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NLRP3 and potassium efflux drive rapid IL-1β release from primary human monocytes during *Toxoplasma gondii* infection

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Abstract

IL-1β is produced by myeloid cells and acts as a critical mediator of host defense during infection and injury. We found that the intracellular protozoan parasite *Toxoplasma gondii* induced an early IL-1β response (within 4 hr) in primary human peripheral blood monocytes isolated from healthy donors. This process involved up-regulation of *IL-1β*, *IL-1RN* (IL-1 receptor antagonist), and *NLRP3* transcripts, *de novo* protein synthesis, and the release of both pro- and mature-IL-1β from infected primary monocytes. The released pro-IL-1β was cleavable to mature, bioactive IL-1β in the extracellular space by the protease caspase-1. Treatment of primary monocytes with the NLRP3 inhibitor MCC950 or with extracellular potassium significantly reduced IL-1β cleavage and release in response to *T. gondii* infection, without affecting the release of TNF-α, and indicated a role for the inflammasome sensor NLRP3 and for potassium efflux in *T. gondii*-induced IL-1β production. Interestingly, *T. gondii* infection did not induce an IL-1β response in primary human macrophages derived from the same blood donors as the monocytes. Consistent with this finding, *NLRP3* was down-regulated during the differentiation of monocytes to macrophages and was not induced in macrophages during *T. gondii* infection. To our knowledge, these findings are the first to identify NLRP3 as an inflammasome sensor for *T. gondii* in primary human peripheral blood cells and to define an upstream regulator of its activation through the release of intracellular potassium.

Introduction

Toxoplasma gondii is an obligate intracellular food-borne parasite that infects approximately one third of the global population (1). Although *T. gondii* typically causes an asymptomatic infection in healthy individuals, infection in immune compromised individuals can cause severe or life-threatening disease, highlighting the importance of active immune surveillance in host defense against *T. gondii*.

IL-1β is a cytokine that is produced predominantly by cells of the myeloid lineage and is a major driver of inflammation during infection and injury. Evidence indicates that IL-1β plays a protective role in host defense against *T. gondii* both *in vivo* and in *in vitro* infection

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models. Mice that are administered recombinant IL-1β during infection are significantly protected from a lethal dose of C57 strain *T. gondii* (2). IL-1β is also required for IL-12-mediated production of protective IFN-γ in a SCID mouse model of *T. gondii* infection (3). In addition, treatment of infected rat retinal vascular endothelial cells with recombinant IL-1β reduces *T. gondii* replication, via a mechanism that is independent of nitric oxide production (4).

The most well studied pathway for IL-1β production involves a two-signal model, which is the predominant mode of activation in mouse macrophages. In this model, danger- or pathogen-associated molecular patterns (DAMPs or PAMPs) prime the cell through Toll-like receptors (TLRs) and NF-κB signaling, leading to the transcriptional activation of the *IL-1β* gene. IL-1β is then synthesized as a zymogen, pro-IL-1β, which is proteolytically processed by a multi-protein complex in the cell called the inflammasome (5). The inflammasome is activated by a sensor protein from the NOD-like receptor (NLR) or AIM2-like receptor family (6). The sensors are pattern recognition receptors, and the stimuli for many of these receptors are microbial ligands. Interestingly, NLRP3, the most well characterized inflammasome sensor, is activated by a variety of stimuli, including extracellular ATP, microbial toxins such as nigericin, and crystalline particles (7). Although the precise mechanisms of NLRP3 activation by these diverse agonists remain undefined, recent evidence indicates that they converge on potassium efflux (8).

Activation of the canonical inflammasome triggers assembly of the sensor, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and the protease caspase-1. Autoproteolysis of pro-caspase-1 through a proximity-induced model results in cleavage of pro-IL-1β (35 kD) into mature, bioactive IL-1β (17 kD) (9). In a parallel pathway, caspase-11 in mice or caspase-4 and -5 in humans are activated by bacterial ligands through a noncanonical inflammasome to process IL-1β and IL-1α (10). The direct detection of cytosolic LPS by caspases-11, -4, and -5 induces pyroptosis (11) and IL-1β release through a pathway involving cleavage of the recently identified gasdermin D protein (12, 13).

Several studies have investigated the pathways that regulate *T. gondii* induction of IL-1β in rodent models. This work has demonstrated that NLRP1 is a sensor for *T. gondii* in mice (14, 15) and in rats (16, 17), and NLRP3 also mediates IL-1β production in mice (15). In addition, IL-1 receptor and IL-18 receptor knock-out mice are acutely susceptible to *T. gondii* infection (15), underscoring the protective role of IL-1β in host defense against *T. gondii*. Collectively these studies indicate inflammasome activation in the murine IL-1β response to *T. gondii*, although the molecular trigger or parasite ligand that initiates inflammasome assembly remains unknown.

T. gondii induction of IL-1β in humans has primarily been studied using human monocytic cells. Monocytes are peripheral blood leukocytes that patrol the vasculature and migrate rapidly to sites of infection or injury (18). They play a critical role in host defense against a variety of pathogens by functioning as effector cells and by differentiating into protective macrophages and dendritic cells (DC) (19). Monocytes are among the first immune cells recruited to the site of *T. gondii* infection and are important mediators of innate immunity

against the parasite (20, 21). During *T. gondii* infection, the production of IL-1β by the MonoMac human monocytic cell line limits parasite replication, and knock-down of the inflammasome sensor NLRP1 (NALP1) in these cells reduces IL-1β production and restores parasite growth (22). In the human monocytic cell line THP-1, IL-1β mRNA is induced by the *T. gondii*-secreted dense granule protein 15 (GRA15) through NF-κB signaling, and IL-1β release from these cells is mediated by the inflammasome components ASC and caspase-1 (23). These findings indicate inflammasome activation in monocytic cell lines during *T. gondii* infection; however, the regulation of the IL-1β response in primary human monocytes has not been defined. We report here that *T. gondii* infection of primary human peripheral blood monocytes results in the induction of IL-1β mRNA, protein synthesis, cleavage, and release within four hours. In addition, we demonstrate that this rapid response is specific to primary monocytes, is not observed in human macrophages from the same donor or in monocyte cell lines, and that the inflammasome sensor NLRP3 is differentially induced in monocytes and macrophages during *T. gondii* infection. Finally, we define a role for NLRP3 and for potassium efflux in *T. gondii*-induced IL-1β cleavage and release. These data are the first to identify NLRP3 as an inflammasome sensor for *T. gondii* in primary human cells and to define a mechanism for its activation through the release of intracellular potassium.

Materials and Methods

Primary cell isolation and culture

Human whole blood was collected by the Institute for Clinical and Translational Science at the University of California from healthy adult donors who provided written informed consent. Blood was collected according to the guidelines of and with approval from the University of California, Irvine, Institutional Review Board. PBMC were isolated from whole blood by density centrifugation using lymphocyte separation media (MP Biomedicals, Santa Ana, CA). Monocytes were enriched from peripheral blood mononuclear cells by counterflow elutriation, as previously described (24) and stained for purity after every isolation. This protocol typically resulted in >94% pure monocyte cultures (the range was from 85–99%) based on CD11b$^+$ and CD3$^-$CD20$^-$CD56$^-$ staining. Freshly isolated monocytes were resuspended in RPMI 1640 (Hyclone, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, without (R-0% medium) or with 10% (R-10% medium) heat-inactivated fetal bovine serum (FBS; Omega Scientific, Tarzana, CA). Monocytes were used immediately after isolation for experiments or differentiated into macrophages with 25 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF; Gemini Bio-Products, West Sacramento, CA) for 6 days, with addition of fresh media and M-CSF at day 3. Human monocyte-derived macrophages were re-plated on day 6, incubated overnight, and utilized for experiments on day 7 of culture.

