Immediate IFNγ production determines host compatibility differences between *Toxoplasma gondii* and *Neospora caninum* in mice

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T. gondii parasitizes mammalian hosts indiscriminately and is adapted to survive in rodents which are critical for transmission of *Toxoplasma gondii* to the definitive feline host via predation. As described in Chapter 1, this relationship has been extensively studied as a model for immune responses to parasites, yet the molecular basis of this unique lack of host specificity is unknown. *Neospora caninum* provides a natural contrast to T. gondii, in that it is a closely related coccidian parasite of ruminants and canines, but is not naturally transmitted by rodents. In Chapter 2, we compared mouse innate immune responses to *N. caninum* or *T. gondii* and found marked difference in cytokine levels and parasite growth kinetics during the first 24 hours post-infection. *N. caninum*-infected mice produced significantly higher levels of IFNγ as early as 4hpi, but IFNγ was significantly lower in *T. gondii*-infected mice during the first 24hpi. "Immediate" IFNγ production was not detected in MyD88$^{-/-}$ mice. However, unlike IFNγ$^{-/-}$ mice, MyD88$^{-/-}$ mice survived *N. caninum* infections. Measures of parasite burden showed MyD88$^{-/-}$ mice were more susceptible to *N. caninum* infections than WT mice, and control of parasite burden correlated with serum IFNγ production 3-4 days after infection. Immediate IFNγ was partially dependent on the *T. gondii* mouse profilin receptor TLR11 but ectopic expression of *N. caninum* profilin in *T. gondii* had no impact on early IFNγ production or parasite proliferation. Our data indicate that *T. gondii* is capable of evading host detection during the first hours after infection while *N. caninum* is not,

and this is likely due to early MyD88-dependent recognition of ligands other than profilin. In Chapter 3, we compared innate immune cell populations and determined that the frequency of IFNγ^+ peritoneal natural killer T (NKT) cells 4hpi was significantly higher in *N. caninum* infections compared with *T. gondii* and uninfected mice. We identified two main populations of NKT cells producing IFNγ: CD3+ NKT and CD4+ NKT cells. In Chapter 4 we describe future work that will determine if T cells are required for immediate IFNγ production, and identify a role for CD1d-restricted NKT cells in this process.

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1.0 Introduction

1.1 *Toxoplasma gondii* and *Neospora caninum*

Toxoplasma gondii is an apicomplexan parasite that can infect and cause disease in all warm-blooded animals including birds and marine species (Boothroyd, 2009). *T. gondii* causes toxoplasmosis and can spread to any mammal via ingestion of oocysts from the definitive host (felids) or tissue cysts from chronically infected intermediate hosts (Stelzer *et al.*, 2019). *T. gondii* can cause severe disease in immune-compromised humans and is an important biotic constraint on agriculturally important animals (Burnett *et al.*, 1998; Luft *et al.*, 1983; A. Wolf, Cowen, & Paige, 1939). Due to oral infectivity and ability to contaminate food and water supplies, *T. gondii* is also a biodefense concern (Aramini *et al.*, 1999; Morris *et al.*, 2012; Northrop-Clewes & Shaw, 2000). *T. gondii* is the only known apicomplexan parasite that is capable of pan-mammalian infection and transmission, although *N.* caninum (a closely related parasite) is known to infect and cause pathology in multiple ruminant and canine species. Neosporosis is the major cause of bovine abortion, resulting in losses of over a billion dollars worldwide in cattle industries (Dubey, Schares, & Ortega-Mora, 2007; Goodswen, Kennedy, & Ellis, 2013). *N. caninum* is thought to be the most efficient vertically transmitting bovine parasite known, and some herds experience up 95% transmission (Buxton, McAllister, & Dubey, 2002; Dubey, Buxton, & Wouda, 2006; González-Warleta *et al.*, 2018; Williams & Trees, 2006). Although *T. gondii* is also capable of vertical transmission, the dominant mode of transmission for *T. gondii* is through oral consumption of parasites either in the form of tissue cysts or environmentally stable oocysts.

During an oral infection in their mammalian host, *T. gondii* and *N. caninum* quickly invade host cells in the gut and cross the epithelium to access the lamina propria where they encounter tissue-dwelling innate immune cells (I. R. Dunay & Sibley, 2010). Immune cell activation and cytokine production is critical for host survival during both *T. gondii* and *N. caninum* infections. As a local immune response is initiated (Fig 1), cytokines produced locally impact cells of the innate immune cell compartment both at the site of infection and in the bone marrow (M. H. Askenase et al., 2015). Over the course of the acute infection, the parasites will spread throughout the body of the host, including crossing the blood brain barrier to infect brain tissue (Collantes-Fernandez *et al.*, 2012; Feustel, Meissner, & Liesenfeld, 2012). Of note, both *N. caninum* and *T. gondii* are capable of crossing the placental barrier to infect the fetus (Maley *et al.*, 2006; Robbins, Zeldovich, Poukchanski, Boothroyd, & Bakardjiev, 2012). The mechanisms driving efficient transmission, persistence, and recrudescence of *N. caninum* in asymptomatic cows is a fundamental unsolved problem in bovine neosporosis and currently there is no treatment (Dubey *et al.*, 2007; Guido, Katzer, Nanjiani, Milne, & Innes, 2016; Horcajo, Regidor-Cerrillo, Aguado-Martinez, Hemphill, & Ortega-Mora, 2016; Nishikawa, 2017).

Despite the difference in host range and dominant transmission routes, at the genomic level *T. gondii* is quite similar to *N. caninum.* Genomic comparisons revealed significant genetic conservation, including 85% synteny and over 90% protein coding gene conservation between species (Adam James Reid et al., 2012). Morphologically, *N. caninum* and *T. gondii* tachyzoites are nearly indistinguishable, necessitating the development of species-specific primers to identify the infecting species or to detect cross-species contaminations in the lab. *N. caninum* and *T. gondii* share the ability to infect a diverse range of cells *in vitro,* and the mechanism for host cell invasion

is conserved (Besteiro, Dubremetz, & Lebrun, 2011; Kemp, Yamamoto, & Soldati-Favre, 2013). Both parasite species secrete a large number of host-interacting proteins into the extracellular or intracellular milieu before and after infection to aid in parasite invasion and survival (Besteiro *et al.*, 2011; Black & Boothroyd, 2000; English, Adomako-Ankomah, & Boyle, 2015). *N. caninum* and *T. gondii* do not require phagocytosis to enter the host cell and instead infect by forming a moving junction and actively invade cells (Morisaki, Heuser, & Sibley, 1995). Following host cell penetration, both *N. caninum* and *T. gondii* reside and replicate inside a parasitophorous vacuole (PV) within the host cell that provides protection from intracellular host defenses and allows parasites to evade recognition (Besteiro *et al.*, 2011; Frenkel & Dubey, 1975; Pittman & Knoll, 2015). Differences in their transmission, host range, and ability to cause disease combined with their similarity in morphology, molecular infection mechanisms, and genetic conservation provide a unique comparative system to uncover parasite-specific mechanisms modulating early host responses.

N. caninum infections are reported frequently in domesticated and wild animals, yet few instances of clinical neosporosis have been identified in the wild. Detection methods usually involve PCR or serology to identify *N. caninum* infections. While these methods identify parasite DNA in the host, or antibodies specific to the pathogen, they are not necessarily indicative of a viable or successful infection. In fact, *N. caninum* isolates obtained from natural infections were derived from a small number of ruminant and canine species, the secondary and primary hosts respectively. Viable tachyzoites have only been isolated from a small number of species including: dogs, sheep, cattle, and water buffalo (Al-Qassab, Reichel, & Ellis, 2010). Although there are no reports of naturally occurring neosporosis in rodents, *N. caninum* DNA has been reported in rodents as well as a variety of other species (M. C. Jenkins et al., 2007). These disparities in clinical

disease, isolation of viable parasites, and prevalence of *N. caninum* DNA is not fully understood. However, information derived from experimental mouse models, combined with information from experimental and natural infections in bovine hosts, has shed some light into how innate immune responses mediate control of *N.* caninum (Teixeira *et al.*, 2005).

Twenty years ago, Khan *et al.*, evaluated the host cytokine response to *N. caninum* in mouse splenocytes harvested from infected mice (I. A. Khan, Schwartzman Jd Fau - Fonseka, Fonseka S Fau - Kasper, & Kasper, 1997). They found that IL-12 and IFNγ transcripts increased dramatically by 6 hours after infection, and then rapidly declined by 24 hours, yet the mechanism underlying this immediate host response and how it impacts parasite proliferation *in vivo* remains poorly understood. While many of the parasite effector genes (Adam James Reid et al., 2012) and host immune responses are conserved (Buchmann, 2014) between *T. gondii* and *N. caninum*, the unique properties that result in *T. gondii* evading host protective responses while *N. caninum* is unsuccessful (in all but a few host species) and controlled by host protective immunity remains ambiguous. As a model for proinflammatory immune response (TH1-type) with IL-12 and IFNγ as key cytokines mediating host protective immunity emerges for *N. caninum* infections (Baszler, Long Mt Fau - McElwain, McElwain Tf Fau - Mathison, & Mathison, 1999; I. A. Khan et al., 1997; Long, Baszler Tv Fau - Mathison, & Mathison, 1998), a better understanding of the underlying mechanism, how it impacts parasite virulence, and how it differs from *T. gondii* infections will inform future work in controlling toxoplasmosis and neosporosis.

1.2 Host response

Multicellular organisms exhibit innate defense mechanisms paramount to their survival during invasive microbial insult, and thus, many components of pathogen recognition and host responses are deeply conserved (Hoffmann, Kafatos, Janeway, & Ezekowitz, 1999). Innate signaling mechanisms discern "self" from "non-self" molecules and the evolution of innate pathogen recognition predates the divergence of vertebrate and invertebrate species, over 0.5 billion years ago (Kimbrell & Beutler, 2001). Innate immunity is comprised of physical barriers, humoral components, and cell-mediated immune responses. Conserved components in mammals and birds include physical barriers like skin and specialized mucosa to prevent infection. Shared humoral components include antimicrobial peptides, and complement to combat extracellular invasive microbes. There is also substantial conservation of cytokines and chemokines to communicate with, attract, or activate innate immune cells. Phagocytic myeloid cells, non-specific cytotoxic cells, and innate lymphoid cells are conserved in birds and mammals and are important for host responses during intracellular infections (Riera Romo, Pérez-Martínez, & Castillo Ferrer, 2016).

The evolution of the adaptive immune system is a more recent development in animal evolution and is a powerful tool employed by higher vertebrates to protect from invading microbes, yet adaptive immunity depends on the innate immune system to both recognize and activate responses to successfully challenge infection (Mogensen, 2009). Despite the critical role of innate recognition for host survival, our knowledge of innate responses to eukaryotic parasites immediately after infection has lagged behind that of adaptive responses. How the innate immune cell compartment changes in response immediately after infection and how these changes contribute to pathology and parasite survival remains largely unknown. The tight regulation of the

innate response is vital for pathogen clearance and changes in this regulation can alter the outcome of infection making a non-permissive host susceptible (Maizels & McSorley, 2016).

The capability of a new virus to sweep across the globe with deadly acute pathology caused by immune cells and the cytokines they produce (Cao, 2020; Merad & Martin, 2020) inspired people world-wide to search for information about immune responses and inflammatory cytokines (Fig 2) and research efforts to understand the mechanisms of the cytokine-induced pathology are well underway (Merad & Martin, 2020; Soy *et al.*, 2020; Zhou *et al.*, 2020).

The emerging culprit, as with other coronaviruses, appears to be excessive inflammatory responses and the dysregulation of interferon pathways (Frieman, Heise, & Baric, 2008; Merad & Martin, 2020; Zhou *et al.*, 2020) The pandemic of 2020 brought the gaps in our understanding of innate immunity sharply into focus (McKechnie & Blish, 2020), and (in addition to vaccine development) there is an increasing sense of urgency to harness innate cell-mediated immune mechanisms to combat the devastating acute immunopathology (Angka, Market, Ardolino, & Auer, 2020). Our bodies are able to detect and respond to pathogens immediately, yet we often don't know we are sick for days after pathogen encounters. A better understanding of cellular mechanisms immediately after pathogen encounter will enable us to develop earlier detection and control methods for a broad range of pathogens, as well as honing our vaccine strategies by creating better adjuvants and innate immune-activating components. Decades of discoveries have characterized host responses to the eukaryotic parasite, *T. gondii*, yet early innate recognition mechanisms have only recently become a focused interest. Much of the research concentrates on acute infections beginning 3-5 days after infection (Hunter, Candolfi, Subauste, Van Cleave, & Remington, 1995; Nakano *et al.*, 2001), however we have shown the murine host has already mounted a robust immune response by this time, as evidenced by large amounts of inflammatory

cytokines detected at the site of infection and systemically within hours of infection (Coombs *et al.*, 2020).

1.3 Toll-like receptors

Toll-like receptors were first identified in *Drosophila melanogaster* with early work showing the Toll gene played a key role in establishing dorsal-ventral polarity in the developing *D. melanogaster* embryo (Anderson, Jürgens, & Nüsslein-Volhard, 1985). In the subsequent years, TLR signaling has been rigorously investigated in numerous vertebrate and invertebrate systems, determining that TLRs are a multi-gene family of receptor molecules conserved through metazoans from invertebrate to vertebrate animals (Roach et al., 2005). Although initially identified for their role in developmental processes, TLRs are notable for their role in pathogen recognition and initiation of immune signaling pathways.

Many pathogen recognition pathways rely on germline encoded receptors that are able to recognize invading microbes and initiate inflammatory responses within minutes of infection (Janeway & Medzhitov, 2002). Innate pattern recognition receptors (PRRs) that recognize infectious agents include this well-described family of toll-like receptors (TLRs). The evolutionarily-conserved TLRs interact with a broad range of equally conserved pathogen molecules called pathogen-associated molecular patterns (PAMPS), highlighting the co-evolutionary relationship between the host immune response and invasive microbes. PAMPs are molecules that are shared among numerous microorganisms and are often required for survival. Well known PAMPS include bacterial endotoxin lipopolysaccharides (LPS), flagellin, and nucleic acids.

By recognizing a myriad of immunostimulatory pathogen molecules, mammalian TLRs initiate signal cascades resulting in the production of antimicrobial molecules or immune signaling proteins like chemokines and cytokines (Fischer, Nitzgen, Reichmann, & Hadding, 1997; Gaddi & Yap, 2007; Gazzinelli *et al.*, 1994). Additionally, TLR expression can be upregulated by cytokine exposure allowing for increased recognition of microbial invaders. TLRs consist of an extracellular ligand binding domain, a transmembrane domain, and an intracellular domain that binds downstream signaling molecules to initiate cell-mediated immune (CMI) responses. Comparisons of mammalian TLRs identified significant conservation between species. Humans and cattle (Menzies & Ingham, 2006) have 10 functional TLRs but lack TLR11 and TLR12. Mice have 12 TLRs, lacking TLR10 but express both TLR11 and TLR12 (Kawasaki & Kawai, 2014). The significant conservation and deep evolutionary roots of vertebrate TLRs indicates mammalian hosts share pathogen recognition mechanisms and therefore recognize and respond to the same pathogen molecules. One well-characterized example of this is TLR4-mediated recognition of bacterial lipopolysaccharide (LPS) in numerous animal and cell models, including mice and humans (Cronin, Turner, Goetze, Bryant, & Sheldon, 2012; Müller *et al.*, 2017; Roach *et al.*, 2005). The differences in TLR repertoire between host species, i.e. mice express TLR11 but in humans the gene harbors several premature stop codons, highlights the evolutionary pressure imposed on the immune system by pathogens common to the host species (Bryant & Monie, 2012).

TLR signal transduction requires conserved downstream adapter proteins, MyD88 or TRIF and invading microbes can be recognized by cell-surface TLRs or endosomal TLRs (Hernandez *et al.*, 2016; Kawasaki & Kawai, 2014). Thus far, TLR4 is thought to be uniquely capable of signaling through either MyD88 or TRIF. However, PAMP recognition via TLR4/MyD88 is a cell surface recognition event and TLR4/TRIF appears to be an endosomal PAMP-derived recognition

event and thus are thought to be spatially separated signaling mechanisms (Deguine & Barton, 2014). TLR signaling is important for determining the type of immune response initiated and the outcome can differ depending on the PAMP, even when signaling through the same TLR. For example, TLR2 activation with *S. mansoni* results in dendritic cell (DC) induction of a TH2 response, (van der Kleij *et al.*, 2002) yet *T. cruzi* stimulation of TLR2 results in a DC induction of a Th1 immune response (Ouaissi *et al.*, 2002). The heterodimerization of TLR2 with either TLR1 or TLR6 is thought to impact the type of immune response elicited by TLR2 activation. *T. gondii* and *N. caninum* stimulate TLR-specific immune responses, yet the parasite PAMPs remain largely uncharacterized. *T. gondii* is known to interact with TLR2, TLR4, and TLR11/12 (Debierre-Grockiego *et al.*, 2007; Hou, Benson, Kuzmich, DeFranco, & Yarovinsky, 2011; Salazar Gonzalez *et al.*, 2014), although only one *T. gondii* PAMP has been identified and characterized, actin-binding protein profilin (A. Alicia Koblansky et al., 2013; F. Yarovinsky et al., 2005). *T. gondii* profilin interacts with TLR11/12 and is necessary for parasite recognition in murine macrophages and DCs (Raetz *et al.*, 2013) *N. caninum* interactions with TLR2 have been described (Mineo, Oliveira, Gutierrez, & Silva, 2010), but *N. caninum* PAMPs have yet to be identified and characterized. Thus, TLRs activate robust antimicrobial immune responses through complex signaling pathways dependent on TLRs interacting with other TLRs, adaptor proteins, and pathogen molecules.

1.3.1 Functional changes in TLRs and host specificity

Pathogens exert high levels of selection on their hosts, and in turn the adapting host exerts Pathogens exert high levels of selection on their hosts, and in turn the adapting host exerts selective pressure on the pathogen. This reciprocal adaption (or coevolution) underlies host specificity and

is defined by ever-evolving host-pathogen interactions (Papkou *et al.*, 2019). TLRs are thought to have evolved from a gene duplication event prior to the divergence of deuterostomes and protostomes creating two ancient TLR families. Subsequent neofunctionalization and further gene duplication resulted in the highly conserved TLR repertoire for pathogen recognition currently observed throughout vertebrate species (Hughes & Piontkivska, 2008; Ishengoma & Agaba, 2017; Liu, Zhang, Zhao, & Zhang, 2020). Although TLRs are conserved, the ligand binding domain varies between species and may contribute to innate mechanisms underlying host specificity.

Several comparisons across species suggest TLR intracellular domains are largely conserved and under purifying selection, while the extracellular leucine-rich motifs are under positive selection and harbor important differences between mammalian species (Fornůsková *et al.*, 2013; Hughes & Piontkivska, 2008; Jungi, Farhat, Burgener, & Werling, 2011). Recent comparisons between cattle and related ungulate species identified high levels of conservation in the overall domain architecture, but also identified species-specific differences in TLR ligand-binding domains (Ishengoma & Agaba, 2017). They identified recurring positive selection in all TLR genes, with TLR4 exhibiting the highest numbers of codons under significant levels positive selection (Ishengoma & Agaba, 2017). In agreement with other reports, they determined cell surface TLRs have more positive selecting sites than endosomal TLRs (Fornůsková et al., 2013; Ishengoma & Agaba, 2017; Liu et al., 2020).

The concentration of positively-selected codons in the PAMP-interacting region of vertebrate TLRs highlights the coevolutionary relationship between pathogen molecules and host innate recognition motifs (Liu et al., 2020; Roach et al., 2005). Functional changes in TLR ligand binding between species may alter the host ability to recognize and respond to microbial insult. Comparisons of the molecular evolution of mammalian TLR2 suggested that ruminant TLR2 is

under less selective pressure than other mammalian TLR2 genes. Bovine TLR2 harbors important nonsynonymous polymorphic sites unique to cattle that localize to the ligand binding domain. Predictions at one polymorphic site of interest in bovine TLR2 identify differences in charge in amino acid residues known to be important for ligand binding in human TLR2, but further investigations are needed to determine if this has any functional impact (Jann, Werling, Chang, Haig, & Glass, 2008). However, functional differences between recognition responses of murine and human TLR2s have been described, supporting the possibility of host specific TLR function due to variation in extracellular domains (Grabiec *et al.*, 2004).

Recent advances characterizing bovine TLRs identified bovine TLR2 expression in multiple dendritic cell populations, even at steady state (non-activated). Dendritic cells are important for host recognition and response to pathogens and are key antigen presenting cells (APCs). DCs exist in various states of maturity, and expression of TLRs can vary based on DC subset. DCs act as sentinels and identify invading pathogens and migrate to lymph nodes via afferent lymph vessels enabling interactions with T and B cells to initiate the adaptive immune response. Recent work characterized bovine TLR expression at steady-state in 4 subsets of afferent lymph dendritic cells, finding that TLR2 was expressed at higher levels compared to all other TLRs in each subset (Werling *et al.*, 2017). Others have shown that murine TLR2 recognizes *N. caninum* and activates APCs *in vitro* and mice lacking TLR2 have higher parasite burden during acute and chronic infections, however TLR2 is not required for survival of *N. caninum* infections in mice (Mineo *et al.*, 2010). Future work is necessary to identify the *N. caninum* effector molecule interacting with TLR2 and it would be interesting to determine if there are differences in *N. caninum* PAMP/Host-TLR interactions comparing natural bovine hosts with other animals that *N. caninum* does not naturally infect, like mice.

1.4 Interferon gamma and interleukin 12

Interferon gamma (IFNγ) has been identified as an important immune-modulating cytokine in many mammals including mice and cattle (Innes *et al.*, 1995; Tau & Rothman, 1999). It is considered to be the master regulator of inflammation and impacts numerous cells and pathways. During parasitic infections, IFNγ is produced by lymphocytes like γδ T cells, natural killer (NK) cells, NKT cells, and αβ T cells. IFNγ is a potent inducer of MHC II expression. IFN-γ is required for mice to survive both *N. caninum* and *T. gondii* infections and is considered a key mediator of both innate and adaptive immune responses to both parasites (Baszler *et al.*, 1999; Coombs *et al.*, 2020; Correia *et al.*, 2015; Suzuki, Orellana, Schreiber, & Remington, 1988). During acute intraperitoneal (IP) *T. gondii* infections, γδ T cells, NK cells and NKT cells accumulate in the peritoneal cavity within 7 dpi (Nakano *et al.*, 2001). γδ T cells are an important source of acute IFNγ production contributing to host resistance in mice whereas NKT cells may function to negatively regulate cell mediated immunity (CMI) during acute *T. gondii* infection (Nakano *et al.*, 2001). Adaptive compartment CD4+ T helper cells and CD8+ killer T cells produce IFNγ in the mid-to late acute phase of infection, beginning 4-5 days after infection. IFNγ is also produced by natural killer (NK) cells throughout the acute infection. CD4+ and CD8+ T cell and NK cell IFNγ production is substantially upregulated by the production of interleukin 18 (IL-18) or interleukin 12 (IL-12) by antigen presenting cells who act as early responders to parasitic infections (I. A. Khan, Matsuura, & Kasper, 1994; I. A. Khan et al., 1997).

IL-12p70 is considered biologically active due to its ability to polarize a proinflammatory (Th-1) immune response. IL-12p70 is a heterodimer composed of the IL-12p35 and IL-12p40 subunits which, as monomers, are considered inactive. IL-12p40 is also a subunit of IL-23, which

is an important cytokine produced during inflammatory disease (Duvallet, Semerano, Assier, Falgarone, & Boissier, 2011). Both human and mouse macrophages and DCs contain biologically active membrane-bound IL-12p70 that can be immediately released upon exposure to *Leishmania donovani* (Quinones *et al.*, 2000). During a *T. gondii* infection neutrophils are a significant source of pre-formed IL-12p70, allowing for the rapid release of biologically active IL-12p70 while bypassing the immediate requirement of *de novo* synthesis via transcription (Bliss, Butcher, & Denkers, 2000). Interestingly, a recent screen testing the response of murine BMDM to numerous potent TLR agonists (TLRs 1/2, 3, 4, 2/6, 7 and 9) found that nearly all TLR agonists were capable of inducing IL-12p40 production in the absence of IFNγ, but only LPS induced proinflammatory-polarizing IL-12p70 in the absence of IFNγ (Müller *et al.*, 2017).

IL-12 is an important cytokine for controlling *T. gondii* and *N. caninum* in mouse infections although there is growing evidence that the mouse immune system can mount an IFNγ-driven immune response in the absence of IL-12, and this may be protective against *N. caninum* and not *T. gondii* (Coombs et al., 2020; Ferreirinha et al., 2018; Hou et al., 2011; I. A. Khan et al., 1994; Scanga et al., 2002; Carolyn R. Sturge et al., 2013). Mice lacking IL-12p40 are still able to produce IFNγ within hours of LPS stimulation, although much less than WT mice indicating that TLR-driven IFNγ is still produced in the absence of IL-12p40 (Magram *et al.*, 1996). Likewise, infections of IL-12p40-/- mice with either *T. gondii* or *N. caninum* results in the production of IFNγ (Ferreirinha *et al.*, 2018; Jankovic *et al.*, 2002). Although very high levels of IL-12 and IFNγ mRNA in spleen cells (I. A. Khan et al., 1997) and protein in peripheral serum (Coombs *et al.*, 2020) are detected within hours of *N. caninum* infections in mice, *T. gondii* infections result in minimally to non-detectable levels of serum IFNγ until 2-3 dpi despite the production of IL-12. Recent work has demonstrated that acute *N. caninum* parasite burden at 3- and 7-days post

infection is lower in IL-12p40-/- mice that have been immunized with *N. caninum* tachyzoite lysate preparations compared to WT mice, and the control of parasite burden is dependent on IFNγ production (Ferreirinha *et al.*, 2018). One mechanism of IFNγ-mediated protection to *N. caninum* infections in mice involves CD8+ T cell immunity. CD8+ T cells are activated and expanded in *N. caninum*-infected mice as early as 4dpi (Correia *et al.*, 2015). Although transferring CD8+ T cells from infected mice prolonged survival of IL-12 unresponsive mice (C57BL/10 ScCr), CD8+ T cells from uninfected mice also exhibited limited protection mediated by IFNγ (Correia *et al.*, 2015). This implies that innate CD8+ T cells may contribute to *N. caninum* incompatibility in a murine host but CD8+ T cell-dependent immunity cannot protect mice that lack IL-12 signaling. Thus, while there is evidence for both IL-12/IFNγ inter-dependence in response to both species, there is also evidence that IFNγ, in the absence of IL-12, responds to acute infections of both parasites, and controls *N. caninum* infections.

1.5 MyD88 and IFNγ

MyD88 is a highly conserved downstream signaling protein that is important for mammalian TLR signal transduction for most TLRs and plays a central role in innate immune responses (Deguine & Barton, 2014). Mice lacking the TLR downstream signaling protein, MyD88 (MyD88-/-), fail to produce either IL-12p40 or IL-12p70 in response to *N. caninum* or *T. gondii.* In chapter 2 we detail our findings that only *T. gondii*-infected MyD88-/- mice uniformly succumb to infection while *N. caninum* infected MyD88-/-mice have increased parasite burden but are able to survive high doses of parasite. Our results support previous findings that mice lacking MyD88 have an increased susceptibility to *N. caninum* (Mineo, Benevides, Silva, & Silva, 2009)

but differ in our finding that MyD88-/- mice indeed can survive *N. caninum* infections. Although TLR-driven mechanisms of host response to *N. caninum* are less known, some work suggests TLR2 activation contributes to proinflammatory immune response in mice but further investigation is necessary to determine if TLR2 recognition contributes to immediate IFNγ production and early control of parasite burden in mice (Mineo *et al.*, 2010). In contrast, a large body of work has demonstrated that MyD88-dependent TLR signaling is critical for an effective host response to *T. gondii* in a mouse model (Hou *et al.*, 2011; Scanga *et al.*, 2002; Sukhumavasi *et al.*, 2008). Infections with either parasite in MyD88-/- mice results in IL-12p40 and IL-12p70 independent production of IFNγ 3-6 dpi, suggesting but this IFNγ production is not sufficient to rescue host survival during *T. gondii* infections. These findings support the hypothesis that MyD88 specific production of IL-12 and the subsequent IFNγ/IL-12 axis is critical for survival of *T. gondii* in mice and not *N. caninum*, addressed in chapter 2.

MyD88-independent host response mechanisms have also been explored during parasite infections in mice. TLR3 signals through downstream adaptor protein TRIF and plays a role, albeit differential, in host recognition for both *N. caninum* and *T. gondii* (Beiting *et al.*, 2014; Miranda *et al.*, 2019). Our recent work addressed in Chapter 2 demonstrated that TLR3 is not required for immediate IFNγ production or control of parasite burden during *N. caninum* infections in mice (Coombs *et al.*, 2020). Additionally, we found that TRIF-deficient mice mount a robust IFNγ-driven response within hours of infection and control parasite burden within the same timeframe as WT mice (Fig 3). Taken together, these findings suggest that important differences in innate immune responses underlie host specificity and that MyD88-independent IFNγ production is insufficient to confer protection to murine hosts infected with *T. gondii.* Additionally, IFNγ produced in a Myd88-inependent mechanisms is able to control parasite burden and increase

survival during *N. caninum* infections. The seemingly disparate host responses to these two similar parasites poses an interesting area of future study to elucidate the intricate innate immune response mechanisms and differences in TLR recognition and parasite antigens.

1.6 Innate cell mediated immunity

Ongoing discoveries and active research in the field result in a continuously shifting paradigm of innate immunity. Innate and adaptive immune responses are no longer viewed as separate, and instead are acknowledged to have overlapping cells and mechanisms (Gasteiger *et al.*, 2017). A number of innate lymphoid cells (ILCs) and innate-like cells including natural killer-like T cells (NKTs) share characteristics of both innate and adaptive compartment cells (Bennett, Round, & Leung, 2015; Vivier *et al.*, 2018). Myeloid cells and natural killer cells (NKs) can exhibit innate memory termed "trained immunity" via epigenetic and transcriptional changes or can exhibit lasting function changes after pathogen encounters (Domínguez-Andrés & Netea, 2020). Additionally, both mouse and human "natural antibodies" have been well described that recognize antigens prior to host exposure (Holodick, Rodríguez-Zhurbenko, & Hernández, 2017). Although there is some overlap in cellular function, innate cell-mediated immune (CMI) responses are critical for adequate adaptive responses to pathogens, including *T. gondii* (Ivanova *et al.*, 2019; Janeway & Medzhitov, 2002).

Several innate immune cells are known to respond to parasitic infections including inflammatory monocytes, dendritic cells (DC), macrophages, natural killer cells (NK), neutrophils, innate lymphoid cells (ILC's) and innate-like T cells (Gazzinelli *et al.*, 1994). Innate immune cells

can act as antigen presenting cells (APCs), have direct killing mechanisms, or produce key inflammatory cytokines. IFNγ is the main mediator of *N. caninum* infections but little is known about the cellular mechanism of IFNγ-mediated protection. Several innate cells have been identified that seem to contribute to host protection during *N. caninum* infections, but the mechanisms of IFNγ-driven control remain unknown. The differences in how *T. gondii* and *N. caninum* interact with host innate immune cells to elicit cell-mediated immunity provides a unique comparative system to investigate host-parasite interactions within the innate cell compartment.

1.6.1 Monocytes, macrophages and dendritic cells (DCs)

Monocytes are hematopoietic cells and are a substantial component of the innate phagocytic immune response to many pathogens including *T. gondii* (I. R. Dunay & Sibley, 2010). Circulating monocytes are highly responsive and quickly mobilize to the site of infection where they exert effector functions (Detavernier *et al.*, 2019). During *T. gondii* infections, inflammatory monocytes migrate to the site of infection and differentiate into macrophages and DCs that then rapidly respond to infection by producing cytokines like IL-12 (Goldszmid *et al.*, 2012). In chapter 2 we discuss differences in IL-12 levels during *N. caninum* and *T. gondii* infection, and in chapter 3 we compare populations of macrophages and dendritic cells which are the major sources of IL-12 during acute *T. gondii* infection (Hunter, Candolfi, *et al.*, 1995; Scanga *et al.*, 2002). Immature monocytes egress from the bone marrow and are recruited to the site of infection, a process that is dependent on the chemokine MCP-1 (CCL2). While monocytes and dendritic cells appear to enhance the innate immune response to *N. caninum* (Abe, Tanaka, Ihara, & Nishikawa, 2014), it is unknown if early recruitment of monocytes and macrophages to the site of infection is required for control of *N. caninum* burden.

1.6.2 Innate lymphoid cells

Innate lymphoid cells (ILC's) are a recent classification of a group of innate cells that are functionally comparable to T cells but do not undergo clonal selection and expansion (Eberl, Colonna, Di Santo, & McKenzie, 2015). ILC's are classified into three subgroups (Type I, II, and III) based on their cytokine expression profiles. ILC's are activated by signals in the local extracellular *milieu* including cytokines and microbial products. Group I ILC's constitutively express the T-bet transcription factor, a key factor driving Th-1 differentiation (Robinette & Colonna, 2016), and produce IFNγ in response to stimulation from myeloid-derived cytokines like IL-12 or IL-18 (Vivier *et al.*, 2018). The most well-described ILCs are natural killer cells which are classified into the type-I ILC subgroup (Vivier *et al.*, 2018). In mice, group I ILC's are NK cells and ILC1 cells, and while both produce IFNγ ILC1s lack cytotoxicity. NK cells have long been recognized for their role in early immune responses to apicomplexan parasites including *T. gondii* (Hauser, Sharma, & Remington, 1983; Ivanova *et al.*, 2019). Conventional NK cells are thought to be the only ILC with cytotoxic ("killer") mechanisms and are often described as the innate counterpart of CD8+ cytotoxic cells (Vivier *et al.*, 2018). ILC1 cells produce IFNγ and TNFα in response to *T. gondii* infections (I. R. Dunay & Diefenbach, 2018). Recent work demonstrated that *T. gondii* infections induce NK cells to permanently convert into ILC1-like cells that gain circulatory abilities and persist after resolution of infections (Park *et al.*, 2019). The ILC1-like cells exhibited expansion during repeated *T. gondii* infections. This fact, combined with the observations that NK cell populations decrease over time during *T. gondii* infections while ILC1-like cells increase, suggests that *T. gondii* infections are capable of altering the functional programming of important proinflammatory innate immune cells.

1.6.3 Natural killer (NK) cells

Natural killer cells are important early responders to parasitic infections based on potent cytotoxic responses and also produce key inflammatory cytokines like IFNγ. NK cell responses are not antigen-specific and are guided by complex inhibitory and activating receptors and interactions. Classical NK cell mechanisms require activation after microbial insult reliant on by cytokines like IL-12, IL-15 and IL-18 that are produced by macrophages or DCs (Newman & Riley, 2007). NK-derived IFNγ is critical for IL-12 production by monocyte-derived DCs during *T. gondii* infections (Goldszmid *et al.*, 2012; Ma *et al.*, 1996). It is now appreciated that NK cells can also be activated by directly sensing PAMPS via TLRs and other pattern recognition receptors (Becker *et al.*, 2003; Boysen, Klevar, Olsen, & Storset, 2006; Sivori *et al.*, 2004).

