Immediate interferon gamma induction determines murine host compatibility differences between Toxoplasma gondii and Neospora caninum

Immediate IFNγ controls N. caninum in mice

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Abstract

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.

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 (1, 2). 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*.

**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 (3-6). 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 (7-9). *N. caninum* is the causative agent of neosporosis, a disease that causes fetal death and neonatal mortality (10) in bovine (11), ovine (12), and canine hosts (13). *N. caninum* is remarkably similar in morphology and genomic synteny (85%) to *T. gondii*, (6, 14, 15) but there are important differences in their
disease pathology. Virulent strains of *T. gondii* are known to cause disease worldwide in all animals (8, 9), 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 (11, 16), although *N. caninum* is far more successful in cattle which are considered poor hosts for *T. gondii*. Mice are an important natural host for *T. gondii* and infections have been well documented in the laboratory and in nature (20-24), and mice infected with even 1 tachyzoite of virulent *T. gondii* strains will become morbid (25-27). Alternatively, mice infected with up to 1 million tachyzoites of *N. caninum* strains do not exhibit any observable morbidity (1, 28, 29). 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 (30-32). Interferon gamma (IFNγ) has been identified as an important immune-modulating cytokine in mice and cattle during *N. caninum* infections (31, 33) and investigations in both mice and cattle show that production of IFNγ is an important protective mechanism during neosporosis (32, 34). While IFNγ can protect against abortion in cattle, high levels of IFNγ at the materno-fetal interface increase fetal death (35, 36). 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 (38). IFNγ is also required for resistance to *N. caninum* in mice (37), 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; (39)) and N. caninum Nc1:Luc (1). 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 (1). 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 (2) and the extensive use of a mouse model to develop vaccines and treatment for bovine neosporosis (40, 41). 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.

**Results**

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. (26, 42, 43) Parame burden correlates directly with photon flux, and is calculated by quantifying photonic emission after luciferase substrate injection in live animals. (44) 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 (26). 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 (39, 45). To compare these strains directly we infected mice intraperitoneally (IP) with $10^6$ luciferase-expressing tachyzoites and performed *in vivo* BLI (Fig 1A). Quantification of bioluminescent images (Fig 1B) 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 1B). 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 1B). 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 (39) as well as *N. caninum* (46). 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) (26, 47, 48). 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 2A TgS1T:Luc n=2, Nc1:Luc n=3; Fig S1 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 2A) 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 1C*).

*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 1A,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 S2A,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 S2A,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 S2C,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 S2).

To confirm these results, we infected 3 BALB/C mice per group with 1x10⁶ 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 2B) and IFNγ (Fig 2C) as early as 4 hpi, while we detected significantly less serum IL-12p40 (P<0.001; Fig 2B) and detected no IFNγ (Fig 2C) 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 S1B 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 2D, 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 (29,
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 2E) and IFNγ (Fig 2F) 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 2F). 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.

**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 (37). Previous work has shown that mice deficient in IFNγ (both IFNγ depleted and *IFNγ*−/−) are also susceptible to *N. caninum* parasites (32, 50), but the impact of IFNγ deletion on parasite kinetics during *N. caninum* infection is unknown. To quantify this, we injected 10⁶ 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 3A, C), and monitored mouse survival (Fig 3B). As expected, mice lacking IFNγ succumbed to *N. caninum* infection in a similar timeframe as *T. gondii* (Fig 3B). 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 3C) despite exhibiting similar levels of burden and proliferation in the first 24 hours (Fig 3C, inset). These results were confirmed by an additional experiment (n=3 per mouse strain and parasite species, Fig 3B, S3A),
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 10⁴ 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 3D*). 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 susceptibility in *N. caninum* compared to *T. gondii* (*Fig 3D*), 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 susceptibility.

**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 (51) 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 4A) 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 4B). 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 4C,D) and remained significantly higher over the course of the infection (Fig 4C,D). In contrast to our experiments in IFNγ−/− mice (Fig 3), parasite burden declined between days 4 and 7 pi in IL12p40−/−. An additional experiment (Fig 4B, n=3 per mouse strain) confirmed our results and the previously reported susceptibility of IL-12p40−/− mice to *N. caninum* (51). In both our experiments, IL-12p40−/− mice infected with *N. caninum* produced no detectable serum IFNγ (Fig 4E).

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 (52). 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 5A) or parasite burden (Fig 5B) 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 5A) and also had similar parasite burden over the course of the experiment (Fig 5C). 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.

