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NLRP1 Is an Inflammasome Sensor for *Toxoplasma gondii*

Sarah E. Ewald,[a] Joseph Chavarria-Smith,[b] John C. Boothroyd[a]

Department of Microbiology and Immunology, Stanford School of Medicine, Stanford, California, USA[a]; Department of Molecular and Cell Biology, University of California Berkeley, Berkeley, California, USA[b]

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 germfree animals. Interleukin 1β (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 bone marrow-derived macrophage (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 to a host-protective innate immune response to the parasite.

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% (United States) 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 immunocompetent hosts are largely asymptomatic, infection of immunosuppressed individuals results in encephalitis and 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 in 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 coopt 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 caspase 1 or caspase 11 directly or via the linker ASC, leading to multimerization of inflammasome complexes. The downstream secretion of interleukin 1 (IL-1)-family cytokines and pyroptotic cell death lead 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 caspase 1-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

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Address correspondence to John C. Boothroyd, john.boothroyd@stanford.edu.
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identified through the use of viral and bacterial infection models, and very little is known about their role in the detection of eukaryotic pathogens. Recently, Gov and colleagues demonstrated that type II strains of *Toxoplasma* elicit IL-1β secretion from human monocytes (14). This is consistent with genome-wide association study (GWAS) results that identified single nucleotide polymorphisms (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 enhances *Toxoplasma* growth (15, 16). However, a definitive study to determine if there is a sensor in the host cell cytosol that detects *Toxoplasma* directly and 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, and B6.129P2-P2X7 receptor knockout (KO) mice were purchased from Jackson Laboratories. Caspase 1/11 double-KO mice were bred in the Stanford animal facility or purchased from Jackson Laboratories. Germfree or restricted-flora Swiss Webster mice were bred and maintained in microisolators in the Stanford Gnotobiotic Facility. Restricted-flora mice were monitored to ensure that they did not acquire opportunistic bacterial species (beta-hemolytic *Streptococcus species*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) but otherwise had 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* strain used was NC-1. 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 25-gauge-syringe lysis in complete Dulbecco's modified Eagle medium (cDMEM; Gibco) plus 10% fetal bovine serum (FBS; HyClone), 100 μg penicillin-streptomycin (Gibco), and 1 mM sodium pyruvate (Gibco). Mouse bone marrow-derived macrophages (BMDMs) were generated from femurs of 6- to 8-week-old mice and differentiated for 6 days in cDMEM plus 20% supernatant from L929 cells expressing mouse macrophage colony-stimulating factor (MCSF). Rat BMDM medium was supplemented with 30 ng/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 0.02×10^6 per well in 96-well plates or 0.05×10^6 per well in 24-well plates in cDMEM. At 12 h or 2 h prior to infection, medium was exchanged for cDMEM containing 100 ng/ml ultrapure lipopolysaccharide (LPS) or 500 ng/ml Pam3CSK4. Intracellular parasites or mock-infected human foreskin fibroblast (HFF) controls (UI-SUP) were syringe lysed through a 25-gauge 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 a multiplicity of 3:1, 5:1, or 10:1 (parasite to host cells) for various times. ATP (5 mM; Invivogen) and lethal toxin, composed of 1 μg/ml each lethal factor (LF) and protective antigen (List Biological Laboratories), were used as controls. *Salmonella enterica* serovar Typhimurium SL13344 was grown overnight in LB at 37°C with aeration, diluted 1:40 in fresh LB, and grown 3 to 4 h before infection at a multiplicity of infection (MOI) of 10:1 (*Salmonella* to host cells). Supernatants were harvested at various time points and diluted 1:3 in 0.1% bovine serum albumin–phosphate-buffered saline (BSA-PBS) for the lactate dehydrogenase (LDH) assay (Cell Cytotox 96; Promega). Remaining supernatants were flash frozen or used fresh for IL-1β enzyme-linked immunosorbent assay (ELISA) (mouse–IL-1β Duoset [R&D] and rat IL-1β Ready-Set-Go [eBioscience]).

