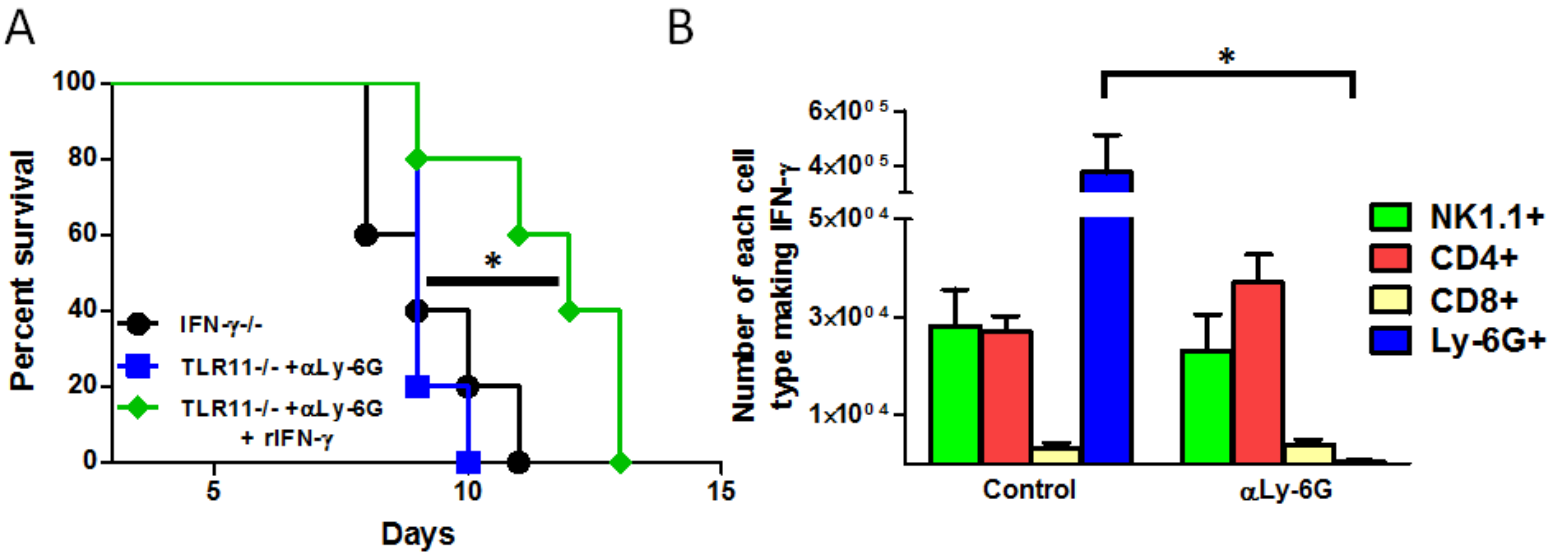


Fig. 3-12: Neutrophils are protective during *T. gondii* infection.
TLR11-/- mice were infected i.p. with *T. gondii* and (A) were additionally treated with αLy-6G antibody (blue) or a combination of αLy-6G antibody and recombinant IFN-γ (rIFN-γ) (green). The survival of the TLR11-/- mice was compared to similarly infected IFN-γ-/- mice (black). $p \leq 0.02$. (B) Absolute quantification of IFN-γ-positive NK, T cells and neutrophils present in the PEC on day 5 post-infection during Ly-6G depletion. The data shown are one of two independent experiments with 5 mice per group. Error bars shown are the means ± s.e.m.

Fig. 3-13

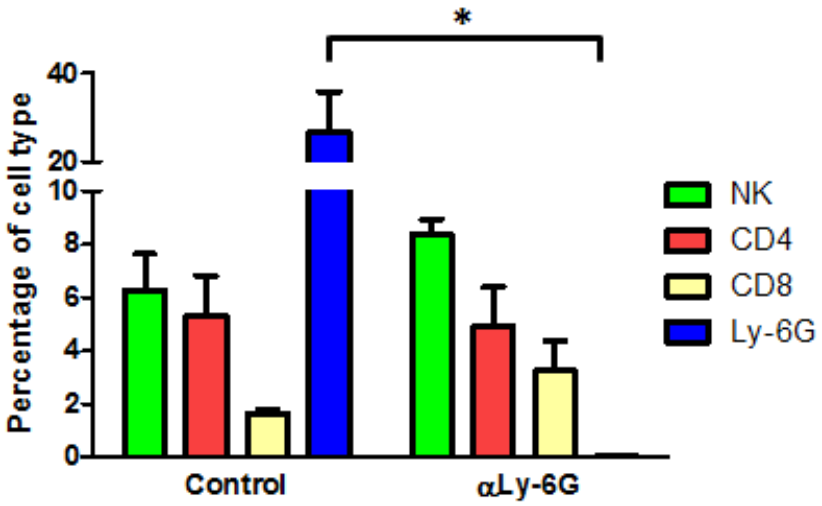


Fig. 3-13: Depletion of Ly-6G+ neutrophils does not eliminate NK or T cells. TLR11−/− mice were infected i.p. with 20 cysts of the ME49 strain of T. gondii, and the efficacy of depletions during survival experiments in Fig. 5A were assessed by the relative (percentage) quantification of NK, T cells, and neutrophils present in peritoneal cavity on day 5. These data shown are one of two independent experiments with five mice per group, and the error bars shown are the means ±SEM and $P \leq 0.04$.

CHAPTER IV

Results Part 2 – Neutrophil-derived IFN-γ is pre-stored during development.

The information in this chapter has not yet been published.

The previous work in chapter III established that neutrophils make IFN-γ independent of TLR ligand recognition (Sturge et al., 2013). While this work pointed towards a role for IL-1β and TNF in observing neutrophil IFN-γ during infection, the mechanisms behind neutrophil-derived IFN-γ has yet to be established. Therefore the aim of my continuing work is to determine when and how neutrophils are making IFN-γ.

Neutrophils make IFN-γ prior to *in vitro* stimulation or infection.

Production of cytokines such as IFN-γ generally occurs in response to stimuli, where the stimulus induces expression or stabilization of the cytokine's transcript and then the protein is produced; this process takes some time to occur and is normally highly regulated to prevent aberrant expression of cytokines which are associated with autoinflammatory and autoimmune diseases. Typically with Th1 cells in culture that will be assessed for cytokine production by flow cytometry, stimulation is given in the presence of GolgiPlug or Brefeldin A protein transport inhibitors. This causes the newly produced proteins to be trapped inside of the cell that made them which can then be observed by appropriate antibody staining and florescent excitation with a flow cytometer. We used these basic techniques to investigate when neutrophils are producing IFN-γ. Since our previous results (Fig. 3-3A) indicated that

wild-type C57BL/6 mice also have IFN-γ-positive neutrophils, we utilized these mice in experiments.

PECs, including neutrophils, isolated from *T. gondii* infected mice were incubated with or without the addition of GolgiPlug and examined for IFN-γ positivity. As expected, the number of IFN-γ-positive NK cells and T cells from infected PECs increases with GolgiPlug incubation (Fig. 4-1). However, here was no change in the levels of IFN-γ observed for neutrophils with or without GolgiPlug incubation (Fig. 4-1). These results indicated that neutrophils were not making any additional IFN-γ during *in vitro* culture incubations. It is possible that *in vitro* culture lacking additional stimuli, were insufficient to trigger additionally cytokine production from neutrophil. Therefore experimental conditions were expanded to include stimulation with PMA and ionomycin – small molecules that trigger non-specific activation of many immune cells – and increasing concentrations of cycloheximide (CHX), a *de novo* protein synthesis inhibitor. T cells from infected mice incubated in culture and stimulated with PMA and ionomycin produced large amounts of IFN-γ by *de novo* protein synthesis, which is inhibited with increasing concentrations of CHX (Fig. 4-2A). However, increasing concentrations of CHX did not inhibit neutrophil IFN-γ in a dose-dependent manner (Fig. 4-2B). Additionally, stimulation with PMA and ionomycin resulted in a consistent decrease in IFN-γ observed in neutrophils regardless of CHX concentration (Fig. 4-2B). Taken together these data are strong evidence that neutrophils are making their IFN-γ before removal from the *T. gondii* infected mice.

Neutrophils make IFN-γ during hematopoiesis.

To address the question of when neutrophils are making IFN- γ, we examined our *in vivo* model. C57BL/6 mice were infected with *T. gondii* and 5 days post-infection PEC, blood, and bone marrow were examined for neutrophils and IFN-γ-positivity. Neutrophils stained positive for IFN-γ in all locations examined (Fig. 4-3A) at roughly the same mean fluorescence intensity (MFI) (Fig. 4-3B). This result is surprising, since on day 5 post-infection with Me49 *T. gondii*, tachyzoites are still mostly confined to the site of infection (in the peritoneal cavity), suggesting that indirect sensing of infection is responsible for their IFN-γ positivity. However, examination of the same locations in naïve C57BL/6 mice also revealed IFN-γ+ neutrophils in the blood and bone marrow but not in the PECs (Fig. 4-3C). The lack of IFN-γ+ neutrophils in the PEC of naïve mice is not surprising, given that neutrophils are not present. Additionally, in the bone marrow there was also an unusual population of Ly-6G-negative IFN-γ+ cells and these cells appeared to be acquiring Ly-6G positivity (Fig. 4-3A,C). Together these data suggest two possibilities: 1. That the naïve mice have a preexisting infection or colonization with a microbial pathogen, or 2. That neutrophils become IFN-γ positive without pathogen stimulation during their development in the bone marrow.