NK cells are important sources of IFNγ during acute *T. gondii* infections and numerous groups have reported that NK-derived IFNγ is produced via an IL-12-dependent mechanism during acute *T. gondii* infections (Gazzinelli *et al.*, 1994; Hunter, Bermudez, Beernink, Waegell, & Remington, 1995; Hunter, Candolfi, *et al.*, 1995; Hunter, Chizzonite, & Remington, 1995; Sher, Oswald, Hieny, & Gazzinelli, 1993). Recent work determined that NK cells from *T. gondii*-infected mice produced significantly less IFNγ than NK cells in uninfected mice (Sultana *et al.*, 2017), suggesting *T. gondii* effectors might impact NK function to increase parasite survival. While *T. gondii* infections result in IFNγ production in response to IL-12 production, recent work demonstrated that *N. caninum* can directly stimulate NK cells to produce IFNγ and also exert cytotoxicity against *N. caninum*-infected fibroblasts (Boysen *et al.*, 2006; Nishikawa *et al.*, 2010). Further investigation is necessary to determine if *N. caninum* can stimulate NK cells *in vivo*. The ability of NK cells to act as effector cells in addition to direct killing ability make them a potentially

valuable tool in disease treatment. Thus, understanding how parasites manipulate effector function and avoid cellular killing mechanisms is an important avenue for further exploration.

1.7 Unconventional T cells

Innate-like T cells, or unconventional T cells, develop both intra- and extrathymically. These T-cell lineage cells include the heterogenous family of natural killer T cells (NKTs) and γδ T cells (Pellicci, Koay, & Berzins, 2020). Unconventional T cells expand the repertoire of antigen classes that are recognized and are often described as cells that connect innate and adaptive compartments (Hedges, Lubick, & Jutila, 2005; Holderness, Hedges, Ramstead, & Jutila, 2013). Unconventional T cells combined with ILCs like ILC1 and NK cells constitute significant portions of IFNγ producing cells early during infection to numerous pathogens (Fig 3). They express TLRs and are responsive to effector signals via cytokine and NK activating receptors.

1.7.1 γδ T cells

γδ T cells are important epithelial-resident cells that are active in pathogen response and control and can function as an early innate responder to parasite infections (Inoue, Niikura, Mineo, & Kobayashi, 2013). γδ T cells have been described in multiple species, including mice, humans, and cattle although there are important differences in populations and function (Holderness *et al.*, 2013). In humans and mice, γδ T cells account for 5% of circulating lymphocytes but are 60-70% of circulating lymphocytes in cattle (Guzman et al., 2014; Hedges et al., 2005; B. L. Plattner & Hostetter, 2011). Human and bovine γδ T cells produce cytokines including IFNγ in response to

experimental antigen stimulation and respond to unconventional antigens including protein and non-protein antigens, and soluble antigen fractions (B. L. Plattner & Hostetter, 2011). Neither bovine or human γδ T cells are MHC I or II restricted and in addition to γδTCR expression, they also express a variety of TLRs in humans, mice and ruminants (Pietschmann *et al.*, 2009). Both human and bovine γδ T cells respond directly to PAMP stimulation in the absence of APCs, but the PAMP-induced upregulation of chemokines is substantially more robust in bovine γδ T cells compared to human γδ T cells (Hedges *et al.*, 2005). Bovine immune systems do not appear to have functioning Foxp3 regulatory T cells, and instead there is growing evidence that γδ T cells function as a major subset of regulatory T cells in the peripheral blood in cattle.

1.7.2 Natural killer T cells

Natural killer T cells (NKTs) are a subset of unconventional T cells with diverse functional capability. As the name suggests, NKTs exhibit both NK and T cell characteristics. NKT cells can activate both innate and adaptive immune responses and uniquely identify unconventional lipid-based antigens through CD1d antigen presentation. NKTs are activated to produce significant amounts of immunomodulating cytokines, as well as exerting cytotoxicity to numerous microbes (Wu & Van Kaer, 2011). NKTs can be subdivided broadly into two subgroups based on their TCR repertoire. While T cells exhibit significant TCR diversity, NKTs are more restricted in TCR expression. NKTs Type 1 NKTs are called invariant NKTs (iNKTs) based on limited diversity in TCRαβ receptor expression while type II NKTs exhibit a more diversity (Pellicci *et al.*, 2020; Shissler, Singh, & Webb, 2020). Both type I and II are CD1d-restricted and are considered classical

and non-classical NKTs, respectively (Fig 4). Invariant NKTs can exert regulatory functions during immune responses stimulating either TH1 or TH2 immune functions by producing either IFNγ, or IL-4 (Liao, Zimmer, & Wang, 2013) and can be activated by CD1d-antigen presentation or by cytokines like IL-12 and IL-18 (B. J. Wolf, Choi, & Exley, 2018). Additionally, iNKTs induce APCs to produce IL-12 in an IFNγ-dependent manor (Y. F. Yang, Tomura, Ono, Hamaoka, & Fujiwara, 2000). Invariant NKTs (iNKTs) upregulate expression of multiple TLRs after TCR activation, and these TCR-activated iNKTs can be directly stimulated with TLR ligands to produce cytokines (Kulkarni *et al.*, 2012). For example, iNKTs can be directly activated via TLR4 engagement to increase IFNγ production (Kim, Kim, Kim, Oh, & Chung, 2012) Thus, iNKTs can be stimulated directly by TLR, indirectly by antigen presentation, or co-stimulated to produce IFNγ in response to TLR activation and or/TCR engagement. NKT cells are also thought to express CD44, CD122, CD25 and CD69 similarly to T cells with memory function (Wu & Van Kaer, 2011) and may also have memory-like function in addition to also exerting innate-like function.

In mice, type I NKTs (iNKTs) are usually CD4+ while type II NKTs can be CD4+, CD8+, or neither (Liao *et al.*, 2013). This differs from humans where Type I NKTs that are CD8+ have been identified (Liao *et al.*, 2013). There is a third subtype of NKTs although less characterized than type I and II NKTs (Kronenberg & Gapin, 2002) now referred to as NKT-like cells (Godfrey, MacDonald, Kronenberg, Smyth, & Van Kaer, 2004). Unlike NKTs, NKT-like cells are CD1d-independent and although similarly produce IFNγ, NKT-like cells do not do not produce IL-4 (Godfrey *et al.*, 2004; Wang *et al.*, 2015). This suggests NKT-like cells do not polarize TH2 responses and instead promote a TH1 (proinflammatory) program. Recent work identified a subset

of NKT-like cells that are CD8+ and exert cytotoxic killing activity against antigen presenting DCs (Wang *et al.*, 2015).

1.8 CD1d antigen presentation

CD1 family proteins are structurally similar to MHC class I proteins and also function as antigen presenting molecules that are important during innate immunity. Although similar to MHC class I antigen presentation, the antigen-binding pockets of CD1 molecules are deep and hydrophobic, allowing CD1 family proteins to bind to lipid and non-lipid antigens. CD1 family proteins are known to present lipid, lipopeptide, glycolipid and aromatic compound antigens to T cells and NKT cells, including γδ T cells (Skold & Behar, 2003). Notably, iNKTs (type I NKTs) require CD1d for development, and mice lacking CD1d (CD1d-/-) lack iNKTs (Kumar *et al.*, 2017; Skold & Behar, 2003). CD1 genes are present in most mammalian species and based on homology to 4 distinct human genes are grouped into group 1 (CD1a, CD1b, and CD1c) and group 2 (CD1d). Interestingly, group 1 CD1 proteins are present in humans and cattle but not mice or rats. Conversely, group 2 CD1d is functional in humans and mice, and not in bovine species. CD1d-restircted NKT cells have a well-established role in host defenses to invasive microbes and activate innate and adaptive immune responses (Skold & Behar, 2003). Yet it appears that bovine immune response lacks this mechanism (Nguyen *et al.*, 2013; Van Rhijn *et al.*, 2006). In mice and humans but not cattle, CD1 proteins present ligands to γδTCR (Holderness *et al.*, 2013). CD1d deficient mice (CD1d-/-) are acutely susceptible to sublethal *T. gondii* infections characterized by significant increases in serum IFNγ and not IL-12p40 during acute infections, measured 8dpi. CD1d-/- mice present with modest increases in parasite burden compared to WT and increased numbers of

activated CD4+ cells. Depletion of CD4+ cells increases survival of CD1d-/- *T. gondii* infected mice (Smiley *et al.*, 2005). Thus, it appears that in the absence of CD1d-restricted mechanisms, *T. gondii* infections result in significantly higher production of IFNγ, and lethal susceptibility. This combined with the increase in parasite burden in CD1d-/- infections supports the existence of an unknown parasiticidal Cd1d-restricted mechanism. Early work identifying a MHCII-independent role for CD4+ NKTs in generating CD8+T cell effector cells during *T. gondii* infections also supports this hypothesis (E. Y. Denkers, Scharton-Kersten, Barbieri, Caspar, & Sher, 1996). NK and NKT cells have been identified as primary effector cells in bovine infections. Although NK and NKT cells are important effector cells during *N. caninum* infections, deletion of NK and NKT cells (not NK cells alone) resulted in increased parasite burden and depletes activation of CD4+T cells.

1.9 A comparative roadmap

Thanks to decades of substantive *T. gondii* research, we understand a great deal about *T. gondii* infections in the mouse model. By combining mechanistic information from *T. gondii* infections with what is known (or unknown, as is often the case) in corresponding *N. caninum* infections, we have identified key cell-mediated immune mechanisms to test for potential differences that alter host susceptibility to *N. caninum*. We know that early production of the chemokine monocyte chemoattractant protein-1 (MCP-1; also known as CCL2), is required for monocytes to be recruited from the bone marrow to the site of infection, and that this is important for host survival during a *T. gondii* infection. We also know that dendritic cells and macrophages produce IL-12 in response to *Toxoplasma* infections through TLR activation. *T. gondii*-induced

IL-12 drives production of Interferon gamma from cells like ILCs and NK cells. Although neutrophils produce IFNγ in response to *T. gondii* infections, the mechanisms are independent of IL-12 production. IFNγ is usually detectable usually 2-3 days after *T. gondii* infection. Interferon gamma is considered the master regulator of inflammation, and its production is tightly regulated. It is important for upregulation of MHC II molecules and activating and upregulating cytotoxicity in macrophages. Both IL-12 and IFNγ are required for survival of *Toxoplasma* and *N. caninum* infections. This work examines these known *T. gondii* mechanisms (Fig 5) by comparing infections of *T. gondii* and *N. caninum* to identify host responses unique to *N. caninum*.

1.10 Figures

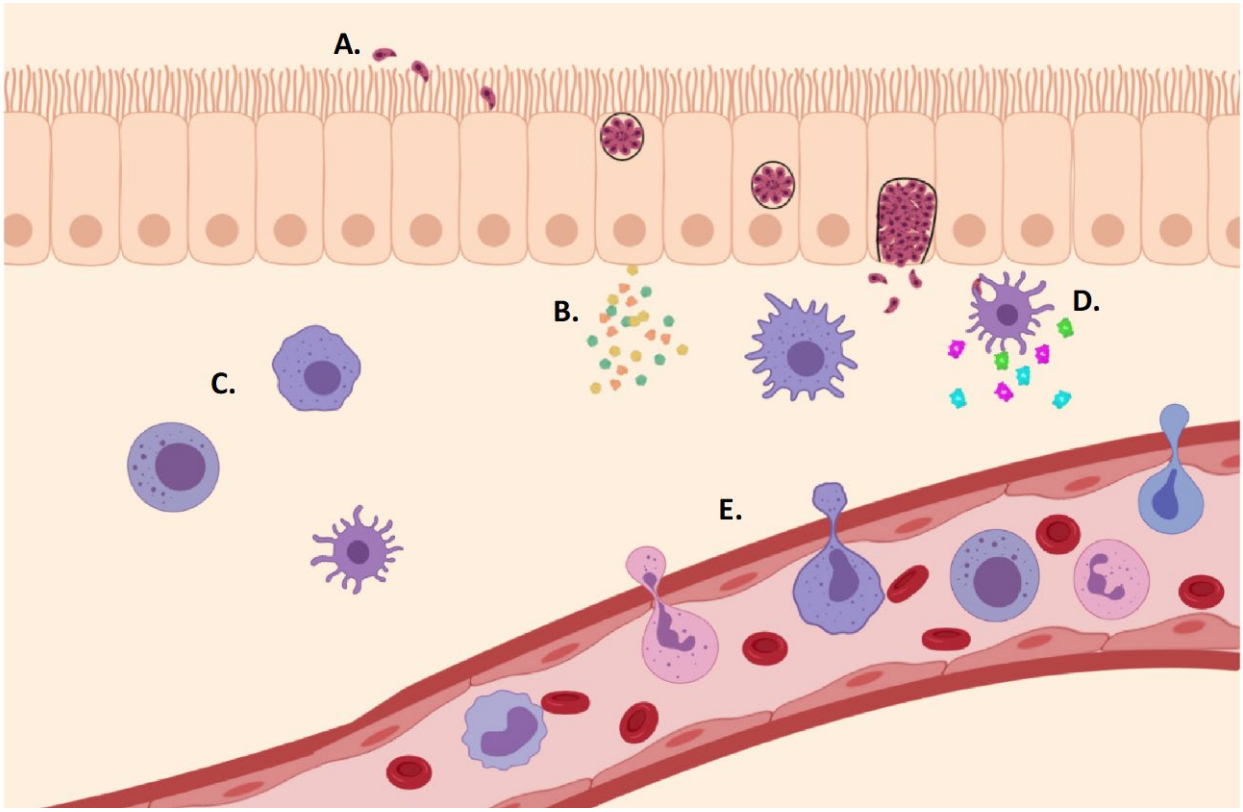

Figure 1 Early infection and innate immune response

T. gondii tachyzoites actively invade host cells where they form a protective vacuole called a parasitophorous vacuole (PV) within the host cell. They replicate inside of the PV protected from intracellular killing mechanisms. Ultimately, they egress and migrate to invade neighboring cells. B) Local cells respond to _T. gondii_ infections by producing chemokines that activate and attract tissue resident innate cells. C) Tissue dwelling innate cells like dendritic cells and macrophages respond to local infection via phagocytic and/or TLR mechanisms. D) DCs and macrophages produce cytokines in response to _T. gondii_ infections including IL-12. E) Hematopoietic cells infiltrate the site of infection in response to chemokine and cytokine signals.

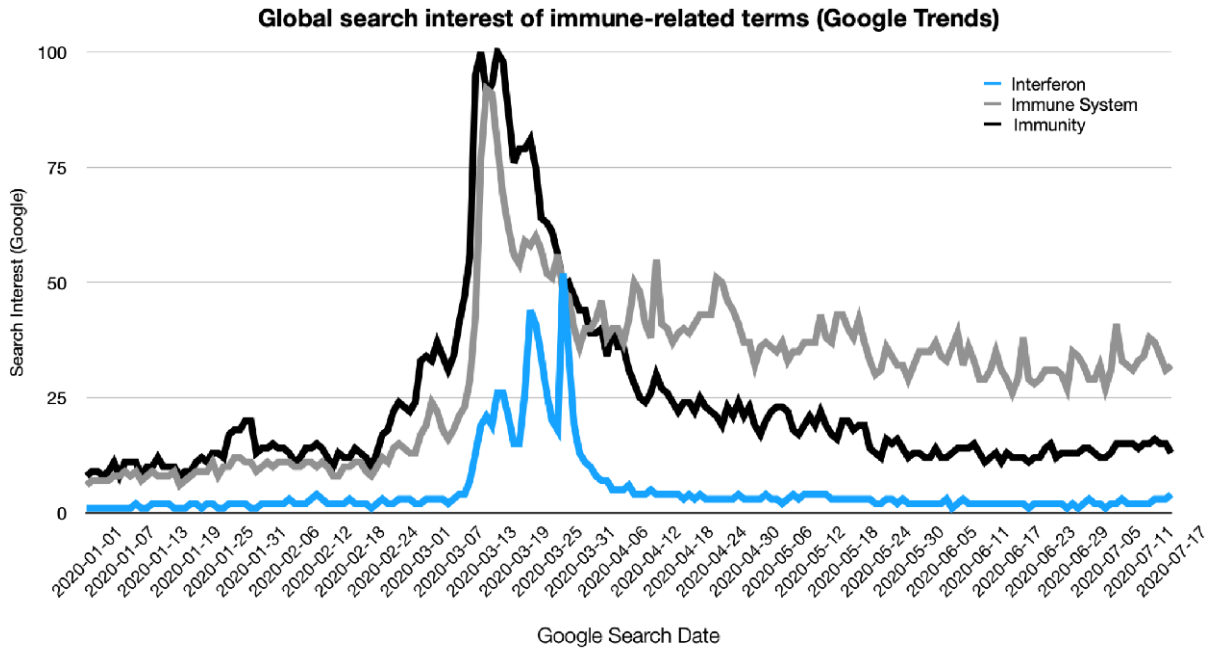

Figure 2 Global search interest of immune-related terms

Global interest in search terms Interferon (blue) Immune system (gray) and Immunity (black) from 01/01/2020 to 07/17/ 2020. Data is from google trends and is indexed from 0-100 based on the popularity of search term compared with all other search terms in a given timeframe. A value of 100 indicates peak popularity compared with other search trends within the that timeframe. Source: Google Trends

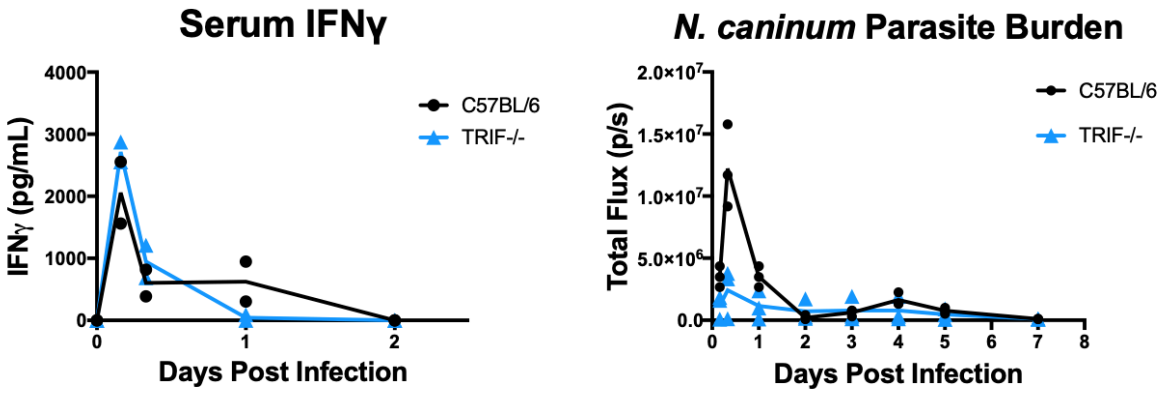

Figure 3 *N. caninum* infection in TRIF knockout mice

Bioluminescent imaging and IFNγ concentration in *N. caninum* infections in or TRIF knockout mice and IFNγ concentration. 6-8 week old TRIF knockout ($TRIF^{-/-}$, blue) or C57BL/6 (black) mice were injected IP with 10^6 Nc1:Luc tachyzoites. A) Serum IFNγ (pg/mL) during the first 2 days of infection, analyzed by ELISA. A two-way RM ANOVA alpha=0.05 with Dunnett multiple comparisons test determined no significance. B) Quantification of bioluminescent imaging through 7 days of infection (photons/s). Data was log transformed and two-way RM ANOVA matching: stacked and alpha=0.05 with Dunnett multiple comparisons test. P value adjusted for multiple comparisons. Statistical significance between C57BL/6 and $TRIF^{-/-}$ infections at 8 h.p.i. p=0.0067.

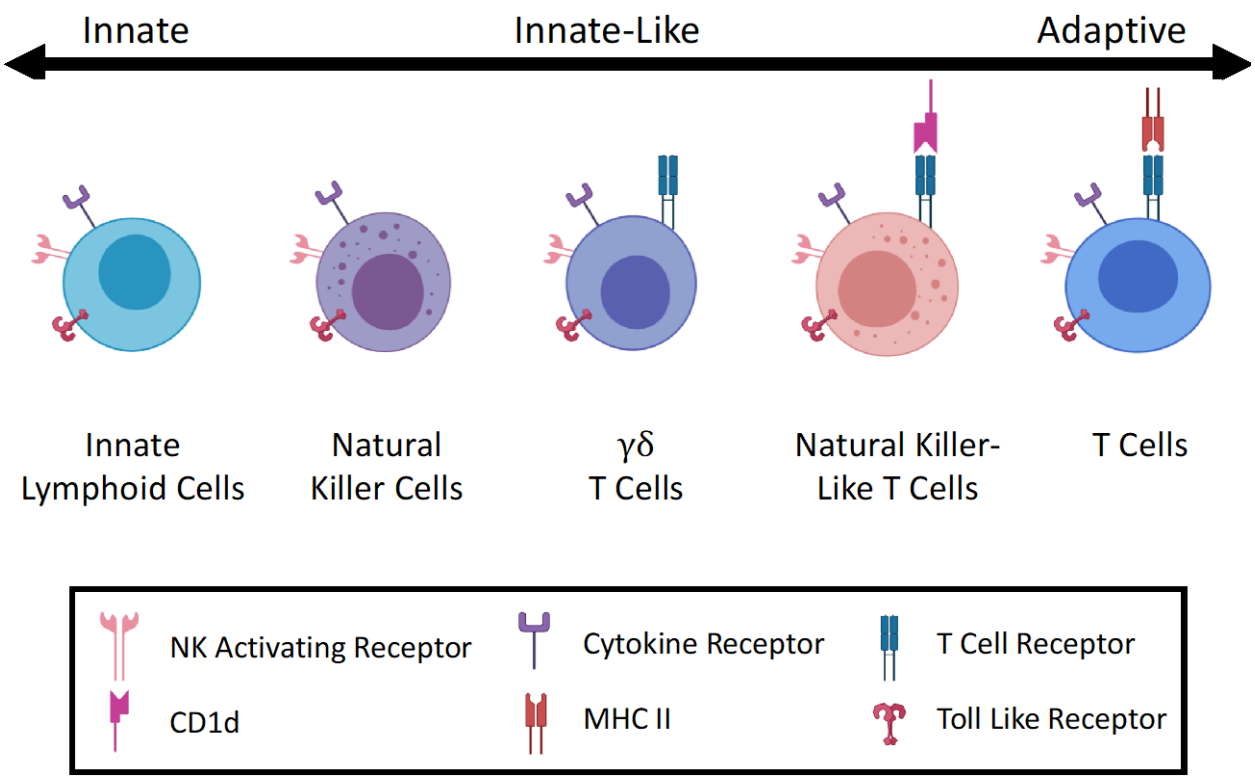

Figure 4 Interferon gamma producing cells

Adapted from (Fan & Rudensky, 2016)

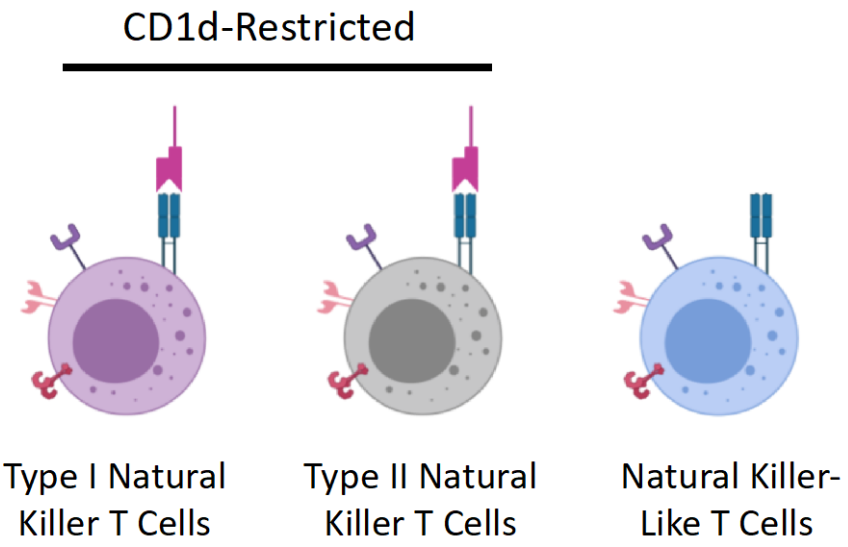

Figure 5 Natural killer T and natural killer-like T cells

Adapted from (Fan & Rudensky, 2016; Kumar *et al.*, 2017). NKT cells express diverse TCRs, and can be CD1d-restricted. NKT-like cells are CD1d-independent and the antigen restriction mechanisms are unknown.

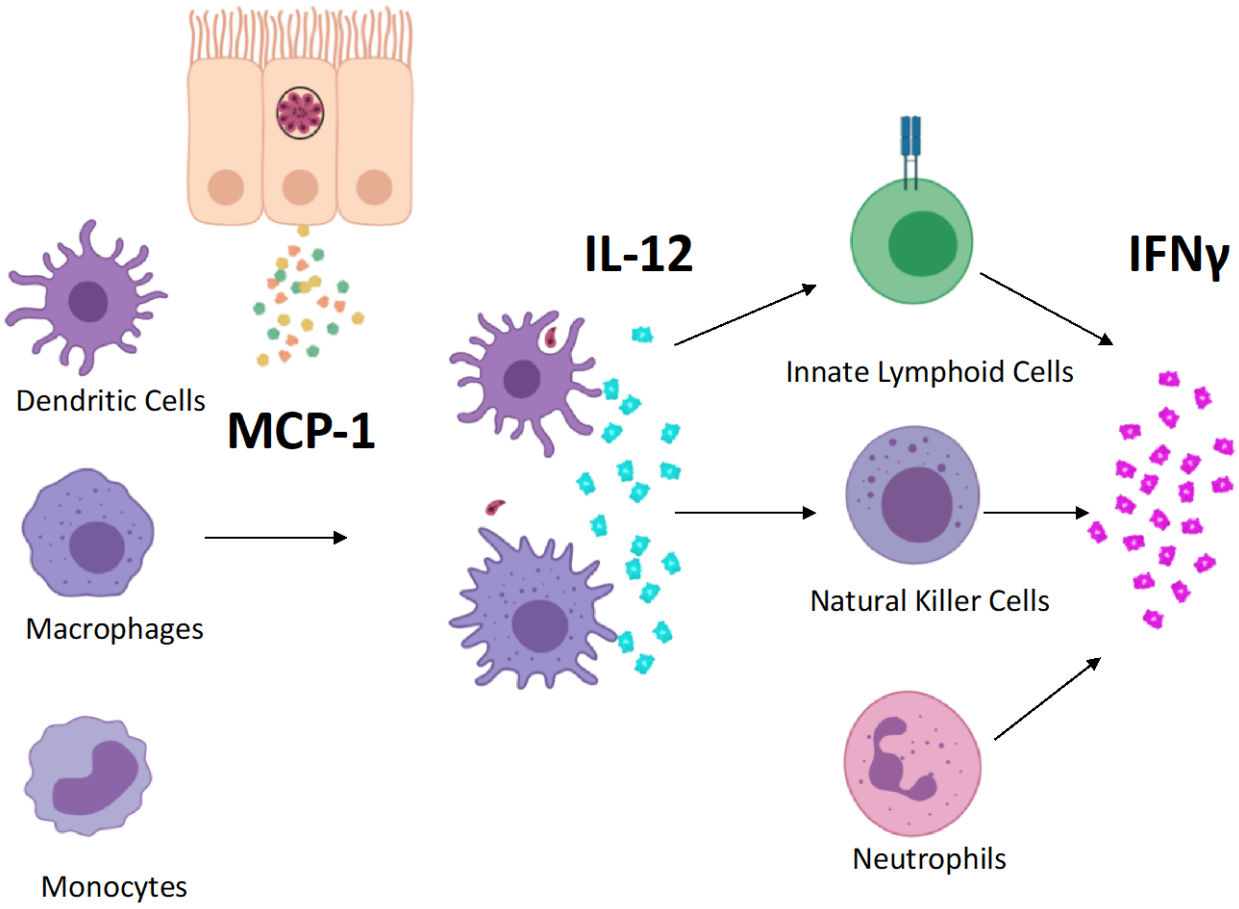

Figure 6 Early immune response to *T. gondii* in mice

MCP-1 production recruits monocytes to the site of infections. Monocyte-derived macrophages and dendritic cells produce IL-12 in response to *T. gondii* infections. IL-12 induces IFNγ production within 2-4 days after infection. Neutrophils produce IL-12 independent IFNγ. IFNγ production upregulates numerous anti-parasitic immune responses to control *T. gondii* proliferation.

2.0 Differences in murine host compatibility between *T. gondii* and *N. caninum* is determined by immediate induction of IFNγ

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2.1 Preface

Rodents are critical for transmission of *Toxoplasma gondii* to the definitive feline host via predation and this relationship has been extensively studied as a model for immune responses to parasites. *Neospora caninum* is a closely related coccidian parasite of ruminants and canines, but is not naturally transmitted by rodents. We compared mouse innate immune responses to *N. caninum* or *T. gondii* and found marked difference in cytokine levels and parasite growth kinetics during the first 24 hours post-infection (hpi). *N. caninum*-infected mice produced significantly higher levels of IL-12 and IFNγ as early as 4hpi, but IFNγ was significantly lower or undetectable in *T. gondii*-infected mice during the first 24hpi. "Immediate" IFNγ and IL-12p40 production was not detected in MyD88$^{-/-}$ mice. However, unlike IL12p40$^{-/-}$ and IFNγ$^{-/-}$, MyD88$^{-/-}$ mice survived *N. caninum* infections at the dose used in this study. Serial measures of parasite burden showed MyD88$^{-/-}$ were more susceptible to *N. caninum* infections than WT mice, and control of parasite burden correlated with a pulse of serum IFNγ 3-4 days post-infection in the absence of detectable

IL-12. Immediate IFNγ was partially dependent on the *T. gondii* mouse profilin receptor TLR11 but ectopic expression of *N. caninum* profilin in *T. gondii* had no impact on early IFNγ production or parasite proliferation. Our data indicate that *T. gondii* is capable of evading host detection during the first hours after infection while *N. caninum* is not, and this is likely due to early MyD88-dependent recognition of ligands other than profilin.

2.2 Author summary

Most parasite species exhibit selective host ranges and infect only one or a small number of intermediate hosts. There are few examples of molecular host range determinants in eukaryotic pathogens, but they are critical for our understanding of the barriers that prevent host jumping or host range expansion. *Toxoplasma gondii* and *Neospora caninum* are parasites with extensive similarity at the genetic level, but with a markedly distinct ability to infect rodents in the wild and in the laboratory (Collantes-Fernandez et al., 2012; English et al., 2015). The mechanisms that determine this difference in host range are unknown. Here we show that *N. caninum* infection is rapidly controlled in mice while *T. gondii* is not due to a robust and rapid induction of protective host cytokines. These data suggest that the host range differences between *T. gondii* and *N. caninum* are due to previously unrecognized mechanisms of immediate recognition of *N. caninum* and avoidance or suppression of that response by *T. gondii.*

2.3 Introduction

In contrast to many viruses and a handful of bacterial pathogens, very little is known about the molecular biology of host-pathogen compatibility in eukaryotic parasites, including *Toxoplasma gondii*. Apicomplexan parasites are formidable eukaryotic pathogens responsible for diseases including malaria, cryptosporidiosis, neosporosis, and toxoplasmosis, which kill millions of people and animals worldwide each year (S. Berger, 2010; S. A. Berger, 2011; Carme, Demar, Ajzenberg, & Dardé, 2009; Kemp et al., 2013). Among these important intracellular pathogens, *Toxoplasma gondii* is the only species capable of infecting, causing disease in, and being transmitted by every warm-blooded animal tested to date (Boothroyd, 2009; Carlson-Bremer *et al.*, 2015; Innes, 2010). *N. caninum* is the causative agent of neosporosis, a disease that causes fetal death and neonatal mortality (Silva & Machado, 2016) in bovine (Dubey et al., 2006), ovine (González-Warleta *et al.*, 2018), and canine hosts (Dubey, Carpenter, Speer, Topper, & Uggla, 1988). *N. caninum* is remarkably similar in morphology and genomic synteny (85%) to *T. gondii,* (Besteiro et al., 2011; Kemp et al., 2013; A. J. Reid et al., 2012) but there are important differences in their disease pathology. Virulent strains of *T. gondii* are known to cause disease worldwide in all animals (Boothroyd, 2009; Innes, 2010), but *N. caninum* is restricted by a comparatively smaller host range. *N. caninum* causes disease in a limited number of closely related ruminant or canine hosts (Dubey *et al.*, 2006; Williams & Trees, 2006), although *N. caninum* is far more successful in cattle which are considered poor hosts for *T. gondii*.(Dubey *et al.*, 2005; Esteban-Redondo & Innes, 1997; Nunes *et al.*, 2015) Mice are an important natural host for *T. gondii* and infections have been well documented in the laboratory and in nature (Dubey et al., 2014; Gazzinelli et al., 1994; Suzuki et al., 1988; Suzuki et al., 2000; Felix Yarovinsky, 2014), and mice infected with even 1 tachyzoite of virulent *T. gondii* strains will become morbid (A. Khan, Taylor,

Ajioka, Rosenthal, & Sibley, 2009; Reese, Zeiner, Saeij, Boothroyd, & Boyle, 2011; Saeij, Boyle, Grigg, Arrizabalaga, & Boothroyd, 2005). Alternatively, mice infected with up to 1 million tachyzoites of *N. caninum* strains do not exhibit any observable morbidity (Dubey et al., 2007; English et al., 2015; Mols-Vorstermans, Hemphill, Monney, Schaap, & Boerhout, 2013). A large body of work has shown that, in mice, proinflammatory responses are the only effective immune response to *N. caninum,* and control is dependent on cytokine production (Aguado-Martínez, Basto, Leitão, & Hemphill, 2017; Baszler et al., 1999; I. A. Khan et al., 1997). Interferon gamma (IFNγ) has been identified as an important immune-modulating cytokine in mice and cattle during *N. caninum* infections (Baszler *et al.*, 1999; Innes *et al.*, 1995) and investigations in both mice and cattle show that production of IFNγ is an important protective mechanism during neosporosis (Correia *et al.*, 2015; I. A. Khan *et al.*, 1997). While IFNγ can protect against abortion in cattle, high levels of IFNγ at the materno-fetal interface increase fetal death (López-Gatius *et al.*, 2007; Maley *et al.*, 2006). IFNγ is required for acute control of *T. gondii* and is dependent on an IFN-γ-driven, cell-mediated immune (CMI) responses where production of IFNγ is primarily derived from interleukin-12 (IL-12) stimulated Natural Killer cells (NK) and T lymphocytes (Gazzinelli, Mendonca-Neto, Lilue, Howard, & Sher, 2014). IFNγ is also required for resistance to *N. caninum* in mice (Schroder, Hertzog, Ravasi, & Hume, 2004), but the precise mechanisms of resistance and when this cytokine is most critical for resistance is not known. Our previous work identified a dramatic difference in infection outcome in Balb/c mice between an avirulent *T. gondii* strain Tg:S1T:Luc:DsRed (TgS1T:Luc; generated from a cross between a Type II and Type III T. gondii strain; (J. P. Saeij et al., 2006)) and *N. caninum* Nc1:Luc (English *et al.*, 2015). After infection with 10^6 tachyzoites, parasite-derived luciferase signal increased similarly between strains during the first 24 hpi, followed by a rapid decrease of *N. caninum* bioluminescent signal while *T. gondii*

signal continued to increase (English *et al.*, 2015). The immediate control of *N. caninum* parasite proliferation *in vivo* was previously unappreciated, despite previous work demonstrating *N. caninum*-derived bioluminescent signal decreased between 1 and 2 days post infection (Collantes-Fernandez *et al.*, 2012) and the extensive use of a mouse model to develop vaccines and treatment for bovine neosporosis (Hemphill, Aguado-Martinez, & Muller, 2016; Horcajo et al., 2016). The mechanism(s) underlying the dramatic difference between *T. gondii* and *N. caninum* growth during the acute infection are unknown. Here, we compare host innate immune responses and parasite proliferation kinetics *in vivo* to identify differences in the host response to *N. caninum* and *T. gondii* during the first 24 hpi that determine this dramatic difference in infection outcome.