**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 (53). *T. gondii* infection in *MyD88*^−^ mice is characterized by uncontrolled parasite proliferation and defective IL-12 production (54). Since *N. caninum* induces a potent pro-inflammatory immune response within hours of infection when compared to *T. gondii* infections in mice (Fig 2), we sought to determine if host TLR signaling was required for early IL12 and IFNγ induction (Fig 2C,F and S1B) and control of *N. caninum* proliferation (Fig 2A,D and S1A), *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 6C n=2 per mouse strain; Fig S3B n=3 per mouse strain; n=3 per mouse strain, *data not shown* and Fig 6F n=3 per mouse strain). We performed three of
the experiments in our facility (Fig 6A) and our collaborator performed the last experiment in another lab at a different institution (Fig 6F). Our results demonstrate that MyD88 is not required for survival of sublethal (10^6) IP infections of N. caninum (Nc1:Luc) in mice (Fig 6A,F), a result that is in conflict with at least one previous report (51). 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 6B,C S3B), with the only significant difference being higher in WT compared to MyD88−/− at 12 hpi (Fig 6C). At later stages of infection parasite burden was significantly higher in MyD88−/− mice compared to WT (Fig 6C S3B), 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; (51)). 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 6E, 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 6D,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 6D,G, S3E). This increased IFNγ preceded a reduction of N. caninum-derived bioluminescent signal between days 4-6 pi in MyD88−/− mice (Fig 6C,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 (55) 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; (56)). 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 S4A,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 10⁶ *N. caninum* tachyzoites and monitored parasite burden (Fig S4C, TLR3−− n=2 and WT n=3; Fig S4E, 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 S4C), or 4 hpi (*P*<0.05, FigS4E). We also found that, unlike MyD88−/− mice, TLR3−− mice were capable of
producing immediate (4 hpi) IFNγ in response to *N. caninum* infection (Fig S4D,F). TLR3−/− did produce significantly (*P* ≪ 0.05) less IFNγ at 4 hpi compared to control mice in one (Fig S4D) out of two (Fig S4F) 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.

**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 (57) in mice, generating a potent TLR11-dependent IL-12 response (58). *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 7A) and both also have tachyzoite transcript abundance levels in to top 5% of all genes queried by RNAseq ((15) 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 (57), all TLR11+/− mice survived *N. caninum* infection (Fig 6F) and we found that by 4 hpi,
both TLR11−/− and MyD88−/− mice had significantly lower serum IFNγ compared to WT mice (Fig 6G). However, by 24 hpi serum IFNγ levels in TLR11−/− mice rose to levels that were similar to that observed in WT mice (Fig 6G). 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).

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 (58). Alignments indicate profilin is highly conserved between T. gondii and its close relatives (59), although it does have 4 amino acid polymorphisms in a region of the protein previously shown (59) to be required for TLR11 activation (green box, Fig 7A). 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 (60) 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 7B). We also found that
both proteins were detectable at an expected apparent molecular weight (20-30 KDa) 
by Western blot (Fig 7C). 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 7C). 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 7D). Regarding 
IFNγ production, we observed no significant differences in serum IFNγ levels at most 
queried timepoints (Fig 7E). The only exception was a significantly higher abundance of 
IFNγ in Tg:TgProHA-infected mice compared to Tg:NcProHA-infected mice at 48hpi 
(Fig 7E). 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 D), 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) (61, 62), 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 7F), 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.

**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 (63, 64). 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 (39, 65, 66) and dense granule proteins like IST (67, 68). 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 3C). 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 3C). 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 (69), but can be actively suppressed by *T. gondii* in a variety of contexts, suggesting the existence of an ongoing molecular arms race (70, 71). 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; (51)) 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 6). 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 (56)).

Despite this discrepancy other aspects of these studies (51, 56) 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 6C, S3B). *N. caninum* infection led to increased IL-12p40 and IFNγ in peritoneal exudates by day 3 pi, and this induction was dependent on MyD88 (51). 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 (51). 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 (72), 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 (10⁶ 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 6D). In the current study, the comparatively higher lethality in MyD88−/− mice observed in (51) 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 (10⁶ trypan blue-negative parasites versus 10⁶ 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 10⁷ tachyzoites; (56)). 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 1 interferons were being significantly induced by *N. caninum* in mice (in contrast to human cells; (55)).

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 (51) 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 (61, 73, 74).

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 (59). 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 (75). In these studies, recombinant *N. caninum* profilin (rNCPro) was unable to induce sufficient cell-mediated responses to provide protection in mice (76), 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 (77). 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 (78). During the acute phase of *T. gondii* infection, much of the IFNγ is produced by natural killer cells (23, 38), although neutrophils have recently been implicated in providing TLR11-independent IFNγ during *T. gondii* infection (79). 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.

**Materials and Methods**

**Parasite maintenance and preparation**

Parasite strains (*Toxoplasma gondii*: TgS1T:Luc:DsRed, TgS23:Luc:DsRed, TgMe49; *Neospora caninum*: Nc1:Lac: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% CO₂ incubators at 37 degrees. Monolayers were washed with PBS (for \textit{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 \textit{in vivo} or \textit{in vitro} assays as described below. Plaque assays were routinely used to verify similarity of the inocula.