To examine the possibility that the microbiota or their products are involved in neutrophil-derived IFN-γ, we obtained germ-free mice from our scientific collaborator's laboratory, Dr. Lora Hooper. We compared our conventionally raised C57BL/6 mice to the C57BL/6 mice that had been raised entirely germ-free in a sterile bubble. Both C57BL/6

colonies, regardless of colonization status, had numerous IFN-γ-positive neutrophils in their bone marrow (Fig. 4-4). Additionally, germ-free mice had the same IFN-γ-positive Ly-6G-negative cells, some of which appear to become Ly-6G positive (Fig 4-4), supporting the hypothesis that neutrophils become IFN-γ-positive during their development regardless of microbial stimuli.

To examine neutrophil development more closely, we adopted a way to examine the developing immature neutrophils by flow cytometry, rather than by morphological examination of their nucleus (Fig. 4-5A). The developed gating strategy excludes other potentially IFN-γ-positive cells, such as NK, T, and B cells as well as earlier hematopoietic cells (Fig 4-5A). The group who reported this strategy demonstrated successful separation of each immature neutrophil stage by morphological nuclear examination and expression patterns of known granule proteins in their sorted cells (Satake et al., 2012). To confirm our successful application of this gating strategy, immature neutrophil populations were sorted and examined for their expression of several known granule proteins by qRT-PCR. Sorted immature neutrophil populations expressed the appropriate relative amounts of mRNA for each protein examined – proteinase 3, a primary granule protein, expressed mostly in early promyelocytes, lactoferrin, a secondary granule protein, expressed mostly in metamyelocytes, and gelatinase, a tertiary granule protein, expressed mostly in late band cells (Fig 4-5B). Examination of the immature neutrophil stages for IFN-γ-positivity by flow cytometry revealed that neutrophils become IFN-γ-positive mostly in the promyelocyte stage, exhibiting a roughly equal split of IFN-γ-positive and negative cells (Fig. 4-5B). Subsequent immature neutrophil stages were uniformly IFN-γ-positive (Fig. 4-5B). However a small

amount of IFN-γ-positivity was observed in the myeloblast stage (Fig. 4-5B) which could represent the true beginning of IFN-γ transcript translation or could be an artifact of applying a defined gating strategy to a non-discrete population continuum.

This low level of IFN-γ-positivity in immature neutrophils leads to the question of whether precursors to the neutrophil linage are making IFN-γ. To examine earlier hematopoietic cells, we adopted a previously utilized flow cytometric gating strategy established (Buechler et al., 2013). This gating strategy allows for the identification of the relatively scarce hematopoietic cell groups, Linage negative sca-1+ c-kit+ (LSKs), as well as subsequent multipotent cells such as common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs) (Fig. 4-6A). Examination of the rare LSK cells, which included the long-term and short-term HSC populations (LT-HSC and ST-HSC) as well as multi-potent progenitors (MMPs), showed that they did not stain positive for IFN-γ by flow cytomtry (Fig 4-6B). Additionally, the immediate precursor to all granulocytes, the GMPs, also did not stain positive for IFN-γ (Fig. 4-6B). These data suggest that this early IFN-γ from neutrophils is restricted to the neutrophil-specific linage development.

Neutrophils IFN-γ is contained in granules.

These data indicate that neutrophil-derived IFN-γ is first seen during immature neutrophil development at the promyelocyte stage (Fig 4-5B) and morphologically this IFN-γ was observed in discrete puncta (Fig 3-6E), therefore we hypothesized that neutrophil IFN-γ could be stored in granules. It is thought that developing immature neutrophils begin packaging granules as they transition from myeloblast to promyelocytes and that granule

formation continues through the metamyelocyte stage (Greer, 2013). To examine the localization of IFN-γ more closely, immunofluorescence was used to visualize the location of IFN-γ in comparison to other known granule proteins. C57BL/6 bone marrow Ly-6G+ neutrophils and immature neutrophils were magnetic bead sorted, centrifuged onto slides, and stained with a combination of IFN-γ and myeloperoxidase, lactoferrin, or gelatinase which are considered markers for primary, secondary, or tertiary granules respectively (Fig 4-7A-C). Visualization of IFN-γ and granule proteins revealed overlap between IFN-γ and myeloperoxidase indicated in yellow (Fig. 4-7A overlay). Lactoferrin and gelatinase had distinct localization that did not appear to overlap with IFN-γ (Fig. 4-7B-C overlays). Of note in (Fig. 4-7C), there is an immature neutrophil, likely a myelocyte, which stains IFN-γ+ but is not yet positive for gelatinase, further supporting the observation that IFN-γ is made relatively early in neutrophil development. Quantitative Colocalization Analysis (QCA) analysis of IFN-γ and select granule proteins statistically indicates that IFN-γ colocalizes with myeloperoxidase but not gelatinase, as indicated by a Mander's coefficient of >0.5 for green to red values (Table 4-1). These data indicate that IFN-γ is in large part contained within the primary myeloperoxidase containing granules. However Mander's coefficients, which can be interpreted as percentage overlap, also indicate a roughly 17% average overlap between IFN-γ and gelatinase, which may be explained by the fact that defined granule proteins contents are more promiscuous than was first appreciated since they are made in a continuous manner during development (Borregaard, 2010).

If neutrophil IFN-γ is indeed contained in primary granules as these data suggest, then stimuli that promotes degranulation should result in degranulation of IFN-γ. Neutrophils degranulate in response to a number of stimuli including bacterial ligands such as TLR ligands or fMLP (Amulic et al., 2012). The cell-intrinsic events that lead to degranulation require increases in intracellular calcium (Lacy, 2006). The propensity at which a particular granule type can be exocytosed is inversely correlated with the order in which they were produced, with primary granules being the hardest to mobilize and secretory granules being the easiest (Amulic et al., 2012; Lacy, 2006). Therefore, in order to examine if degranulation stimuli would result in IFN-γ exocytosis, bone marrow from C57BL/6 mice were harvested and exposed to stimuli known to cause degranulation. Stimulation for one hour with fMLP and LPS resulted in a slight loss of IFN-γ signal compared to media only controls (Fig. 4-8A.). Additionally a slight loss of myeloperoxidase signal was observed with fMLP and LPS stimulation (Fig. 4-8B) while a large loss of the tertiary granule protein gelatinase signal was seen (Fig. 4-8B) which is consistent with the knowledge that tertiary granules require less stimuli for degranulation than primary granules. Stimulation with fMLP, LPS, PMA, and ionomycin together resulted in a nearly complete loss of IFN-γ signal and myeloperoxidase signal by flow cytometry (Fig. 4-8A-B) and complete loss of gelatinase signal (Fig. 4-8B). Furthermore, stimulation with fMLP, LPS, PMA, and ionomycin resulted in a small but detectable amount of IFN-γ released into the culture supernatant (Fig. 4-8C) which was statistically significant compared to media alone or fMLP and LPS stimulation. Taken together these data indicate that neutrophil-derived IFN-γ is largely contained in primary granules (also called azurophilic granules).

Neutrophil-derived IFN-γ is not regulated by T-bet.

IFN-γ in T cells is known to be largely regulated by the transcription factor T-bet; for example, T-bet is absolutely essential for CD4+ T cell IFN-γ production. However in other cells types additional transcription factors are also involved in IFN-γ production including several STAT family members and the transcription factor Eomes, which is in the same family as T-bet (Szabo et al., 2002; Thierfelder et al., 1996). We next investigated if these transcription factors were involved in IFN-γ production by neutrophils. Initial experiments were done to compare C57BL/6 and T-bet-/- mice examining their blood for Ly-6G+ neutrophils and IFN-γ. Regardless of genotype, neutrophils were IFN-γ+ as compared to isotype control (Fig. 4-9A), indicating that lack of T-bet did not affect neutrophil-derived IFN-γ.

Additional experiments were performed to examine other major transcription factors as possible candidates for neutrophil-derived IFN-γ regulation. We examined immature neutrophil developmental stages for expression of transcription factors with the assumption that any transcription factors regulating IFN-γ should be highly expressed in promyelocytes (Fig. 4-5) and likely decrease as neutrophils mature (Fig. 4-8). Relative expression of T-bet, STAT1, STAT3, and STAT4 showed various degrees of increasing expression during neutrophil linage commitment while expression of ID2, an essential NK cell development transcription factor, remained relatively unchanged (Fig. 4-9B). The relative expression of Eomes is low but displays expression in the promyelocyte stage with a relative decrease in later developmental stages (Fig. 4-9B). While the relative expression of Eomes is purely a

correlation with IFN-γ expression, these data suggests that Eomes is a potential transcription factor to investigate further.

Discussion of the Results: Part 2.