2.4 Results

2.4.1 The mouse host response controls *N. caninum* proliferation within 24 h post-infection

In vivo bioluminescent imaging (BLI) is a powerful tool to detect differences in pathogen burden during live infections in mice (Dellacasa-Lindberg, Hitziger, & Barragan, 2007; Miller *et al.*, 2013; Saeij *et al.*, 2005). Parasite burden corelates directly with photon flux, and is calculated by quantifying photonic emission after luciferase substrate injection in live animals (Subauste, 2012). By employing this non-invasive technique, we were able to compare the kinetics of *T. gondii* and *N. caninum* proliferation within a single mouse throughout infection. First, we selected *T. gondii* strains with well characterized differences in virulence, selecting high (TgS23:Luc) and low (TgS1T:Luc) virulence *T. gondii* strains to compare with the *N. caninum* strain Nc1:Luc (Saeij *et al.*, 2005). We selected these F1 progeny for our comparisons because S23 has the “virulent

allele" for 5 genes known to impact disease outcome and mortality in mice, while S1T has the "avirulent allele" at these same 5 loci (Grigg, Bonnefoy, Hehl, Suzuki, & Boothroyd, 2001; J. P. Saeij et al., 2006). To compare these strains directly we infected mice intraperitoneally (IP) with 10^6 luciferase-expressing tachyzoites and performed *in vivo* BLI (**Fig 7A**). Quantification of bioluminescent images (**Fig 7B**) showed that both *T. gondii* strains and *N. caninum* grew at similar rates during the first 20 hpi, after which both *T. gondii* strains continued to proliferate and *N. caninum* was controlled. We observed no statistically significant differences in parasite burden in the first 20 hour comparing all 3 infections (**Fig 7B**). We observed statistically significant differences in parasite burden between TgS1T:Luc and TgS23:Luc, with the latter more virulent *T. gondii* strain having higher parasite burden compared to TgS1T:Luc strain by 72 hpi (**Fig 7B**). Our results were replicated in a second experiment again using 3 mice per group (data not shown) and are consistent with known virulence differences between the *T. gondii* strains (J. P. Saeij et al., 2006) as well as *N. caninum* (Goodswen et al., 2013). This suggested that *N. caninum* is limited in its capacity to establish infections in mice after the first 20 h, even compared to the less virulent *T. gondii* strain (TgS1T) (Saeij *et al.*, 2005; Sibley & Boothroyd, 1992; Sibley, LeBlanc, Pfefferkorn, & Boothroyd, 1992). To confirm that *N. caninum* parasite growth is controlled within the first 24 hours of infection, we performed 3 additional experiments comparing TgS1T:luc and Nc1:Luc BLI during the acute infection (**Fig 8A** TgS1T:Luc n=2, Nc1:Luc n=3; **Fig 14** n=3 per parasite species; data not shown n=3 per parasite species). *N. caninum* parasite burden was significantly lower compared to *T. gondii* ($P<0.001$) during days 3-6 pi (**Fig 8A**) demonstrating the reproducibility of this observation. Together, these results demonstrate that *N. caninum*-derived luciferase signal peaks at 24 hpi and therefore appears to be rapidly controlled, while *T. gondii* continues to proliferate for up to 6-8 dpi. Importantly, when we compared *in vitro* growth

rates of avirulent TgS1T:Luc and Nc1:Luc strains in Human Foreskin Fibroblasts (HFF) we found no significant differences in parasite derived fluorescence at 24 or 48 hpi (**Fig 7C**).

2.4.2 *N. caninum* infections in mice induce significantly higher levels of pro-inflammatory cytokines within the first 24 hours of infection compared to *T. gondii*

Given the clear differences in proliferation *in vivo* between *N. caninum* and *T. gondii* (**Fig 7A,B**), we hypothesized that the host innate response might be responsible for the observed *in vivo* proliferation differences. To test this hypothesis, we performed an initial screen using a Luminex multiplex assay to compare levels of 32 mouse chemokines and cytokines by comparing analyte fluorescence in *T. gondii* to *N. caninum* infections. Mice (n=3 per species) were infected IP with 10^6 tachyzoites of either TgS1T:Luc or Nc1:Luc and serum or peritoneal lavage samples were collected at 14 hpi and screened for analyte differences between parasite species (**Fig 15A,B**). These comparisons revealed that *N. caninum* infection induced a distinct cytokine/chemokine profile very early after infection compared to *T. gondii*, including several critical inflammatory cytokines known to be important for controlling intracellular parasite infections. Of note, we observed large (>10-fold) differences in serum IFN-γ, IL-12p70, IL-12p40, and CCL2 (MCP) compared to *T. gondii*-infected mice, while other queried cytokines like IP-10 and MIG were either poorly induced or induced similarly by both species (**Fig 15A,B**). Next, we wanted to screen additional time points to see how early these changes were detectable in our cytokine panel and so we tested 2 mice per parasite species and timepoint at 4 or 8 hpi and analyzed serum with the same Luminex panel. We observed similar differential production of cytokines at both 4 and 8 hpi comparing *T. gondii* and *N. caninum* (**Fig 15C,D**). This screen suggested that IL-12 and IFNγ

production occurred in response to *N. caninum* infection as early as 4 hpi, while these cytokines were lower or undetectable at these time points in *T. gondii*-infected mice (**Fig 15**).

To confirm these results, we infected 3 BALB/C mice per group with $1x10^6$ Nc1:luc or TgS1T:Luc tachyzoites, collected serum and measured IL-12p40 and IFNγ at multiple timepoints in the first 24 hours of infection by ELISA. In *N. caninum*-infected mice we detected serum IL-12p40 (**Fig 8B**) and IFNγ (**Fig 8C**) as early as 4 hpi, while we detected significantly less serum IL-12p40 ($P<0.001$; **Fig 8B**) and detected no IFNγ (**Fig 8C**) in *T. gondii*-infected mice during the first 24 hpi. To confirm that TgS1T:Luc infected mice did not induce early IFNγ, the experiment was repeated twice with similar results (**Fig 15B** n=3 per parasite species; and n=3 per parasite species *data not shown*).

To ensure that this growth and cytokine response phenotype was not specific to BALB/C mice, we also compared *T. gondii* and *N. caninum* infections in C57BL/6 mice (**Fig 8D**, n=3 per parasite species). We observed that similar to BALB/C infections, C57BL/6 mice controlled *N. caninum* infections within 24 hpi, and *N. caninum* burden was significantly lower at 5 dpi in C57BL/6 mice. This confirms the findings of others that BALB/C and C57BL/6 mice have similar susceptibility to *N. caninum* infection (Baszler *et al.*, 1999; Ellis *et al.*, 2010; Mols-Vorstermans *et al.*, 2013). We also observed cytokine profiles in C57BL/6 mice that were similar to those observed in BALB/C mice. Specifically, we detected significantly higher levels of Il-12p40 (**Fig 8E**) and IFNγ (**Fig 8F**) during the first 24 hpi of *N. caninum* infections compared to *T. gondii* ($P=<0.01$, $P=<0.001$) and, as for BALB/C, IFNγ was not detectable in *T. gondii*-infected mouse serum in the first 24 hours of infection (**Fig 8F**). Thus, using two different detection methods (ELISA and Luminex) we observed consistent differences in cytokine production in *N. caninum* infections in either mouse strain tested.

2.4.3 IFNγ is required for control of *N. caninum* proliferation

IFNγ is a critical cytokine for both innate and adaptive immunity and is required for acute control of *T. gondii* infections in mice (Schroder *et al.*, 2004). Previous work has shown that mice deficient in IFNγ (both IFNγ depleted and *IFNγ*$^{-/-}$) are also susceptible to *N. caninum* parasites (I. A. Khan *et al.*, 1997; Nishikawa *et al.*, 2001), but the impact of *IFNγ* deletion on parasite kinetics during *N. caninum* infection is unknown. To quantify this, we injected 10^6 tachyzoites of either TgS1T:Luc or Nc1:Luc intraperitoneally into either IFNγ$^{-/-}$ or WT mice (n=3 per mouse strain and parasite species), calculated parasite burden using BLI (**Fig 9A**,**C**), and monitored mouse survival (**Fig 9B**). As expected, mice lacking IFNγ succumbed to *N. caninum* infection in a similar timeframe as *T. gondii* (**Fig 9B**). Surprisingly, we found that *N. caninum* parasite burden significantly exceeded that of *T. gondii* at days 4 and 5 post infection in IFNγ$^{-/-}$ mice (**Fig 9C**) despite exhibiting similar levels of burden and proliferation in the first 24 hours (**Fig 9C**, inset). These results were confirmed by an additional experiment (n=3 per mouse strain and parasite species, **Fig 9B, 16A**), showing that IFNγ knockout mice are more susceptible to *N. caninum* than they are to *T. gondii*.

One reason for the dramatic differences in parasite control during the first 24 hpi could be due to differences in susceptibility to IFNγ-induced parasitocidal mediators between *T. gondii* and *N. caninum*. To compare IFNγ susceptibility we performed *in vitro* growth assays in mouse embryonic fibroblast cells (MEFs) in the presence or absence of IFNγ. MEF monolayers in 96 well plates were infected with 10^4 tachyzoites of either TgS1T:Luc or Nc1:Luc for 24 h and then treated with either 100 U/mL or 0 U/mL of recombinant mouse IFNγ (**Fig 9D).** We quantified parasite numbers by measuring total DsRed-derived fluorescence (as both Nc1:Luc and S1T:Luc also expressed DsRed off of the same promoter; see Materials and Methods). We did not find any

evidence for higher IFNγ susceptibly in *N. caninum* compared to *T. gondii* (**Fig 9D**), in that both parasite species had similar growth profiles in the presence of mouse IFNγ. These results were confirmed in one additional experiment (*data not shown*). These data suggest that differences in *in vivo* proliferation between *T. gondii* and *N. caninum* in WT and IFNγ$^{-/-}$ mice are unlikely to be due to differences in IFNγ susceptibly.

2.4.4 IL-12p40 is less critical for host resistance to *N. caninum* than IFNγ

Previous work has shown IL-12p40$^{-/-}$ mice are susceptible to *N. caninum* infection (Mineo et al., 2009) but the impact of IL12p40 deletion on infection kinetics was not known. To investigate the role of IL-12p40 in *N. caninum* proliferation kinetics we first performed an exploratory experiment to test the susceptibility of IL-12p40 knockout (IL-12p40$^{-/-}$) mice to Nc1:Luc and monitored bioluminescence and survival (n=2). We infected WT or IL-12p40$^{-/-}$ mice with 10^6 *N. caninum* tachyzoites and measured parasite burden (**Fig 10A**) and quantified serum IFNγ. As expected, based on prior work, IL-12p40$^{-/-}$ mice were more susceptible to *N. caninum* infection compared to WT mice, as illustrated by increased mortality (**Fig 10B**). Increased mortality correlated with significantly higher *N. caninum* parasite burden in IL-12p40$^{-/-}$ mice compared to WT mice as early as 1-2 dpi (**Fig 10C,D**) and remained significantly higher over the course of the infection (**Fig 10C,D**). In contrast to our experiments in IFNγ$^{-/-}$ mice (**Fig 9)**, parasite burden declined between days 4 and 7 pi in IL12p40$^{-/-}$. An additional experiment (**Fig 10B**, n=3 per mouse strain) confirmed our results and the previously reported susceptibility of IL-12p40$^{-/-}$ mice to *N. caninum* (Mineo *et al.*, 2009). In both our experiments, IL-12p40$^{-/-}$ mice infected *with N. caninum* produced no detectable serum IFNγ (**Fig 10E**).

2.4.5 CCL2/MCP gene deletion does not alter the outcome of *N. caninum* infections

In our Luminex-based cytokine screen, we observed that the chemokine CCL2/MCP had an induction profile similar to both IFNγ and IL-12, in that it was immediately induced (within 24 hpi) during *N. caninum* infections and not until later (e.g., 48 hpi) in infections with *T. gondii.* We reasoned that CCL2/MCP might be important for host control of *N. caninum* proliferation, as it has been previously shown that loss of CCL2/MCP enhanced susceptibility to *T. gondii* due to its role in recruiting $GR1^{+}$ inflammatory monocytes to the site of infection (Paul M. Robben, Marie LaRegina, William A. Kuziel, & L. David Sibley, 2005). To test how genetic ablation of this chemokine would impact proliferation of either parasite, we compared TgS1T:Luc and Nc1:Luc infections in MCP knockout ($MCP^{-/-}$) mice and measured bioluminescent signal throughout the infection (n=3 per mouse strain and parasite species). We found no difference in survival (**Fig 11A**) or parasite burden (**Fig 11B**) for *N. caninum* infections between WT C57BL/6 and $MCP^{-/-}$ mice. Similarly, $MCP^{-/-}$ mice infected with *T. gondii* did not exhibit significant differences in mortality compared to WT mice (**Fig 11A**) and also had similar parasite burden over the course of the experiment (**Fig 11C**). These data show that MCP plays a minor, if any, role in mouse resistance to *N. caninum* (Nc1) or *T. gondii* (S1T) during the acute phase of infection at the doses used in these assays.

2.4.6 MyD88 is required for immediate induction of IL12 and IFNγ in response to *N. caninum* infection and control of *N. caninum* during the first 24 hpi

It is well-established that *MyD88* is required for resistance to *T. gondii* infections in mice (Scanga *et al.*, 2002). *T. gondii* infection in $MyD88^{-/-}$ mice is characterized by uncontrolled parasite

proliferation and defective IL-12 production (Eric Y. Denkers, 2010). Since *N. caninum* induces a potent pro-inflammatory immune response within hours of infection when compared to *T. gondii* infections in mice (**Fig 8**), we sought to determine if host TLR signaling was required for early IL12 and IFNγ induction (**Fig 8C,F** and **14B**) and control of *N. caninum* proliferation (**Fig 8A,D** and **14A**), *in vivo*. We first performed an exploratory experiment and infected 2 mice lacking MyD88 or WT (C57BL/6) mice with 10^6 Nc1:Luc:DsRed (Nc1:Luc) tachyzoites. Both MyD88$^{-/-}$ mice survived the Nc1:Luc infection. We then performed 3 additional experiments to confirm the accuracy of our results. We observed that 100% of *N. caninum*-infected MyD88$^{-/-}$ mice survived in all four experiments (Fig **12C** n=2 per mouse strain; Fig **16B** n=3 per mouse strain; n=3 per mouse strain, *data not shown* and **Fig 12F** n=3 per mouse strain). We performed three of the experiments in our facility (**Fig 12A)** and our collaborator performed the last experiment in another lab at a different institution (**Fig 12F**). Our results demonstrate that MyD88 is not required for survival of sublethal (10^6) IP infections of *N. caninum* (Nc1:Luc) in mice (**Fig 12A,F**), a result that is in conflict with at least one previous report (Mineo *et al.*, 2009). However, when we quantified parasite burden using *in vivo* BLI we found that parasite burden was mostly similar between WT and MyD88$^{-/-}$ mice during the first 24hpi (**Fig 12B**,**C 16B**), with the only significant difference being higher in WT compared to MyD88$^{-/-}$ at 12 hpi (**Fig 12C**). At later stages of infection parasite burden was significantly higher in MyD88$^{-/-}$ mice compared to WT (**Fig 12C 16B**), suggesting that MyD88$^{-/-}$ mice have deficiencies in controlling *N. caninum* proliferation during the acute infection (consistent with prior reports of MyD88-/- mice having increased susceptibility to N. caninum; (Mineo *et al.*, 2009)). When we examined serum cytokine levels we found that in MyD88$^{-/-}$ mice infected with *N. caninum* did not produce detectable serum IL-12p40 or IL-12p70 throughout the infection (**Fig 12E, S3C,D**). We also did not detect IFNγ during the

first 24 hours in *N. caninum*-infected MyD88$^{-/-}$ mice (in contrast wo WT mice; **Fig 12D,G, S3E**) indicating a dependence of immediate serum IL-12 and IFNγ on MyD88. Surprisingly, however, we observed significantly increased IFNγ at D3-5 pi in MyD88$^{-/-}$ mice but not in control mice (**Fig 12D,G, S3E**). This increased IFNγ preceded a reduction of *N. caninum*-derived bioluminescent signal between days 4-6 pi in MyD88$^{-/-}$ mice (**Fig 12C,D S3B,E**). Thus, while MyD88$^{-/-}$ mice did not produce IFNγ during the first 3 days of *N. caninum* infection, and this coincided with significantly higher parasite burden compared to WT mice, MyD88$^{-/-}$-mice did produce IFNγ later during the acute infection (4-5 dpi) and this correlated with what appeared to be a resolution of the infection. Importantly, we did not detect serum IL-12 (p40 or p70) at any timepoint during *N. caninum* infection in MyD88 knockout mice, suggesting that this "pulse" of IFNγ was IL12-independent.

Previous work *in vitro* has shown that *N. caninum* infection induced Type I interferon responses that are dependent on TLR3 signaling (Beiting *et al.*, 2014) in human cells. Additionally, TLR3 has been shown to be important for mouse resistance to *N. caninum* (although with a different *N. caninum* strain and at a 10-fold higher dose than those used here; (Miranda *et al.*, 2019)). To investigate the possibility that type 1 interferons might also be produced during the immediate host response to *N. caninum* we tested serum samples from our *in vivo* experiments for detectable IFNα and IFNβ during the first 48 hpi after both *T. gondii* and *N. caninum* infections in BALB/c mice. We also tested serum and peritoneal lavage samples from MyD88-/- and WT C57BL/6 mice infected with *N. caninum.* We observed no significant induction of either cytokine at 4 or 8 hpi, and only slight (~100 pg/mL) induction of IFNα in one *N. caninum*-infected BALB/c mouse at 24 hpi **(Fig 17A,B).** These data suggest that type I interferons are unlikely to play a major role in driving immediate innate responses to *N. caninum*. Next, we investigated the possibility

that TLR3 signaling played a role in driving early IFNγ induction during *N. caninum* infection. We performed 2 experiments infecting TLR3 knockout ($TLR3^{-/-}$) and WT (C57BL/6) mice with 10^6 *N. caninum* tachyzoites and monitored parasite burden (**Fig 17C,** $TLR3^{-/-}$ n=2 and WT n=3; **Fig 17E**, $TLR3^{-/-}$ n=3 and WT n=2). Overall, we found that $TLR3^{-/-}$ mice were not more susceptible to *N. caninum* compared to WT mice. In fact, we found that *N. caninum* parasite burden was significantly *lower* in $TLR3^{-/-}$ mice compared to control mice at either 8 hpi (*P=<0.0001*, **Fig 17C**), or 4 hpi (*P<0.05,* Fig17E). We also found that, unlike $MyD88^{-/-}$ mice, $TLR3^{-/-}$ mice were capable of producing immediate (4 hpi) IFNγ in response to *N. caninum* infection (**Fig 17D,F**). $TLR3^{-/-}$ did produce significantly (*P=<0.05*) less IFNγ at 4 hpi compared to control mice in one (**Fig 17D**) out of two (**Fig 17F**) experiments. Overall, we found that TLR3-signaling is not required for most, if not all, immediate induction of IFNγ in response to *N. caninum*. These results indicate that while previous reports suggest TLR3 and IFNα/IFNβ may have a role in response of human cells to *N. caninum*, we did not find any evidence that these pathways are required for immediate IFNγ induction nor in host resistance to *N. caninum* in the mouse model of infection.

2.4.7 Mice deficient in TLR11 do not produce immediate IFNγ during *N. caninum* infections

T. gondii profilin, an essential actin-binding protein required for parasite invasion, is recognized by TLR11 (F. Yarovinsky *et al.*) in mice, generating a potent TLR11-dependent IL-12 response (F. Plattner et al., 2008). *N. caninum* has a gene that is a clear ortholog of *T. gondii profilin*, and *N. caninum* profilin (*NCLIV_000610*) shares over 95% amino acid identity with T. *gondii* profilin (**Fig 13A**) and both also have tachyzoite transcript abundance levels in to top 5% of all genes queried by RNAseq ((A. J. Reid et al., 2012) and ToxoDB.org). However, to date it is

not known if TLR11 is important for innate immune recognition during *N. caninum* infection. To address the possibility that *N. caninum* profilin is responsible for immediate host recognition resulting in the robust production of IFNγ and IL-12 within hours of infection, we infected 4 TLR11$^{-/-}$, 3 MyD88$^{-/-}$, or 3 WT mice with 10^6 Nc1:Luc and tested serum samples for cytokine induction throughout the infection. As with *T. gondii* infection (F. Yarovinsky *et al.*), all TLR11$^{-/-}$ mice survived *N. caninum* infection (**Fig 12F**) and we found that by 4 hpi, both TLR11$^{-/-}$ and MyD88$^{-/-}$ mice had significantly lower serum IFNγ compared to WT mice (**Fig 12G**). However, by 24 hpi serum IFNγ levels in TLR11$^{-/-}$ mice rose to levels that were similar to that observed in WT mice (**Fig 12G**). This suggested that unlike TLR3, TLR11 may play at least a partial role in immediate induction of IFNγ during *N. caninum* infection, but there may be additional MyD88-dependent host factors required (such as TLR12).

2.4.8 Expression of *N. caninum* profilin in *T. gondii* Me49 does not result in early induction of IFNγ

Serum IFNγ was not detected in mice lacking TLR11 or MyD88 during the first few hours of infection, consistent with deficiencies in innate TLR sensor recognition mechanisms. *T. gondii* profilin is known to interact with mouse TLR11 resulting in an IL-12-driven immune response (F. Plattner et al., 2008). Alignments indicate profilin is highly conserved between *T. gondii* and its close relatives (Kaury Kucera *et al.*, 2010), although it does have 4 amino acid polymorphisms in a region of the protein previously shown (Kaury Kucera *et al.*, 2010) to be required for TLR11 activation (green box, **Fig 13A**). We hypothesized that the immediate induction of IFNγ in *N. caninum* infected mice was due to the observed differences in the profilin sequences. To test this hypothesis, we expressed C-terminally HA tagged versions of either *N. caninum* or *T. gondii*

profilin in *T. gondii* (ME49 strain) off of a highly active GRA1 promoter (Y. Adomako-Ankomah, Wier, Borges, Wand, & Boyle, 2014) and quantified its impact on the host innate response and parasite proliferation during acute infections in mice. Using immunofluorescence microscopy, we showed that both *T. gondii* and *N. caninum* HA-tagged profilin were expressed at similar levels in *T. gondii* and localized to the parasite cytoplasm (**Fig 13B**). We also found that both proteins were detectable at an expected apparent molecular weight (20-30 KDa) by Western blot (**Fig 13C**). Normalization to *T. gondii* SAG1 indicated that the *T. gondii* profilin gene was expressed at ~2-fold higher levels compared to the *N. caninum* gene (**Fig 13C**). When we infected 3 mice per parasite strain with 10^6 *T. gondii* tachyzoites expressing either *T. gondii* TgME49:TgProHA (Tg:TgProHA) or *N. caninum* TgME49:NcProHA (Tg:NcProHA) profilin, we observed no significant differences in parasite burden during the first 48 hpi, but we did observe a slight but significant reduction in Tg:NcProHA burden compared to Tg:TgProHA burden (**Fig 13D).** Regarding IFNγ production, we observed no significant differences in serum IFNγ levels at most queried timepoints (**Fig 13E)**. The only exception was a significantly higher abundance of IFNγ in Tg:TgProHA-infected mice compared to Tg:NcProHA-infected mice at 48hpi (**Fig 13E**). Consistent with the cytokine data, we also found no differences in host survival (all infected mice succumbed to the infection by day 8 pi as expected for this dose and strain), but our observation of significantly reduced parasite burden for Tg:NcProHA compared to Tg:TgProHA at both and 3 and 5 dpi (**Fig 13D**), suggests that there may be moderate differences in *N. caninum* and *T. gondii* profilin that might impact infection outcome. However these differences do not correlate with any differences in the production of IFNγ and are therefore unlikely to be the main driver of dramatic phenotypic differences in host response and resistance to these two parasite species. Since we saw no difference in IFNγ production during the first 24 hours of infection we sought to confirm this

negative result and eliminate the possibility that profilin release was somehow altered in our transgenic parasites. Since profilin activity can be assayed directly by injection of soluble tachyzoite antigen (STAg) (A. A. Koblansky et al., 2013; Tosh et al., 2016), we injected 3 mice per parasite strain with STAg equivalent to 10^7 parasites of either Tg:TgProHA or Tg:NcProHA and quantified IFNγ production. We observed no statistically significant differences in serum IFNγ concentration between mice injected with parasites expressing HA-tagged *T. gondii* or *N. caninum* profilin (**Fig 13**F), suggesting that differences in the profilin coding sequence between these species are not sufficient to account for their dramatic differences in IFNγ induction and host control during the first 24 hpi in mice.

2.5 Discussion

The *T. gondii/N. caninum* comparative system provides a unique opportunity to address the question of host compatibility at the molecular level using the genetically tractable mouse model to compare two parasites with a close phylogenetic relationship, conserved gene content, and yet dramatic differences in disease outcome. Our results add to a growing body of work indicating that both *T. gondii* and *N. caninum* rely on IFNγ for resistance to infection and is consistent with the key role that this cytokine plays in immunity to intracellular pathogens (McCabe, Luft, & Remington, 1984; Olle, Bessieres, Malecaze, & Seguela, 1996). What we found to be surprising was the fact that *N. caninum* induced a potent IFNγ response in the hours immediately following infection while *T. gondii* did not, a fact that has not been appreciated prior to our comparative analyses. This is somewhat paradoxical since *T. gondii* infection is characterized by remarkably high IFNγ levels in the later stages of infection which exceed those

observed in *N. caninum* infections by ~10 fold. At this stage of infection *T. gondii* growth is protected to varying degrees by an extensive array of *T. gondii*-secreted effectors that disrupt IFNγ-mediate parasite killing such as the rhoptry kinases 5, 18 and 17 (Behnke et al., 2015; J. P. Saeij et al., 2006; Taylor et al., 2006) and dense granule proteins like IST (Gay *et al.*, 2016; Olias, Etheridge, Zhang, Holtzman, & Sibley, 2016). The lack of IFNγ induction during the first 48 h after *T. gondii* infection has not previously been examined in a comparative context as we have done here, and leading to an important question as to how *T. gondii*, but not *N. caninum*, avoids and/or suppresses this robust and rapid IFNγ response.

In IFNγ$^{-/-}$ mice, *N. caninum*-derived luciferase activity rose to levels that were 10-fold higher than those observed for *T. gondii* infected IFNγ$^{-/-}$ mice (**Fig 9C**). It is unlikely that this difference in luciferase signal was due to differences in luciferase production by *N. caninum* compared to *T. gondii,* rather than a difference in actual parasite burden, since we observed similar baseline luciferase levels between these parasite species during the first 24 hpi in WT mice and as late as 2 dpi in IFNγ$^{-/-}$ mice (**Fig 9C**). The difference in burden observed in IFNγ$^{-/-}$ mice might seem surprising given the lack of host-pathogen compatibility between *N. caninum* and the mouse, but it makes sense in the context of 1) a reliance on IFNγ for protection and 2) there having been little, if any, opportunity for host-pathogen co-evolution between *N. caninum* and the murine host. Compared to its extensive interactions with *T. gondii,* the mouse has not developed countermeasures against *N. caninum* outside of IFNγ production since, as we clearly show, this is a highly effective mechanism of *N. caninum* control and overall fails to control *T. gondii*. Once this immunological barrier is removed, the markedly enhanced replication of *N. caninum* may be due to the absence of any additional parasite-specific defenses. From our comparative studies, an important question emerges as to what additional countermeasures are restricting *T. gondii* growth

in the absence of IFNγ. The mouse may recognize other *T. gondii*-derived antigens via innate immune receptors, leading to the recruitment of other parasiticidal host cells and/or production of other proinflammatory cytokines with effector function. One such effector could be nitric oxide which is important for resistance against chronic *T. gondii* infection in mice (Tanaka, Nagasawa H Fau - Fujisaki, Fujisaki K Fau - Suzuki, Suzuki N Fau - Mikami, & Mikami, 2000), but can be actively suppressed by *T. gondii* in a variety of contexts, suggesting the existence of an ongoing molecular arms race (Scharton-Kersten *et al.*, 1997; Tobin & Knoll, 2012). The *N. caninum/T. gondii* comparative model may be an effective system to identify what these differences may be, and allow the discovery of unique, IFNγ-independent mechanisms of intracellular eukaryotic pathogen elimination.

Prior work showed that MyD88 was required for mouse survival to infection with 10^6 "viable" tachyzoites of *N. caninum* (NC-1 strain; (Mineo *et al.*, 2009)) where the number of "viable" tachyzoites was determined using trypan blue exclusion. In the current study we found that all MyD88$^{-/-}$ mice survived infection with 10^6 *N. caninum* tachyzoites (**Fig 12**). This discrepancy is most likely due to differences between the studies in how the dose was determined. For our studies we did not normalize input parasites by dye exclusion, but based on plaque assays for these studies we typically observe ~10-20% viability of input parasites. Therefore it is likely that our "effective" dose is significantly lower than 10^6 and this is why we did not observe any mortality while others did (including a more recent study showing 100% mortality in MyD88$^{-/-}$ mice infected with 10^7 total *N. caninum* tachyzoites (Miranda *et al.*, 2019)).

Despite this discrepancy other aspects of these studies (Mineo *et al.*, 2009; Miranda *et al.*, 2019) are consistent with our results. For one, our data clearly show increased susceptibility to *N. caninum* in MyD88 knockout mice based on higher parasite burden at the later stages of acute

infection (**Fig 12C, 16B**). *N. caninum* infection led to increased IL-12p40 and IFNγ in peritoneal exudates by day 3 pi, and this induction was dependent on MyD88 (Mineo *et al.*, 2009). IL-12p40 was also higher in serum taken from *N. caninum*-infected WT mice by day 3 pi compared to day 0, and this increase persisted over the course of the experiment and was also dependent on MyD88. In contrast to our work, serum IFNγ was not detected in this study until D7 pi and was only moderately dependent on MyD88 (Mineo *et al.*, 2009). Our study supplements this prior work by showing that ***immediate*** IL-12 (both p40 and p70) and IFNγ are highly dependent on MyD88, and that ***late*** IFNγ is observed in response to *N. caninum* ONLY in MyD88$^{-/-}$ mice and therefore not dependent on MyD88. Although IL-12p40 is also a subunit for IL-23 (Oppmann *et al.*, 2000), our results demonstrate that the biologically active form, IL-12p70, is absent during the immediate infection in the absence of MyD88. Our work establishes two phases of the IFNγ response to *N. caninum*. 1) An immediate response that can be sufficient to effectively control the parasite under the dosing regimen used (10^6 total tachyzoites). 2) If the infection is not effectively controlled by this immediate response (as in MyD88$^{-/-}$ mice), a later wave of IFNγ can promote parasite control and mouse survival (**Fig 12D**). In the current study, the comparatively higher lethality in MyD88$^{-/-}$ mice observed in (Mineo *et al.*, 2009) suggests that lack of immediate control in MyD88$^{-/-}$ mice led to increased proliferation of the parasite at levels that overwhelmed the mouse by day 12-15 pi due to the higher effective inoculum (10^6 trypan blue-negative parasites versus 10^6 total tachyzoites in the present study). Differences in inoculum and parasite strain also may contribute to differences between the present study and another showing that TLR3 knockout mice were more susceptible to *N. caninum* compared to WT (in this case after infection with 10^7 tachyzoites; (Miranda *et al.*, 2019)). In our study, we observed small differences in parasite burden and IFNγ 4hpi, and no impact on parasite control in TLR3$^{-/-}$ mice, as *N. caninum* infections were controlled

within the same timeframe as WT mice. We also failed to detect any evidence that type I interferons were being significantly induced by *N. caninum* in mice (in contrast to human cells; (Beiting *et al.*, 2014)).

It is interesting that in our study the same pattern did not occur in IL-12p40$^{-/-}$ mice. While these mice failed to mount an immediate response to *N. caninum,* we never observed detectable levels of serum IFNγ. Overall if the differences in parasite preparation are taken into account, our study and the previous report (Mineo *et al.*, 2009) are complementary, in that ours focuses on differences in the immediate (i.e., within 24 h) responses to infection while the prior study focuses more on later responses (i.e., day 3 pi onward).

Since the immediate IFNγ response elicited by *N. caninum* was dependent upon MyD88 and IL-12, we reasoned that TLR11 recognition of *N. caninum* may be an important sensor required for IFNγ production in the first 24 hours, thus contributing to *N. caninum* incompatibility in mice. Although early (4 hpi) IFNγ production was not detected in TLR11$^{-/-}$ mice, TLR11$^{-/-}$ mice did produce serum IFNγ beginning at 24 hpi , suggesting that TLR11 is not the only MyD88-dependent recognition mechanism capable of contributing to the production of critical IFNγ required to control *N. caninum.* The most likely candidate for mediating this immediate response to *N. caninum* is TLR12, another MyD88-dependent TLR known to be capable of recognizing *T. gondii*-derived PAMPs, including the TLR ligand profilin (A. A. Koblansky et al., 2013; K. Kucera et al., 2010; Raetz et al., 2013).

