Human Innate Immunity to *Toxoplasma gondii* Is Mediated by Host Caspase-1 and ASC and Parasite GRA15

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ABSTRACT Interleukin-1β (IL-1β) functions as a key regulator of inflammation and innate immunity. The protozoan parasite *Toxoplasma gondii* actively infects human blood monocytes and induces the production of IL-1β; however, the host and parasite factors that mediate IL-1β production during *T. gondii* infection are poorly understood. We report that *T. gondii* induces IL-1β transcript, processing/cleavage, and release from infected primary human monocytes and THP-1 cells. Treating monocytes with the caspase-1 inhibitor Ac-YVAD-CMK reduced IL-1β release, suggesting a role for the inflammasome in *T. gondii*-induced IL-1β production. This was confirmed by performing short hairpin RNA (shRNA) knockdown of caspase-1 and of the inflammasome adaptor protein ASC. IL-1β induction required active parasite invasion of monocytes, since heat-killed or mycalolide B-treated parasites did not induce IL-1β. Among the type I, II, and III strains of *T. gondii*, the type II strain induced substantially more IL-1β mRNA and protein release than did the type I and III strains. Since IL-1β transcript is known to be induced downstream of NF-κB signaling, we investigated a role for the GRA15 protein, which induces sustained NF-κB signaling in a parasite strain-specific manner. By infecting human monocytes with a *GRA15*-knockout type II strain and a type I strain stably expressing type II *GRA15*, we determined that *GRA15* is responsible for IL-1β induction during *T. gondii* infection of human monocytes. This research defines a pathway driving human innate immunity by describing a role for the classical inflammasome components caspase-1 and ASC and the parasite GRA15 protein in *T. gondii*-induced IL-1β production.

IMPORTANCE Monocytes are immune cells that protect against infection by increasing inflammation and antimicrobial activities in the body. Upon infection with the parasitic pathogen *Toxoplasma gondii*, human monocytes release interleukin-1β (IL-1β), a "master regulator" of inflammation, which amplifies immune responses. Although inflammatory responses are critical for host defense against infection, excessive inflammation can result in tissue damage and pathology. This delicate balance underscores the importance of understanding the mechanisms that regulate IL-1β during infection. We have investigated the molecular pathway by which *T. gondii* induces the synthesis and release of IL-1β in human monocytes. We found that specific proteins in the parasite and the host cell coordinate to induce IL-1β production. This research is significant because it contributes to a greater understanding of human innate immunity to infection and IL-1β regulation, thereby enhancing our potential to modulate inflammation in the body.

*T*oxoplasma gondii is an obligate intracellular parasite that has a remarkable ability to infect virtually any nucleated cell of almost any warm-blooded mammal, including humans (1). The parasite is a successful pathogen with a global distribution: at least one-third of the world population is estimated to be seropositive (2). *T. gondii* can be transmitted either horizontally, through the ingestion of tissue cysts or oocysts in contaminated food or water, or vertically, from mother to fetus, in the case of primary maternal infection during pregnancy (3). Infections are usually asymptomatic in healthy individuals. However, primary infection or reactivation of latent infection in immunocompromised individuals, such as AIDS patients or patients undergoing immunosuppressive therapy, can result in life-threatening disease (4, 5).

A strong T-cell mediated response is critical for protective immunity against *T. gondii* (6, 7). However, several studies also indicate that innate immunity, and in particular the activity of inflammatory monocytes, plays an important role in host defense against *T. gondii*. Upon recruitment to the site of infection, monocytes differentiate into macrophages and interleukin-12 (IL-12)-producing dendritic cells (8). The Gr1^{+} inflammatory subset of monocytes is recruited during the early stage of *T. gondii* infection in a low-dose nonlethal intraperitoneal infection model (9), but mice deficient in the CCR2 chemokine receptor or its ligand, monocyte chemoattractant protein 1 (MCP-1; CCL2), fail to recruit monocytes and succumb to infection (10). Gr1^{+} monocytes have also been shown to mediate mucosal immunity to *T. gondii*, as CCR2^{−/−} and MCP-1^{−/−} mice exhibit increased mortality and pathology in response to oral *T. gondii* challenge but are rescued by the adoptive transfer of Gr1^{+} monocytes (11). In humans, among human peripheral blood leukocytes, monocytes are pref-

erentially infected by *T. gondii* (12). These data suggest that monocytes play a critical role in controlling the parasite and become infected, but there is little known about the mechanisms mediating innate immunity to the parasite in human cells.

IL-1β is an inflammatory cytokine that has been described as a "master regulator" of inflammation, since it can activate downstream inflammatory genes (13). *T. gondii* has been shown to induce IL-1β in multiple human cell types, including monocytes, foreskin fibroblasts, and retinal pigment epithelial cells *in vitro* (14–17). Exogenous administration of IL-1β is protective against lethal *T. gondii* infection *in vivo* (18). Additionally, IL-1β is required for IL-12-mediated resistance to *T. gondii* in an *in vivo* SCID mouse model (19). These data suggest that *T. gondii*-induced IL-1β mediates protection against the parasite, but pathways that lead to IL-1β production in human cells during *T. gondii* infection are not well understood.

The current model for IL-1β regulation involves two signals, whereby Toll-like receptor (TLR) activation (signal one) and NF-κB signaling induce IL-1β transcription. The mRNA is subsequently translated into pro-IL-1β, and a second signal activates the inflammasome, a multiprotein complex, to proteolytically process the zymogen into mature, bioactive IL-1β, which can be released from cells (20). The classical inflammasome consists of a NOD-like receptor (NLR) sensor, the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and the protease caspase-1 (21). Myeloid cells, in particular, produce IL-1β in response to a variety of intracellular pathogens as a critical means of host defense. Recently, Witola et al. (14) found that susceptibility to human congenital toxoplasmosis mapped to alleles of the *NALP1* (*NLRP1*) gene, which encodes the sensor for the NLRP1 inflammasome, suggesting a role for the inflammasome in the innate immune response to *T. gondii*.

Collectively, this research suggests that monocytes and IL-1β contribute to immunity to *T. gondii*. In the current study, we provide important mechanistic insight into the regulation of IL-1β during *T. gondii* infection of primary human monocytes and THP-1 cells by defining both parasite and host factors involved in IL-1β synthesis, processing, and release. We show that unlike IL-1β, IL-18 mRNA levels are not induced by *T. gondii* infection. Using both pharmacological inhibitors and short hairpin RNA (shRNA) knockdown, we demonstrate that *T. gondii*-induced IL-1β production is dependent on the classical inflammasome components caspase-1 and ASC. Monocyte production of IL-1β required active parasite invasion, and among the type I, II, and III strains of *T. gondii*, only the type II strain induced substantial levels of IL-1β. Additionally, we demonstrate a role for a specific parasite factor, dense granule protein GRA15, in *T. gondii* induction of IL-1β.