Host cell and parasite culture

Human foreskin fibroblasts (HFF; ATCC, Manassas, VA) were cultured in D-10% medium: Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. THP-1 and U937 monocytic cell lines were cultured in R-10% medium.

T. gondii tachyzoites were maintained by serial passage in confluent monolayers of HFF. Type II (*Prugniaud*, (25)) and type II *GRA15*KO (26) parasites constitutively expressing green fluorescent protein (GFP) were used.

Cell and parasite lines were cultured at 37°C in 5% CO_2 incubators. All cultures were tested bimonthly and confirmed to be free of mycoplasma contamination.

Cell Infection, Stimulation, and Harvest

T. gondii-infected HFF monolayers were washed with D-3% medium: DMEM supplemented with 3% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. HFF monolayers were then scraped and syringe-lysed. Lysed tachyzoite cultures were washed with D-3%, passed through a 5.0 µm filter (EMD Millipore, Billerica, MA), and washed again. This resulted in parasite cultures that were free of host cell debris and soluble factors. Purified *T. gondii* tachyzoites were immediately added to host cells at a multiplicity of infection (MOI) of 1 to 3 in biological triplicates. All infections were performed with the type II *Prugniaud* strain of *T. gondii* unless otherwise specified. "Mock" infection was the addition of the equivalent volume of media (without parasites) to cells.

Cells were stimulated with 100 ng/ml ultrapure lipopolysaccharide (LPS; LIST Biological Laboratories, Campbell, CA) for 4 or 16 hours (hr), depending on the experiment, 100 µg/ml monosodium urate crystals (MSU; Invivogen, San Diego, CA) for 4 hr, and 5 mM of adenosine triphosphate (ATP; Sigma-Aldrich, St. Louis, MO) for the last 30 min of culture, as indicated.

At the indicated time point, monocytes or macrophages were pelleted by centrifugation at 5,000 to 10,000 rpm for 5 to 10 min. Collected cells were stained, fixed, or lysed accordingly, as described below. For cell surface expression analysis, adherent macrophages were harvested by incubating with non-enzymatic Cellstripper (Mediatech, Manassas, VA) for 5 to 10 min, pelleted by centrifugation, and stained as described below.

Inhibitors

Cycloheximide (CHX; Chem Service, West Chester, PA), a protein translation inhibitor, and MCC950 (Adipogen, San Diego, CA), a small molecule that selectively inhibits NLRP3, were resuspended in deionized water. Monocytes were pre-treated with twice the final concentration of CHX or MCC950, or with the equivalent volume of deionized water, for 30 min to one hour and then infected or stimulated as described above at a final concentration of 5 µg/ml CHX or 5 µM MCC950. To block potassium efflux, potassium chloride (Fisher Scientific, Waltham, MA) was added to the cultures at 15 min post-infection or post-stimulation at a final concentration of 50 mM.

Flow cytometry

For cell surface staining assays, cells were blocked with Human TruStain FcX (BioLegend, San Diego, CA) on ice for 10 min and then stained with control Ig or the following anti-human antibodies (all from BioLegend, unless otherwise indicated): α-CD56-APC

(HCD56), α-CD11b-PE or -APC (ICRF44), α-CD14-FITC (M5E2) or -PE/Cy7 (HCD14), α-CD16-PE/Cy7 (3G8), α-CD3-PE (UCHT1), α-CD20-PE/Cy7 (2H7), α-HLA-DR,P,Q-FITC (Tu39; BD Biosciences, San Jose, CA), α-CD40-PE (5C3), or α-CD86-biotin (IT2.2). Cells were stained with primary antibodies on ice for 30 min and then washed with FACS buffer (PBS with 2% FBS) and subsequently incubated with streptavidin-FITC secondary antibody as needed. After the final wash, cells were fixed with 2 to 4% paraformaldehyde (PFA).

For intracellular cytokine staining, cells were fixed with 2 to 4% PFA, blocked with Human TruStain FcX as described above, permeabilized with 0.1% Triton-X for 10 min, stained with control Ig-PE or anti-IL-1β-PE (CRM56, eBioscience, San Diego, CA) antibodies for 30 min, and then washed with FACS buffer.

To measure infection efficiency, cells were fixed as described above at the harvest time point.

Samples were analyzed by flow cytometry on a FACS Calibur flow cytometer using Cell Quest software (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo software (Treestar, Ashland, OR) and cells were first identified based on their forward and side scatter profile and subsequently analyzed for cell surface marker expression, intracellular cytokine expression, or GFP signal.

Quantitative real-time PCR (Q-PCR)

At the harvest time point, total RNA was harvested using the RNeasy kit (Qiagen, Germantown, MD) and treated with DNase I (Life Technologies, Carlsbad, CA) to remove any contaminating genomic DNA. Complementary DNA was generated using the Superscript III First-Strand Synthesis kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions and subsequently used as template in quantitative real-time PCR (Q-PCR). Q-PCR was performed in triplicate using a Bio-Rad iCycler PCR system and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer pairs used were either previously published sequences, IL-1β (27), NLRP3 (28), GAPDH (29), or self-designed, IL-1 receptor antagonist (IL-1RN): ATGGAGGGAAGATGTGCCTGTCCTG (sense) and GCGAAGCGCTTGTCCTGCTTT (anti-sense). All primer pairs spanned intron-exon boundaries whenever possible and bound to all isoforms of the gene, where applicable. All primers were commercially synthesized by Integrated DNA Technologies (Coralville, IA).

Q-PCR data was analyzed using the threshold cycle (C_T) method as previously described (30), and gene expression data is shown normalized to that of the housekeeping gene, GAPDH. In all Q-PCR assays, complementary DNA generated in the absence of reverse transcriptase as well as water in the place of DNA template were used as negative controls, and these samples were confirmed to have no amplification.

ELISA and Western blotting

Human IL-1β, IL-6, and TNF-α protein released into the supernatant were measured using the ELISA MAX Deluxe kits (BioLegend, San Diego, CA) according to the manufacturer's

instructions. In samples indicated as "n.d.: not detected," the signal was below the threshold of detection.

At the harvest time point, cells were lysed by addition of 2X Laemmli buffer containing 10% β-mercaptoethanol. For experiments where supernatant was analyzed by Western blotting, serum-free R-0% medium was used during the infection and supernatant was concentrated using Amicon Ultra Centrifugal filters (EMD Millipore, Billerica, MA) according to manufacturer's instructions. Concentrated supernatant was diluted with 2X Laemmli buffer containing 10% β-mercaptoethanol. Samples were boiled at 100°C for 10 to 15 min and then separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) for immunoblotting. Membranes were blotted for IL-1β (3ZD from the National Cancer Institute Biological Resources Branch) or β-actin (AC-15; Sigma-Aldrich, St. Louis, MO) using the SNAP i.d. Protein Detection System (EMD Millipore, Billerica, MA) according to manufacturer's instructions. Primary antibodies were followed by horseradish peroxidase-conjugated secondary antibodies (BioLegend, San Diego, CA) and membranes were developed with SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, Carlsbad, CA) or Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) substrate. Signal was detected using a Nikon camera as previously described (31).