Immunoblots. 293T cells were seeded at 1×10^6 cells per well in 6-well plates and infected as described above. Cells were scraped in PBS, pelleted, and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with EDTA, complete protease inhibitor (Roche), and phenylmethylsulfonyl fluoride (PMSF) (Sigma). Samples were divided in half. To resolve cleaved NLRP1, samples were denatured at 95°C for 5 min; to resolve full-length NLRP1, samples were not. To measure supernatant proteins, medium was replaced with 1.8 ml of serum-free, phenol red-free DMEM just prior to infection. For the time zero time point, plates were infected on ice in cold medium and parasites were allowed to settle for 30 min; then monolayers/supernatants were harvested immediately. Supernatants were cleared of gross cell debris by centrifugation at $3,000 \times g$ and 4°C for 5 min, and soluble proteins were precipitated in a final concentration of 10% trichloroacetic acid (TCA) overnight. Precipitate was pelleted by centrifugation at $12,000 \times g$ for 20 min at 4°C, washed with 100% acetone, pelleted as described above, and air dried. Cell pellets and supernatant pellets were resuspended in 8 M urea (no boiling). Following the addition of SDS loading dye, all protein samples were separated on 4 to 12% bis-Tris gels (Invitrogen) by SDS-PAGE and then transferred to nitrocellulose membranes. The following antibodies were used to probe protein levels by immunoblotting: anti-hemagglutinin-horseradish peroxidase (anti-HA-HRP) (Bio-Rad), anti-green fluorescent protein (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 5 μl/ml propidium iodide (BioLegend) in PBS for 10 min prior to fixation. Coverslips were mounted in Vectashield (Vector Laboratories) imaged on an Olympus BX60 upright fluorescence microscope with a 20× or 40× objective.

***In vivo* infections.** To generate cysts, 6- to 8-week-old female CBA/J mice were infected with 1,000 tachyzoites of strain Me49-GFP-Luc (Me49 engineered to express GFP and luciferase) intraperitoneally (IP). At 4 to 8 weeks following infection, brains were harvested, disrupted through a 50-μm filter, and washed 3 times in PBS, and cyst walls were stained with rhodamine-labeled dolichos (Vector Laboratories), and the number of cysts was determined by counting double-positive cysts at a ×40 magnification. Prior to infection, 8- to 10-week-old male mice were cross-housed on dirty bedding for 2 weeks to normalize commensal microbiota. Mice were starved overnight and fed between 200 and 250 Me49-GFP-Luc cysts on 1/4 of a 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 min in isoflurane, and imaged for 4 min on an IVIS system. To image organs, mice were injected 10 min prior to euthanasia, and organs were imaged using an IVIS 200 imaging system. Images were analyzed with Living-Image software and ImageJ. For quantification, brain cysts were harvested and stained as described for CBA/J mice except that brain mash was fixed for 20 min in 2.5% formaldehyde in PBS prior to rhodamine-labeled dolichos staining.

Twelve-week-old male Swiss Webster mice housed in germfree or restricted-flora microisolators were used in gnotobiotic experiments. Brain cysts (or mock-infected brains) were prepared under sterile conditions, and then vials containing infectious doses were decontaminated for 2 h prior to infection on 1/4 of a piece of mouse diet. Parasite load and dissemination were monitored by BLI as described above. Sera were collected