To the best of our knowledge, these data are the first to definitively show that IFN-γ is produced in immature neutrophils. These results explain a number of individual reports in the literature which have observed IFN-γ in neutrophils during various infection models including *T. gondii, Salmonella, Listeria, Nocardia, Streptococcus,* and *M. tuberculosis* (Bold and Ernst, 2012; Ellis and Beaman, 2002; Kirby et al., 2002; Matsumura et al., 2012; Sturge et al., 2013; Yin and Ferguson, 2009). One reason why the concept of neutrophil IFN-γ has not been well accepted in the past is due to the relatively low cytokine levels observed by flow cytometry in comparison to the 'traditional' IFN-γ producing cells (such as NK cells and CD4 T cells) (Fig. 3-2B, 3-3, and 4-3). Indeed, rough estimates from our ELISA data (Fig. 4-8) indicate that neutrophils contain 21pg of IFN-γ/10^6 cells or that each neutrophil has approximately seventy molecules of IFN-γ that could be degranulated (calculation not shown). Moreover those that study IFN-γ, especially in T cells, know that the typical pathway of cytokine secretion for IFN-γ is constitutive exocytosis. Constitutive exocytosis is dependent on receptor-mediated stimulation followed by transcription and *de novo* synthesis of cytokines for export (Stanley and Lacy, 2010). In some cases, the contribution of neutrophil-derived IFN-γ was dismissed entirely as non-specific binding, in part because both naïve and infected neutrophils stained IFN-γ-positive (Bold and Ernst,

2012). Our data indicate that naïve neutrophils contain pre-formed IFN-γ in their granules which are mobilized upon a receptor-ligand signal, making this a form of regulated exocytosis. Interestingly, there is precedence for pre-formed cytokines in neutrophils – a previous report indicated that TGFα is stored in tertiary granules at low levels of 2.5pg of TGFα/10^6 cells (Calafat et al., 1997). There is also some evidence that neutrophils may pre-store IL-12, although we have not been able to confirm this in our lab, and the specific location of stored IL-12 was not addressed in their investigations (Bliss et al., 2000). It is logical that IFN-γ, which is a potent inflammatory cytokine especially compared to TGFα, would be stored in earlier granules which require more stimulation to fully degranulate. Another possibility to consider is that the uncontrolled cell lysis of neutrophils due to a pathogen can release granule contents, now including IFN-γ, which would serve to alert nearby cells to an infection.

The implications of pre-stored IFN-γ in neutrophils are enormous; the majority of bacterial and intracellular parasite infections result in neutrophil recruitment and neutrophils are the most abundant first responder during these infections. Moreover, we know that IFN-γ can feed back to reinforce IL-12 responses and innate immunity. Specifically in the context of *T. gondii* infection, mice blocked for IFN-γ or depleted of NK cells (early producers of IFN-γ) demonstrate impaired maturation of DCs and inflammatory monocytes, increased numbers of resident macrophages, and decreased IL-12 production by DCs (Goldszmid et al., 2012). On the molecular level it has been shown that IFN-γ enhances the production and transcription of IL-12 via induction of IRF8, a major transcriptional factor regulating IL-12p40 and IL-12p35 transcription (Ma et al., 1996; Raetz et al., 2013b). The ability of

monocytes to respond to IFN-γ priming is necessary for their increased activation and IL-12 production by monocyte-derived DCs in response to *T. gondii* infection (Goldszmid et al., 2012; Raetz et al., 2013b). This response is so important that *T. gondii* has a mechanism to impair chromatin remodeling at STAT1-regulated promoters thereby impairing infected macrophages responses to IFN-γ priming (Lang et al., 2012). Additionally, our lab has found that depletion of Ly-6G+ neutrophils during *T.gondii* infection in TLR11-/- mice results in decreased CD4+ T cell IFN-γ responses on day 7 (data not shown), again implicating neutrophils and potentially their early IFN-γ in the generation of later essential Th1 responses. These data compel us to think differently about the dogma of linear Th1 immunity; Th1 immunity should be regarded as a circular system with pathogen stimuli leading independently to IL-12 and IFN-γ, IL-12 leading to IFN-γ, and IFN-γ leading to IL-12 (Fig. 4- 10).

The mechanisms specifically driving expression of neutrophil IFN-γ during development are still unknown. This question may be technically difficult to address, especially if transcription factors essential for host development or neutrophil-specific linage development are required for IFN-γ expression; this is potentially the case for further Eomes investigations, as complete knockout of the gene is embryonically lethal. Future experiments to continue this work will need examine potential transcription factor regulation of neutrophil-derived IFN-γ utilizing RNA-seq and explore epigenetic changes to the DNA and histones around the IFN-γ loci which allow for IFN-γ expression. We anticipate that the IFN-γ locus in neutrophils will be found to be more ‘open’, much like an NK cell (Tato et al., 2004), during early neutrophil development, but that it likely acquires some inhibitory

histone marks by the time neutrophils are mature to prevent constitutive expression, much like other granule proteins (Ciavatta et al., 2010).

In summary, neutrophil-derived IFN-γ is produced in the promyelocyte stage and prestored in granules for later release. These data and others changes the way we think about neutrophils in the disease state – in addition to being potent antimicrobial effectors they are an important source of immediate cytokines to initiate immune responses.

Fig. 4-1

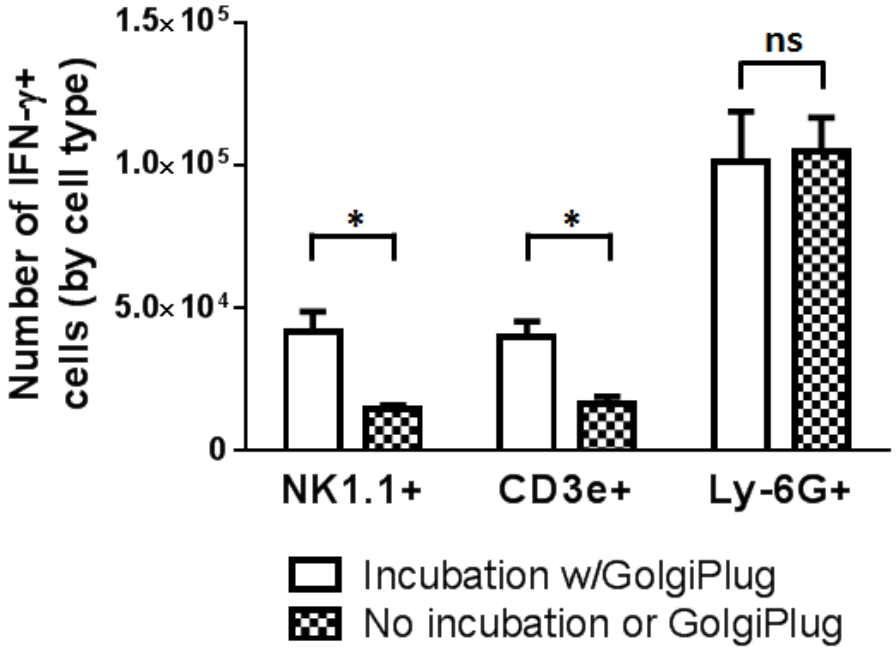


Fig. 4-1: Incubation with GolgiPlug is not required for Neutrophil-derived IFN-γ. B6 mice were infected i.p. with *T. gondii* and PECs were harvested day 5 post-infection. PECs were incubated at 37C for 5 hours in media with GolgiPlug (Brefeldin A) (white) or placed in media on ice for 5 hours (checkered). Cells were assessed by flow cytometry using the markers NK1.1 (NK cells), CD3e (T cells), or Ly-6G (Neutrophils) and IFN-γ positivity.

Fig. 4-2

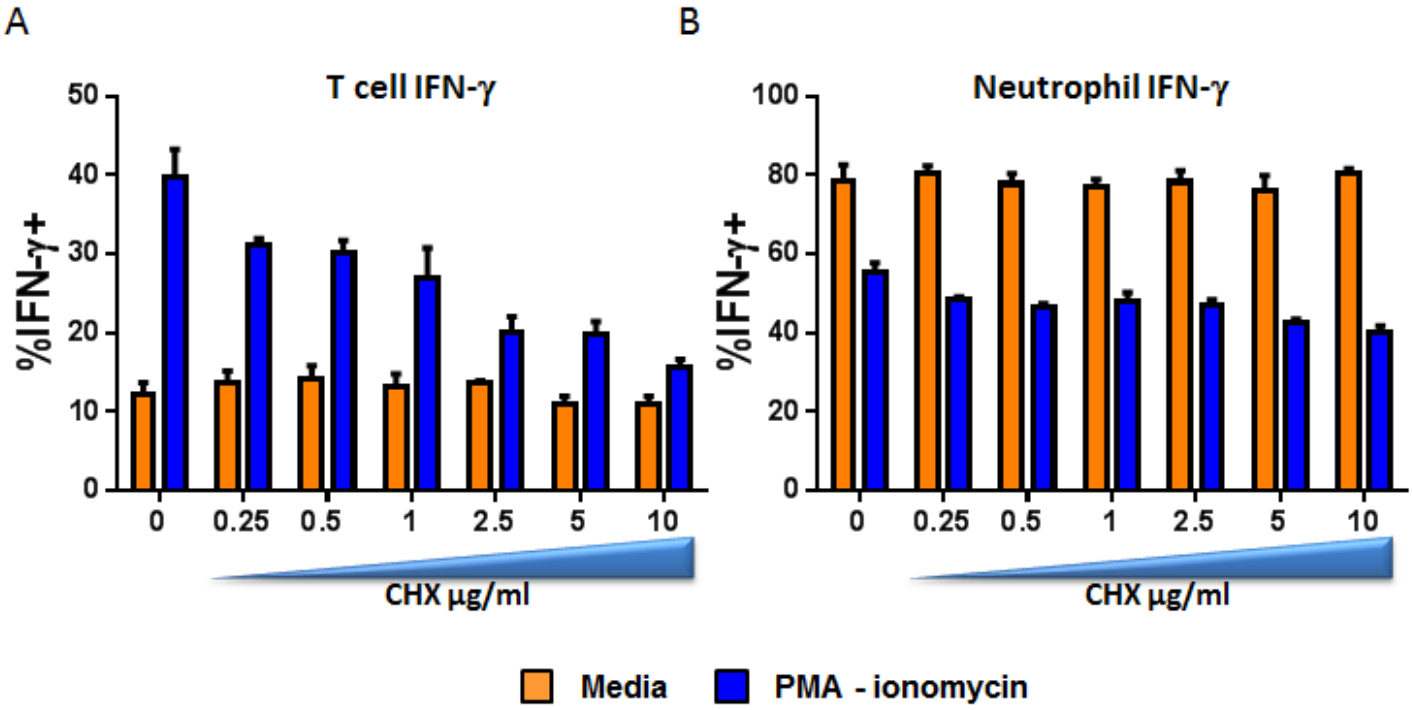


Fig. 4-2: IFN-γ seen in mature neutrophils is not made by de novo protein synthesis. B6 mice were infected i.p. with *T. gondii* and PECs were harvested day 5 post-infection. PECs were incubated for 5 hours in media with GolgiPlug with or without stimulation (PMA-ionomycin) and with increasing concentrations of Cycloheximide (CHX), a de novo protein synthesis inhibitor. The percentage of IFN-γ positive (A) T cells or (B) Neutrophils were assessed by flow cytometry.

