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Regulation of the NLRP3 inflammasome and IL-1β production and release during *Toxoplasma gondii* infection of human monocytes

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

William J. Pandori

Dissertation Committee:
Associate Professor Melissa B. Lodoen, Chair
Associate Professor Naomi S. Morrissette
Professor David A. Fruman
Professor Eric M. Pearlman

2020

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DEDICATION

To

My family, my friends and everyone who has helped me along the way

Thank you for everything

To the past

"The past is never dead. It's not even past."
— William Faulkner, Requiem for a Nun

To the present

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."
— Marie Curie

To the future

"Somewhere, something incredible is waiting to be known."
— Carl Sagan

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ACKNOWLEDGEMENTS

Many people and organizations deserve acknowledgement for the work presented here. First off, I need to acknowledge my parents for their support of my education and their help in developing my curious and scientific mind early in life. My family has afforded me many privileges, without which I do not think completing a doctoral degree would have been possible. I would like to acknowledge my father for promoting and helping in the development of my critical thinking capabilities. My mother deserves acknowledgement for helping to foster my love for science. She would do experiments with me at home, found science summer camps that I loved, celebrated and promoted my interests in school and learning and even acquired additional science curriculum for me. My younger sister Laura has also always inspired me to work hard and strive to be my best; in part because she has always been better than me in everything we have ever done. Thanks for that. My whole extended family has supported and encouraged this endeavor as well. Thank you all.

My teachers and mentors deserve acknowledgement as well. I would like to thank my university professors Milton Saier and James Kadonaga for believing in me and encouraging my pursuit of science. A great deal of my development as a research scientist, and my desire to pursue research is owed to Drs. David Hess and Trang Vo. They were both patient with me, encouraging and taught me the basic skills I have relied on throughout my doctoral program.

I also owe a great deal to Dr. David Fruman. He took a chance on me as a researcher when no one else did. I am incredibly appreciative of the lifeline he gave me, and fortunately for me he was also able to be a great resource and mentor just down the hall from lab. Thank you for the advice and direction you have given me. Also, thank you for helping to fund the GPS-STEM program at the school which has also been fun and useful for my development.

Dr. Eric Pearlman deserves special acknowledgement as well for his guidance and promotion of me as a student. Dr. Pearlman has afforded me some amazing opportunities through his running of the Institute for Immunology and allowing me to rotate in his lab while it was just starting at UCI. His work has afforded me some wonderful opportunities to attend conferences, receive a training grant and meet leading researchers in my field. Thank you for your advice on my projects as well.

The last member of my committee, Dr. Naomi Morrissette also requires special recognition. I love the joint lab meetings we have together, and I need to thank you for being a mentor who I discuss academic and non-academic issues with. When Dr. Lodoen was on sabbatical, Dr. Morrissette made sure to spend more time with each of the students in our lab, and the time you spent with me was extremely helpful.

My PI, Dr. Melissa Lodoen, has been an amazing influence on my development and I consider myself quite lucky that I joined her lab. I have grown in many ways under her direction. I feel as though I have been able to improve many aspect of my academic and non-academic life due to some of her guidance and following her example. I am especially grateful for her drive and encouragement to always perform at our best, and the massive amount of time she has dedicated to her students. She has always made time for us when we needed it. Dr. Lodoen's has treated every one of her students with kindness and respect. Thank you for fostering a fun and friendly lab environment, and for all the diligent work and time you have spent trying to help me improve as a scientist.

All of the members of the Lodoen lab during my time there have also meant a lot to me. Particularly I owe thanks to Drs. Lanny Gov and Tatiane Soares de Lima. They both helped train me and gave me the tools necessary to succeed in my graduate research. Drs. Armond Franklin-Murray and Christine Schneider-Lewis were always one step ahead of me on my path to graduation, and as such they were wonderful resources and a great help. Christine thank you

for knowing the answers to all my questions. Armond, thank you for your advice and making lab such a fun place. Tiffany Kao, an undergraduate researcher I worked with, has generated a significant amount of data presented here, and drove me to be a better teacher and mentor. Her dedication to our work routinely inspired me. Also, I would like to acknowledge Evelyn Hoover, who has been step in step with me through our time in lab. It has been great to be lab mates, and I have appreciated our conversations and the assistance and council we have been able to provide for each other. Sharmila Mallya, who has been with me through my time in both Dr. Fruman's and Dr. Lodoen's labs has also generated a significant amount of this dissertation's data. Sharmila, thank you for making my work life better in so many ways. Sharmila's expertise helped jumpstart a lot of these projects, and we would not have accomplished nearly as much without her assistance. Steph, Stephanie and Olivia, it has been great to work with you all, and just like Tiffany, you each have helped push me to be a better mentor and teacher. Good luck to each of you.

The contributions of Drs. Andrea Tenner and Rosa Andrade and their labs have also helped us in many ways over the past 5 years, especially through sharing of equipment. I also must acknowledge the nurses, blood donors and directors of the healthy blood donor program, without whom this research would not have been possible.

Finally, and most importantly, I want to acknowledge my wife Lauren. You have continually inspired me and shown me what is possible. You have supported me through everything, and I promise to always be there for you in return. Partners forever.

Financial support was provided by the University of California, Irvine, National Institutes of Health (NIH) R01AI120846, American Cancer Society, RSG-14-202-01-MPC, and NIH T32AI060573-12.

VITA
William J. Pandori

EDUCATION

2015-2020	*Ph.D.* Biological Sciences, University of California, Irvine
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RESEARCH EXPERIENCE

2015-Present	Graduate Research Assistant, University of California, Irvine Research Focus: Define the mechanisms by which *Toxoplasma gondii* activates the NLRP3 inflammasome and IL-1β production and release from human monocytes. Principal Investigator and Thesis Mentor: Dr. Melissa B. Lodoen
2014-2015	Research Volunteer, University of California, Irvine Research Focus: Explore mechanisms of chemotherapy resistance to maintenance drugs in B-cell leukemia Principal Investigator: Dr. David Fruman Mentor: Dr. Trang Vo
2012-2014	Undergraduate Research Assistant, University of California San Diego Research Focus: investigate evolutionary history of the human brain and architectural changes in the brains of people with William's Syndrome Principal Investigator and Mentor: Dr. Katerina Semendeferi
2010-2012	Undergraduate Research Assistant, University of California San Diego Research Focus: Study deficiencies in neuronal purine metabolism in Lesch-Nyhan Syndrome Principal Investigator: Dr. Theodore Freidman Mentor: Dr. Ghiabe Guibinga
2010	Undergraduate Research Assistant, University of Santa Clara Research Focus: Sequence and analyze the genomes of drug resistant strains of *Neisseria gonorrhea* circulating in the San Francisco bay area Principle Investigator and Mentor: Dr. David Hess

PUBLICATIONS

[7] **William Pandori**, J.P. Lawrence, William Gaieck, Maria Montchal, and Evelyn Valdez-Ward. "Science Policy for Scientists: A Simple Task for Great Effect" PNAS (2020) 1073/pnas.2012824117

[6] **William J. Pandori**, Tatiane S. Lima, Sharmila Mallya, Tiffany H. Kao, Lanny Gov, and Melissa B. Lodoen. "Toxoplasma gondii activates a Syk-CARD9-NF-κB signaling axis and gasdermin-D independent release of IL-1β during infection of primary human monocytes." *PLoS Pathogens* (2019) 15(8): e1007923.

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[1] David Hess, Abel Wu, Daniel Golparian, Sarah Esmaili, **Will Pandori**, Emilee Sena, Jeffrey D. Klausner, Pennan Barry, Magnus Unemo, and Mark Pandori. "Genome sequencing of a Neisseria gonorrhoeae isolate of a successful international clone with decreased susceptibility and resistance to extended-spectrum cephalosporins." *Antimicrobial Agents and Chemotherapy* 56, no. 11 (2012): 5633-5641.

ORAL AND POSTER PRESENTATIONS

2020	10th annual Southern California Eukaryotic Pathogens Symposium, Riverside, CA (talk)
2019	17th annual UC Irvine Immunology Fair, Irvine, CA (poster)
2019	La Jolla Immunology Conference, La Jolla, CA (poster)
2019	Immunology LA Conference, Los Angeles, CA (poster)
2019	15th Biannual International Congress on Toxoplasmosis, Quindio, Colombia (talk)
2019	Gordon Research Conference on Phagocytes, Waterville Valley, NH (talk & poster)
2019	Biological Sciences Seminar Series (Santa Clara University), Santa Clara, CA (talk)
2018	16th annual UC Irvine Immunology Fair, Irvine, CA (talk & poster)
2018	La Jolla Immunology Conference, La Jolla, CA (poster)
2018	8th annual Southern California Eukaryotic Pathogens Symposium, Riverside, CA (talk)
2018	Immunology LA Conference, Los Angeles, CA (talk)
2018	29th annual Woods Hole Immunoparasitology Conference, Woods Hole, MA (talk)
2018	Annual Federation of Clinical Immunology Societies (FOCIS) Conference, San Francisco, CA (talk & poster)
2017	15th annual UC Irvine Immunology Fair, Irvine, CA (talk & poster)
2017	Immunology LA Conference, Los Angeles, CA (poster)
2017	7th annual Southern California Eukaryotic Pathogens Symposium, Riverside, CA (poster)
2016	14th annual UC Irvine Immunology Fair, Irvine CA (poster)

HONORS AND AWARDS

2020	Krishna and Sujata Tewari scholar award for excellence in graduate research
2019	Best student presentation at Gordon Research Seminar (Phagocytes)
2019	Travel award for Santa Clara University Biological Sciences Seminar Series
2018	Travel award for 2018 FOCIS conference
2017-2019	T32 NIH Immunology Training Grant recipient

MENTORING AND TEACHING

Mentored 1 undergraduate student (now in Ph.D. program at UC San Diego) and 2 rotation students currently in the Lodoen Lab

2019	Biochemistry – Graduate TA UC Irvine
2019	Immunology – Graduate TA UC Irvine
2018	Parasitology – Graduate TA UC Irvine
2018	Microbiology Lab – Graduate TA UC Irvine

SERVICE AND LEADERSHIP

(2017-2020) DECADE Peer Mentor Program

- Completed the DECADE mentoring program course and served as a graduate student mentor for an undergraduate research student.

(2017-2020) Science Policy Advocate

- Co-authored an op-ed on how to get involved in science policy.
- Worked with local “March for Science” organizers
- Helped to run and host a podcast series on science policy
- Volunteered with American Cancer Society, helped develop a presentation on importance of cancer research and met with congressional representative to discuss a palliative care bill.
- Completed American Institute of Biological Sciences science communication bootcamp and discussed importance of basic research and NIH and NSF funding with congressional staff and representatives

ABSTRACT OF THE DISSERTATION

Regulation of the NLRP3 inflammasome and IL-1β production and release during *Toxoplasma gondii* infection of human monocytes

by
William Pandori

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2020

Associate Professor Melissa B. Lodoen, Chair

Toxoplasma gondii is an obligate intracellular eukaryotic parasite that is estimated to infect one-third of the global population and is especially life-threatening in developing fetuses and immunocompromised individuals. Innate immune cells contribute to host defense against *T. gondii* infection. Specifically, monocytes are rapidly recruited to sites of infection and CCR2 or CCL2 KO mice are more susceptible to *T. gondii* infection. Monocytes protect against infection by initiating a robust inflammatory response which is in part mediated by release of IL-1β during activation of the NLRP3 inflammasome. IL-1β is also implicated in the development of many autoimmune disorders like rheumatoid arthritis and atherosclerosis, but there is still much that is unknown about how human monocytes produce, process and release IL-1β.

We have identified that *T. gondii* induces IL-1β production via a Syk-CARD9-NF-κB signaling axis in primary human peripheral blood monocytes. Syk was rapidly phosphorylated during *T. gondii* infection of primary monocytes, and inhibiting Syk with the pharmacological inhibitors R406 or entospletinib, or genetic ablation of Syk in THP-1 cells, reduced IL-1β release. Inhibition of Syk in primary cells or deletion of Syk in THP-1 cells decreased parasite-induced transcription and production of pro-IL-1β protein. Inhibition of PKCδ, CARD9/MALT-1 and IKK also reduced p65 phosphorylation and pro-IL-1β production in *T. gondii*-infected primary monocytes, and genetic knock-out (KO) of PKCδ or CARD9 in THP-1 cells also reduced

pro-IL-1β protein levels and IL-1β release during *T. gondii* infection, indicating that Syk functions upstream of this NF-κB-dependent signaling pathway for IL-1β transcriptional activation.

We have also found that primary human monocytes treated with a caspase-8 inhibitor released less IL-1β after *T. gondii* infection than control cells. Similarly, caspase-1 and caspase-8 KO human monocytic THP-1 cells, but not caspase-4 or -5 KO cells were impaired in their release of IL-1β after infection compared to empty vector (EV) THP-1 cells. We found caspase-8 deficiency did not significantly affect the *pro-IL-1β* transcripts or production of pro-IL-1β protein. Similarly, caspase-8 had no significant affect on NLRP3 inflammasome activation or cleavage of pro-IL-1β to mature IL-1β during *T. gondii* infection. Instead, caspase-8 deficiency appeared to stunt the release mechanism of IL-1β from infected cells as mature-IL-1β would accumulate intracellularly in these KO cells.

IL-1β release from *T. gondii*-infected primary human monocytes did require an NLRP3-ASC-caspase-1 inflammasome. While the release mechanism of IL-1β during NLRP3 inflammasome activation often requires cleavage of gasdermin D (GSDMD), formation of pores in the cell membrane and induction of an inflammatory form of cell death known as pyroptosis, human monocytes released IL-1β independent of these factors. Taken together, our data indicate that *T. gondii* induces a Syk-CARD9/MALT-1-NF-κB signaling pathway and activation of the NLRP3 inflammasome for the release of IL-1β in a cell death- and GSDMD-independent manner. This research also describes a novel role for caspase-8 in IL-1β release from *T. gondii*-infected monocytes and contributes to the growing notion that IL-1β can be released from human myeloid cells independent of cell death.

Collectively, this research expands our understanding of the molecular basis for human innate immune regulation of inflammation and host defense during parasite infection.

Chapter 1

Introduction:

Monocytes and the regulation of inflammation in response to *Toxoplasma gondii* infection

Myeloid cell biology, differentiation and polarization

Myeloid cells, cells derived from the common myeloid progenitor stem cell population in the bone marrow, include megakaryocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, monocytes and some macrophages and dendritic cells (Fig.1.1). Megakaryocytes and erythrocytes contribute to clotting and the transport of oxygen through the body. Mast cells, basophils and eosinophils make large contributions to the allergic response and immune control of extracellular parasitic worms. Neutrophils, the most numerous innate immune cell in the blood, are short-lived cells that are professional phagocytes and contain granules used to trap and kill invading pathogens. Dendritic cells, on the other hand, are much longer lived, and while they do maintain phagocytic capabilities, are the major cells involved in antigen presentation and training of the adaptive immune system (1–3).

Macrophages and monocytes share some of the same functions as other myeloid cells but also play distinct roles in the immune response to pathogens and the homeostasis of various tissues. Macrophages are long-lived cells that can be derived either from peripheral blood monocytes or a preliminary wave of stem cells from the yolk sac early in development (4,5). Yolk sac derived macrophages constitute many of the tissue resident macrophage populations, including microglia, alveolar macrophages, Langerhans and Kupffer cells (5). These tissue resident cells are maintained by local stem cell populations and display distinct transcriptional profiles while also performing distinct functions (3,4,6). Peripheral blood monocytes do seed other macrophage and dendritic cell populations in organs like the gut and can even replace tissue resident cells originally derived from the yolk sac if the resident stem cell populations are depleted (7,8).

Macrophages utilize their phagocytic and cytokine producing capabilities to contribute to the innate immune response to pathogens in tissue and help to maintain tissue homeostasis (4,5,9). Macrophages are excellent scavengers and remove dead cells and debris through the

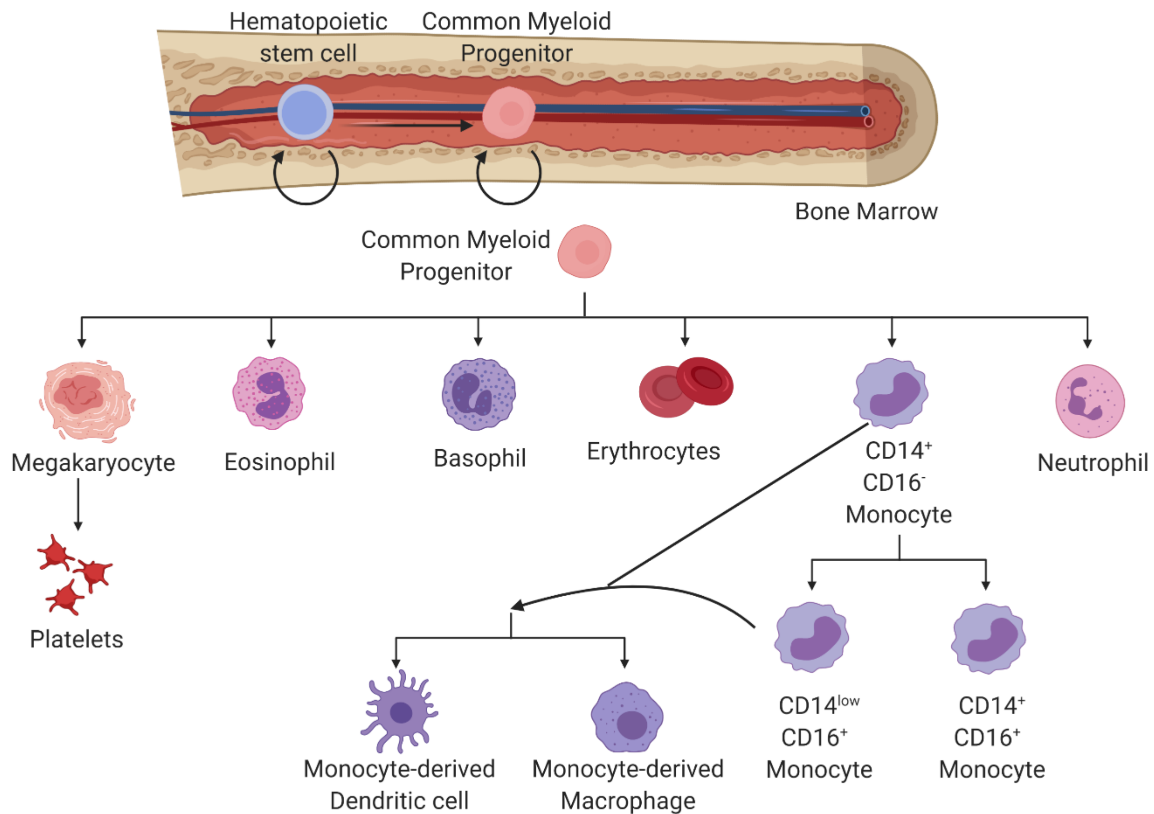


Fig. 1.1. Hematopoiesis of Myeloid Cells. The hematopoietic stem cell (HSC) resides in the bone marrow where it self-renews and can differentiate to the common lymphoid progenitor or the common myeloid progenitor. The common myeloid progenitor will also self-renew in the bone marrow, but it only contains the potential to differentiate into any myeloid cell. Most of these differentiation steps occur within the bone marrow. $CD14^{+}CD16^{-}$ classical inflammatory monocytes are produced within the bone marrow, but they appear to differentiate to $CD14^{low}$ $CD16^{+}$ non-classical patrolling monocytes and $CD14^{+}CD16^{+}$ intermediate monocytes in the vasculature. The classical inflammatory and non-classical patrolling monocytes also have the potential to differentiate to monocyte-derived macrophages and dendritic cells in peripheral tissues.

engagement of specialized receptors, in a process called efferocytosis (9). Macrophages largely recognize apoptotic cells through binding of phosphatidylserine on the cell surface. There are many receptors that can bind this “eat me” signal, including Mer-TK, Tim family proteins, integrin αvβ3/5, and RAGE. Calreticulin, a different “eat me” signal is also recognized by CD91 in macrophages. Engagement of each of these receptors ultimately leads to RAC1 activation, changes in actin dynamics, and the engulfment and recycling of the dead cells. Conversely, CD47 functions as a “don’t eat me” signal that helps to differentiate self from non-self. CD47 will bind SIRPα on macrophages leading to signaling through ITIMs that results in decreased phagocytosis through inhibition of cytoskeleton rearrangements. Additional receptors called scavenger receptors help to bind and uptake cell debris and other smaller harmful products like heat shock proteins, LDLs and Amyloid-β (9–13).

Macrophages can also sense extracellular viral, bacterial, fungal and parasitic pathogens by directly binding to the pathogen, binding of pathogen products or through sensing of other PAMPs and DAMPs associated with the pathogen which activate intracellular and extracellular receptors. These receptors include Toll-like receptors (TLRs) that can be extracellular or intracellular, intracellular Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and sensors of intracellular DNA and RNA like STING and RIG-I respectively. Once stimulated, these receptors can lead to production and release proinflammatory cytokines like IL-1β, IL-6, IL-12 and TNF-α, which contribute to the control of pathogens by activating other cells of the immune system or the induction of antimicrobial processes (1,9,14–18).

However, macrophages do not serve only a proinflammatory role during infection. Macrophages are extremely plastic cells and upon stimulation can be temporarily “polarized.” Historically, macrophages were described as polarizing towards a more inflammatory Th1-like M1 phenotype or anti-inflammatory Th2-like M2 phenotype (10,19–21). Polarization to the M1 phenotype can be induced through LPS stimulation, culturing with GM-CSF or exposure to IFNγ

produced by T cells and NK cells (20). In addition to the release of proinflammatory cytokines, M1 polarized macrophages will upregulate MHCII expression on their cell surface and produce greater amounts of ROS to assist with the killing and processing of pathogens (20). These cells can cause serious damage to surrounding tissues if left unchecked, so as might be expected, the M2 alternatively activated macrophages can directly counteract the effects of M1 macrophages (9,19–21). M2 macrophages are characterized by the production and release of anti-inflammatory cytokines and effectors that promote tissue repair such as IL-10, TGF-β, CCL22 and VEGF. Instead of the transcription factors that are indicative of M1 macrophages like STAT1 and NF-κB, M2 macrophages rely on STAT3, STAT6 and cMyc. These cells do retain phagocytic capabilities and are especially adept at clearing cellular debris and promoting the regeneration of nearby tissues (9,19–21).

With advances in sequencing it has also become apparent that there are many different transcriptional states of stimulated macrophages that cannot be described simply as M1 or M2. Instead macrophages display a much wider spectrum of transcriptional states (22). For example, M2 macrophages can be subdivided into M2a, M2b, M2c and M2d subsets (19). The M2b subset, although clustering with other M2 macrophages, does still produce the proinflammatory cytokines IL-1β and TNF-α, and plays a significant role in clearance of bacterial and parasitic infections. Meanwhile the M2d subset makes more IL-10 and VEGF helping to promote angiogenesis (19–21). The capabilities of these polarized cells, and the combinations of stimulations and environmental cues that promote these polarizations, are still being elucidated and will hopefully lead to the development of more precise therapeutics that can influence targeted subsets of macrophages only in specific settings or conditions.

Monocyte biology and subsets

Monocytes are also descended from the common myeloid progenitor in the bone marrow. After leaving the bone marrow, monocytes mostly circulate in the blood, where they constitute roughly 10% of all PBMCs in humans (23). Once in circulation they can reseed monocyte-derived macrophage populations, differentiate into dendritic cells, respond to infection and inflammatory signals, and patrol the vasculature (2,23–25). Monocytes are also much shorter-lived cells than macrophages and dendritic cells. Approximately half of circulating monocytes will die and be recycled within 24 hours of leaving the bone marrow, while the other half undergoes differentiation in tissues (26). The induction of inflammation can also extend the lifespan of monocytes, as the inflammatory machinery inside the cells can inhibit apoptosis until inflammation is resolved (15).

Human monocytes are normally separated into three subsets depending on their expression of the cell surface markers CD14 and CD16, or two subsets in mice based on their expression of Ly6C. Classical inflammatory monocytes are identified as $CD14^{+}$ and $CD16^{-}$ and the mouse counterpart is $Ly6C^{hi}CX3CR1^{int}CCR2^{+}CD62L^{+}CD43^{low}$. Intermediate monocytes are $CD14^{+}$ and $CD16^{+}$, whereas nonclassical patrolling monocytes are $CD14^{low}$ and $CD16^{+}$. The murine counterpart for the nonclassical population is $Ly6C^{low}CX3CR1^{hi}CCR2^{low}CD62L^{+}CD43^{+}$ (Table 1.1)(25,27–29). Beyond just CD14 and CD16, CCR2, HLA-DR, CD36 and CD11c have also been used to discriminate among these three populations with more accuracy (25,30). In humans the classical inflammatory monocytes constitute about 85% of circulating monocytes, whereas the intermediate monocytes are about 5%, and nonclassical patrolling monocytes are about 10% of circulating monocytes (24,25). Monocyte depletion experiments have also suggested that the classical inflammatory population appears to be mobilized from the bone marrow, whereas the other populations differentiate from these classical monocytes within the vasculature (Fig 1.1)(24,31,32).

With the development of CyTOF and advances in single-cell sequencing, up to 8 human monocyte subsets have now been described (30). The classical monocytes can be separated into 4 subsets and the nonclassical monocytes can be split into 3 subsets (30). Focus on the roles of each of these subsets of cells is critical for the understanding of different monocyte-driven diseases as, similar to macrophages, each subset can perform unique functions. For example, nonclassical patrolling monocytes have long been associated with the development of atherosclerosis (1,30,32), but other findings show that while a $Slan^{+}CXCR6^{+}$ nonclassical patrolling monocytes monocyte population is expanded in coronary artery disease another nonclassical $Slan^{+}CXCR6^{-}$ subset increases its efferocytosis capabilities, which has previously been associated with protection from disease progression (30).