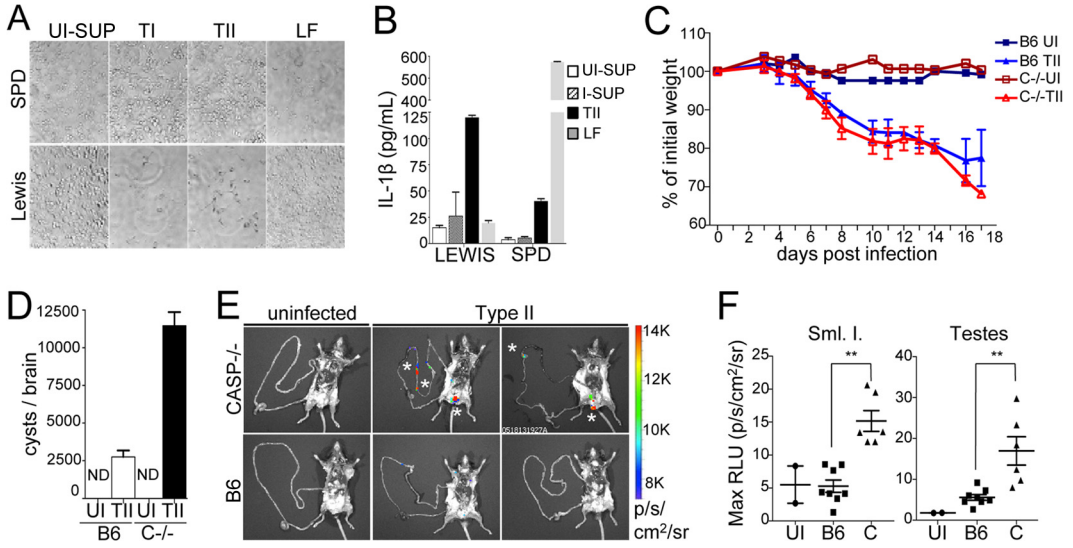

FIG 1 *Toxoplasma* elicits an inflammasome response in mice and rats. (A and B) BMDMs from *Toxoplasma*-restrictive Lewis rats or permissive Sprague-Dawley (SPD) rats were treated overnight with 100 ng/ml LPS, then infected with RH (type I [TI]) or Me49 (type II [TII]) parasites (MOI, 3:1), and then treated with supernatant from mock-infected HFFs (UI-SUP), supernatant from the TII *Toxoplasma* preparation (I-SUP), or 1 μg/ml lethal toxin (LF). At 10 hpi, cell monolayers were imaged at a magnification of ×20 (A), and IL-1β levels in supernatants were measured by ELISA (B). Data are representative of 3 independent experiments. (C to E) B6 or caspase 1/11 KO ($C^{-/-}$) mice were infected orally with 250 Me49-GFP-Luc cysts (type II) or were uninfected (UI). (C) No significant difference in weight was observed throughout acute infection. (D) At 18 dpi, brain cyst burden was determined by rhodamine-labeled dolichos staining of brain mash and counting of cysts at a magnification of ×60. ND, none detected. (E) At 18 dpi, parasite burden was determined by BLI in B6 or $CASP^{-/-}$ mice. Asterisks indicate regions of BLI above background in the small intestines or testes. (F) Quantification of BLI in the small intestines (Sml. I.) or testes as maximum relative light units (RLU) (photons). Data are representative of 3 individual experiments with 5 to 8 mice per genotype per experiment. Error bars show standard errors of the mean (SEM). **, $P < 0.005$.

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 F_1 crosses between *Toxoplasma*-restrictive Lewis rats and *Toxoplasma*-permissive BN rats, Cavailles and colleagues determined that a single 1.7-centimorgan (cM) region of rat chromosome 10, referred to as the *toxo1* locus, controls the parasite resistance phenotype (18). 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 the *Toxoplasma*-restrictive response of Lewis rats and rat macrophages.

To examine inflammasome activation, bone-marrow-derived macrophages (BMDMs) from Lewis and Sprague-Dawley rats (which 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 h. 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 (Fig. 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 (Fig. 1B). IL-1β secretion and cell death could be observed in the absence of Toll-like receptor (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) carryover 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 caspase 1/11 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 (Fig. 1C) or parasite dissemination, measured by bioluminescent intensity (BLI) (data not shown), was observed throughout the acute stages of infection between caspase 1/11 KO