Fig. 4-3

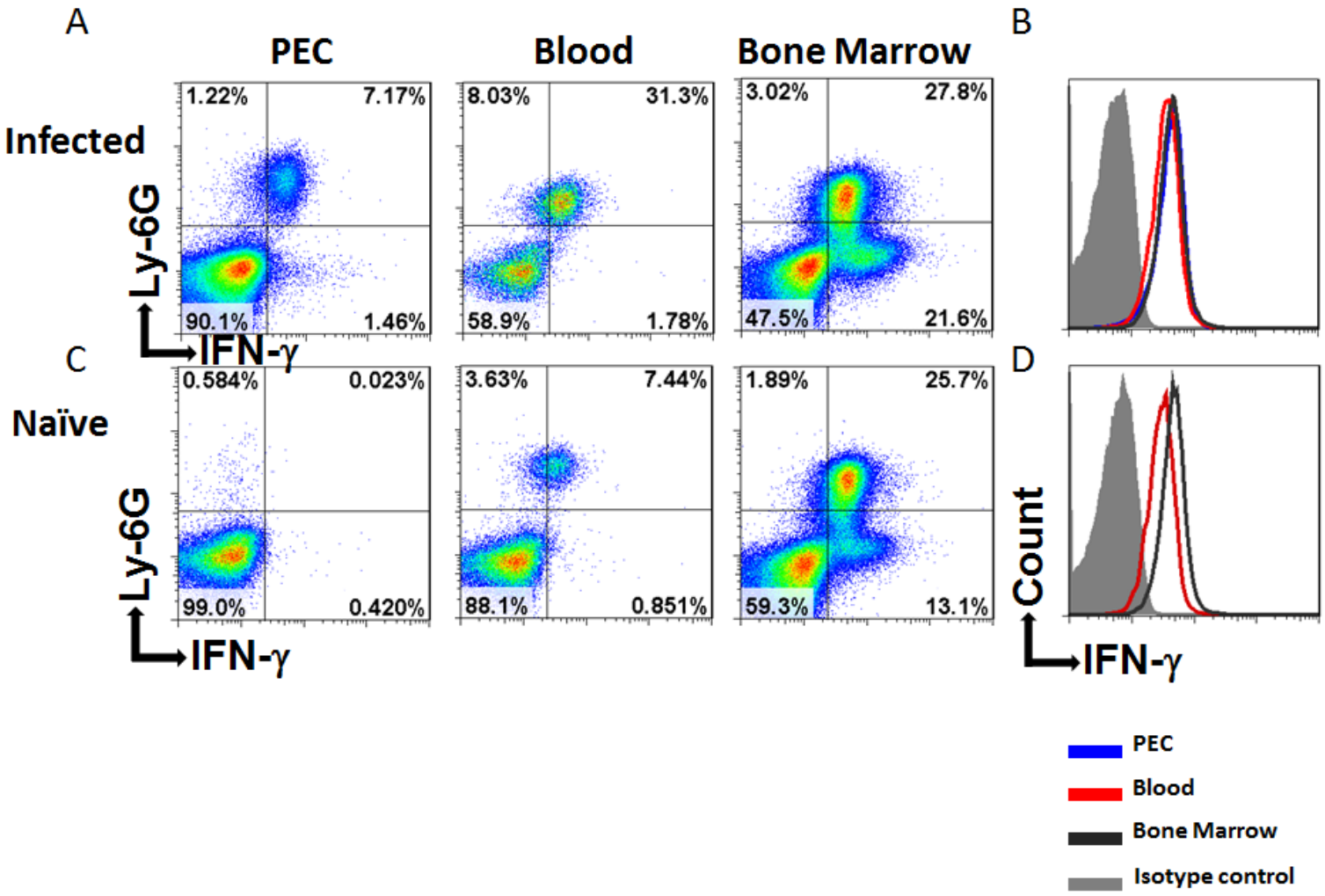


Fig. 4-3: Neutrophils are IFN-γ positive at other anatomical locations in the mouse regardless of infection status.
(A-B) B6 mice were infected i.p. with *T. gondii* or were kept naïve (C-D) and PECs were harvested day 5 post-infection. PEC, Blood, and Bone Marrow were examined (A-C) for the presence of Ly-6G+ neutrophils and IFN-γ positivity. (B-D) are gated on neutrophils and are historgrams of their MFI of IFN-γ, PEC (blue), Blood (red), Bone Marrow (black), and isotype control of Bone Marrow samples (grey). Note there is no PEC (blue) in (D) due to a lack of neutrophils to gate on in naïve PEC.

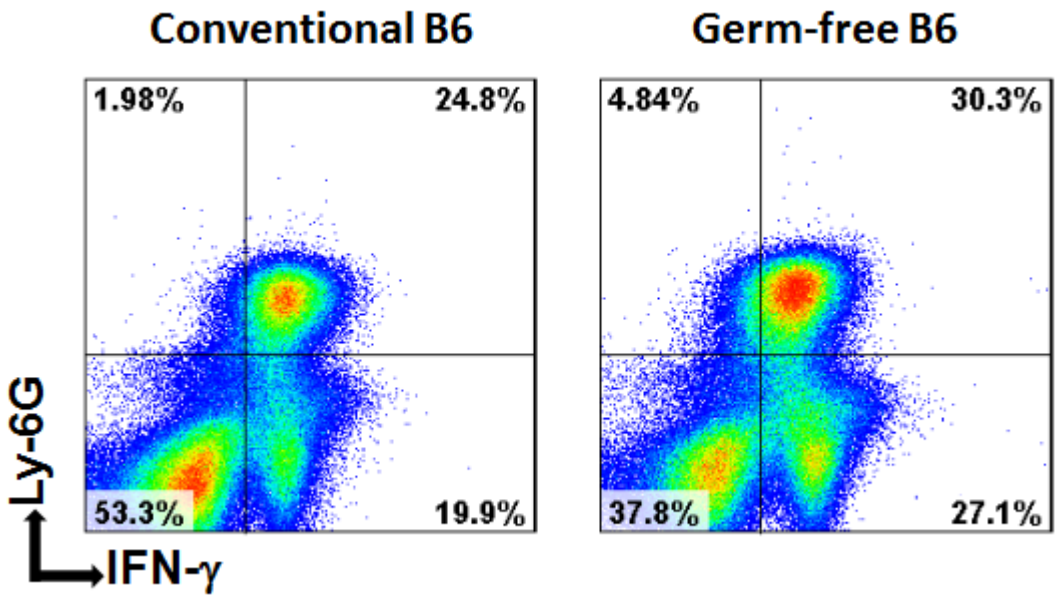


Fig. 4-4: Neutrophils are IFN-γ positive in germ-free mice.
Bone marrow from naïve B6 mice from our conventional animal facility or raised in germ-free bubbles were examined for the presence of IFN-γ positive neutrophils.