Classical inflammatory monocytes display higher expression of CCR2 than other subsets and are therefore much better at migrating towards CCL2 and CCL3 gradients. This likely not only helps monocytes leave the bone marrow in large numbers during infection, but it also directs classical inflammatory monocytes to injured or inflamed tissues where they can differentiate (25,28). Experiments involving monocyte depletion in the blood suggest that these classical inflammatory monocytes, in part due to their prevalence compared to other monocyte subsets, are the primary precursors of monocyte-derived tissue resident macrophages (31,32). Therefore, this population of cells has a major influence not only on inflammation in the blood and at endothelial barriers, but also on continuation and resolution of inflammation as they differentiate into other effector cells. Classical monocytes are also much more adept in production of ROS than other subsets and produce higher levels of inflammatory cytokines like IL-6 and the chemokine CCL2 upon stimulation (25,27,33).

Compared to other monocyte subsets, much less is known about intermediate monocytes. They present more antigen on MHC class II compared to other subsets, produce proinflammatory cytokines upon stimulation, and interestingly, are more susceptible to HIV

infection due to higher expression of CCR5 (25,27,33,34). Expansion of the intermediate monocyte population has also been associated with rheumatoid arthritis and sepsis (25,27,34). This population greatly expands during systemic infection or inflammation and correlates with worse clinical outcome during sepsis (34,35). Collectively the data on these cells suggest that they significantly contribute to inflammation in response to pathogens but may lack some of the necessary functions to help resolve inflammation. However, investigations have also found that intermediate monocytes produce the majority of anti-inflammatory IL-10 compared to other monocyte subsets during TLR stimulation. These diverse and seemingly contradictory roles of intermediate monocytes warrant further investigation, but the lack of an equivalent cell population in mice makes this research difficult (34,35).

Nonclassical patrolling monocytes express higher levels of CX_3CR1, which binds to fractalkine, and likely assists in their patrolling behaviors, adherence to the endothelium, and diapedesis into tissues expressing CX_3CL1 (fractalkine) (25,33). Nonclassical monocytes can also present antigen, but are more associated with anti-inflammatory behaviors, wound healing, and angiogenesis than other monocyte subsets (25,33). They do not produce nearly as high of levels of proinflammatory cytokines compared to classical monocytes upon stimulation, and, as their name suggests, this subset is thought to patrol the endothelium in search of infection or injury (25,27,33). Therefore, they are among the first cells to detect an insult in the vasculature and play an important role in the recruitment of other monocytes and neutrophils through TNF-α secretion (25,27). Like classical inflammatory monocytes, nonclassical patrolling monocytes can also differentiate in tissues to give rise to monocyte-derived macrophages and dendritic cells (25,27). Whereas classical monocytes are major contributors to the control of several fungal and parasitic infections, nonclassical monocytes appear to be just as critical for the control of viral infection, likely due to their increased ability to present antigen, differentiate to dendritic cells, and identify infected cells through their patrolling behavior (34,36).

Table 1.1. Human and mouse monocyte subsets and their properties

Monocyte Subset	Classical Inflammatory	Intermediate	Nonclassical Patrolling
Cell Surface Markers	(Human) $CD14^{+}CD16^{-}$ (Mouse) $Ly6C^{hi}CX3CR1^{int}CCR2^{+}$ $CD62L^{+}CD43^{low}$	(Human) $CD14^{+}CD16^{+}$ (Mouse) Not well defined	(Human) $CD14^{low}CD16^{+}$ (Mouse) $Ly6C^{low}CX3CR1^{hi}CCR2^{low}$ $CD62L^{+}CD43^{+}$
Most prominent response to stimulation	IL-6, IL-10, CCL22, ROS	Low IL-6 and IL-10	TNFα, IL-1β
% of Monocyte Population	~85%	~5%	~10%
Distinguishing functions	• Recruited by CCL2 and CCL3 binding CCR2 • Major producer of both inflammatory and anti-inflammatory cytokines • Largely phagocytic • Circulates in vasculature • Differentiates to mostly macrophages and some dendritic cells	• Mixture of proinflammatory and anti-inflammatory cytokine release • Correlated with sepsis and rheumatoid arthritis	• Recruited by Cx3CL1 binding Cx3CR1 • Produces mostly inflammatory cytokines and chemokines • Attracts neutrophils to sites of infection • Patrols on vasculature • Performs antigen presentation • Differentiates to mostly dendritic cells and some macrophages

Taken together, human monocyte subsets display remarkable heterogeneity in their surface marker expression and function. Classical monocytes exhibit a more pro-inflammatory phenotype via their ability to secrete soluble mediators and to differentiate into monocyte-derived DCs to bridge innate and adaptive immune responses. Intermediate monocytes are specialized in antigen presentation and play important roles in rheumatoid arthritis and sepsis, and nonclassical monocytes contribute to continual surveillance of the endothelium, recruitment of neutrophils through TNF-α release and the antiviral response (Table 1.1). Once we reach a better understanding of the capabilities of each monocyte subset and how they contribute to their roles, it may be possible to specifically manipulate them to influence these processes.

Monocyte sensing of pathogens

In addition to functioning as the progenitors of some macrophages and dendritic cells, monocytes are also fully functional immune cells in their own right. Similar to macrophages, monocytes are dynamic responders during infection with a wide variety of pathogens and utilize many of the same cell surface and intracellular receptors to respond to these pathogens. Monocytes are present in draining lymph nodes during steady state conditions and can present antigen through MHC class II, like dendritic cells, though MHC class II is more highly expressed in dendritic cells and macrophages than it is in monocytes (6,37–40). Human monocytes also respond to some infections in ways that are unique compared to macrophages.

Monocytes sense viral pathogens through TLR2, which can sense vaccinia virus through recognition of viral glycoproteins and other viral components at the plasma membrane, and TLR3 recognition of dsRNA, TLR7/8 recognition of ssRNA, or TLR9 recognition of dsDNA in the endosome (14,18). These receptors lead to MyD88 and TRIF/TRAF activation inducing NF-κB and IRF3/7 translocation resulting in the production of inflammatory cytokines like TNF-α, IL-1β, and IL-6, as well as type-1 interferons that participate in antiviral defense and the recruitment of

leukocytes to areas of infection (14,18,33,36,41). Dengue, West Nile virus, measles, and Herpes simplex virus all activate TLR2 on monocytes, and monocytes have been shown to be critical for control of both Dengue and West Nile virus in mouse models of infection (14,18,36,41). Monocytes can also recognize cytosolic dsDNA through receptors such as AIM2 (42).

The inflammation induced in response to viral infection coupled with DAMPs released from infected and dead cells can become pathogenic if left unchecked. For example, during influenza infection of the lungs, type-1 interferons stimulate nearby cells to produce MCP-1 (CCL2), which acts to attract more classical inflammatory monocytes to areas of infection (1,14). Although they do assist in the destruction of virus, an accumulation of pro-inflammatory monocytes in the lungs has been implicated as a major driver of lung injury during infection and is correlated with morbidity and mortality during infection (43). Indeed, it appears that proinflammatory monocytes also contribute to pathology in the brain during LCMV infection and pathology in the lungs during SARS-COV-2 infection (44–46).

Monocytes also respond to bacterial infection through recognition of bacteria or bacterial products sensed by intracellular and extracellular TLRs and the NLRC4 inflammasome which senses cytosolic flagellin and the type 3 secretion system (47–50). Depletion of CCR2, and therefore depletion of circulating monocytes, makes mice more susceptible to many bacterial infections, including infection with the Gram-positive bacteria *Listeria monocytogenes* (50–52). TLR signaling in monocytes was also essential for defense against *L. monocytogenes*, suggesting that the initial monocyte sensing and response to infection, and not only activation of monocytes by cytokines from other innate immune cells, was required for controlling *L. monocytogenes* (50). Monocytes can also directly kill bacterial pathogens through engagement of Fcγ receptors, leading to phagocytosis, formation of the phagolysosome, production of ROS, and activation of iNOS and nitric oxide species (49,52,53).

Monocytes also express receptors that facilitate detection of fungal pathogens or fungal components. Whereas TLRs, complement receptors, Fcγ receptors and the NLRP3 inflammasome have all been implicated in the control of fungal infections (54–56), cell surface C-type lectin receptors (CLRs) are the major receptors that bind specific fungal products to induce an immune response (16,49,55,57,58). In monocytes the CLR Dectin-1 recognizes β-1,3-glucans present in the cell wall of *Aspergillus* and *Candida*, whereas Dectin-2 and Mincle bind α-mannan (16,58,59). Unlike TLRs, CLRs do not activate MyD88 or TRIF to induce downstream signaling. Instead, CLRs recruit spleen tyrosine kinase (Syk) through their ITAM domains, which can activate downstream PKCδ-CARD9-BCL10-MALT1 signaling leading to NF-κB translocation and the production of proinflammatory cytokines like TNF-α, IL-1β, and IL-12 (60).

Deletion of CCR2 or any combination of CLRs, TLR2, TLR4 and NLRP3 have all been associated with greater fungal burden in mice during challenge (49,55–58). Human susceptibility to *Candida* infections has been correlated to SNPs in cytokines produced by monocytes, and monocyte-produced TNF-α in a GWAS study (61–64). Again, these data indicate that monocyte direct sensing of these pathogens significantly contributes to host defense against fungal infection.

Parasitic helminths and protozoa also cannot escape recognition by monocytes. Given the diversity of parasites, the recognition mechanisms used to alert monocytes and the rest of the immune system to infection can vary greatly between each parasite. Therefore, it is perhaps unsurprising that multiple studies have implicated activation of complement receptors, Fcγ receptors, scavenging receptors, TLRs, and NLRs in monocytes as being critical for host defense during parasitic infections (49,52,65–68). In response to large multicellular helminths such as *Schistosoma mansoni*, inflammatory monocytes are recruited from the bone marrow or periphery in response to a CCL2 gradient (52,69). At sites of infection, these inflammatory

monocytes will become polarized and start to release Th2 related cytokines such as IL-4 and IL-13. Monocytes will also differentiate to M2 polarized macrophages in areas of infection to assist with production of RALDH2 and PD-L2, which also help to coordinate the immune response against helminths (49,52,68–70). Monocytes and macrophages will attempt to recruit more neutrophils and eosinophils to sites of infection and either "trap" helminths in large granulomas or release cytotoxic granules that can kill these large multicellular parasites (49,52,71,72).

Regarding protozoan infections, monocytes participate in the control of various *Plasmodium* species in the blood (39,73,74). During infection with *Leishmania*, they also contribute to activation of the adaptive immune response through antigen presentation, cytokine production and produce ROS and iNOS (75–77). However, sometimes these responses can be so intense that they cause tissue damage and harm to the host. For example, inhibition of some inflammatory monocyte activity through depletion of CCR2 or addition of IL-10 helps to dampen inflammatory signaling and reduce the severe tissue damage that routinely occurs during infection with *Trypanosoma brucei* or *Plasmodium berghei* cerebral infections (78–80). Monocytes and monocyte-induced responses are also important in protection against infection with the obligate intracellular protozoan parasite *Toxoplasma gondii*, as CCL2 KO and CCR2 KO mice are more susceptible to *T. gondii* infection (49,81,82). CCL2 or CCR2 KO mice do not release monocytes from the bone marrow and lack circulating monocytes and monocyte derived macrophages and dendritic cells.

***Toxoplasma gondii* biology and immunity**

Toxoplasma gondii is a single-celled eukaryotic parasite. It was first identified in 1908 independently by Alphonso Splendore (83) and Charles Nicolle and Louis Manceaux (84) and belongs to the phylum *Apicomplexa,* making it closely related to other apicomplexans like *Plasmodium*, the causative agent of malaria. *T. gondii* is a remarkable pathogen in that it can

infect any nucleated cell in any warm-blooded animal (85). This foodborne pathogen is also estimated to infect 1/3rd of the global population, making it one of the most successful human pathogens (86,87).

The life cycle of *T. gondii* starts in the digestive tract of felids, the only known definitive host for the parasite, within which *T. gondii* undergoes sexual replication (88). After sexual replication in the cat intestine, oocysts containing sporozoites are shed through the feline digestive tract (89). The oocyst is highly infectious after it sporulates at ambient temperatures, and it can remain viable and infectious for long periods of time in the environment (90) where oocysts contaminate water supplies, attach to crops, or be ingested by other animals which then serve as intermediate hosts of the parasite (91). Upon consumption, digestive enzymes activate the sporulated oocyst. The sporozoites emerge from the oocyst, infect the intestine and convert to rapid growing tachyzoites (91–93). Tachyzoites, indicators of acute *T. gondii* infection, will then invade host cells in the intestine where they replicate asexually, eventually lysing these host cells and allowing for dissemination into the vasculature and distant tissues like the brain and heart (94–98). During acute infection tachyzoites can also cross the placenta of pregnant women, leading to vertical transmission of infection, which can be life-threatening to the developing fetus (99–103). Subsequent dissemination cues from the immune response and gene expression changes in the parasite then drive the highly proliferative and destructive tachyzoites to stage convert to encysted and slow-growing bradyzoites (104–106). Bradyzoite-containing tissue cysts in intermediate hosts are also orally infectious. Consumption of raw or undercooked meat from infected animals leads to infection in other intermediate hosts and can lead to reinfection of a felid where the parasite life cycle can start anew (Fig. 1.2) (85,88,91).

In immune competent infected hosts, acute infection inevitably leads to a chronic infection, since the tissue cysts persist in long-lived tissues and are never fully cleared by the

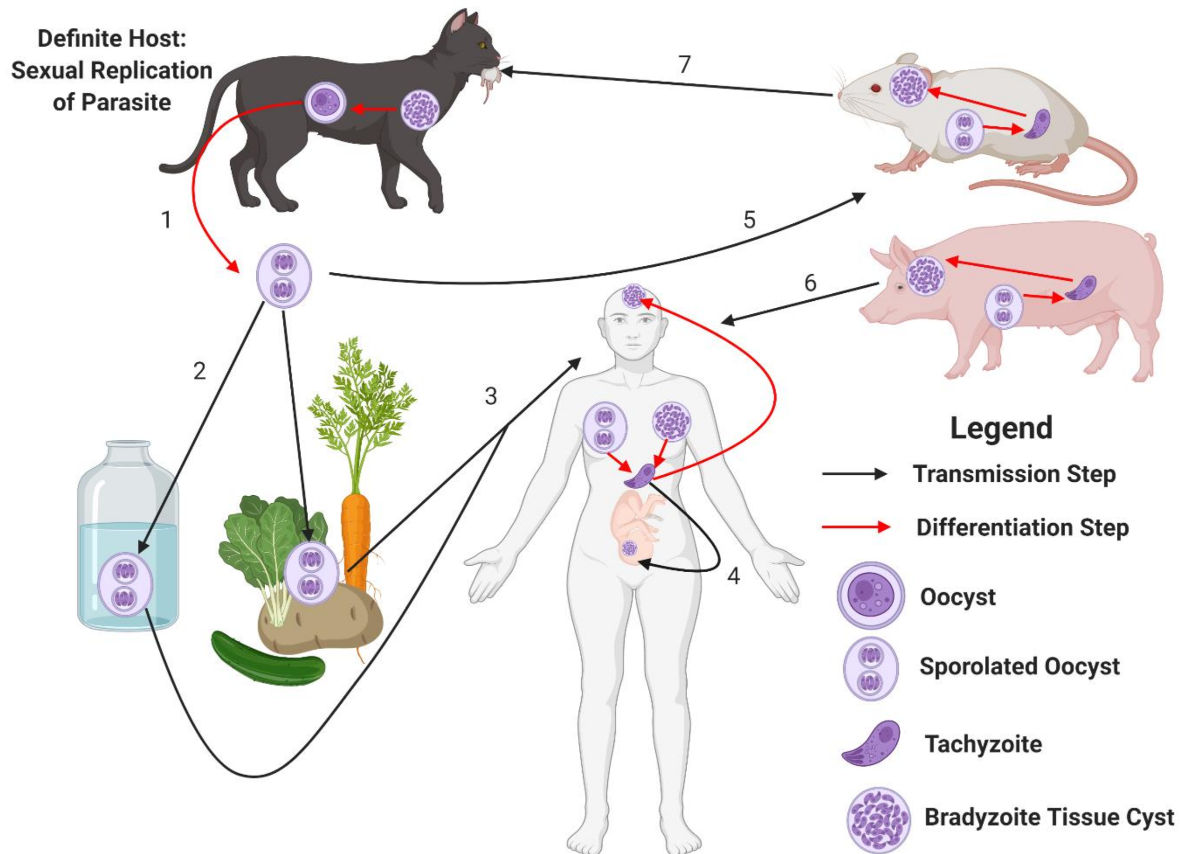


Fig. 1.2. Life cycle and dissemination of *Toxoplasma gondii*. *Toxoplasma gondii* undergoes sexual replication within the gut of a felid and (1) is excreted as an oocyst. The oocyst then sporulates in the environment. In the environment the oocyst can (2) contaminate food and water supplies, which can then be (3) consumed by intermediate hosts such as humans. Upon consumption the sporozoites infect the gut, differentiate to tachyzoites and infect tissues such as the muscle, brain and heart. The tachyzoite can also be (4) vertically transmitted from mother to fetus across the placenta. The immune response will trigger tachyzoites to differentiate to bradyzoites, which form cysts in long-lived tissues. Other intermediate hosts like rodents and livestock can also become infected by (5) consuming sporulated oocysts in the environment. (6) Consumption of undercooked meat from intermediate hosts containing the parasite can also lead to infection. If a felid (7) consumes an oocyst containing sporozoites in the environment those sporozoites will undergo sexual recombination in the gut, but if a bradyzoite is consumed from infected prey animals, the bradyzoite will differentiate to sporozoites in the gut before undergoing sexual recombination, perpetuating the life-cycle and infection-cycle of the parasite.

immune response. This may partially account for the high prevalence of *T. gondii* infection globally. Moreover, if an infected individual becomes immunocompromised, the encysted bradyzoites will differentiate back into tachyzoites and cause a reoccurrence of infection that may not be restrained (106,107). Indeed, toxoplasmosis and Toxoplasmic encephalitis are a major cause of mortality in AIDS patients and organ and bone marrow transplant recipients (108–111).

Given the sheer numbers of new *T. gondii* infections each year, *T. gondii* is still a leading cause of hospitalizations and deaths due to a foodborne pathogen in the United States. It has been classified as a neglected parasitic infection by the CDC (112,113). Combinations of pyrimethamine and sulphadiazine are used to treat acute *T. gondii* infection, but these treatments have serious side effects, and if administered too late will not control the infection (114–117). Fortunately, the human immune response to *T. gondii* infection is robust, and acute infection typically presents only as mild flu-like symptoms in immune competent individuals (118).

During *T. gondii* infection, monocytes traffic to areas of infection in response to CCL2 gradients (81,82,119–121). However, while monocytes can phagocytose *T. gondii* at sites of infection, parasite invasion of monocytes occurs faster (122,123), and monocytes are preferentially infected by the parasite compared to other PBMCs. To infect its host cell, the tachyzoite stage of *T. gondii* secretes adhesins from parasite organelles called micronemes and rhoptries to facilitate attachment to the host cell membrane. The interaction of the rhoptry neck protein (RON2) with AMA1 enables the parasite to establish invasion of host cells (124–126). *T. gondii* actively invades host cells using actin-based machinery. During this process the host cell membrane is invaginated as the parasite pulls itself into the host cell. This excludes integral proteins from the membrane in the process of forming the parasitophorous vacuole (PV). shedding many markers on the plasma membrane along the way. The (PV) forms a relatively

immune silent niche in which the parasite replicates within the cytosol (122,123,127,128). From this PV the parasite will also feast on nutrients within the cytosol and alter host cell immune functions by secreting effector proteins from the dense granules, GRAs (129–133). GRA proteins, like GRA15 and GRA24, can modulate NF-κB and MAPK signaling pathways within host cells and induce or reduce the inflammatory response that monocytes are able to mount against infection (130,134,135).

In addition to replicating within monocytes, *T. gondii* can also likely use the motility of immune cells it has invaded to assist its dissemination to other tissues (136,137). In fact, the parasite can induce a hypermotility phenotypes in its host cells by altering integrin adhesions dynamics, which may contribute to its ability to cross formidable biological barriers such as the blood brain barrier or the maternal-fetal interface (138,139).

Despite *T. gondii's* ability to infect monocytes and hijack their function, monocytes and monocyte-induced responses have also been highly implicated in protection against infection, as CCL2 KO and CCR2 KO mice are more susceptible to *T. gondii* infection (81,82,120,121,140). Ultimately, immune protection against *T. gondii* infection requires production of IL-12 from monocyte-derived dendritic cell and macrophage populations as well as an IFN-γ from NK cells, T cells, and neutrophils (120,141–144). IFN-γ exerts its antimicrobial functions through production of ROS and nitric oxide, amino acid starvation, and activation of GTPases in mice which can directly disrupt the PV and lead to clearance of the intracellular parasites (Fig. 1.3)(129,143,145–148).

Infections of several TLR KO and MyD88 KO mice have revealed that these receptors and the common MyD88 adaptor protein contribute to IL-12 production by myeloid cells and ultimately IFN-γ production by T cells during *T. gondii* infection. Knockout mice are highly susceptible to infection, suggesting direct sensing of the parasite or parasite products through TLRs by myeloid cells is important for mediating immunity against infection (95,144). Follow up

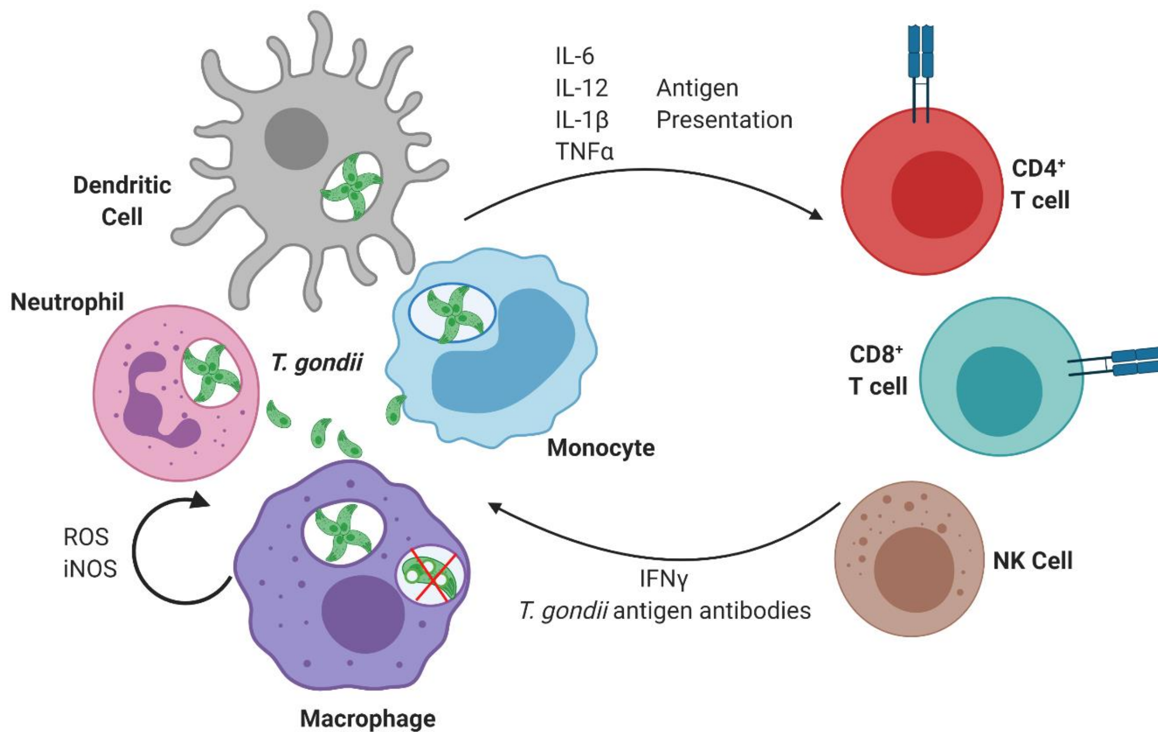


Fig. 1.3. Immune response to *T. gondii* infection. *T. gondii* tachyzoites can both invade and be phagocytosed by innate immune cells such as neutrophils, dendritic cells, macrophages and monocytes. Together these cells will then produce and release cytokines such as IL-6, IL-12, IL-1β and TNF-α. These cytokines and presentation of antigen through MHC class II can help to prime and activate CD4+ T cells, CD8+ T cells and NK cells. The lymphocytes produce IFN-γ and recognize *T. gondii* antigens. IFN-γ further activates myeloid cells and helps induce anti-microbial responses like the activation of several GTPases and production of ROS and nitric oxide. Eventually B cells will also produce and release *T. gondii* antigen-specific antibodies.

experiments in mice showed various TLRs, including TLR2, TLR4, TLR7 and TLR9 could detect soluble parasite antigens leading to production of IL-12 and TNF-α during infection. Specifically, TLR11 and TLR12 heterodimers bind to *T. gondii* profilin, leading to downstream inflammatory cytokine production, and deletion of parasite profilin leaves mice more susceptible to infection (149–153).

However, how human myeloid cells sense the parasite and induce an inflammatory response is still puzzling. TLR11 and TLR12 are not functional in humans, as they are represented by a pseudogene (154), and other human TLRs do not appear to bind *T. gondii* profilin or other *T. gondii*-derived products (129,149,152,155,156). Yet, human monocytes, but not mouse monocytes or human macrophages infected with *T. gondii* can induce rapid NF-κB activation and release of IL-1β, which depends on activation of the NLRP3 inflammasome (66,134,157–160). This suggests that there are TLR-independent sensing mechanisms for *T. gondii* infection at work in human monocytes. The identity of these hypothetical receptors, and the differences between human monocytes and macrophages or murine monocytes that can account for these differing responses to infection remain large questions in the field of *T. gondii* immunity. Further investigation into how the NLRP3 inflammasome is activated in these cells during infection may shed light on the sensing mechanisms that human monocytes use to respond to *T. gondii* infection.

NLRP3 sensing of *T. gondii* and IL-1β production

Work from our lab has shown that nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing protein 3 (NLRP3) is required for the IL-1β response to *T. gondii* infection in human monocytes (66,134,157). NLRP3, like other NLRs, is a cytosolic sensor protein that can sense a diverse set of PAMPs and DAMPs (161,162). NLRP3 is the best studied of the NLRPs and can be activated by the largest variety of stimuli. Once

stimulated, NLRP3 will oligomerize into a multi-protein complex call an inflammasome, which serves to process and release the pro-inflammatory IL-1 family cytokines (163–166).

