NLRP1 is an inflammasome sensor for *Toxoplasma gondii*

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Running Head: An NLRP1 inflammasome detects Toxoplasma

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Abstract

The obligate intracellular parasite *Toxoplasma gondii* is able to infect nearly all nucleated cell types of warm-blooded animals. This is achieved through the injection of hundreds of parasite effectors into the host cell cytosol allowing the parasite to establish a vacuolar niche for growth, replication and persistence. Here we show that *Toxoplasma* infection actives an inflammasome response in mice and rats, an innate immune sensing system designed to survey the host cytosol for foreign components leading to inflammation and cell death. Oral infection with *Toxoplasma* triggers an inflammasome response that is protective to the host, limiting parasite load and dissemination.

*Toxoplasma* infection is sufficient to generate an inflammasome response in germ-free animals. IL-1β secretion by macrophage requires the effector caspases-1 and -11, the adapter ASC and NLRP1, the sensor previously described to initiate the inflammasome response to *Bacillus anthracis* lethal factor. The allele of NLRP1b derived from 129 mice is sufficient to enhance the B6 BMDM inflammasome response to *Toxoplasma* independent of the lethal factor proteolysis site. Moreover, N-terminal processing of NLRP1b, the only mechanism of activation known to date, is not observed in response to *Toxoplasma* infection. Cumulatively, these data indicate that NLRP1 is an innate immune sensor for *Toxoplasma* infection, activated via a novel mechanism that corresponds with a host-protective innate immune response to the parasite.

Introduction
Toxoplasma gondii is among the most successful protozoan parasites, exhibiting an extremely broad host range. While rodents are thought to be a natural host for the parasite, Toxoplasma is also a relevant human pathogen, with infection rates ranging from 10% (U.S.) to 65% (France and Brazil) of the population worldwide (1). Toxoplasma is acquired through ingestion of oocysts or tissue cysts (bradyzoites) in food or contaminated water. Once they have crossed the intestinal barrier the parasites convert into rapidly dividing tachyzoites that traffic through the host in infected leukocytes (2). In order to complete the infectious cycle, the parasite must convert to the bradyzoite form, most abundant in the brain and muscle. While immuno-competent hosts are largely asymptomatic, infection of immuno-suppressed individuals results in encephalitis, heart and lung damage. As such, the ability to raise an effective immune response is critical to the survival and success of both the host and the parasite. Cumulatively, these observations suggest that mammalian hosts have been under a stringent selective pressure to develop specific mechanisms to sense and suppress the activity of Toxoplasma.

The parasite’s ability to invade and persist within host cells, often for the life of an individual, is achieved through the secretion of a large number of molecules into the host cell cytosol. Many of these secreted/injected proteins function as “effectors”, intersecting host signaling pathways like ROP16 which acts as a mimic of host JAK kinases, phosphorylating STAT-3 and STAT-6 (3, 4). ROP5 and ROP18 cooperate to interfere with immunity-related GTPases blocking their attack of the parasitophorous vacuole membrane (5, 6). Dense granule proteins have recently been shown to enter the host cytoplasm where they can interact with crucial host functions (7, 8). This biology is...
conserved across the three major strains of *Toxoplasma* that predominate Europe and
North America, referred to as Type I, Type II and Type III, though, allelic differences in
some of the secreted proteins appears to dictate differences in the virulence between
types. The full extent of how *Toxoplasma* uses secretion to co-opt the host cell is not yet
known, especially as regards interaction with the innate immune surveillance systems.

Inflammasomes are sensor systems used to survey the host cell cytosol, a
normally sterile compartment, for microbial ligands or abnormal host cell biology as
signatures of infection. The ability to recognize and respond to a diverse set of stimuli is
achieved through the modular use of multiple sensors belonging to the NLRP and the
PYRIN/HIN families. These sensors interact with caspase1 or caspase11 directly or via
the linker ASC leading to multimerization of inflammasome complexes. The downstream
secretion of IL-1-family cytokines and pyroptotic cell death leads to the recruitment of
other inflammatory cells to the region and limits intracellular pathogen replication.

NLRP1, the first inflammasome characterized, is activated by *Bacillus anthracis* the
causative agent of anthrax (9). N-terminal cleavage of the sensor by the proteolytic
component of anthrax lethal toxin, the so-called “lethal factor” (LF), leads to robust
ASC- and caspase1-dependent IL-1β release and cell death (10, 11). This sensing
mechanism is responsible for an “early-response phenotype” characterized by ataxia,
loose stool and dilated blood vessels in response to live *Bacillus anthracis* spores that
restricts bacterial dissemination but can lead to host death in response to the purified
toxin (12, 13).