Fig. 4-5

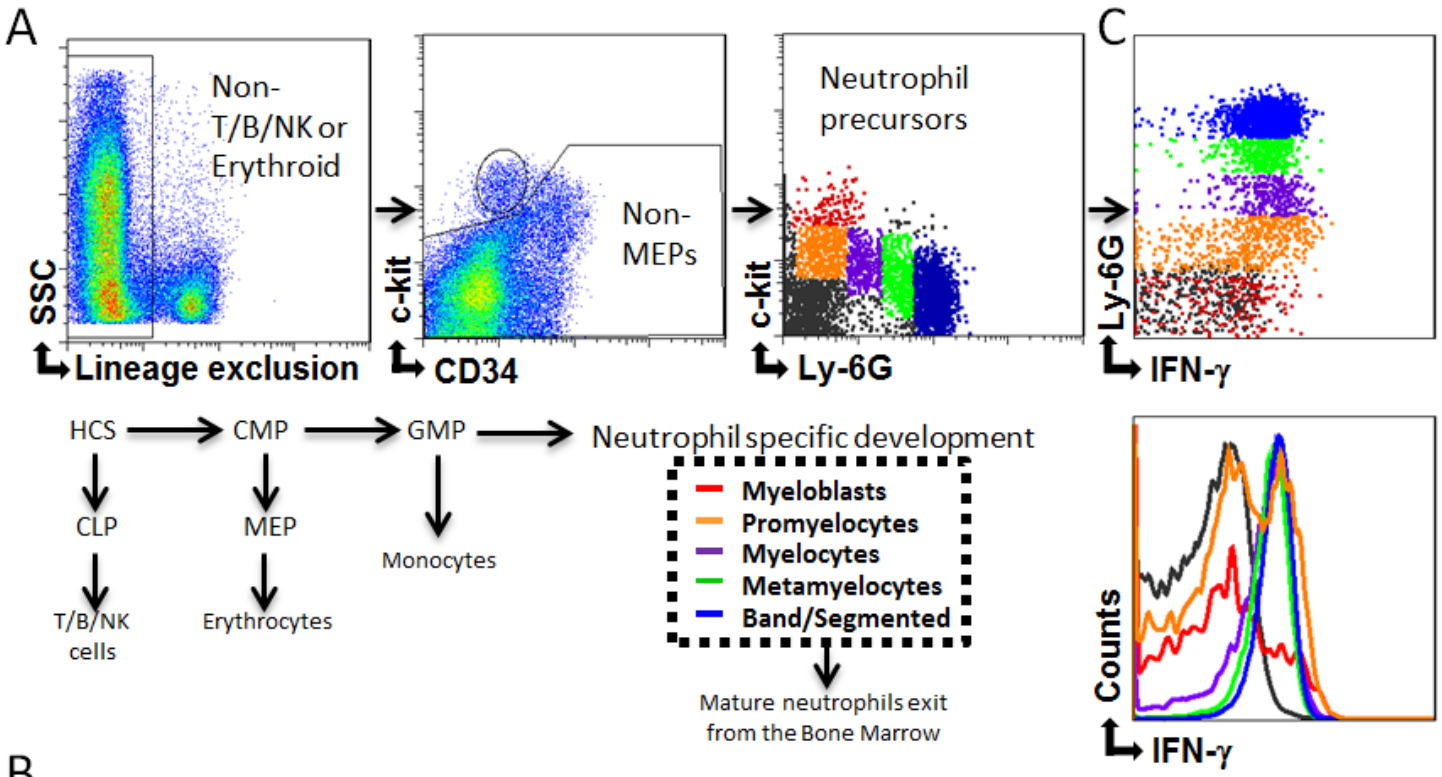


Fig. 4-5: Developing neutrophils become IFN-γ-positive in the promyelocyte stage.
Bone marrow from B6 mice were harvested and examined for different stages of their development. The neutrophil-specific linage develops from the granulocyte-monocyte progenitor (GMP) through a series of stages, myeloblasts (red), promyelocytes (orange), myelocytes (purple), metamyelocytes (green), band/segmented neutrophils (blue). The gating strategy excludes other potentially IFN-γ-positive cells by linage negative selection (non -CD3e, CD19, NK1.1, and Ter119) and early linage cells as shown, with the gating of the neutrophil precursor populations shown in the third box from the left, in the colors indicated above. Each colored population was examined for expression of IFN-γ in the fourth box and in the histogram below it. (B) Relative expression of granule proteins, Proteinase 3, Lactoferrin, and Gelatinase, characteristic of different neutrophil precursor developmental stages. (C). Expression of IFN-γ in neutrophil precursors.

Fig. 4-6

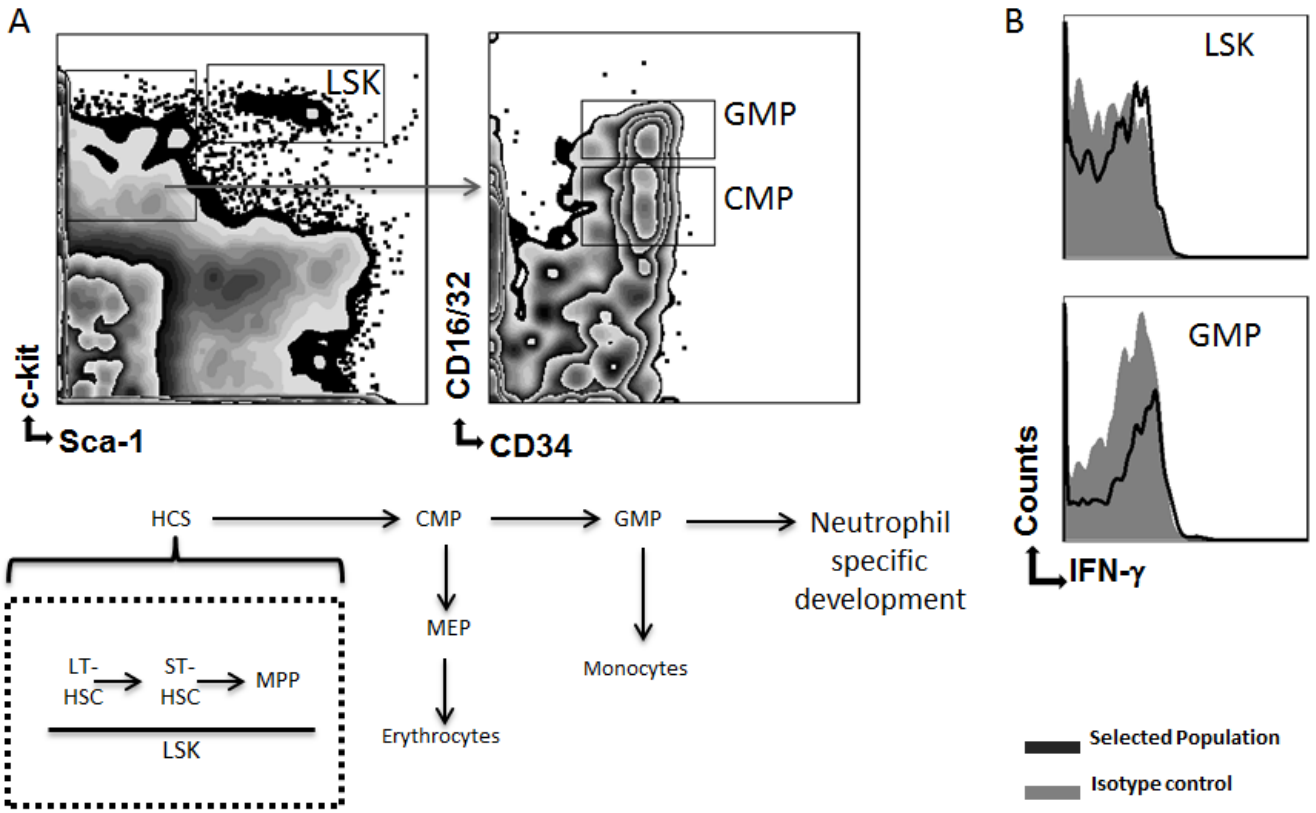


Fig. 4-6: The hematopoietic linage prior to neutrophil development is not IFN-γ-positive. Bone marrow from B6 mice were harvested and examined for different stages of hematopoietic development. The neutrophil-specific linage develops from the granulocyte-monocyte progenitor (GMP) and prior to that the common-myeloid progenitor (CMP) and Linage-negative Sca-1-positive c-kit-positive (LSKs). Having excluded the linage-specific markers, CD3e, CD19, NK1.1, F4/80, Gr-1, CD11b, and Ter119, the LSK and GMP populations were identified: LSK (c-kit^{hi} Sca-1+), GMP (c-kit^{hi} Sca-1- CD34+ CD16/32^{hi}). (B) LSKs and GMPs were examined for IFN-γ positivity (black) and isotype control (gray) shown in histograms.

Fig. 4-7

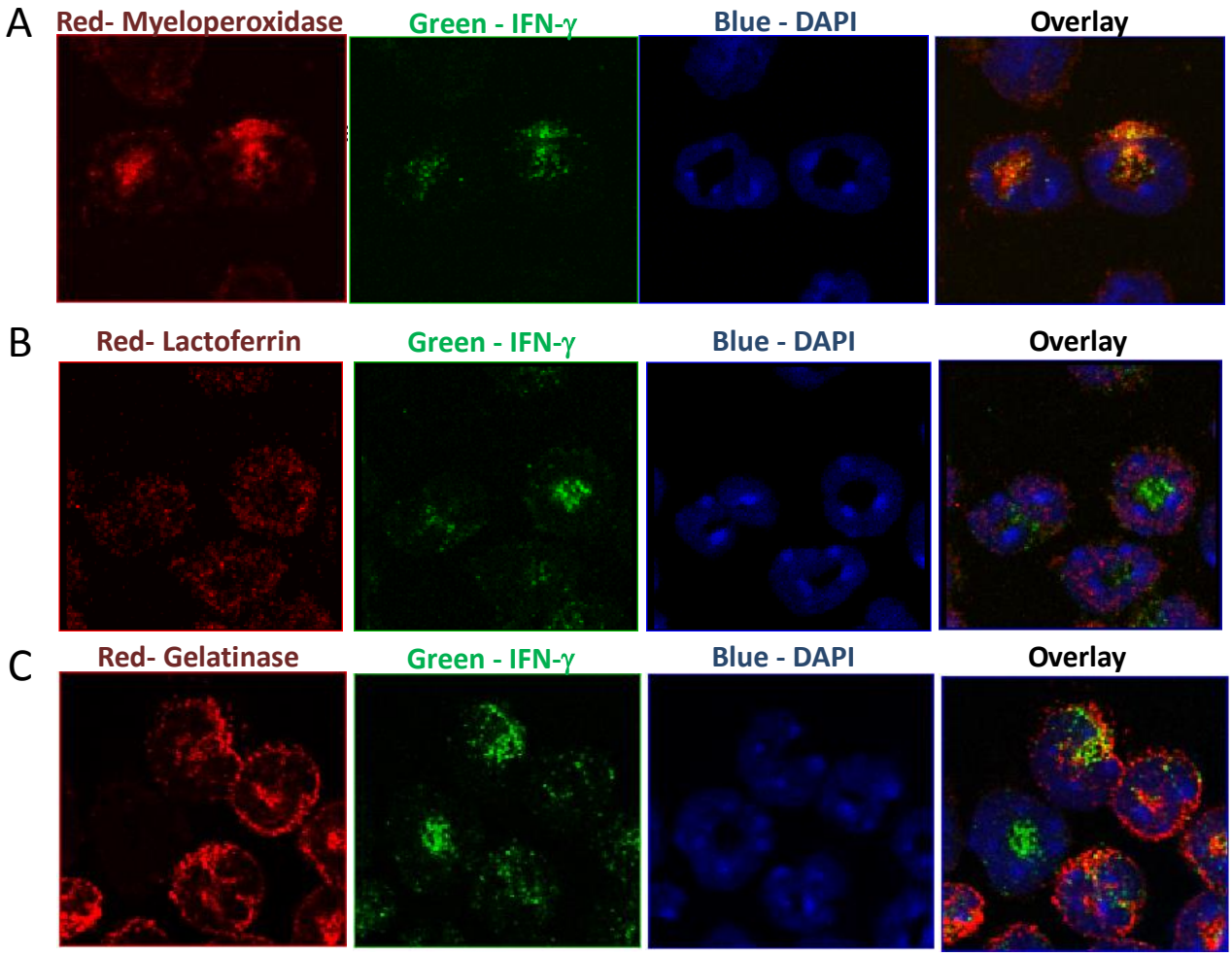


Fig. 4-7: Neutrophil IFN-γ colocalizes with granule proteins.
Ly-6G+ neutrophils were magnetic bead purified from the bne marrow of C57BL/6 mice and fixed to slides and stained with IFN-γ-Alexa488 (green) and DAPI (blue). They were additionally stained anti-myeloperoxidase (A), anti-Lactoferrin (B), or anti-Gelatinase (C) and a secondary antibody conjugated to Alexa568 (red). Individual channels are shown as well as an overlay of representative cells. Overlay between red and green is seen in yellow.