To induce formation of the NLRP3 inflammasome and release of bio-active IL-1β, both a priming signal and activation signal are required. The priming signal, which could be stimulation of a TLR, CLR or other immune receptor, leads to NF-κB activation and the production of *NLRP3* and *IL-1β* transcripts and pro-IL-1b protein (166–168). After priming, the second activation signal, which for NLRP3 could be various PAMPs or DAMPs, such as extracellular ATP or monosodium urate (MSU) crystals, induces potassium efflux (162,165,167). The potassium efflux will generate structural changes in NEK7, which normally binds NLRP3 in an inactive state, now allowing it to oligomerize with other NLRP3 units (161,169–171). NLRP3 contains a pyrin domain, which binds the adaptor protein ASC. ASC acts as a bridge between the NLRP3 oligomers and caspase-1, which it binds using a CARD domain (172–174). This brings caspase-1, which is a zymogen, into close contact with itself allowing for auto-proteolytic processing. Now active, caspase-1 cleaves pro-IL-1β, which is biologically inactive, into mature IL-1β, which can now be released from the cell to bind its receptor (Fig. 1.4)(14,175–177).

Curiously, a single priming signal, like LPS stimulation or *T. gondii* infection, can also induce activation the NLRP3 inflammasome and release of bioactive IL-1β without the need for a second signal (171,178–181). So far this phenotype has only been observed in human monocytes and neutrophils, but not in murine monocytes or human macrophages. Human monocytes also activate a non-canonical inflammasome where stimulation with intracellular LPS binding endosomal TLR4 activates caspases 4 and 5 and NLRP3 resulting in IL-1β release, again without a secondary activating signal (179,182–185). An alternative NLRP3 inflammasome has also been characterized in human monocytes in which extracellular LPS alone can activate the NLRP3 inflammasome in a process that now requires caspase-8 but is independent of potassium efflux (178).

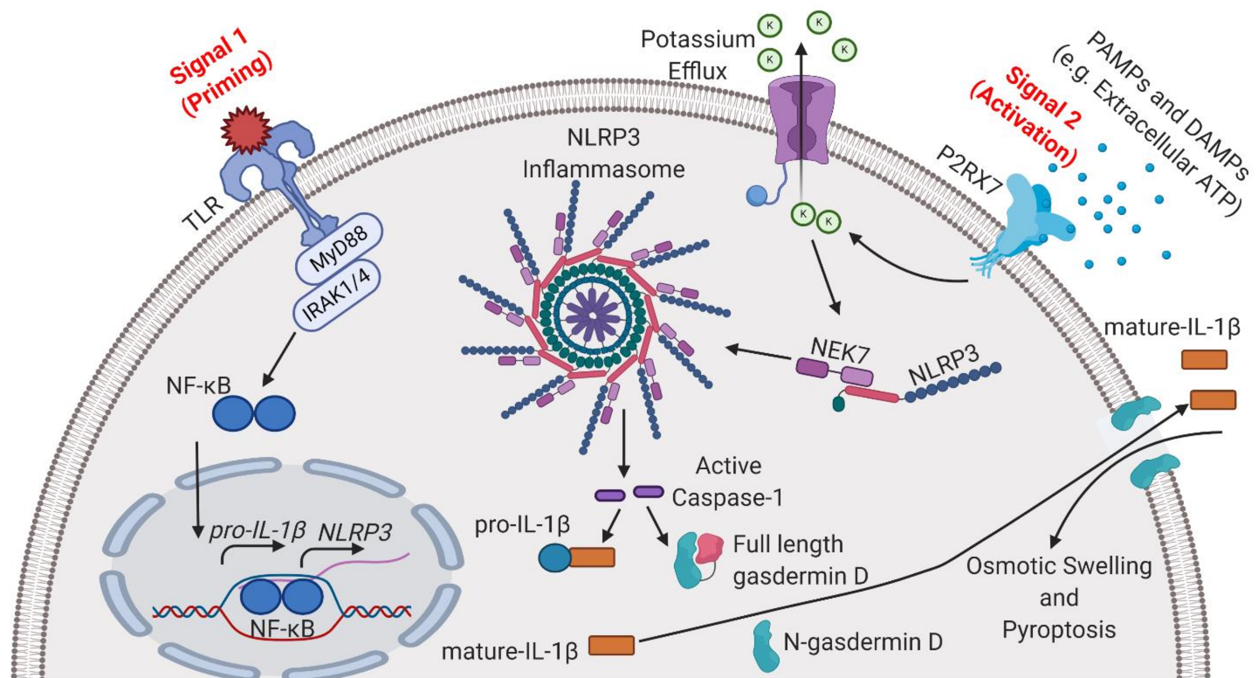


Fig. 1.4. Two step model of NLRP3 inflammasome regulation and IL-1β release. The canonical two step NLRP3 inflammasome requires a priming and activation signal. The priming signal induces NF-κB translocation and transcription of *pro-IL-1β* and *NLRP3*. The activating signal 2 converges on potassium efflux, which causes structural changes in NEK7. This structural change frees the NLR region of NLRP3 and allows it to nucleate into an inflammasome complex with ASC and caspase-1. Caspase-1 undergoes autoproteolysis for activation and cleaves pro-IL-1β to mature IL-1β. Active caspase-1 also cleaves gasdermin D. The N-terminal fragment of gasdermin D binds to the inner plasma membrane and forms a pore structure. The pore then allows for passive release of IL-1β from viable cells and osmosis-driven cell swelling followed by lysis in an inflammatory form of cell death called pyroptosis.

Inflammasome activity and downstream IL-1β and IL-18 release have been shown to contribute to control of *T. gondii* infection in mice and humans (134,186–189). A GWAS study identified SNPs in inflammasome components that increased susceptibility to congenital toxoplasmosis (159), addition of recombinant IL-1β is beneficial for mouse survival during *T. gondii* infection (187), and NLRP3 has been shown to contribute to clearance of intracellular *T. gondii* from macrophages through production of ROS (186). Contributions of the NLRP3 inflammasome to control of infection may also be explained by IL-1 family cytokine release contributing to IFN-γ production by T cells and NK cells during infection (Fig. 1.4).

These data indicate that human monocytes are uniquely sensitive to NLRP3 stimulation, and by understanding the mechanisms that drive priming and activation of the inflammasome during *T. gondii* infection we may learn more about how human monocytes sense and induce an effective inflammatory response during a challenge with this parasite.

Interleukin-1β history and contributions to immunity and pathology

IL-1β was first purified in 1977 and cloned in 1984 (190–192). These steps were revolutionary as they showed that a single endogenous molecule, and not a mixture of several factors, could be a pyrogen (induce fever). In fact, IL-1β is the single most potent known pyrogen and, as discussed, significantly contributes to protection against infections with many different pathogens (190,193). The cloning of IL-1β also soon led to the cloning of its receptor (194), and the observation that the cytoplasmic domain of this receptor closely resembled the Toll protein in *Drosophila* triggered the investigations into human TLRs and their effects in immunity. The powerful stimulating properties of IL-1β has even led to its use in human patients to promote bone marrow recovery (195).

However, due to its potency, IL-1β is also associated with the development of several autoimmune disorders. Diseases linked to aberrant IL-1β production include adult onset Still's

disease, rheumatoid arthritis, atherosclerosis, type 2 diabetes, CAPS, Alzheimer's disease and gout (166,195–205). Anti-IL-1 antibodies like canakinumab, Rilonacept, a soluble IL-1 receptor which can neutralize soluble IL-1, and anakinra, a modified IL-1 receptor antagonist, have been approved for treatment of rheumatoid arthritis and tested in Still's, CAPS, multiple myeloma, type 2 diabetes, and gout (203,206,207). Anakinra has also been given to patients after myocardial infarction and has reduced the severity of heart failure in these patients (208). Chronic IL-1β production can lead to VEGF activation, angiogenesis and the advancement of some solid tumor growth, suggesting that anti-IL-1 therapies may also be beneficial to some cancer patients (193,195,209). These data show that there is a real need to better understand how immune cells, like monocytes, produce and release IL-1β during diverse stimulations and disease states. This information will likely assist in the development of new and better anti-IL-1 therapies. One issue with current anti IL-1 treatments is that they work by inhibiting IL-1 from binding to its receptor, after it has been produced and released. These drugs cannot be everywhere at once, and as such, they do not completely block the effects of IL-1. Ideally, newer treatments should seek to specifically block pathogenic IL-1 production and release.

Since the NLRP3 inflammasome responds to the most diverse set of stimuli, the molecular mechanisms that lead to its priming and activation are intensely studied. As mentioned above, human monocytes can activate the NLRP3 inflammasome in some non-canonical ways by utilizing caspases other than caspase-1, such as caspases-4, -5 and -8 (175,178,179,182,183,210–215). While it is known that IL-1β release from *T. gondii*-infected human monocytes depends on NRLP3 and caspase-1 (66,134), it was not clear what signals led to priming and activation of this inflammasome or if other caspases might also be involved in its regulation.

Caspases in cell death and inflammation

Caspases are proteases that have classically been separated into two categories based on their ability to influence inflammation or cell death. Caspases-1, -4 and -5 are considered inflammatory caspases (179,185,216–218) based on their ability to facilitate IL-1β maturation through direct cleavage of the substrate or activation of an inflammasome complex (Table 1.2). Once IL-1β is cleaved it still must be released from the cell; however IL-1β and other IL-1 family member proteins are not released through Golgi-mediated exocytosis like many other inflammatory cytokines (219–221). While several hypotheses for the mechanism of IL-1β release were proposed, it took almost 2 decades from the cloning of the cytokine to show that IL-1β can be released through an inflammatory form of cell death called pyroptosis (217,222). Pyroptosis occurs when activated caspase-1 cleaves gasdermin D into a C-terminal and N-terminal fragment. The N-terminal fragment, now free of the inhibitory C-terminal fragment, will then bind to the cytoplasmic side of the cell membrane and form pores (223–225). These pores allow for passive release of intracellular cytosolic contents like IL-1β (226,227). They also allow for osmosis-driven cell swelling and lysis. This lysis dumps out remaining cytosolic contents, including any remaining IL-1β into the extracellular space (Fig. 1.4)(217,223,225,228). Pro-IL-1β released into the supernatant this way can also be cleaved extracellularly to produce more active cytokine (66,229).

Apoptotic caspases consist of the initiator caspases-2, -8, -9, and -10 and the executioner caspases-3, -6 and -7. Although caspase-9 is traditionally considered an initiator of intrinsic apoptosis driven by BIM, BID and BAX signaling at the mitochondria, and caspase-8 is considered the initiator of extrinsic apoptosis which can be driven by TNF receptor and FADD signaling in the death-inducing signaling complex (DISC) located at the plasma membrane (230,231), they can also be coordinately activated (232). Both caspases serve to activate caspases-3 and -7 which execute the final stages of apoptosis (230,231)(Table 1.2).

Table 1.2. Classifications of apoptotic and inflammatory human caspases

Type of Cell Death	Role	Protease
Apoptotic	Initiator	Caspase-2
		Caspase-8
		Caspase-9
	Executioner	Caspase-10
		Caspase-3
		Caspase-6
		Caspase-7
Pyroptotic	Inflammatory	Caspase-1
		Caspase-4
		Caspase-5
		Caspase-8

Caspase-8 also negatively regulates necroptosis, an inflammatory form of cell death, through its inhibition of RIPK1, thus inhibiting MLKL activity, which is the executioner of necroptosis (216,233–235). Recently, caspase-8 has also been shown to contribute to IL-1β release from monocytes and therefore function as an inflammatory caspase as well (175,178,214,234).

Recent investigations into caspase-8 have revealed that it can influence NLRP3 inflammasome activity at nearly every step. Caspase-8 can contribute to production of IL-1β transcripts, activation of the inflammasome complex, and in scenarios where caspase-1 is inactive, it can directly cleave IL-1β and gasdermin D, inducing pyroptosis or activating caspase-3 which then cleaves gasdermin E for a delayed pyroptotic cell death and release of IL-1α (175,210–215,234,236–238). Interestingly, caspase-8 activity in the alternative NLRP3 inflammasome, and caspases-4 and -5 activity in the noncanonical inflammasome, have been tied to IL-1β release that is independent of cell death and gasdermin D-mediated pore formation (178,179,182,184). Likewise, many studies have suggested that IL-1β can be released from myeloid cells through some cell death independent mechanisms, such as release in exosomes or through secretory autophagy (157,219–221,239–244)

Secretory autophagy (SA) refers to the unconventional secretion of cargo from a cell that relies on autophagy associated proteins (ATGs) or other autophagy machinery. During secretion of IL-1β by SA, IL-1β binds LC3B, which then traffics it to the plasma membrane. Various TRIM and SNARE proteins then assist in the formation of a phagosome around this cargo, but this phagosome does not fuse with a lysosome for degradation. Instead the phagosome is secreted into the extracellular space where IL-1β and any other cargo can perform biological functions (220,241–244). Release by secretory autophagy is not specific to IL-1 family member cytokines. All types of cargo, even full organelles or intact pathogens can be secreted from cells in this manner. In particular, the release of prion-like or aggregating proteins such as Amyloid-β have also been observed in SA (241–243,245). What is clear is that investigation of SA is still in its

infancy and any new insights into its regulation could be extremely informative and beneficial to therapeutic development.

Considering the high prevalence of *T. gondii* and the significant burden it creates through morbidity and mortality, further investigation into the mechanisms by which the host immune response controls this infection is still warranted. In addition, since *T. gondii* induces IL-1β release from human monocytes, uncovering the mechanisms that lead to this release will improve our understanding of IL-1β and inflammasome biology and may ultimately assist in the treatment of other diseases, as discussed above. Thus, our overarching goal of this dissertation is to investigate the cell biology and molecular mechanisms that drive IL-1β release from *T. gondii*-infected human monocytes. In our investigation of these mechanisms we have attempted to address the questions of how *T. gondii* infection may be sensed by human monocytes at early stages of infection, how it induces priming and activation of the NLRP3 inflammasome, what caspases may contribute to IL-1β release from *T. gondii*-infected human monocytes, and the mechanism of IL-1β release from infected monocytes. The data presented aims to answer each of these questions and also contribute more broadly to the fields of innate immunity and inflammation.

Chapter 2

Toxoplasma gondii activates a Syk-CARD9-NF-κB signaling axis and gasdermin D-independent release of IL-1β during infection of primary human monocytes

The contents of this chapter were previously published as (157)

Introduction

Toxoplasma gondii is an obligate intracellular foodborne parasite capable of infecting and replicating in any nucleated cell of its infected hosts (85). Global estimates suggest that as much as a third of the world population is chronically infected with this parasite and that over thirty million people become ill from *T. gondii* infections each year (112,113). While a robust immune response typically controls the infection, *T. gondii* poses severe health risks to immunocompromised individuals and to the developing fetus during congenital disease (86,246). In particular, $CD4^+$ and $CD8^+$ T cells and the production of IFN-γ are required for protection against *T. gondii* infection (247,248). Innate immune cells also contribute significantly to host defense against *T. gondii* through the production of IL-12 and cell intrinsic mechanisms of host defense (249). Monocytes, in particular, are among the first cells recruited to sites of *T. gondii* infection and are critical for parasite control during both the acute and chronic stages of disease (81,82,120,121,250,251).

IL-1β is a potent pro-inflammatory cytokine that is induced by infection and injury and coordinates both the innate and adaptive immune responses (193). Uncontrolled production of IL-1β has been implicated in the pathogenesis of a variety of diseases such as atherosclerosis, arthritis, diabetes, inflammatory bowel disease, and Alzheimer's disease (166,196), indicating that IL-1β production and release must be tightly controlled to maintain healthy immune function, during both homeostasis and infection.

Myeloid cells, such as macrophages and monocytes, are major producers of IL-1β during infection or injury. Macrophages regulate IL-1β via a two-signal model. The first signal (Signal 1) is typically induced by Toll-like receptor (TLR) engagement and MyD88 signaling that results in NF-κB nuclear translocation and *IL-1β* transcription (252). IL-1β is then translated as a biologically inactive pro- protein that cannot bind to the IL-1 receptor until it is cleaved into the mature form by a protease, such as caspase-1. The second signal (Signal 2) activates a

multiprotein complex called the inflammasome, of which at least five have been described, which leads to caspase-1 activation and IL-1β cleavage and release (171). Interestingly, inflammasomes are differentially regulated in macrophages and monocytes (181), and even in human and mouse monocytes: human monocytes activate the inflammasome and release IL-1β in response to LPS alone, using a “one-step” pathway, whereas mouse monocytes stimulated with LPS require an additional Signal 2 for IL-1β cleavage and release (178). These differences in response to stimulation may reflect unique species- and cell-specific strategies for the regulation and induction of inflammation. Inflammasome activation and IL-1β production are also differentially regulated depending on the nature of the stimulus, which can be as diverse as pathogen infection, microbial products, or sterile inducers of inflammation.

Unlike most cytokines, IL-1β does not possess a signal peptide or traffic through the standard secretory pathway (221). Instead the best-characterized mechanism of IL-1β release from myeloid cells is through an inflammatory form of cell death known as pyroptosis (222). Activation of the noncanonical or canonical NLRP3 inflammasome induces pyroptosis through caspase-11- or caspase-1-mediated cleavage of gasdermin D (GSDMD) (184,253). The cleaved N-terminal domain of GSDMD then inserts into the plasma membrane, where it forms pores through which IL-1β can pass. These pores allow for an influx of extracellular fluid, cell swelling, and eventually pyroptosis, which can release any remaining IL-1β into the extracellular space (223,254,255). Recent work has shown GSDMD-dependent pore formation can also mediate IL-1β release from viable “hyperactivated” cells (226), suggesting that GSDMD could serve as a critical mechanistic unifier for the release of IL-1β from both pyroptotic and viable, non-pyroptotic cells.

IL-1β production contributes to host control of *T. gondii* infection (159,187,188,256), and we have previously shown that *T. gondii* infection of primary human monocytes induces the production of IL-1β transcripts and activation of the NLRP3 inflammasome (66,134). However, *T. gondii* infection does not activate any known human TLRs, and the signaling pathways

involved in TLR-independent IL-1β production during infection, particularly in human cells, however, remain poorly defined. In the current study, we demonstrate that primary human monocytes infected with *T. gondii* produced IL-1β through a Syk-PKCδ-CARD9/MALT-1-NF-κB signaling pathway and activated the NLRP3 inflammasome for IL-1β release from viable cells in a GSDMD-independent manner. Moreover, we have defined differences in the role of Syk in *T. gondii*-infected compared to LPS-stimulated primary human monocytes: during *T. gondii* infection, Syk was critical for pro-IL-1β synthesis, whereas in LPS-stimulated monocytes, Syk predominantly mediated IL-1β release. These studies detailing the activation and regulation of the IL-1β pathway during infection and in response to microbial products further our understanding of how primary human immune cells regulate inflammation when activated by diverse stimuli.

Results

T. gondii infection activates the canonical NLRP3 inflammasome and induces the release of bioactive IL-1 from primary human monocytes

To investigate IL-1β production and release from primary human monocytes during *T. gondii* infection, we isolated monocytes from healthy blood donors as previously described (Fig. 2.S1) (66) and immediately infected the cells *in vitro* with GFP-expressing *T. gondii* or treated them with an equal volume of culture medium (mock). At 4 hours post-infection (hpi), IL-1β release into the supernatant was detected by ELISA, and the response of cells from individual human donors was compared (each dot represents a unique donor) (Fig. 2.1A). The pretreatment of primary monocytes with MCC950, an NLRP3 inhibitor; YVAD, a caspase-1 inhibitor; or KCl, which prevents K^+ efflux and inhibits activation of the canonical NLRP3 inflammasome (162), all resulted in a significant decrease in IL-1β release from infected primary

human monocytes from multiple independent donors (Fig. 2.1A). Notably, none of these treatments or inhibitors affected the efficiency of infection, as determined by the percent of GFP^+ (infected) cells in the culture (Fig. 2.S2A) or the ability of the parasite to replicate in and lyse human foreskin fibroblasts (HFFs), as determined by plaque assays (Fig. 2.S2B). The transfer of supernatants from *T. gondii*-infected, but not mock-treated monocytes to the HEK-Blue reporter cell line resulted in reporter cell activation, indicating the release of functional IL-1 from the infected primary monocytes (Fig. 2.1B).

There are three subsets of peripheral blood monocytes that have been described in humans, which are defined by their relative expression of CD14 and CD16 (33)(Fig. 2.1C). Recent publications have shown that the $CD14^+CD16^-$ inflammatory subset of monocytes is associated with increased and chronic inflammation and the development of arthritis (257,258). Using intracellular cytokine staining (ICCS) to compare IL-1β production in each subset, we found that *T. gondii* infection stimulated all three subsets to produce IL-1β by 4 hpi (Fig 2.1D). We also observed that in each of the five donors analyzed, the $CD14^+CD16^-$ inflammatory monocytes exhibited the highest percentage of IL-1β^+ cells (Fig 2.1D). Collectively, these data demonstrate that *T. gondii* triggers the production of IL-1β in all subsets of primary peripheral blood human monocytes and activates the canonical NLRP3 inflammasome for the release of bioactive IL-1 by 4 hpi.

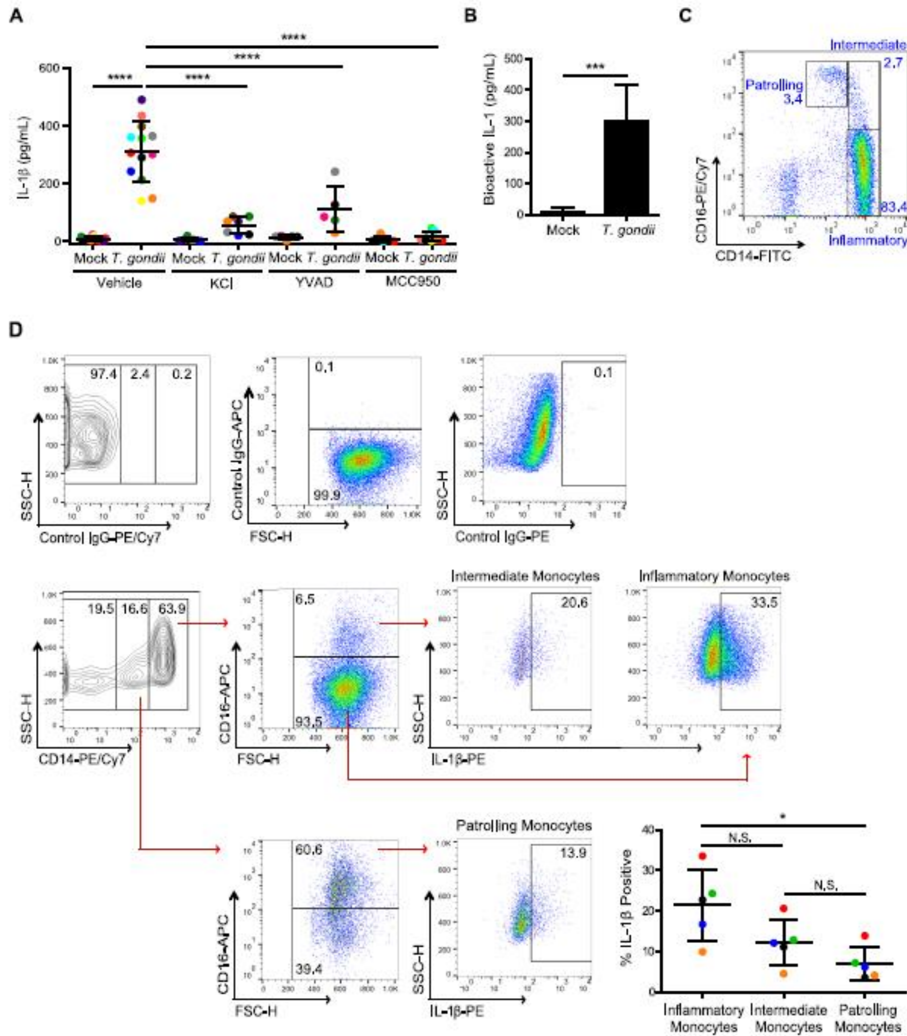


Fig. 2.1. *T. gondii*-infected human monocytes release bioactive IL-1β through the NLRP3 inflammasome. (**A**) Primary human peripheral blood monocytes were pretreated with 50 mM potassium chloride, 50 μM YVAD, 2 μM MCC950, or vehicle control for 40 min. Cells were then mock treated or infected with *T. gondii* for 4 h, and the levels of IL-1β in the supernatant were measured by ELISA. Each dot represents the response of an individual donor's cells. (**B**) Primary monocytes were mock treated or infected with *T. gondii* for 4 h, and bioactive IL-1 in the supernatant was detected using HEK-Blue IL-1R reporter cells. (**C**) Primary monocytes were analyzed by flow cytometry, and the three monocyte subsets (patrolling, intermediate and inflammatory monocytes) were observed based on CD14 and CD16 expression. (**D**) Primary monocytes were infected with *T. gondii* for 4 h, fixed, permeabilized, stained with control Ig or anti-IL-1β, anti-CD14 and anti-CD16 antibodies, and analyzed by flow cytometry. The representative gating strategy is shown (left). The percentage of IL-1β positive cells was measured in the three subsets of monocytes (right). Data in (A) reflect combined results of 5-13 experiments with independent donors. For (B), combined data from 5 donors are shown. In (C), a representative plot from over 70 monocyte isolation experiments is shown. In (D), combined results from 5 experiments with different donors is shown. Values are expressed as the mean ± SD, **P*<0.05, ****P*<0.001, *****P*<0.0001 (one-way ANOVA followed by a Tukey post-test in A, B, and D).