For extracellular pro-IL-1β cleavage experiments, supernatant was concentrated as described above and subsequently left untreated and harvested immediately, or left untreated, treated with caspase assay buffer (Enzo, Farmingdale, NY) alone as a vehicle control, or treated with 100 units of recombinant caspase-1 (Enzo, Farmingdale, NY) diluted in caspase assay buffer and incubated at 37°C for 2 hr. Treated samples were then diluted with 2X Laemmli buffer containing 10% β-mercaptoethanol, boiled, and processed for Western blotting as described above.

Results

T. gondii infection induces an early IL-1β response in primary human monocytes

Primary human monocytes were isolated from healthy donor blood by counterflow elutriation and assessed for purity. A representative analysis is shown in Figure 1A. In order to distinguish monocytes from NK cells, which both express CD11b and CD16 (32), the cells were first evaluated for expression of the NK cell marker CD56. 96.6% of the cells were found to be CD56−, indicating few NK cell contaminants. Using sequential gating, we found that 91.8% of the CD56− population represented CD11b+ monocytes. Of these cells, we identified three monocyte subsets based on differential expression of CD14 and CD16, with the classical monocytes (CD14^{high} CD16−) representing the dominant subset (Fig. 1A), as previously reported (33, 34). In each experiment, enriched monocyte cultures were also evaluated for CD3 and CD20 expression to identify any T or B cell contaminants, respectively, and were confirmed to typically contain less than 4% of these cells collectively (data not shown). For all the presented experiments, the primary human monocytes were used in infection experiments immediately after isolation.

We previously demonstrated that *T. gondii* infection induces IL-1β transcripts in primary human monocytes and the THP-1 monocytic cell line at late time points (16 and 24 hours post-infection, hpi, respectively) (23). Since IL-1β is an early regulator of inflammation, we investigated the kinetics of the response in primary monocytes at early time-points after *T. gondii* infection. As early as 1 hpi, *T. gondii*-infected cells upregulated IL-1β mRNA 27-fold over mock-infected cells. Although the magnitude of the induction declined by 4 hpi (Fig. 1B), *T. gondii*-infected monocytes maintained higher IL-1β transcripts than mock-infected cells out to 16 hpi (Fig S1A). Interestingly, *T. gondii* infection of primary human monocytes also resulted in upregulation of transcripts for the IL-1 receptor antagonist (IL-1RN), a naturally occurring antagonist of IL-1β that functions as a negative regulator of IL-1 receptor signaling, with slightly delayed kinetics. Parasite-mediated induction of IL-1RN mRNA increased between 1 and 4 hpi, with a 16-fold induction over mock-infected cells by 4 hpi (Fig. 1C).

THP-1 and U937 cells are human monocytic cell lines that are commonly used as models for primary human monocytes. We investigated the induction of IL-1β mRNA in these cell lines and found that although statistically significant, these cell lines induced substantially less IL-1β mRNA upon *T. gondii* infection at early time points, with only 3.2 to 7-fold induction over mock-infected cells between 1 to 4 hpi. However, at 24 hpi *T. gondii* strongly induced IL-1β mRNA over mock-infected THP-1 cells (610-fold), as previously reported (23), and more weakly induced IL-1β mRNA in U937 cells (12-fold) (Fig. 1D). These data suggest that while human monocytic cell lines respond similarly to primary human monocytes, the kinetics and magnitude of the response differs, and primary cells are more rapidly responsive to infection.

T. gondii induction of IL-1β protein occurs in primary human monocytes but not in macrophages derived from the same donor

After observing that primary human monocytes upregulate IL-1β mRNA within 1 hpi, we next investigated whether IL-1β protein was detectable in this time scale after *T. gondii* infection by using intracellular cytokine staining (ICCS). Primary human monocytes were infected with *T. gondii* parasites that constitutively express GFP, which allows for the detection of cells harboring parasites (GFP+) by using flow cytometry. In contrast to mock-infected cells, *T. gondii*-infected monocyte cultures had detectable levels of intracellular IL-1β protein as early as 1 hpi. The percent of IL-1β+ cells increased from 3.8% at 1 hpi to 15.3% by 4 hpi. Notably, the IL-1β+ population was comprised predominantly of GFP+ cells, and not the GFP− bystander cells, suggesting that IL-1β production was associated with the presence of intracellular *T. gondii* (Fig. 2A). The range of GFP+ signal intensities indicates cells with varying numbers of intracellular parasites. However, at these early time points, there does not appear to be a correlation between intracellular parasite burden and the detection of IL-1β. Notably, these time points were all examined prior to parasite replication, which occurs at ~6–8 hpi in these cells. We next investigated IL-1β release into the culture supernatant and found that in contrast to mock-infected cells, parasite-infected cultures had detectable levels of IL-1β in the supernatant by ELISA as early as 2 hpi, and this increased at 4 hpi (Fig. 2B), consistent with the intracellular cytokine staining for IL-1β (Fig. 2A). Not surprisingly, neither the THP-1 nor U937 monocytic cell lines released detectable levels

of IL-1β protein upon *T. gondii* infection between 1 and 4 hpi (Fig. S2), although THP-1 cells did release detectable levels by 24 hpi (Fig. S2). We have previously shown that in THP-1 cells, *T. gondii* induction of IL-1β is mediated by dense granule protein GRA15 at 24 hpi (23), and we have confirmed a role for GRA15 in *T. gondii*-induced IL-1β in primary human monocytes at late time points (Fig. S3).

Since much of the research on the inflammasome has involved studies on macrophages, we next compared the IL-1β response of primary human monocytes and monocyte-derived macrophages from the same donor. Differentiated macrophages were first phenotyped and confirmed to be CD86^low, HLA-DR/P/Q^+, CD40^+, CD14^+, and CD11b^+, as previously reported (35) (Fig. 3A). In contrast to human monocytes, *T. gondii* infection did not induce IL-1β transcripts in macrophages derived from the same blood donor (Fig. 3B). However, macrophages and monocytes up-regulated IL-1β in response to the classical two-step inflammasome stimuli, LPS and ATP (Fig. 3B and Fig. S1B), indicating that macrophages were capable of responding to canonical inflammasome stimuli within the same 4 h time scale. These data demonstrate that human monocytes and monocyte-derived macrophages differentially regulate IL-1β in response to *T. gondii* infection.

Both pro-IL-1β and mature IL-1β are released from infected monocytes, and pro-IL-1β is cleavable extracellularly

IL-1β is produced as a zymogen, pro-IL-1β, which is post-translationally processed into mature IL-1β, the biologically active form of the protein. Neither commercial ICCS antibodies nor ELISA kits distinguish between the pro- and mature forms of IL-1β. In order to investigate the processing of pro-IL-1β upon *T. gondii* infection, we analyzed the lysate and supernatant samples by Western blot. In the lysate, pro-IL-1β was detectable as early as 1 hpi, and this signal increased over time, with mature IL-1β detectable at 2 and 4 hpi (Fig. 4A). In the supernatant, IL-1β signal increased over time, and interestingly, both the pro- and mature forms of IL-1β were found (Fig. 4A). These data are consistent with previous studies showing that primary human monocytes release both pro- and mature IL-1β in response to LPS (36).