Table 4-1: Quantitative Colocalization Analysis (QCA) analysis of IFN-γ and select granule proteins

Slide	Red Stain	Green Stain	Red to Green Mander M1	Green to Red Mander M2	Does IFNγ (Green) Colocalize with Red
1	Myeloperoxidase	IFNγ	0.4067	0.5639	yes
2	Myeloperoxidase	IFNγ	0.3169	0.6335	yes
3	Gelatinase	IFNγ	0.162	0.2349	no
4	Gelatinase	IFNγ	0.1889	0.1137	no

Mander coefficient value >0.5 is considered colocalization

Fig. 4-8

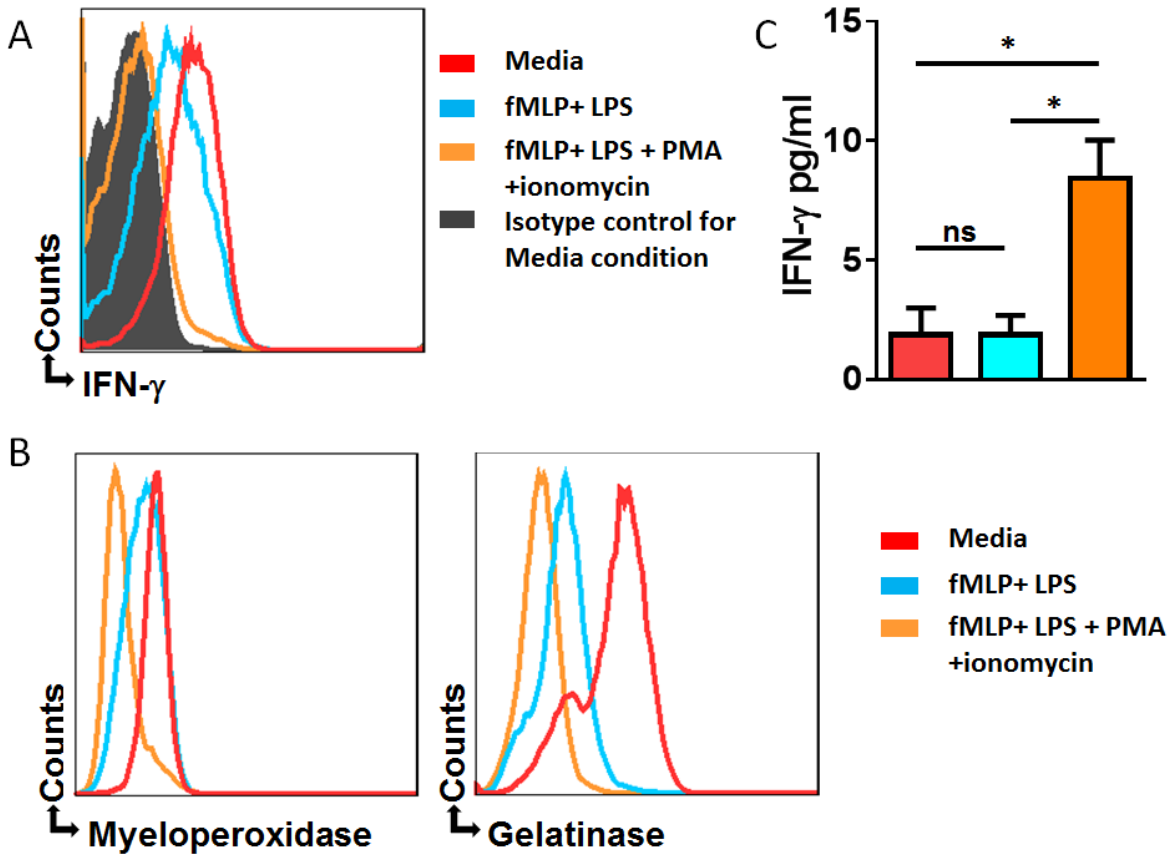


Fig. 4-8: Degranulation stimuli induce exocytosis of neutrophil-derived IFN-γ.
Bone marrow from C57BL/6 mice were incubated for one hour with the following stimuli: media alone (red), fMLP + LPS (blue), or fMLP + LPS + PMA + ionmycin (orange). (A) Cells were gated on Ly-6G+ neutrophils and examined for IFN-γ or (B) myeloperoxidase and gelatinase. (C) Supernatants from these experiments were saved for ELISA.

Fig. 4-9

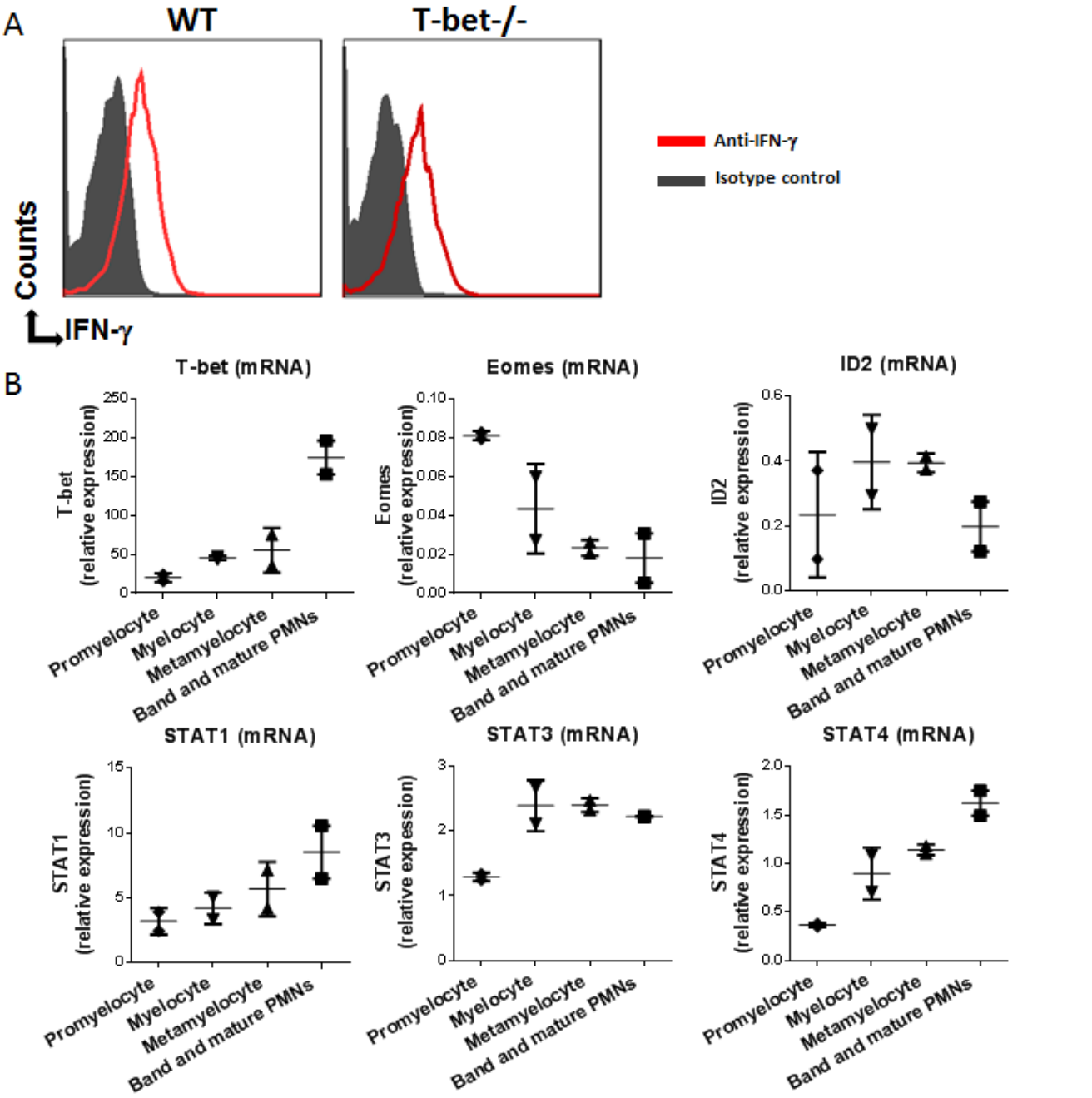
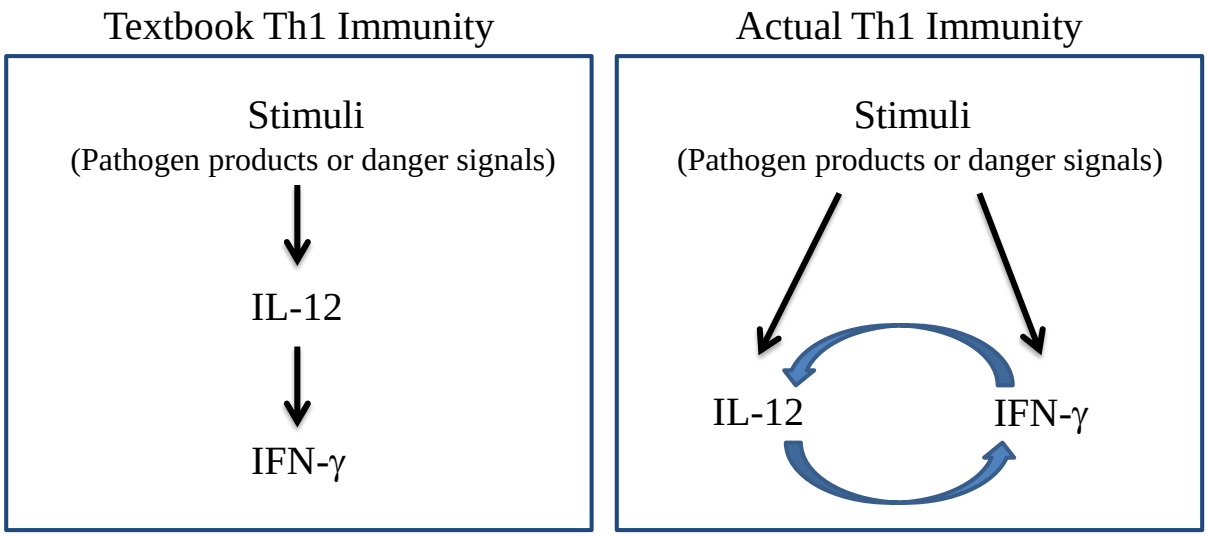