IL-1β release from primary human monocytes is dependent on Syk

Syk is a tyrosine kinase that is expressed in hematopoietic cells and is involved in NLRP3 activation during fungal infection, viral infection, and in response to LPS stimulation (179,259–261). However, the role of Syk in IL-1β regulation during parasite infection is unknown. Interestingly, rapid phosphorylation of Syk at tyrosine 525/526 was detected by phospho-flow cytometry (Fig. 2.2A) and Western blotting of lysates (Fig. 2.2B) from monocytes that were infected with *T. gondii* or treated with LPS, as a positive control. The phosphorylation of Syk was reduced in the presence of the Syk-specific inhibitor R406 (Fig. 2.2B). To investigate a potential role for Syk in IL-1β release during *T. gondii* infection, primary human monocytes were pre-treated with the Syk inhibitor R406 or a vehicle control, and either infected with *T. gondii* or treated with LPS. LPS stimulation induced significantly more IL-1β release than *T. gondii* infection, but R406 treatment significantly reduced IL-1β release from primary human monocytes treated with either stimulus at the 4 hour time-point (Fig. 2.2C). Titration of R406 revealed a dose-dependent effect of the Syk inhibitor (Fig. 2.2D). Importantly, pretreatment of monocytes with the R406 inhibitor did not reduce the infection efficiency of the parasite or the GFP median fluorescence intensity (MFI) of monocytes infected with GFP-expressing parasites at either 4 hpi or 16 hpi (Fig. 2.S3A). In addition, R406 did not decrease the ability of *T. gondii* to grow in and lyse HFFs, as measured by plaque assays (Fig. 2.S3B), or the viability of monocytes (Fig. 2.S3C). Furthermore, an independent Syk inhibitor, entospletinib, which is currently in use in clinical trials for leukemia (262), also reduced IL-1β release from *T. gondii*-infected monocytes in a dose-dependent manner (Fig. 2.2D), without affecting parasite infection efficiency (Fig. 2.S2A) or viability (Fig. 2.S2B). Collectively, these data indicate on-target effects of the Syk inhibitors and demonstrate that *T. gondii* infection induces IL-1β release from primary human monocytes in a Syk-dependent manner.

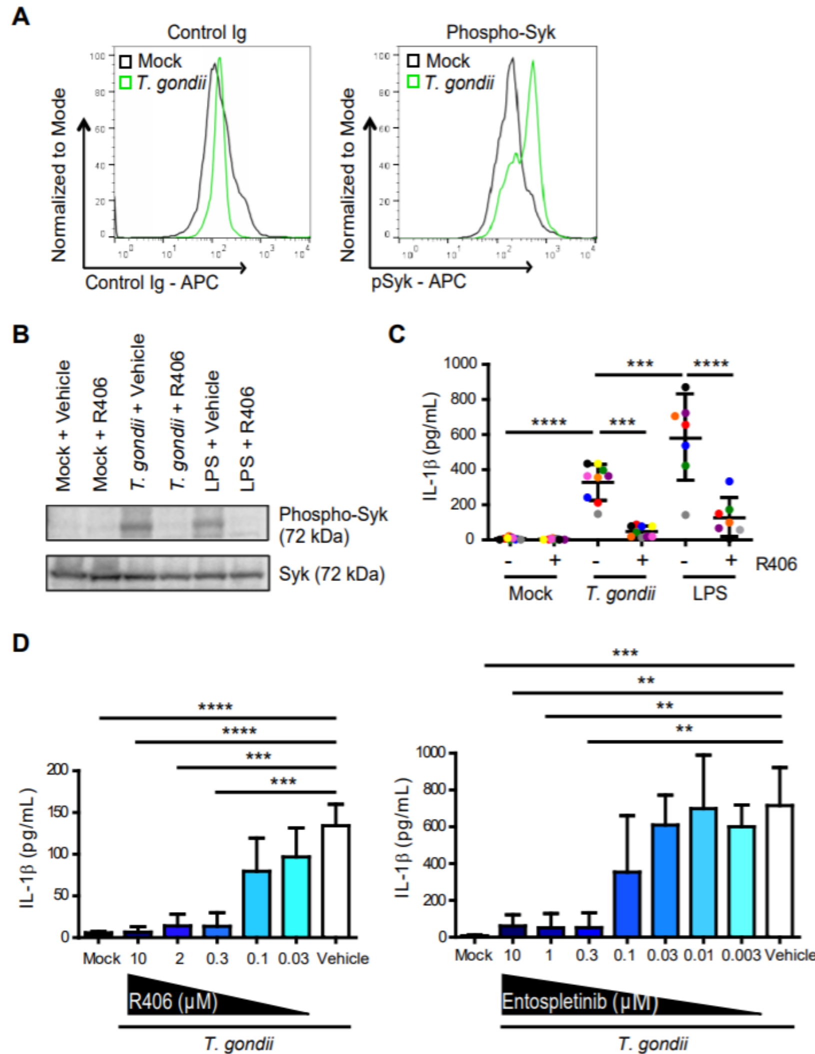


Fig. 2.2. Syk is activated during *T. gondii* infection of primary human monocytes. (**A**) Primary human monocytes were mock treated or infected with *T. gondii*. After 30 min, cells were permeabilized, stained with control Ig or anti-phospho-Syk (Tyr525/526) antibody, and flow cytometry was performed. (**B**) Primary monocytes were treated with 2 μM R406 or vehicle control and then mock treated, infected with *T. gondii*, or stimulated with LPS (100 ng/ml) for 30 min. Total Syk and phospho-Syk (Tyr525/526) in the cell lysate were visualized by Western blotting. (**C**) Primary monocytes were pretreated with 2 μM R406 or vehicle control for 40 min and then mock treated, infected with *T. gondii*, or stimulated with LPS (100 ng/ml) for 4 h, and the levels of IL-1β in the supernatant were measured by ELISA. (**D**) Primary monocytes were pretreated with different concentrations of R406 (0.03-10 μM), entospletinib (0.003-10 μM), or vehicle control for 40 min. Monocytes were then mock treated or infected with *T. gondii* for 4 h, and the levels of IL-1β in the supernatant were measured by ELISA. Representative plots of 5 experiments are shown in (A). In (B), a representative Western blot from 4 experiments is shown. Data in (C) and (D) reflect combined results of 7 and 3 experiments with independent donors, respectively. Values are expressed as the mean ± SD, ***P*<0.01, ****P*<0.001, *****P*<0.0001 (one-way ANOVA followed by a Tukey post-test in panel C and a Dunnett post-test in panel D).

Syk is required for IL-1β transcript production in *T. gondii*-infected primary human monocytes

Syk has been proposed to play two roles in the regulation of IL-1β production in other models: inducing NF-κB translocation and IL-1β transcription via the PKCδ-CARD9/MALT-1-NF-κB pathway or indirectly activating NLRP3 inflammasome assembly via ASC phosphorylation and oligomerization (172,263). In the lysates and supernatants of primary human monocytes pretreated with the Syk inhibitor R406, we observed a marked reduction in both pro- and mature IL-1β protein levels compared to infected monocytes treated with the vehicle control (Fig. 2.3A). In contrast, R406 pre-treatment of LPS-stimulated primary monocytes did not reduce the levels of pro-IL-1β protein in the cell lysates (Fig. 2.3A). Notably, the NLRP3 and caspase-1 inhibitors MCC950 and YVAD, respectively, had no effect on pro-IL-1β synthesis or release from primary monocytes, as expected (Fig. 2.3B). ICCS of primary human monocytes infected with *T. gondii* or treated with LPS in the presence or absence of R406 indicated that Syk inhibition reduced the percentage of intracellular IL-1β^{+} *T. gondii*-infected monocytes but did not decrease the percentage of intracellular IL-1β^{+} monocytes stimulated with LPS (Fig. 2.3C).

To directly examine the effect of Syk inhibition on IL-1β and NLRP3 transcript levels, qPCR was performed on samples from human monocytes infected with *T. gondii* or treated with LPS in the presence or absence of R406. These data corroborated the Western blot and ICCS data and demonstrated that R406 decreased *IL-1β* and *NLRP3* transcripts at 1 and 4 hpi in *T. gondii*-infected monocytes (Fig. 2.3D). Interestingly, R406 treatment also reduced *IL-1β* and *NLRP3* transcripts in LPS-stimulated monocytes (Fig. 2.3D), despite having little to no effect on pro-IL-1β levels in these cells (Fig. 2.3A). Together these data suggest that Syk signaling is critical for production of *IL-1β* and *NLRP3* transcripts in *T. gondii*-infected primary human monocytes, and therefore appears to act in the priming stage of IL-1β production during infection.

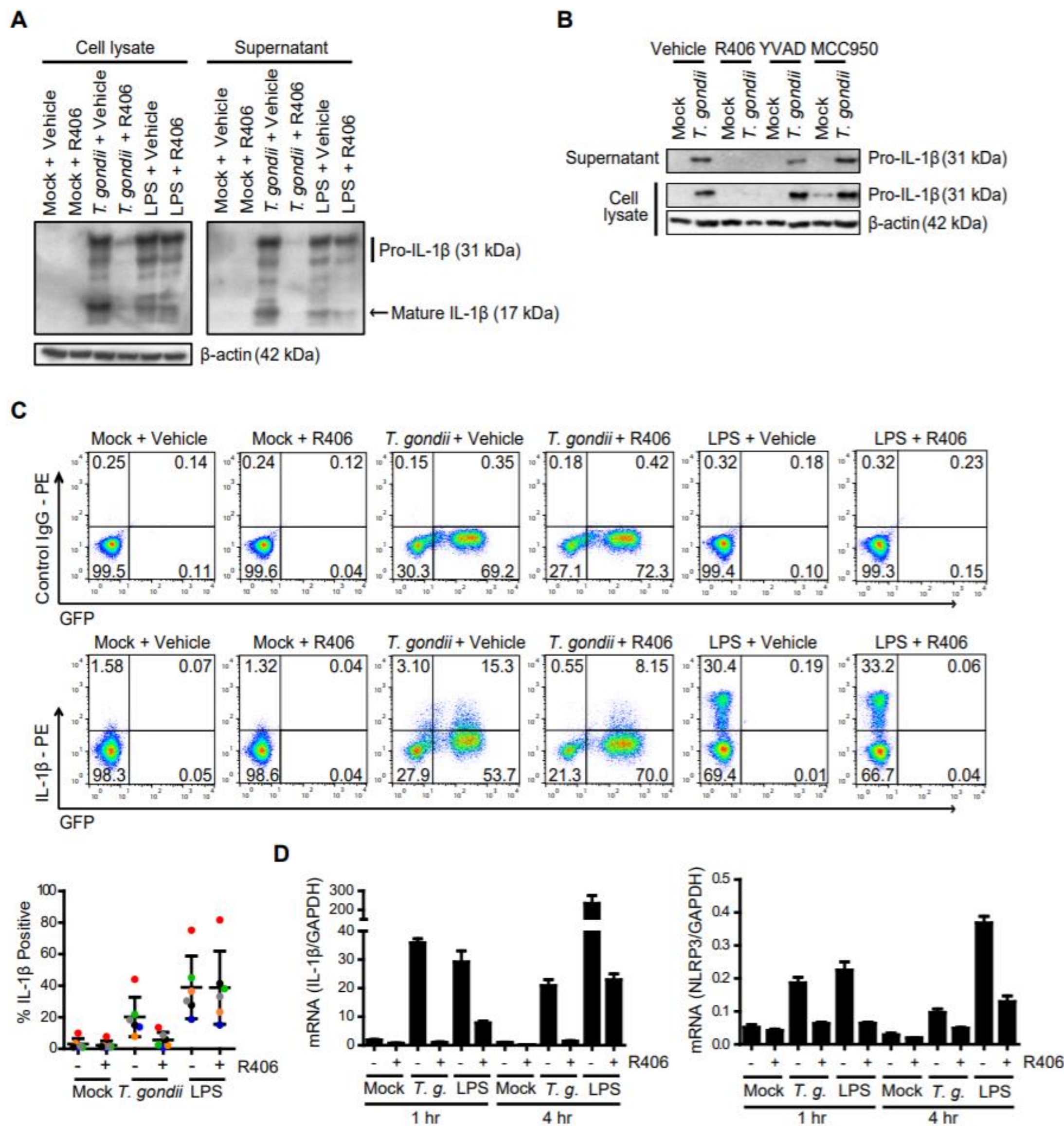


Fig. 2.3. Syk is required for IL-1β production in *T. gondii*-infected human monocytes. (**A**) Primary human monocytes were pretreated with 2 μM R406 or vehicle control for 40 min and then mock treated, infected with *T. gondii*, or stimulated with LPS (100 ng/ml) for 4 h. Pro- and mature IL-1β in the cell lysate and supernatant, and β-actin in the lysate were visualized by Western blotting. (**B**) Primary monocytes were pretreated with 2 μM R406, 50 μM YVAD, 2 μM MCC950, or vehicle control for 40 min. Cells were then mock treated or infected with *T. gondii* for 4 h, and pro-IL-1β in the cell lysate and supernatant was visualized by Western blotting. (**C**) Cells were pretreated with 2 μM R406 or vehicle control for 40 min and then mock treated, infected with *T. gondii*, or stimulated with LPS (100 ng/ml). After 4 h, cells were fixed, permeabilized, stained with control Ig or anti-IL-1β, and analyzed by flow cytometry. The percentage of IL-1β positive cells in each condition was plotted. (**D**) qPCR was performed with primers specific for *IL-1β* and *NLRP3*, and transcript levels relative to those of *GAPDH* are graphed. Representative Western blots from 4 (A and B) experiments are shown. For the graph in (C), data show combined results from 6 experiments with independent donors, and representative FACS plots are shown. In (D), representative data from 4 experiments are shown. Values are expressed as the mean ± SD.

IL-1β synthesis and release are reduced during *T. gondii* infection of Syk knockout THP-1 cells

Since primary human monocytes cannot be genetically manipulated (or reliably cultured *in vitro* for more than ~24 hours), we examined a role for Syk in the human monocytic cell line THP-1. These cells also release IL-1β in response to *T. gondii* infection, but with delayed kinetics compared to primary monocytes (66,134). Similar to primary monocytes, *T. gondii* infection induced Syk phosphorylation in THP-1 cells (Fig. 2.S4A), and pre-treatment of THP-1 cells with R406 resulted in the release of significantly less IL-1β than in infected THP-1 cells treated with the vehicle control (Fig. 2.4A). To complement the R406 inhibitor experiments, THP-1 cells were transduced with lentivirus carrying guide RNAs targeting Syk for CRISPR/Cas9-mediated genome editing. As a control, THP-1 cells were transduced with an empty vector (EV) lacking the Syk targeting sequence. Unlike in the wild-type (WT) parental THP-1 cells, Cas9 was detected in the EV control and Syk KO lines, and Syk was absent only from the KO line (Fig. 2.4B). This Syk KO clone harbors mutations in the SH2 domain, resulting in a frameshift mutation that alters the amino acid sequence of the targeted exon (Fig. 2.S4B and 2.S4C).

The control EV line and the Syk KO THP-1 cells were infected with *T. gondii* and examined for IL-1β production. qPCR analysis revealed reduced levels of *IL-1β* mRNA in the Syk KO THP-1 cells compared to the EV control cells (Fig. 2.4C). Similarly, reduced levels of pro-IL-1β protein were detected in the Syk KO cells compared to the EV control cells during infection (Fig. 2.4D), suggesting that Syk functions upstream of *IL-1β* transcription and pro-IL-1β protein synthesis in THP-1 cells, similar to the effects of the R406 and entospletinib inhibitors in primary human monocytes. Finally, Syk KO THP-1 cells released less IL-1β in the supernatant, as detected by ELISA, than the control EV cells during *T. gondii* infection (Fig. 2.4E). Collectively, these data indicate that *T. gondii* infection induces IL-1β synthesis and release from both primary human monocytes and THP-1 cells in a Syk-dependent manner.

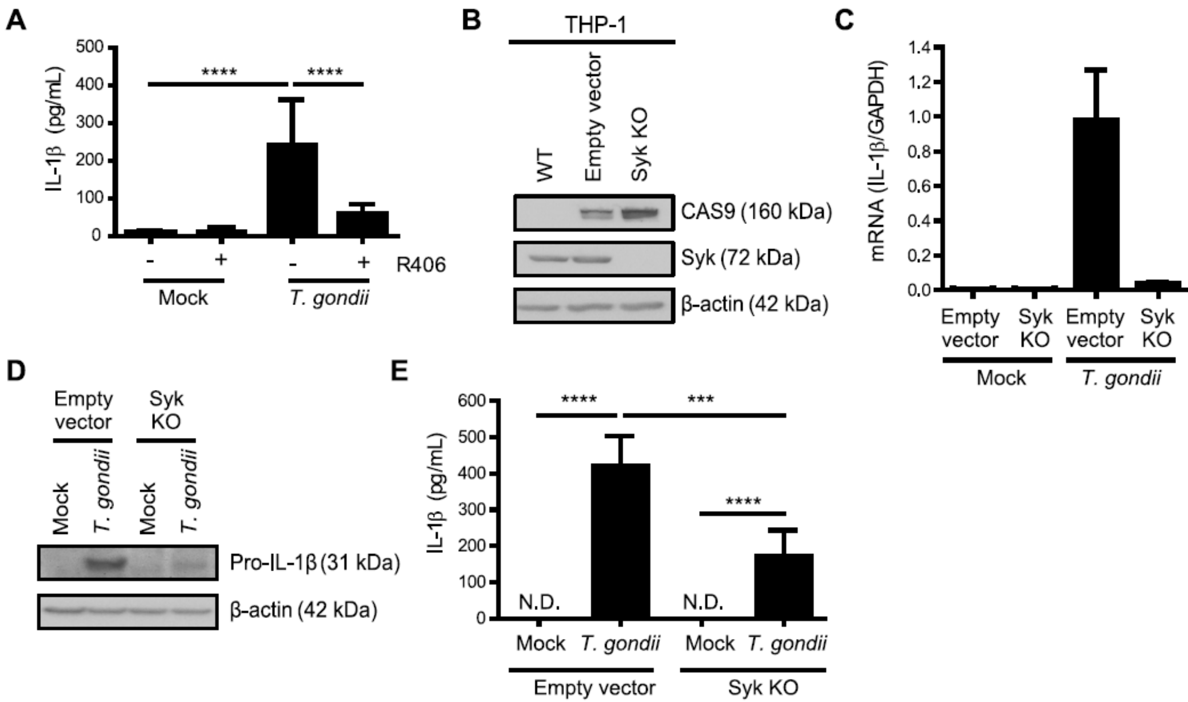


Fig. 2.4. Syk contributes to IL-1β production in *T. gondii*-infected monocytic THP-1 cells. (**A**) THP-1 cells were pretreated with 2 μM R406 or vehicle control for 40 min and then mock treated or infected with *T. gondii* for 18 h, and the levels of IL-1β in the supernatant were measured by ELISA. (**B**) Lysates from wild-type (parental) THP-1 cells, control Empty Vector THP-1 cells, and Syk KO THP-1 cells were blotted with antibodies to visualize Cas9, Syk, and β-actin. (**C**) Empty Vector THP-1 cells and Syk KO THP-1 cells were mock treated or infected with *T. gondii* and qPCR was performed with primers specific for *IL-1β*. Transcript levels relative to those of *GAPDH* are graphed. (**D** and **E**) Empty Vector or Syk KO THP-1 cells were mock treated or infected with *T. gondii*, and pro-IL-1β and β-actin in the cell lysate were visualized by Western blotting (**D**) or the levels of IL-1β in the supernatant were measured by ELISA (**E**). In (A) and (E), combined data from 4 experiments are shown. Representative Western blots (B and D) and qPCR (C) from 4 experiments are shown. Values are expressed as the mean ± SD, ****P*<0.001, *****P*<0.0001 (one-way ANOVA followed by a Tukey post-test in A and E).

T. gondii-infected monocytes activate a Syk-PKCδ-CARD9/MALT-1-NF-κB signaling pathway for the production and release of IL-1β

While it has been well documented that LPS activates a MyD88-IRAK1/4-TRAF6 pathway resulting in NF-κB nuclear translocation, Syk has been shown to activate an alternative PKCδ-CARD9/MALT-1-NF-κB signaling pathway (263). To investigate a potential role for this pathway in IL-1β production during *T. gondii* infection of human monocytes, we examined the activation of PKCδ and the NF-κB subunit p65 and found that *T. gondii* infection induced phosphorylation of both PKCδ and p65 in primary human monocytes (Fig. 2.5A and 2.5B). Treatment of monocytes with R406; Go6983, a PKC inhibitor which is active against PKCδ; MI2, a MALT-1 and CARD9 complex inhibitor; or PS1145, an IKK inhibitor, prior to *T. gondii* infection all significantly reduced p65 phosphorylation induced by *T. gondii* infection (Fig. 2.5B), indicating that the inhibitors all targeted a pathway upstream of NF-κB activation. Similar to the Syk inhibitor, the PKCδ, CARD9/MALT-1, and IKK inhibitors all reduced pro-IL-1β protein production in *T. gondii*-infected primary human monocytes at 4 hpi (Fig. 2.5C). Consistent with these data, IL-1β release was significantly reduced in primary human monocytes treated with these inhibitors compared to vehicle control-treated monocytes during infection, as determined by ELISA (Fig. 2.5D). Importantly, the inhibitors did not reduce infection efficiency at 4 hpi (Fig. 2.S2A) or affect the ability of the parasites to replicate in and lyse HFFs at the concentrations used (Fig. 2.S2B). Together, these data suggest that primary human monocytes rely almost completely on signaling through a Syk-PKCδ-CARD9/MALT-1-NF-κB signaling pathway for IL-1β production during *T. gondii* infection.

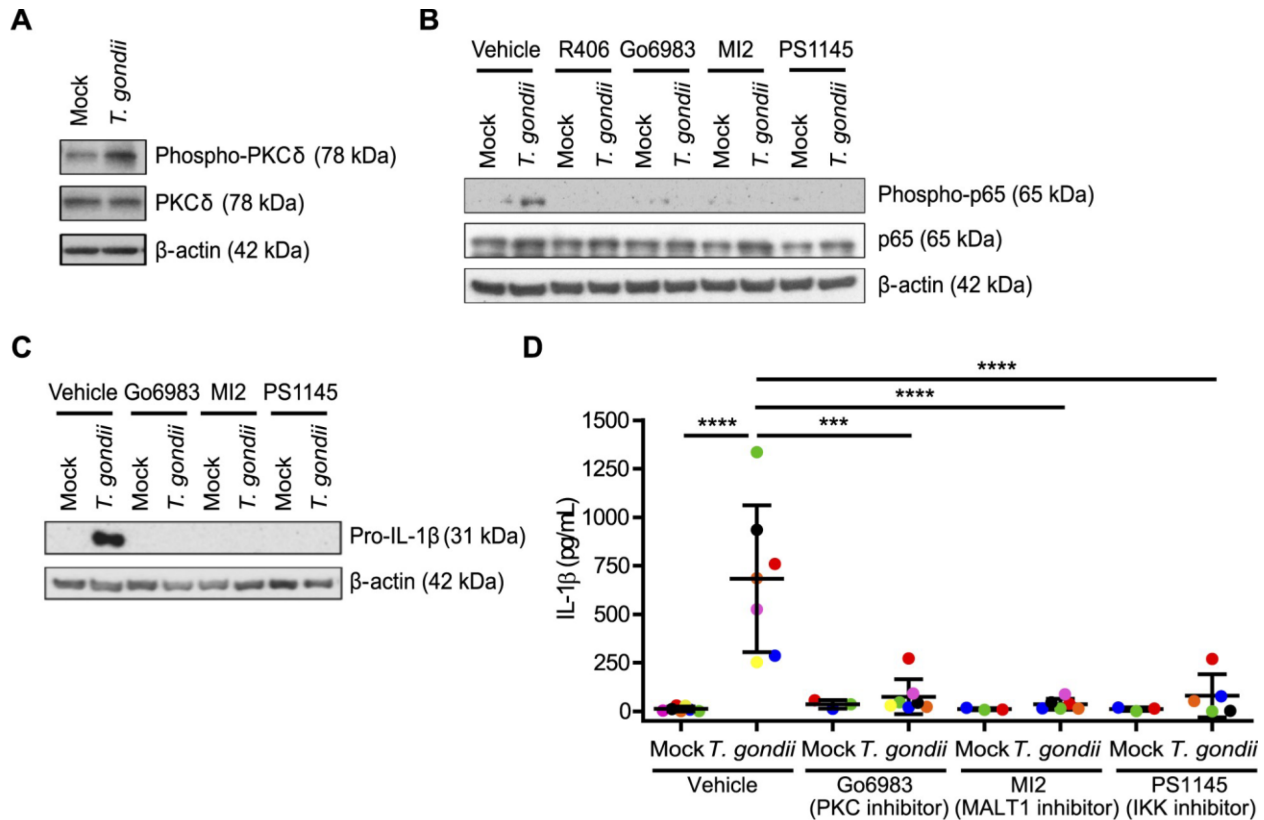


Fig. 2.5. The Syk-PKCδ-CARD9/MALT-1-NF-κB pathway is activated in *T. gondii*-infected human monocytes. (**A**) Primary monocytes were mock treated or infected with *T. gondii* and the lysates were blotted for total and phospho-PKCδ and for β-actin. (**B**) Primary monocytes were pretreated with 2 μm R406, 300 nM Go6983, 3 μM MI2, 100 nM PS1145, or vehicle control for 40 min then mock treated or infected with *T. gondii* for 1 h. Total and phospho-p65 (Ser536) and β-actin in the cell lysate were visualized by Western blotting. (**C**) Pro-IL-1β and β-actin were visualized by Western blotting of lysates from primary monocytes that were mock treated or infected with *T. gondii* in the presence of the vehicle control or the indicated inhibitors. (**D**) The levels of IL-1β in the supernatant of mock or *T. gondii*-infected primary monocytes in the presence or absence of the indicated inhibitors were measured by ELISA. Data in (D) are combined results of 3-7 experiments with independent donors. Representative Western blots from 3 (A and C) and 4 (B) experiments are shown. Values are expressed as the mean ± SD, ****P*<0.001, *****P*<0.0001 (one-way ANOVA followed by a Tukey post-test).