To investigate whether the *T. gondii*-induced pro-IL-1β in the supernatant was cleavable, cell-free supernatant was harvested at 4 hpi and either left untreated, or treated with caspase assay buffer (vehicle control) or with recombinant caspase-1 in assay buffer. These differentially treated supernatants were then either harvested immediately or incubated in the absence of cells. *T. gondii*-infected monocytes released substantially more pro-IL-1β and mature IL-1β protein into the supernatant than mock-treated cells (Fig. 4B). Incubating these supernatants with or without assay buffer did not increase the post-translational processing of pro-IL-1β. However, incubating *T. gondii*-infected supernatant in the presence of recombinant caspase-1 resulted in almost complete conversion of pro-IL-1β into mature IL-1β (Fig. 4B), suggesting that the pro-form of IL-1β protein is indeed cleavable in the extracellular space.

Rapid *T. gondii*-mediated IL-1β induction involves *de novo* protein synthesis

Although monocytes from some healthy blood donors produced a low level of IL-1β in the absence of *in vitro* infection or stimulation, we consistently observed that IL-1β was up-regulated by *T. gondii* infection at the mRNA and protein levels. To test the extent to which *T. gondii*-induced IL-1β protein was newly synthesized, we pre-treated the monocytes with cycloheximide (CHX), a small molecule that inhibits the elongation step of eukaryotic protein translation (37), and then infected the cells with *T. gondii*. We first confirmed by flow cytometry that the infection efficiency between control monocytes and CHX-treated monocytes was comparable (Fig. S4). In contrast to control cells, monocytes infected in the presence of CHX had reduced IL-1β in both the lysate and the supernatant (Fig. 5A). Furthermore, monocytes infected in the presence of CHX released 73.5% less IL-1β into the supernatant compared to control cells (Fig. 5B). Collectively, these data indicate that primary human monocytes rapidly synthesize IL-1β mRNA and protein upon *T. gondii* infection.

T. gondii induced IL-1β is dependent on NLRP3 and potassium efflux

Cleavage of pro-IL-1β into mature IL-1β occurs through the action of the multi-protein inflammasome complex and is triggered by a sensor, frequently of the NLR family. The role of NLRP3 in *T. gondii* infection has been demonstrated in mouse macrophages (15), but whether this inflammasome sensor plays a role in human cells, and in monocytes in particular, has not been examined. We found that *T. gondii* infection of primary human monocytes resulted in the rapid induction of *NLRP3* transcripts, with a 4.9-fold induction by 2 hpi. By 4 hpi, the transcript levels were comparable with mock-infected cells (Fig. 6A). Interestingly, human macrophages from the same donor did not up-regulate *NLRP3* in response to *T. gondii* infection (Fig 6A). These data suggest that *T. gondii* upregulates *NLRP3* and *IL-1β* transcripts in parallel in monocytes but not in macrophages (Figs. 1B and 6A).

In both the classical and alternative inflammasome models, IL-1β regulation in response to LPS has been shown to be dependent on NLRP3. However, the role of potassium efflux is a distinguishing factor between these two pathways, with the newly described alternative pathway operating independently of potassium efflux (38). To formally test a role for NLRP3 and potassium efflux in *T. gondii*-mediated induction of IL-1β in primary human monocytes, we infected the cells in the presence of MCC950, a selective small molecule inhibitor specific to NLRP3 but not the other inflammasome sensors (39). We also infected monocytes in the presence of high potassium to block potassium efflux, as previously reported (38). We confirmed that the infection efficiency was robust and comparable across the treatments, with 72–77% of cells harboring GFP[+] parasites regardless of treatment condition (Fig. 6B). This is an important control to ensure that any effects of MCC950 and high potassium on *T. gondii*-mediated IL-1β production were not due to effects of the treatments on parasite infection itself. In contrast to cells infected in the presence of the vehicle control, cells infected in the presence of either MCC950 or high potassium released significantly less IL-1β into the supernatant: MCC950 treatment reduced IL-1β release by 82.2%, and media with high potassium reduced IL-1β by 87.2% compared to vehicle control-treated cells (Fig. 6C). Furthermore, by examining the supernatants by Western blot,

we determined that MCC950 and high potassium markedly reduced the cleavage of pro-IL-1β into mature IL-1β (Fig 6D). To confirm that these treatments did not globally impair monocyte cytokine production, we examined the production of TNF-α and found that *T. gondii*-induced TNF-α production was not affected by either MCC950 or high potassium (Fig. 6E). These data indicate that NLRP3 functions as an inflammasome sensor for *T. gondii* in primary human cells. Moreover, the increase in *NLRP3* transcripts in infected monocytes but not in macrophages from the same donor (Fig. 6A) may contribute to the inability of human macrophages to release IL-1β in response to *T. gondii* infection alone.

Finally, we confirmed that the MCC950 and high potassium treatments effectively targeted the inflammasome. As noted above, LPS alone can activate the one-step, alternative inflammasome, resulting in IL-1β release that is dependent on NLRP3 but independent of potassium efflux (38). We demonstrate that LPS stimulation of primary human monocytes resulted in IL-1β release, which was decreased in the presence of MCC950 but unaffected by high potassium, as expected (Fig. 6F). The combination of LPS and monosodium urate crystals (MSU) is known to activate the classical, two-step inflammasome. LPS/MSU treatment resulted in IL-1β protein induction that was dependent on both NLRP3 and potassium efflux (Fig. 6F), consistent with what has previously been reported for the two-step inflammasome (38). Collectively, these data suggest that *T. gondii* induction of IL-1β in primary human monocytes activates the classical inflammasome and does so in a manner dependent on both NLRP3 and potassium efflux.

Discussion

The inflammasome was defined in 2002 as a molecular platform that mediates the cleavage and release of IL-1β through the activity of the protease caspase-1 (5). Since the initial identification of the inflammasome complex, many studies have further dissected the molecular pathways that trigger the processing of IL-1β. Much of our understanding in this area comes from experiments in murine models, which confer the benefit of genetic tractability. Consistent with this, inflammasome sensors for *T. gondii* have been most extensively studied in rats and mice. A genetic locus that confers resistance of Lewis (LEW) rats to *T. gondii*, named *Toxo1* (40), was found to overlap with the locus that controls the sensitivity of rat macrophages to the anthrax lethal toxin (LT) (16, 17). Genetic mapping studies in recombinant inbred rats allowed for the identification of *Nlrp1a* as the *Toxo1* locus (16), and macrophages expressing *Nlrp1* sequences from resistant rat strains undergo rapid caspase-1 dependent pyroptosis and IL-1β release in response to *T. gondii* infection, thereby limiting parasite proliferation (17). In the case of LT, the bacterial toxin is a protease that cleaves NLRP1, activating this inflammasome sensor (41, 42). Studies in mouse macrophages, however, have revealed that *T. gondii* infection does not induce cleavage of the N-terminal domain of NLRP1, indicating a novel mechanism of activation of this inflammasome sensor by the parasite (14). Subsequent studies in mice also identified NLRP3 as a sensor for *T. gondii*, and demonstrated that mice deficient in either NLRP1 or NLRP3 succumbed more rapidly to acute *T. gondii* infection than wild-type mice (15), reinforcing a protective role for inflammasome activation during *T. gondii* infection.