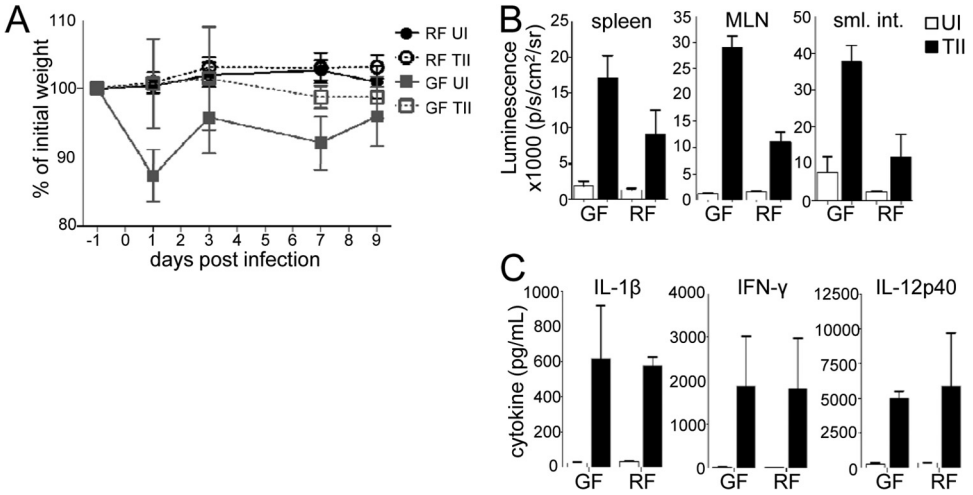

FIG 2 *Toxoplasma* is sufficient to trigger inflammasome activation in the absence of commensal microbiota. Germfree (GF) or restricted-flora (RF) mice were infected orally with 230 Me49-GFP-Luc cysts (type II [TII]) or mock infected (UI). (A) Mouse weight was monitored over the course of infection. (B) At 9 dpi, mice were sacrificed, and parasite load was determined by BLI in spleen, mesenteric lymph nodes (MLN), and small intestines (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 show SEM.

and B6 controls. Strikingly, however, caspase 1/11 KO mice harbored 5 times more brain cysts than B6 controls at the onset of chronic infection, 18 days postinfection (dpi) (Fig. 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 caspase 1/11 KO mice by BLI (Fig. 1E, white asterisks, and F). Cumulatively, these data strongly suggest that caspase 1/11-mediated inflammasome responses control parasite burden and/or dissemination in the mouse.

***Toxoplasma* alone is sufficient to trigger an inflammasome response in germfree 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 caspase 1/11 KO mice are consistent with a requirement for the inflammasome in regulating the outcome of *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.

Germfree (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 (Fig. 2A). Parasite load was determined 9 dpi by BLI. Importantly, *Toxoplasma* was detected in the small intestines, mesenteric lymph nodes, and spleens of GF mice, suggesting that the parasites are able to colonize and disseminate to these organs in the absence of commensal microbiota (Fig. 2B). In addition to generating comparable levels of gamma interferon (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 (Fig. 2C). The infection status of the mice was verified by serology, and the germfree (excepting *Toxoplasma*) status of the GF mice 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 caspase 1/11 activation (Fig. 3A; also, see Fig. S1 in the supplemental material) (22). Although these mice do not harbor commensal bacteria, exposure to endotoxin and other microbial components in the mouse diet likely primes pro-IL-1β transcription (signal 1 for inflammasome activation), while IL-1β cleavage and secretion are 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 caspase 1/11- 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 medium or complete medium 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 (Fig. 3A) or LPS (Fig. 3C) was required for robust IL-1β secretion (7, 24). In mouse macrophages, gamma interferon exposure stimulates transcription of immunity-related GTPases (IRGs) that can coat the parasitophorous vacuole and lead to parasite clearance (25, 26); gamma interferon could not, however, efficiently prime IL-1β secretion in response to all three types of *Toxoplasma* (see Fig. S1 in the supplemental material). 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 (PI) compared to unin-