To date, the majority of cytosolic sensor systems have been found through the use
of viral and bacterial infection models and very little is known about their role in the
detection of eukaryotic pathogens. Recently Lodoen and colleagues demonstrated that Type II strains of *Toxoplasma* elicit IL-1β secretion from human monocytes (14). This is consistent with GWAS results that identified SNPs in *nlrp1* and the NLRP3 inflammasome activator *p2x7r* associated with susceptibility to congenital Toxoplasmosis, and the finding that NLRP1 knockdown in a human monocyte line enhanced *Toxoplasma* growth (15, 16). However, a definitive study to determine if there is a sensor in the host cell cytosol that detects *Toxoplasma* directly or a description of the role inflammasome plays in *Toxoplasma* infection have not been completed. The work described here addresses both these questions.

**Materials and Methods**

**Animals**

CBA/J, BALB/cJ, C57BL/6J, 1291/SvImJ, B6129PF1, B6.129P2-P2X7 receptor KO mice were purchased from Jackson Laboratories. Caspase1/11 double KO mice were bred in the Stanford animal facility or purchased from Jackson Laboratories. Germ-free or restricted-flora Swiss Webster mice were bred and maintained in micro-isolators in the Stanford Gnotobiotic Facility. Restricted-flora mice were monitored to ensure that they do not acquire opportunistic bacterial species (beta hemolytic *Streptococcus* species, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) but otherwise have a diverse range of commensal phyla comparable to conventionally raised mice. Sprague Dawley and Lewis rats were purchased from...
Taconic. All animal protocols were approved by Stanford University's Administrative Panel on Laboratory Animal Care (Animal Welfare Assurance # A3213-01, protocol # 9478). All animals were housed and treated in accordance with AAALAC and IACUC guidelines at the Stanford School of Medicine Veterinary Service Center.

Parasites, cells and cell lines

The following parasite strains were used Type I, RH88; Type II, Pru or Me49; Type III, CEP. The *Neospora caninum* used was the NC-1 strain. RH88 and Me49 strains stably expressing green fluorescent protein and luciferase were previously described (17). Parasites were passaged intracellularly in human foreskin fibroblasts (ATCC) and passaged by 25G syringe lysis in complete DMEM (cDMEM, Gibco) plus 10% FBS (HiClone), 100ug Penicillin-Streptomycin (Gibco) and 1mM Sodium Pyruvate (Gibco).

Mouse BMDMs were generated from femurs of 6-8 week old mice, and differentiated 6 days in cDMEM plus 20% supernatant from L929 cells expressing mouse MCSF. Rat BMDM media was supplemented with 30ng/mL recombinant rat MCSF (ProSpec).

Immortalized BMDMs stably expressing NLRP1 constructs were previously described (10). NLRP1 constructs expressed in the MSCV2.2 retroviral vector were transiently transfected in 293T/17 cells (ATCC) using lipofectamine LTX (Invitrogen) according to the manufacturer’s directions.

Supernatant ELISAs and LDH assays

BMDMs were plated at 0.02x10⁶ per well in 96-well plates or 0.05x10⁶ per well in 24-well plates in cDMEM. 12 hours or 2 hours prior to infection media was exchanged for cDMEM containing 100ng/mL ultra-pure LPS or 500ng/mL Pam3CSK4. Intracellular parasites or mock-infected HFFs controls (UI-SUP) were syringe-lysed through a 25G
needle in fresh cDMEM, washed in cDMEM and counted on a hemocytometer.

Infectious doses were washed and pelleted. A volume of supernatant equivalent to parasite pellet volume was reserved following the final spin and used as an “infected supernatant” control (I-SUP). Cells were infected at the multiplicity of 3:1, 5:1 or 10:1 (parasite: host cells) for the times indicated. 5 mM ATP (Invivogen) or Lethal Toxin composed of 1 µg/mL each lethal factor (LF) and protective antigen (List Biological Laboratories) were used as controls. *Salmonella typhimurium* SL13344 was grown overnight in LB at 37°C with aeration, diluted 1:40 in fresh LB and grown 3-4 hour before infection at an MOI of 10:1 (*Salmonella: host cells*). Supernatants were harvested at the indicated time points and diluted 1:3 in 0.1%BSA-PBS for LDH assay (*Cell Cytotox 96, Promega*). Remaining supernatants were flash frozen or used fresh for IL-1β ELISA (mouse-IL-1β duoset R&D, rat IL-1β Ready-Set-Go eBioscience).