Knockout of PKCδ and CARD9 in THP-1 cells decreases *T. gondii*-induced IL-1β production

To investigate roles for PKCδ and CARD9 in IL-1β production during *T. gondii* infection using a genetic approach, THP-1 cells were subjected to CRISPR/Cas9-mediated genome editing using guide RNAs targeting each of these two proteins. Cas9 protein was detected in the EV control cells and the KO cells, and PKCδ and CARD9 were absent or severely reduced in each of the respective KO populations (Fig. 2.6A). The faint detection of PKCδ in the PKCδ KO population (Fig. 2.6A and 2.6C) may reflect the fact that these cells represent a mixed population, rather than a clonal KO line. Infection of either the PKCδ KO or CARD9 KO THP-1 cells with *T. gondii* resulted in reduced IL-1β release compared to infection of the EV cells (Fig. 2.6B). In addition, *T. gondii*-infected PKCδ KO and CARD9 KO THP-1 cells contained less pro-IL-1β protein in the cell lysates than *T. gondii*-infected EV THP-1 cells (Fig. 2.6C). These data indicate that both PKCδ and CARD9 contribute to IL-1β synthesis and release from THP-1 cells, consistent with the results obtained using inhibitors of these proteins in primary human monocytes.

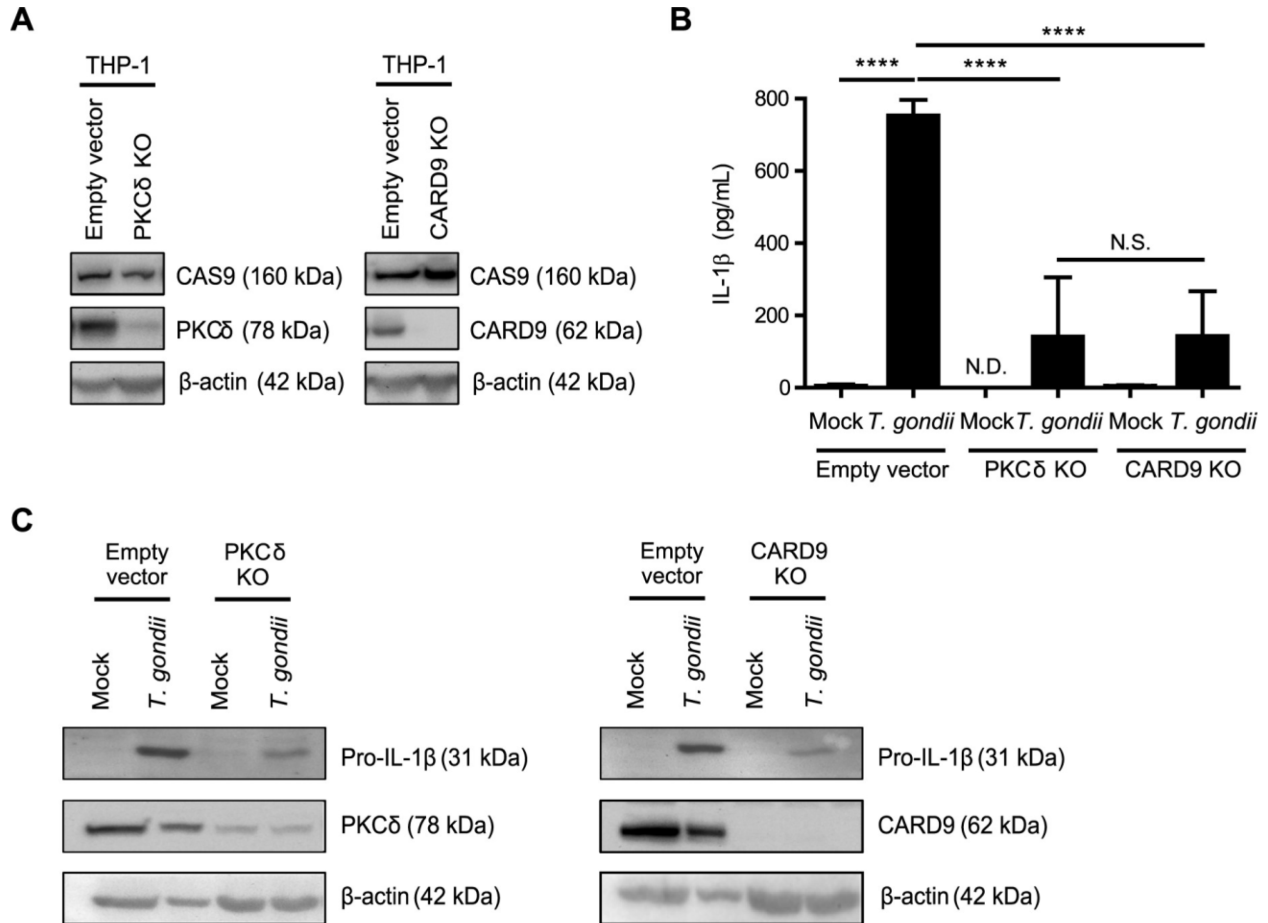


Fig. 2.6. PKCδ and CARD9 contribute to IL-1β production in *T. gondii*-infected monocytic THP-1 cells. (**A**) Cell lysates from control Empty Vector THP-1 cells, PKCδ KO, and CARD9 KO THP-1 cells were blotted with antibodies against Cas9, PKCδ, CARD9, and β-actin. (**B**) Empty Vector, PKCδ KO, and CARD9 KO THP-1 cells were mock treated or infected with *T. gondii* for 18 h, and the levels of IL-1β in the supernatant were measured by ELISA. (**C**) The same experiments were also used to visualize pro-IL-1β, β-actin, PKCδ, and CARD9 in the cell lysates by Western blotting. Data in (B) are combined results of 4 experiments. Representative Western blots from 4 (A and C) experiments are shown. Values are expressed as the mean ± SD, $^{****}P<0.0001$ (one-way ANOVA followed by a Tukey post-test in panel B).

T. gondii-infected and LPS-stimulated human monocytes release IL-1β via a mechanism that is independent of cell death and GSDMD.

The most well characterized mechanism of IL-1β release due to inflammasome activation is an inflammatory form of cell death, marked by cell membrane pore formation, cell swelling, and lysis, termed pyroptosis (222). To address a potential role for pyroptosis in IL-1β release during *T. gondii* infection, primary human monocytes were mock treated, infected with *T. gondii*, or stimulated with LPS or LPS and ATP, as controls. The cells were then stained with propidium iodide (PI), which passes through small pores in the plasma membrane and binds to DNA. At 4 hpi, when bioactive IL-1β release was detected (Fig. 2.1B), the viability of the *T. gondii*-infected monocyte population, as measured by the percentage of PI^+ cells, was indistinguishable from mock-treated cells (Fig. 2.7A), and the addition of the Syk inhibitor R406 did not alter the percentage of PI^+ cells. In contrast to *T. gondii* infection and LPS stimulation, a high level of cell death was detected when cells were treated with the canonical inflammasome activator, LPS and ATP (Fig. 2.7A). Titrating the ATP in LPS-stimulated cells triggered cell death in a dose-dependent manner (Fig. 2.S5). The addition of extracellular glycine, which inhibits ion flux, thereby halting cell swelling and the completion of pyroptosis, reduced IL-1β release from LPS and ATP-stimulated cells but not from LPS-stimulated or *T. gondii*-infected cells (Fig. 2.7B). In contrast, Syk inhibition with R406 did reduce IL-1β release in the LPS-stimulated and *T. gondii*-infected cells, without affecting cell death (Fig. 2.7A and 2.7B). Interestingly, the stimulus of LPS and ATP, which induced cell death, led to the release of more than ten times the amount of IL-1β from primary human monocytes than *T. gondii* infection or LPS stimulation alone (Fig. 2.7B). These results support the idea that the degree of pyroptosis and IL-1β release during stimulation may relate to the intensity of the stimulus encountered by the cells (219).

Although we did not detect significantly more cell death among *T. gondii*-infected monocytes compared to mock-treated cells, we formally tested a role for GSDMD by infecting

wild-type (WT) and GSDMD knockout THP-1 cells (264) with *T. gondii* and examining IL-1β release by ELISA (Fig. 2.7C). Notably, the GSDMD KO cells were not impaired in their release of IL-1β during *T. gondii* infection compared to WT THP-1 cells (Fig. 2.7C), and the viability of these cells was not significantly different than that of mock-treated THP-1 cells at the same time-point (Fig. 2.7C). Furthermore, whereas LPS and ATP stimulation of primary human monocytes led to the cleavage of GSDMD from the full-length 60 kDa protein to the N-terminal p30 fragment, neither LPS nor *T. gondii* infection resulted in increased GSDMD cleavage at 4 hpi, and Syk inhibition with R406 did not affect GSDMD cleavage in the *T. gondii* or LPS conditions (Fig. 2.7D). These data further support the conclusion that LPS and *T. gondii* trigger IL-1β release from human monocytes independent of GSDMD cleavage, pore formation, and pyroptosis.

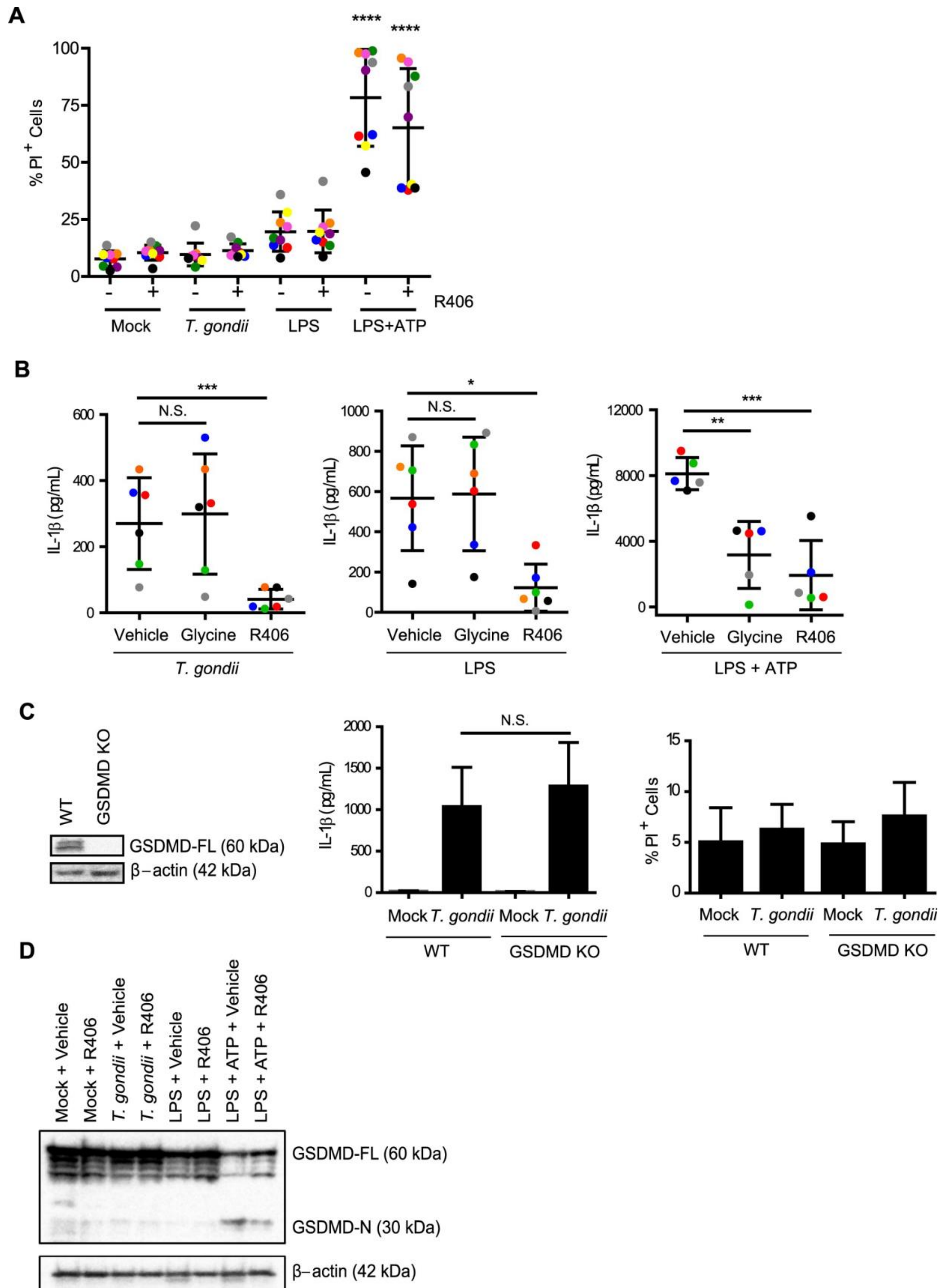


Fig. 2.7.

Fig. 2.7. *T. gondii*-induced IL-1β release is independent of cell death and gasdermin D (GSDMD). (**A**) Primary human monocytes were pre-treated with 2 μM of the Syk inhibitor R406 or vehicle control (DMSO) for 40 min, mock treated, infected with *T. gondii*, stimulated with LPS (100 ng/ml), or stimulated with LPS (100 ng/ml) plus ATP (5 mM) for 4 h (ATP was added for the last 30 minutes of treatment). Cell viability by PI staining was then measured by flow cytometry. (**B**) Primary monocytes were pretreated with 5 mM glycine, 2 μM R406, or vehicle control for 40 min. Cells were then infected with *T. gondii*, stimulated with LPS alone or LPS+ATP for 4 h, and the levels of IL-1β in the supernatant were measured by ELISA. (**C**) Lysates from wild type and GSDMD knockout (KO) THP-1 cells were blotted with anti-GSDMD or anti-β-actin antibodies. The WT or GSDMD KO cells were mock treated or infected with *T. gondii* for 18 h. The levels of IL-1β in the supernatant were measured by ELISA, and cell viability by PI staining was measured by flow cytometry. (**D**) GSDMD-FL (full-length) and GSDMD-N (N-terminal) in the cell lysate were visualized by Western blotting. Data in (A) and (B) reflect combined results of 9 and 5 experiments with independent donors, respectively. For (C), combined data from 6 and 13 experiments is shown for the IL-1β release ELISA and PI staining experiments respectively. In (D) a representative Western blot from 4 experiments is shown. Values are expressed as the mean ± SD, *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$ (one-way ANOVA followed by a Tukey post-test in A, B and C). In (A), the LPS+ATP treated conditions contained a significantly higher percentage of PI^{+} cells than all of the first six conditions, which were not significantly different from each other.

Discussion

Syk is a tyrosine kinase expressed in immune cells and is typically activated by receptors or receptor-associated adaptor proteins with cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) (265). Syk is known to be critical for lymphatic development, inflammatory signaling, and inflammasome activation (266). We now demonstrate that inhibition of Syk in primary human monocytes and genetic deletion of Syk in monocytic THP-1 cells both significantly reduced *IL-1β* transcripts and pro-IL-1β production during *T. gondii* infection, indicating a role for Syk in IL-1β synthesis in parasite-infected cells. Among other signaling pathways, Syk can signal through PKCδ and CARD9 to induce NF-κB activation (263,267), and indeed, inhibitors against PKCδ, CARD9/MALT-1, and IKK, or genetic deletion of PKCδ and CARD9 in THP-1 cells revealed the importance of this pathway in IL-1β synthesis in *T. gondii*-infected monocytes, as depicted in Figure 2.8. Syk also contributed to *NLRP3* transcript induction in response to *T. gondii* infection, further supporting a role for Syk in the priming of both IL-1β and NLRP3. In contrast, Syk appeared to be less important for the production of pro-IL-1β in LPS-stimulated primary human monocytes, but rather, seemed to contribute more substantially to IL-1β release from these cells. LPS-stimulated monocytes likely rely more heavily on canonical NF-κB signaling downstream of TLR4 and MyD88 for IL-1β transcription, and on Syk for processing and release of IL-1β. Indeed, it has been shown that Syk can lead to activation of the NLRP3 inflammasome through the indirect phosphorylation of the inflammasome adaptor protein ASC (172,268). ASC phosphorylation induces its oligomerization, facilitating activation of the NLRP3 inflammasome and caspase-1 (173,269). Collectively, our data indicate that immune cells responding to pathogens harboring multiple PAMPs or vitaPAMPs will likely regulate IL-1β differently than cells responding to a single PAMP or stimulus. For example, primary human monocytes utilize Syk signaling through ERK1/2 to produce IL-1β when dengue virus is in complex with antibody (261), and our research shows that during *T. gondii* infection Syk activates a separate signaling pathway to

reach the same net result. Thus, examining the production and processing of IL-1β and other cytokines during infection with various live pathogens may unveil new pathways of regulation that could be critical for enhancing or dampening inflammation during different types of infections.

In mice, *T. gondii* is sensed by TLR11/12 recognition of the parasite actin-binding protein profilin (153,270,271); interestingly, however, these TLRs are not functional in humans. And although Syk can be activated downstream of TLR4 (260,272), there is no known human TLR that has been shown to recognize *T. gondii*, suggesting that *T. gondii*-induced Syk signaling occurs via a different receptor. Recent work has shown that *T. gondii*-infected cells release alarmin S100A11, which binds to the RAGE receptor on monocytes (67), yet no innate immune sensor has been shown to directly bind to *T. gondii* PAMPs. Our data suggest that an ITAM-bearing receptor or adaptor protein that activates Syk may serve as a potential recognition receptor, and this possibility is under investigation. We have previously identified a partial role for the parasite-secreted protein GRA15 in IL-1β production in primary human monocytes (134), and Syk signaling may synergize with GRA15 to induce maximal priming of IL-1β production.

Although IL-1β production was detected in all three subsets of monocytes by ICCS, in each donor examined, the $CD14^{+}CD16^{-}$ inflammatory monocytes produced more IL-1β in response to *T. gondii* than the other monocyte subsets. In human blood, this inflammatory subset of monocytes is present in significantly greater numbers than the other subsets. Recent research indicates that this $CD14^{+}CD16^{-}$ population of inflammatory monocytes is largely responsible for pathogenic inflammation in arthritis and sepsis (257,258), and our data are consistent with these findings. An intriguing possibility is that inflammatory monocytes regulate the expression or function of the receptors or signaling molecules involved in *T. gondii*-induced IL-1β production differently than the other monocyte subsets, rendering them more responsive to inflammatory stimuli.

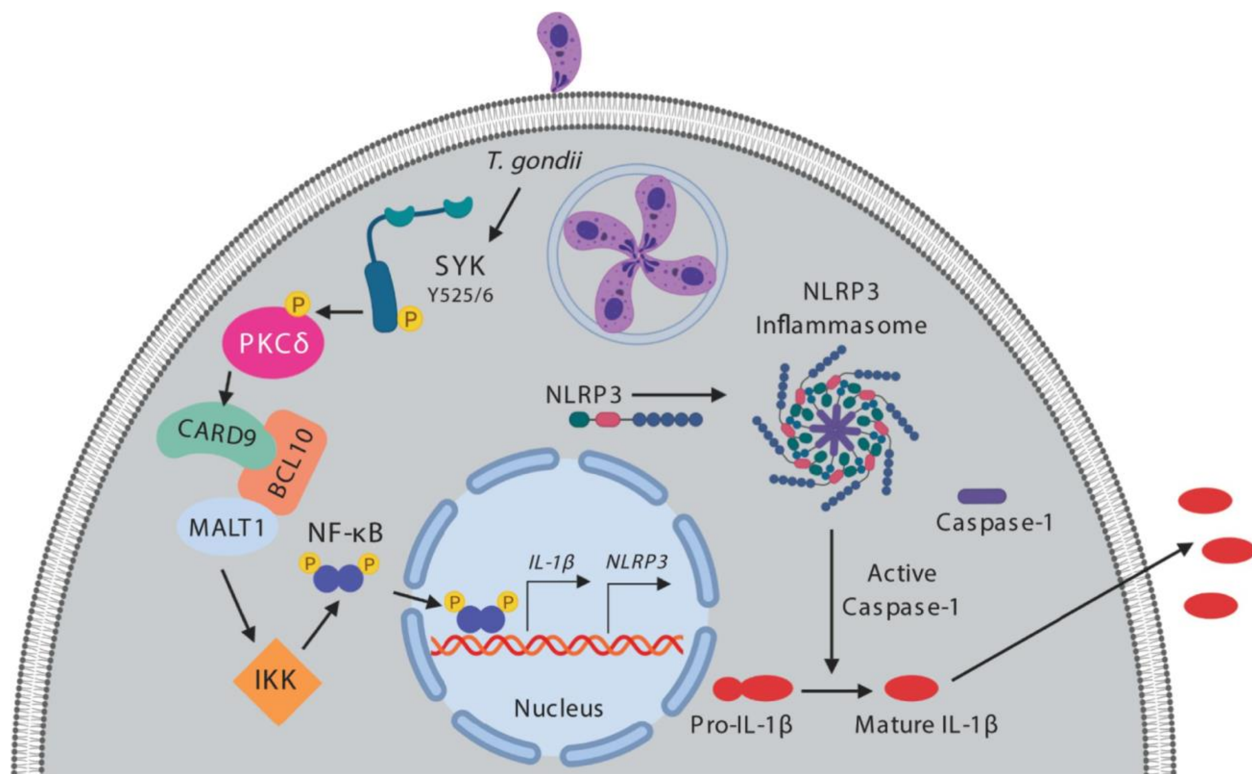


Fig. 2.8. Model for *T. gondii*-induced IL-1β production in primary human monocytes. *T. gondii* infection induces phosphorylation and activation of Syk at tyrosine 525/6 (Y525/6). Syk activation subsequently leads to the phosphorylation of PKCδ, which activates the CARD9/BCL10/MALT1 complex and downstream IKK. This leads to phosphorylation of the p65 subunit of NF-κB and the translocation of NF-κB into the nucleus where it initiates transcription of *IL-1β* and *NLRP3*. NLRP3 then associates with ASC and caspase-1 to form the canonical NLRP3 inflammasome. Pro-caspase-1 is cleaved into activate caspase-1, which then cleaves pro-IL-1β to mature IL-1β. The mature, bioactive IL-1β exits the cell by an unknown mechanism, independent of pyroptosis, gasdermin D, or pore formation in the plasma membrane.

Since the discovery that the fungal metabolite brefeldin A, which inhibits conventional protein secretion, did not inhibit IL-1β secretion from stimulated immune cells (221), the potential mechanisms of IL-1β release have been intensely studied. The best characterized mechanism of release occurs through an inflammatory form of cell death marked by cell swelling and lysis, termed pyroptosis (222). Notably, IL-1β can also be released from viable cells in a pyroptosis-independent manner (226). Indeed, this is the case with human, but not mouse, monocytes treated with LPS (178). In 2015, the identification and characterization of GSDMD (184,253), which can be activated by the inflammasome and functions as the effector protein of pyroptosis by forming small pores in the cell membrane (223,254,255), provided a molecular basis for this inflammatory form of cell death. Interestingly, in the context of *T. gondii* infection, GSDMD cleavage and cell death did not appear to drive IL-1β release from primary human monocytes, as there was no difference in the percent of viable *T. gondii*-infected or mock-infected monocytes at 4 hpi, the time-point when functional IL-1β was detected in the supernatant. In addition, glycine treatment, which inhibits ion flux and pyroptosis had no effect on *T. gondii*-induced IL-1β release. While it cannot be completely ruled out that a small number of monocytes that die early during *T. gondii* infection are responsible for all the IL-1β released, this possibility seems unlikely because *T. gondii* continues to live and replicate within human monocytes for at least another 14 hours after maximal IL-1β release is detected, suggesting that the cells do not die rapidly after infection. In examining a role for GSDMD, we found that the cleaved, active N-terminal fragment of GSDMD was not increased in *T. gondii*-infected primary monocyte lysates. Finally, GSDMD KO THP-1 cells released comparable levels of IL-1β to wild-type THP-1 cells during *T. gondii* infection, further suggesting a pyroptosis-independent mechanism of IL-1β release from *T. gondii*-infected monocytes.

Our current findings support and expand on a threshold model in which the amount of IL-1β production and the mechanism of its release are dependent on the stimulus (219). The

amount of IL-1β released by primary human monocytes during LPS stimulation was significantly higher than that released during *T. gondii* infection, and LPS and ATP stimulation together induced almost an order of magnitude more IL-1β release than LPS alone. Notably, only LPS and ATP stimulation triggered significantly more cell death than mock-treated monocytes. These data suggest that perhaps different signaling pathways are activated to induce low, medium, and high amounts of IL-1β release, all depending on the stimulus that a cell encounters. The use of a variety of stimuli that can lead to the same response, but perhaps through different mechanisms, will be a valuable tool in developing a more comprehensive understanding of how human immune cells regulate inflammation. This work also demonstrates that IL-1β production can be uncoupled from IL-1β release during LPS stimulation of primary human monocytes and highlights GSDMD-independent mechanisms of IL-1β release in the context of viable cells. Collectively, the current findings not only provide a more detailed understanding of how human innate immune cells regulate inflammation but also shed light on the pathways that contribute to host defense against a parasite pathogen of global importance.

Supporting Information

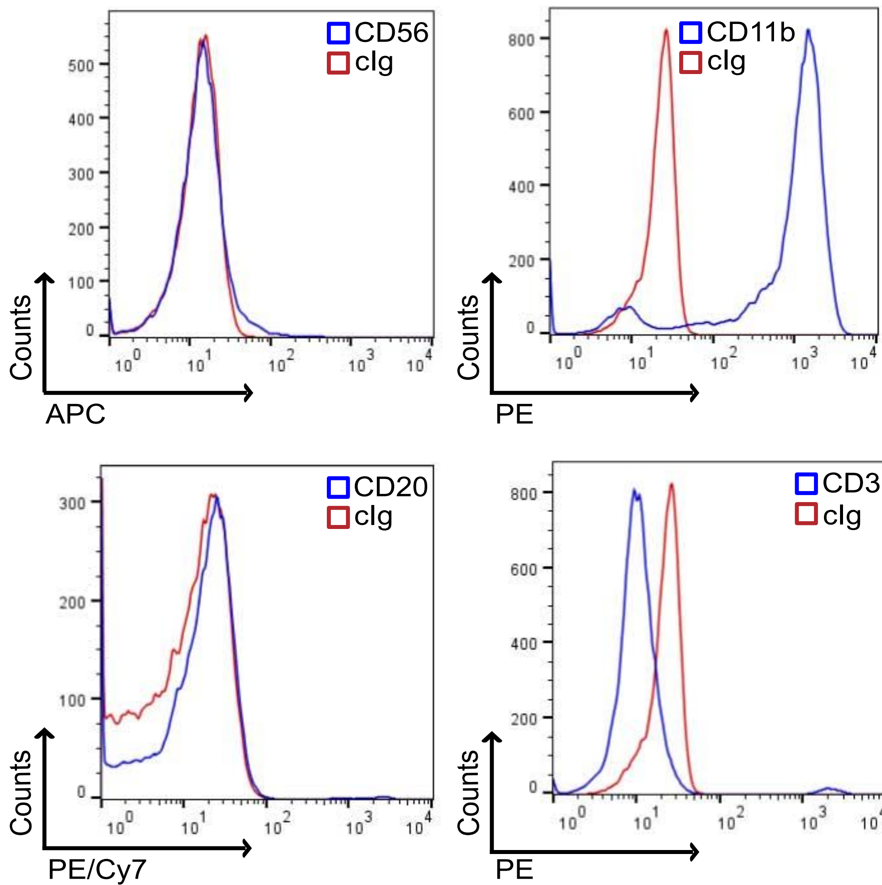


Fig. 2.S1 Phenotype of primary human monocytes. Primary monocytes were enriched from PBMCs of healthy blood donors by counterflow elutriation and analyzed for purity. Cells were stained with anti-CD56, anti-CD11b, anti-CD20, anti-CD3 or isotype controls (cIg) for each antibody, and flow cytometry was performed. The results of a representative analysis from > 70 independent monocyte isolation experiments are shown.