RESULTS

***T. gondii* infection induces IL-1β in human monocytes.** Since monocytes are critical for controlling *T. gondii* infection, and IL-1β plays an important role in protective immunity, we investigated the regulation of the IL-1 family members IL-1β and IL-18 in response to parasite infection in human monocytes. We found that infection by type II *T. gondii* (*Prugniaud* strain) highly upregulated IL-1β transcript levels in both primary monocytes and THP-1 cells compared to mock-infected cells (Fig. 1A and B, respectively). In contrast, IL-18 transcripts were not induced upon parasite infection and even appeared to be slightly reduced in in-
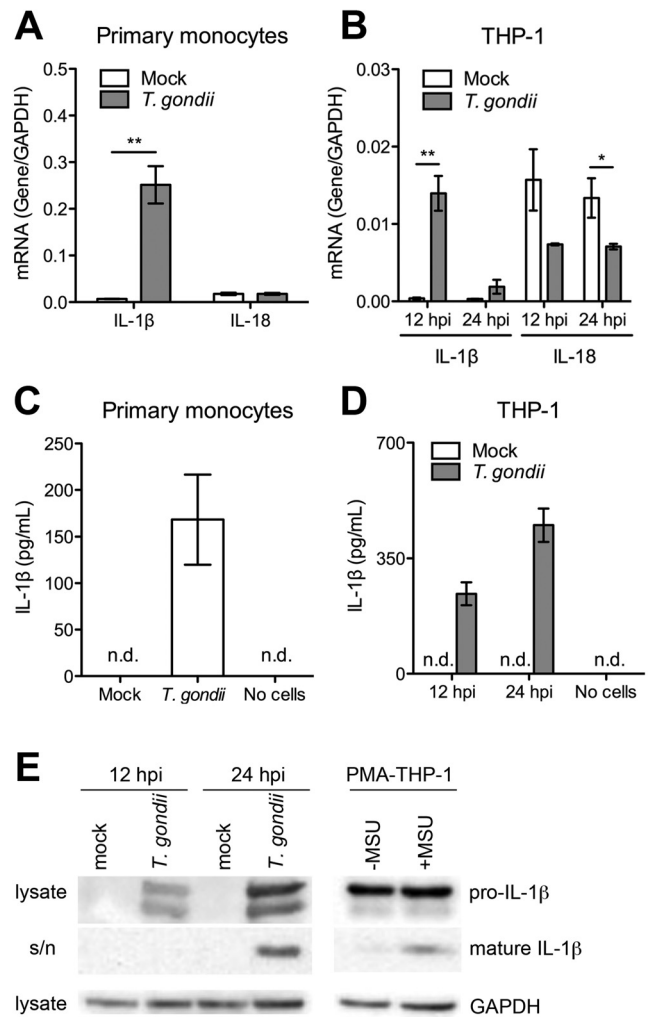

FIG 1 IL-1β and IL-18 synthesis in primary human monocytes and THP-1 cells in response to *T. gondii* infection. Primary monocytes or THP-1 cells were either mock infected or infected with GFP-expressing type II *T. gondii*. (A and C) Primary monocytes were infected for 16 h. (B, D, and E) THP-1 cells were infected for 12 or 24 h. (A and B) Quantitative real-time PCR (Q-PCR) was performed with primers specific for IL-1β or IL-18. The transcript levels relative to those of GAPDH are shown. (C and D) IL-1β released into the culture supernatant was measured by ELISA. "No cells" indicates samples in which parasites were added to wells without monocytes and were cultured in parallel as a negative control. n.d., not detected. (E) Pro-IL-1β and mature IL-1β in the cell lysate and culture supernatant (s/n) were visualized by Western blotting. As a positive control for IL-1β detection, THP-1 cells were primed with PMA and treated with MSU. For panels A to D, error bars represent the standard deviations of biological triplicates. These experiments were performed 3 (A and B), 5 (C), 8 (D), and 6 (E) times. Representative experiments are shown. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test).

fected THP-1 cells (Fig. 1A and B). These data suggest that IL-1β and IL-18 transcripts are not coordinately regulated during *T. gondii* infection.

We next examined the release of IL-1β into the culture supernatant of infected cells by enzyme-linked immunosorbent assay (ELISA). IL-1β signal was below the level of detection in mock-infected cultures, as expected. In contrast, parasite infection induced IL-1β release in primary monocytes (Fig. 1C; see also Fig. S1 in the supplemental material) and THP-1 cells (Fig. 1D),

and this induction increased over time (Fig. 1D). By examining monocytes from multiple donors, we found that monocytes from all donors produced IL-1β in response to *T. gondii*, although there was donor-to-donor variability in the magnitude of the IL-1β response (see Fig. S1). As a control to confirm that the detected IL-1β was produced by the infected monocytes and not carried over from the parasite cultures, we also added parasites to wells without monocytes (labeled as "no cells"). In all experiments, the IL-1β signal from these control samples was below the level of detection, confirming that the IL-1β detected was indeed produced by the infected monocytes (Fig. 1C and D).

Unlike many other cytokines, IL-1β lacks a classical signal sequence and does not traffic through the Golgi-endoplasmic reticulum (ER) secretion pathway. IL-1β is synthesized as a zymogen, pro-IL-1β, which is posttranslationally processed into mature IL-1β (22). To investigate the processing of pro-IL-1β in *T. gondii*-infected cells, we examined the forms of IL-1β present in the lysate and supernatant of infected monocytes by Western blotting. We detected pro-IL-1β in the cell lysate and mature IL-1β in the supernatant of parasite-infected cells (Fig. 1E). THP-1 cells primed with phorbol myristate acetate (PMA) and stimulated with monosodium urate (MSU) crystals have been shown to release mature IL-1β in a caspase-1 inflammasome-dependent manner (23), and we included this treatment as a positive control (Fig. 1E). Notably, mock-infected cultures did not harbor a pool of pro-IL-1β or mature IL-1β (Fig. 1E), suggesting that parasite-induced IL-1β protein was newly synthesized and processed upon infection.

T. gondii induces the release of IL-1β from human monocytes in an ASC-dependent manner. Since *T. gondii* infection of monocytes induced the cleavage of pro-IL-1β and the release of mature IL-1β (Fig. 1E), we next investigated the host cell pathways responsible for this posttranslational processing. The canonical pathway by which IL-1β cleavage occurs is through the inflammasome (21). We first investigated the role of ASC in *T. gondii*-mediated IL-1β processing, since it is an adaptor protein common to multiple inflammasomes (21, 24, 25). THP-1 cells were stably transduced with a lentivirus containing an shRNA targeting ASC (shASC) or a nontargeting shRNA (shNeg) as a control. By Western blotting, we confirmed >80% knockdown of ASC in the shASC cells compared to the shNeg cells (Fig. 2A). Since the parasites constitutively express green fluorescent protein (GFP), the percentage of GFP$^+$ cells in the culture can be used as a measure of infection efficiency. Infection of the shASC and shNeg cells with *T. gondii* resulted in similar infection efficiencies (Fig. 2B). When we measured the levels of IL-1β released from these cells after *T. gondii* infection, we observed a 72% reduction in the amount of IL-1β released into the supernatant of the infected shASC cells compared to that of the infected shNeg cells (Fig. 2C). We also infected THP-1 cells stably expressing an shRNA targeting ASC or expressing a mutated shASC control shRNA that were independently generated and provided by the Ting lab (26) and found that *T. gondii* also induced IL-1β release in an ASC-dependent manner in these cells (data not shown). We conducted control experiments to confirm that the ASC knockdown cells could still respond to other stimuli. Since ASC has been shown to play a role in the production of multiple cytokines in response to a variety of TLR ligands (26), we stimulated the shASC and shNeg cells with PMA and examined the upregulation of CD11c as a readout of responsiveness. CD11c was similarly upregulated on the surface of
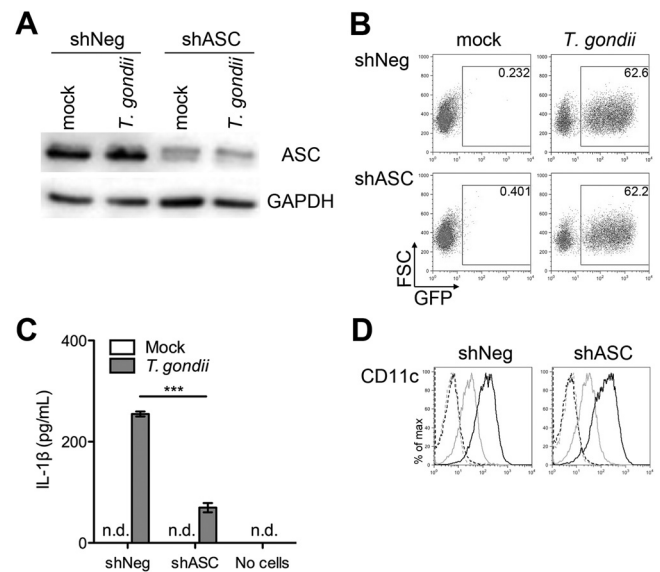