**Immunoblots**

293T cells were seeded at 1x10^6 cells per well in 6-well plates and infected as described above. Cells were scraped in PBS, pelleted and lysed in RIPA buffer (50mM Tris pH 8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with EDTA, complete protease inhibitor (Roche) and PMSF (Sigma). Samples were divided in half, to resolve cleaved NLRP1 samples were denatured at 95°C for 5 minutes, to resolve full-length NLRP1 samples were not. To measure supernatant proteins media was replaced with 1.8mL of serum-free, phenol red-free DMEM just prior to infection. For the “time 0” time-point, plates were infected on ice in cold media and parasites were allowed to settle for 30 minutes then monolayers/supernatants were harvested immediately. Supernatants were cleared of gross cell debris by centrifugation at
3000g, 4°C for 5 minutes, and soluble proteins were precipitated in a final concentration of 10% TCA overnight. Precipitate was pelleted by centrifugation at 12000g for 20 minutes at 4°C, washed with 100% acetone, pelleted as above and air dried. Cell pellets and supernatant pellets were re-suspended in 8M Urea (no boiling). Following addition of SDS loading dye all protein samples, were separated on 4-12% BIS-TRIS gels (Invitrogen) by SDS PAGE then transferred to nitrocellulose membranes. The following antibodies were used to probe protein levels by immunoblot: anti HA-HRP (Biorad), anti-gfp (Abcam), anti-SAG1, and anti-GAPDH (Sigma-Aldrich).

**Microscopy**

Cells were imaged in tissue culture dishes or plated on coverslips coated with poly-D-lysine (Sigma). Cells were fixed in 2.5% formaldehyde in PBS or stained with 5µL/mL propidium iodide (BioLegend) in PBS for 10 minutes prior to fixation. Coverslips were mounted in Vectashield (Vector Laboratories) imaged on an Olympus BX60 upright fluorescence microscope with a 20x or 40x objective.

**In vivo infections**

To generate cysts 6-8 week-old female CBA/J mice were infected with 1000 Me49-gfp-luc tachyzoites IP. 4-8 weeks following infection brains were harvested, disrupted through a 50 µm filter, washed 3 times in PBS and stained with dolichos-rhodamine (Vector labs) and the number of cysts were determined by counting double-positive cysts at 40x magnification. Prior to infection 8-10 week-old male mice were cross-housed on dirty bedding for two weeks to normalize commensal microbiota. Mice were starved over night and fed between 200 and 250 Me49-gfp-luc cysts on ¼ piece of mouse diet. Weights and health were monitored daily. For bioluminescence imaging...
(BLI), mice were injected with 200 µL of a 15 mg/mL stock solution of luciferin (Xenogen), anesthetized 10 minutes in isoflurane and imaged for 4 minutes on an IVIS system. To image organs, mice were injected 10 minutes prior to euthanasia and organs were imaged as described. Images were analyzed with LivingImage software and ImageJ. For quantification, brain cysts were harvested and stained as described for CBA/Js except that brain mash was fixed for 20 minutes in 2.5% formaldehyde in PBS prior to dolichos-rhodamine staining.

12-week-old male Swiss Webster mice housed in germ-free or restricted-flora micro isolators were used in gnotobiotic experiments. Brain cysts (or mock infected brains) were prepared under sterile conditions then vials containing infectious doses were decontaminated for 2 hrs prior to infection on ¼ piece of mouse diet. Parasite load and dissemination were monitored by BLI as described above. Sera were collected at sacrifice and cytokines were measured by Luminex bead assay (Illumina) at the Stanford Human Immune Monitoring Center.

Results

Macrophages from rat strains resistant to Toxoplasma secrete IL-1β and pyroptose in response to the parasite. Inbred strains of rats fall into two major groups with respect to their ability to restrict Toxoplasma early in acute infection or permit high cyst burdens. In an analysis of F1 crosses between Toxoplasma-restrictive Lewis rats and Toxoplasma-permissive BN rats, Cavailles and colleagues determined that a single 1.7cM region of rat chromosome 10, referred to as the toxo1 locus, controls the parasite-
Peritoneal exudate cells isolated from these rats and infected *in vitro* were able to restrict parasite replication in a manner that correlated with decreased host cell number over the course of the experiment. Interestingly, the *toxo1* interval overlaps with the rat susceptibility locus to anthrax lethal factor (LF), mapped in a cross between BN and SHR rats, that contains and is controlled by NLRP1 (19). Based on these observations, we hypothesized that the inflammasome may be involved in *Toxoplasma*-restrictive response of Lewis rats and rat macrophages.