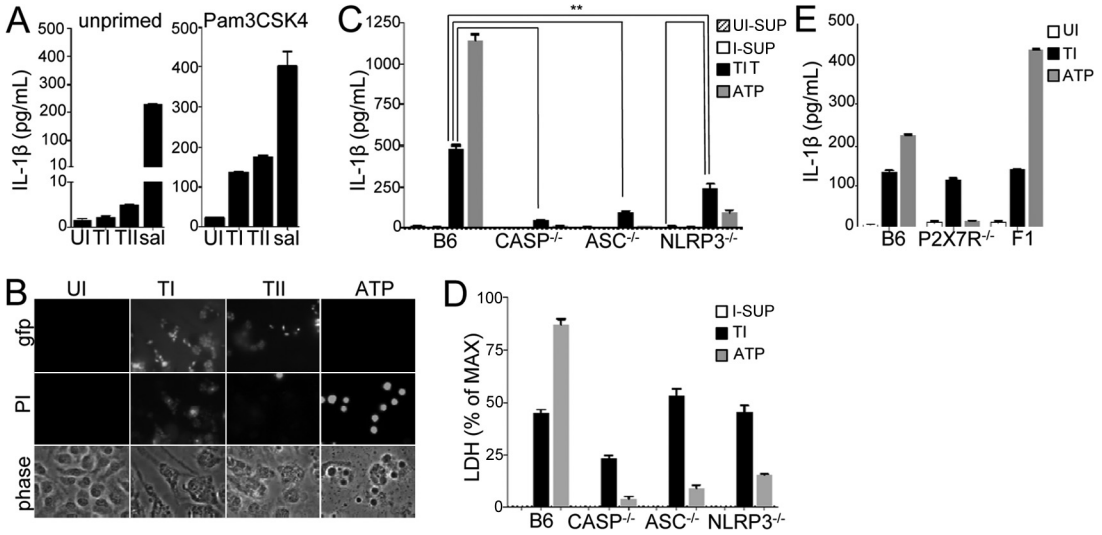

FIG 3 *Toxoplasma* elicits caspase 1/11- and ASC-dependent but NLRP3-independent IL-1β secretion from BMDMs. (A) BMDMs from B6 mice were primed overnight with 500 ng/ml Pam3CSK4 and infected with RH (type I [TI]), Pru (type II [TII]) (MOI, 3:1), or *Salmonella* (sal; MOI, 10:1) or not infected (UI). (B) At 20 h after infection with TI (RH-GFP-Luc) or TII (Me49-GFP-Luc) parasites, 6 h after treatment with 5 μM ATP, or with no infection (UI), BMDM monolayers were imaged at a magnification of ×60. GFP, propidium iodide (PI), and phase images are shown. (C and D) BMDMs from B6, ASC KO, caspase 1/11 KO ($CASP^{-/-}$), and NLRP3 KO mice were pretreated 2 h with 100 ng/ml LPS and infected with RH (TI) (MOI, 5:1) or an equivalent volume of supernatant from uninfected HFFs (UI-SUP) or the TI culture (I-SUP) for 15 h. An ATP control (5 mM) was added 3 h prior to harvesting of supernatants for ELISA (C) or LDH release assay (D). (E) BMDMs from B6.129P2-P2X7 receptor KO mice ($P2X7R^{-/-}$) or B6 or B6129PF1 (F_1) controls were treated as described for panel A with RH (TI) (MOI, 3:1), treated with 1 mM ATP, or not infected (UI). IL-1β was measured in supernatants by ELISA at 20 hpi. Data are representative of 3 to 5 individual experiments; error bars show SEM. **, $P < 0.01$ (Student's *t* test).

fected controls (Fig. 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 did not occur in the majority of B6 BMDMs following *Toxoplasma* infection. Since we consistently noted that responses to type I and type II strains of *Toxoplasma* following TLR priming were equally robust, we focused our 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 caspase 1/11 double-KO mice as well as ASC KO mice, consistent with the interpretation that the IL-1β secretion observed is inflammasome mediated (Fig. 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 caspase 1/11 and ASC independent (Fig. 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 of the low-grade membrane permeability noted in PI staining experiments (Fig. 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 breakdown of endosomal membranes, can trigger the NLRP3 inflammasome. While IL-1β was reduced in NLRP3 KO mice, a significant portion of IL-1β was NLRP3 independent compared to infected supernatant and uninfected HFF controls (Fig. 3C). Moreover, LDH release into the supernatant appeared to be entirely NLRP3 independent (Fig. 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 (Fig. 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 (Fig. 3C to 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 min before or after lethal factor treatment (see Fig. S2A and B in the supplemental material).