Given the dependence of immediate IFNγ during *N. caninum* infection on MyD88 and TLR11, profilin was a likely candidate gene to mediate the observed differences in host response and resistance to *N. caninum* infection. We were encouraged to test this hypothesis based on the existence of multiple amino acid differences between the predicted *T. gondii* and *N. caninum*

profilin gene products in a β-hairpin region of the protein shown previously to be required for interactions with TLR11 (Kaury Kucera *et al.*, 2010). Since our aim was to identify any differences in the biology of *N. caninum* profilin compared with *T. gondii* in the immediate production of IFNγ, we reasoned that transgenic expression of *N. caninum* profilin would reveal dominant antigenic properties associated with innate IFNγ production. By using a parasite expression system, we remove issues that arise from bacterial expression constructs including bacterial activation of host immune responses and post-translational modification differences. Using both live parasites as well as STAg preparations, we did not observe differences in cytokine production or parasite burden consistent with the immediate induction of IFNγ by *N. caninum* (and not *T. gondii*) being driven solely (or even in part) by differences in the profilin gene. This observation is consistent with prior work testing various apicomplexan profilins as antigen or adjuvant components for bovine vaccines (Mansilla & Capozzo, 2017). In these studies, recombinant *N. caninum* profilin (rNCPro) was unable to induce sufficient cell-mediated responses to provide protection in mice (Mansilla *et al.*, 2016), and although it has been shown that rNcPro produced in *E.coli* results in IFNγ production, non-recombinant protein controls (6, 24hpi) also induced IFNγ production in these experiments (Mark C. Jenkins et al., 2010). Therefore, it seems likely that there are other ligands in *N. caninum* that contribute to immediate cytokine induction and control of *N. caninum* and their identification may require further characterization through biochemical purification and mass spectrometry. These may be additional TLR ligands or other microbe-associated molecular pattern-bearing molecules that require, at a minimum, the adaptor protein MyD88.

We do not yet know what cell type in the mouse is producing the immediate (4hpi) IFNγ in response to *N. caninum*. Recent work investigated IFNγ-producing cells in adipose tissue at

later timepoints (24 hpi, 7dpi, 21dpi and 12 months) after infection, but have not identified sources if IFNγ immediately after infection responsible for controlling *N. caninum* parasite burden(Teixeira *et al.*, 2016). During the acute phase of *T. gondii* infection, much of the IFNγ is produced by natural killer cells (Gazzinelli *et al.*, 2014; Felix Yarovinsky, 2014), although neutrophils have recently been implicated in providing TLR11-independent IFNγ during *T. gondii* infection (C. R. Sturge et al., 2013). Regardless of the nature of the *N. caninum*-derived signal, it is a critical determinant of the incompatibility between *N. caninum* and the mouse. While it could be recognized via similar mechanisms used for *T. gondii* recognition, our results suggest that the mechanisms may be distinct or at least not fully overlapping. This idea finds support given the extensive co-evolution that has occurred between *T. gondii* and the murine host and the dramatic and highly protective response to *N. caninum* that *T. gondii* manages to circumvent. Once molecules required for parasite recognition and immunity are identified and compared between *T. gondii* and *N. caninum,* it will be possible to dissect the molecular evolutionary events that led to the divergent host ranges of these two important animal parasites.

2.6 Materials and methods

2.6.1 Parasite maintenance and preparation

Parasite strains (*Toxoplasma gondii*: TgS1T:Luc:DsRed, TgS23:Luc:DsRed, TgMe49; *Neospora caninum*: Nc1:Luc:DsRed) were maintained by serial passage in human foreskin fibroblasts (HFFs) isolated from pooled donated foreskins from newborns in ~2003 at Stanford Hospital. These tissues are 100% de-identified and do not entail any human subject research.

HFF's were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) (cDMEM). Cells were grown in humidified, 5% CO_2 incubators at 37 degrees. Monolayers were washed with PBS (for *in vivo* infections) or cDMEM (for cell culture infections), lysed by serial passage through 25- and 27-gauge needles, pelleted by centrifugation at 800 x *g* for 10 minutes, resuspended in 3 mL PBS or cDMEM, and then quantified by counting on a hemocytometer. Parasites were then either concentrated by centrifugation or serially diluted in PBS or cDMEM. Parasites were used at various concentrations for *in vivo* or *in vitro* assays as described below. Plaque assays were routinely used to verify similarity of the inocula.

2.6.2 Soluble tachyzoite Antigen (STAg) preparation

Soluble tachyzoite preparations of $1x10^7$ parasites/mL were prepared based on published protocols and specifically as follows: Monolayers of well infected confluent HFF cells were washed, scraped, and lysed using a 25g and 27g needle and then filtered through a 5 µm filter. Parasites were counted, resuspended in PBS at the desired concentration, and then frozen at -80°C. After thawing on ice, preparations were sonicated using a Sonic Dismembrator Model FB10 (Fisher Scientific) for 20 second bursts on ice (4 times) with 1-minute rests (amplitude=20). After sonication, the samples were centrifuged for 5 minutes at 10,000 x g. The supernatant was removed and 200 µL was injected intraperitoneally into each mouse**.**

2.6.3 Transgenic *T. gondii* and *N. caninum* strains

T. gondii strains and TgS23:GFP:Luc were described previously (Saeij *et al.*, 2005). For *T. gondii* strain TgS1T, we identified it as a potentially avirulent strain based on its genotype at key virulence loci. Specifically, based on existing F1 progeny genotype and virulence phenotype data from two distinct II x III crosses (J. P. Saeij et al., 2006; Sibley et al., 1992). We identified TgS1T as one of the strains lacking "virulent alleles" for 5 previously characterized virulence quantitative trait loci (J. P. Saeij et al., 2006). *T. gondii* S1T and *N. caninum* NC1 were generated by transfection with a plasmid encoding dsRED, clickbeetle luciferase and a bleomycin resistance gene, selected using 2-3 rounds of bleomycin exposure (previously described in (English *et al.*, 2015; Kamau *et al.*, 2012)), and cloned by limiting dilution. All luciferase-positive clones were screened for similar levels of luciferase activity prior to use in *in vivo* bioluminescence experiments.

To generate exogenous profilin expression constructs and transgenic parasites, *N. caninum* (Liverpool strain) or *T. gondii* (ME49) profilin genes were PCR amplified from genomic DNA using primers gaaatcaagcaagatgcaATGTCCGACTGGGACCCTG and acgtcgtacgggtacCCAGACTGGTGAAGATACTCGG for *T. gondii* profilin and gaaatcaagcaagatgcaATGTCGGACTGGGATCCC and acgtcgtacgggtacCCAGACTGGTGAAGGTAC for *N. caninum* profilin. DNA fragments were cloned downstream of the GRA1 promoter and in frame with a C-terminal HA tag into pGRA-HA-HPT using Gibson assembly after plasmid digestion with NsiI and NcoI (J. P. Saeij et al., 2006) To generate transgenic parasites, $2x10^7$ ME49:Δ*HPT*:Luc parasites were passed through 25 and 27 gauge needles and pelleted at 800xg for 10 minutes. Parasites were resuspended in GSH, ATP and Cytomix (0.15mM $CaCl_2$; 120mM KCl; 25mM HEPES; 2mM EDTA; 5mM $MgCl_2$;

10mM KPO_4; pH to 7.6) and electroporated with 30-50 µg of the relevant plasmid at 25µF and 1.6Kv. Transfected parasites were selected using cDMEM supplemented with MPA/Xan. Clones were obtained by limiting dilution and confirmed by immunofluorescence (IFA) and western blot, using SAG1 as a loading control.

2.6.4 Animal experiments

Experiments were performed with 4-8wk old female mice of the following strains: C57BL/6J, BALB/cJ, C.129S7(B6)-*Ifng*^{tm1Ts}/J and B6.129S7-*Ifng*^{tm1Ts}/J, B6.129S1-*Il12b*^{tm1Jm}/J, B6.129S4-Ccl2tm1Rol/J, and B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J obtained from Jackson Laboratories with the exception of the TLR11$^{-/-}$ mice which have been described previously (F. Yarovinsky *et al.*) and maintained by the Yarovinsky lab.

2.6.5 *In vivo* bioluminescence assays

We infected 4-8 week old female mice (Balb/c, C67BL/6 and various knockout lines as indicated; Jackson Laboratories) via intraperitoneal (IP) injection of 10^6 luciferase-expressing (Luc) parasites in 200 µL sterile PBS. Acute infections were monitored using bioluminescent imaging by injecting mice with 3 mg D-Luciferin in 200 uL sterile PBS. Images were taken using the IVIS Lumina II imaging system (Xenogen Corporation) for 4 minutes using maximum binning. Images were analyzed using Living Image software to calculate total flux (photons/s) across the entire body of the mouse.

2.6.6 *In vitro* bioluminescence assays

For *in vitro* growth assays, confluent HFFs or MEFs in 96-well plates were infected with 10^4 *T. gondii* or *N. caninum* parasites per well and quantified using a fluorescent imaging reader (BioTek Cytation5). For HFF growth comparison, cells were infected and fluorescence was measured after infection, and 24-48hpi. For fluorescent assays, murine embryonic fibroblasts were treated with 100 units/ml of recombinant mouse IFNγ (R&D) 24 hours after inoculation with 10^4 parasites. Cells were inoculated with 10^4 TgS1T:Luc:DsRed or Nc1:Luc:DsRed parasites per well and fluorescence was read at specified timepoints throughout the experiment (3, 24, 48 hpi).

2.6.7 Cytokine and chemokine detection and analysis

Mouse chemokine and cytokine levels were measured using Enzyme-Linked Immunosorbent Assays (ELISA) or by Luminex processed at the University of Pittsburgh Medical Center CFP Luminex Core Laboratory. Cytokine response profile is a commercially available panel that measures 32 mouse chemokines and cytokines. Commercially available ELISA kits were obtained from BD Biosciences (IL-12p40 and IFNγ) and used according to manufacturer instructions.

2.6.8 Immunofluorescence

Coverslips (12mm) were seeded with HFFs in 24 well plated and grown in CDMEM as described above. Coverslips were infected with Me49 *T. gondii* parasites exogenously expressing *T. gondii or N. caninum* profilin (Tg:TgProHA, Tg:NcProHA). Infected HFF coverslips were

incubated overnight, washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes. Following fixation, cells were blocked and permeabilized in PBS containing 5% BSA 0.1% triton for 1 hour. Fixed cells were then stained with commercially obtained antibodies for HA.

2.6.9 Western blot

Parasites were grown in HFF culture as described above. Cultures were lysed using a 5 µm syringe to release parasites, filtered to remove host cell debris, washed in PBS and then suspended in lysis buffer. Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and blocked for 1 hour in 5%BSA in PBS-Tween20 (PBS-T). Primary antibody incubation was performed in blocking buffer for 60 min followed by three washes in PBS-T. Anti-HA antibody (Anti-HA High Affinity rat monoclonal clone 3F10 Sigma-Aldrich #11867431001) and *T. gondii* SAG1 antibody (monoclonal mouse D61S, ThermoFisher #MA5-18268) were used at 1:1000. Secondary antibody incubation was performed with horseradish peroxidase-conjugated secondary antibodies to the respective primary antibodies in blocking buffer for 45 min. Bands were visualized with West Pico chemiluminescent substrate (ThermoFisher).

2.6.10 Statistical analysis

All statistical analysis was performed using Prism 7 (GraphPad Software, Inc). Statistical tests were chosen based on experimental design. Analyses included: two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test, two-way repeated measure ANOVA (alpha=0.05) with uncorrected Fisher's LSD test, or two-way repeated measure ANOVA

(alpha=0.05) with Dunnett multiple comparisons test. Statistical methods are described in the figure legends where appropriate. All bioluminescent data were log-transformed prior to analysis.

2.6.11 Ethics statement

All animal procedures in this study met the standards of the American Veterinary Medical Association and were approved locally under the University of Pittsburgh Institutional Animal Care and Use Committee protocol number #12010130. Animal protocol meets National Institutes of Health (NIH) Public Health Policy (PSH) on Humane Care and Use of Laboratory Animals and United States Department of Agriculture (USDA) Animal Welfare Regulation (AWR) guidelines.

2.6.12 Acknowledgments

This work was supported by NIH grant RO1AI114655 (JPB) and NIH AI121090 and AI136538 (FY). This project used the UPMC Hillman Luminex Core facility that is supported in part by award P30CA047904.

2.7 Figures

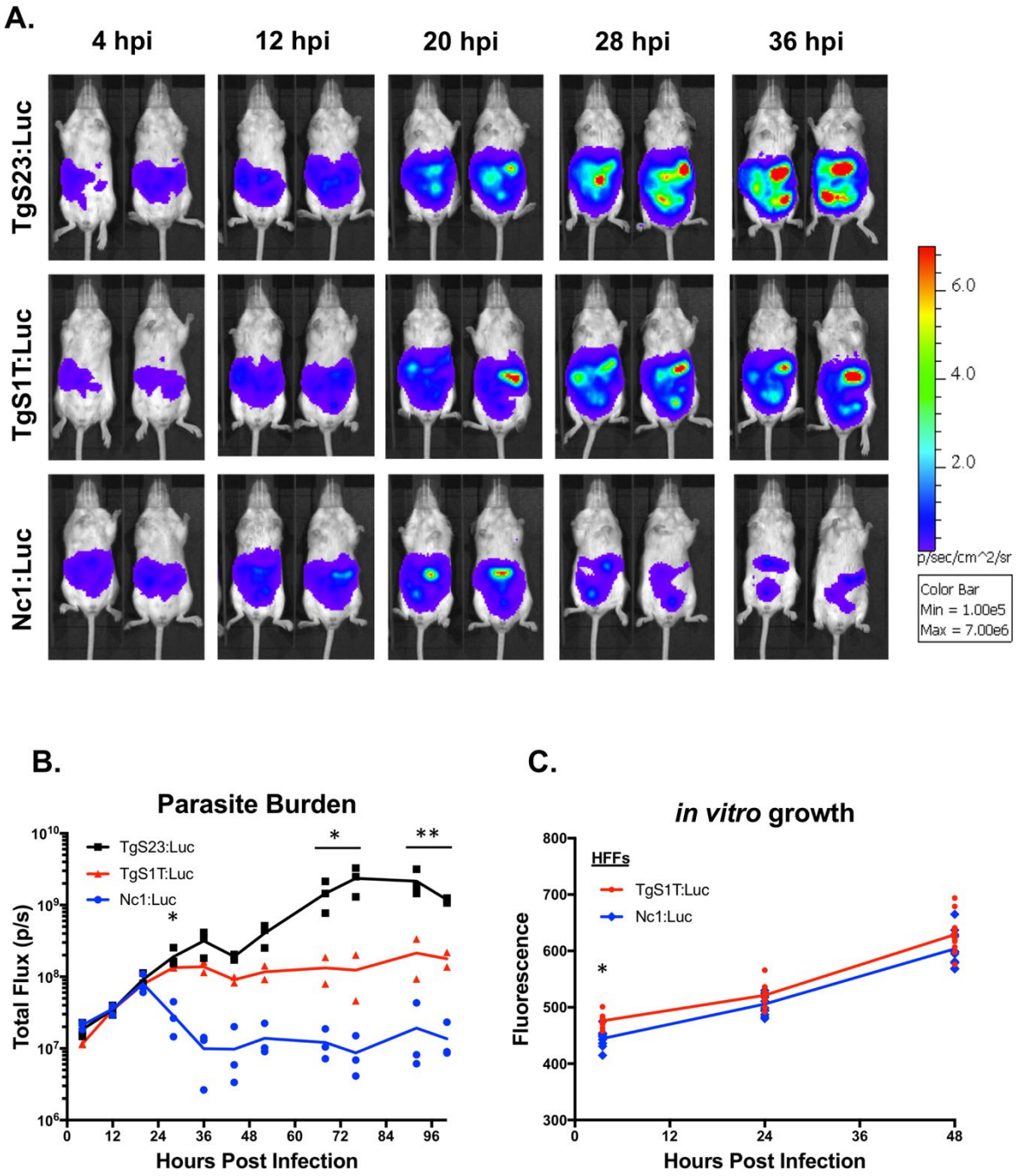

Figure 7 *T. gondii* and *N. caninum* growth rates *in vitro* and *in vivo*

A) *In vivo* bioluminescence imaging of mice infected with 10^6 parasites of luciferase expressing *T. gondii* strains Tg:S1T:Luc, TgS23:Luc, or *N. caninum* strain Nc1:Luc. Images were taken every 8 hours, starting 4 hours post infection (hpi) B) Quantification of images where each data point represents the total flux (photons/s) of an infected mouse (Red TgS1T:Luc, n=2; Black TgS23:Luc, n=3; Blue Nc1:Luc, n=3). C) *In vitro* growth assay of TgS1T:Luc:DsRed (Tg:S1Tluc), and Nc1:Luc:DsRed (Nc1:Luc). Human foreskin fibroblasts (HFFs) were inoculated with 10^4 parasites/well in 96 well plates and fluorescence was measured at indicated timepoints. Imaging data were log transformed and both *in vitro* and *in vivo* assays were analyzed using a two-way repeated measures ANOVA (alpha=0.05) with Sidak's multiple comparisons test was performed. *p=<0.05, **p=<0.01, ***p=<0.001

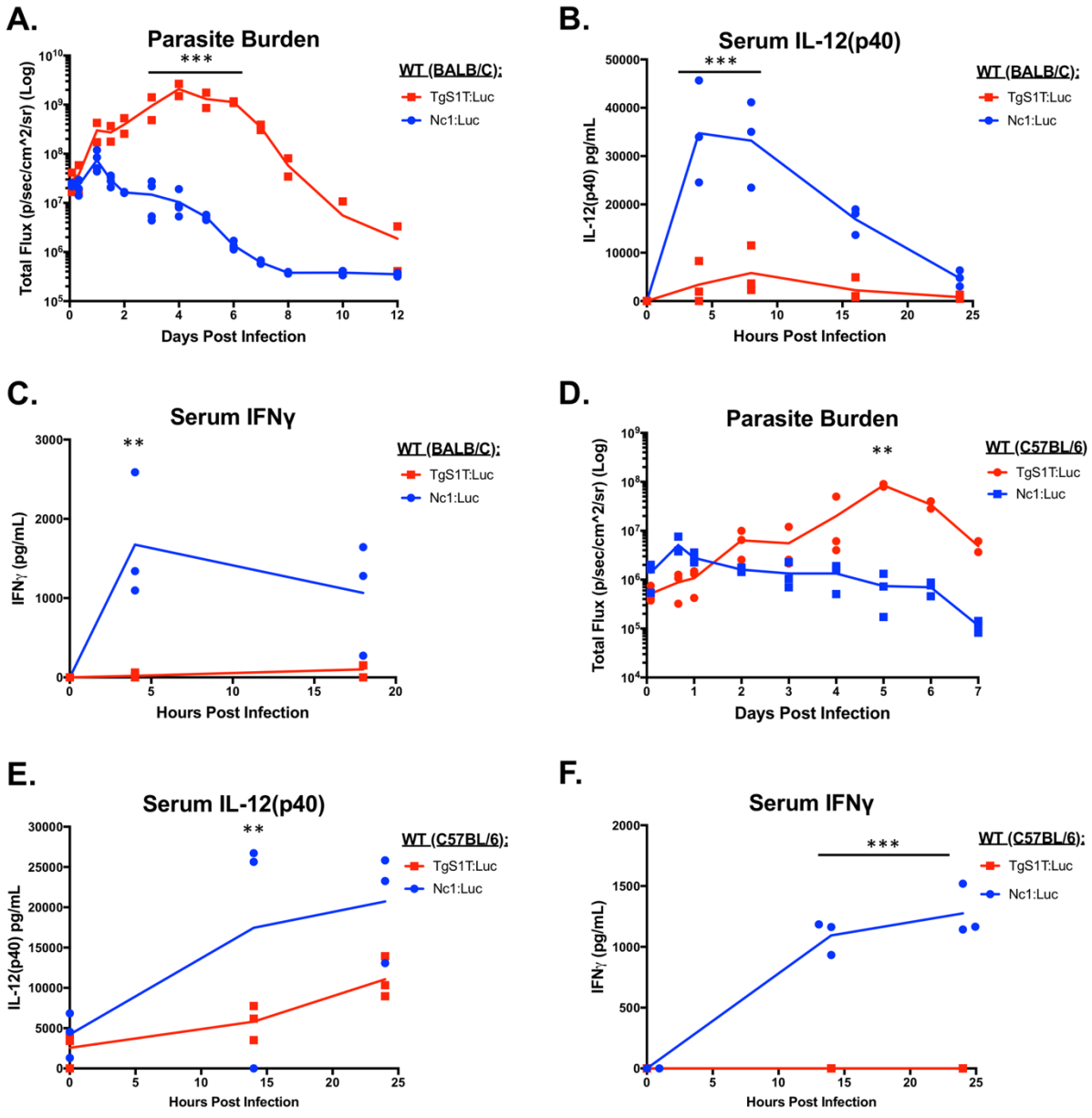

Figure 8 Parasite burden and day 0-1 cytokine levels in mice infected with *N. caninum* (Nc-1) or *T. gondii* (S1T)

Six week old BALB/c (A-C) or C57BL/6 (D-F) mice were IP injected with either 10^6 TgS1T:Luc (red) or Nc1:Luc (Blue) tachyzoites. Bioluminescent imaging was used to monitor parasite burden throughout the infection. Serum was collected and analyzed for IL-12p40 or IFNγ by ELISA. A) Quantification of bioluminescence during *in vivo* infections in BALB/c mice (Red TgS1T:Luc n=2, Blue Nc1:Luc n=3;

experiment repeated in Fig 14). B,C) Cytokine quantification using ELISA for mouse IL-12p40 (B) or IFNγ (C) n=3 per parasite species. D-F) Same as A-C but in C57BL/6 mice (n=3 per species; this experiment was performed only once). For all experiments, imaging data were log transformed and then a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test was performed. Cytokines were analyzed by a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test. *p=<0.05, **p=<0.01, ***p=<0.001.

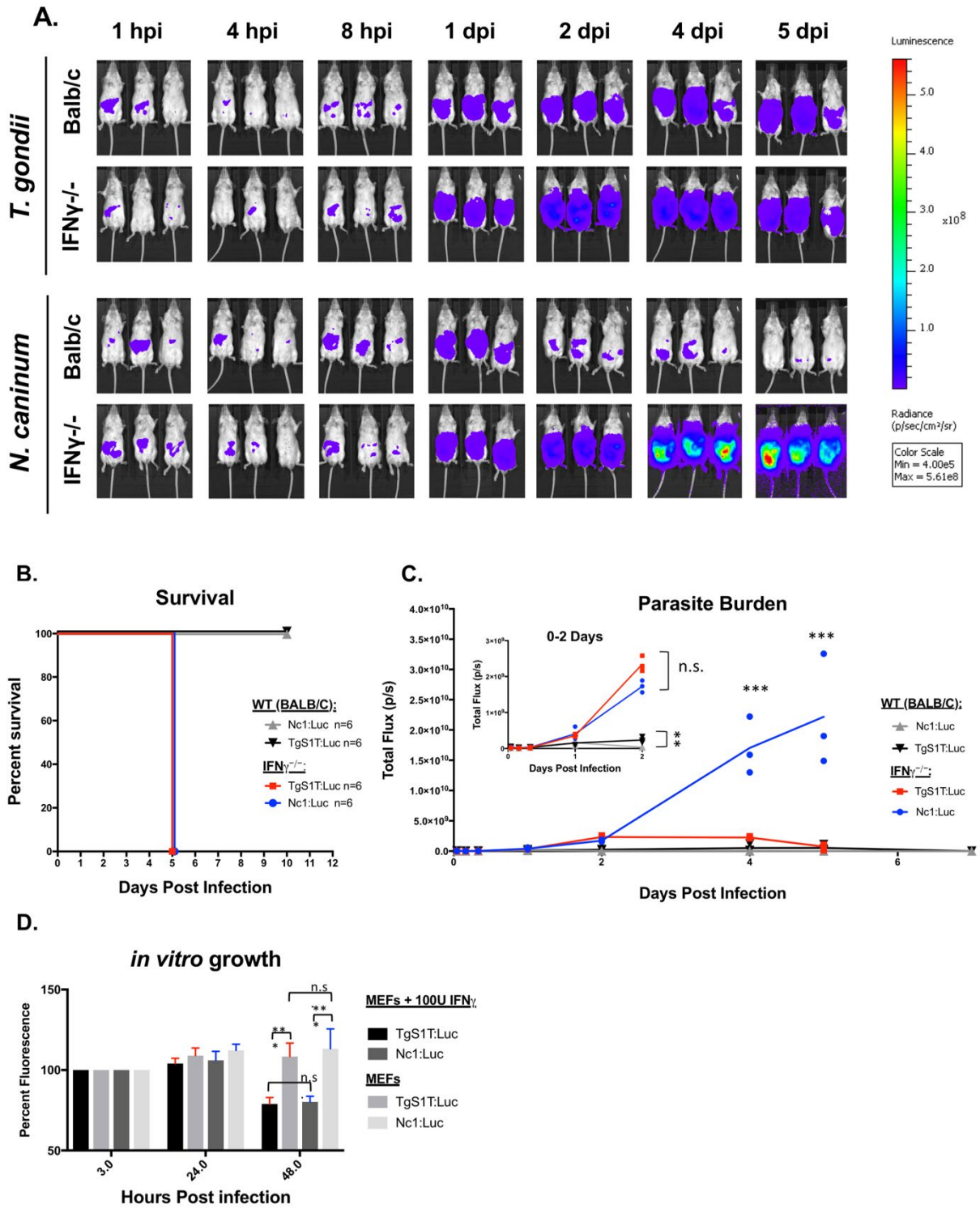

Figure 9 IFNγ is required for control of *N. caninum* proliferation during acute infections in BALB/c IFNγ-/- mice

Six week old female interferon gamma knockout mice (IFNγ$^{-/-}$) and BALB/c control mice were infected with 10^6 TgS1T:Luc or 10^6 Nc1:Luc tachyzoites IP. A) bioluminescent images showing parasite-derived luciferase signal at selected time points. B) Combined survival of IFNγ$^{-/-}$ or BALB/c mice infected with *T. gondii* or *N. caninum* (Data shown is from two experimental replicates each with n=3 per parasite/mouse strain). C) BALB/c background IFNγ$^{-/-}$ (n=3 per parasite species) or WT BALB/c mice (n=3 per parasite species) were infected as described, Experiment repeated fig 16A. D) Mouse embryonic fibroblast cells were treated with 100 units of recombinant IFNγ (R&D) 24 hours after being inoculated with 10^4 tachyzoites of either TgS1T:Luc or Nc1:Luc. Parasite-derived DsRED fluorescence (both strains also express DsRED; see Methods) was quantified at 3 and 24 hpi, then IFNγ was added and fluorescence was quantified 24 hours after addition of IFNγ (48 hours after inoculation). The first data point was normalized to 100% for display purposes. Statistical analysis was performed using a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test on the raw (non-normalized) data. *p=<0.05, **p=<0.01, ***p=<0.001

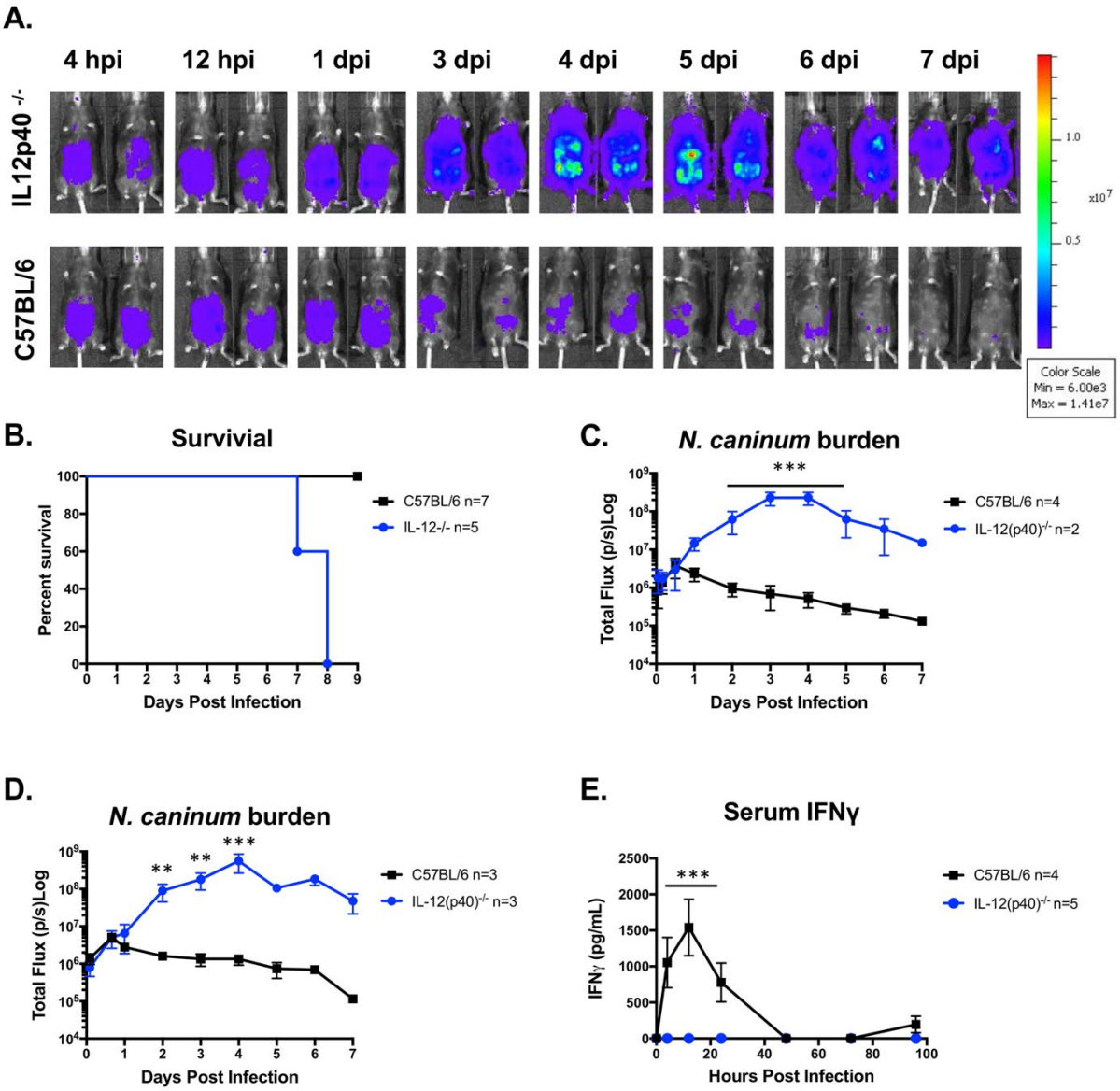

Figure 10 IL12p40 knockout mice fail to control *N. caninum* infection, although with different infection dynamics

Six week old C57BL/6 (black) or IL12p40 knockout (IL12p40$^{-/-}$) (blue) mice were injected IP with 10^6 Nc1:Luc tachyzoites. A) Bioluminescence imaging data during days 0-7 post-infection. Hours post infection (hpi) and days post infection (dpi) are indicated (n=2 per mouse strain). B) Combined survival of IL12p40$^{-/-}$ mice compared to control mice infected with *N. caninum.* Data shown is from two experiments (Blue IL12p40$^{-/-}$ n=5, Black WT n=7) C) Quantification of bioluminescence data measuring parasite burden (total flux p/s) over time (n=2 per mouse strain. D) Quantification of bioluminescent data from experimental repeat (Blue

IL-12p40$^{-/-}$ n=3, Black WT n=3). E) Serum samples were collected at 0, 8, 12, 24, 48, 72, and 96 h.p.i. and analyzed with a mouse IFNγ ELISA. No IFNγ was detected in IL12p40$^{-/-}$ mice. Graph combines two experiments (Blue, IL-12p40$^{-/-}$ mice n=5 and Black, WT n=4). Control mice (n=4) for the experiment in Fig 10D were infected at the same time as multiple knockouts and data from serum taken from these same mice is also shown in Fig 12D. Cytokines were analyzed by a two-way repeated measures ANOVA (alpha=0.05) with Sidak's multiple comparisons test. Imaging data were log transformed and then a two-way repeated measure ANOVA (alpha=0.05) and Sidak's multiple comparisons test was performed. *p=<0.05, **p=<0.01, ***p=<0.001.

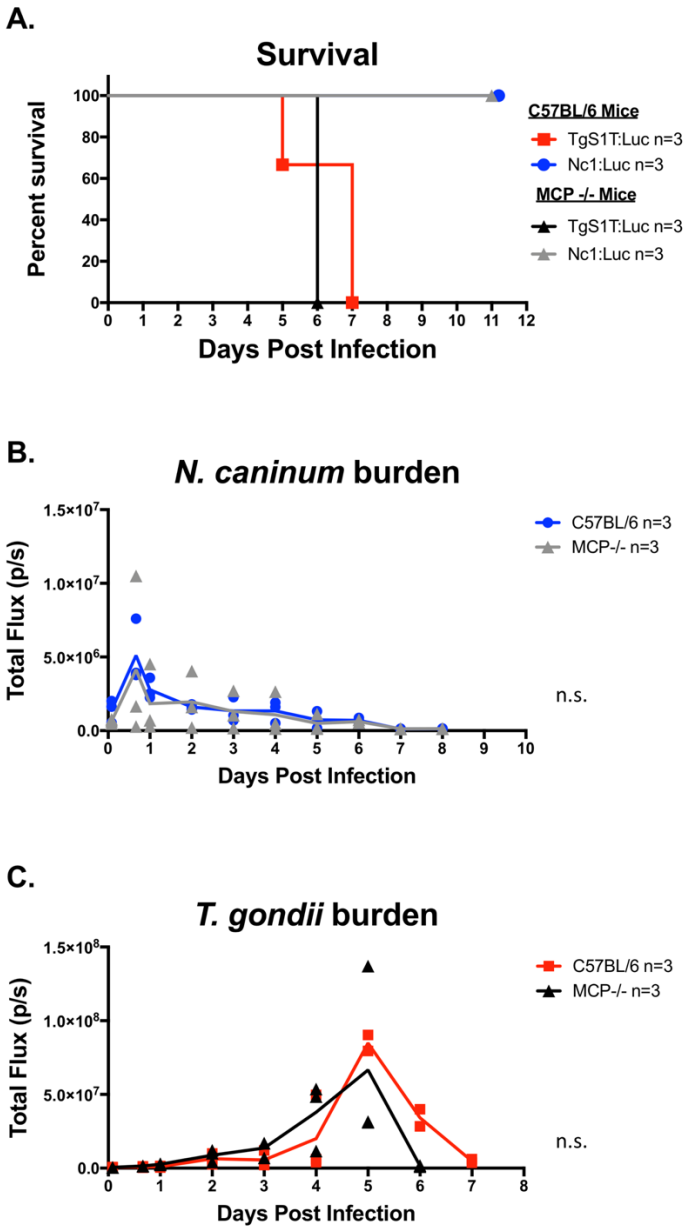

Figure 11 *In vivo* bioluminescence imaging comparing *N. caninum* and *T. gondii* acute infection in MCP (CCL2) knockout or WT C57BL/6 mice

MCP knockout mice (MCP$^{-/-}$ n=3 per parasite species) and C57BL/6 (control) mice (n=3 per parasite species) were infected with 10^6 tachyzoites of either *N. caninum* or *T. gondii*. A) Survival of mice throughout the acute infection. B) Parasite burden of Nc1:Luc infection in either C57BL/6 (blue) or MCP$^{-/-}$ (gray). Data were log transformed and a two-way repeated measure ANOVA (alpha=0.05) with Dunnett multiple comparisons test was performed. No statistically significant differences were found in the MCP$^{-/-}$ comparison for Nc1 infection.