To examine inflammasome activation, bone-marrow-derived macrophages (BMDMs) from Lewis and Sprague Dawely rats (that phenotypically copy the BN strain of rat and express an identical version of *nlrp1*) were pretreated with LPS overnight and infected with Type I (RH) or Type II (Me49) *Toxoplasma* for 10 hrs. Consistent with the previous report that Lewis cell cultures decrease in cell number following *Toxoplasma* infection, we observed rapid and uniform cell death across infected Lewis BMDM cultures that phenotypically resembled pyroptosis (Figure 1A) (18). Lewis BMDMs did not pyroptose in response to LF, as previously reported (19). Importantly, Sprague Dawley BMDMs did not die in response to the parasite, although they were sensitive to LF. When supernatant IL-1β levels were examined by ELISA, we found that Lewis BMDMs secrete robust IL-1β in response to the parasite but not LF, and vice versa for Sprague Dawley BMDMs (Figure 1B). IL-1β secretion and cell death could be observed in the absence of TLR priming, although cytokine secretion was more robust following LPS or PAM3CysK4 treatment, indicating that *Toxoplasma* was sufficient to trigger an inflammasome response in Lewis BMDMs (data not shown). IL-1β secretion was not induced by treatment with supernatants from uninfected HFF cultures (UI-SUP), or an
equivalent amount of supernatant from Toxoplasma-infected HFF cultures (I-SUP) which served as controls for cytokine or damage associated molecular pattern (DAMP) carry over from parasite harvest. These results are consistent with the interpretation that the cell death response in Toxoplasma-restrictive rat macrophages is inflammasome-mediated which correlates with parasite restriction in vivo.

**Inflammasome activation is protective following oral Toxoplasma infection.**

To directly examine the inflammasome response to Toxoplasma we turned to the mouse model where many genetic and experimental tools have been developed to study inflammasomes. For this, we infected B6 mice or caspase1/11 KO mice with 250 Type II (Me49 engineered to express gfp and luciferase, Me49-gfp-luc) brain cysts orally. No significant difference in weight loss (Figure 1C) or parasite dissemination measured by bioluminescent intensity (BLI, data not shown) was observed throughout the acute stages of infection comparing caspase1/11 KO and B6 controls. Strikingly, however, caspase1/11 KO mice harbored 5 times more brain cysts than B6 controls at the onset of chronic infection, 18 days post infection (dpi; Figure 1D). Whereas by this time B6 mice appeared to have cleared most of the infection from the periphery, parasites were still detected in the gut and testes of caspase1/11 KO by BLI (Figure 1E, white asterisks; Figure 1F). Cumulatively, these data strongly suggest that caspase1/11-mediated inflammasome responses control parasite burden and/or dissemination in the mouse.

**Toxoplasma alone is sufficient to trigger an inflammasome response in germ-free mice.** An important caveat with the in vivo experiments is that oral infection with Toxoplasma triggers transient disruptions in the intestinal epithelium. Commensals have been documented to disseminate from the gut to the liver and spleen, trigger TLR
responses and generate commensal-specific memory T-cell populations (20, 21). While our experiments using caspase1/11 KO mice are consistent with a requirement for the inflammasome in regulating the outcome to *Toxoplasma* infection, it is likely that both the parasite and intestinal flora contribute to this response. For this reason, we turned away from continued experiments infecting mice deficient in components of the inflammasome and decided to directly address the question of whether *Toxoplasma* alone is sufficient to trigger an inflammasome response in mice.

Germ-free (GF) or restricted-flora (RF) outbred Swiss Webster mice were infected with 230 Type II (Me49-gfp-luc) brain cysts or equivalent amounts of uninfected brain. In comparison to what is normally observed in inbred strains, no appreciable change in weight was observed in RF or GF mice throughout the 9 days of the experiment (Figure 2A). Parasite load was determined 9 dpi by BLI. Importantly, *Toxoplasma* was detected in the small intestine, mesenteric lymph node and spleen of GF mice, suggesting that the parasites are able to colonize and disseminate to these organs in the absence of commensal microbiota (Figure 2B). In addition to generating comparable levels of IFN-γ and IL-12, hallmarks of a response to *Toxoplasma* infection, GF mice were able to generate a robust IL-1β response to parasite infection (Figure 2C). Infection status of the mice was verified by serology and the “germ-free” status of the GF mice (excepting *Toxoplasma*) was confirmed by PCR at sacrifice (data not shown). As described in the literature, our in vitro experiments suggest that TLR priming is required to produce the pro-IL-1β substrate for caspase1/11 activation (Figure 3A, Supplementary Figure 1) (22). Although these mice do not harbor commensal bacteria, exposure to endotoxin and other microbial components in the mouse diet likely prime pro-IL-1β
transcription (signal 1 for inflammasome activation) while IL-1β cleavage and secretion is a response to *Toxoplasma* (signal 2)(22). While we cannot conclude that the response observed in the GF mice is driven by the same sensing mechanism as in conventional mice, for example neutrophil serine proteases have also been shown to process pro-IL-1β, these experiments show that infection with *Toxoplasma* is sufficient to trigger an IL-1β response in these animals (23).