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 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*

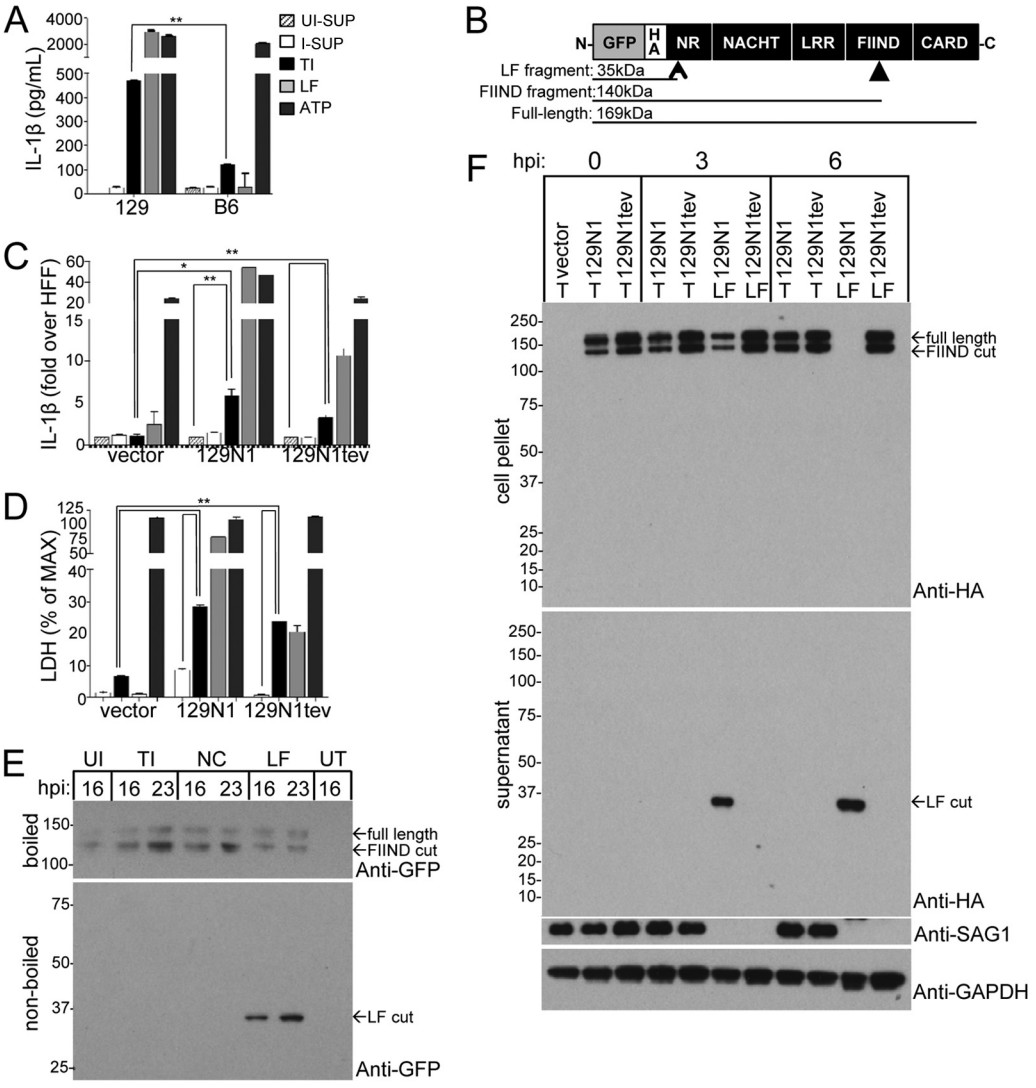

FIG 4 The 129 allele of NLRP1b enhances the inflammasome response to *Toxoplasma* independently of N-terminal proteolysis. (A) BMDMs derived from B6 or 129S1/SvImJ (129) mice were primed 2 h prior to infection with 100 ng/ml LPS, infected with RH (type I [TI]; MOI, 5:1) parasites, and treated with equivalent volumes of uninfected HFF supernatant (UI-SUP) or TI supernatant (I-SUP) controls. At 6 h prior to harvest, 1 μg/ml lethal toxin (LF) or 5 mM ATP was 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. The arrowhead indicates the lethal factor proteolysis site; the solid triangle indicates the FIIND domain autoproteolysis site. (C) B6 immortalized BMDMs (iBMDMs) transduced with $NLRP1b^{129}$ (129N1), $NLRP1b^{129}$-TEV (129N1tev), or empty vector control (vector) were infected as described for panel 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 show SEM. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). (E) 293T cells transiently transfected with GFP-HA-tagged $NLRP1b^{129}$ or untransfected (UT) were infected with RH (TI) or *Neospora caninum* (NC) (MOI, 5:1) or treated with 1 μg/ml lethal toxin (LF) or were uninfected (UI). Cell pellets were harvested 16 or 23 hpi, and NLRP1 processing was analyzed by immunoblotting with a GFP-specific antibody. Data are representative of five experiments. (F) B6 iBMDMs described for panel C were infected with RH (TI; MOI, 5:1) or treated with 1 μg/ml lethal factor (LF). Cell pellets and concentrated supernatants were harvested 0, 3, or 6 hpi and analyzed by immunoblotting with an HA-specific antibody. Antibodies to SAG1 (*Toxoplasma*) and GAPDH (mouse) were loading controls. Data are representative of 3 experiments.

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 (Fig. 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 (Fig. 4A).

To directly test the possibility that the 129 allele of *nlrp1b* ($NLRP1b^{129}$) was sufficient to drive a more robust response to the parasite, we infected B6 immortalized BMDMs (iBMDMs) that were engineered to express $NLRP1b^{129}$ tagged at the N terminus with GFP and HA (Fig. 4B). Following retroviral gene transfer, the transduced iBMDM population was approximately 30% GFP positive by fluorescence-activated cell sorting (data not shown). B6 iBMDMs expressing $NLRP1b^{129}$ generated five times more IL-1β than B6 iBMDMs transduced with the empty-vector control (Fig. 4C). Lethal factor is a zinc metalloprotease that activates NLRP1b by cleaving the sensor in the N terminus (Fig. 4B). Pre-