FIG 2 *T. gondii* induction of IL-1β in shASC cells. THP-1 cells stably transduced with a nontargeting shRNA control (shNeg) or an shRNA targeting ASC (shASC) were mock infected or infected with GFP-expressing type II *T. gondii*. (A) The degree of ASC knockdown was visualized by Western blotting. (B) At 24 hpi, the percentage of GFP$^+$ (infected) cells in the culture was measured by flow cytometry. (C) IL-1β released into the supernatant upon *T. gondii* infection was measured by ELISA. "No cells" indicates samples in which parasites were added to wells without monocytes and were cultured in parallel as a negative control. n.d., not detected. Error bars represent the standard deviations of biological triplicates. (D) shNeg or shASC cells were either left unstimulated (gray histograms) or were stimulated (black histograms) with 0.5 μM PMA for 24 h. The cells were stained with a control Ig (dashed histograms) or anti-CD11c antibodies (solid histograms) and examined by flow cytometry. These experiments were performed 4 (A to C) or 3 (D) times, and representative experiments are shown. Data shown in panels B and C are from the same experiment. ***, $P < 0.001$ (Student's *t* test).

the shASC and shNeg cells after PMA stimulation (Fig. 2D), indicating that the ASC knockdown cells were responsive to stimuli. Collectively, these data demonstrate that the inflammasome adaptor protein ASC mediates the release of IL-1β from *T. gondii*-infected monocytes.

T. gondii-induced IL-1β production is dependent on the protease caspase-1. We next sought to define the specific protease involved in *T. gondii*-induced IL-1β processing, and we focused on the prototypical protease, caspase-1. We detected elevated levels of mature caspase-1 in the culture supernatant of *T. gondii*-infected monocytes compared to mock-infected cells, suggesting caspase-1 activation in infected cells (Fig. 3A). To test a role for caspase-1, we first used acetyl-YVAD-chloromethylketone (Ac-YVAD-CMK), a cell-permeable tetrapeptide inhibitor that binds to the active site of caspase-1 and prevents substrate interaction (22). We confirmed by flow cytometry that the caspase-1 inhibitor did not affect the infection efficiency (Fig. 3B) or cell viability (data not shown) compared to the dimethyl sulfoxide (DMSO) vehicle control. *T. gondii*-mediated IL-1β release was significantly reduced in the presence of the caspase-1 inhibitor compared to the DMSO control, and this reduction was observed at all time points assayed (Fig. 3C).

To complement the experiments using the pharmacological inhibitor, we used a genetic approach to confirm a role for caspase-1 in regulating *T. gondii*-induced IL-1β. We stably trans-
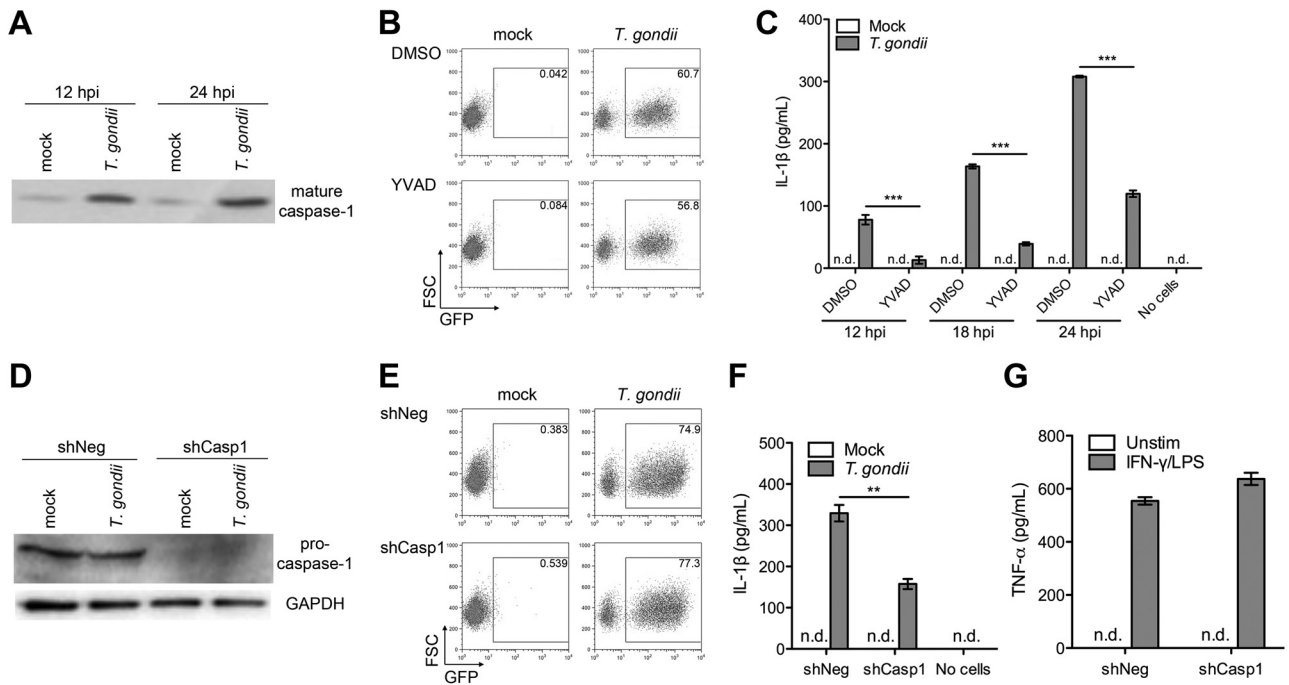
FIG 3 Role of caspase-1 in parasite-mediated IL-1β release. THP-1 cells were mock infected or infected with GFP-expressing type II *T. gondii* for 24 h. (A) Caspase-1 in the culture supernatant was visualized by Western blotting. (B and C) THP-1 cells were pretreated with either DMSO (vehicle control) or 20 to 50 μM Ac-YVAD-CMK (YVAD), a specific caspase-1 inhibitor, and infected as described above. (D to F) THP-1 cells were stably transduced with either a nontargeting shRNA control (shNeg) or an shRNA targeting caspase-1 (shCasp1) and infected as described above. (D) The degree of caspase-1 knockdown in the cell lysate was visualized by Western blotting. (B and E) At 24 hpi, infection efficiency was measured by flow cytometry. (C and F) The amount of IL-1β released into the culture supernatant was measured by ELISA. "No cells" indicates samples in which parasites were added to wells without monocytes and were cultured in parallel as a negative control. (G) shNeg or shCasp1 cells were left unstimulated or were stimulated with 100 U/ml IFN-γ and 1 μg/ml LPS for 24 h, and the amount of TNF-α released into the culture supernatant was measured by ELISA. n.d., not detected. Error bars represent the standard deviations of biological triplicates. Data are representative of 4 (A), 3 (B, C, and G), and 4 (D to F) independent experiments. Data shown in panels E and F are from the same experiment. **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test).