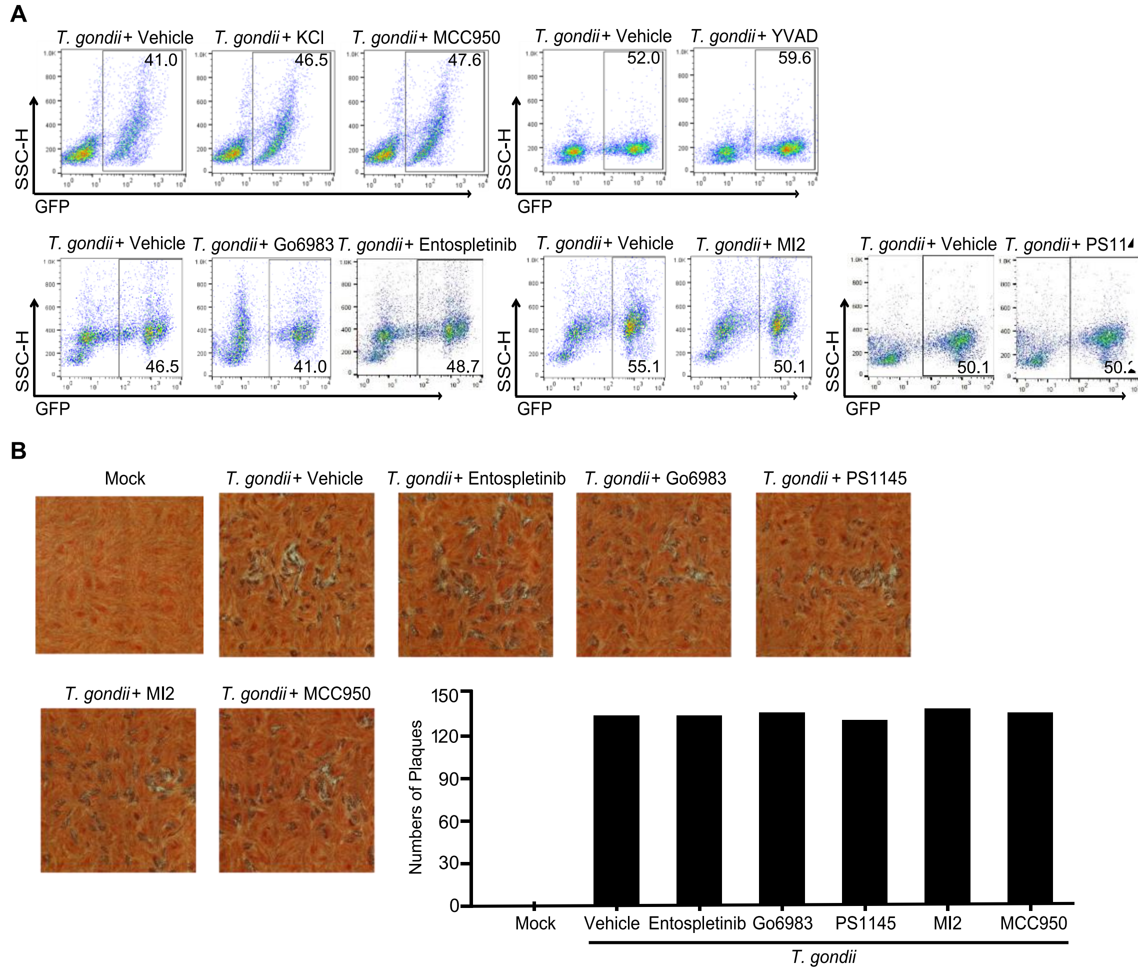


Fig. 2.S2 Effect of inhibitors on infection efficiency and parasite viability. (**A**) Primary human monocytes were pretreated with 50 mM potassium chloride, 2 µM MCC950, 50 µM YVAD, 0.3 µm entospletinib, 300 nM Go6983, 3 µM MI2, 100 nM PS1145, or vehicle control for 40 min. Cells were then infected with T. gondii for 4 h, and infection efficiency (% of GFP+ cells) was analyzed by flow cytometry. (B) HFFs were grown in 6-well plates and pre-treated with 2 µM MCC950, 0.3 µm entospletinib, 300 nM Go6983, 3 µM MI2, 100 nM PS1145, or vehicle control for 40 min before infection with *T. gondii*. Plaque assays were conducted and plaques in each condition were counted. For (A), representative plots from 2-9 independent donors are shown. 1 representative plaque assay of 3 independent experiments is shown in (B).

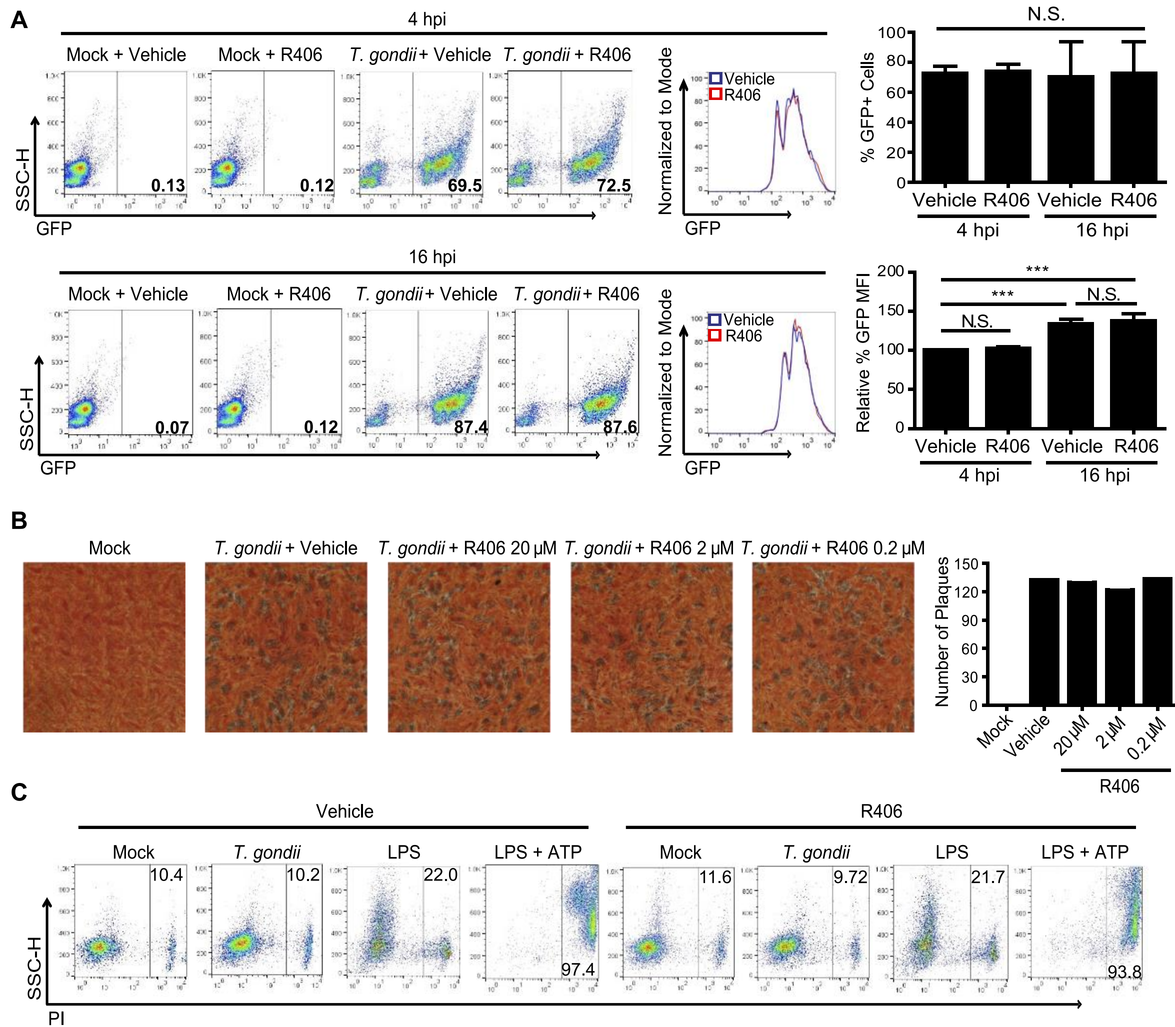


Fig. 2.S3 Effect of the Syk-specific inhibitor R406 on infection efficiency and parasite viability. (**A**) Primary human monocytes were pretreated with 2 μM R406 or vehicle control for 40 min and infected with *T. gondii* for 4 h or 16h. The infection efficiency (% of GFP^{+} cells) and the median fluorescence intensity (MFI) of the GFP^{+} population was analyzed by flow cytometry. (**B**) HFFs were grown in 6-well plates and pre-treated with a titration of R406 or vehicle control for 40 min before infection with *T. gondii*. Plaque assays were conducted, and plaques in each condition were counted. (**C**) Primary human monocytes were treated with a vehicle control or R406 and either infected with *T. gondii,* stimulated with LPS alone, or LPS+ATP, and stained with propidium iodide. The cells were analyzed by flow cytometry for cell viability. These data show the representative FACS plots for the graph shown in Figure 7A. For (A), a representative set of FACS plots is shown, and the GFP MFI and infection efficiency graphs reflect data from 3 independent experiments. 1 representative plaque assay of 2 experiments is shown in (B). Values are expressed as the mean ± SD, ****P*<0.001, (one-way ANOVA followed by a Tukey post-test in panel A).

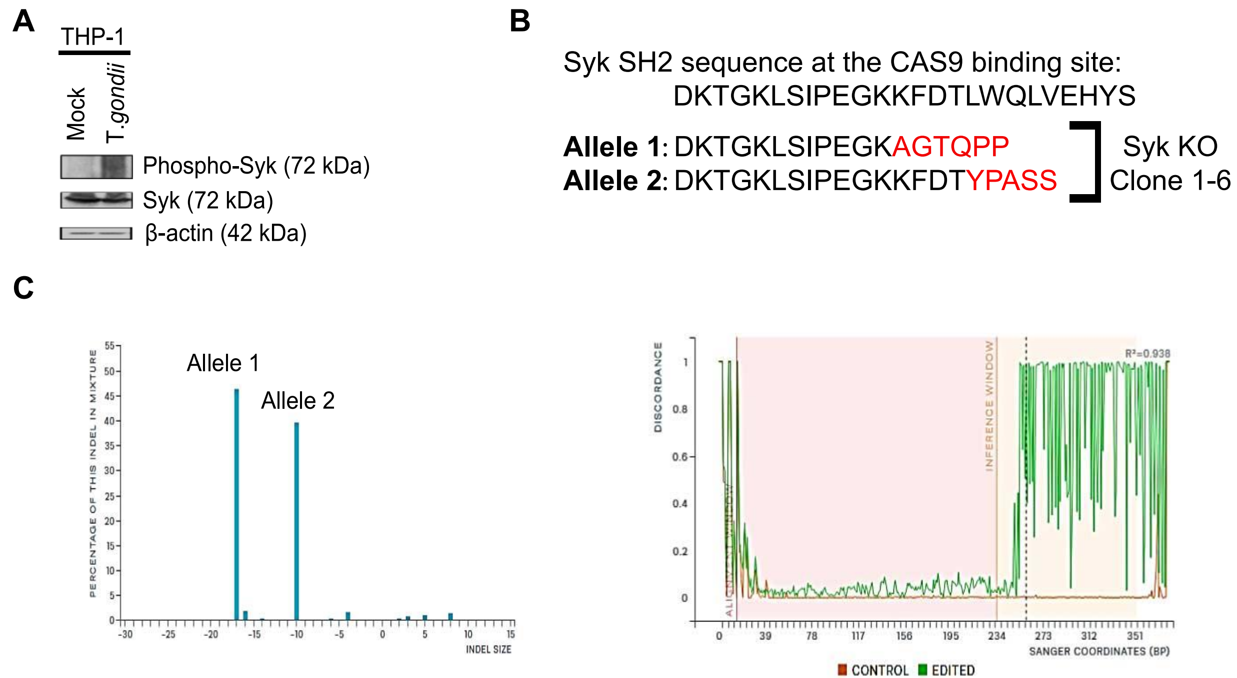


Fig. 2.S4 Generation of Syk KO clone in THP-1 cells. (**A**) THP-1 cells were mock treated or infected with *T. gondii* for 30 min. Total Syk, phospho-Syk (Tyr 525/526), and β-actin in the cell lysates were visualized by Western blotting. (**B**) Syk KO clone 1-6 contains an indel in both alleles (biallelic indel) that introduces a frameshift mutation in the second SH2 domain. The wild-type amino acid (aa) sequence of Syk near the Cas9 binding site is shown above, and the aa sequences of the two alleles in the KO clone are shown below, with the mutated sequences shown in red. (**C**) Interference of CRISPR edits (ICE) software analysis of Syk clone 1-6 generated an indel frequency plot (left) showing the relative frequency of each indel based on their number of nucleotides (indel sizes), with equal frequencies of the two indels for the biallelic KO clone. Discordance plots (right) show the alignment of bases between the wild-type unedited sequence (red) and the KO sequence (green), with discordance observed near the Cas9 cut site. Vertical dotted lines denote the expected cut site.

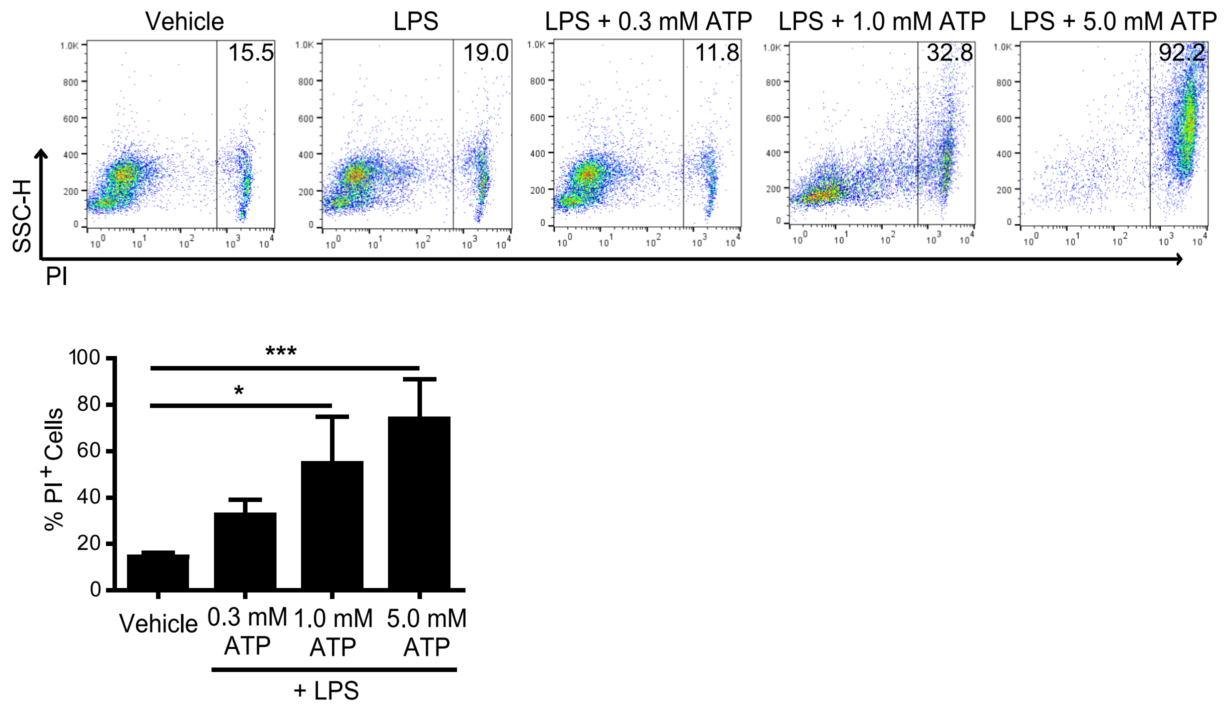


Fig. 2.S5 ATP triggers cell death in a dose-dependent manner. Primary monocytes were stimulated with LPS (100 ng/ml) alone or in combination with ATP (0.3, 1.0, or 5.0 mM), or vehicle control for 4 h, and stained with propidium iodide (PI). Cell viability was analyzed by flow cytometry. Values are expressed as the mean ± SD from experiments with n = 3 independent donors. **P*<0.05, ****P*<0.001 (one-way ANOVA followed by a Tukey post-test).

Materials and Methods

Ethics Statement

Human whole blood was collected by the Institute for Clinical and Translational Science (ICTS) at the University of California, Irvine 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 (HS #2017-3753).

Mammalian and *T. gondii* cell culture

Primary human monocytes were isolated from human whole blood collected by the Institute for Clinical and Translational Science (ICTS) at the University of California, Irvine from healthy adult donors. PBMCs were isolated from whole blood by density gradient centrifugation using lymphocyte separation media (MP Biomedicals, Santa Ana, CA). Monocytes were enriched from PBMCs by counterflow elutriation, as previously described (66), and stained for purity after isolation. This protocol typically resulted in >90% pure monocyte cultures (ranging from 85-95%) based on $CD11b^{+}$ and $CD3^{-}CD20^{-}CD56^{-}$ staining (S1 Fig). Freshly isolated monocytes were resuspended in RPMI 1640 (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and either 10% heat-inactivated FBS (Omega Scientific, Tarzana, CA) (R-10%) or no serum (R-0%). Monocytes were used immediately after isolation for experiments.

The human monocytic THP-1 cell line and the gasdermin D knock-out (GSDMD KO) THP-1 cells, a gift from Dr. Derek Abbott (Case Western Reserve University) (264), were cultured in R-10% (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The Syk KO, CARD9 KO, and PKCδ KO THP-1 cells were cultured in R-10% (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml puromycin.

Human foreskin fibroblasts (HFFs; from the lab of Dr. John Boothroyd, Stanford University School of Medicine) were cultured in D-10% medium: DMEM (HyClone) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. *T. gondii* tachyzoites were maintained by serial passage in confluent monolayers of HFFs. Type II (*Prugniaud*) (273) parasites constitutively expressing GFP were used.

All mammalian 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.

Generation of Knockout (KO) Cell Lines

Knockout THP-1 cells were generated using the Lenti-CRISPR-Cas9 system. Guide RNAs (sgRNA) targeting human Syk, PKCδ, or CARD9 were cloned into the LentiCRISPR v2 plasmid (Feng Zhang, Addgene plasmid #52961). Virus was generated by transfecting the sgRNA plasmid constructs into HEK 293T cells along with the psPAX2 packaging (Didier Trono, Addgene plasmid #12260) and pCMV-VSVG envelope (Bob Weinberg, Addgene plasmid #8454) plasmids. Viral supernatants collected 48 hr post-transfection were used to infect THP1 cells by spinfection at 1800 rpm for 1 hr. Single-cell Syk knockout clones were generated by limiting dilution in 96-well plates under puromycin selection. Single-cell clones were sequenced after PCR amplification of a 500 bp region near the Cas9 binding site. Interference of CRISPR edits (ICE) analysis software (Synthego) was used to characterize the indel for each clone. A Syk KO clone (named 1-6) with a biallelic indel-induced frame-shift mutation in the second SH2 domain was used for subsequent experiments. The PKCδ and CARD9 KOs are comprised of mixed cell populations. All the mixed populations and clones were screened via Western blot for the presence of Cas9 and the absence of the gene targeted for deletion. The sequences for the guide RNAs were as follows: Syk: GAAAGAAGTTCGACACGCTC, PKCδ: AGTTCTTACCCACGTCCTCC, and CARD9: ATCGTTCTCGTAGTCCGACA.

Infection and stimulation of monocytes

Primary human monocytes and monocytic THP-1 cells were resuspended in R-0% or R-10% medium directly after isolation and incubated with small molecule inhibitors or equal volumes of vehicle and incubated for 40 minutes at 37°C. *T. gondii*-infected HFF were washed with D-10% medium, scraped, and syringe lysed. Lysed tachyzoites were washed with R-0%, passed through a 5-μm filter (EMD Millipore, Billerica, MA), and washed with R-0% medium 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 2. All infections were performed with GFP-expressing type II *T. gondii*. "Mock" infections were samples in which an equivalent volume of culture medium without parasites was added to the cells. Cells were stimulated with 100 ng/ml ultrapure *E. coli* LPS (List Biological Laboratories, Campbell, CA) and 5 mM ATP (Sigma-Aldrich, St. Louis, MO) for the last 30 min of culture, as indicated. "Mock" treatment was the addition of the equivalent volume of media (without parasites or LPS) to cells. At the indicated time point, monocytes were pelleted by centrifugation at 500 x g for 5 min. Collected cells were stained, fixed, or lysed accordingly, as described below.

Inhibitors

MCC950 (Adipogen, San Diego, CA), glycine (Fisher Scientific, Waltham, MA) and potassium chloride (Fisher Scientific) were resuspended in deionized water. Go6983 (Selleck Chemicals, Houston, TX), Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK or YVAD) (Cayman Chemical, Ann Arbor, MI), MI2 (Tocris Bioscience, Bristol, UK), PS1145 (Cayman Chemical), R406, and entospletinib (Selleck Chemicals), were all resuspended in DMSO. Monocytes were treated with the inhibitors or with an equivalent volume of the appropriate vehicle, for 40 min at 37°C and then infected or stimulated as described above. MCC950, Go6983, MI2, PS1145, R406 and entospletinib were all added to infected cells in half log

titrations to determine the concentrations of the inhibitors that did not induce cell death or reduce infection efficiency.

Plaque Assays

HFF were plated on 6-well plates and grown to confluence for two days. HFF were pretreated with specific inhibitors for 40 min and then infected with freshly-lysed *T. gondii* tachyzoites for 5-7 days, followed by fixation with 10% Neutral Buffered Formalin. Staining was done using 600 µg/ml of Neutral Red solution overnight. The plaques were manually counted and imaged using a Leica DMi8 microscope with a DMC 5400 camera.

Flow cytometry

To measure infection efficiency, cells were harvested at the time points listed and immediately analyzed by FACS to determine the percent of GFP^{+} (*T. gondii*-infected) cells. To measure cell viability, cells were harvested, washed and resuspended in FACS buffer (2% FBS in PBS) containing propidium iodide and analyzed by flow cytometry without fixation. For cell surface staining, 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 Abs (all from BioLegend, unless otherwise indicated): anti-CD56–allophycocyanin (HCD56), anti-CD11b–PE or, anti-CD14–FITC (M5E2) or -PE/Cy7 (HCD14), anti-CD16-PE/Cy7 or -APC (3G8), anti-CD3–PE (UCHT1), or anti-CD20–PE/Cy7 (2H7). Cells were stained with the Abs on ice for 30 min, washed with FACS buffer, and either run live or fixed with 2-4% paraformaldehyde. For intracellular cytokine staining (ICCS), cells were fixed and permeabilized with 100 µL of BD Cytofix/Cytoperm solution (BD Biosciences, Franklin Lakes, NJ) for 20 minutes on ice. After incubation, cells were washed with FACS buffer containing 0.1% Triton-X, blocked with Human TruStain FcX as described above, stained with control Ig-PE or anti-IL-1β–PE (CRM56; eBioscience, San Diego, CA), control Ig-PE/Cy7 or anti-phospho-Syk (Y525/526)-

PE-Cy7 (C87C1; Cell Signaling Technologies, Danvers, MA) Abs for 30 min, and washed with FACS buffer.

Samples were analyzed by flow cytometry on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). 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 (qPCR)

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. cDNA was synthesized using the Superscript III First-Strand Synthesis kit (Life Technologies), according to the manufacturer's instructions, and subsequently used as template in quantitative real-time PCR (qPCR). qPCR was performed in triplicate using a Bio-Rad iCycler PCR system (Bio-Rad, Hercules, CA) and iTaq Universal SYBR Green Supermix (Bio-Rad). Previously published sequences for *IL-1β, NLRP3* and *GAPDH* primers were used (66). 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). qPCR data were analyzed using the threshold cycle method, as previously described (66), and gene expression data are shown normalized to that of the housekeeping gene GAPDH. In all qPCR assays, cDNA 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

Human IL-1β protein released into the supernatant was measured using ELISA MAX Deluxe kits (BioLegend), according to the manufacturer's instructions. Signal from ELISA plates was read with a Spectra Max Plus 384 plate reader (molecular Devices, San Jose, CA) using SoftMax Pro Version 5 software (molecular Devices), and the threshold of detection was 0.5 pg/ml.