Although the mechanism of activation of these inflammasomes during *T. gondii* infection remains unknown, some clues may be gleaned from the parasite signals that contribute to IL-1β release. Active parasite invasion is necessary, since inhibiting invasion by pre-treatment of the parasites with the actin polymerization inhibitor Mycalolide B prevents IL-1β release (17, 23). Interestingly, in studies in mouse and rat macrophages, robust production of IL-1β in response to *T. gondii* requires a priming signal with a TLR ligand, such as LPS or PAMCysK, as *T. gondii* alone induces little (14) to undetectable (17) IL-1β cleavage and release. In contrast, our data presented here show that human monocytes produce IL-1β in response to *T. gondii* infection alone. These data suggest that the differences observed between mouse/rat macrophages and human monocytes may be a difference between species. Indeed, there is precedent for species differences in the responses of human, murine, and porcine monocytes to LPS: murine monocytes require two signals for IL-1β production, whereas porcine and human monocytes release IL-β in response to LPS alone (38). Our data support the idea that the differences observed are perhaps more likely due to a difference between cell types, since we found that human monocytes produce IL-1β in response to *T. gondii* infection alone, but human macrophages derived from the same donor do not. Indeed, it has long been known that monocytes and macrophages differentially regulate the pathway of IL-1β release in response to LPS (43). Since TLR ligands provide "signal one" for transcriptional induction of IL-1β, the need for this priming signal in macrophages suggests that *T. gondii* itself is not sufficient to provide a strong signal one in these cells. In addition to inducing IL-1β transcription, TLR agonists contribute to the transcriptional activation of components of the inflammasome that are important for IL-1β cleavage. During the differentiation of human monocytes into macrophages, we found that the cells down-regulated the expression of NLRP3, which we identified as a sensor for *T. gondii* infection. It is perhaps due to changes in the expression of inflammasome components during differentiation from monocytes to macrophages that leads to the requirement for a stronger signal one in macrophages infected with *T. gondii*. As a result, monocytes may be more poised to synthesize and release IL-1β rapidly during infection. These differences are not likely due to the *in vitro* culture and differentiation of macrophages, as differences in IL-1β regulation have also been observed in comparisons between primary human peripheral blood monocytes and autologous freshly isolated alveolar macrophages (43). This difference in the primed state of monocytes and macrophages may have evolved to suit the different niches that these cells occupy. Monocytes are more prevalent in the vascular compartment, and their secretion of IL-1β would lead to a rapid systemic response that also activates the vascular endothelium, whereas as tissue-resident cells, the release of inflammatory mediators by macrophages may have more local effects in the surrounding tissue.

Beyond the canonical mechanism of inflammasome activation, recent work has revealed the existence of additional, alternative pathways of inflammasome activation, particularly in human monocytes. Whereas mouse macrophages generally require two signals to produce IL-1β, human monocytes have long been known to release IL-1β in response to LPS alone. It was demonstrated that human monocytes have constitutively active caspase-1, which obviates the need for a second signal (44). A non-canonical inflammasome has also been described in human monocytes in which LPS can serve both as a priming signal and an

activator of NLRP3 through caspases-4 and -5 (45). More recently, a one-step or alternative inflammasome in human monocytes that is activated by LPS and mediated by TLR4, TRIF, RIPK1, and FADD/Caspase-8 also bypasses the need for a second signal (38).

Although inflammasome activation and IL-1β production occur during *T. gondii* infection, the precise trigger(s) for this response remain elusive. Mouse macrophages primed with TLR agonists release IL-1β in response to multiple different strains of *T. gondii* (15, 17), indicating that if there is a parasite factor that activates the inflammasome, it is shared among these strains. Unlike LPS, infection with *T. gondii* provides a complex array of PAMPs for pattern recognition receptors as well as parasite-secreted effector proteins that are injected into the host cell during invasion and intersect signal transduction and transcriptional pathways (46). Parasite-secreted effectors have also been shown to cross the parasitophorous vacuole membrane and enter the cytosol (47). Given the cytosolic localization of some parasite effectors, it is possible that a yet to be identified parasite protein activates an inflammasome sensor directly. We have demonstrated a role for the parasite-secreted GRA15 protein in IL-1β production in THP-1 cells (23) and in primary human monocytes (present study), but this secreted effector likely acts at the level of IL-1β transcript induction, rather than inflammasome activation, since it is a known inducer of NF-κB activation (26). Interestingly, GRA15 plays a more significant role in IL-1β production in THP-1 cells than in primary monocytes, and this is likely due to the differential kinetics of the response in these cells. Although both cell types produce IL-1β in response to *T. gondii*, primary monocytes release IL-1β within 2–4 hours, whereas THP-1 cells require 18–24 hours. GRA15 was identified as an effector protein that sustains NF-κB activation at late time points during infection, consistent with the kinetics of IL-1β release from THP-1 cells. In addition, primary monocytes are significantly more phagocytic than THP-1 cells, and in our experiments, ~30% of the intracellular *T. gondii* in primary monocytes are the result of phagocytosis events (data not shown). Indeed, phagocytosis of *T. gondii* by primary monocytes likely contributes to some of the IL-1β signal observed, as phagocytosis induces a stronger cytokine response than does invasion by the parasite (48).

Alternatively, inflammasome activation during *T. gondii* infection may be the result of a host cell process that is induced in response to the infection. Indeed, we have found that potassium efflux plays a critical role in *T. gondii* activation of the NLRP3 inflammasome in human monocytes. Long before the identification of the inflammasome, it was well established that potassium depletion from cells induced IL-1β maturation (49) (50). And more recently, it was determined that the diverse activators of the NLRP3 inflammasome converge on potassium release (8). A role for potassium in *T. gondii*-induced IL-1β release is particularly interesting in light of the known effects of ion flux in cells infected with *T. gondii*. It is the loss of intracellular potassium that triggers the parasite to egress from host cells (51). The reduction in intracellular potassium results in an increase in cytosolic calcium, which is sensed by *T. gondii* and activates the actin-myosin machinery that powers motility for egress (52). Notably, the release of potassium in infected human monocytes is sufficient to trigger inflammasome activation and IL-1β release, but does not induce parasite egress. These data suggest that there is a threshold effect, and the decrease in potassium ion concentration that is necessary for NLRP3 inflammasome activation does not reach the level required for parasite egress.