duced THP-1 cells with an shRNA targeting caspase-1 (shCasp1) and confirmed efficient knockdown of caspase-1 by Western blotting. As shown in Fig. 3D, caspase-1 levels in the shCasp1 cells were below the level of detection, whereas caspase-1 was expressed in the shNeg control cells. In addition, we confirmed that the infection efficiency (Fig. 3E) and cell viability (data not shown) were comparable between the shCasp1 cells and the shNeg cells. *T. gondii*-induced IL-1β protein release was decreased by 52% in the caspase-1-knockdown cells compared to the shNeg control cells (Fig. 3F). As a positive control, we measured the amount of tumor necrosis factor alpha (TNF-α) released into the culture supernatant following gamma interferon (IFN-γ) and lipopolysaccharide (LPS) stimulation of these cells and confirmed that these stimuli induced comparable levels of TNF-α in the shNeg and shCasp1 cells (Fig. 3G). These findings are consistent with the caspase-1 inhibitor data and indicate that caspase-1 plays a role in IL-1β production during *T. gondii* infection of human monocytes.

T. gondii-mediated IL-1β induction requires active parasite invasion. To better understand how *T. gondii* induces IL-1β in monocytes, we sought to determine the parasite factors required for induction. *T. gondii* infection of host cells occurs in an orchestrated cascade of events, in which the parasite first attaches to the host cell membrane, secretes parasite effectors into the host cell, and then actively invades (27). *T. gondii* can be treated with pharmacological inhibitors that impair invasion. To formally test if

IL-1β induction required parasite invasion, we cultured monocytes with parasites that were heat killed, treated with DMSO (vehicle control), or pretreated with mycalolide B. Heat-killed parasites allow us to test whether host cell sensing of the parasite is sufficient to mediate IL-1β induction. Mycalolide B irreversibly inhibits actin polymerization and allows the parasites to attach to the host cell but prevents their active invasion. The parasites were pretreated with mycalolide B and then washed before addition to host cells. We first evaluated the effectiveness of these treatments by immunofluorescence microscopy. GFP-expressing *T. gondii* were used. Samples were stained with an antibody against *T. gondii* surface antigen-1 (SAG-1; red) without permeabilization, thus allowing us to distinguish between intracellular (GFP⁺) and extracellular (GFP⁺ and SAG-1⁺) parasites. As expected, DMSO-treated parasites successfully invaded and replicated within monocytes and did not stain positively for SAG-1 (Fig. 4A, row 2). By differential interference contrast (DIC) microscopy, heat-killed parasites remained visibly extracellular (Fig. 4A, row 3). These parasites did not stain positively for SAG-1, presumably due to epitope denaturing from the heat treatment. As expected, parasites pretreated with mycalolide B were localized around the host cell and appeared to be attached but clearly remained extracellular, as indicated by positive SAG-1 staining (Fig. 4A, row 4). We also evaluated the effectiveness of these treatments on a population level by flow cytometry (Fig. 4B). When monocytes were infected with DMSO-treated parasites, approximately 60% of the
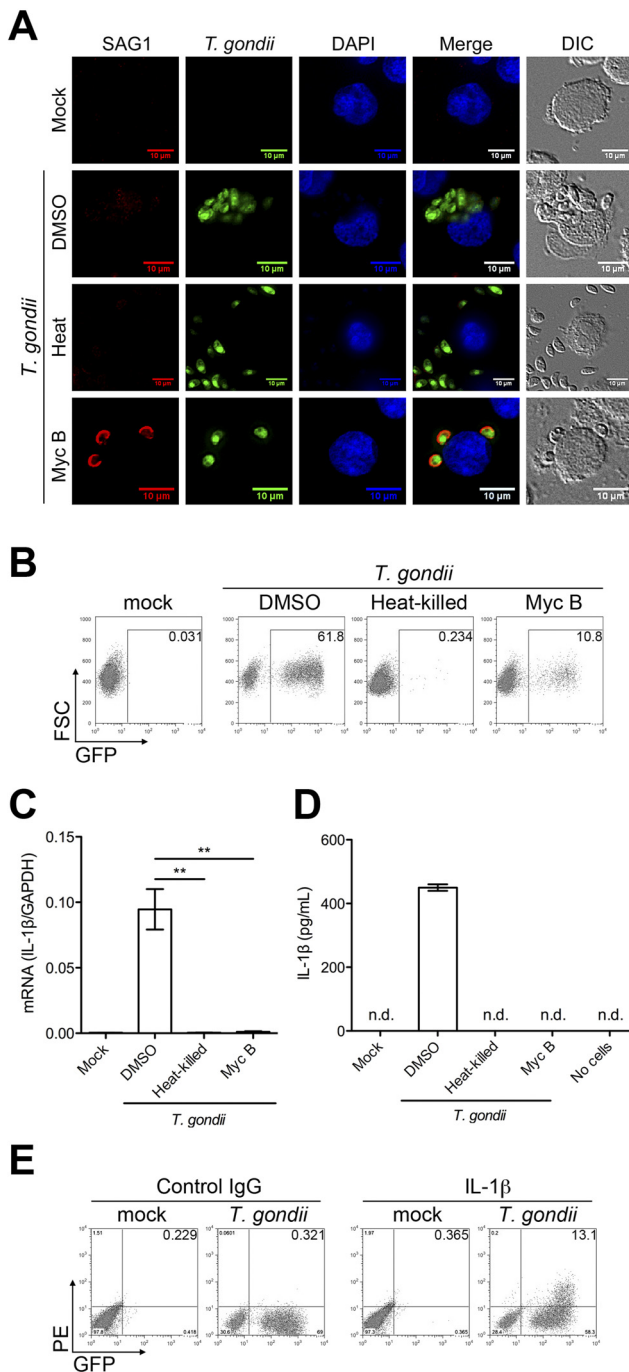
FIG 4 Effects of blocking parasite invasion on IL-1β production. (A and B) Parasites were heat killed, treated with DMSO (vehicle control), or pretreated with 3 μM mycalolide B (Myc B) and added to THP-1 cells for 24 h. Mock-infected cells were cultured in parallel. Unpermeabilized cells were fixed and stained with an antibody against *T. gondii* SAG-1 (shown in red) and examined by immunofluorescence assay (A) or analyzed by flow cytometry (B). In panel A, the scale bar represents 10 μm. (C) IL-1β mRNA levels were measured by Q-PCR, and transcript levels relative to those of GAPDH are shown. (D) The amount of IL-1β released into the culture supernatant was measured by ELISA. "No cells" indicates samples in which parasites were added to wells without monocytes and were cultured in parallel as a negative control. n.d., not detected. Error bars represent the standard deviations of biological triplicates. (E) THP-1 cells were either mock infected or infected with GFP-expressing type II *T. gondii*. At 24 hpi, the cells were fixed, permeabilized, and stained with a control Ig or an antibody against IL-1β and analyzed by flow cytometry.

(Continued)

cells were infected. As predicted, heat-killed parasites did not infect the cells. When the parasites were pretreated with mycalolide B, the percentage of GFP⁺ cells was reduced by approximately 82% compared to the DMSO control. Collectively, our data indicate that these treatments effectively impaired invasion.