vious work has shown that when the primary cleavage site is converted to the consensus sequence for TEV protease (NLRP1b^{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^{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 (Fig. 4C and D). While the level of IL-1β production in response to *Toxoplasma* was slightly less in NLRP1b^{129}-TEV than NLRP1b^{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 *Toxoplasma*-sensitive and -resistant rat strains indicated that only 5 nonconserved regions are present, all within the N terminus of NLRP1, which thus could be responsible for the difference in responsiveness to *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/LPRV in the LF-sensitive, *Toxoplasma*-resistant alleles (10, 11, 30). This region is markedly different in the *Toxoplasma*-sensitive alleles of NLRP1, which have QVEQSFLG instead in the corresponding position. Cumulatively, these results are consistent with the interpretation that NLRP1b^{129} is sufficient to enhance the inflammasome response to *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 *Toxoplasma* infection. We next asked if NLRP1b^{129} is cleaved in response to the parasite. When 293T cells were transiently transfected with NLRP1b^{129}, both the 160-kDa full-length protein and the ~140-kDa fragment, the result of FIIND domain auto-proteolysis that is necessary but not sufficient for sensor activation, were detected (Fig. 4E and B) (31). As previously reported, lethal factor treatment led to accumulation of an N-terminal ~35-kDa fragment (Fig. 4E). However, NLRP1b^{129} proteolysis was not observed in response to *Toxoplasma* after 16 or 23 h of infection (Fig. 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 35-kDa N-terminal fragment of the sensor into the supernatant (Fig. 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. In 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 (Fig. 4F). While we cannot definitively rule out an undetectable level of cleavage, these data are consistent with the interpretation that NLRP1 activation in response to the *Toxoplasma* occurs via a novel, nonproteolytic 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 *nlrp1b* is not responsive to LF, it is possible that NLRP1b^{B6} is sensitive to *Toxoplasma* infection and accounts for the inflammasome response observed in 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 did 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 *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 flagellin or the type III secretion apparatus to trigger a caspase 1-dependent inflammasome through NLRC4 (32, 33). There is evidence that the non-lethal-toxin-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 *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, nonproteolytic, posttranslational 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 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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