Although multiple mechanisms of IL-1β release have been demonstrated, including the release of microvesicles, secretory lysosomes, and pyroptosis (53), the pathway by which *T. gondii* induces IL-1β release remains to be determined. In our studies, *T. gondii* infection of human monocytes did not lead to pyroptosis, despite activation of caspase-1, indicating an uncoupling of the pathway for caspase-1 activation and cell death. Similarly, pyroptosis does not occur during *T. gondii* infection of mouse macrophages (14, 15) or in rat macrophages from *T. gondii*-susceptible strains of rats (e.g., Sprague-Dawley) (17). In addition, we demonstrate the release of significant amounts of pro-IL-1β from human monocytes, which is cleavable in the extracellular space. It has been demonstrated previously that human monocytes release pro-IL-1β (36), as well as caspase-1 (54) in response to LPS and ATP, respectively. An intriguing possibility is that *T. gondii* has evolved a mechanism to protect the cell from death, despite inducing an IL-1β response. Indeed, the activation of NLRP1 by *T. gondii* in rat macrophages from the resistant LEW rat strain triggers a rapid pyroptosis and IL-1β release. As a result, the parasites lose their intracellular niche and fail to establish an infection (16, 17). In this scenario, the pathogen is cleared by the host response. Given the variety of pathways by which *T. gondii* manipulates and modulates the host cell environment, the parasite may very well have evolved a mechanism to maintain the integrity of its host cell and persist intracellularly, despite the host cell mounting a protective cytokine response. Future work defining the molecular interplay between the parasite and its host cell during the induction of inflammatory responses will likely reveal myriad other interesting features of this successful global pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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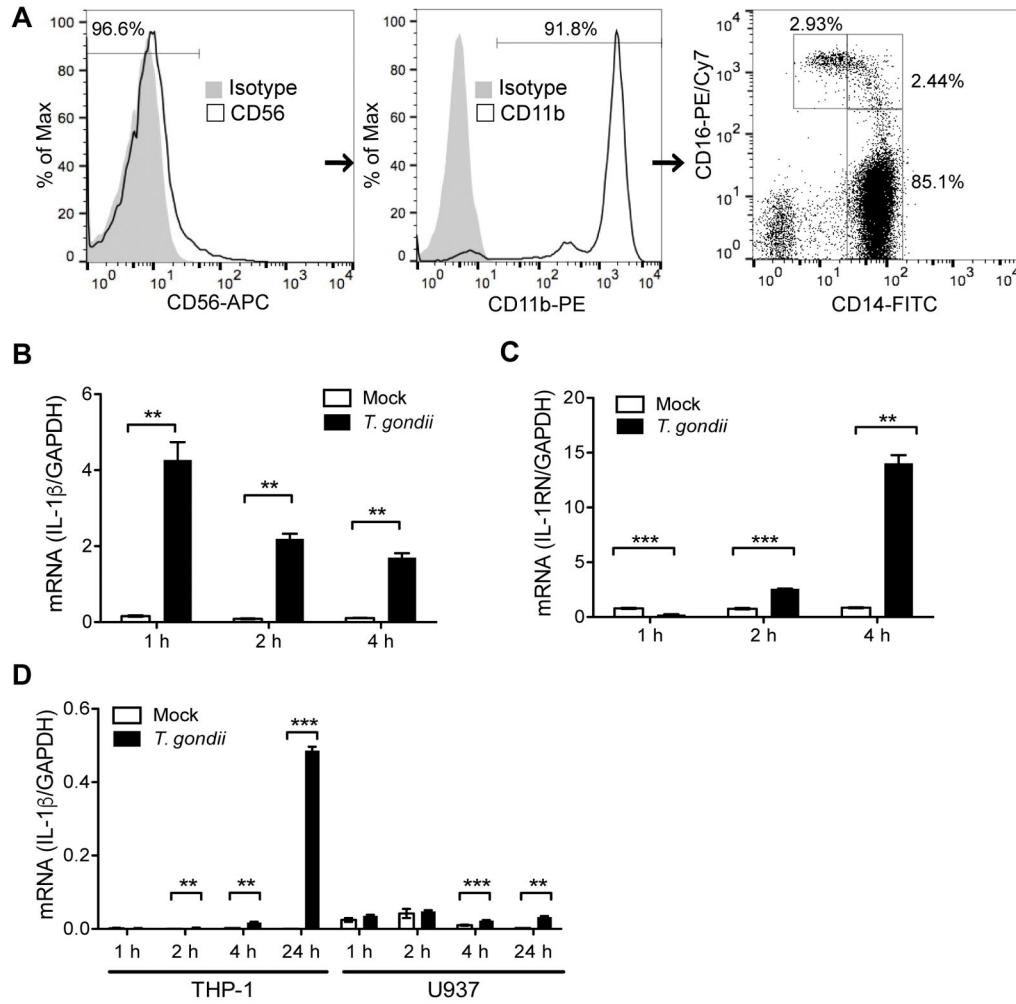
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Fig 1.

Analysis of IL-1β and IL-1RN transcripts in primary human monocytes and monocytic cell lines. (A) Primary monocytes were enriched from the PBMC of healthy donors and phenotyped for purity by flow cytometry. Sequential gating analysis was performed as follows: cells were identified by forward and side scatter and examined for CD56 expression. CD56- cells were analyzed for expression of CD11b. CD11b+ cells were analyzed for CD14 and CD16 expression. Gates were drawn based on the staining of the isotype control antibody. Representative analysis from 24 independent donors is shown. (B-C) Isolated monocyte cultures were either mock infected or infected with *T. gondii* for 1 to 4 hours (h). Q-PCR using primers specific to IL-1β or IL-1 receptor antagonist (IL-1RN) was performed and transcript levels relative to GAPDH are shown. (D) THP-1 or U937 monocytic cell lines were mock infected or infected with *T. gondii* for 1, 2, 4, or 24 h. *IL-1β* transcripts were measured by Q-PCR and the levels relative to *GAPDH* are shown. For panels B to D, error bars represent the standard deviations of technical triplicates. Experiments were performed with (B) 4 to 6 independent donors/time point, (C) 3 to 5 independent donors/time point, and (D) 3 independent times. Representative experiments are shown. **$p<0.01$; ***$p<0.001$ (Student's *t* test).
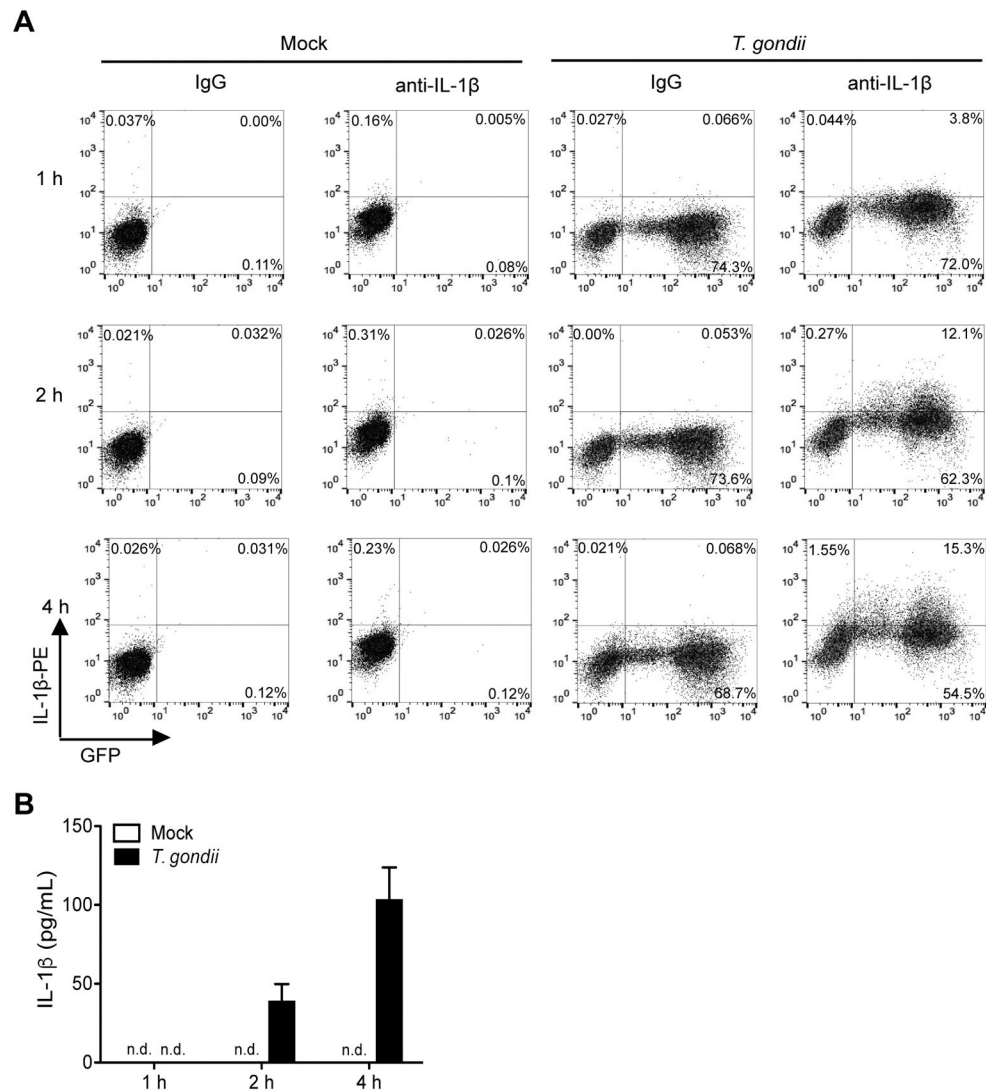
A