Interestingly, heat-killed and mycalolide B-treated parasites did not induce any IL-1β mRNA or protein release compared to the DMSO control-treated parasites (Fig. 4C and D, respectively). These data suggest that host cell sensing of the parasite and/or parasite attachment to host cells was insufficient for IL-1β transcript induction and IL-1β release. Moreover, these data indicate that *T. gondii*-mediated IL-1β transcript induction and protein release from human monocytes required active parasite invasion.

To investigate if uninfected bystander cells in an infected cell culture were capable of producing IL-1β, we fixed and permeabilized the cells after parasite infection and performed intracellular cytokine staining for IL-1β. This method allowed us to examine IL-1β production on a single-cell basis and to gate specifically on the infected (GFP⁺) or uninfected (GFP⁻) cells. As demonstrated in Fig. 4E, intracellular IL-1β was detected only in the infected cell population and specifically in the cells harboring the greatest number of parasites (i.e., those cells with the highest GFP intensity). These data indicated that IL-1β was produced specifically in parasite-infected cells and was not produced in response to a soluble host cell factor released during infection. We cannot distinguish if the IL-1β-expressing GFPʰⁱᵍʰ population was the result of parasite replication within the cell or was due to multiple invasion events of a single cell. However, taken together, these data indicate that *T. gondii* induction of IL-1β requires active parasite invasion and that IL-1β production is linked to high parasite burden.

T. gondii induction of IL-1β in human monocytes is strain specific and mediated by GRA15. In Europe and North America, there are three dominant clonal lineages of *T. gondii* with <5% genetic divergence (28), but these strains differ dramatically in their virulence in mice (29). To investigate if *T. gondii* induces IL-1β in a parasite strain-specific manner, we infected monocytes with parasites from each of these three clonal lineages, type I, II, and III. For each experiment, we observed that the cells maintained high viability in all samples (data not shown), and the infection efficiencies were similar across all three strains (Fig. 5A). This was an important control, since we had observed that IL-1β production was linked to high intracellular parasite burden (Fig. 4E); if the monocytes were not comparably infected with the different parasite strains, any differences in IL-1β production may be due simply to differences in infection efficiency rather than to genetic differences among the strains. Infection with type II parasites substantially induced IL-1β mRNA (327-fold) and protein release compared to mock-infected cells (Fig. 5B and C, respectively). In contrast, IL-1β mRNA was increased only weakly in cells infected with type I and III parasites (5- and 1.8-fold, respectively) compared to mock-infected cells. These data indicate that *T. gondii* induced IL-1β in a strain-specific manner in human monocytes.

It has previously been demonstrated that the type II strain, but not the type I or III strains, of *T. gondii* activates sustained NF-κB

Figure Legend Continued

These experiments were performed 2 (A), 4 (B and C), 3 (D and E) times. Representative experiments are shown. **, *P* < 0.01 (Student's *t* test).
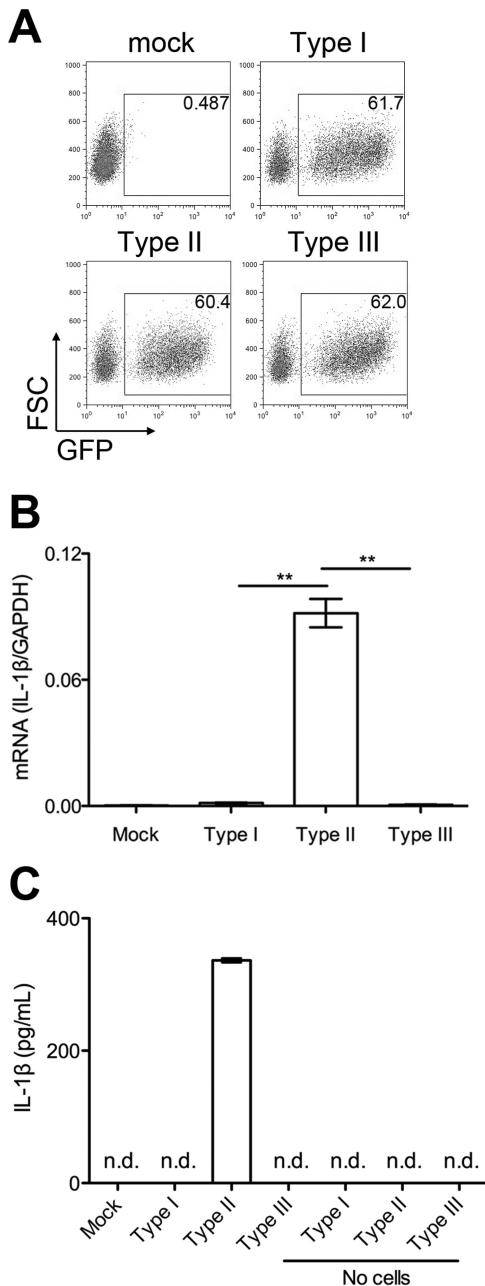
FIG 5 Strain specificity of *T. gondii*-induced IL-1β in human monocytes. THP-1 cells were infected with the indicated strain of GFP-expressing parasites (type I, II, or III) for 24 h. (A) Infection efficiency was measured by flow cytometry. (B) IL-1β mRNA levels were measured by Q-PCR and normalized to GAPDH. (C) The amount of IL-1β in the culture supernatant was measured by ELISA. "No cells" indicates samples in which parasites were added to wells without monocytes and were cultured in parallel as a negative control. Error bars represent the standard deviations of biological triplicates. These experiments were performed 7 (A and C) and 3 (B) times. A representative experiment is shown. **, $P < 0.01$ (Student's *t* test).

nuclear translocation in infected cells due to the type II allele that encodes the dense granule protein GRA15 (30). Since IL-1β transcription is known to be activated downstream of NF-κB signaling, we hypothesized that the parasite factor GRA15 may mediate IL-1β induction during monocyte infection with type II strains.

To test this possibility, we infected human monocytes with either type I, type I stably expressing a type II allele of *GRA15* (type I *GRA15*$_{II}$), type II, or type II *GRA15*-knockout (type II *GRA15*KO) parasites. We confirmed by flow cytometry that the infection efficiency and cell viability were comparable across all samples (Fig. 6A and D and data not shown). As expected, monocytes infected with type I parasites did not substantially upregulate IL-1β mRNA (4-fold over mock-infected cells) or release IL-1β (Fig. 6B and C). In contrast, type I parasites expressing a type II *GRA15* markedly induced IL-1β mRNA (159-fold over mock-infected cells) and protein release from monocytes (Fig. 6B and C), suggesting that GRA15$_{II}$ is sufficient to mediate IL-1β induction in human monocytes infected with type I *T. gondii*. Additionally, type II *GRA15*-knockout parasites were dramatically impaired in their ability to induce IL-1β mRNA (9-fold over mock-infected cells) and protein release, compared to type II parasites (568-fold over mock-infected cells) (Fig. 6E and F). Collectively, these data demonstrate that GRA15$_{II}$ is necessary and sufficient for IL-1β induction in human monocytes.