Western blots

At the harvest time point, cells were lysed by addition of 2X Laemmli buffer containing 10% 2-ME. For experiments in which supernatant was analyzed, serum-free R-0% medium was used during the infection, and supernatant was concentrated using Amicon Ultra Centrifugal filters (EMD Millipore, Burlington, MA), according to the manufacturer's instructions. Concentrated supernatant was diluted with 2X Laemmli buffer containing 10% 2-ME. Samples were boiled at 100°C for 10 to 15 min, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) for immunoblotting. Membranes were blocked for 1 h at room temperature (RT) with blocking buffer: 5% non-fat milk or 5% bovine serum albumin (BSA) (Fisher Bioreagents). Membranes were then incubated with primary antibodies diluted in blocking buffer for 1 h at RT or overnight at 4°C. Membranes were probed with antibodies against NF-κB p65 (D14E12; Cell Signaling), phospho-NF-κB p65 (Ser536) (93H1; Cell Signaling), total Syk (2712S; Cell Signaling), phospho(Tyr525/526)-Syk (2711S; Cell Signaling), gasdermin D (NPB2-33422; Novus Biologicals, Littleton,CO), or β-actin (AC-15; Sigma-Aldrich). Membranes were blotted for IL-1β (3ZD from the National Cancer Institute Biological Resources Branch) using the SNAP i.d. Protein Detection System (EMD Millipore), according to the manufacturer's instructions. Primary Abs were followed by HRP-conjugated secondary Abs (BioLegend), and membranes were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Carlsbad, CA),

Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, U.K.) substrate or ECL Prime Substrate (Thermo Fisher Scientific). Signal was detected using a Nikon camera, as previously described (66). Quantification analysis of blots was performed using ImageJ software.

HEK-Blue reporter cell assay

HEK-Blue IL-1 reporter cells (Invivogen, San Diego, CA), which respond to IL-1 binding to the IL-1R, were incubated in D-10% medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, Normocin (100 μg/ml), Hygromycin B Gold (200 μg/ml) and Zeocin (100 μg/ml). For the detection of IL-1 released from THP-1 cells and primary human monocytes, HEK-Blue cells were resuspended at a concentration of 500,000 cells/ml and added to flat-bottom 96-well plates. Supernatants collected from THP-1 cells or primary monocytes were added to the HEK-Blue cells and incubated for 24 hours at 37°C. The HEK-Blue cell supernatant was combined with the QUANTI-Blue detection reagent (Invivogen), incubated at 37°C for 1 to 3 hours, and then quantified with a Spectra Max Plus 384 plate reader (Molecular Devices, San Jose CA) using SoftMax Pro Version 5 (Molecular Devices) software.

Statistics

Statistical analyses were performed using GraphPad Instat software. Analysis of variance (ANOVA) followed by Tukey's or Bonferroni's test, as indicated, were used for comparison between means. Differences were considered significant when the *P* value was <0.05.

Acknowledgements

We would like to thank members of the Lodoen, Tenner, Nelson, and Morrissette labs, as well as Dr. Eric Pearlman and Dr. Martin Minns for helpful discussion on the project. We would also like to thank Dr. Derek Abbott at Case Western Reserve University for the gift of the GSDMD KO THP-1 cells. We are grateful to the administration and excellent nurses of the UC Irvine Institute of Clinical and Translational Sciences (ICTS) for maintaining the Healthy Blood Donor program, as well as all the individuals who donated blood for this study. This work was supported by NIH R01AI120846 to (M.B.L.), American Cancer Society RSG-14-202-01-MPC (to M.B.L.), and NIH T32AI060573-12 (to W.J.P.)

Chapter 3

Caspase-8 and the NLRP3 inflammasome are required for IL-1β release from viable cells during *Toxoplasma gondii*-infection of human monocytes

Introduction

Caspases are a set of cysteine-aspartic proteases that can function in inflammation and cell death. Caspases-1, -4 and -5 are considered inflammatory caspases, as their activities lead to the cleavage of the proinflammatory cytokine pro-IL-1β into its active form, mature IL-1β (179,253,274,275). Cell death-related caspases include the initiator caspases-2, -8, -9, and -10 and the executioner caspases-3, -6 and -7. Although caspase-9 is traditionally considered an initiator of intrinsic apoptosis and caspase-8 an initiator of extrinsic apoptosis, they can also be coordinately activated (232). Both caspases serve to activate caspases-3 and -7, which execute the final stages of apoptosis (230,231). Caspase-8 also negatively regulates an inflammatory form of cell death termed necroptosis through its inhibition of RIPK1 and therefore RIPK3 and MLKL (236). More recently it has been revealed that caspase-8 can also function as an inflammatory caspase by influencing IL-1β release through priming and activation of the NLRP3 inflammasome as well as cleaving pro-IL-1β and gasdermin D, in addition to its previously described role as an initiator caspase in apoptosis (175,178,213,231,236,238,276).

The proinflammatory cytokine and potent human pyrogen IL-1β is critical for protection against many bacterial, viral, fungal, and parasitic pathogens (191,193). However, given its potency, IL-1β can also cause pathological consequences and has been implicated in the development of autoimmune diseases, such as rheumatoid arthritis, atherosclerosis, type II diabetes, CAPS, Alzheimer's disease, and gout (196,199,200,202). Although the modified IL-1 receptor antagonist, anakinra, has been approved for the treatment of rheumatoid arthritis, there is no treatment that specifically inhibits the aberrant production of IL-1β. In this pursuit, a significant amount of research has focused on understanding the steps required for the production and release of IL-1β.

Similar to the caspases, pro-IL-1β is translated as an inactive protein, and must be proteolytically processed, most commonly by caspase-1, to exert its biological effects

(176,177,274). Caspase-1 is activated by multiprotein complexes called inflammasomes, which consist of NOD-like receptor proteins (NLRPs) that sense intracellular stimuli and bind the adaptor protein ASC, which contains a caspase-recruitment domain (CARD) (168,277). ASC, once nucleated in these large complexes, binds pro-caspase-1, bringing the protease into close physical contact with itself and allowing for auto-proteolysis and activation of caspase-1 (171,173). Once cleaved, IL-1β does not exit the cell through the conventional secretory pathway via the Golgi (221). Instead, caspase-1 also cleaves gasdermin D (GSDMD), which forms pores in the cell membrane and allows IL-1β to pass through these pores from still viable cells (226,278). Eventually these pores allow for osmosis-driven cell swelling and lysis, during which intracellular contents, including additional IL-1β, are released from the dying cells in a form of inflammatory cell death called pyroptosis (222,253,255).

The NLRP3 inflammasome is the best studied inflammasome and responds to a wide variety of stimuli. Canonical NLRP3 inflammasome activity in most cells requires two separate signals. First, a priming signal, such as LPS binding to TLR4, is required to induce NF-κB activation and translocation and the transcriptional activation of pro-IL-1β and NLRP3 (171). Next an activating signal, such as exposure to extracellular ATP or nigericin, is required to induce conformational changes in the NLR which then facilitates its oligomerization and formation of the active inflammasome, which cleaves caspase-1 and IL-1β (170,171).

While this model of IL-1β regulation has mostly held true in murine and human macrophages, human, but not murine, monocytes and neutrophils release IL-1β and activate the NLRP3 inflammasome in response to a single priming stimulus without a secondary activating signal (178,181,221,275). During non-canonical NLRP3 inflammasome activation, human monocytes recognize intracellular LPS, which activates caspases-4 and -5. These caspases are then responsible for caspase-1 activation and the cleavage of IL-1β (179,184,275). Human monocytes can also activate an alternative inflammasome, in which

extracellular LPS stimulation alone signals through caspase-8, which contributes to NLRP3 inflammasome activation through its interactions with FADD and RIPK1 (178). The alternative NLRP3 inflammasome also induces IL-1β release independent of cell death or potassium efflux, two markers of the canonical NLRP3 inflammasome (178).

To further investigate the requirements for inflammasome activation during infection, we infected primary human peripheral blood monocytes with the protozoan parasite *Toxoplasma gondii*, which induces the release of IL-1β (134). *T. gondii*, a member of the phylum *Apicomplexa* and closely related to *Plasmodium* species, is a eukaryotic single-celled obligate intracellular parasite that is estimated to infect one-third of the global population (86). *T. gondii* infection is life-threating for immunocompromised individuals and developing fetuses, and also causes significant morbidity in immunocompetent hosts, as it is a leading cause of hospitalization due to a foodborne pathogen in the United States (99,108,113). $CD4^+$ and $CD8^+$ T cells are required for protection against infection through the production of IFN-γ, but innate immune cells, particularly human monocytes and IL-1β also significantly contribute to immune control of infection (81,247,279). Monocytes are among the first cells recruited to sites of infection. They are also preferentially infected by the parasite compared to other PBMCs and once infected rely on the NLRP3 inflammasome to produce and release bioactive IL-1β (66,134,157). Considering that infected human monocytes produce and release IL-1β during a successful immune response against this pathogen, these cells provide an excellent and biologically relevant system to study IL-1β production and release.

It is still unclear if caspases other than caspase-1 contribute to IL-1β release from *T. gondii*-infected human monocytes, and the precise mechanism of IL-1β release from *T. gondii*-infected monocytes has yet to be defined. We previously showed that IL-1β is released from *T. gondii*-infected human monocytes in a manner dependent on priming through a Syk-PKCδ-CARD9-MALT1-NF-κB signaling pathway, and activation of an NLRP3 inflammasome that

requires caspase-1, ASC, and potassium efflux (66,157). IL-1β was released from these cells via a process that was independent of cell death or GSDMD (157). Here we demonstrate that caspase-8, but not caspases -4 or -5, is also required for IL-1β release from *T. gondii*-infected cells. In addition, caspase-8 appeared to function in a novel mechanism of controlling the release of IL-1β from viable cells, rather than cleavage of IL-1β. This process was also independent of caspase-3 or GSDMD. The studies presented here expand on the known inflammatory roles for caspase-8 and defines a novel role for caspase-8 in IL-1β release from viable cells.

Results

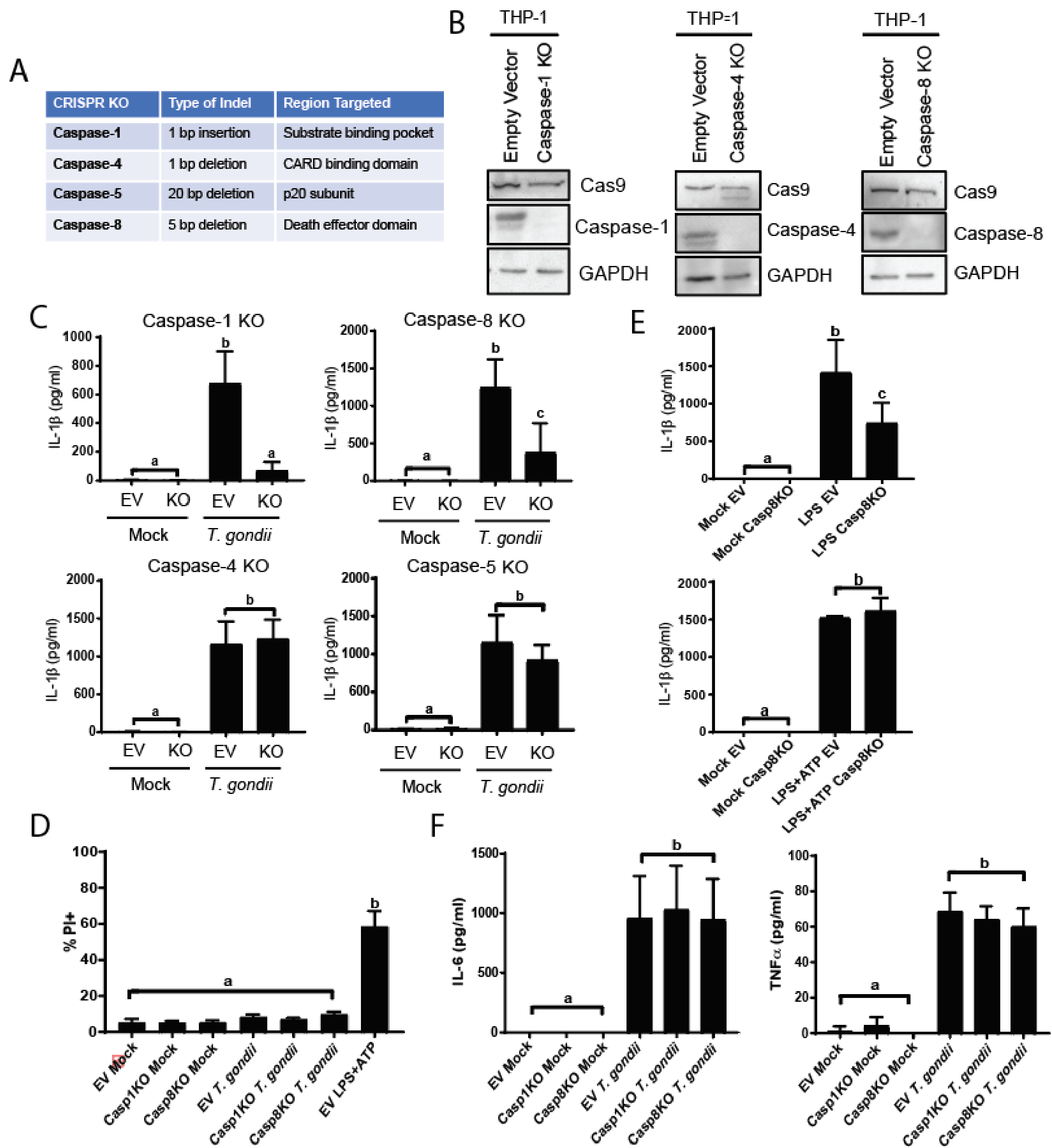
Caspase-1 and -8, but not caspase-4 or -5, contribute to IL-1β release from *T. gondii*-infected human monocytes

Caspases-1, -4, -5 and -8 have each been shown to contribute to IL-1β release from human monocytes and macrophages during activation of the canonical, noncanonical or alternative NLRP3 inflammasomes in response to LPS stimulation and infection with pathogens (171,175,178,182,275). We and others have previously reported a role for caspase-1 in *T. gondii*-induced IL-1β production (66,134,157,159). To determine if other caspases are involved in the release of IL-1β in human monocytes infected with *T. gondii*, caspase-1, -4, -5, and -8 knockout (KO) and empty vector (EV) human monocytic THP-1 cells were generated using CRISPR/Cas9 genome editing, as previously described (157). After selection and clonal expansion of stable KO and EV cell lines, the clones were sequenced to identify the mutations introduced by the CRISPR/Cas9 system. The caspase-1 KO clone contained a 1 base pair (bp) insertion in the substrate binding pocket, the caspase-4 KO clone contained a 2 bp deletion in the CARD-binding domain leading to a premature stop codon, the caspase-5 KO clone

contained a 20 bp deletion in the p20 subunit leading to a premature stop codon, and the caspase-8 clone held a 5 bp deletion in the death effector domain (Fig. 3.1A). Western blot analysis of the EV and KO clones demonstrated that caspase-1, -4, and -8 were undetectable in cell lysates of their respective KO clones, and Cas9 was detectable in both EV and KO cells (Fig. 3.1B). Since we could not detect caspase-5 by Western blot using several commercially available antibodies, as has been previously reported (280), the KO clone was verified by sequencing.

THP-1 KO and EV clones were infected with a type II *Prugniaud*, GFP-expressing strain of *T. gondii* or exposed to an equal volume of media (mock), and the supernatants were analyzed by ELISA. At 16 hours post infection (hpi) *T. gondii*-infected caspase-1 and -8 KO cells, but not caspase-4 or -5 KO cells, released significantly less IL-1β compared to the mock cells or the *T. gondii*-infected EV cells (Fig. 3.1C). The reduced IL-1β release was not due to reduced infection of the KO lines, as there was no difference in the percent of infected (GFP^+) cells in each condition (Fig. 3.S1A). To evaluate an effect of infection on cell death in the EV and caspase-1 and -8 KO cells, propidium iodide (PI) staining was used. Flow cytometry analysis revealed that there were no significant changes in viability between the infected and mock-treated conditions (Fig. 3.1D). As a positive control for cell death, the EV cells were treated with dual LPS and ATP stimulation, which activates the canonical NLRP3 inflammasome. inducing pyroptosis and a significant increase in PI^+ cells.

Caspase-8 KO THP-1 cells also released less IL-1β than EV cells during LPS stimulation (Fig. 3.1E), but caspase-8 was dispensable for IL-1β release during LPS and ATP stimulation, as expected (Fig. 3.1F), reinforcing that caspase-8 is not required for IL-1β release from human monocytes during priming and activating stimulation of the canonical NLRP3 inflammasome. The role of caspase-8 in cytokine release appeared to be specific to IL-1β, as caspase-8 KO THP-1 cells released comparable levels of IL-6 and TNF-α to the EV cells during *T. gondii* infection (Fig. 3.1G)



CRISPR KO	Type of Indel	Region Targeted
Caspase-1	1 bp insertion	Substrate binding pocket
Caspase-4	1 bp deletion	CARD binding domain
Caspase-5	20 bp deletion	p20 subunit
Caspase-8	5 bp deletion	Death effector domain

Fig. 3.1

Fig. 3.1. IL-1β release during *T. gondii* infection of THP-1 cells is dependent on caspase-1 and caspase-8. (A) Summary of mutations in caspase KO THP-1 cells. **(B)** Empty vector (EV) control and caspase-1, -4 and -8 KO THP-1 cells were lysed and probed for Cas9, GAPDH, caspase-1, -4, and -8 by Western blot. **(C)** EV and caspase KO THP-1 cells were mock treated or infected with *T. gondii* (MOI = 2) for 16 hours. IL-1β in the supernatant was detected by ELISA. Results of 4, 8, 5 and 6 experiments are plotted for caspase-1, -8, -4 and -5 KO THP-1 cells respectively. **(D)** EV and caspase KO THP-1 cells were mock treated, infected with *T. gondii* (MOI = 2) for 16 hours or stimulated with 100 ng/mL of LPS for 16 hours and 5 mM ATP for the last 30 minutes of stimulation. Cells were stained with propidium iodide (PI), and the percentage of PI^+ cells in each condition was quantified by flow cytometry. Results of 7 independent experiments are plotted. **(E)** EV and caspase-8 KO THP-1 cells were mock treated or stimulated with 100 ng/mL of LPS for 16 hours. IL-1β in the supernatant was detected by ELISA. Results of 4 experiments are plotted. **(F)** EV and caspase-8 KO THP-1 cells were mock treated or stimulated with 100 ng/mL of LPS for 16 hours and 5 mM ATP for the last 30 minutes of stimulation. IL-1β in the supernatant was detected by ELISA. Results of 3 experiments are plotted. **(G)** EV and caspase-8 KO THP-1 cells were mock treated or infected with *T. gondii* (MOI = 2) for 16 hours. TNF-α and IL-6 in the supernatant were detected by ELISA. Results of 4 experiments are plotted. Values are expressed as the mean ± SD. Letters denote statistically significant differences between conditions not containing the same letter, with the cutoff for significance at $P < 0.05$ (one-way ANOVA followed by a Tukey post-test).

Caspase-8 contributes to IL-1β release from primary human monocytes during *T. gondii* infection

Given the reduction in IL-1β release observed during *T. gondii* infection of caspase-8 KO THP-1 cells, we investigated whether primary human monocytes also relied on caspase-8 for IL-1β release during infection. Primary human monocytes were isolated from healthy blood donors as previously described (66,157), and since these cells cannot be reliably genetically manipulated, the caspase-8-specific inhibitor IETD was used. In addition to its role in apoptosis, caspase-8 also functions in preventing necroptosis by inhibiting RIPK1 (236). Therefore, caspase-8-inhibited cells were also treated with Necrostatin-1 (Nec1), a RIPK1 inhibitor, to prevent necroptosis in the absence of caspase-8 activity. An equivalent volume of DMSO was used as the vehicle control. As we have previously reported (66,134,157), *T. gondii* infection but not mock infection of primary human monocytes induced the release of IL-1β by 4 hpi, as detected by ELISA (Fig. 3.2A). Nec-1 treatment had no effect on IL-1β release in mock or infected conditions. In contrast, *T. gondii*-infected cells treated with IETD and Nec1 released significantly less IL-1β compared to cells treated with Nec1 or vehicle control alone (Fig. 3.2A). To visualize the release of 17 kDa mature IL-1β from cells, Western blot was used to detect mature IL-1β in the cell supernatants at 4 hpi. The combination of caspase-8 and necroptosis inhibition significantly reduced mature IL-1β in the supernatant at 4 hpi (Fig. 3.2B). These data corroborate the ELISA results and indicate that caspase-8 contributes to IL-1β release from primary human monocytes during *T. gondii* infection. As a positive control, primary human monocytes were treated with LPS, which has been shown to induce IL-1β release via a caspase-8-dependent mechanism (178). As expected, Nec1 and IETD treatment also reduced IL-1β release during LPS stimulation of primary monocytes (Fig. 3.2C).

Interestingly, infection of murine macrophages with another closely related apicomplexan parasite, *Neospora caninum*, has also recently been shown to induce IL-1β

release through activation of the NLRP3 inflammasome (281,282). Pretreatment of primary human monocytes with NLRP3, caspase-1 and -8 specific inhibitors prior to infection with *N. caninum* also reduced IL-1β release, indicating that this release was reliant on NLRP3, caspase-1 and -8, similar to *T. gondii* infection (Fig. 3.S2). These data suggest that caspase-8 is required for IL-1β release from primary human monocytes during infection with multiple apicomplexans.

Caspase-8 has been suggested to contribute to IL-1β release through activation of caspase-3, which subsequently can cleave gasdermin E and induce cell death and IL-1β release (175). However, pretreatment of monocytes with the caspase-3 inhibitor DEVD did not reduce IL-1β release during *T. gondii* infection (Fig. 3.2D), suggesting that caspase-3 is not involved in *T. gondii*-induced IL-1β release. Moreover, to evaluate the potential effect of Nec1 and IETD treatment on monocyte viability during *T. gondii* infection, cell death was analyzed by the uptake of propidium iodide (PI). As shown in (Fig. 3.2E), *T. gondii* infection did not result in increased cell death, as we have previously reported. IETD treatment of mock or infected cells resulted in elevated cell death, as expected, since caspase-8 also functions in inhibiting RIPK1/3-mediated necroptosis. However, the addition of Nec-1 to IETD-treated cells restored their viability (Fig. 3.2E). These data indicate that the reduction in IL-1β release in caspase-8-inhibited cells was not due to an effect on cell viability. Furthermore, although it is possible that off-target effects of the caspase-1 and caspase-8 inhibitors on the parasite's ability to infect monocytes result in decreased IL-1β release during stimulation of primary monocytes, pretreatment with YVAD or IETD + Nec1 had no significant effect on parasite infection efficiency, or host cell death during the period of infection (Fig. 3.2E and Fig. 3.S1B)(157).

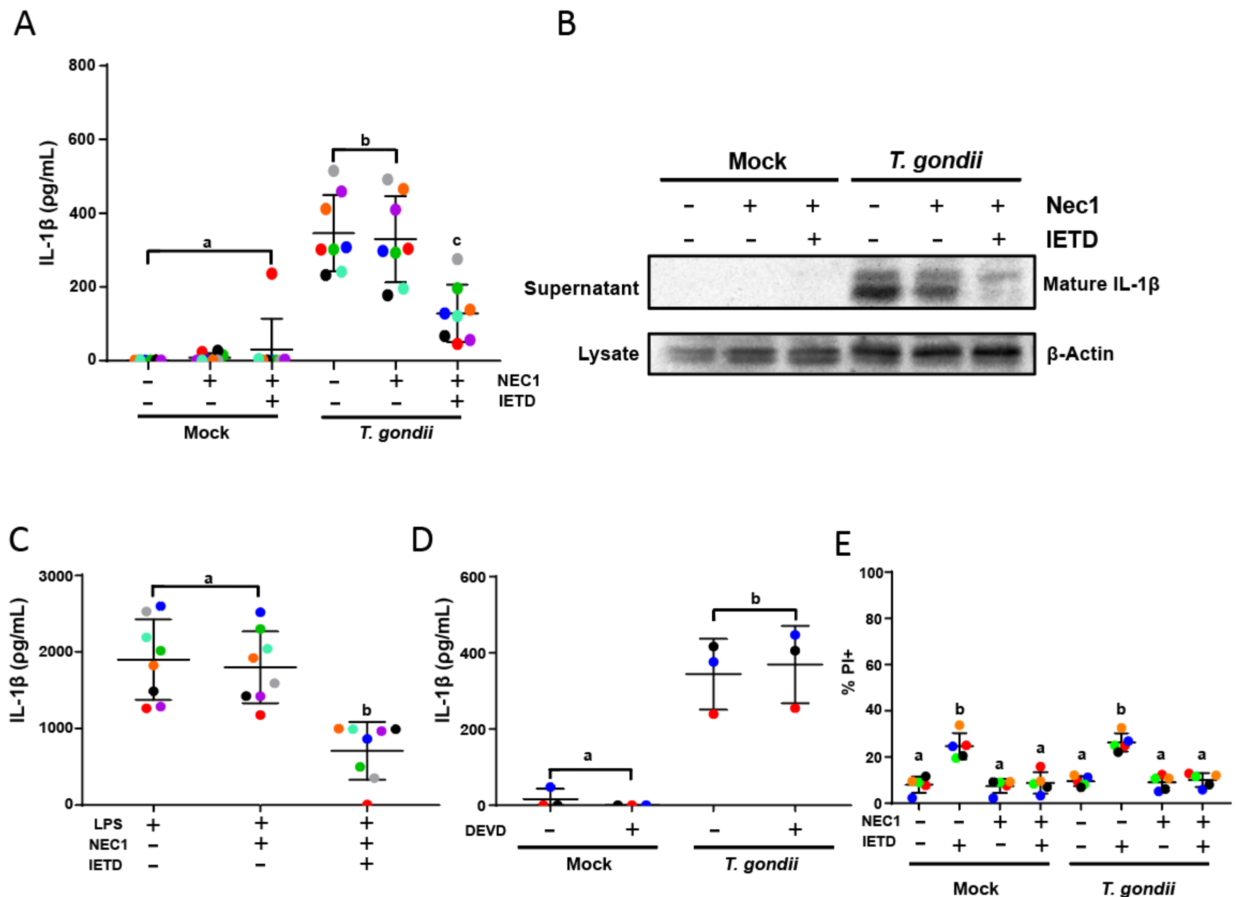


Fig. 3.2. Primary human monocytes utilize caspase-8 for IL-1β release during *T. gondii* infection. Primary human monocytes were pretreated with 10 ng/mL Nec1 for 30 min before incubating the cells with 10 μM IETD, or vehicle control for 30 min. Cells were then mock treated or infected with *T. gondii* (MOI = 2) for 4 hours. **(A)** IL-1β in the supernatant was measured by ELISA. Each color-coded dot represents a separate human blood donor. The results of 9 independent experiments are plotted. **(B)** Mature IL-1β in the supernatants and β-Actin from corresponding cell lysates were detected by Western blot. A representative blot of 2 experiments is shown. **(C)** Primary human monocytes were stimulated with 100 ng/mL LPS for 4 hours. 5 mM ATP was added for the last 30 minutes of culture. Supernatants were used for detection of IL-1β by ELISA, and the results of 3 experiments are plotted. **(D)** Primary monocytes were pre-treated with 3 μM DEVD for 30 minutes, then infected with *T. gondii* or mock infected for 4 hours. IL-1β was detected in supernatants by ELISA and the results of 3 experiments are plotted. **(E)** Primary human monocytes were treated as described in (A-C) and harvested at 4 hpi. Cells were stained with PI, and the percentage of PI^{+} cells was quantified by flow cytometry. The results of 5 experiments are graphed. Values are expressed as the mean ± SD. Letters denote statistically significant differences between conditions not containing the same letter with the cutoff for significance at $P < 0.05$ (one-way ANOVA followed by a Tukey post-test).