C) TgS1T:Luc infections in C57BL/6 (red) or MCP$^{-/-}$ (black). Data were log transformed and a two-way repeated measures ANOVA with Dunnett multiple comparisons test was performed. No statistically significant differences were found in the MCP$^{-/-}$ comparison for TgS1T infection

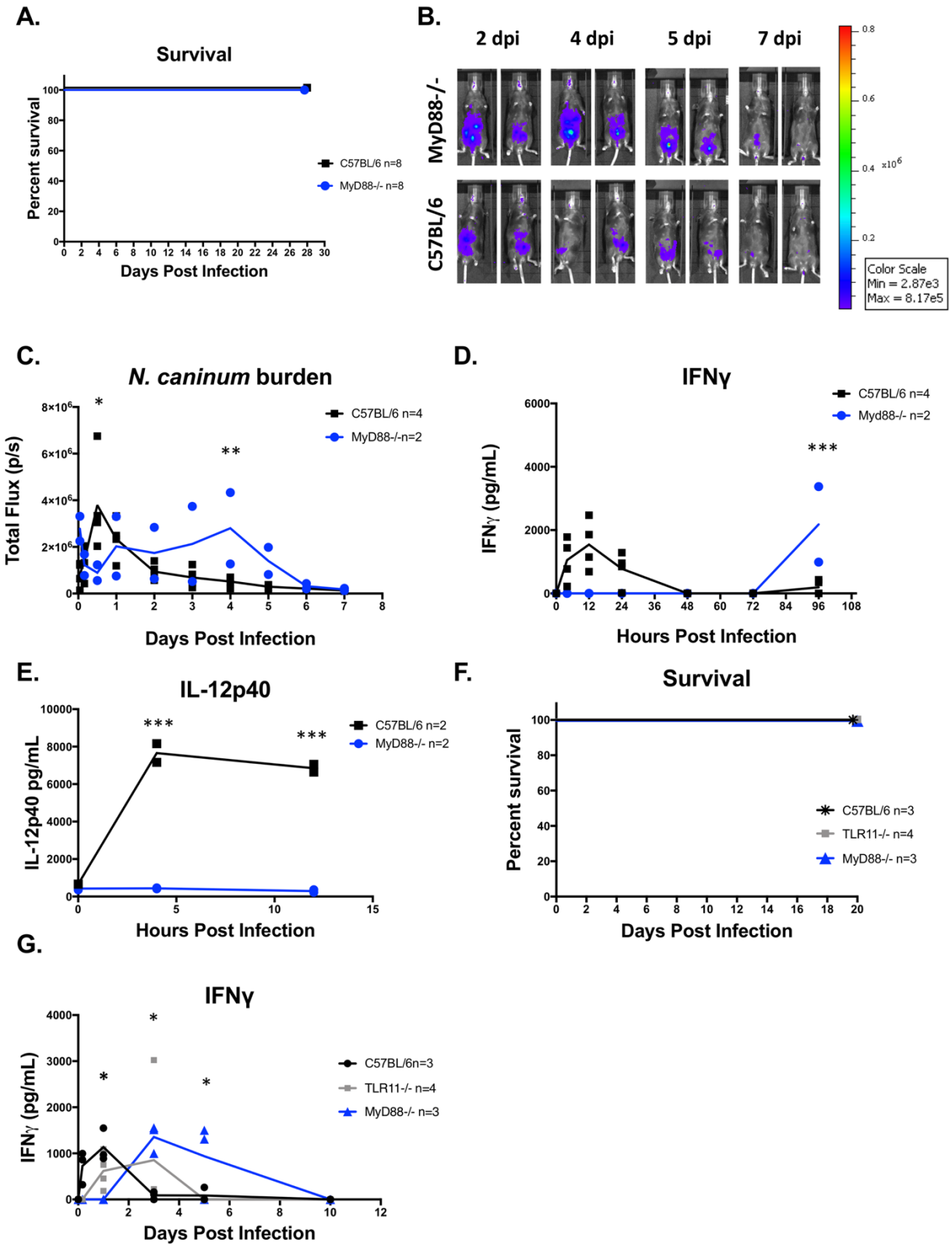

Figure 12 *In vivo* bioluminescence imaging and cytokine production in MyD88 or TLR11 knockout mice

A) Combined survival of MyD88$^{-/-}$ (blue) or C57BL/6 (black) mice infected with 10^6 Nc1:Luc tachyzoites from 3 experiments (n=8 per mouse strain). B) Bioluminescence imaging of Nc1:Luc infections. The control mice shown are also shown in Fig 10A, as multiple knockout mice were tested at the same time. C) Quantification of bioluminescence imaging (n=2 per mouse strain, repeated experiment shown in Fig 16B). Bioluminescence data were log transformed and two-way repeated measure ANOVA (alpha=0.05) with Dunnett multiple comparisons test was performed. D,E) Serum samples were collected at 0, 8, 12, 24, 48, 72, and 96 h.p.i. and analyzed for IFNγ (D) or IL-12p40 (E) by ELISA (n=2 per mouse strain and time point; repeated experiment shown in Fig 16). F) Survival data for MyD88$^{-/-}$ (n=3), TLR11$^{-/-}$ (n=4) and WT (C57BL/6) mice (n=3) infected with Nc1:Luc. G) IFNγ concentration in serum samples from Nc1:Luc infections in TLR11$^{-/-}$ (grey), MyD88$^{-/-}$ (blue) or WT (C57BL/6; black) mice. Cytokine data were analyzed using a two-way repeated measures ANOVA (alpha=0.05) with Sidak's multiple comparisons test. *p=<0.05, **p=<0.01, ***p=<0.001.

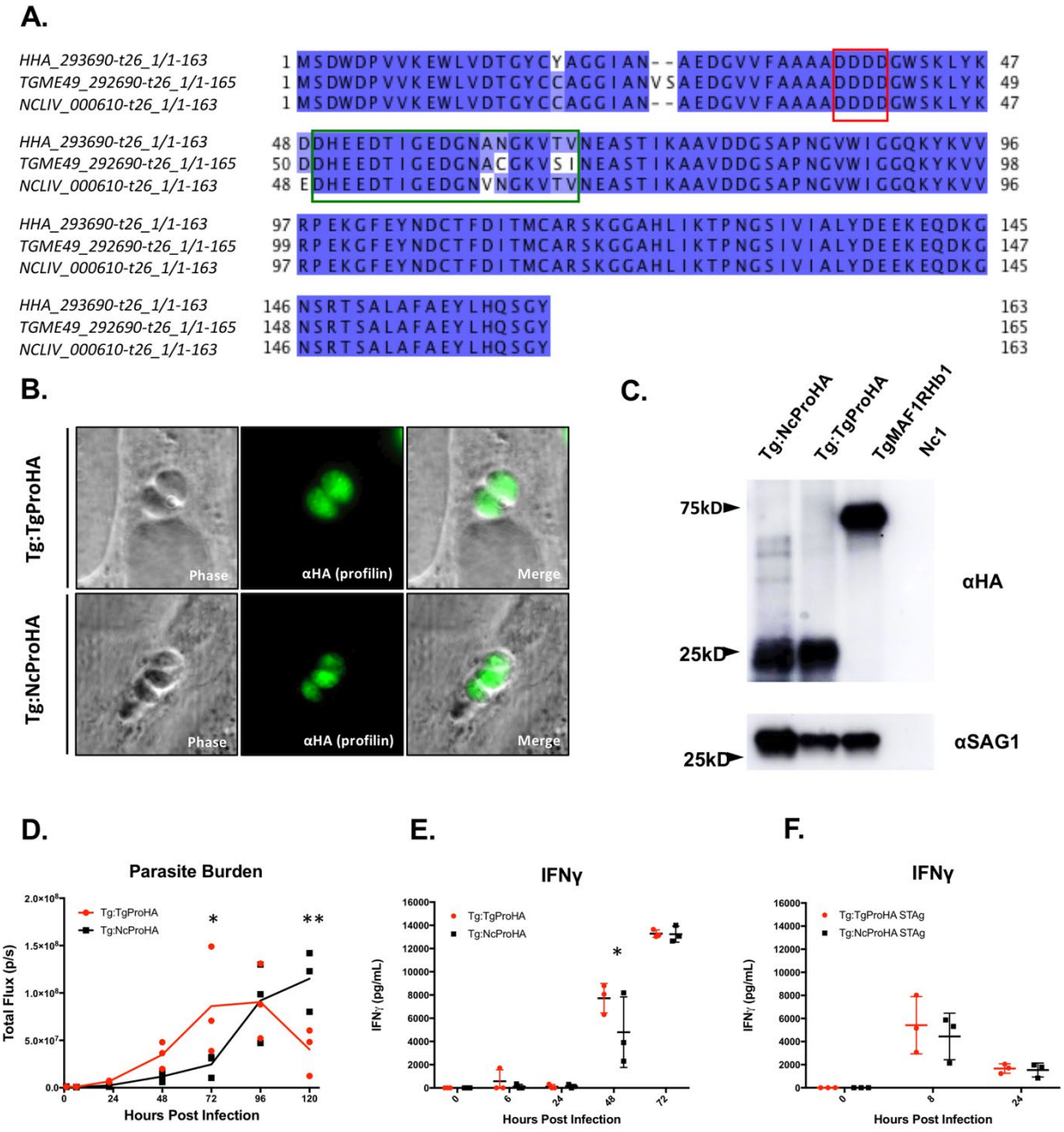

Figure 13 IFNγ production and *in vivo* bioluminescent imaging of transgenic *T. gondii* ME49 expressing either *T. gondii* or *N. caninum* profilin

A) Alignment of *T. gondii* profilin predicted amino acid sequence with those from near-relatives *N. caninum* and *H. hammondi*. Putative substrate-binding motifs identified by Kucera and colleagues 2010 (Kaury Kucera *et al.*, 2010) are outlined as follows: Red box indicates acidic loop (AL) and green box indicates β-hairpin. Sequences obtained from ToxoDB.org: *H. hammondi* HHA_293690, *T. gondii* TGME49_293690 and

N. caninum **NCLIV_000610. B) Anti-HA immunofluorescence assay (IFA) of Me49:TgProfilinHA (Tg:TgProHA; top row) or Me49:NcProfilinHA (Tg:NcProHA; bottom row) showing expected cytoplasmic localization . C) Western blot using anti-HA (top blot) or anti-SAG1 (bottom blot) as a loading control. Arrows indicate size in kD. (D-F) Six to eight-week-old BALB/c mice were infected with 10^6 tachyzoites (n=3 per parasite strain) or injected with equivalent 10^6 soluble tachyzoite antigen preparations (STAg) from indicated parasite strains (n=3 per parasite strain). D) Quantification of bioluminescent imaging (total flux; photons/s) throughout infection of mice with Tg:TgProHA (red) or Tg:NcProHA (black). Data were log transformed and a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test (analyses performed on days 0-5). E) IFNγ ELISA of samples collected throughout the infection of Tg:TgProHA (red) or Tg:NcProHA (black). A two-way repeated measure ANOVA (alpha=0.05) and Sidak's multiple comparisons test was performed. F) IFNγ ELISA of samples collected after injection of Tg:TgProHA STAg (red) or Tg:NcProHA STAg (black). A two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test was performed (analysis for 0-48 hours). No statistically significant differences were identified between mice injected with Tg:TgProHA STAg and Tg:NcProHA STAg (F). *p=<0.05, **p=<0.01, ***p=<0.001.**

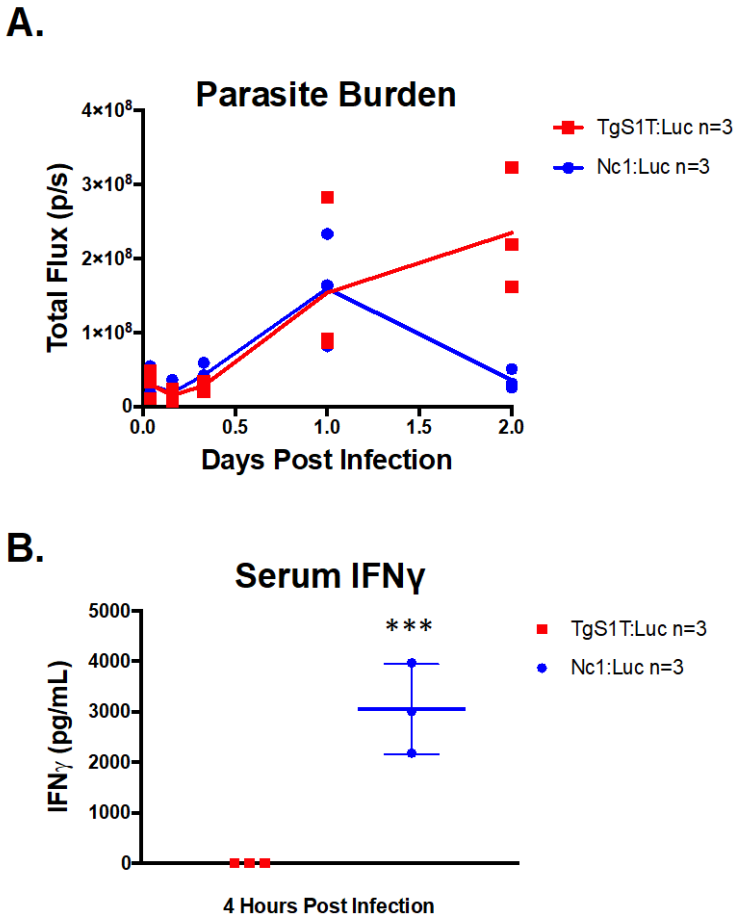

Figure 14 Parasite burden and early cytokine levels in mice infected with *N. caninum* (Nc-1) or *T. gondii* (S1T)

Six week old BALB/c mice were IP injected with either 10^6 TgS1T:Luc (red) or Nc1:Luc (Blue) tachyzoites. Bioluminescence imaging was used to monitor parasite burden throughout the infection. Serum was collected and analyzed for IFNγ by ELISA. A) Quantification of bioluminescence during *in vivo* infections in BALB/c mice (n=3 per parasite species) B) Cytokine quantification using ELISA for mouse serum IFNγ (n=3 per parasite species). All imaging data were log transformed and then a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test was performed. Cytokines were analyzed by a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test. *p=<0.05, **p=<0.01, ***p=<0.001.

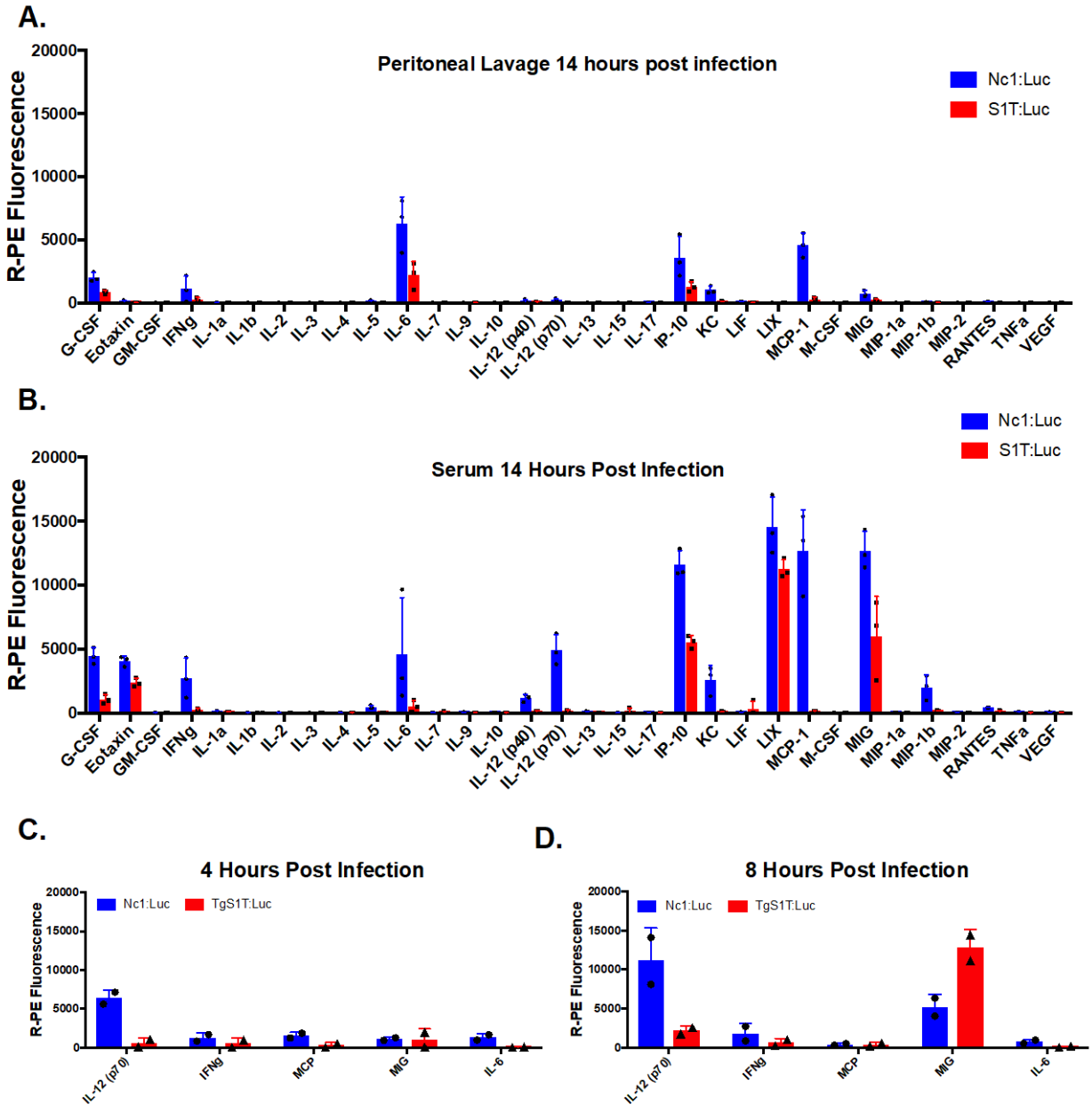

Figure 15 Early cytokine levels in mice infected with *N. caninum* or *T. gondii*

Six-week old BALB/C mice injected IP with either 10^6 TgS1T:Luc (red) or Nc1:Luc (Blue) tachyzoites. A) Peritoneal lavage samples were collected at 14 hours after injection and supernatant was analyzed for multiple cytokines; n=3 per parasite species. B-D) Blood samples were collected at 14 (B, n=3 per parasite species), 4 (C, n=2 per parasite species), or 8 (D, n=2 per parasite species) hours post-infection. Blood samples and lavage supernatant were analyzed using a mouse cytokine/chemokine multiplex assay (Luminex) and 32 mouse analytes were tested.

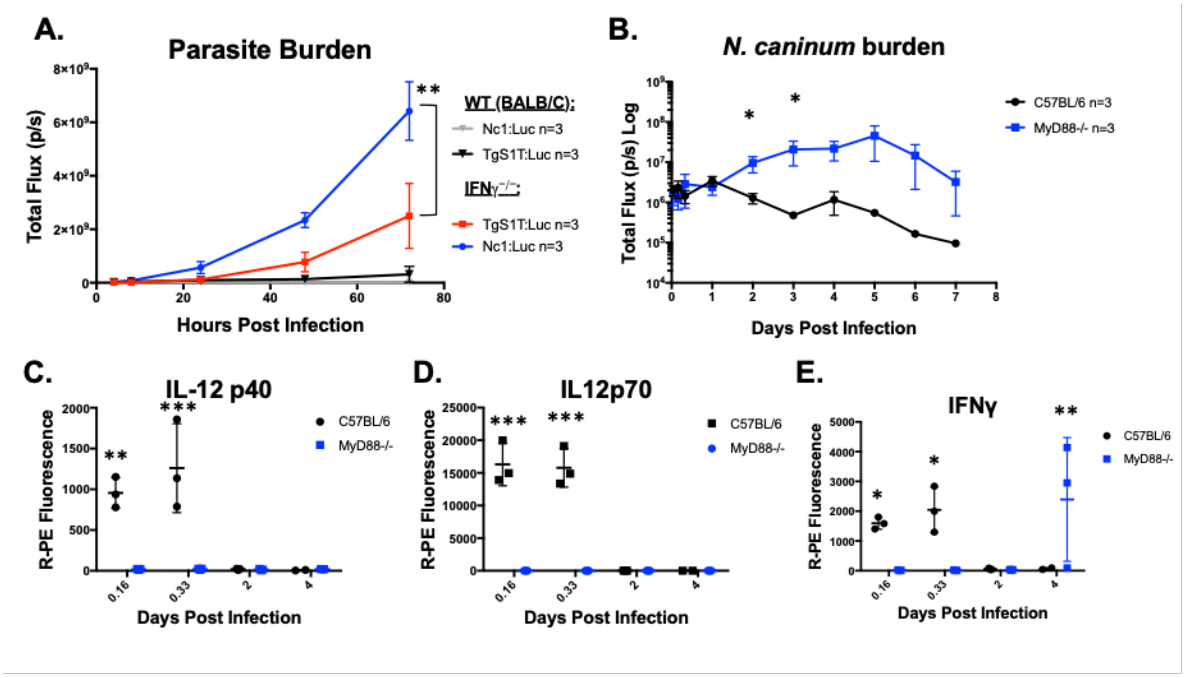

Figure 16 Parasite burden and early cytokine levels in IFNγ or MyD88 knockout mice infected with *N. caninum* (Nc-1) or *T. gondii* (S1T)

Six week old BALB/c or IFNγ knockout (IFNγ$^{-/-}$) mice or six week old C57BL/6 or MyD88 knockout (MyD88$^{-/-}$) mice were injected IP with 10^6 Nc1:Luc or TgS1T:Luc tachyzoites. A) BALB/c or IFNγ$^{-/-}$ mice: quantification of bioluminescence imaging days 0-7 post-infection (n=3 per mouse strain per parasite). B) C57BL/6 or MyD88$^{-/-}$ mice: quantification of bioluminescence imaging of MyD88$^{-/-}$ (n=3) or C57BL/6 (n=3) mice infected with 10^6 Nc1:Luc tachyzoites. All BLI data were log transformed and two-way repeated measure ANOVA (alpha=0.05) with Dunnett multiple comparisons test was performed. (C-E) Serum samples were collected at 4, 8, 48, and 96 hpi (0.16, 0.33. 2, and 4 dpi) and analyzed using a 32 plex Luminex cytokine response panel. Data shown is for C) IL-12p40, D) IL-12p70 and E) IFNγ (n=3 per mouse strain). Cytokines were analyzed by a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test. *p=<0.05, **p=<0.01, ***p=<0.001.

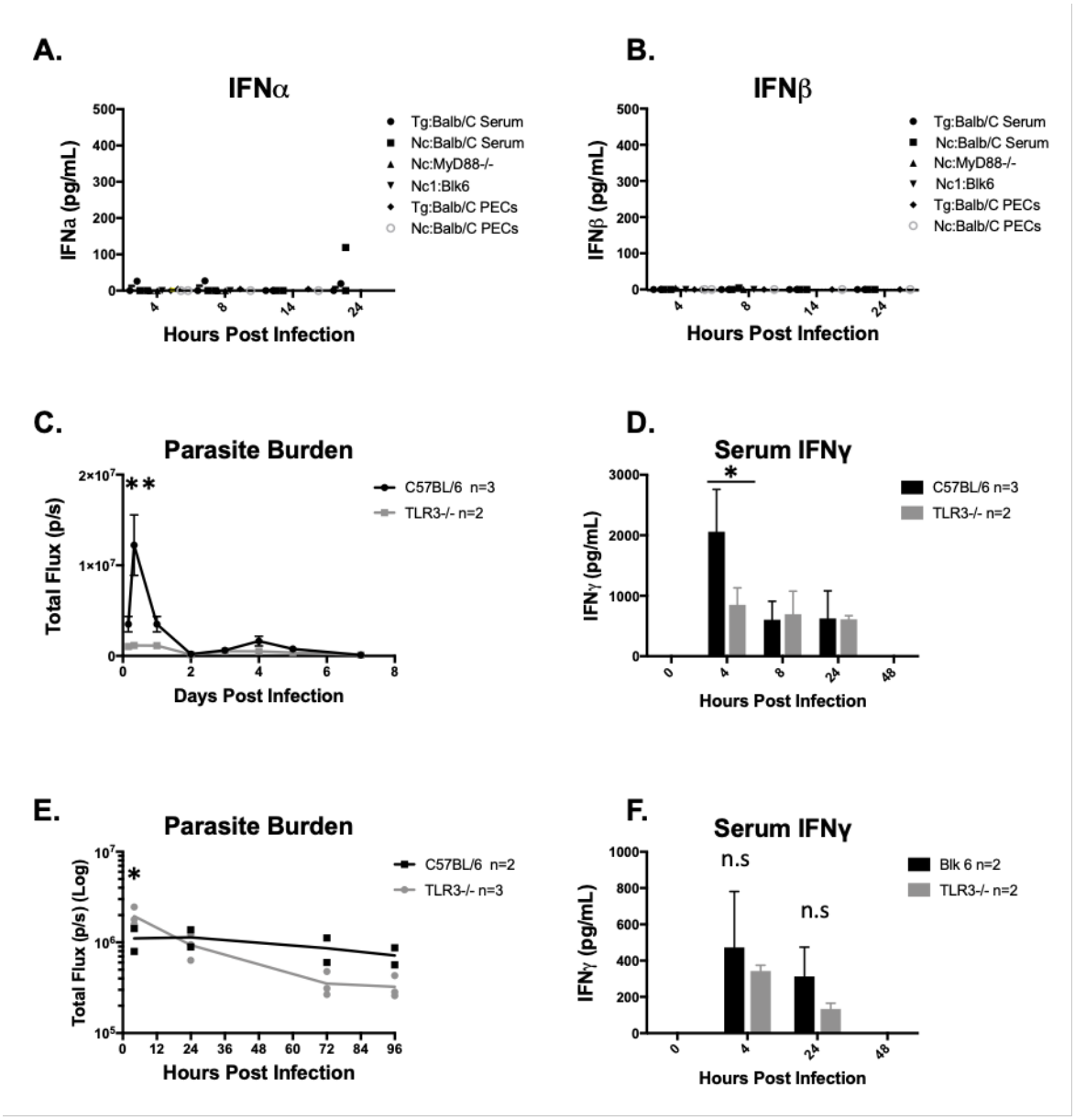

Figure 17 Bioluminescent imaging and IFNγ concentration in *N. caninum* infections in TLR3 knockout mice and interferon gamma concentration in multiple infections

A) Interferon alpha (IFNα) or B) Interferon beta (IFNβ) concentration in serum or peritoneal lavage supernatant (PECs) from multiple mouse strains infected with with either Nc1:Luc (NC) or TgS1T:Luc (Tg). Sample size was either 3 for each mouse strain/parasite species combination except for MyD88-/- and C57BL/6, where the sample size was n=2. Samples were analyzed with a multiplex assay (Luminex) and concentration was calculated using a standard curve. C) Six to eight week old TLR3 knockout mice (TLR3$^{-/-}$, gray n=2) or WT C57BL/6 (black n=3) mice were infected IP with 10^6 Nc1:Luc tachyzoites. Quantification of

bioluminescence imaging was conducted for 7 days of infection (photons/s). Data were log transformed and two-way repeated measure ANOVA (alpha=0.05) with Dunnett multiple comparisons test was performed. TLR3$^{-/-}$ infections had significantly less parasite burden compared to control infections at 8 hpi (*p=<0.01*). D) Serum IFNγ (pg/mL) during the first 48 hours of infection, analyzed by ELISA. Data were analyzed by a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test. TLR3$^{-/-}$ mice had significantly less (*p=<0.05*) than control mice at 4 hpi. E) As in C, 6-8 week old TLR3 knockout mice (TLR3$^{-/-}$, gray n=3) or WT C57BL/6 (black n=2) mice were injected IP with 10^6 Nc1:Luc tachyzoites. Quantification of bioluminescence imaging was performed through 96 hours of infection (photons/s). Data were log transformed and two-way repeated measure ANOVA (alpha=0.05) with Dunnett multiple comparisons test was performed. TLR3$^{-/-}$ infections had significantly less parasite burden when compared to control infections at 4 hpi (P=<0.01). F) Serum IFNγ (pg/mL) during the first 48 hours of infection, analyzed by ELISA. Data were analyzed by a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test. No statistically significant differences in IFNγ levels were observed. *p=<0.05, **p=<0.01, ***p=<0.001

3.0 *Neospora caninum* infections in mice induce immediate production of IFNγ by NK, NKT and NKT-like cells

3.1 Introduction

N. caninum and *T. gondii* are closely related apicomplexan parasites with substantial genetic similarity but distinct host compatibilities. *T. gondii* is capable of infecting all warm-blooded animals and causes disease in numerous species including humans. *N. caninum* infects and causes disease in ruminant and canine species and is specifically problematic to cattle and dairy industries worldwide. A large body of work has identified natural killer (NK) cells as IFNγ producers during acute *T. gondii* infections and describe an IL-12-dependent mechanism for NK IFNγ production (Gazzinelli et al., 1994; Hunter, Bermudez, et al., 1995; Hunter, Candolfi, et al., 1995; Hunter, Chizzonite, et al., 1995; Sher et al., 1993). More recent work demonstrated that NK cells from *T. gondii*-infected mice produced significantly less IFNγ than NK cells in uninfected mice (Sultana *et al.*, 2017), suggesting that *T. gondii* can downregulate effector function in NK cells. Although fewer studies have addressed NK cell activity during *N. caninum* mouse infections, NK cells were identified as important early responders during bovine neosporosis and are detected within days of infection in circulation (Klevar *et al.*, 2007). Importantly, bovine NK cells can be directly stimulated by *N. caninum* to produce IFNγ and exert cytotoxicity against *N. caninum*-infected cells (Boysen et al., 2006).

Dendritic cells (DCs) and macrophages also play a crucial role in controlling *T. gondii* infections, both as antigen presenting cells (APCs) and as major sources of IL-12 (Gazzinelli et al., 1994; Hou et al., 2011). Additionally, monocytes are required for host survival to *T. gondii* and

are key to expanding macrophages and DC populations during infection (Ildiko R. Dunay, Fuchs, & Sibley, 2010). Inflammatory monocytes migrate to the site of infection and differentiate into macrophages and DCs that then rapidly respond to infection through phagocytosis, antigen presentation, and cytokine production. Although there are tissue-resident populations of both cell types, it is primarily inflammatory (infiltrating) monocyte-derived cells that produce IL-12 at the site of infection (Goldszmid *et al.*, 2012). NK-derived IFNγ is critical for monocyte differentiation at the site of infection and is also required for local production of IL-12 by monocyte-derived DCs (Goldszmid *et al.*, 2012; Ma *et al.*, 1996). Recent work identified bone marrow NK cells producing IFNγ prior to the onset of systemic inflammation during acute *T. gondii* infections. Importantly, they showed that NK-derived-IFNγ in the bone marrow phenotypically alters monocytes before they egress, ultimately changing their function in target tissues (M. H. Askenase *et al.*, 2015). These examples highlight how local infections alter monocyte cell fate during hematopoiesis, and how IFNγ and IL-12 production can impact innate cell populations prior to systemic inflammatory responses.

The difference between *T. gondii* and *N. caninum* host compatibility in a mouse model is determined by early production of the inflammatory cytokine IFNγ (Coombs et al., 2020). Comparisons between *T. gondii* and *N. caninum* revealed a robust proinflammatory cytokine profile is produced as early as early as 4 hours after infection during a *N. caninum* infection, including significant levels of IFNγ. Although *T. gondii* infections induce IFNγ, it is usually not detected until 2-5 days after infection, long after *N. caninum* infections have been controlled. Several immune cells produce IFNγ in response to *T. gondii* infections and innate immune mechanisms have recently been reviewed (Sasai & Yamamoto, 2019). ILC1, NK cells, iNKT cells, and both CD8 and CD4 T cells produce IFNγ during *T. gondii* infections (I. R. Dunay &

Diefenbach, 2018). Other groups have also identified γδ T cells as IFNγ producers during *T. gondii* infections (Kasper *et al.*, 1996). As we previously reported in Chapter 2, IFNγ is detectable in the serum 2-3 days after infection, and thus changes in the frequencies of cells capable of producing IFNγ during the first few hours of infections are largely unknown. Although the immediate control of *N. caninum* proliferation and production of IFNγ is well-established (Coombs et al., 2020; English et al., 2015; Innes et al., 1995), the changes in innate cell populations, which cells are responsible for driving robust cytokine production, and how that differs from *T. gondii* infections remains unknown. Here, we identify the cellular sources of immediate IFNγ during an *N. caninum* infection and characterize key differences in the immediate, cell-mediated immune response that results in host compatibility differences between *T. gondii* and *N. caninum* in mice. Since early production of IFNγ and IL-12 differ between *T. gondii* and *N. caninum* infections, we reasoned that immediate production of these cytokines may be due to a difference in the frequencies of IFNγ+ of cells present at the site of infection. To address this hypothesis, we compared innate cell populations at the site of infection and in the spleen within hours of infection with *T. gondii* or *N. caninum*.

3.2 Results

3.2.1 Macrophage and dendritic cell populations are not increased at the site of infection or in the spleen 4 hours after infection

Our previous work demonstrated that *N. caninum* induces substantially more IL-12 as early as 4 hpi compared to *T. gondii* infections (Coombs et al., 2020). Since macrophages and dendritic

cells are an important source of IL-12 during acute *T. gondii* infections, we hypothesized that increased IL-12 could be indicative of a differential increase in DCs or macrophages at the site of infection. To determine if *N. caninum* infections resulted in significantly more macrophages or DCs at the site of infection when compared to *T. gondii* we selected the *N. caninum* strain Nc-1 and two strains of *T. gondii* with known virulence differences in mice. Tg:S1T is a low virulence F1 progeny clone from a type II x III cross that carries "avirulent alleles" for 5 key virulence loci that impacts survival in mice (J. P. J. Saeij et al., 2006). Additionally, we chose the type II Tg:Me49 strain since type-II parasites exhibit higher virulence in mice and frequently cause disease in humans (Howe & Sibley, 1995). Importantly, type II tachyzoites exhibit stronger associations with $CD11c^+$ DC cells than type I, and both type II and III *T. gondii* tachyzoites induce higher migratory frequencies in dendritic cells than type I parasites (Lambert, Vutova, Adams, Loré, & Barragan, 2009). Since numerous studies have identified $CD11c^+$ DCs as a crucial source of IL-12 during acute *T. gondii* infections (Hou et al., 2011; Scanga et al., 2002; F. Yarovinsky et al., 2005), we first sought to compare populations of $CD11c^+$ DCs (CD45+, CD11b-, MHC II+) between *T. gondii* and *N. caninum*-infected mice using flow cytometry. Although previous work suggests that DCs are important for IL-12 production during acute *T. gondii* infections, macrophages are also a source of IL-12 (Morgado *et al.*, 2014) and depletion of macrophages leads to neosporosis in mice (Abe et al., 2014). To identify macrophages we employed a similar staining and gating strategy previously described (Rose, Misharin, & Perlman, 2012) including Ly6G/Ly6C markers (Fig 24).