To confirm that type II *T. gondii* induces NF-κB nuclear translocation in human monocytes, THP-1 cells were mock infected or infected with type II or type II *GRA15*KO parasites, and the localization of the p65 subunit of NF-κB was examined by microscopy. Nuclear p65 was clearly detectable in monocytes infected with type II *T. gondii*, whereas p65 remained predominantly in the cytoplasm of uninfected cells or cells infected with type II *GRA15*KO (Fig. 6G). We also quantified the mean fluorescence intensity (MFI) of nuclear p65 in over 330 cells and found a significant increase in p65 signal in the nucleus of monocytes infected with type II parasites, as compared to uninfected monocytes in the same culture (2.4-fold), mock-infected cells (3.6-fold), or monocytes infected with type II *GRA15*KO parasites (4.9-fold) (Fig. 6H). These data indicate that *T. gondii* GRA15$_{II}$ mediates NF-κB nuclear translocation in human monocytes.

DISCUSSION

IL-1β is a proinflammatory cytokine that plays a critical role in host defense and innate immunity. Given its potent inflammatory activities, the production of IL-1β can also trigger immune pathology and tissue damage, reinforcing the importance of IL-1β regulation for maintaining innate immune function without excessive inflammation. The synthesis and processing of IL-1β have been extensively studied in the mouse, but there are differences in the pathways involved in IL-1β release in humans and mice (31) and among different cell types (32). The mechanisms that mediate IL-1β induction and regulation during human innate immune responses are far less understood.

IL-1β and IL-18 are related cytokines that share structural homology and are processed by caspase-1 (22, 33, 34). Although both IL-1 family members contribute to inflammation, their gene expression and synthesis are known to differ. Unlike IL-1β, which is induced upon stimulation, IL-18 mRNA is constitutively expressed in freshly isolated, unstimulated human peripheral blood mononuclear cells (PBMC) and whole blood, and preexisting IL-18 precursor protein is present in unstimulated cells and is not further elevated upon stimulation (35). In primary human monocytes and THP-1 cells, we found that *T. gondii* infection induced IL-1β but not IL-18 mRNA levels, and even appeared to reduce IL-18 transcripts, suggesting differential regulation of these cytokines during *T. gondii* infection.
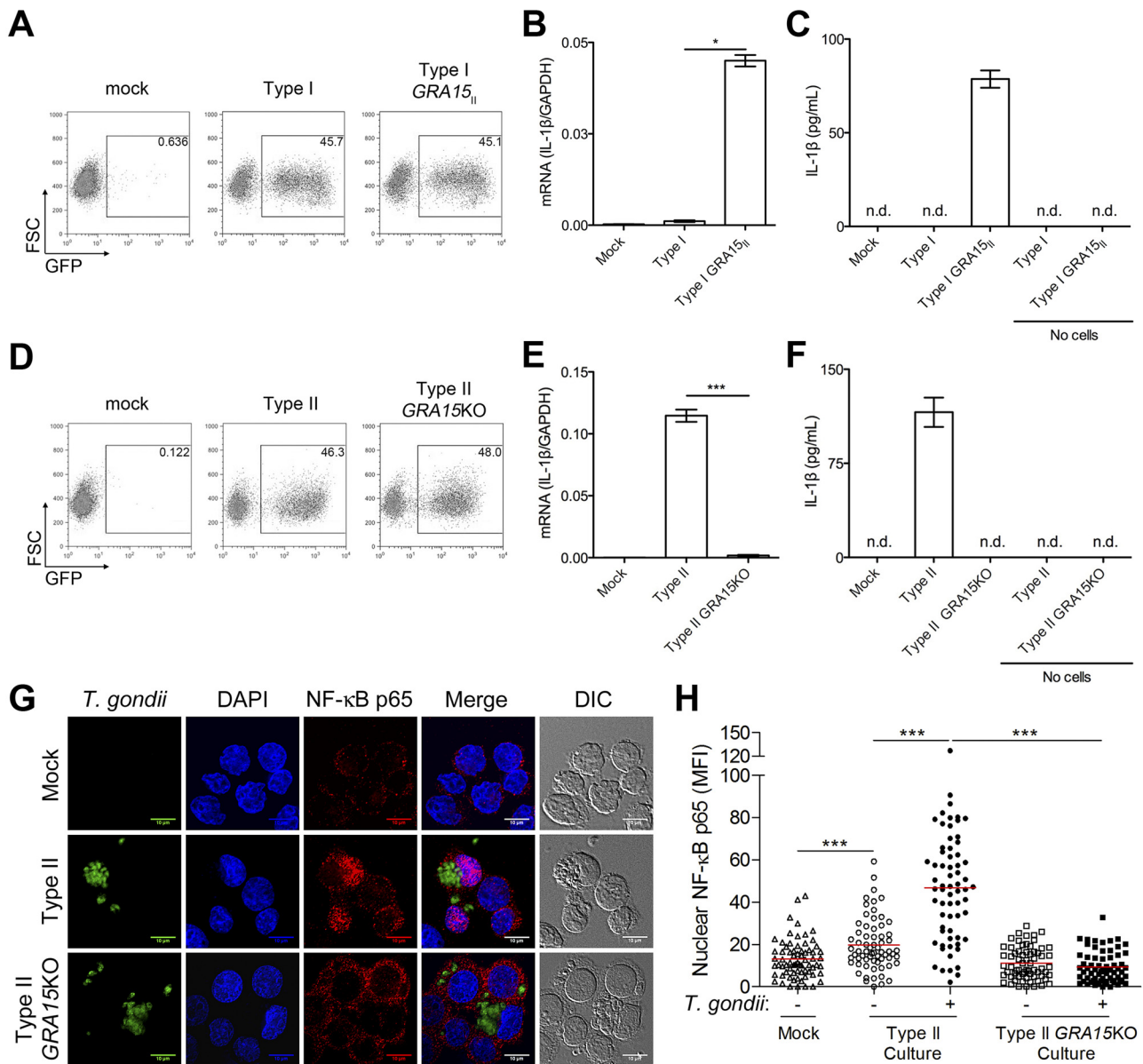
FIG 6 Role of GRA15 in *T. gondii*-mediated IL-1β induction in human monocytes. THP-1 cells were mock-infected or infected with the indicated strain of GFP-expressing parasites and examined at 24 hpi. (A and D) The infection efficiency was measured by flow cytometry. (B and E) IL-1β mRNA levels were measured by Q-PCR and normalized to GAPDH. (C and F) The amount of IL-1β in the culture supernatant was measured by ELISA. "No cells" indicates samples in which parasites were added to wells without monocytes and were cultured in parallel as a negative control. n.d., not detected. (G) Cells were fixed, permeabilized, and stained with an antibody against the p65 subunit of NF-κB and examined by immunofluorescence assay. (H) The mean fluorescence intensity (MFI) of nuclear p65 was quantified for 70 mock-infected cells, 132 cells from type II-infected cultures, and 132 cells from type II *GRA15*KO-infected cultures. Red bars indicate the average MFI. Error bars represent the standard deviations of biological triplicates. These experiments were performed 7 (A and C), 3 (B and E), and 4 (D and F) times and 1 (G and H) time. Representative experiments are shown. *, $P < 0.05$; ***, $P < 0.001$ (Student's *t* test).