B

Fig. 2.
Rapid induction of IL-1β protein in *T. gondii*-infected primary human monocytes. Primary monocytes were infected with GFP-expressing *T. gondii* parasites for 1, 2, or 4 h. At each time point, (A) cells were permeabilized and stained with a control antibody or an anti-IL-1β antibody and analyzed by flow cytometry or (B) supernatant was collected and analyzed for IL-1β protein release by ELISA. n.d., not detected. Error bars represent the standard deviation of biological triplicates. These experiments were performed with (A) 3 independent donors or (B) 7 to 11 independent donors per time point. Representative experiments are shown.
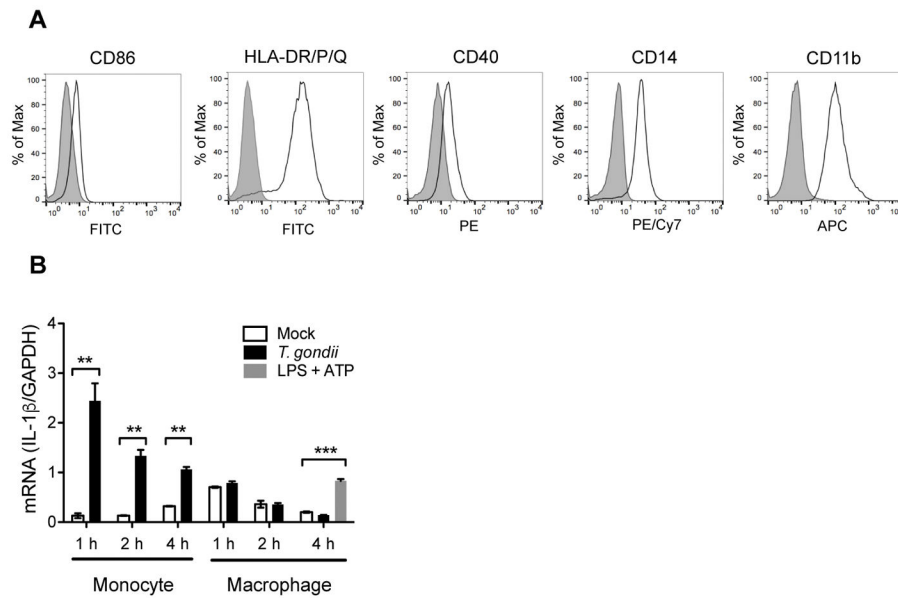
Fig. 3.

Comparison of IL-1β detection in *T. gondii*-infected primary human monocytes and macrophages from the same donors. Monocyte-derived macrophages were differentiated *in vitro* with M-CSF for 6 days. (A) On day 7, macrophages were phenotyped for purity and confirmed to express low levels of surface CD86 and to express HLA-DR/P/Q, CD40, CD14, and CD11b. Isotype controls are shown in shaded gray. (B) Primary human monocytes and macrophages derived from the same donor were mock infected or infected with *T. gondii* for 1 to 4 h, or stimulated with LPS/ATP for a total of 4 h. *IL-1β* transcripts were measured by Q-PCR and the levels relative to *GAPDH* are shown. Error bars represent the standard deviations of technical triplicates. Data shown are representative of (A) 2 donors or (B), 2 to 4 donors per time point. **p<0.01; ***p<0.001 (Student's *t* test).

A

B
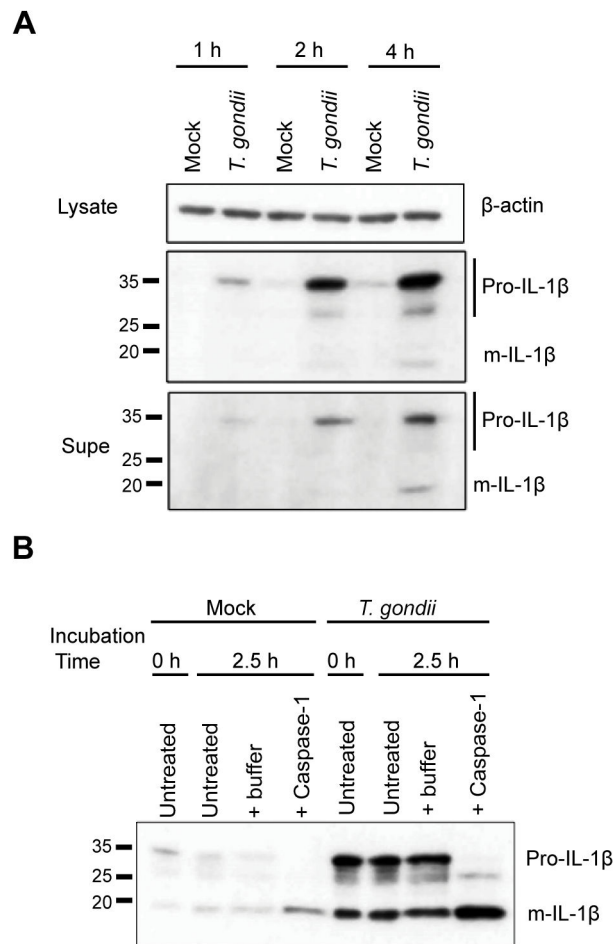
Fig. 4.