We infected Balb/cJ mice via intraperitoneal (IP) injection with 10^6 *N. caninum* (Nc:Nc1) or *T. gondii* (Tg:S1T or Tg:Me49) tachyzoites and peritoneal cells and spleens were collected for flow cytometry 4 h post-infection (p.i.). We observed no statistically significant differences in the numbers of DCs (Fig 18A) or macrophages (Fig 18B) present at the site of infection or in the

spleen between infected and uninfected mice or between parasite infections. These results were confirmed with additional experiments comparing *T. gondii* (Tg:S1T and Nc:Nc1) and also Tg:Me49 and Nc:Nc1, (data not shown). This indicates that the differences in IL-12 during *T. gondii* and *N. caninum* infection we previously reported (Coombs *et al.*, 2020) are not likely due to an increase in macrophages (CD45+, CD11b+, Ly6G-, CD19-, Ly6c-, MHCII+) or DCs (CD45+, CD11b-, MHC II+) at the site of infection early after infection.

3.2.2 *T. gondii* and *N. caninum* induce similar frequencies of peritoneal monocytes within 4 hours after infection

Inflammatory monocytes are recruited to the site of infection and are crucial for early control of *T. gondii* infections. MCP-1 is produced early during infection and is required for monocytes to egress from the bone marrow (I. R. Dunay *et al.*, 2008; I. R. Dunay & Sibley, 2010). Due to a lack of monocyte egress, mice lacking MCP-1 are acutely susceptible to *T. gondii* infections (P. M. Robben, M. LaRegina, W. A. Kuziel, & L. D. Sibley, 2005), including low-virulence strains (Coombs *et al.*, 2020). We previously demonstrated differences in susceptibility of MCP-1 knockout mice to *T. gondii* and *N. caninum* infections. Despite robust production of MCP-1 during *N. caninum* infections, mice lacking MCP-1 are still resistant to *N. caninum* infections and control parasite proliferation within 24 hours of infection (Coombs *et al.*, 2020). Since we observed differences in MCP-1 production and susceptibility in MCP-1 knockout mice, we hypothesized there would be differences in early monocyte populations comparing *T. gondii* and *N. caninum.* In addition to identifying macrophages, the staining and gating strategy described above (Fig 24) also identifies monocytes (CD45+, CD11b+, $Ly6G^{Low}$, CD19-, $Ly6C^{High}$). Unlike DC and macrophage populations, we observed a significant ($p<0.05$) increase in the number of

peritoneal monocytes comparing infected and uninfected mice, however there was no significant difference between *T. gondii* and *N. caninum* infections (Fig 18C). As with macrophages and DCs, there were no significant differences across all spleen samples (Fig 18F). These data suggest that monocyte populations are not significantly increased in *N. caninum* infections compared with *T. gondii* despite substantially more proinflammatory cytokines and chemokines being produced early after IP infection with *N. caninum*.

3.2.3 *N. caninum* infections exhibit significantly more DX5+ IFNγ+ peritoneal cells 4 hours after infection compared to *T. gondii*-infected and naïve mice

Our previous work in Chapter 2 described MyD88-dependent production of IFNγ during *N. caninum* infections that was detected both at the site of infection and in the serum during the first few hours of infection, but the nature and quantity of IFNγ-producing cells immediately after infection remained unknown (Coombs *et al.*, 2020). Therefore, we sought to compare the frequency of IFNγ-producing cells with the expectation that the number of peritoneal cells producing IFNγ would be significantly elevated in *N. caninum* infections compared to *T. gondii* and naïve mice. To quantify the number of IFNγ-producing cells we performed intracellular cytokine staining and flow cytometric analysis on *T. gondii* and *N. caninum*-infected or naïve mouse samples. We gated cells to remove debris and clumps as described and identified CD45+ IFNγ+ cells (Fig 25). As expected, we observed significantly ($p<0.01$) elevated frequencies of IFNγ-producing peritoneal cells (CD45+, IFNγ+) in *N. caninum*-infected mice compared to naïve and *T. gondii*-infected mice (Fig 19AB, Fig 26 B,C), while no significant differences were observed in spleen samples (Fig 19A,C). *T. gondii* infections did not exhibit statistically significant

differences when compared to naïve mice, indicating that *N. caninum* infections uniquely increase the percent of IFNγ-producing cells as early as 4 hours after IP infection.

Since NK and NKT cells produce IFNγ during microbial infections (Ishikawa *et al.*, 2000), and DX5/Cd49b is considered a pan NK marker and is especially useful in identifying NKT and NK cells in NK1.1-negative Balb/cJ mice (Ivanova *et al.*, 2019; Werner *et al.*, 2011), we sought to compare expression of CD49b using the DX5 antibody in the CD45+ IFNγ+ cell population. We found *N. caninum* infections resulted in significantly ($p<0.01$) more IFNγ-producing CD45+ IFNγ+ DX5+ peritoneal immune cells than *T. gondii* and naïve mice (Fig 20A,B), and no significant differences in CD45+ IFNγ+ DX5+ spleen cells (Fig 20A,C). Moreover, our analysis of two separate experiments (n=8 Tg:S1T infected mice, n=7 Nc:Nc1 infected mice) revealed that nearly all (~94-99% Fig 23A) peritoneal CD45+ IFNγ+ cells were DX5+, suggesting that most, if not all, IFNγ-producing cells are likely NK or NKT cells early after infection (Male & Brady, 2017; Werner *et al.*, 2011). Based on reproducibility of these results demonstrated in two independent experiments, we conclude that IFNγ+ cells are largely DX5+ in both *N. caninum* and *T. gondii* infections at the 4 h timepoint, and *N. caninum* infections have significantly more DX5+ IFNγ+ cells than naïve and *T. gondii* infected mice.

3.2.4 *T. gondii* infections exhibit fewer peritoneal IFNγ+ NK cells compared to uninfected and *N. caninum*-infected

NK cells were originally identified based on their ability to lyse virus-infected cells and tumor cells (Vivier *et al.*, 2018) and their activity is governed by a multiple activating and inhibitory signaling pathways orchestrated by surface molecules that differ in expression based on species, function, development, and target tissue (Abel, Yang, Thakar, & Malarkannan, 2018). The

heterogeneity of NK marker expression has made NK cells more elusive to characterize than other innate cells (Lankry, Gazit, & Mandelboim, 2010) and flow cytometry panels utilize a number of NK lineage-exclusionary markers like CD3 and TCRβ combined with species/strain appropriate NK markers to evaluate NK cell populations (Veluchamy *et al.*, 2017). To identify NK cells in the present study, we identified IFNγ-producing NK cells as CD45+, IFNγ+, DX5+, TCRβ-, CD90-, CD3- (Gazzinelli *et al.*, 1994; Lin, Nieda, Rozenkov, & Nicol, 2006; Werner *et al.*, 2011). As described above, we compared peritoneal and spleen cells from Balb/cJ mice infected IP with 10^6 *N. caninum* (Nc:Nc1) *T. gondii* (Tg:S1T or Tg:Me49) with naïve mice. Our analysis revealed that NK cells (CD45+, IFNγ+, DX5+, TCRβ-/CD90-, CD3-) in *N. caninum* infections were not significantly elevated above uninfected samples. Surprisingly, *T. gondii*-infected mice had significantly fewer peritoneal IFNγ+ NK cells than uninfected mice (Fig 21A,B). As with other cell populations, we saw no significant difference in the frequency of spleen NK cells (Fig 21A,D). This suggests that *T. gondii* infections lower the number of peritoneal IFNγ+ NK cells, rather than *N.* caninum significantly increasing them.

3.2.5 *N. caninum* infections induce significantly more IFNγ+ peritoneal natural killer T cells (NKTs) cells than T. gondii within hours of infection

Natural killer T cells (NKTs) are a heterogenous populations of innate-like T cells that can be categorized based on cytokine production, antigen recognition, MHC/CD1d restriction, and TCR expression (Godfrey et al., 2004). Although NK cells are early responders during parasitic infection, numerous studies have also highlighted important roles for NKT and during *T. gondii* (Nakano *et al.*, 2001; Smiley *et al.*, 2005) and *N. caninum* infections (Nishikawa *et al.*, 2010). Based on the diverse TCR repertoire expressed on NKT cells, we included multiple markers to

identify NKT cells. We identified CD3+ NKT-like peritoneal cells that were TCRβ-/CD90- (Fig 21A,C), and CD4+ TCRβ+/CD90+ NKT cells (Fig 22A,B) as the two largest subpopulations of NKT cells producing IFNγ within hours of *N. caninum* infection. Additionally, there was a smaller number of CD8+ TCRβ+/CD90+ NKT cells that were also IFNγ+ (Fig 5A,C). In the first two subgroups (Cd4+ NKT and CD3+ NKT), *N. caninum* infections exhibited significantly higher frequencies of peritoneal IFNγ+ NKTs than *T. gondii* and uninfected mice (Fig 21 and 22, CD4+ NKTs $p<0.01$, CD3+NKTs $p<0.001$). As with our other comparisons we found no significant differences in spleen samples (Fig 21D,E, Fig 22D,E). To validate these results, we also analyzed data from two independent experiments to determine the frequencies of NK and NKT cells in the IFNγ+ cell compartment (Fig 23). We determined that NK cells were on average 64% of all peritoneal IFNγ+ cells in *T. gondii* infections. In contrast, NK cells were a significantly lower percentage of all peritoneal IFNγ+ cells in *N. caninum* infection ($p<0.01$), with NK cells accounting for only 47% of IFNγ+ cells (Fig 23B,C). When all NKT cells were grouped together (CD3+NKTs, CD4+NKTs, CD8+NKTs), they accounted for only 28% of *T. gondii* peritoneal IFNγ+ cells, while they accounted for 44% of the peritoneal IFNγ+ compartment in response to *N. caninum* ($p<0.05$). Taken together, we can conclude that IFNγ host responses to *N. caninum* are exhibit more IFNγ-producing NKTs compared to *T. gondii,* and of the NKTs we identified, CD3+ NKTs are the largest population. The early activation of NKT cells to produce IFNγ within hours of *N. caninum* infections and not *T. gondii* may play a critical role in determining the dramatic differences in host compatibility between these species in mice.

3.3 Discussion

This study compared innate immune cell populations in Balb/cJ mice hours after infection and our findings are in agreement with previous reports identifying a crucial role for NKT cells during *N. caninum* infections in Balb/cJ mice (Nishikawa *et al.*, 2010). Differences in susceptibility of BALB/c and C57BL/6 to *T. gondii* have been well-characterized (Araujo, Williams, Grumet, & Remington, 1976; Brandão *et al.*, 2011). Other work identified an important role for CD4+ NKTs during early *L. major* infections in Balb/cJ mice while C57BL/6 mice are generally resistant (Ishikawa *et al.*, 2000). Therefore it is possible that comparisons in between C57BL/6 and Balb/cJ might reveal differences in NK and NKTs during *N. caninum* infections, but these strains are not differently susceptible to *N. caninum* infections (Long et al., 1998). Although our previous work verified similar cytokine profiles and infection kinetics in Balb/cJ and C57BL/6 mice during *N. caninum* infections (Coombs *et al.*, 2020), further experiments would be needed to verify results of the current study in additional mouse strains.

ILCs are a recently described group of innate cells that modulate immune responses by producing cytokines like IFNγ and TNFα. Group 1 Innate lymphoid cells include NK cells and ILC1s, and both have been identified as important innate sources of IFNγ during *T. gondii* infections (I. R. Dunay & Diefenbach, 2018). Yet the mechanisms that activate ILC and ILC-like cells during *T. gondii* are completely unknown (Sasai & Yamamoto, 2019). Recent advances in NK cell biology have revealed significant diversity in NK cell phenotype and function using transcription factors, NK marker expression, tissue specificity, and effector function. Here, we identify NK cells as CD45+, IFNγ+, DX5+, TCRβ-, CD90-, CD3- that might be similar to conventional NK or cNK cells. Functional assays would be necessary to determine NK subtypes

because NK cell designations are often based on functionality (Erick & Brossay, 2016). Substantial efforts have been made to determine NK subtypes in numerous tissues including the liver, skin, the uterus, submandibular glands, the spleen, kidneys as well as in circulation, yet few have examined peritoneal cells at steady state or early after infection (Abel *et al.*, 2018; I. R. Dunay & Diefenbach, 2018; Erick & Brossay, 2016; Male & Brady, 2017). A more detailed characterization of these cells and comparisons of NK responses to both *N. caninum* and *T. gondii* will be necessary to fully understand the impact changes in NK cells have on infection. Future direction of this work will be to determine if *T. gondii* is subverting NK function within hours of infection, and how that impacts *T. gondii* infection. Our work shows that *T. gondii* infections exhibit fewer IFNγ+ cells compared to uninfected mice, complementing previous reports showing NK cells from *T. gondii*-infected mice produce less IFNγ than uninfected mice (Sultana et al., 2017). We further demonstrate that *N. caninum* infections have the opposite effect, exhibiting elevated numbers of IFNγ+ NK cells compared to *T. gondii* infections. Although we observed a slight increase in NK cells compared to uninfected mice, this difference was not statistically significant. Further experiments are necessary to determine if IFNγ+ NK cells continue to increase in *N. caninum* infections by repeating this experiment at multiple timepoints.

Recent findings revealed *T. gondii* infections drive conversion of NK cells into ILC-like cells and the ILC-like phenotype is maintained after control of the acute infection (Park *et al.*, 2019). One explanation for the lower abundance we observed of NKs (IFNγ+, TCRβ/CD90-, CD3-, DX5+) during *T. gondii* infections is that *T. gondii* begins driving conversion of NK cells within hours of infection resulting in fewer IFNγ+ DX5+ cells. Complementary to this idea, we did not observe significant differences in total DX5+ cell populations (combined IFNγ+ and IFNγ-, data not shown), and instead only observed a difference in the number of DX5+ IFNγ+ cells. Our work

suggests *N. caninum* infections differentially activates DX5+ cells to produce IFNγ, but further characterization of effector function and phenotype of these cells is necessary to address this hypothesis.

NKT cells are also important sources of IFNγ during apicomplexan infections, and previous work investigate NKT cell cytokine production beginning at 7 days after *T. gondii* infections (Nakano *et al.*, 2001; Smiley *et al.*, 2005). However, to our knowledge, this is the first report of *T. gondii* and *N. caninum* altering NK and NKT cell populations immediately after infection. NKT subpopulations exhibit differences in their ability to activate cells as well as differences in their cytokine production profiles (Lin *et al.*, 2006). Previous work demonstrated that iNKTs can be activated by direct TLR stimulation, and indeed express numerous TLRs (P. W. Askenase et al., 2005; Grela et al., 2011; Kim et al., 2012; Slauenwhite & Johnston, 2015) Our work outlined in Chapter 2 determined that immediate production of IFNγ is MyD88-dependent and it will be an important future direction of this work is to identify the TLR mechanism that results in NKT cell IFNγ production. This may be via direct stimulation, or dependent on an accessory cell that is required for NKT activation.

In cattle, CD4+ T cells and γδT cells have been identified as important sources of IFNγ during bovine infections (Marks, Lundén, Harkins, & Innes, 1998) and subsequent efforts have focused on identifying antigens recognized by CD4+T cell antigens (Tuo, Fetterer, Davis, Jenkins, & Dubey, 2005) Importantly, recent work suggests that NKTs (and not NKs) are important for CD4+T cell activation during *N. caninum* infections in mice (Nishikawa *et al.*, 2010). Cellular Infiltration during ruminant pregnancy is thought to be a significant cause of abortive pathology during *N*. caninum infection in numerous species (Buxton et al., 2002). One group identified γδT cells, CD3+ lymphocytes, CD4+T cells as infiltrating cells in the buffalo placentome early during

N. caninum infections (Cantón *et al.*, 2014). In cattle, the cellular infiltrates have been identified as CD3+ Lymphocytes, CD4+ T cells, γδT cells, and to a much lesser extent CD8+ T cells (Maley *et al.*, 2006). Identification of NK cells in cattle has been delayed due to a lack of known bovine NK cell markers, and only recently was one described and incorporated into immune cell studies (Boysen & Storset, 2009; Maley *et al.*, 2006). NK biology in bovine and other ruminant species is uncharacterized compared with humans and mice, and far fewer markers and phenotypic knowledge exists. It is entirely possible that the CD3+ Lymphocytes often described as separate populations from γδT cells, CD4 T cells, and CD8 T cells may include a subset unidentified of NKTs. Further characterization of *N. caninum* and *T. gondii* interactions with NK and NKT cells immediately after infection may enable future identification of similar bovine cells based on cellular interactions with parasite infections.

3.4 Materials and methods

3.4.1 Parasite strains, maintenance and infection

Parasite strains *Neospora caninum* Nc1:LucDsRed (Nc:Nc1), *Toxoplasma gondii* TgS1T:Luc:DsRed (Tg:S1T) and TgMe49 were maintained in human foreskin fibroblasts as previously described (Coombs *et al.*, 2020). Cells and parasites were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) (cDMEM) and grown in humidified, 5% CO_2 incubators at 37 degrees. To obtain parasites for infections, cells were passed through 25- and 27-gauge needles, pelleted by centrifugation and resuspended in 3 mL PBS. Parasites were

quantified using a hemocytometer and concentrations were adjusted by dilution or concentrated with centrifugation.

3.4.2 Animals

Experiments were performed with 6-8-week-old female mice obtained from Jackson Laboratories. The following strains were used: BALB/cJ. All animal procedures in this study met the standards of the American Veterinary Medical Association and were approved locally under the University of Pittsburgh Institutional Animal Care and Use Committee protocol number #12010130. Animal protocol meets National Institutes of Health (NIH) Public Health Policy (PSH) on Humane Care and Use of Laboratory Animals and United States Department of Agriculture (USDA) Animal Welfare Regulation (AWR) guidelines

3.4.3 Sample preparation for flow cytometry

6-8-week-old Balb/C mice were purchased form Jackson laboratories and were infected with 10^6 tachyzoites of *N. caninum* Nc1 strain or *T. gondii* Me49 or S1T strain. Mice were sacrificed 4 hours after infection and peritoneal and spleen samples were collected from infected mice and additionally collected from uninfected (naive) mice for comparisons. Peritoneal cells were collected by peritoneal lavage with 5-10mLs of Ice cold RPMI media containing 3%FBS, 1:1000 50mM BME, 1:100 penicillin (100 U/mL), streptomycin (100 µg/mL), and 1:50 HEPES. PEC cells were then washed and incubated with the selected antibody cocktails. For spleen samples, the spleens were harvested from the infected and naïve mice described above. Spleens were suspended in the media described above and kept on ice. Spleen cells were suspended using

a 70uM filter and washed using centrifugation and media. Red blood cells were lysed using 1 ml ACK lysis buffer and washed again. Step was repeated once if necessary and then incubated with the appropriate antibody cocktail.

3.4.4 Flow cytometry

Splenocytes and PECs were stained using the pertinent cocktail of following antibodies: Cd45.2, CD11b, CD11c, MHC II, Ly6G, Ly6C, CD19, DX5, CD3, TCRβ, CD90, CD4, CD8b, CD8a, IL-12, IFNγ, CD44, CD64, CD103, TNFα, GMCSF, NK1.1, γδTCR. Dead cells were excluded using LIVE/DEAD fixable dead stain (ThermoFisher). All stains were performed in media containing anti-CD16/32 blocking antibody (clone 93, ThermoFisher). For intracellular staining for cytokines, cells were incubated with 5μg/mL Brefeldin-A for 3.5 hours at 37°C, following which they were fixed and permeabilized using the FoxP3/Transcription factor staining buffer set according to the manufacturer's directions.

3.4.5 Statistical analysis

All statistical analysis was performed using Prism 7 (GraphPad Software, Inc). Statistical tests were chosen based on experimental design. Results from flow cytometry experiments were transformed and analyzed using one-way ANOVA with Tukey's multiple comparison test. Bioluminescent and cytokine concentration was analyzed using a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test, Statistical methods are described in the figure legends where appropriate. *p=<0.05, **p=<0.01, ***p=<0.001.

3.5 Figures

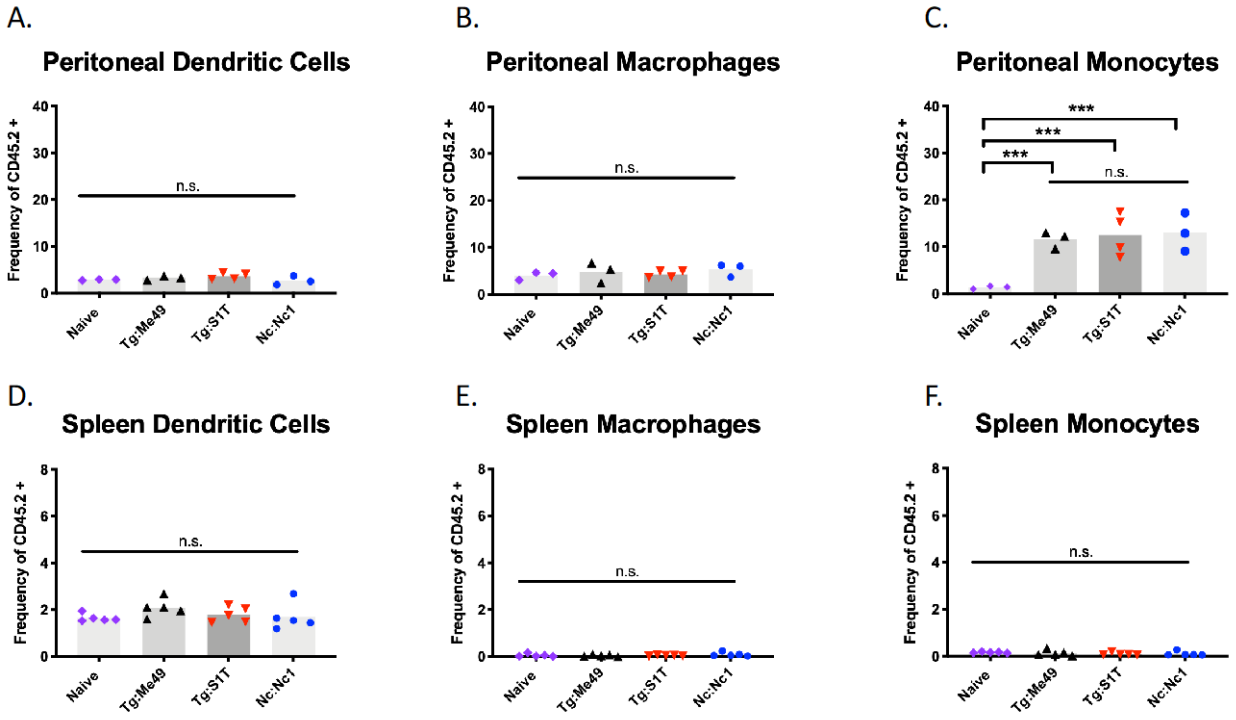

Figure 18 Comparison of peritoneal DC, macrophage, and monocyte populations

6-8 week old BALB/c mice were infected with *T. gondii* (Tg:Me49 black, Tg:S1T,red) or *N. caninum* (Nc:Nc1, blue) and compared with uninfected (naïve, purple) samples. Peritoneal and spleen cells were collected and stained with relevant antibody cocktails. Flow cytometric analysis was used to compare cell populations. A-F) n=3 naïve, n=3 Tg:Me49, n=4 Tg:S1T, n=3 Nc:Nc1. G-I) n=5 naïve, n=5 Tg:S1T, n=5 Nc:Nc1. Cell populations are frequencies of live leukocytes (frequency of CD45.2+) A) peritoneal DCs, D) Spleen DCs. DC's were identified using the gating strategy outlined in Fig 24 and are defined as CD45+, CD11c+, CD11b-, $MHCII^{High}$. B) Peritoneal macrophages, E) Spleen Macrophages. Macrophages were identified using the gating strategy described in Fig 24 and are defined as CD45+, CD11b+, $Ly6G^{Low}$, CD19-, $Ly6C^{Low}$ $MHCII^{High}$. C) Peritoneal monocytes and F) Spleen monocytes. Monocytes were defined by CD45+, CD11b+, $Ly6G^{Low}$, CD19-, $Ly6C^{High}$ $MHC\ II^{Negative}$. Data were transformed and then a one-way ANOVA with Tukey's multiple comparison test was performed to identify significance. Data shown is one experiment and results were confirmed with an additional experiment (data not shown) *p=<0.05, **p=<0.01, ***p=<0.001

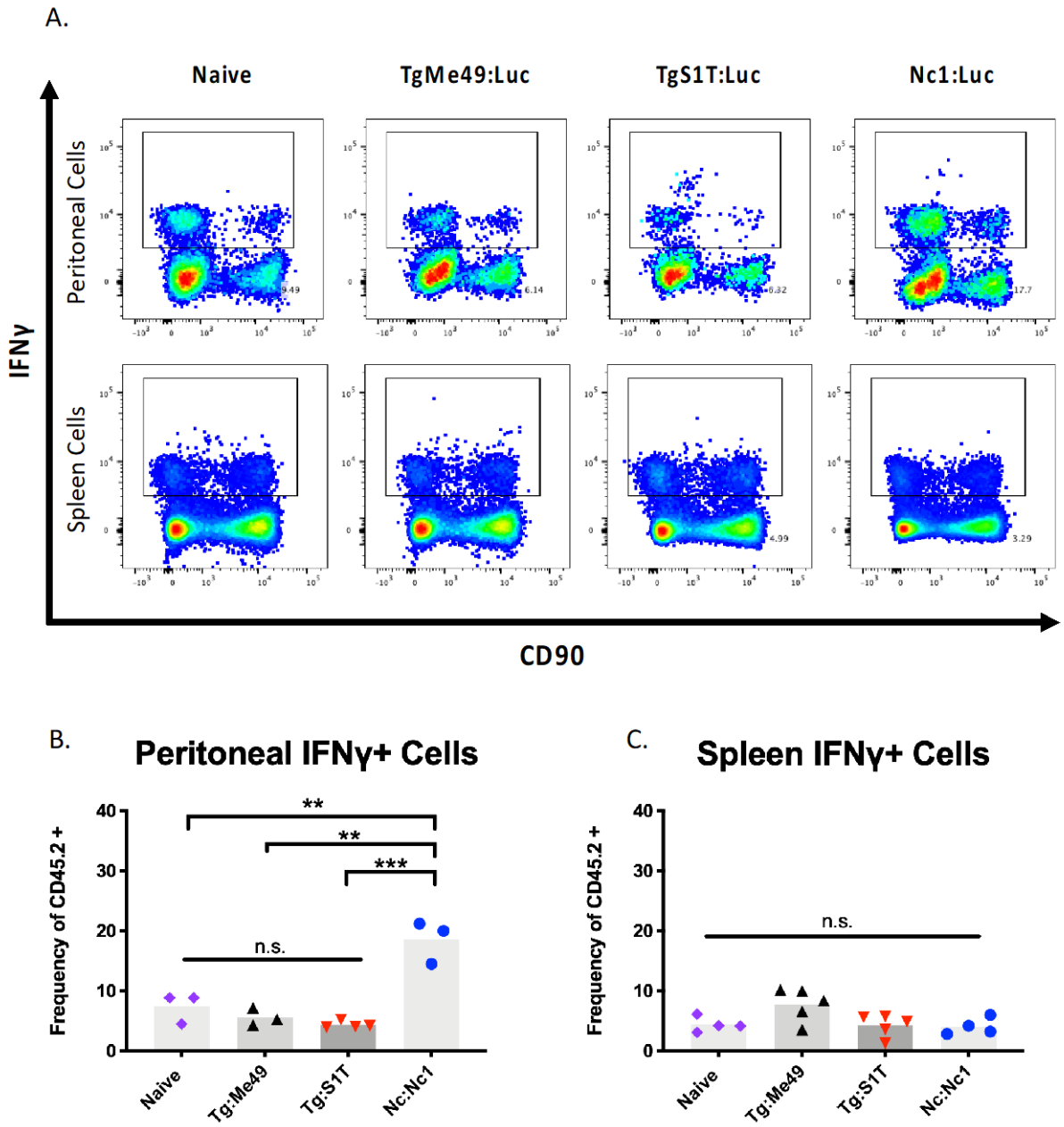

Figure 19 Comparison of IFNγ+ cells

6 week old BALB/c mice were infected with *T. gondii* (Tg:Me49 black, Tg:S1T,red) or *N. caninum* (Nc:Nc1, blue) and compared with uninfected (naïve, purple) samples. Peritoneal and spleen cells were collected and stained with relevant antibody cocktails and intracellular cytokine staining was performed. Flow cytometric

analysis was used to compare cell populations. A-C) n=3 naïve, n=3 Tg:Me49, n=4 Tg:S1T, n=3 Nc:Nc1. A) Representative flow plots of IFNγ+ cells identified using the gating strategy outlined in Fig 25A. B) Frequency of peritoneal CD45+ live cells that are IFNγ+ showing Nc:Nc1 significantly elevated above other samples. C) Frequency of spleen CD45+ live cells that are IFNγ+. Data were transformed and a one-way ANOVA with Tukey's multiple comparison test was performed to identify significance. Data shown is one experiment and results were confirmed with an additional experiment (data not shown). *p=<0.05, **p=<0.01, ***p=<0.001

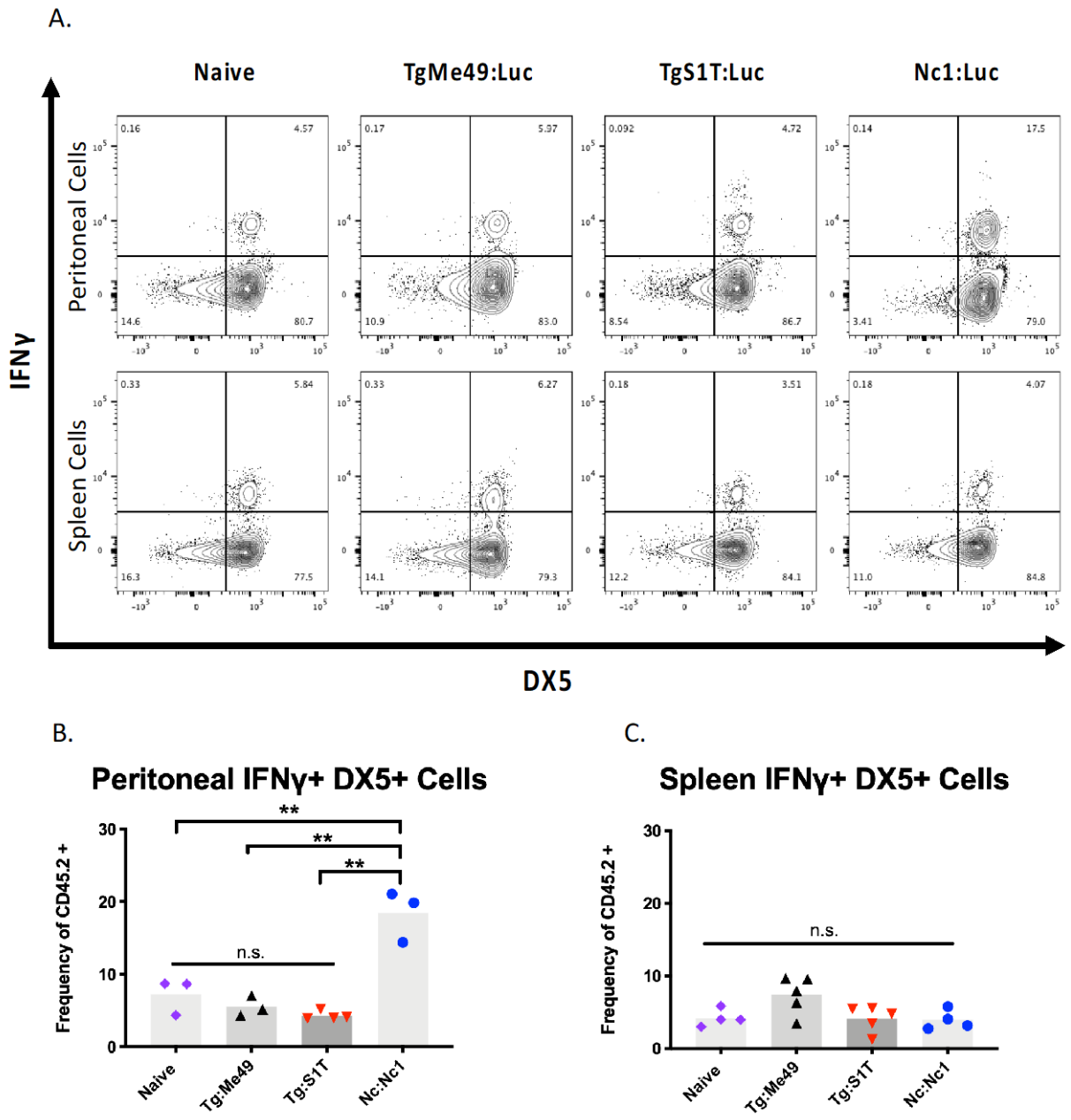

Figure 20 Comparison of IFNγ+ DX5+ cells

6 week old BALB/c mice were infected with *T. gondii* (Tg:Me49 black n=3, Tg:S1T,red n=4) or *N. caninum* (Nc:Nc1, blue n=3) and compared with uninfected (naïve, purple n=3) samples. Peritoneal and spleen cells were collected and stained and intracellular cytokine staining was performed. Flow cytometric analysis was used to compare cell populations. A) Representative flow plots of CD45+ live cells gated by IFNγ and DX5 cells using the gating strategy outlined in Fig 25B. B) Frequency of CD45+ IFNγ+ DX5+ peritoneal cells. Data

were transformed and a one-way ANOVA with Tukey's multiple comparison test was performed to identify significance. Data shown is one experiment and results were confirmed with an additional experiment (data not shown). *p=<0.05, **p=<0.01, ***p=<0.001