Inflammasomes are multiprotein complexes that mediate cytosolic immune surveillance and activate caspase-1 to proteolytically process IL-1β and IL-18 (36). Interestingly, genetic mapping studies have implicated an inflammasome sensor in the control of *T. gondii* in humans. Witola et al. demonstrated that susceptibility alleles for human congenital toxoplasmosis mapped to the *NALP1* (*NLRP1*) gene and that NALP1 contributed to the control of parasite growth in human monocytes (14). Our study extends these findings and provides the first evidence that the inflammasome components ASC and caspase-1 are involved in the innate immune response to *T. gondii* in human monocytes by regulating the release of IL-1β. Interestingly, even though caspase-1 was undetectable in shCasp1 cells, we observed a 52% reduction in IL-1β release after *T. gondii* infection. It is possible that the residual caspase-1 expressed in the knockdown cells is sufficient to mediate pro-IL-1β processing. Alternatively, another enzyme may be involved. In humans, caspase-1, caspase-4, and caspase-5 comprise the group I inflammatory caspases (37). The NALP1-ASC complex has been shown to activate both caspase-1 and caspase-5 in human THP-1 cells, and optimal pro-IL-1β cleavage required

both caspase-1 and caspase-5 (21). Interestingly, caspase-1-deficient (IL-1β-converting enzyme [ICE]$^{-/-}$) mice control infection with a nonlethal dose of type II *T. gondii* (PTGluc strain) comparably to wild-type mice (38). The original caspase-1-knockout mice (39) are now known to also be deficient in caspase-11 (40). It would be interesting to know if there are independent functions for caspase-1 and caspase-11 or a role for the inflammasome in host defense against *T. gondii* infection in mice.

In delineating the parasite factors involved in IL-1β induction in human monocytes, we found that active parasite invasion was necessary for upregulating IL-1β mRNA. These data suggested that extracellular pattern recognition receptor (PRR) sensing of *T. gondii* was insufficient to induce IL-1β and that either intracellular sensing of the parasite or the activity of parasite effector molecules was required. The strain-specific difference in IL-1β induction led us to investigate the dense granule protein GRA15, which activates NF-κB nuclear translocation, since transcription of IL-1β is known to be downstream of NF-κB signaling. Moreover, the effects of GRA15 on NF-κB are independent of MyD88 and TRIF (30), the adaptor proteins that mediate signaling from TLR. GRA15 is secreted into host cells during invasion and is believed to be continuously released from intracellular parasites, contributing to increased nuclear translocation of the NF-κB p65 subunit over time (30). Our finding that mycalolide B-treated parasites do not induce IL-1β transcripts or protein release indicates that these treated parasites may not secrete sufficient GRA15 into the host cells to mediate NF-κB nuclear translocation and IL-1β transcription. This is also consistent with our data showing that intracellular IL-1β is predominantly detected in monocytes with a high parasite burden, which may be the result of parasite replication or of multiple invasion events of the same cell.

At this point, it remains to be determined if *T. gondii* induces IL-1β solely at the level of its transcription, or if the parasite activates multiple steps in the synthesis and processing of IL-1β. Unlike macrophages, human blood monocytes have constitutively active caspase-1 and can release mature IL-1β in response to TLR signals alone without the need for a second signal to activate the inflammasome (32). It is possible that GRA15$_{II}$ activation of IL-1β transcription is sufficient to drive monocyte production of IL-1β. Alternatively, GRA15$_{II}$ may mediate both the induction of IL-1β mRNA via NF-κB and also IL-1β processing by activating the inflammasome through an as-yet-uncharacterized mechanism. A third possibility is that GRA15$_{II}$ induces IL-1β transcription and that another aspect of *T. gondii* infection serves as the "second signal" to activate the inflammasome. This second signal may be a different parasite effector protein, since it is now appreciated that a large number of parasite-secreted proteins gain access to the host cell cytosol (41) and may serve as potential ligands for inflammasome sensors. The second signal could also be provided as an indirect effect of infection, for instance through the release of "danger signals" such as ATP from dying cells. Although we have confirmed high monocyte viability at the end of each infection experiment, it is possible that ATP or other factors are released from the small percentage of dying cells. ATP translocation into cells via the P2X$_7$ receptor (P2X$_7$R) has been shown to activate the inflammasome, leading to IL-1β processing (42), and several studies have indicated a role for the P2X$_7$R in host defense against *T. gondii* (43–45). Each of these potential models reinforces the complex interplay between *T. gondii* and the infected host cell. Moreover, the delicate balance between IL-1β-mediated host pro-

tection and inflammation underscores the value of understanding the pathways that regulate IL-1β production. This study defines both host and parasite factors involved in the regulation of IL-1β in response to *T. gondii* and sheds light on an important mechanism of innate immunity in human cells.

MATERIALS AND METHODS

Host cell and parasite cultures. Human foreskin fibroblasts (HFF) and 293T cells (a gift from David Fruman, University of California, Irvine) were cultured in D-10% medium: Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. HTR-8/SVneo trophoblasts were a gift from Dongbao Chen, University of California, Irvine. THP-1 cells stably expressing an shRNA against ASC (shASC) and a mutant shASC construct were generously provided by Jenny Ting (University of North Carolina, Chapel Hill) and previously published in reference 26. All THP-1 cells were cultured in R-10% medium: RPMI 1640 (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. Where indicated, THP-1 cells were primed with 0.5 μM PMA (Sigma-Aldrich, St. Louis, MO) for 3 h, washed, plated overnight, and stimulated with 100 μg/ml MSU (InvivoGen, San Diego, CA) for 6 h to activate the NLRP3—caspase-1 inflammasome (23). All blood samples were collected at the University of California, Irvine, Institute for Clinical and Translational Science in accordance with guidelines and approval of the University of California, Irvine, Institutional Review Board. Human monocytes were isolated from peripheral blood mononuclear cells (PBMC) using counterflow elutriation, as previously described (46). Freshly isolated cells were resuspended in R-10% medium and used immediately after isolation in infection experiments.

Tachyzoites of type I (RHgfpluc [47]), type II (Prugniaud A7 [48]), type III (cΔLuc123 [49]), transgenic type I *GRA15*$_{II}$ (30), and type II *GRA15*KO (30) *T. gondii* were used for infections. All parasite strains constitutively express green fluorescent protein (GFP) and were maintained as previously described (49). Transgenic type I *GRA15*$_{II}$ and type II *GRA15*KO *T. gondii* tachyzoites were generously provided by Jeroen Saeij (Massachusetts Institute of Technology).

All parasite and mammalian cell cultures were maintained at 37°C in 5% CO$_2$ incubators. All parasite and cell lines were tested monthly for mycoplasma contamination and were confirmed to be negative.

Infections. *T. gondii* tachyzoites were passaged once through the HTR-8/SVneo human trophoblast line prior to infection of human monocytes. This was done because *T. gondii* cultures maintained in HFF produce IL-1β, but those maintained in HTR-8/SVneo cells do not (data not shown). Passaging the parasites through the HTR-8/SVneo cells reduced the likelihood of any potential IL-1β carryover from the HFF cultures into the monocyte infection experiments. Infected HTR-8/SVneo monolayers were syringe lysed, and *T. gondii* tachyzoites were washed with D-3% medium: DMEM supplemented with 3% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. Syringe-lysed parasite cultures were further purified using a PD-10 desalting column (GE Healthcare, Waukesha, WI), washed again with D-3% medium, and resuspended in R-10% medium for use in infections. This isolation process resulted in pure parasite cultures without host cell debris. Monocytes were infected at a multiplicity of infection (MOI) of 1 to 2 in biological triplicates. Samples were harvested at 24 h postinfection (hpi) unless otherwise indicated.

Inhibitors. The caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK; Cayman Chemical, Ann Arbor, MI) was resuspended in dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Waltham, MA). Monocytes were pretreated with the inhibitor at 20 to 50 μM or with an equivalent volume of DMSO as a vehicle control for 30 min and infected, as described above. The inhibitor did not alter the viability of the cells, as indicated by propidium iodide (PI) exclusion.