*Toxoplasma* elicits a caspase1/11- and ASC-dependent inflammasome response from mouse macrophages. To determine if known inflammasome components are required for the response to *Toxoplasma*, BMDMs from B6 mice were plated overnight in complete media or complete media supplemented with TLR ligands, a signal required for transcription of pro-IL-1β and some NLRP sensors, and then infected with Type I (RH) or Type II (Pru or Me49) parasites (22). Although *Toxoplasma* has been reported to stimulate various TLRs, and in the case of Type II infection trigger NF-κB activation directly, TLR priming with Pam3CSK4 (Figure 3A) or LPS (Figure 3C) was required for robust IL-1β secretion (7, 24). In mouse macrophages, interferon-γ exposure stimulates transcription of immunity-related GTPases (IRGs) that can coat the parasitophorous vacuole and lead to parasite clearance (25, 26); interferon-γ could not, however, efficiently prime IL-1β secretion in response to all three Types of *Toxoplasma* (Supplementary Figure 1). Macrophage morphology changed following *Toxoplasma* infection and, consistent with previous reports, there was an increase in host membrane permeability leading to a low but noticeable staining with dyes like propidium iodide when compared to uninfected controls (Figure 3B) (27). However, this staining was distinct from the nuclear PI staining observed following ATP treatment, a well-studied...
inducer of pyroptosis consistent with the conclusion that pyroptosis was not occurring in
the majority of B6 BMDMs following *Toxoplasma* infection. Since we consistently noted
that responses were equally robust to Type I and Type II strains of *Toxoplasma* following
TLR priming, we focused attention on Type I strains for the majority of *in vitro*
experiments.

To determine if known inflammasome components were required for IL-1β
secretion in response to *Toxoplasma*, BMDMs from mice deficient in various
inflammasome components were infected. The IL-1β response to *Toxoplasma* was
reduced to near background levels when we infected BMDMs from caspase1/11 double
KO mice as well as ASC KO mice, consistent with interpretation that the IL-1β secretion
observed is inflammasome-mediated (Figure 3C). Although we noted that *Toxoplasma*
led to a modest release of the cytosolic enzyme lactate dehydrogenase (LDH) into the
culture supernatant, we found that the majority of this release was caspase1/11- and ASC-
independent (Figure 3D). This may be the result of damage to a minority of cells in the
infected culture, for example through spontaneous parasite egress, or the result the low-
grade membrane permeability noted in PI-staining experiments (Figure 3B). Either way,
the LDH release observed appears to be largely inflammasome-independent suggesting
that, in contrast to our observations in the rat where *Toxoplasma* infection induces
pyroptosis, there is not a robust pyroptotic response to *Toxoplasma* in B6 BMDMs.

Host cell damage can lead to elevated levels of extracellular ATP that, along with
other indicators of infection like ROS-production and break-down of endosomal
membranes, can trigger the NLRP3 inflammasome. While IL-1β was reduced in NLRP3
KOs, a significant portion of IL-1β was NLRP3-independent when compared to infected
supernatant and uninfected HFF controls (Figure 3C). Moreover, LDH release into the supernatant appeared to be entirely NLRP3-independent (Figure 3D). Previous reports have shown that triggering the P2X7 receptor with elevated extracellular ATP, an NLRP3 stimulus, prevented *Toxoplasma* growth (15, 28). We found that the response to *Toxoplasma* was entirely independent of the P2X7 receptor (Figure 3E), suggesting that while triggering inflammasome activation through the NLRP3 prevents parasite replication, the inflammasome triggered by the parasite directly has an important, NLRP3-independent component (Figure 3C-E). Similarly, ASC- and caspase-dependent and NLRP3/P2X7R-independent responses were also observed for infections with Type II and Type III *Toxoplasma* strains (data not shown). Of note, *Toxoplasma* was not able to inhibit inflammasome responses generated by exogenous inflammasome triggers. In mixing experiments, *Toxoplasma* infection did not interfere with pyroptosis triggered by the NLRP1 stimulus lethal toxin when infection occurred 30 minutes before or after lethal factor treatment (Supplementary Figure 2A and B).

The 129 allele of NLRP1b is sufficient to enhance the inflammasome response to *Toxoplasma independent of the lethal factor cleavage sites*. The result that a major component of the inflammasome response to *Toxoplasma* in mouse was NLRP3-independent raised the possibility that another sensor directly detects the parasite. Combined with the observation that the restrictive phenotype in Lewis rats mapped to a genomic interval containing NLRP1 and our finding that Lewis BMDMs secrete IL-1β and pyroptose in response to the parasite, we hypothesized that NLRP1 may be involved in the response to *Toxoplasma*. In contrast to the rats, which have a single *nlrp1* gene, mice have an expanded NLRP1 locus encompassing three paralogues of the sensor.
nlrp1a, nlrp1b and nlrp1c. B6 mice express an allele of nlrp1b that does not respond to anthrax lethal factor (29). 129 mice express a lethal-factor-sensitive allele of nlrp1b but do not express nlrp1a or nlrp1c. We observed that BMDMs from 129 mice generate a greater IL-1β response to Type I and Type II Toxoplasma infection than B6 BMDMs (Figure 4A and data not shown). As previously described, B6 BMDMs do not respond to anthrax lethal factor whereas 129 BMDMs are sensitive; however, both respond robustly to extracellular ATP in the context of LPS priming (Figure 4A).