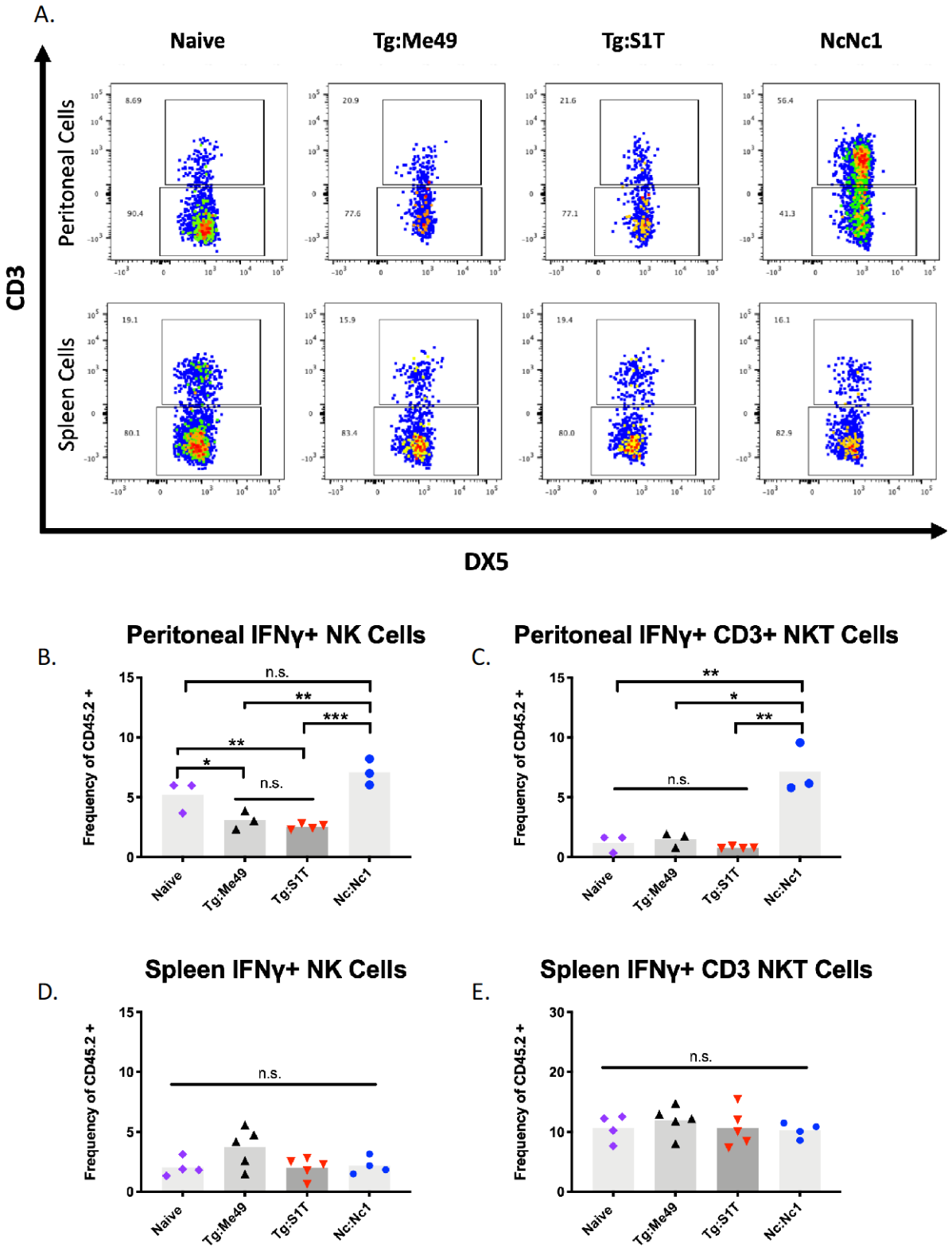

Figure 21 Identification of NK cells and CD3+ NKT cells

6 week old BALB/c mice were infected with *T. gondii* (Tg:Me49 black, Tg:S1T,red) or *N. caninum* (Nc:Nc1, blue) and compared with uninfected (naïve, purple) samples. Peritoneal and spleen cells were collected and stained with relevant antibody cocktails and intracellular cytokine staining was performed, followed by analysis using flow cytometry. A-E) n=3 naïve, n=3 Tg:Me49, n=4 Tg:S1T, n=3 Nc:Nc1. A) Representative flow plots of IFNγ+, DX5+, TCRβ-/CD90-, CD3- NK cells (bottom box) and IFNγ+, DX5+, TCRβ-/CD90-, CD3+ NKT cells (top box) identified using the gating strategy outlined in Fig 26A. B) Frequency of peritoneal CD45+ IFNγ+, DX5+, TCRβ-/CD90-, CD3- NK cells. C) Frequency of peritoneal CD45+, IFNγ+, DX5+, TCRβ-/CD90-, CD3+ NKT cells D) Frequency of spleen CD45+ IFNγ+, DX5+, TCRβ-/CD90-, CD3- NK cells. E)) Frequency of spleen CD45+, IFNγ+, DX5+, TCRβ-/CD90-, CD3+ NKT. Data were transformed and a one-way ANOVA with Tukey's multiple comparison test was performed to identify significance. *p=<0.05, **p=<0.01, ***p=<0.001

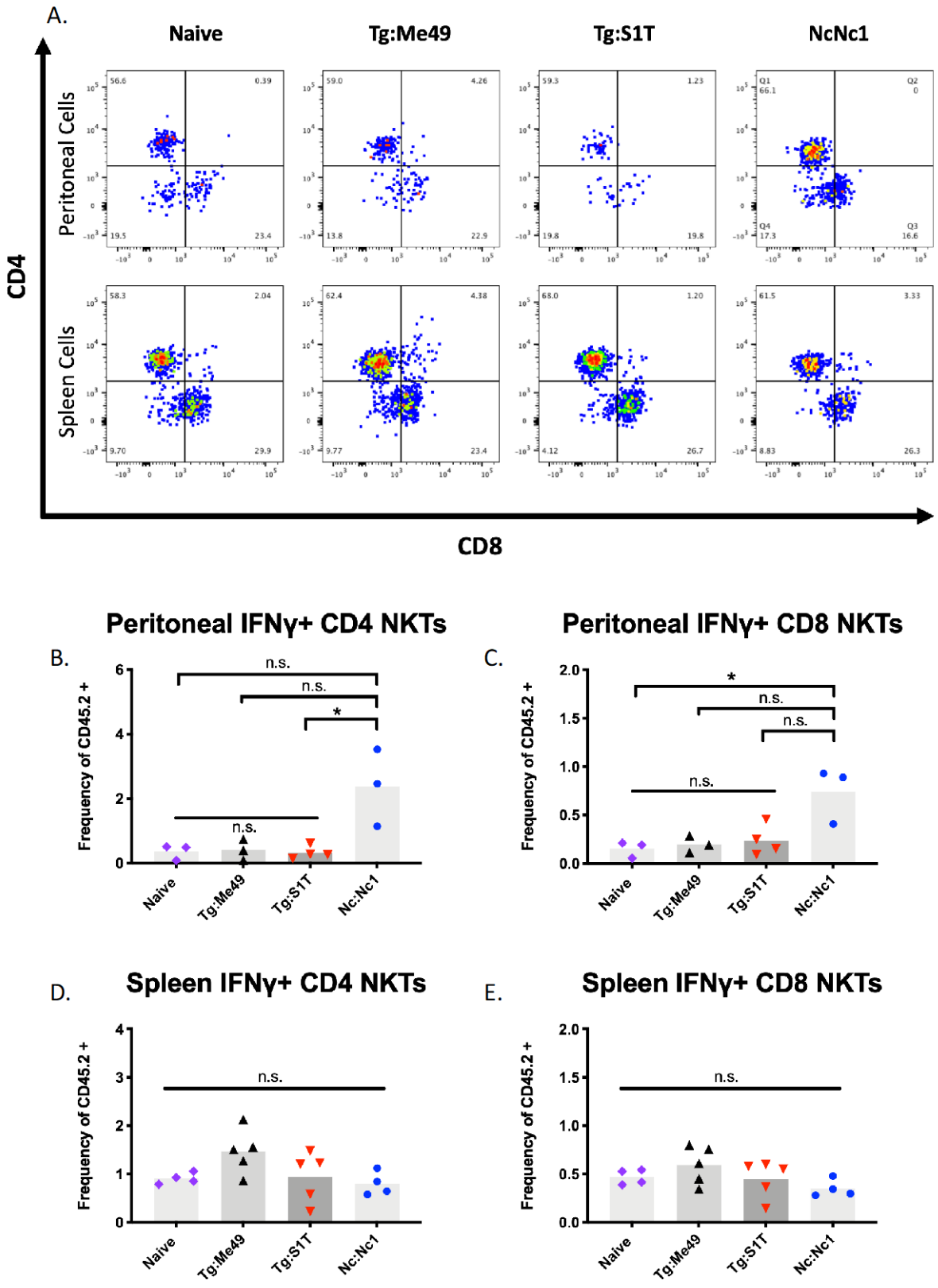

Figure 22 Identification of TCRb+/CD90+ NKT cells

6 week old BALB/c mice were infected with *T. gondii* (Tg:Me49 black, Tg:S1T,red) or *N. caninum* (Nc:Nc1, blue) and compared with uninfected (naïve, purple) samples. Peritoneal and spleen cells were collected and stained with relevant antibody cocktails and intracellular cytokine staining was performed. Flow cytometric analysis was used to compare cell populations. A-E) n=3 naïve, n=3 Tg:Me49, n=4 Tg:S1T, n=3 Nc:Nc1. A) Representative flow plots of IFNγ+, DX5+, TCRβ+/CD90+ CD4+ or CD8+ NKT cells using the gating strategy outlined in Fig 26A. B) Frequency of peritoneal CD45+ IFNγ+, DX5+, TCRβ+/CD90+, CD4+ NKTs. C) Frequency of peritoneal CD45+, IFNγ+, DX5+, TCRβ+/CD90+, CD8+ NKT cells D) Frequency of spleen CD45+ IFNγ+, DX5+, TCRβ+/CD90+, CD4+ NKT cells. E) Frequency of spleen CD45+, IFNγ+, DX5+, TCRβ+/CD90+, CD8+ NKT cells. Data were transformed and a one-way ANOVA with Tukey's multiple comparison test was performed to identify significance. *p=<0.05, **p=<0.01, ***p=<0.001

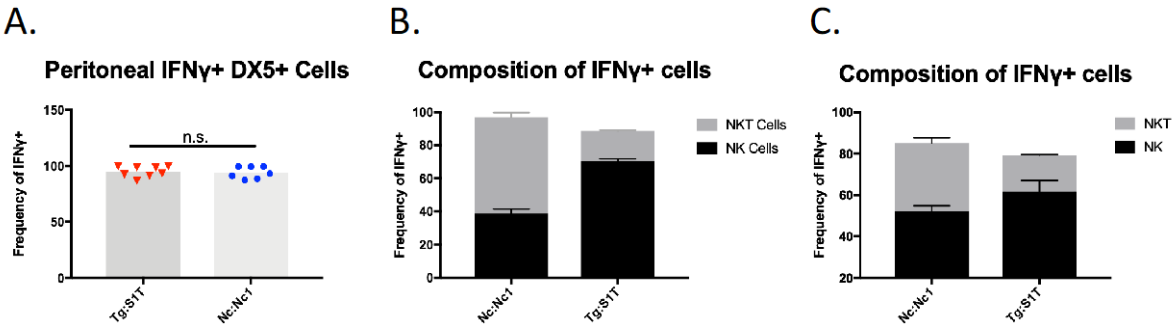

Figure 23 Differences in NK and NKT cell frequencies of total IFNγ+ cells

6-8 week old BALB/c mice were infected with *T. gondii* (Tg:S1T, red) or *N. caninum* (Nc:Nc1, blue). Peritoneal cells were collected and stained with relevant antibody cocktails and intracellular cytokine staining was performed. A) Data is combined from 2 experiments (Total n=7 Nc:Nc1, total n=8 Tg:S1T). A) Frequency of peritoneal IFNγ+ cells that are DX5+ B-C) Frequency of peritoneal IFNγ+ cells that are NK cells (DX5+, TCRβ-/CD90-, CD3-) or NKT cells (TCRβ+/CD90+, CD8+, TCRβ+/CD90+, CD4+, and TCRβ-

/CD90-, CD3+). B) Nc:Nc1 n=4, Tg:S1T n=4. C) Nc:Nc1 n=3, Tg:S1T n=4. One-way ANOVA with Tukey's multiple comparison test was performed to identify significance. *p=<0.05, **p=<0.01, ***p=<0.001.

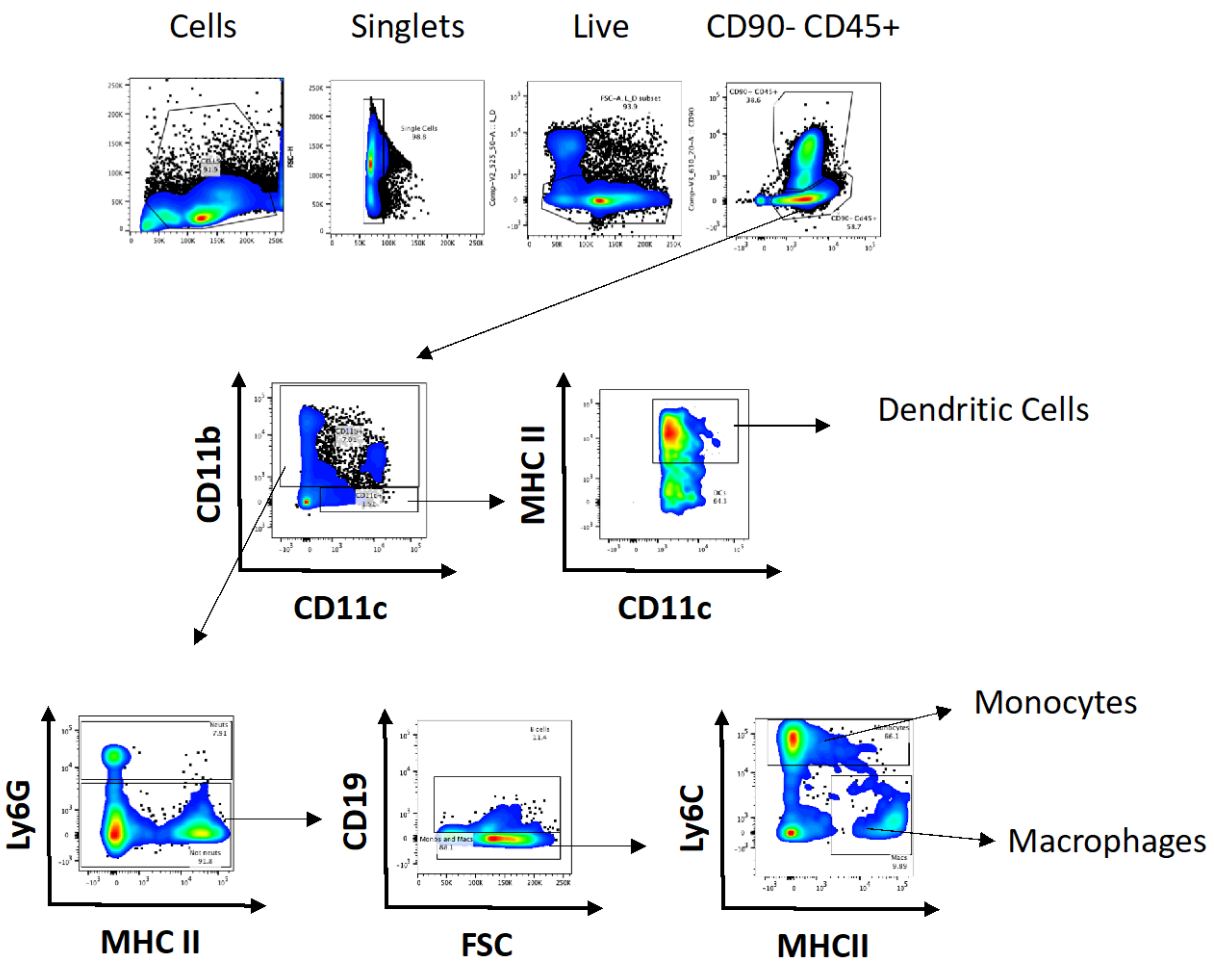

Figure 24 Gating strategy for monocytes, dendritic cells and macrophages

Gating strategy to identify monocyte, dendritic cell and macrophage populations. Clumps and small debris were removed by gating SSC-A x FSC-A. Doublet were removed by gating FSC-H x FSC-W. Live cells were identified using a viability stain (LD) and gated L/D x FSC-A. Leukocytes were then identified based on CD45.2 expression and CD90+ cells were excluded by gating Cd45.2 x CD90. Live CD45+, CD90- cells were gated by CD11b x CD11c to identify CD11b-, CD11c+ cells and CD11b+ cells. CD11b-, CD11c+ cells were then gated with MHC II to identify CD11b-, CD11c+, MHC II+ dendritic cells. CD11b+ cells were further analyzed by excluding Ly6G+ (neutrophils) and excluding CD19+ (B cells). CD11b+, Ly6G-, CD19- cells were gated by $Ly6C^{High}$ (monocytes) and $Ly6C^{Low}$ $MHCII^{High}$ (Macrophages).

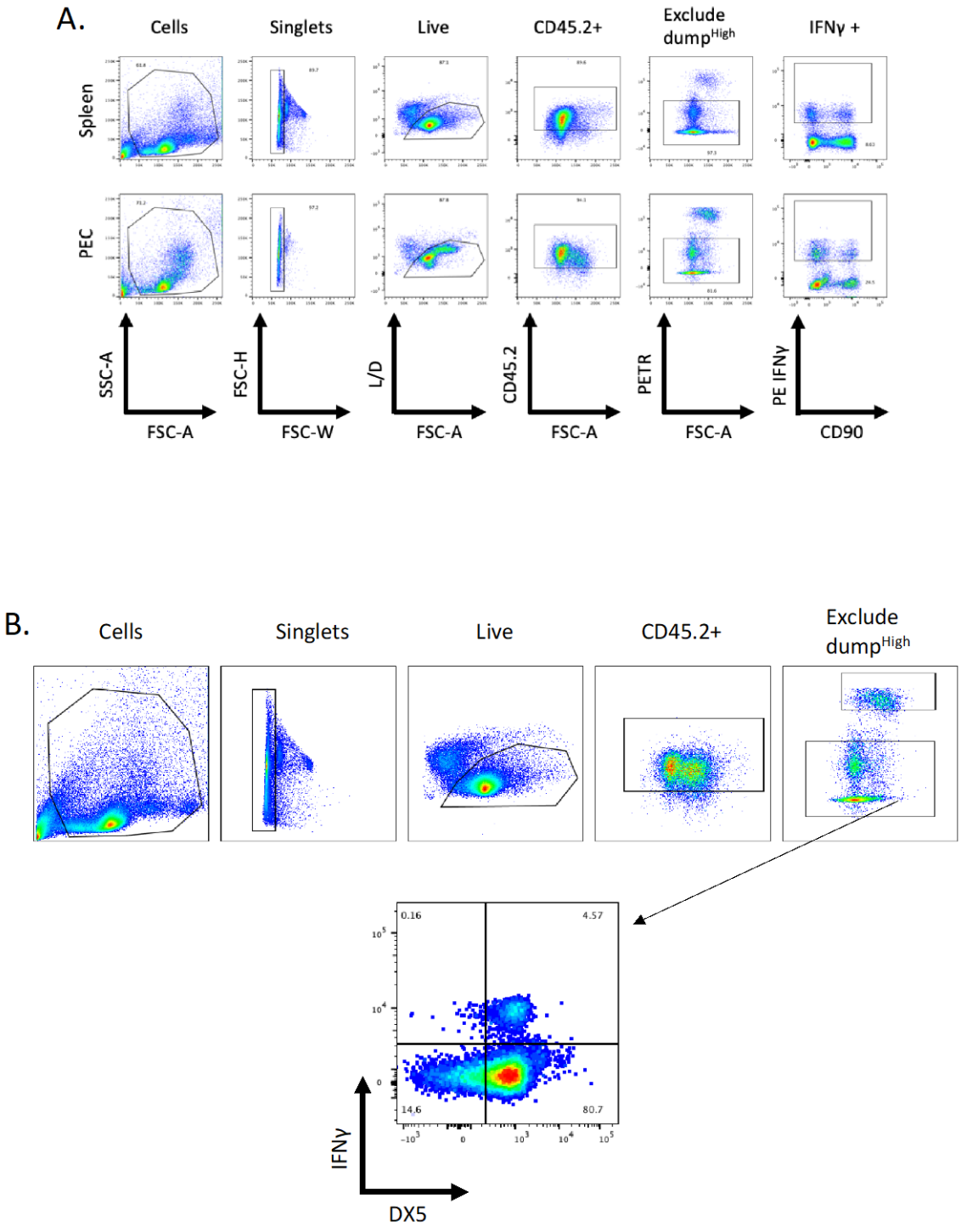

Figure 25 Gating strategy for IFNγ+ cells and total DX5+ (IFNγ+ and IFNγ- cells)

A: Gating strategy to identify IFNγ+ cells. Clumps and small debris were removed first by gating SSC-A x FSC-A. Doublet were removed by gating FSC-H x FSC-W. Live cells were identified using a viability stain (LD) and gated L/D x FSC-A. Leukocytes were then identified based on CD45.2 expression and gated Cd45.2 x FSC-A. To reduce spillover and non-specific staining from tandem dye degradation we excluded cells staining high in our "dump" channel. Cells were then gated for IFNγ+ staining. B: To identify total DX5+ cells, cells were gated as described in A, except cells were gated by IFNγ x DX5.

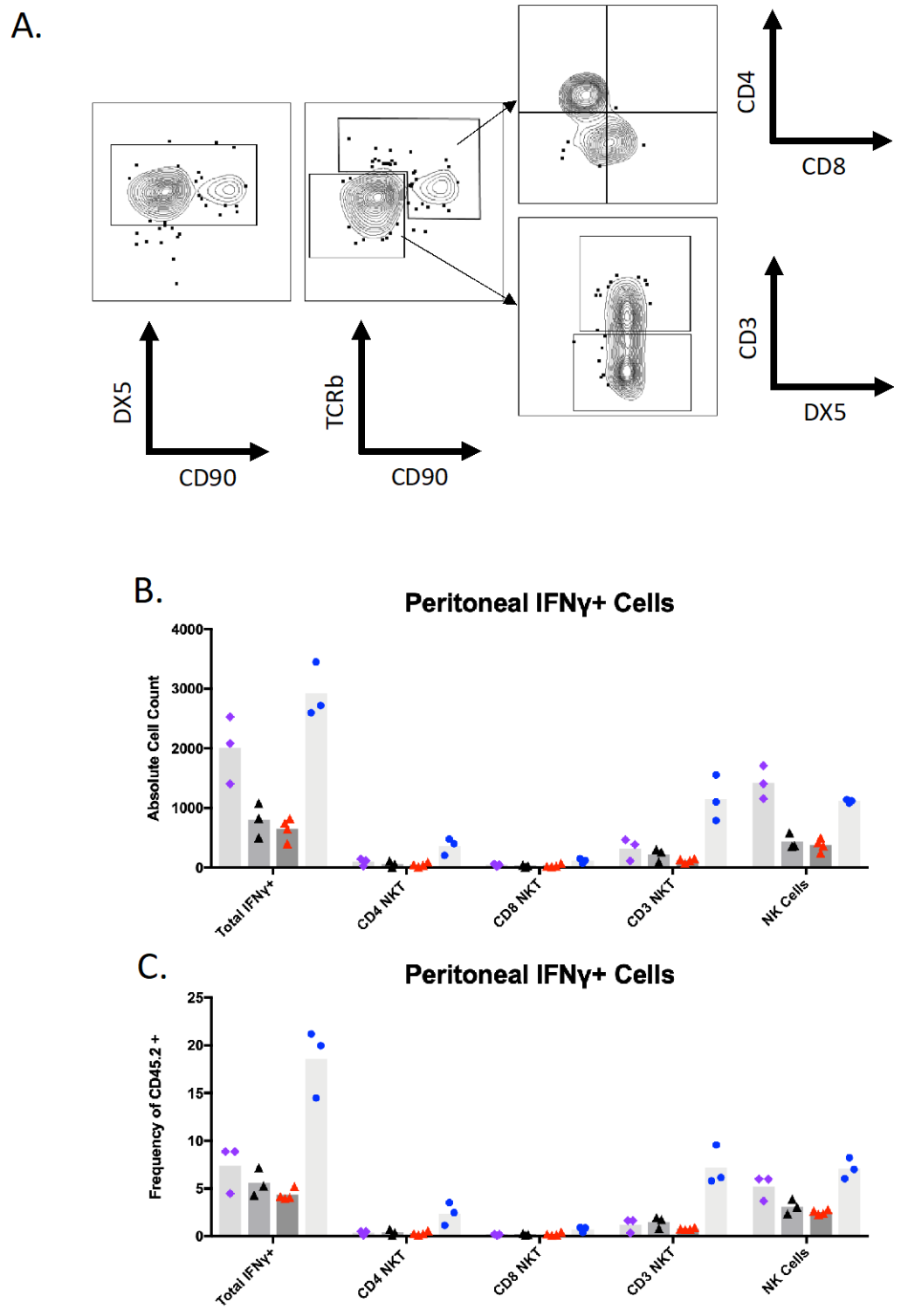

Figure 26 Gating strategy for characterization of IFNγ+ cells)

A) Gating strategy to identify NK and NKT cells. Cells were gated as described in Fig 25 to obtain IFNγ+ cell population. Next, IFNγ+ cells were gated to identify DX5+ cells. IFNγ+, DX5+ cells were then gated into TCRβ+/CD90+ or TCRβ-/CD90- populations. TCRβ-/CD90- were then gated by CD3 to identify NK cells: IFNγ+, DX5+, TCRβ-/CD90-, CD3- or NKT cells: IFNγ+, DX5+, TCRβ-/CD90-, CD3+. TCRβ-+/CD90+ were gated by CD4 and CD3 to identify two additional subsets of NKTs: IFNγ+, DX5+, TCRβ+/CD90+, CD4+ and IFNγ+, DX5+, TCRβ+/CD90+, CD8+. B: Absolute cell counts obtained using the cells using the gating strategy outlined in A. C: Analysis of cell counts obtained in B by taking the cell count of the population / cell count of Cd45+ to obtain the frequency of live leukocytes for each population.

4.0 Conclusions and future directions

4.1 *T. gondii* and *N. caninum*: a powerful comparative system in mice

T. gondii is considered to be one of the most successful eukaryotic parasites on earth with a global distribution and a capacity to infect nearly all warm-blooded animals. *T. gondii* must gain entry into the host cell to survive and replicate, but can infect any nucleated cell once inside the host. The work presented in this dissertation interrogates host-parasite interactions by comparing host responses to *T. gondii* with its close relative *N. caninum* in a murine model. *N. caninum* and *T. gondii* are morphologically and genetically quite similar and share invasion machinery and secretory organelles that are important for host cell manipulation and ultimately for pathogenesis. Despite these substantial similarities, there is a marked difference in their host range and transmission dynamics. While *T. gondii* infects all animals, *N. caninum* has a much narrower host range and is primarily known as a cattle pathogen and infects other ruminant species. While both parasites transmit asexually in secondary hosts or sexually in the definitive host, *T. gondii* is uniquely efficient at direct transmission between intermediate hosts. *N. caninum* is highly efficient at vertical transmission during a primary infection or recrudescence, where latent infections reactivate and the parasite is efficiently transmitted to the fetus.

To compare parasite infections, we used bioluminescent imaging, measures of pathogenesis like weight loss and behavior changes, and cytokine analysis. We engineered transgenic parasites that express the luciferase enzyme and at the time points of interest we inject the mouse with the substrate for luciferase. This allowed us to measure infection dynamics contiguously in the same host and identify changes in real time. With this system, we tested

numerous strains of *T. gondii* with varying virulence, and as expected there are differences in parasite burden between high and low virulence strains of *T. gondii* 3 days after infection (Chapter 2). We found that *T. gondii* and *N. caninum* both proliferate similarly during the first 24 hours of infection, but *N. caninum* parasites do not proliferate beyond 24 hours. We concluded that the mouse rapidly controls *N. caninum* infection within the first 24 hours, and well within the timeframe of the innate immune response. In contrast *T. gondii* successfully proliferates well beyond this 24 h period, ultimately establishing a long-term chronic infection.

4.1.1 Parasite proliferation in the absence of IFNγ is greater in *N. caninum* infections compared to T. gondii infections

By comparing cytokine screens, we identified substantial differences in cytokine and chemokine induction as early as 4 hours after infection. We found very minimal induction of these early cytokines during early *Toxoplasma* infections and by comparison, *N. caninum* infections exhibited elevated levels of an array of cytokines including MCP-1, IFNγ, IL-12p40 and IL-12p70. MCP-1 is required for mice to survive *Toxoplasma* infections (P. M. Robben et al., 2005) but the role of MCP-1 in controlling early *N. caninum* parasite burden was unknown. MCP-1 was detected at high levels both in the serum and at the site of infection at early time points during an *N. caninum* infections compared to *T. gondii*. Using bioluminescence imaging to investigate parasite burden in mice that lacked MCP-1 (MCP-1-/-), we compared *T. gondii* and *N. caninum* acute infections. MCP-1-/- mice were uniformly susceptible to *T. gondii,* as previous reports suggested. However, when we infected MCP-1-/- mice with *N. caninum*, we did not observe any differences in parasite burden or survival compared to WT infections, indicating that MCP-1 is not necessary for controlling parasite burden. Since MCP-1 is required for recruiting monocytes from the bone

marrow to the site of infection, these knockout mice would be expected to have reduced monocyte infiltration into the peritoneal cavity. These data suggest that infiltrating monocytes do not play a critical role in controlling *Neospora caninum* burden. To compare the populations of monocytes, dendritic cells and macrophages between *T. gondii*-infected, *N. caninum*-infected, and uninfected mice we performed flow cytometric analysis at the same 4-hour timepoint at which we observed elevated cytokines during *N. caninum* infections. We compared two strains of *T. gondii* with distinct virulence phenotypes to *N. caninum*-infected mice or to naïve (uninfected) mice and quantified dendritic cells, macrophages, and monocytes. We observed no differences in the number of dendritic cells or macrophages between infected and uninfected mice. The number of monocytes were elevated in all infected treatment groups compared to uninfected mice, but there were no significant differences between parasite species or strains. Thus, at this early point of infection where we see robust cytokine production, monocyte populations are not different between *T. gondii* and *N. caninum*. This data combined with our findings that MCP-1 is not required for controlling *N. caninum* growth suggests that the early infiltration of monocytes is not important for controlling *N. caninum* infections. This represents an important, and previously unknown, distinction between *T. gondii* and *N. caninum* infections in mice.

Since *N. caninum* infections induce proinflammatory cytokine responses as early as 4 hours after infection while *T. gondii* does not, and MCP-1 was not important for controlling *N. caninum* burden as described in chapter 2, we focused on the IL-12/IFNγ axis to look for differences using our bioluminescent system and cytokine analysis. It is well-established that mice lacking IL-12 or IFNγ are highly susceptible to microbial pathogens and both *T. gondii* and *N. caninum* are lethal to mice lacking IFNγ or IL-12p40. Our infections in IL-12p40-/- mice with 10^6 tachyzoites resulted in lethality by 7-9 days after infection. Similarly, in IFNγ-/- mice we observed acute lethality by

5-6 days-post-infection. Since previous reports suggested similar morbidity in these mouse models, we hypothesized that we would see equivalent parasite burden comparing *T. gondii* and *N. caninum* infections. To determine if *N. caninum* proliferation is similar to *T. gondii* infections in the absence of IL-12 or IFNγ we quantified parasite burden using bioluminescence and monitored mouse health and morbidity. Both parasite species establish similar infections and are similar in number at 24 hours post-infection. After 24 hours in a wild-type mouse, *N. caninum* burden has declined substantially, while in the absence of IFNγ, *N. caninum* continues to proliferate. Surprisingly, in IFNγ-KO mice, *N. caninum* expands to significantly higher levels than *T. gondii* based on raw luminescence signal (Chapter 2). This demonstrates that IFNγ is critical for control of *N. caninum* in mice and the removal of IFNγ from the system impacts *N. caninum* growth substantially more than *Toxoplasma*. We found the same was true in both BALB/cj and C57BL/6 strains. Moreover, we recently found that parasite burden is also substantially higher in *N. caninum* infection than *T. gondii* infection in IFNγ-receptor deficient mice (IFNγR-/-; Fig 27), further confirming that IFNγ and the downstream signaling it induces is paramount to control of *N. caninum* in mice, while for *T. gondii* there appears to be another layer of control even in the absence of this critical cytokine and it's effectors.

We compared infections in mice lacking IL-12p40 (IL-12p40-/-) which were available in a C57BL/6 background. Consistent with what we expected based previous work and the prevailing dogma, we saw similar effects of IFNγ and IL-12p40 deletion in T. gondii. In contrast, *N. caninum* infections in IL-12p40-/- mice peaked with significantly higher parasite burden compared to *T. gondii*. However, we did observe lower *N. caninum* parasite burden in the IL-12p40-/- mice when compared to the IFNγ-/- mice suggesting that IL-12p40-/- mice are less susceptible to *N. caninum*

infections than IFNγ-/- mice. Across multiple experiments, we consistently observed uniform lethality to *N. caninum* infections in IL-12p40-/- mice but that the parasite burden would start to decline indicating the host began to control the infection. These results suggest IL-12p40-/- mice are more resistant to *N. caninum* than IFNγ-/-, based on our observations of lower parasite burden, longer survival and what appeared to be some control of parasite expansion, although not enough to rescue survival. This suggests that IFNγ has a unique relationship with *N. caninum* infections when compared to the extensive literature on this cytokine in *T. gondii*. Additionally, our finding that other key inflammatory cytokines like interleukin-1 alpha and beta (IL-1α/β) production was not significantly increased (Fig 15A) in the first 14 hours by either *T. gondii* or *N. caninum* further supports our finding that IFNγ production is uniquely produced to control *N. caninum* and not *T. gondii* infections in the first 24 hours if infection. By comparing the effects of IFNγ treatment *in vitro* (Chapter 2) we determined that *N. caninum* and *T. gondii* are equally susceptible to the effects of IFNγ indicated by a similar reduction in proliferation after treatment. Therefore, intracellular IFNγ-driven mechanisms are equally successful against *T. gondii* and *N. caninum* infections. This combined with our findings that IFNγ-/- mice are more susceptible to *N. caninum* proliferation suggests that *N. caninum* infections induce specific, cell-mediate immune responses driven by IFNγ, and in the absence of IFNγ-driven cell mediate mechanisms, *N. caninum* proliferation is unmitigated.

4.2 MyD88 is not required for survival of *N. caninum* infections in mice

During a *T. gondii* infection, IL-12 drives IFNγ production in a TLR-dependent manner and therefore we sought to investigate Toll-Like Receptor signaling during *N. caninum* infections.

Toll-like receptors are highly conserved proteins that are expressed on the cell surface and inside endosomal compartments of almost all cells. TLRs initiate immune responses by recognizing molecules that are specific to pathogens that are called PAMPS (pathogen associated molecular patterns). TLRs interact with their PAMP ligand and then either heterodimerize or homodimerize to initiate signaling cascades. TLRs signal through downstream adaptor proteins, and all of the known mouse TLRs except TLR3 can signal through MyD88. In fact, MyD88 is required for all mouse TLRs with the exception of TLR3 which is TRIF-dependent, and TLR4 which can use either TRIF or MyD88. Numerous groups have shown that MyD88 is required for the mouse to survive a *T. gondii* infections and TLR activation leads to IL-12 production. Not surprisingly, even the low virulent *T. gondii* strain S1T was uniformly lethal to MyD88-/- mice, however in our infection model using the same doses we found MyD88-/- mice to be resistant to *N. caninum* infections. Numerous experimental replicates confirmed our findings that MyD88-/- mice survive *N. caninum* infections with 10^6 Nc:Nc1 tachyzoites. Additionally, our bioluminescent system enabled us to show that *N. caninum* established a successful infection in MyD88-/- mice and *N. caninum* continues to proliferate several days longer than in wild-type comparisons. Our results demonstrate that MyD88 is not required for survival after infection with 10^6 N. caninum, but that MyD88-/- mice are more susceptible based on prolonged parasite proliferation in this background compared to WT. Our cytokine analysis on the serum from our experiments revealed that WT mice infected with *N. caninum* exhibited an early spike of IFNγ, as expected. In mice lacking MyD88, we observed a loss of the initial production of IFNγ, and instead observed a later spike at 3-5 days after infection. Similar to the immediate IFNγ production in WT mice, this later spike in IFNγ correlated very well with the timing of parasite burden control (Chapter 2). Thus, in the absence of early IFNγ, we see a second wave of IFNγ that is MyD88 independent and controls *N. caninum*

infections. It will be interesting to identify IFNγ producing cells at this later timepoint and determine if NKT cells contribute to the late wave of IFNγ.