Fig. 4-9: T-bet and other common IFN-γ-regulating transcription factors do not seem to play a role in Neutrophil-derived IFN-γ.
(A) Blood from WT or T-bet-/- mice were examined for Ly-6G+ neutrophils and IFN-γ by flow cytometry. Histogram showed anti-IFN-γ (red) as compared to isotype control (black). (B) Relative expression of transcription factors T-bet, Eomes, ID2, STAT1, STAT3, and STAT4, in different immature neutrophil populations by qRT-PCR.

Fig. 4-10: Models of Th1 immunity



CHAPTER V

EXTENDED DISCUSSION

A novel role for neutrophils during *T. gondii* infection.

The research presented in this thesis establishes a novel role for neutrophils during infection. Specifically during *T. gondii* infection, IFN-γ-positive neutrophils rapidly infiltrate into the site of infection (Fig. 3-2). Neutrophils in culture exposed to *T.gondii* tachyzoites release IFN-γ into the supernatant even in the absence of TLR11 (Fig. 3-7). This suggests that neutrophils recognize a non-profilin component of *T. gondii* or perhaps a more general danger signal associated with infection; these are not mutually exclusive possibilities for two reasons: 1) We know that fMLP and LPS stimulation causes minor degranulation of IFN-γ-containing granules, but that signals eliciting a stronger Ca^{2+} flux can cause almost complete degranulation of IFN-γ-containing primary granules (Fig. 4-8). 2) Recent work by other laboratories has shown that NLRP1 and NLRP3 (which have been shown to be expressed in neutrophils (Guarda et al., 2011)) recognize an unknown ligand during *T. gondii* infection leading to inflammasome activation (Cirelli et al., 2014; Gorfu et al., 2014). Priming events for specific inflammasome activation in the context of *T. gondii* infection are not understood but are discussed in (Fig. 5-1) – our updated model of IFN-γ-mediated resistance to *T gondii*. Regardless of the specific mechanism, we know that early neutrophil infiltrate and IFN-γ are important for survival during i.p. *T. gondii* infection, as depletion of neutrophils using anti-

Ly6G (1A8) results in early mortality in mice, but can be partially rescued by the addition of IFN-γ (Fig. 3-12).

The large variety of cell types capable of making IFN-γ illustrates the importance of this cytokine in immunity but also make it difficult to assess the overall contribution of neutrophil-derived IFN-γ during infection. Additionally, most *T. gondii* infection data in the literature are in C57BL/6 mice which have a stronger overall Th1 response compared to other mouse strains along with an intact TLR11 recognition system and consequent NK cell IFN-γ response to provide early protection again *T. gondii* (Sturge and Yarovinsky, 2014). The generation of a conditional IFN-γ- or IFN-γ receptor-deficient mouse model may provide the opportunity to dissect this dilemma in *T. gondii* and other infection models.

The future nomenclature of neutrophils will be defined by their 'Th' cytokine production phenotypes

We now know that neutrophils are capable of making IFN-γ, which is generally considered an adaptive CD4+ T cell cytokine and defines the idea of 'Th1' immunity. Overtime, CD4+ T cells have been classified into different differentiation phenotypes, Th1, Th2, Th9, Th17, Th22, T_{reg}, and more, based on the characteristic cytokines they secrete and defined transcription factors that regulate their development. Historically, the initial expansion of T cell classifications (Th1 vs Th2) occurred after there was a general consensus in the immunology community that the T cell network was well understood (Liew, 2002). Much like the original T cell definition, most immunologists view neutrophils as one defined population, yet there is increasing evidence that neutrophils are also heterogeneous.

Concurrent with our findings of neutrophil IFN-γ, another group found that neutrophils can make IL-22 (Zindl et al., 2013). In their model, neutrophils infiltrating into sites of DSS-induced colonic damage and are stimulated by local IL-23 to secrete IL-22, which is beneficial for epithelial cell barrier function and proliferation. Production of IL-22 from neutrophils in response to IL-23 is augmented by addition of TNF. This finding makes these neutrophils functionally similar to but also distinct from Th22 cells which produce IL-22, as well as some IL-13 and TNF. This year another laboratory found that neutrophils produce another 'Th' cytokine, IL-17 (Taylor et al., 2014a). Infection models utilizing pathogenic fungi *Aspergillis fumigatus* or *Fusarium oxysporumn* found that neutrophils produced IL-17, which shared a similar protective role with IL-17 produced by Th17 (Taylor et al., 2014a; Taylor et al., 2014b). Neutrophil IL-17 was not pre-formed; instead, *de novo* IL-17 production was stimulated by several factors including IL-6, IL-23, IL-17, and dectin-2 receptor recognition of fungal products (Taylor et al., 2014b). Interestingly, a subpopultion of neutrophils were found to be RORγt-positive in naive bone marrow and IL-17 producing neutrophils expressed RORγt transcript, the transcription factor traditionally defining Th17 development (Taylor et al., 2014b). These data and others illustrate that neutrophils are transcriptionally active cells capable of making diverse cytokines dependent on their environment.

In the context of infections, neutrophil-derived cytokines such as IFN-γ, IL-22 and IL-17 are beneficial, but these cytokines may also be detrimental in the case of sterile inflammation. In a model of acute kidney ischemia-reperfusion injury (IRI), neutrophils rapidly infiltrate after reperfusion and secrete IL-17 (Li et al., 2010). Many chemokines and

cytokines including IL-17 itself contribute to neutrophil infiltration after reperfusion. This duality of IL-17 complicates the interpretation of experiments utilizing anti-IL-17 treatment or chimeric mice with IL-17-/- hematopoietic cells; experiment with both these models show improved kidney function after kidney IRI but it is difficult to separate the significance of neutrophil infiltration vs neutrophil-derived IL-17 to these systems (Li et al., 2010). Blocking IL-12, IL-23, or CXCR2 also resulted in less neutrophil infiltration and improved kidney recovery (Li et al., 2010). Additional data with the same injury model found that infiltrating neutrophils were IFN-γ-positive (Li et al., 2007). A closer examination of their work reveals that their data supports our conclusions indicating pre-formed IFN-γ, but their utilization of anti-Gr-1 (RB6-8C5) rather than anti-Ly-6G (1A8) lead them to see a negative population (likely inflammatory monocytes) which they may have interpreted as IFN-γ-negative neutrophils. Blocking neutrophil infiltration, in this case by depleting NKT cells and their cytokines, also results in improved kidney function compared to controls (Li et al., 2007). Taken together these data suggest that neutrophil infiltration increases reperfusion damage in this injury model, but the data implicating neutrophil IL-17 or IFN-γ are only correlation.

Much like CD4+ T_{reg} cells which can suppress immune responses, there is a population of neutrophils implicated in immune suppression. MDSC of granulocytic origin (neutrophils) or monocytic origin were discovered in the context of cancer for their ability to suppress the immune response to tumors. There is not currently a definitive way to segregate these neutrophil subpopulations, but the literature suggests that differential CD244 expression may be of some use (Youn et al., 2012). However, unlike T_{reg} cells which

suppress immune responses through the cytokines IL-10 and TGFβ, suppression of T-cell responses by MDSCs occurs through secretion of arginase-1 and reactive oxygen species (ROS). The main purpose of arginase-1 and ROS is to inhibit pathogen growth rather than to suppress T cell functions by depleting amino acids and increasing oxidizing agents. Yet both of these things modify the microenvironment and indirectly decrease T cell proliferation (Pillay et al., 2013). Ironically, MDSC are loosely defined as their own cell type but production of arginase-1 and ROS is a characteristic of all neutrophils and monocytes.