To directly test the possibility that the 129 allele of nlrp1b (NLRP1b\textsuperscript{129}) was sufficient to drive a more robust response to the parasite, we infected B6 immortalized BMDMs (iBMDMs) that were engineered to express NLRP1b\textsuperscript{129} tagged at the N-terminus with GFP and HA (Figure 4B). Following retroviral gene transfer the transduced iBMDM population was approximately 30% GFP positive by FACS (data not show). B6 iBMDMs expressing NLRP1b\textsuperscript{129} generated five times more IL-1β than B6 iBMDMs transduced with the empty vector control (Figure 4C). Lethal factor is a zinc metalloprotease that activates NLRP1b by cleaving the sensor in the N-terminus (arrowhead, Figure 4B). Previous work has shown that when the primary cleavage site is converted to the consensus sequence for TEV protease (NLRP1b\textsuperscript{129}-TEV), lethal factor cannot efficiently process the sensor, though inefficient secondary cleavage can occur at a neighboring hydrophobic residue, resulting in a reduction in IL-1β secretion and cell death (10). Interestingly, B6 iBMDMs stably expressing NLRP1b\textsuperscript{129}-TEV also responded to Toxoplasma, generating significantly more IL-1β secretion and LDH release than B6 iBMDMs expressing the empty vector or the HFF- and the SUP-treated controls (Figure 4 C, D). While the level of IL-1β production in response to Toxoplasma was slightly less
in NLRP1b\textsuperscript{129}-TEV than NLRP1b\textsuperscript{129} this was not significant and may be due to minor differences in transduction efficiency between stable iBMDM lines. Consistent with this result, alignment of the NLRP1 sequences from \textit{Toxoplasma}-sensitive and -resistant rat strains indicated that only 5 non-conserved regions are present, all within the N-terminus of NLRP1, which thus, could be responsible for the difference in responsiveness to \textit{Toxoplasma}. Moreover, lethal factor has a preference for a highly hydrophobic residue at the P1’ position, cleaving between P1 Pro44 and P1’ Leu45 in the sequence RPRP\textsubscript{1}LPRV in the LF-sensitive, \textit{Toxoplasma}-resistant alleles; (10, 11, 30). This region markedly different in the \textit{Toxoplasma}-sensitive alleles of NLRP1 which have, instead, QVEQSFLG in the corresponding position. Cumulatively, these results are consistent with the interpretation that NLRP1b\textsuperscript{129} is sufficient to enhance the inflammasome response to \textit{Toxoplasma} in B6 BMDMs in a manner that does not require the major lethal factor proteolysis site.

The N-terminus of NLRP1 is not processed following \textit{Toxoplasma} infection.

We next asked if NLRP1b\textsuperscript{129} is cleaved in response to the parasite. When 293T cells were transiently transfected with NLRP1b\textsuperscript{129} both the 160kDa full-length protein and the \textsim{140}kDa fragment, the result of FIIND domain auto-proteolysis that is necessary but not sufficient for sensor activation, were detected (Figure 4E ‘FIIND cut’, Figure 4B filled arrowhead) (31). As previously reported, lethal factor treatment led to accumulation of an N-terminal \textsim{35}kDa fragment (Figure 4E ‘LF cut’). However, NLRP1b\textsuperscript{129} proteolysis was not observed in response to \textit{Toxoplasma} after 16 or 23 hours of infection (Figure 4E). A similar result was observed with Type II parasites (Me49, data not shown) as well as a
closely related organism, *Neospora caninum* (NC), which lacks many of the virulence strategies important to *Toxoplasma* infection.

Given that 293T cells lack necessary components for inflammasome activation, we looked at NLRP1 processing in the B6 iBMDMs stably transduced with NLRP1b^{129} or NLRP1b^{129}-TEV that have an enhanced response to the parasite. Similar to our results in 293T cells, LF treatment of B6 iBMDMs expressing NLRP1b^{129} led to a reduction in the full-length and FIIND-domain-cleaved bands in the cell pellet which correlated with secretion of the 35kDa N-terminal fragment of the sensor into the supernatant (Figure 4F). This was dependent on the LF cleavage sites, as no NLRP1b cleavage was observed in iBMDMs transduced with NLRP1b^{129}-TEV following LF treatment. By contrast, *Toxoplasma* infection did not lead to a shift in the levels of the full-length or FIIND domain-cleaved bands in the cell pellet or accumulation of a N-terminal cleavage product in the supernatants (Figure 4F). While we cannot definitively rule an undetectable level of cleavage, these data are consistent with the interpretation that NLRP1 activation in response to the *Toxoplasma* occurs via a novel, non-proteolytic mechanism.