T. gondii tachyzoites were prepared as described above and differentially treated prior to use in infections. Parasites were heat killed by boiling at 100°C for 15 min. Mycalolide B (Myc B; Enzo Life Sciences, Farmingdale, NY), an irreversible actin polymerization inhibitor (50), was resuspended in DMSO. Parasites were pretreated with 3 to 5 μM of mycalolide B for 10 min at room temperature (RT) and then washed twice. As a control, parasites were treated with an equivalent volume of DMSO for 10 min at RT. These treated parasites were then added to the monocytes.

Q-PCR. Total RNA was extracted using the RNeasy minikit (Qiagen, Germantown, MD) and treated with DNase I (Life Technologies, Carlsbad, CA) to reduce genomic DNA contamination. cDNA was synthesized using the Superscript III First-Strand synthesis kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, and quantification of cytokine gene expression was performed using an iCycler PCR system (Bio-Rad, Hercules, CA) and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The threshold cycle ($\Delta\Delta C_T$) method was used for data analysis (51), and the data shown are the transcript expression levels relative to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. Primers used were either previously published sequences, IL-1β (52) and GAPDH (53), or self-designed, IL-18: GGCTGCTGAACCAGTAGAAGACAAT (sense) and AGGCCGATT TCCTTGGTCAATGAAG (antisense). All primer pairs spanned intron-exon boundaries. All primers were commercially synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA). As negative controls, water or cDNA synthesized in the absence of reverse transcriptase were used in place of DNA template. No amplification of product was observed in reactions with these negative controls. All reactions were performed in triplicate, and error bars reflect the standard deviation of these triplicates.

Preparation of cell lysates, Western blotting, and ELISA. Whole-cell lysates were generated by the addition of 2× Laemmli buffer containing 10% β-mercaptoethanol to cell pellets. For experiments in which supernatant was concentrated and analyzed by Western blotting, serum-free medium was used. Supernatant was concentrated using Amicon Ultra Centrifugal filters (EMD Millipore, Billerica, MA) according to the manufacturer's instructions and diluted with 2× Laemmli buffer containing 10% β-mercaptoethanol. Cell lysates and concentrated supernatant were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Membranes were blotted with antibodies against IL-1β (clone 3ZD from the National Cancer Institute Biological Resources Branch), GAPDH (ZG003; Life Technologies, Carlsbad, CA), caspase-1 (catalog no. 2225; Cell Signaling Technology, Danvers, MA), or ASC (AL177; Enzo Life Sciences, Farmingdale, NY). Primary antibodies were followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were developed with Amersham ECL Prime (GE Healthcare, Waukesha, WI) and detected using a Nikon camera as previously described (54). Released IL-1β and TNF-α protein levels were measured using the Ready-Set-Go ELISA kit (eBioscience, San Diego, CA), according to the manufacturer's instructions. In samples indicated as "not detected," the IL-1β signal was below the threshold of detection.

Flow cytometry. In every infection experiment, the cells were analyzed by flow cytometry for viability and infection efficiency. At the harvest time point, the cells were collected, resuspended in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] with 2% FBS), and stained with PI (eBioscience, San Diego, CA). Data were immediately acquired on a FACSCalibur flow cytometer with Cell-Quest software (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR). Samples were gated on PI⁻ cells, followed by forward and side scatter (FSC and SSC, respectively), and then on GFP⁺ (infected) cells.

For cell surface marker staining, cells were resuspended in FACS buffer containing human Fc receptor binding inhibitor (eBioscience, San Diego, CA) and incubated on ice for 10 min. The cells were then stained with control IgG1 conjugated to allophycocyanin (APC; TG5/CCR2) or anti-

CD11c conjugated to APC (3.9), incubated on ice for 30 min, and washed. For intracellular cytokine staining, cells were fixed with 4% paraformaldehyde (PFA), blocked with human Fc receptor binding inhibitor as described above, permeabilized with 0.1% Triton-X, and stained with a mouse IgG2a κ isotype control (eBioscience, San Diego, CA) or an anti-IL-1β antibody (CRM56; eBioscience, San Diego, CA) conjugated to phycoerythrin (PE). Data were acquired and analyzed with software described above. Antibodies were purchased from BioLegend (San Diego, CA), except where indicated.

Immunofluorescence microscopy. Coverslips were coated with 20 μg/ml fibronectin for 1 h. Cells were infected as described above and added to fibronectin-coated coverslips for the last hour of culture. At 24 hpi, cells were fixed with 4% PFA and blocked with 5% normal goat serum and 1% bovine serum albumin for 1 h at RT to prevent nonspecific binding. For *T. gondii* SAG-1 staining, cells were stained with a rabbit polyclonal antibody against *T. gondii* surface antigen-1 (55) without permeabilization. For examining NF-κB nuclear translocation, cells were permeabilized with 0.5% Triton-X for 20 min at RT and then stained with a rabbit monoclonal antibody against the p65 subunit of NF-κB (C22B4; Cell Signaling Technology, Danvers, MA). The primary antibodies were followed by an Alexa Fluor 594-conjugated secondary antibody (Life Technologies, Carlsbad, CA). Coverslips were mounted onto glass slides using Vectashield with DAPI (4′,6-diamidino-2-phenylindole; Vector Labs, Burlingame, CA). Images were acquired using a Nikon Eclipse Ti inverted microscope with a 40× or 60× objective and NIS-Elements acquisition software (Nikon Instruments, Melville, NY). MFI from p65 signal in the THP-1 nuclei minus background MFI was calculated for 334 cells chosen at random using ImageJ software.

shRNA plasmid construction and lentiviral transduction. shRNA knockdown was performed using lentiviral transduction. A nontargeting control hairpin cloned into the pLKO.1 puro backbone (shNeg, plasmid 17920 deposited by Sheila Stewart [56]) and the psPAX2 viral packaging plasmid (plasmid 12260 deposited by Didier Trono) were purchased from Addgene (Cambridge, MA). pCMV-VSVg viral envelope plasmid was generously provided by Jessica Hamerman. shRNA target sequences have previously been published and are as follows: shASC, GCTCTTCAGTTT CACACCA (26); shCasp1, GTGAAGAGATCCTTCTGTA (57). shASC and shCasp1 were cloned into the pLKO.1 puro backbone. 293T cells were cotransfected with the shRNA, psPAX2, and pCMV-VSVg plasmids using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Lentiviral supernatants were harvested on days 2 and 3 posttransfection. THP-1 cells were infected with lentivirus by centrifugation at 2,500 rpm for 2 h at 25°C in the presence of 8 μg/ml hexadimethrine bromide (Sigma-Aldrich, St. Louis, MO). Thirty hours posttransduction, target cells were selected with 2 μg/ml puromycin (Thermo Fisher Scientific, Waltham, MA). Western blotting was performed after 5 days in puromycin selection to confirm knockdown of the targeted protein. In addition, knockdown was confirmed in each experiment performed with the transduced cells. Where indicated, shRNA-expressing THP-1 cells were stimulated with 100 U/ml IFN-γ and 1 μg/ml LPS or 0.5 μM PMA for 24 h as a positive control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00255-13/-/DCSupplemental.

Figure S1, TIF file, 0.4 MB.

ACKNOWLEDGMENTS

scher, Tenner, and Nelson labs, and in particular Marie Benoit, for helpful discussion on this project. We also thank Dongbao Chen, David Fruman, Jenny Ting, and Jeroen Saeij for generously sharing cell and parasite lines and Elizabeth Clarke for primary monocyte isolation.

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