Pro- and mature IL-1β are detected in *T. gondii* infected supernatant and pro-IL-1β is cleavable. Primary human monocytes were mock infected or infected with *T. gondii*. (A) At 1 to 4 hpi, pro- and mature IL-1β (m-IL-1β) in the cell lysate and supernatant (supe) were visualized by Western blotting. β-actin from the cell lysate was used as a loading control. (B) At 4 hpi, supernatant was harvested, concentrated, and either left untreated, treated with caspase assay buffer (vehicle control), or treated with recombinant caspase-1 protein. Differentially treated supernatant was then incubated at 37 °C for 0 or 2.5 h and analyzed by Western blotting for pro- and mature IL-1β. Data shown are representative of (A) 3 to 6 independent donors per time point or (B) 3 independent donors.
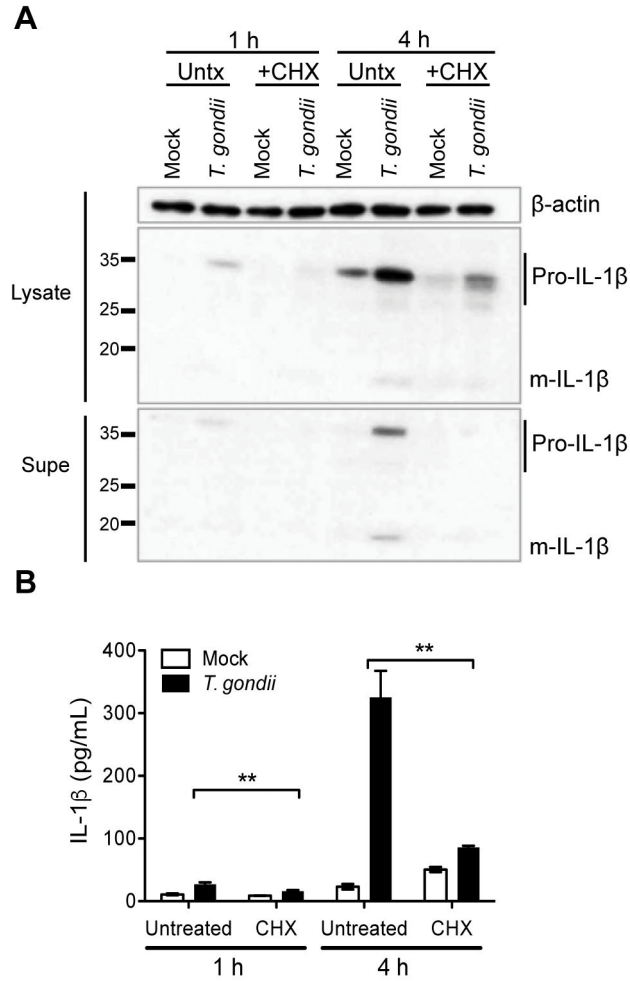
A

B

Fig. 5.

T. gondii- induced IL-1β is dependent on *de novo* synthesis. Primary human monocytes were either left untreated (untx) or were pre-treated with cycloheximide (CHX) and then mock infected or infected with *T. gondii* for 1 or 4 h. (A) Pro- and mature IL-1β (m-IL-1β) were visualized by Western blotting in both lysate and supernatant (supe). (B) IL-1β protein released into the supernatant was measured by ELISA. Error bars represent the standard deviation of biological triplicates. These experiments were performed with (A) 3 or (B) 4 independent donors and representative experiments are shown. **p<0.01 (Student's *t* test).
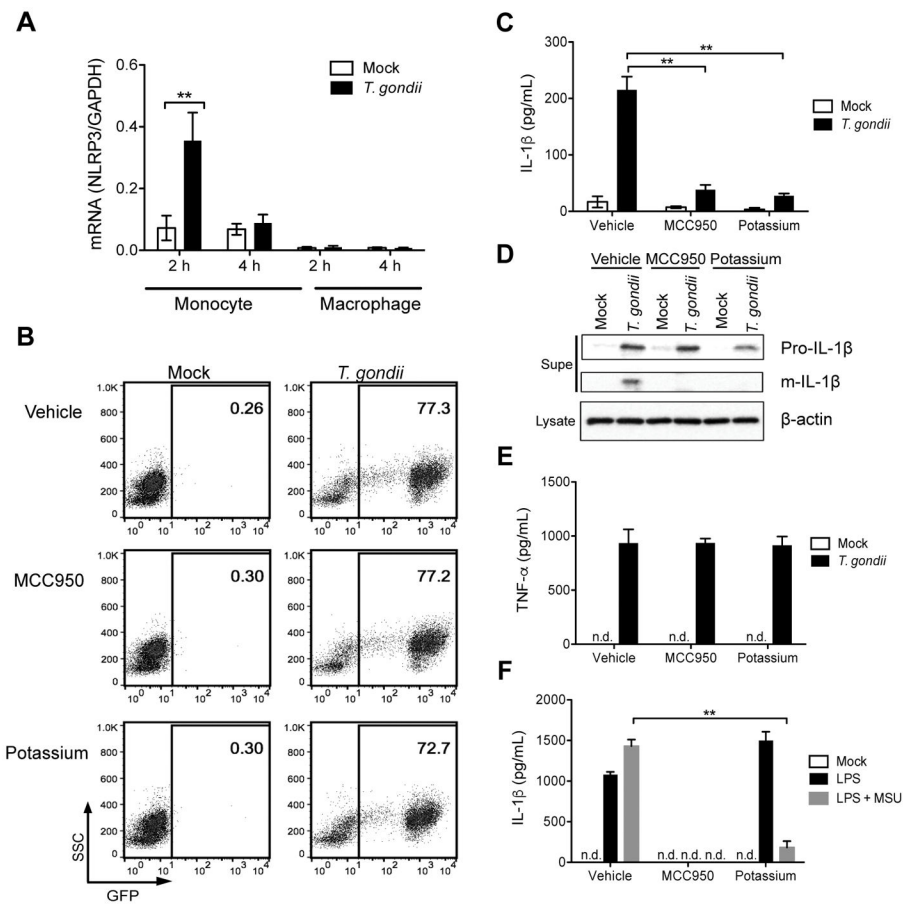
Fig. 6.

T. gondii induced IL-1β in primary human monocytes is dependent on NLRP3 and potassium efflux. (A) Primary human monocytes and macrophages derived from the same donor were mock infected or infected with *T. gondii* for 2 or 4 h. *NLRP3* mRNA levels were measured by Q-PCR and transcript levels relative to *GAPDH* are shown. Error bars represent standard deviation of technical triplicates. (B to F) Primary human monocytes were either pre-treated with vehicle control or 5 μM MCC950, an inhibitor specific to NLRP3, or were treated with 50 mM potassium chloride. (B to E) Cells were mock infected or infected with GFP-expressing *T. gondii* for 4 h. (B) Cells were analyzed by flow cytometry for infection efficiency. (C) Levels of IL-1β in the supernatant were measured by ELISA. (D) Levels of pro- and mature IL-1β in the supernatants or β-actin in the cell lysates were determined by Western blotting. (E) Levels of TNF-α in the supernatant were measured by ELISA. (F) Primary human monocytes were stimulated with LPS alone or LPS in combination with monosodium urate crystals (MSU) for 16 h and levels of IL-1β in the supernatant were measured by ELISA. n.d., not detected. Error bars represent the standard deviation of biological triplicates. These experiments were performed with (A) 2, (B to C) 5, (D) 4, (E) 4, or (F) 3 independent donors and representative experiments are shown. **p<0.01 (Student's *t* test).