**Discussion**

The result that the mouse NLRP1b^{129} can drive an inflammasome response to *Toxoplasma* in the absence of the lethal factor proteolysis sites, and likely receptor processing in general, is consistent with our observation that Lewis rats, which express an allele of NLRP1 that cannot be processed by LF, are highly responsive to *Toxoplasma*. Interestingly, while NLRP1b^{129} is sufficient to enhance the response to *Toxoplasma*, B6
mice still generate a robust, albeit lower, response to the parasite. Although the B6 allele of \textit{nlrplb} is not responsive to LF, it is possible that NLRP1b\textsuperscript{B6} is sensitive to \textit{Toxoplasma} infection and accounts for the inflammasome response observed on this background.

Given that NLRP1a is a nearer homologue of rat and human NLRP1, it is also possible that this paralog or possibly even another NLRP plays a role in the B6 response to the parasite; we think this is unlikely, however, as we do not see an additive effect in the response from BMDMs derived from an F1 cross between B6 and 129 mice (data not shown).

The precise mechanism of NLRP1 activation in response to the parasite and whether this is a response exclusive to \textit{Toxoplasma} or conserved across other protozoan parasites remain to be determined. To date, only two mechanisms of inflammasome activation have been described: NLRP1 proteolysis by lethal factor and Naip binding directly to flagelin or the type III secretion apparatus to trigger a caspase1-dependent inflammasome through NLRC4 (32, 33). There is evidence that the lethal toxin non-responsive BALB/c allele can still be processed by lethal factor, albeit at a different site from that observed for the sensitive NOD/129 allele, suggesting that not all proteolytic events are sufficient for sensor activation (34). While it is clear that \textit{Toxoplasma} invasion, growth and persistence within the cell require sustained interaction with the host cell cytosol, the catalogue of parasite components injected into the host cell is far from complete. A secreted component may be detected by NLRP1 directly or it may be that a secreted effector leads to other, non-proteolytic, post-translational modifications to the sensor.
In addition to the expansion in the nlrp1 locus observed in mice, nlrp1 exhibits a high degree of allelic variation across inbred rodent strains and the human population. It may be that interactions with different pathogens stabilized genetic diversity in nlrp1 and that a portion of this diversity is due to interaction with *Toxoplasma*. For example, the immunity-related GTPases (IRGs) attack the vacuole of Type II parasites and lead to parasite clearance, whereas Type I and Type III parasites express versions of the secreted effectors ROP5 and ROP18 that interact directly with the IRGs and subvert attachment to the vacuole (5, 6, 35). Mice are predicted to encode 21 immunity-related GTPases (IRGs), and *Toxoplasma* expresses between 4 and 10 tandem repeats of ROP5, depending on Type, evidence of an “evolutionary arms race” between parasite effectors and host sensing machinery (36, 37).

In humans, a growing number of GWAS findings have linked polymorphisms in NLRP1 with hereditary predispositions to vitiligo, psoriasis, systemic lupus erythematosus and rheumatoid arthritis among other autoimmune diseases (38). It remains to be seen if alleles that confer responsiveness to *Toxoplasma*, *Bacillus anthracis* or other pathogens come at the cost of susceptibility to autoimmunity. Identifying the pathogens that trigger NLRP1 and their mechanisms of activating the sensor may provide valuable insight into the how some alleles of nlrp1 contribute to autoimmune disorders and provide a cornerstone for developing better therapeutics to treat NLRP1-driven autoimmune diseases.

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References


Figure 1. Toxoplasma elicits an inflammasome response in mice and rats.