4.3 *N. caninum* profilin is not immunodominant in a *T. gondii* infection but TLR11 is required for immediate IFNγ production

Since MyD88 is clearly required for early production of IFNγ during *Neospora* infections, we next wanted to investigate TLR11 signaling, since it has a very well-defined role in driving cytokine production during *T. gondii* infections. TLR11-/- mice are not commercially available, therefore we established a collaboration with Dr. Yarovinsky at the University of Rochester. He infected TLR11-/- mice as well as MyD88-/- to verify our previous findings. Cytokine analysis revealed that TLR11-/- mice did not make IFNγ at that early 4-hour time point, but did make it at 24 hours. Therefore, IFNγ induction is delayed in TLR11-/- mice until 24 hours after infection.

Profilin is the only known *T. gondii* PAMP and is known to interacts with TLR11. Since we identified a potential role for TLR11 in immediate IFNγ production, we compared *T. gondii* and *N. caninum* profilin sequences. Previous work from the Kucera group identified a surface exposed motif in *T. gondii* profilin that is recognized by TLR11 (Kaury Kucera et al., 2010). They identified an acidic loop that combined with the B-hairpin motif to interact with TLR11. The only differences we observed in the amino acid sequence between *T. gondii* and *N. caninum* was in the B-hairpin motif. To determine if expression of *N. caninum* profilin was sufficient to induce early IFNγ during *T. gondii* infection we expressed an HA-tagged versions of *N. caninum* profilin in *Toxoplasma* parasites. We hypothesized that if *N. caninum* profilin was immunodominant and induced immediate IFNγ production, *T. gondii* parasites expressing *N. caninum* profilin would

result in immediate IFNγ production. However, *in vivo* we did not find a difference in early IFNγ production when *N. caninum* profilin was expressed in *T. gondii* parasites. To account for the possibility that *T. gondii* might alter the host cell after infection in a way that would subvert the immunodominant effect of *N. caninum* profilin we also tested parasite lysate. Injection of mice with soluble tachyzoite lysate preparations (STAg) did not yield any difference in IFNγ levels between *N. caninum*-profilin and *T. gondii*-profilin expressing *T. gondii* parasites. From this, we concluded that *N. caninum* profilin is not sufficient to induce IFNγ when expressed in *T. gondii.* This suggests that there might be a different *N. caninum* PAMP that induces this robust immediate immune response in a TLR-dependent mechanism. An important future direction of this work will be to identify *N. caninum* PAMP responsible for the immediate IFNγ production in *N. caninum* and to then determine how this PAMP is either uniquely produced and/or bears unique features in *N. caninum* compared to *T. gondii*.

4.4 NK and NKT cells produce IFNγ in response to *N. caninum* infections 4 hours after infections

To identify the cells making IFNγ, we performed flow cytometry and intracellular cytokine staining on peritoneal and spleen cells 4 hours after infection. We compared populations of cells known to produce IFNγ during acute *T. gondii* infections, including NK, NKT, and T cells. We compared two strains of *T. gondii* with different virulence to *N. caninum* and naïve mice. We determined *N. caninum* infections have significantly more IFNγ+ peritoneal cells 4 hours after infection than uninfected mice or *T. gondii* infected mice. When we compared that to spleen samples, we do not see differences between infected and uninfected mice, or between parasite

strains or species. We found that more NK and NKT cells were induced to make IFNγ during *N. caninum* infections and importantly that *T. gondii* infection leads to lower numbers of IFNγ-producing NK cells, in support of recent findings that *T. gondii* infections convert NK cells into ILC-like cells. NK cells are poorly characterized in bovine systems because few NK markers have been identified. NKT cells are even more enigmatic and have yet to be fully characterized, leading some to hypothesize the bovine immune response lacks similar NKT mechanism compared with mice (Boysen & Storset, 2009). If *N. caninum* evolved in a host without NKT mechanisms that exist in mice, it could be hypothesized that NKT mechanism may be an important determinant of host susceptibility to *N. caninum.* In that line of reasoning, differences in how *T. gondii* and *N. caninum* activate NKT cells and the molecular basis of these interactions will provide important insight into the co-evolutionary relationship of host-pathogen interactions that contributes to the broad host range of *T. gondii.*

4.5 Future directions

4.5.1 Investigate NK and NKT cells contribute to control of *N. caninum* infections *in vivo*

Based on our finding that *N. caninum* infections significantly increase the number of IFNγ-producing NKT cells (Chapter 3), we are currently working to define the role of NKT cells in immediate IFNγ production and early control of *N. caninum* parasite burden. To this end, an important future direction of this work will be comparing IFNγ production and parasite burden during acute *T. gondii* or *N. caninum* infection in mice lacking functional T and B cells (Rag1-/-) and mice lacking functional NKT cells (CD1d-/-). Our work presented in this chapter identifies

multiple IFNγ-producing cell types that expressed T cell markers. Future work will test the requirement of functional T cells for immediate IFNγ production and controlling early parasite proliferation. We will infect Rag1-/- mice in a BALB/cJ background to compare infections because these mice lack functional T and B cells, but still exhibit a robust NK cell response. We will also infect CD1d-/- mice to quantify differences between *T. gondii* and *N. caninum* infection kinetics and IFNγ production. By comparing infections in CD1d-/- mice, we will determine if functional NKT cells are required for immediate IFNγ production, and if CD1d-restricted NKT cells are necessary for immediate control of *N. caninum* proliferation. By comparing infections of *T. gondii* and *N. caninum* in these knockout mice we will identify differences in susceptibility and infection outcome. To confirm the importance of NK cells in the early control of *N. caninum* infections, we will also compare infections of *N. caninum* in Rag1-/- mice to infections in Rag2-/- γC -/- mice. Rag2-/- γC -/- mice lack functional T and B cells like Rag1-/- mice, and additionally also lack functional NK cells. This comparison will allow us to identify changes in the infection outcomes during *N. caninum* infections resulting from the loss of T and B cells, and additionally the loss of NK cells.

4.5.2 Identify NKT activation mechanisms during *N. caninum* infections and determine NK cell dynamics in the first 24 hours of *T. gondii* infection

Recent advances identified a unique subset of ILC1-like cells that are expanded and during acute *T. gondii* infections in mice and persist after acute infection resolution (Park *et al.*, 2019). They determined *T. gondii* infections drive conversion of NK cells into ILC1-like cells and investigated timepoints beginning 7 days after infection. This work adds to a growing body of evidence suggesting NK cells exhibit plasticity (Silver & Humbles, 2017) and memory

(O'Sullivan, Sun, & Lanier, 2015) functions. However, they did not address the possibility that *T. gondii* may exert effector function on NK cells rapidly after infection. Our work in Chapter 3 determined that *T. gondii* infections exhibited fewer IFNγ+ NK cells than naïve mice. One important future direction of this work will be to determine how *T. gondii* infections alter NK cell populations immediately after infection. To accomplish this, further characterization of NK cells and ILC1 cell populations will be necessary. Our work in Chapter 3 identified differences in NK and NKT cells comparing *T. gondii* and *N. caninum* infections within hours of infection, and identified subpopulations similar to iNKT and NKT-like cells that produced IFNγ in response to *N. caninum* infections. An important future direction of this work will be to determine the activation mechanisms responsible for NKT cell IFNγ production. Our work in chapter 2 determined that MyD88 is required for immediate IFNγ production, however it remains unknown if NK and NKT cells are directly stimulated to produce IFNγ in a TLR-dependent mechanism or if TLR-driven cytokine production from an accessory cell is required NKT activation.

4.5.3 Identify *N. caninum* protein(s) activating NKT and NK cell IFNγ production

Proinflammatory responses are the only effective immune response to *N. caninum* and control of infection is dependent on cytokine production (Almeria, Serrano-Perez, & Lopez-Gatius, 2017; Correia et al., 2015; Tuo et al., 2005). Investigations in both mice and cattle identified IFNγ as an important immune-modulating cytokine required protective mechanism during neosporosis (Almeria et al., 2017; Baszler et al., 1999; Correia et al., 2015; I. A. Khan et al., 1997). Our previous work identified two temporally distinct waves of IFNγ production during a *N. caninum* infection. Our data suggests that an innate (TLR-driven) mechanism recognizes *N. caninum* and results in IFNγ production, and in the absence of MyD88-dependent IFNγ a later (3-

5 dpi) production of IFNγ that is MyD88-independent is produced and the infection is successfully controlled (Coombs *et al.*, 2020). Despite the crucial role this cytokine plays during *N. caninum* infections, the parasite effector molecules eliciting cytokine production are unknown. Only a handful of *Neospora caninum*-specific antigens are known, and even fewer *N. caninum* proteins have been characterized within the context of the specific immune responses they elicit. Identification of *N.* caninum-specific antigens driving IFNγ production will contribute to better prevention strategies by expanding our current repertoire of immune-modulating proteins, and contribute new molecular tools for further investigation of IFNγ-driven immunity. There is consensus in the field that expanding our current repertoire of immune-modulating proteins combined with research on the host immune responses they elicit will be a powerful advancement in the treatment and prevention of neosporosis (Al-Qassab et al., 2010; Guido et al., 2016; Horcajo et al., 2016; Nishikawa, 2017; Rea & Rae, 2015). An important future direction of this work will be to identify *N. caninum* molecules that stimulate immediate IFNγ production *in vivo.*

4.6 Figures

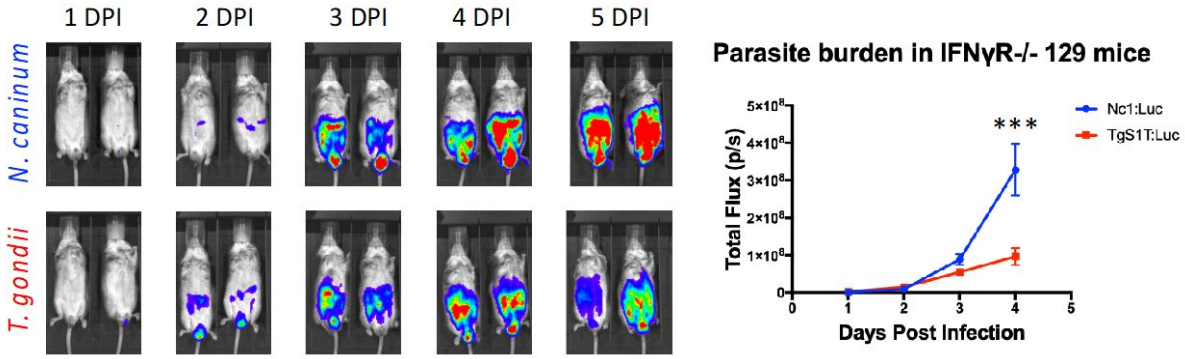

Figure 27 *N. caninum* and *T. gondii* infections in IFNγR knockout mice

Mice were provided by Dr. Melissa Kane (Department of Pediatrics, University of Pittsburgh School of Medicine) and infected as described in the Chapter 2 methods sections 2.6.4 and 2.6.5 and described in chapter 2. Mice lacking the IFNγ receptor (IFNγR-/- 129 strain) were infected with 10^6 *N. caninum* (NC1 strain, blue line and top row of mouse images) or *T. gondii* (S1T strain, red line and bottom row of mouse images). 2 male (shown in bioluminescent images) and 2 female (images not shown, but data included in quantification graph) mice were used per parasite treatment group. Statistical analysis was performed using a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test on the raw (non-normalized) data. *p=<0.05, **p=<0.01, ***p=<0.001

Appendix A Determine the effects of *Toxoplasma*-Specific-Expanded-locus 8 (TSEL8) on parasite growth and virulence

Appendix A.1 Introduction

Advances in sequencing technology enabled investigations into large-scale structural mutations that result in copy number variation (CNV) where locus expansion or contraction changes the number of genes within a locus (Alkan *et al.*, 2009; Medvedev, Stanciu, & Brudno, 2009; Mills *et al.*, 2011). CNV results from duplications and deletions in the genome and the importance of gene duplication and diversification in pathogen virulence and host responses have been characterized in many plants, animals and microbes (Y. Adomako-Ankomah et al., 2014; Denise et al., 2003; Qiu et al., 2012; Reese et al., 2011; Schwartze et al., 2014; Walzer et al., 2013). Large-scale locus expansion in immune and host defense genes are seen from pacific oysters, to yaks and even humans.(Almal & Padh, 2012; Qiu *et al.*, 2012; Sudmant *et al.*, 2010; Zhang *et al.*, 2015) Diversification in the polygenic immunoglobulin receptor gene families increases the ability of the mammalian immune system to recognize new pathogens (Jiang *et al.*, 2012; Parham, Norman, Abi-Rached, & Guethlein, 2011). CNV in immune related genes is an important host tool in the battle with pathogens that cause diseases (Linzmeier & Ganz, 2005; Y. Yang et al., 2007).

Expansions are not exclusive to host genes and are prevalent in pathogen virulence-related genes. High levels of gene expansion in virulence genes have been identified for many pathogens. For example, the CPB gene in the parasite *Leishmania mexicana* is a multi-copy, tandem repeat

gene found to be a potent virulence effector in mouse infections.(Denise *et al.*, 2003) The multi-copy *var* family genes of *Plasmodium* spp. are responsible for the antigenic diversity and immune evasion seen in the pathology of malaria.(Claessens *et al.*, 2014; Hernandez-Rivas *et al.*, 1997) Other examples of pathogen virulence resulting from gene family expansions include the fungal genus *Candida* spp.(Moran, Coleman, & Sullivan, 2011), and the bacteria *Rickettsia* spp.*(Ogata et al., 2005),* the causative agent of typhus and Rocky Mountain spotted fever. The most potent virulence factor identified for *T. gondii* in a mouse infection also exhibits CNV between strains.(Reese *et al.*, 2011).

Despite a high degree of morphologic and genetic similarity, genomic comparisons within *T. gondii* strains and extant sister species *H. hammondi* and *N. caninum* have revealed differences in multi-copy locus expansion. Characterization of differences in locus expansion found 30% of *T. gondii* Expanded Loci (ELs) overlap with *N. caninum* ELs, much lower than expected by overall genomic synteny.(Y. Adomako-Ankomah *et al.*, 2014) Toxoplasma-Expanded Loci (TSELs) are tandem arrays of duplicated genes that are specific to *T. gondii* when compared to close relatives.(Y. Adomako-Ankomah *et al.*, 2014) Some TSELs encode putative secretory proteins that share properties with known virulence effectors.(Yaw Adomako-Ankomah *et al.*, 2016) Although duplicated genes often correspond to pathogen virulence, most TSEL gene remain uncharacterized (Claessens *et al.*, 2014; Denise *et al.*, 2003; Hernandez-Rivas *et al.*, 1997; Moran *et al.*, 2011; Ogata *et al.*, 2005; Reese *et al.*, 2011; Schwartze *et al.*, 2014). By assessing TSEL gene impact on parasite success in acute and chronic infections we can identify non-conserved genes conferring virulence to *T.* gondii and identify underlying genetic differences contributing to the evolution of *T. gondii* pathogenesis. Identifying new virulence factors and phenotypes conferring parasite success will lay the groundwork for further exploration of host range variation

and pathogenesis of *T. gondii* when compared to other apicomplexan parasites. In previous work we identified multiple expanded loci via an *in silico* screen that are unique to *T. gondii* when compared to *H. hammondi* and *N. caninum.*(Y. Adomako-Ankomah *et al.*, 2014) We hypothesized that TSEL8 encoding putative proteins would impact *T. gondii* virulence. We selected TSEL8 with unknown function that exhibits high CNV when compared to extant relatives, and has high levels of transcription in one or both phases of parasite lifecycle.

Appendix A.2 Generation of TSEL8 knockout and wild-type strains

To study the effect of TSEL8 on parasite virulence both *in vitro* and *in vivo* we needed an avirulent strain of *T. gondii* that is amenable to genetic manipulation, contains a selectable marker, and expresses luciferase for *in vivo* bioluminescence imaging. We chose *PruΔku80Δhxgprt*, a *T. gondii* strain with attenuated virulence in mouse infections that lack the Ku80 protein required for non-homologous end joining (NHEJ) to take place (Fox *et al.*, 2011; Knoll & Boothroyd, 1998). This provides a genetic background ideal for double homologous recombination, making genetic manipulations more successful and efficient (Huynh & Carruthers, 2009; Rommereim *et al.*, 2013). Additionally, this strain lacks the *HXGPRT* (Hypoxanthine-guanine phosphorribosyltransferase) gene providing a mechanism for selection (Donald, Carter, Ullman, & Roos, 1996). This transferase is specific to apicomplexans, thus selective media does not affect the host cell (Huynh & Carruthers, 2009). We generated a construct with the *HXGPRT* gene flanked by large homology arms specific to the TSEL8 5' and 3' UTRs (Figure 28A). After transfection parasites expressing the *HXGPRT* gene were selected using mycophenolic acid (MPA) and Xanthine (Xan) media and the cloned by limiting dilution. The TSEL8 knockout construct

utilizes double homologous recombination to replace TSEL8 with a *HXGPRT* gene. However, some parasites incorporate the *HXGPRT* gene outside of the targeted locus. Therefore, we designed query primers to amplify the insertion site and homology arms to verify that the repair template inserted in the right location and orientation (Figure 28B, C). We also verified that the entire locus was knocked out using multiple primer sets specific to TLSE8 (Figure 28B,D,E). We identified clones lacking TSLE8 (TSEL8KO, 28 1E) as well as clones that did not lack TSEL8 (TSEL8WT, Figure 28D) that were used as wild-type controls. Since these parasites were not capable of bioluminescence, we then transfected TSEL8KO and TSEL8WT parasites with the luciferase reporter gene and identified clones with similar luciferase signal for *in vivo* comparisons (Figure 28 F,H).

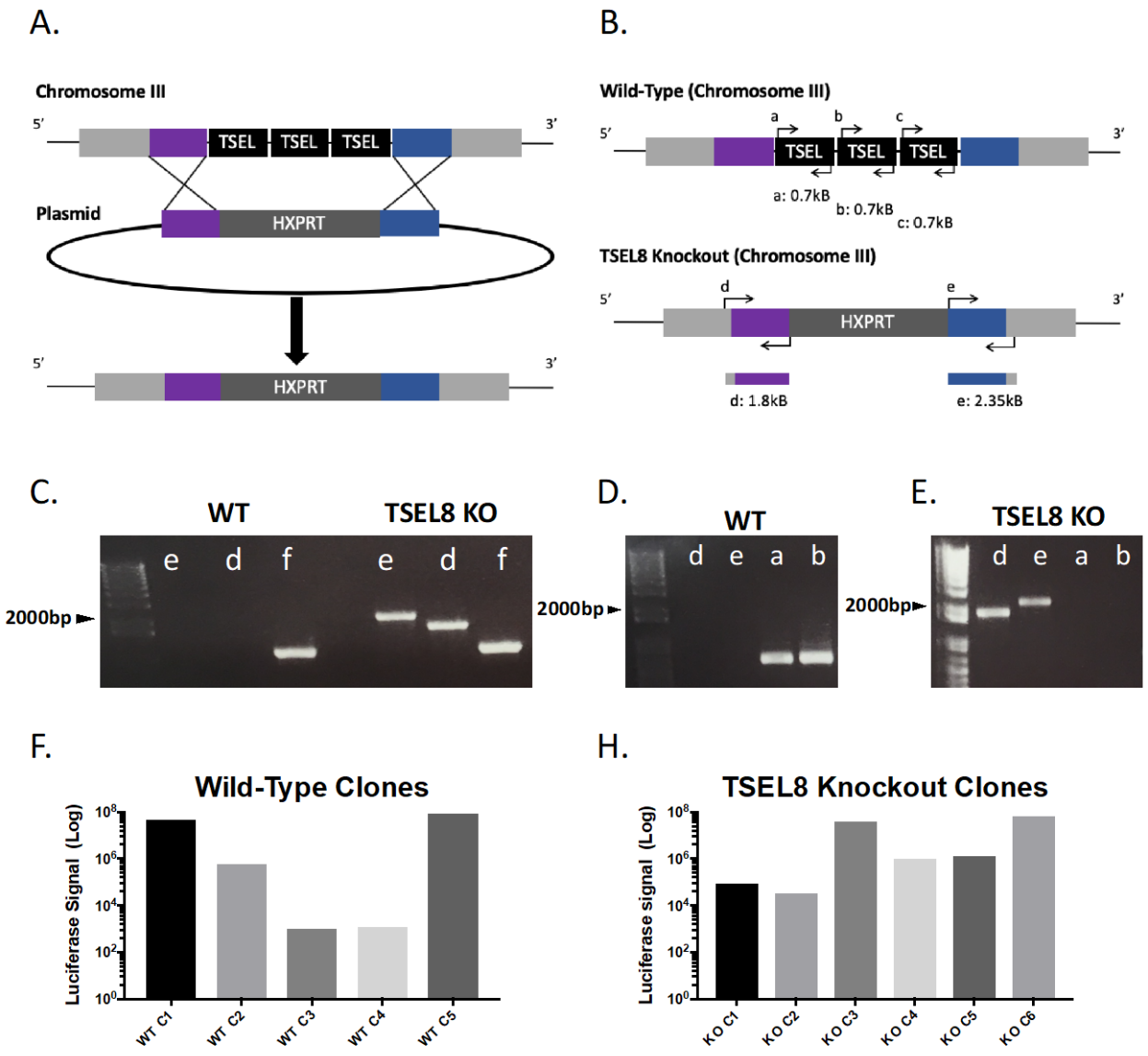

Figure 28 TSEL8 knockout construct and clone verification

(A) Schematic of TSEL8 knockout construct with homology flanks (purple, blue), representative TSEL locus with 3 copies (black) and HXPRT selectable gene (gray). (B) Schematic of the TSEL8 locus knockout construct with primers to amplify the insertion site and homology arms. Directional arrows indicating primers to confirm correct insertion of the HXPRT selection gene and locus knockout, product size is indicated below chromosome schematic. a,b, and c represent TSEL8 primers used to query the internal locus. d and e are query primer to verify the insertion of the repair template in the correct orientation. (C) Agarose gel with PCR products of the wild-type (WT) or knockout (TSEL8KO) clones. No PCR product for HXPRT

insertion in WT (e,d) and PCR product is present for TSEL8KO HXPRT insertion for both sets of query primers (e,d). Control (f) is PCR product for *T. gondii*-specific to verify parasite DNA is present. (D) Agarose gel of PCR product for WT clone demonstrating the presence of internal genes (a,b) and not the HXPRT insertion (d,e). (E) Agarose gel of PCR product of TSEL8KO demonstrating PCR product for both HXPRT query primer sets (d,e) and no internal genes (a,b)

Appendix A.3 *In vivo* and *in vitro* effects of TSEL8 deletion

To identify changes in parasite fitness due to TSEL8 deletion we perform growth assays *in vitro* in Human Foreskin Fibroblast cells (HFFs). Confluent cells in T25 flasks were infected with either 100 TSEL8WT or TSEL8KO parasites and plaques size was measured 7 days after infection. We found no significant differences in plaque size comparing TSEL8KO and TSEL8WT (Figure 29G) in addition to no differences in the number of plaques formed (data not shown). These data demonstrate that both TSEL8KO and TSEL8WT are have similar viability and proliferation capability *in vitro.*

Parasites with similar growth *in vitro* can vary when challenged with a fully competent immune system. Therefore, we compared TSEL8KO and TSEL8WT parasite growth *in vivo* using bioluminescent live imaging. BALB/C mice were infected with either 10^5 TSEL8KO or TSEL8WT parasites and imaged daily during the acute infection (n=5 mice per parasite strain, Figure 29) Parasite burden was quantified and survival was monitored (Figure 29B-E). Quantification of bioluminescent imaging revealed statistically significant parasite burden ($p=<0.05$) 3-7dpi in one replicate (Figure 29B) and 5-6dpi in a second replicate (Figure 29D). Two additional experiments demonstrated the reproducibility of this phenotype with TSEL8KO-infected mice exhibiting significantly higher parasite burden at 4-5dpi (n=5 mice per parasite

strain, figure 30C) and 5dpi (n=5 mice per parasite strain, figure 30E). We found no statistically significant differences in mouse survival (Figure 29C,E) or weight loss (Fig 30B,D) comparing TSEL8KO and TSEL8WT infections. In one experiment (Figure 29E) out of four, TSEL8KO-infected mice had a reduced survival, albeit non-significant. Although we observed a small difference in IFNγ production5dpi (Figure 29F) the difference was not statistically significant. These results indicate that deletion of TSEL8 increases acute proliferation of *T. gondii in vivo* and not *in vitro* suggesting that the gene product(s) of TSEL8 interact with the host during acute infections impacting parasite growth. Further work to identify and characterize the gene product and host response is needed to understand how TSEL8 impacts parasite growth *in vivo* and not *in vitro.*

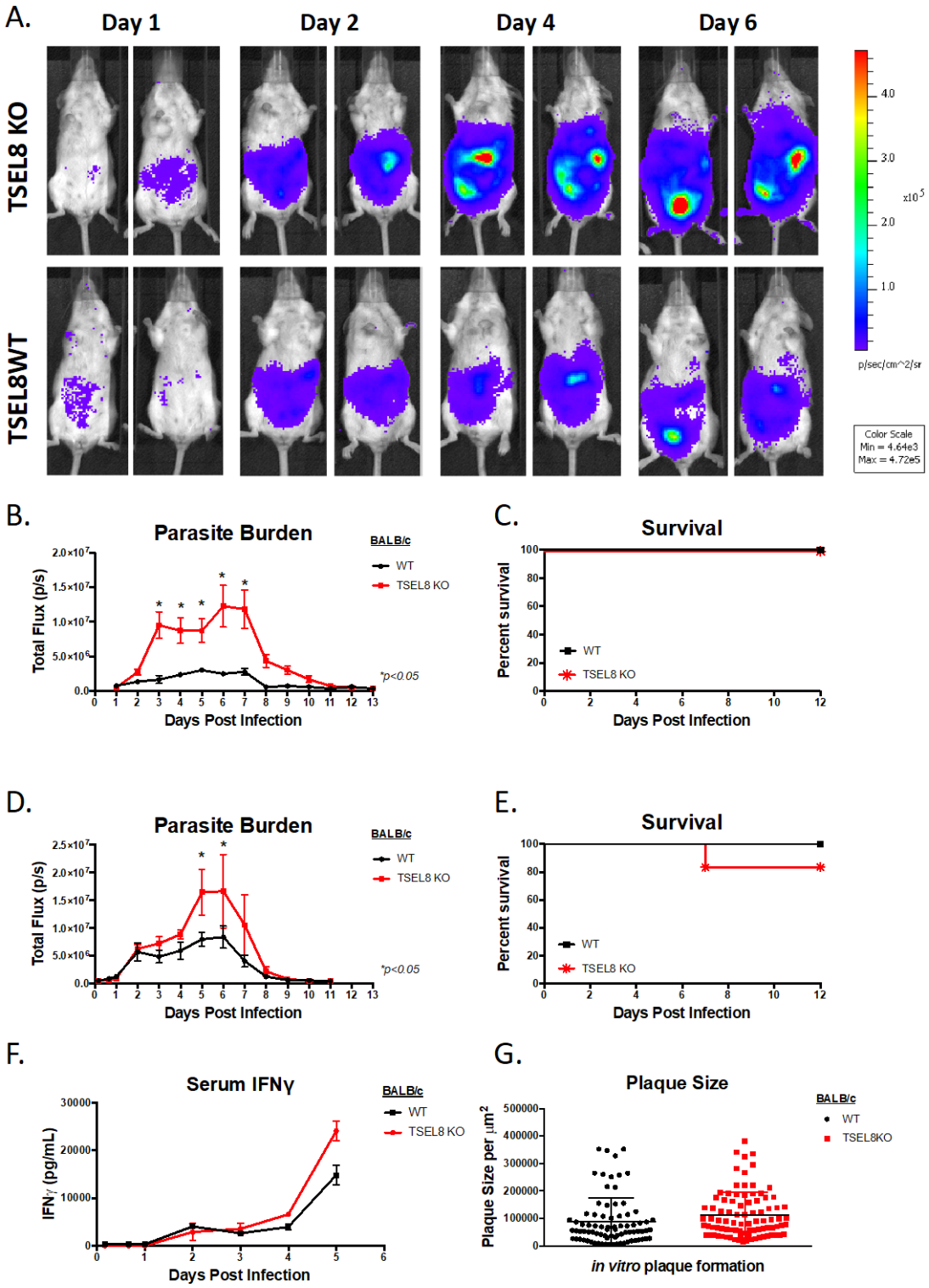

Figure 29 *in vivo* and *in vitro* comparisons of WT and TSEL8KO infections

(A) Representative images 6-week-old Balb/C mice infected IP with 10^5 TSEL8 knockout (TSEL8KO) or wild-type (TSEL8WT) parasite tachyzoites per mouse and were imaged daily. 5 mice per paraite strain (n=5) were infected. Bioluminescent images show parasite-derived luciferase signal at select timepoints. (B and D) Quantification of bioluminescent imaging where total flux is the measurement for parasite burden in the mouse body of TSEL8KO-infection (red line) or TSEL8WT (black line). (C and E) Mouse survival during acute infection TSL8KO (red line) or TSEL8WT (black line). (F) Serum IFNγ was analyzed using ELISA. Samples were collected throughout the acute infection of TSL8KO (red line) or TSEL8WT (black line). (G) Quantification of plaque assay measuring area of plaques comparing TSL8KO (red line) or TSEL8WT (black line). Cytokines and parasite burden were analyzed by a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test. *p=<0.05, **p=<0.01, ***p=<0.001.

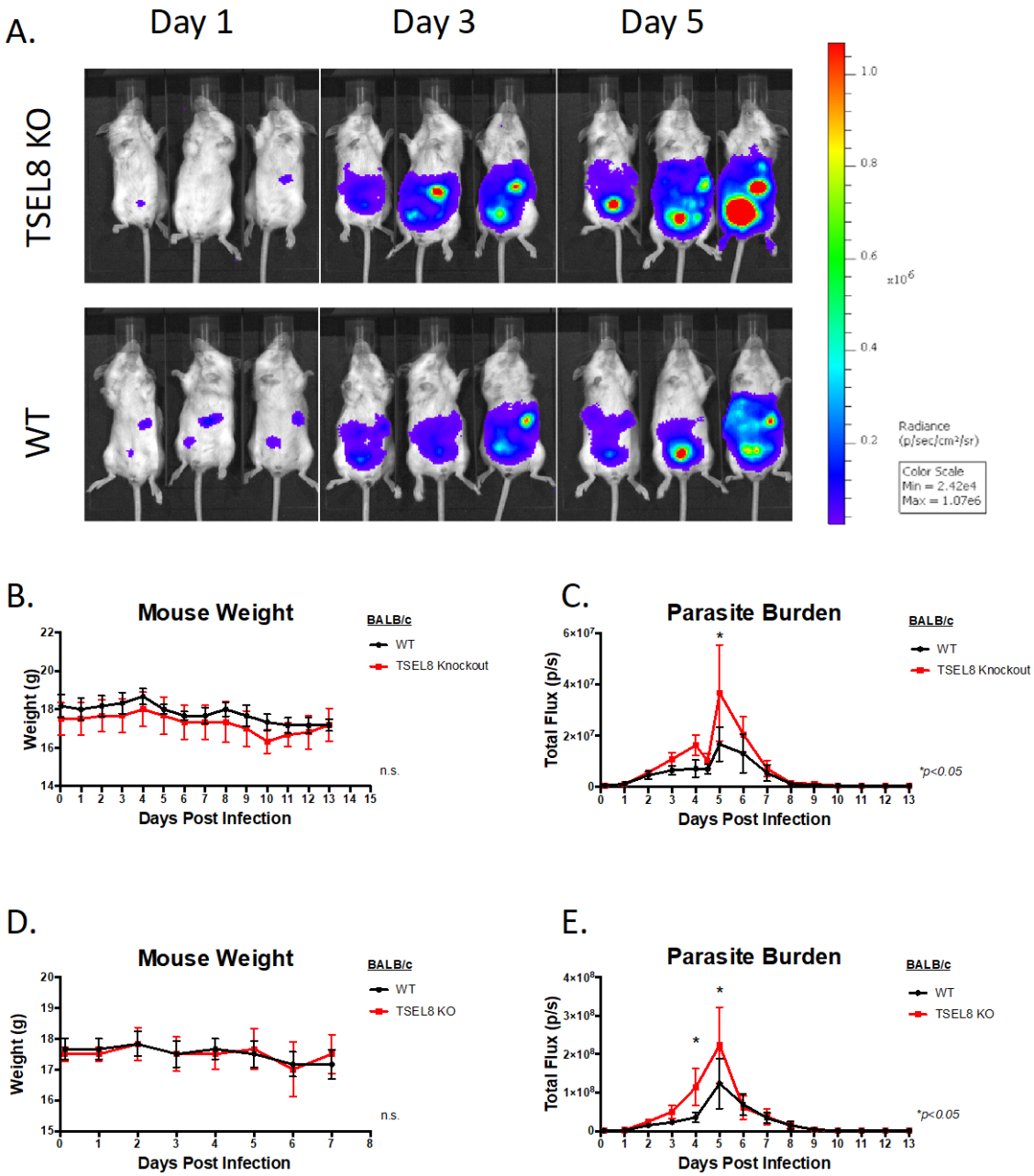

Figure 30 TSEL8KO and WT *in vivo* infections

A) **Representative images 6-week-old Balb/C mice infected IP withTSEL8 knockout (TSEL8KO) or wild-type (TSEL8WT) parasite tachyzoites per mouse and were imaged daily. 5 mice per parasite strain (n=5) were infected. Bioluminescent images show parasite-derived luciferase signal at select timepoints. (B and D) compare mouse weight throughout the acute infection with TSEL8T infections shown in black and TSEL8KO**

infections shown in red. (E and C) Shows quantification of bioluminescent imaging where total flux is the measurement for parasite burden in the mouse body of TSEL8KO-infection (red line) or TSEL8WT (black line). Parasite burden was analyzed by a two-way repeated measure ANOVA (alpha=0.05) with Sidak's multiple comparisons test. *p=<0.05, **p=<0.01, ***p=<0.001*N. caninum* soluble tachyzoite antigen characterization

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