Applying diverse phenotypic definitions to the innate cell populations has recently become common practice: first with the definition of M1 and M2 macrophages, which are defined by the Th1 vs Th2 type of inflammatory environment they produce (Martinez and Gordon, 2014) and second, with the recent addition of innate lymphoid cells (ILCs). ILCs have thus far been grouped into ILC1, ILC2, and ILC3 subpopulations defined by production of the 'Th1' cytokine IFN-γ, 'Th2' cytokines IL-5, IL-9, and IL-13, and 'Th17/22' cytokines IL-17 and IL-22 (Walker et al., 2013). In the future we predict that distinct phenotypes of neutrophils will be recognized and defined, similar to the Th1, Th17, Th22, and T_{reg} phenotypes but without the requirement for differentiation and with additional plasticity (Fig. 5-2).

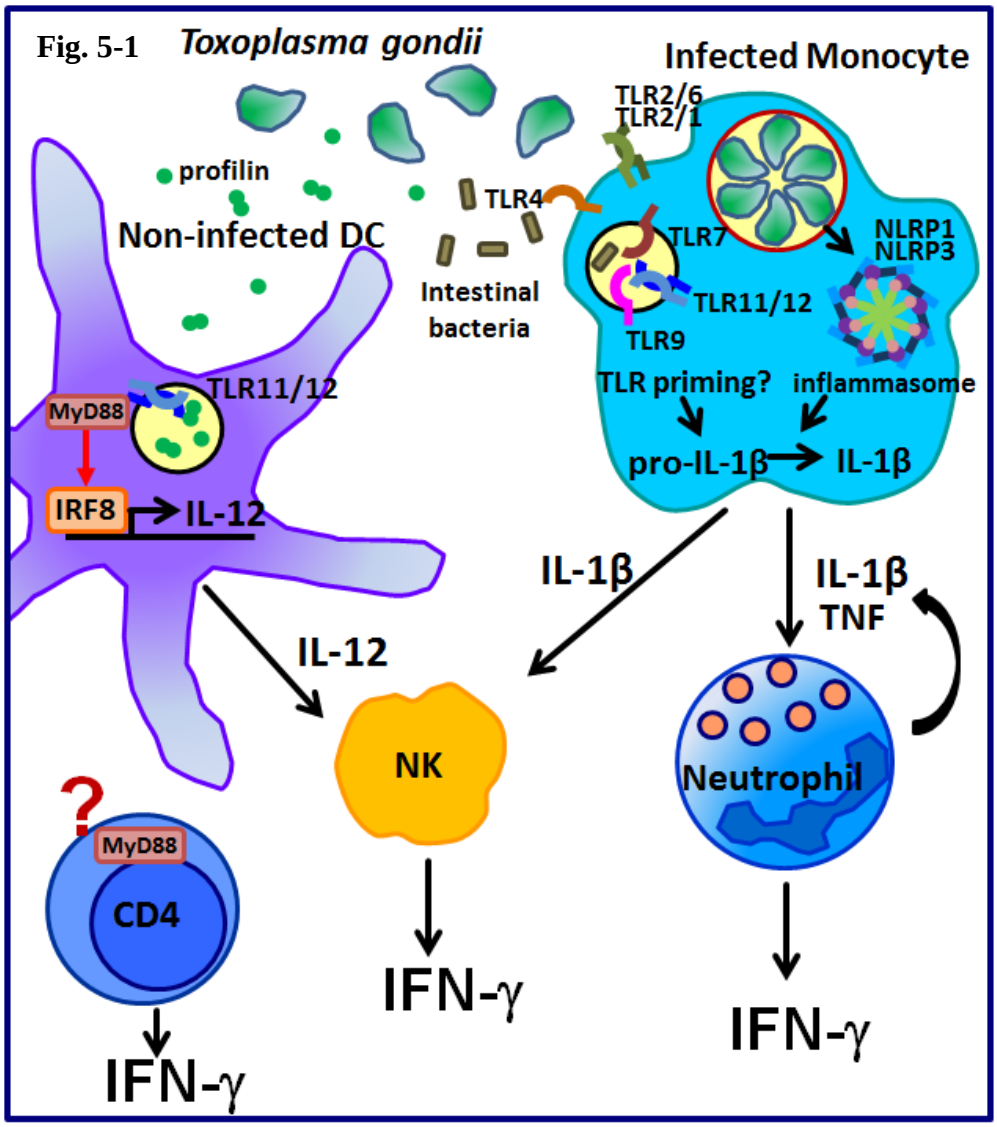
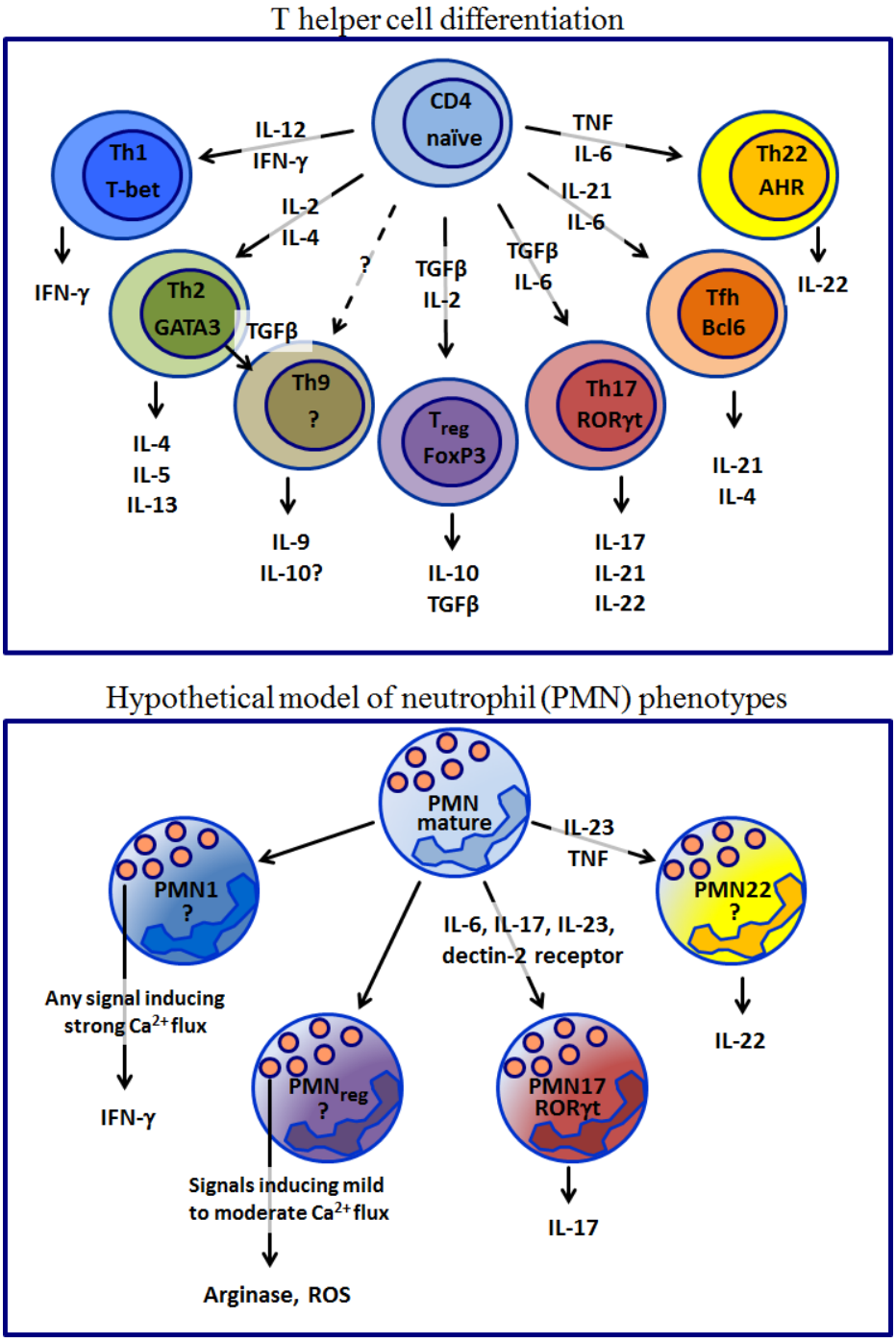


Fig. 5-1: Revised model of TLR-mediated recognition and immune responses to *T. gondii*.
When *T. gondii* infects a mouse, endosomal TLR11/12 complex recognizes a parasitic protein profilin. TLR11 via its adaptor protein MyD88 initiates downstream signaling leading to activation of the transcription factor IRF8. This allows DCs to produce IL-12 in response to *T. gondii* infection. IL-12 mediates activation of NK and their IFN-γ production. Infected macrophages and monocytes may be able to respond to *T. gondii* infection through NLRP1 and NLRP3 inflammasomes. Priming by TLRs is needed to induce pro-IL-1β expression before subsequent cleavage by the inflammasome complex for maturation of IL-1β and secretion. While the specific TLRs involved in priming are not known it is likely initiated by two different events: (i) *T. gondii* itself has ligands for the endosomal TLRs 7/9/11/12 and surface expressed TLR2, (ii) intestinal bacteria can pass the mucosal barrier during *T. gondii*-mediated inflammation and contain ligands for TLR2, TLR4, and TLR9. IL-1β and TNF are involved in IFN-γ seen from neutrophils, and neutrophils themselves can produce IL-1β and TNF resulting in a potential positive feedback. IL-1β production can also enhance the response of NK cells. It was recently shown that T cell IFN-γ is largely dependent on T cell-intrinsic MyD88 rather than MyD88 in other cells types. Whether this MyD88 is dependent on TLRs or the IL-1 family receptors has yet to be investigated. This figure was previously published in (Sturge and Yarovinsky, 2014).

Fig. 5-2: Models of 'Th' immunity – T cells vs hypothetical neutrophil types.



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