A-B, BMDM from Toxoplasma-restrictive Lewis rats or permissive Sprague Dawley (SPD) rats were treated overnight with 100 ng/mL LPS then infected with RH (TI), Me49 (TII) parasites (MOI 3:1), treated with supernatant from mock infected HFFs (HFF), supernatant from the TII Toxoplasma preparation (SUP) or 1 µg/mL lethal toxin (LF). 10 hpi cell monolayers were imaged at 20x magnification (A) and IL-1β levels in supernatants were measured by ELISA (B). Data are representative of 3 independent experiments. C-E, B6 or caspase1/11 KO (C-/-) mice were infected orally with 250 Me49-gfp-luc cysts (TII) or uninfected (UI). C, No significant difference in weight was observed throughout acute infection. D, At 18 dpi brain cyst burden was determined by dolichos-rhodamine staining brain mash and counting at cysts at 60x magnification. ND=none detected. E, 18 dpi parasite burden was determined by BLI in B6 or CASP-/- mice. White asterisks indicate regions of BLI above background in the small intestine or testis. F, Quantification of BLI in the small intestine (left) or testes (right) as maximum relative light units (photons). Data are representative of 3 individual experiments, N=5-8 mice per genotype per experiment. Error bars SEM. ** P<0.005
Figure 2. Toxoplasma is sufficient to trigger inflammasome activation in the absence of commensal microbiota. Germ-free (GF) or restricted-flora (RF) mice were infected orally with 230 Me49-gfp-luc cysts (TII) or mock infected. A, Mouse weight was monitored over the course of infection. B, 9 dpi mice were sacrificed and parasite load determined by BLI in spleen, mesenteric lymph node (MLN) and small intestine (sml. int.). C, IL-1β, IFN-γ and IL-12p40 levels in spleen lysates were determined by Luminex. N=3 mice per condition. Error bars SEM.
Figure 3. *Toxoplasma* elicits caspase1/11- and ASC-dependent but NLRP3-independent IL-1β secretion from BMDMs. **A**, BMDMs from B6 mice were primed overnight with 500 ng/mL Pam3CSK4 and infected with RH (TI), Pru (TI), (MOI 3:1), *Salmonella* (sal, MOI 10:1) or not infected (UI). **B**, 20 hpi with TI (RH-gfp-luc) or TII Me49-gfp-luc, 6 hrs post treatment with 5uM ATP, or not infected (UI) BMDM monolayers were imaged at 60x magnification. GFP (gfp), propidium iodide (PI) and phase images are shown. **C,D**, BMDMs from B6, ASC KO, Caspase1/11 KO (CASPΔ) or NLRP3 KO mice were pretreated 2 hours with 100 ng/mL LPS and infected with RH (TI, MOI 5:1) or an equivalent volume of supernatant from the T1 culture (SUP) for 15 hours. ATP control (5mM) was added 3 hours prior to harvesting supernatants for ELISA (C) or LDH release assay (D). **E**, BMDMs from B6,129P2-P2X7 receptor KO mice (P2X7RΔ), B6 or B6129PF1 (F1) controls were treated as described in A with RH (TI, MOI 3:1), 1 mM ATP or not infected (UI). IL-1β was measured in supernatants by ELISA 20 hpi. Data are representative of 3-5 individual experiments, error bars SEM, **p<0.01 students T-test.
Figure 4. The 129 allele of NLRP1b enhances the inflammasome response to Toxoplasma independent of N-terminal proteolysis. A, BMDMs derived from B6 or 129S1/SvImJ (129) mice were primed 2hr prior to infection with 100 ng/mL LPS infected with RH (TI, MOI 5:1) parasites, treated with equivalent volumes of uninfected HFF supernatant or TI supernatant (SUP) controls. 6 hrs prior to harvest 1 µg/mL lethal toxin (LF) or 5 mM ATP were added. Supernatants were harvested 24 hpi for IL-1β ELISA. B, Schematic of the cleavage fragments from the N-terminally GFP-HA tagged NLRP1 construct. Arrowheads indicate lethal factor proteolysis sites, solid triangle indicates FIIND domain autoproteolysis site. C, B6 immortalized BMDMs (iBMDMs) transduced with NLRP1b129 (129N1), NLRP1b129-TEV (129N1tev) or empty vector control (vector) were infected as described in A. Supernatants were harvested 12 hpi and IL-1β levels were analyzed by ELISA (C) or cell death was analyzed by LDH release (D). Data are representative of 3 experiments, error bars SEM. * p<0.05, **p<0.01 students T-test. E, 293T cells transiently transfected with GFP-HA tagged NLRP1b129 or untransfected (UT)
were infected with RH (TI), *Neospora caninum* (NC) (MOI 5:1) or treated with 1 µg/mL lethal toxin (LF) or uninfected (UI). Cell pellets were harvested 16 or 23 hpi and NLRP1 processing analyzed by immunoblot with a gfp-specific antibody. Data are representative of five experiments. F, B6 iBMDMs described in C were infected with RH (TI, MOI 5:1) or treated with 1 µg/mL lethal factor (LF). Cell pellets (upper panel) and concentrated supernatants (lower panel) were harvested 0, 3 or 6 hpi and analyzed by immunoblot with a HA-specific antibody. Antibodies to SAG1 (*Toxoplasma*) and GAPDH (mouse) were loading controls. Data are representative of 3 experiments.