\textbf{Soluble tachyzoite Antigen (STAg) preparation}

Soluble tachyzoite preparations of $1 \times 10^7$ 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.
Transgenic *T. gondii* and *N. caninum* strains

*T. gondii* strains and TgS23:GFP:Luc were described previously (26). 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 (39, 48). We identified TgS1T as one of the strains lacking “virulent alleles” for 5 previously characterized virulence quantitative trait loci (39). *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 (1, 80)), 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 acgtcgtaggtacCCAGACTGGTGAAAGATACTCGG for *T. gondii* profilin and gaaatcaagcaagatgcaATGTCGGACTGGGATCCC and acgtcgtaggtacCCAGACTGGTGAAAGGTAC 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 (39). To generate transgenic parasites, 2x10^7 ME49:ΔHPT: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.

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 (57) and maintained by the Yarovinsky lab.

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.
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).

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.

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.

**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).

**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.

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.

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References


abortion linked to gamma interferon production in pregnant dairy cows naturally infected with Neospora caninum. Theriogenology 68:1067-1073.


Fig 1. 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

Fig 2. 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 S1). 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.

**Fig 3. 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 figure S3A. 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

Fig 4. 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 10⁶ 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 4D were infected at the same time as multiple knockouts and data from serum taken from these same mice is also shown in Fig 6D. 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.
Fig 5. *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<sup>−/−</sup> n=3 per parasite species) and C57BL/6 (control) mice (n=3 per parasite species) were infected with 10<sup>6</sup> 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<sup>−/−</sup> (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<sup>−/−</sup> comparison for Nc1 infection. C) TgS1T:Luc infections in C57BL/6 (red) or MCP<sup>−/−</sup> (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<sup>−/−</sup> comparison for TgS1T infection.

Fig 6. *In vivo* bioluminescence imaging and cytokine production in MyD88 or TLR11 knockout mice. A) Combined survival of MyD88<sup>−/−</sup> (blue) or C57BL/6 (black) mice infected with 10<sup>6</sup> 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 4A, 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 S3B). 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 S3). **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.

**Fig 7.** 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 (59) 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 10⁶ tachyzoites (n=3 per parasite strain) or injected with equivalent 10⁶ 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. 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. *p=<0.05, **p=<0.01, ***p=<0.001.
Fig. 1

A. 4 hpi  12 hpi  20 hpi  28 hpi  36 hpi

TgS23:Luc
TgS1T:Luc
Nc1:Luc

B. Parasite Burden

Parasite Burden

C. in vitro growth

in vitro growth

HFFs

TgS1T:Luc
Nc1:Luc

Fluorescence

Hours Post Infection
Fig 1. *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
A. Parasite Burden

B. Serum IL-12(p40)

C. Serum IFNγ

D. Parasite Burden

E. Serum IL-12(p40)

F. Serum IFNγ
Fig 2. 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 S1). 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.
Fig. 3

A. 

T. gondii

IFN-γ-

Balb/c

N. caninum

Balb/c

IFN-γ-

B. 

Survival

Percent survival

Days Post Infection

WT (BALB/C): IFNγ-/-:

C. 

Parasite Burden

Total Flux (p/s)

Days Post Infection

WT (BALB/C): IFNγ-/-:

D. 

in vitro growth

Percent Fluorescence

Hours Post infection

MEFs + 100U IFN:

TgS1T:Luc

Nc1:Luc

n=6

n=6

n=6

n=6

****

***

n.s.
Fig 3. 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 10⁶ TgS1T:Luc or 10⁶ 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 figure S3A. D) Mouse embryonic fibroblast cells were treated with 100 units of recombinant IFNγ (R&D) 24 hours after being inoculated with 10⁴ 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
Fig. 4

A. 

I. 12p40
II. C57BL/6

B. Survival

C. N. caninum burden

D. N. caninum burden

E. Serum IFNγ
Fig 4. 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 10⁶ *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 4D were infected at the same time as multiple knockouts and data from serum taken from these same mice is also shown in Fig 6D. 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.
Survival

Days Post Infection

Percent survival

A. Survival

B. N. caninum burden

C. T. gondii burden

Fig 5
Fig 5. 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.
A. Survival

Percent survival against Days Post Infection for C57BL/6 and MyD88-/- mice.

B. Fig. 6

1 dpi 4 dpi 5 dpi 6 dpi 7 dpi

C57BL/6
MyD88-/-

C. N. caninum burden

Total Flux (p/s) against Days Post Infection for C57BL/6 and MyD88-/- mice.

D. IFNγ

IFNγ (pg/mL) against Hours Post Infection for C57BL/6, MyD88-/-, and Myd88-/- mice.

E. IL-12p40

IL-12p40 (pg/mL) against Hours Post Infection for C57BL/6 and MyD88-/- mice.

F. Survival

Percent survival against Days Post Infection for C57BL/6, TLR11-/-, and MyD88-/- mice.

G. IFNγ

IFNγ (pg/mL) against Days Post Infection for C57BL/6, TLR11-/-, and MyD88-/- mice.
**Fig 6. 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 4A, 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 S2B). 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 S2).

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.
**Fig. 7**

### A.

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### B.

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### C.

- αHA (profilin)
- Merged

### D.

- Parasite Burden
- Total Flux (p/s)

### E.

- IFNγ (pg/mL)

### F.

- IFNγ
Fig 7. 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 (59) 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.