Caspase-8 is not required for IL-1β production during *T. gondii* infection of human monocytes

Although caspase-8 appeared to contribute to the release of IL-1β in both primary human monocytes and monocytic THP-1 cells, the mechanism by which this occurred was still unclear. Based on previously published roles for caspase-8 in regulating IL-1β, we hypothesized that caspase-8 could be involved in inducing IL-1β synthesis (238,283) or in activation of the NLRP3 inflammasome (213,234,276).

Caspase-8 has been shown to contribute to *IL-1β* transcript production in murine bone marrow derived macrophages (BMDMs) during *T. gondii* infection (238), so we first examined whether caspase-8 performs a similar function in human monocytes. As expected, *IL-1β* transcripts were induced in THP-1 cells during *T. gondii* infection; however, there was no significant reduction in *IL-1β* mRNA in caspase-8 KO cells in response to *T. gondii* (Fig. 3.3A). In addition, pro-IL-1β protein levels in cell lysates of caspase-8-inhibited primary monocytes or caspase-8 KO THP-1 cells were not significantly different from those in infected EV THP-1 cells or mock-treated primary monocytes (Fig. 3.3B and 3.3C). These data strongly suggest that caspase-8 does not significantly contribute to the induction of *IL-1β* transcripts or production of pro-IL-1β protein during *T. gondii* infection of human monocytes.

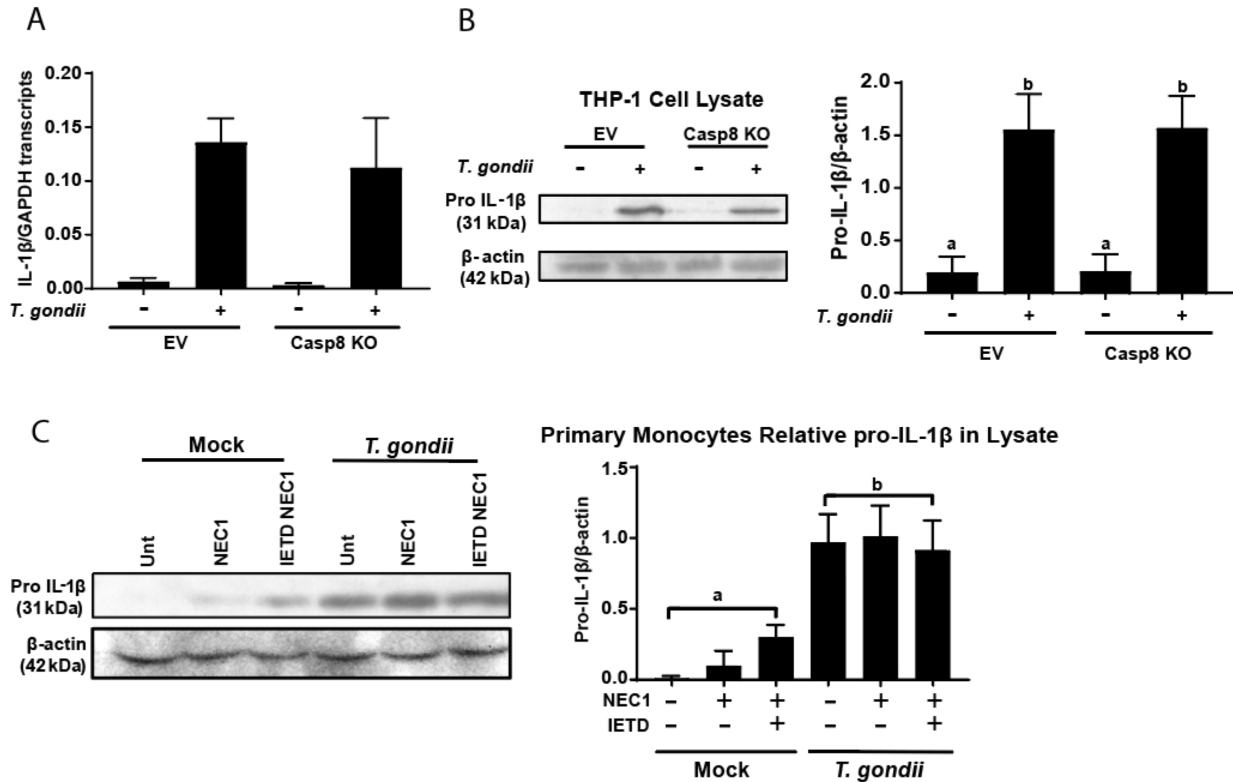


Fig. 3.3. Caspase-8 is not required for IL-1β production during *T. gondii* infection of human monocytes (A, B) EV and Caspase-8 KO THP-1 cells were mock treated or infected with *T. gondii* (MOI = 2). **(A)** Pro-IL-1β and GAPDH transcript levels were measured by qPCR at 6 hpi, and 1 representative experiment of 3 is shown. **(B)** EV and caspase-8 KO cells were harvested at 16 hpi, and cell lysates were probed for pro-IL-1β by Western blot. A representative blot is shown, and pro-IL-1β levels from 4 experiments were quantified by densitometry. **(C)** Primary human monocytes were pretreated with 10 ng/mL Nec1 or an equal volume of DMSO for 30 minutes. Cells were then mock treated or incubated with 10 μM IETD for an additional 30 minutes before infection with *T. gondii* or mock infection. Cells were harvested at 4 hpi. Pro-IL-1β in the cell lysate was analyzed by Western blot. A representative blot is shown, and pro-IL-1β levels from 3 experiments were quantified by densitometry. Values are expressed as the mean ± SD. Letters denote statistically significant differences between conditions not containing the same letter with the cutoff for significance at $P < 0.05$ (one-way ANOVA followed by a Tukey post-test).

Caspase-8 is not required for caspase-1 activity or IL-1β cleavage during *T. gondii* infection of human monocytes

Recent publications have indicated that caspase-8 can function as an inflammatory caspase by activating the non-canonical or alternative NLRP3 inflammasome to induce caspase-1 activation, or, in the absence of caspase-1 activity, caspase-8 can directly cleave IL-1β and gasdermin D (175,178,213,235,276). To investigate whether caspase-8 functions in *T. gondii*-infected human monocytes by activating the inflammasome, a luminescence-based caspase activity assay was used to probe for caspase-1 activity in *T. gondii*-infected THP-1 EV and caspase KO cell lysates. An increase in caspase-1 activity was detected during both *T. gondii* infection and during LPS and ATP dual stimulation of EV control cells, but not in infected or stimulated caspase-1 KO cells (Fig. 3.4A). These data supported the previous findings that caspase-1 is also required for IL-1β release from *T. gondii*-infected human monocytes (Fig. 3.1C)(66,134,157). Notably, there were still comparable levels of active caspase-1 in infected caspase-8 KO cells compared to EV cells during infection (Fig. 3.4A). These data indicate that caspase-1 activation can occur in the absence of caspase-8 during *T. gondii* infection and suggest that NLRP3 activation may be intact in caspase-8 KO cells.

To directly test whether caspase-8 is required for IL-1β cleavage, the EV, caspase-8, and caspase-1 KO cells were mock treated or infected with *T. gondii*, and the cell lysates were examined for pro- and mature-IL-1β by Western blot. As shown in Figure 3, levels of pro-IL-1β were equivalent between the infected caspase-8 KO and EV THP-1 cells. Interestingly, over multiple experiments there was no reduction in the cleavage of IL-1β in the infected caspase-8 KO cells, but there was an accumulation of this cleaved IL-1β in the caspase-8 KO cells compared to infected EV THP-1 cells (Fig. 3.4B). These results were corroborated by ELISAs measuring IL-1β levels in infected EV and caspase-8 KO cell lysates. Infected caspase-8 KO cell lysates contained significantly more IL-1β than infected EV control cells (Fig. 3.4C).

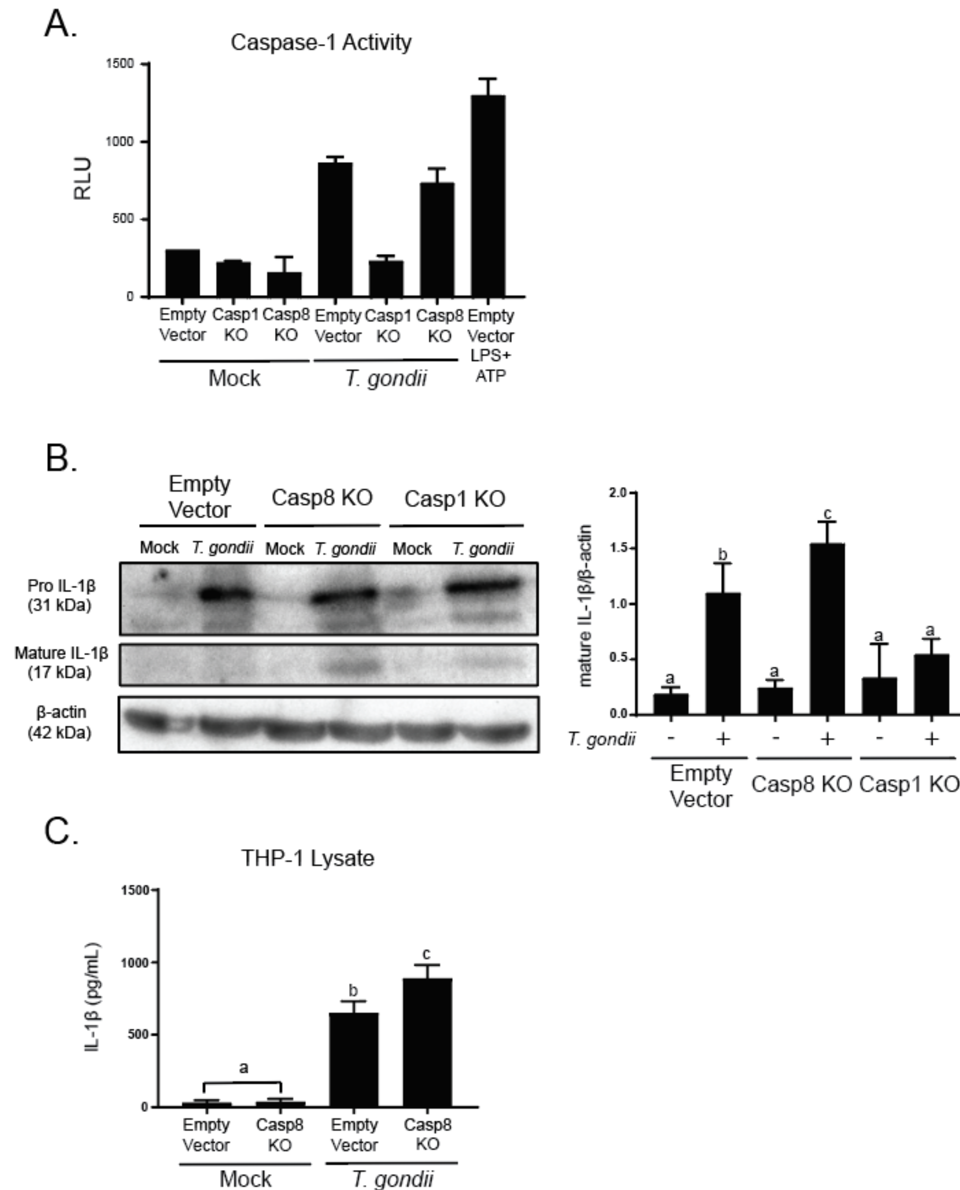


Fig. 3.4. Caspase-8 is not required for pro-IL-1β cleavage or caspase-1 activity during *T. gondii* infection of THP-1 cells (A) EV, Caspase-1 KO and Caspase-8 KO THP-1 cells were mock treated, stimulated with 100 ng/mL LPS for 16 hours and 5 mM ATP for the last 30 minutes of stimulation, or infected with *T. gondii* (MOI = 2) for 16 hours. Cells were collected at 16 hpi, and caspase-1 activity was quantified in the cell pellets using a luminescence-based caspase-glo 1 inflammasome assay. A representative experiment of 3 replicates is shown. **(B)** THP-1 cells were treated as described in (A). Cells were collected at 16 hpi, lysed, and IL-1β and β-actin were probed for by Western blot. A representative blot is shown and mature-IL-1β levels from 5 experiments were quantified by densitometry. **(C)** THP-1 cells were treated as described in (A). Cell pellets were collected at 16 hpi, lysed via 3 rapid freeze thaw cycles, and resulting lysates were probed for IL-1β by ELISA. The results of 4 experiments are plotted. Values are expressed as the mean ± SD. Letters denote statistically significant differences between conditions not containing the same letter with the cutoff for significance at $P < 0.05$ (one-way ANOVA followed by a Tukey post-test).

Considering *T. gondii*-infected human monocytes demonstrated a reliance on caspase-8 for the release of IL-1β but also appear to accumulate mature IL-1β intracellularly, these data strongly suggest that caspase-8 is not essential for IL-1β cleavage in *T. gondii*-infected human monocytes, but instead likely contributes to the cytokine's release from cells.

Caspase-8 regulates the release of mature IL-1β and active caspase-1 from *T. gondii*-infected THP-1 cells

To further confirm the importance of caspase-8 in the release of IL-1β from cells, mature IL-1β in the supernatants of *T. gondii*-infected caspase-1 and -8 KO and EV THP-1 cells were concentrated and visualized by Western blot. There was a significant decrease in the amount of mature IL-1β detected in the supernatants of infected caspase-8 KO cells compared to infected empty vector cells (Fig. 3.5A). As expected, mature IL-1β was not detectable in supernatants of infected caspase-1 KO cells (Fig. 3.5A). However, mature IL-1β release was not completely eliminated in caspase-8 KO cells suggesting that although caspase-8 likely serves a large role in this release process, some mature IL-1β is released independent of caspase-8 activity.

Intriguingly, when concentrated supernatants were probed for caspase-1, the active p10 fragment of caspase-1 could be detected in the supernatant of infected EV cells, but significantly less p10 caspase-1 was found in the supernatants of infected caspase-8 KO cells (Fig. 3.5B). We next examined caspase-1 activity in the supernatants of infected EV, caspase-1, and caspase-8 KO cells using the luminescence-based caspase-1 activity assay, and significantly less active caspase-1 was detected in the supernatants of caspase-8 KO cells (Fig. 3.5C). Since caspase-8 deficiency did not significantly decrease the levels of active caspase-1 in the cell lysates, these data indicate that caspase-8 may also affect the release of other inflammasome machinery, in addition to IL-1β.

During canonical NLRP3 inflammasome activation, IL-1β is released via pyroptosis, an inflammatory form of cell death that relies on gasdermin D-mediated pore formation (222,253,255). Caspase-8 has been demonstrated to cleave GSDMD, albeit with much lower efficiency than caspase-1 (175,235,276). However, *T. gondii* infection of human monocytes did not appear to induce a detectable amount of cell death (Fig. 3.1D and 3.2E)(157), and the resulting IL-1β release is independent of GSDMD and pore formation in the cell membrane (157). To confirm that GSDMD cleavage is not observed during *T. gondii* infection of human monocytes, cell lysates of infected, LPS and ATP-stimulated or mock treated EV and caspase KO THP-1 cells were probed for cleaved GSDMD by Western blot. Cleaved GSDMD was detected in the LPS and ATP dual-stimulated lysates, but was not detected in any of the *T. gondii* infection conditions (Fig. 3.5D), supporting the existence of a caspase-8-dependent release mechanism for IL-1β that is independent of GSDMD, pyroptosis and cell membrane pore formation.

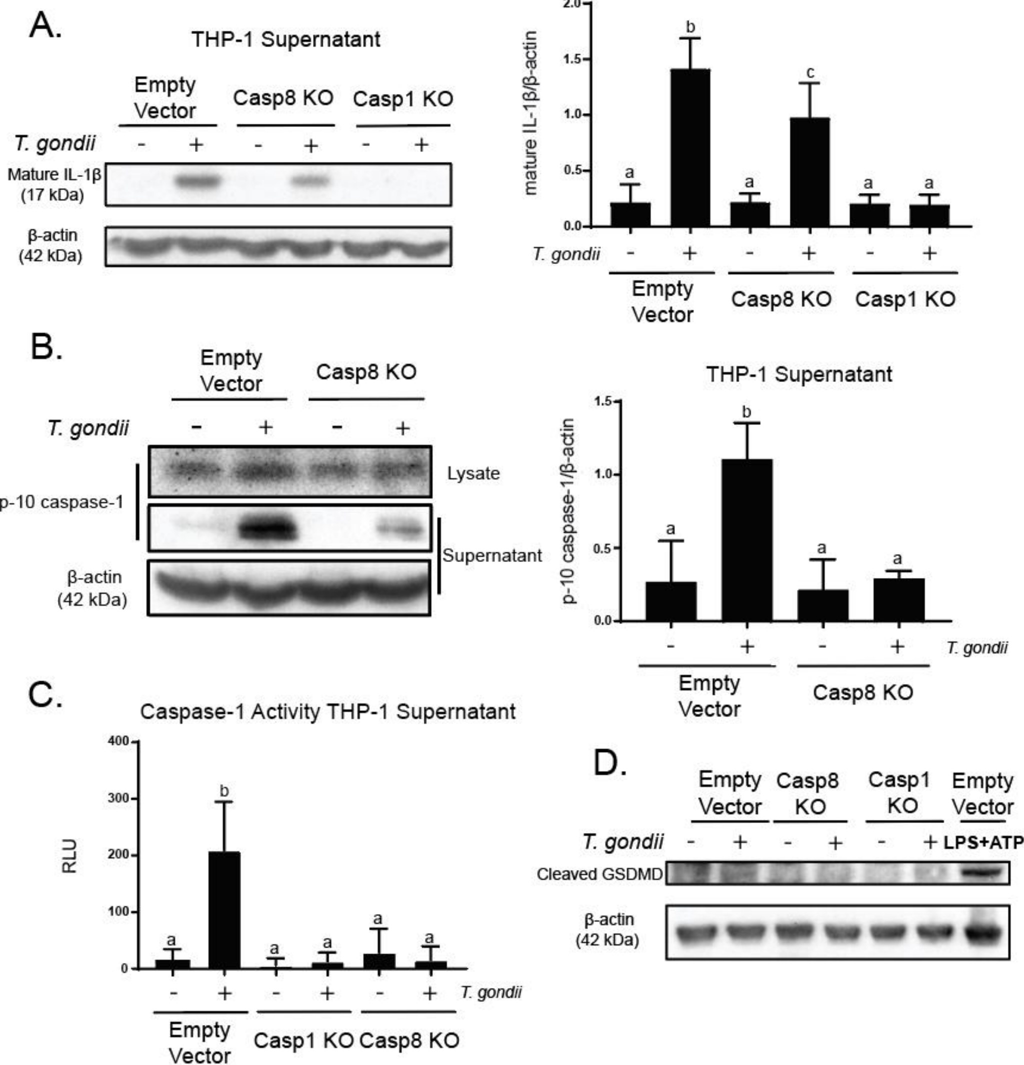


Fig. 3.5. Caspase-8 contributes to the release of mature IL-1β and active caspase-1 from *T. gondii* infected THP-1 cells (A) EV, caspase-1 KO and caspase-8 KO THP-1 cells were mock treated or infected with *T. gondii* for 16 hours. Pro-IL-1β and mature IL-1β in concentrated supernatants and β-actin from corresponding cell lysates were visualized by Western blot. A representative blot is shown and results of densitometry analysis of 5 experiments are quantified. **(B)** THP-1 cell pellets and supernatants were treated and harvested as described in (A). Western blots were used to probe for p10 caspase-1 in supernatants and β-actin in corresponding cell lysates. A representative blot is shown, and results of 3 experiments have been graphed using densitometry analysis. **(C)** Supernatants from (B) were evaluated for caspase-1 activity using a caspase-glo 1 inflammasome assay. Results of 3 experiments are shown. **(D)** THP-1 cells were treated as described in (A), and EV cells were stimulated with 100 ng/mL LPS for 16 hours and 5 mM ATP for the last 30 minutes of stimulation. Western blots were used to probe for cleaved Gasdermin D (GSDMD) and β-actin. A representative blot is displayed, and the results of 5 experiments have been quantified using densitometry analysis. Values are expressed as the mean ± SD. Letters denote statistically significant differences between conditions not containing the same letter with the cutoff for significance at $P < 0.05$ (one-way ANOVA followed by a Tukey post-test).

Discussion

Recent research has revealed that caspase-8 and the NLRP3 inflammasome are at a nexus between the induction of inflammation and the regulation of several types of cell death (216,231,236). In addition to its functions in regulating apoptosis and necroptosis, caspase-8 can contribute to both NLRP3 inflammasome priming and activation (213,237,238,283). In instances of caspase-1 inactivation, caspase-8 can also induce IL-1β and GSDMD cleavage, activation of caspases -3 and -7 leading to GSDME cleavage and IL-1 release, and the induction of pyroptosis (175,235,237). Here we show caspase-8, but not caspases -4 or -5, is a critical mediator of IL-1β release from *T. gondii*-infected human monocytes through a mechanism that is independent of cell death or GSDMD. We did not observe an effect of caspase-8 inhibition on IL-1β transcript or protein production, induction of NLRP3 inflammasome activation, as measured by caspase-1 activity, or the cleavage of pro-IL-1β to mature IL-1β. Instead, loss of caspase-8 function resulted in a deficit in IL-1β release, as mature IL-1β accumulated intracellularly in *T. gondii*-infected caspase-8 KO cells.

Since the isolation of IL-1β in 1977 (191) and its cloning in 1985 (192), the mechanism by which this inflammatory cytokine is released from cells remained unresolved for almost 20 years. Eventually it was shown to be released through a caspase-1-mediated inflammatory cell death named pyroptosis (222), and it was almost another decade until GSDMD was proven to be the final executor of this inflammatory cell death (253,255). During this time, other cell death-independent and gasdermin-independent mechanisms of IL-1β release had been proposed. There is now mounting evidence that indeed IL-1β can be released from viable cells through several different mechanisms, including exosome release and secretory autophagy (219,221,239,284). Whereas *T. gondii* infection of human monocytes induces a cell death-independent release mechanism of IL-1β, caspase-8 has not previously been shown to function in one of these mechanisms.

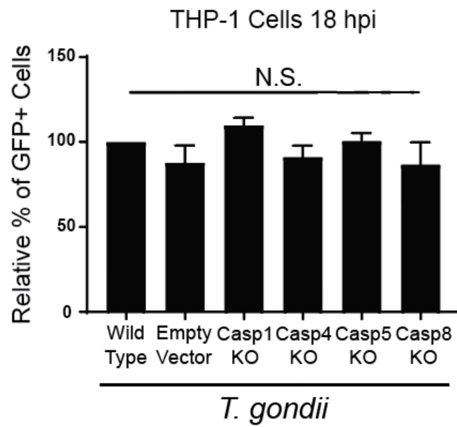
These data, coupled with our previous findings, describe activation of a canonical NLRP3 inflammasome, but the signaling that occurs downstream of inflammasome activation had not previously been defined. *T. gondii* infection of human monocytes appears to activate the canonical NLRP3 inflammasome as inflammasome activity requires potassium efflux and caspase-1 (66,157). However, unlike the canonical NLRP3 inflammasome, IL-1β release is independent of cell death and gasdermin D, and also largely dependent on caspase-8. In contrast to the non-canonical inflammasome, there is not a reliance on either caspases -4 or -5. Also, although the alternative NLRP3 inflammasome requires caspases -1 and -8 for cell death-independent release of IL-1β, again our data suggest that caspase-8 is not required for activation of the inflammasome whereas potassium efflux is. Therefore, it may be inaccurate to describe *T. gondii* infection of human monocytes as activating the traditional canonical, non-canonical or alternative NLRP3 inflammasomes. We also anticipate that future investigators studying NLRP3 inflammasome regulation will observe similar results. As such, *T. gondii* may activate a novel NLRP3 inflammasome that leads to IL-1β release via a caspase-8-dependent, cell death-independent mechanism. This novel NLRP3 inflammasome may be termed the viable caspase-8 reliant NLRP3 inflammasome, or the v8 NLRP3 inflammasome.

Considering the current interest in the field of inflammasome and caspase biology, it is perhaps surprising that this function for caspase-8 in mediating IL-1β release independent of cell death had not been previously described. The decision to use human monocytes and *Toxoplasma gondii* to probe inflammasome function may have helped to reveal this new role for caspase-8. Whereas most investigations in inflammasome biology have been conducted in murine and human macrophages, human monocytes and neutrophils regulate inflammasome activity differently. The non-canonical and alternative NLRP3 inflammasomes were both described in human monocytes. In addition, many investigations utilize single stimuli of inflammasome priming, such as such as LPS or PolyI:C, or activation, like nigericin or MSU

crystals. While these stimulations are effective, live pathogenic stimuli like *T. gondii* may activate distinct signaling pathways and mechanisms of inflammasome activity. For example, it is possible that as yet undefined *T. gondii*-derived factors may inhibit gasdermin D or other inflammasome-related proteins, thereby allowing infected cells to maintain viability and allowing for the activation of secondary or redundant IL-1β release mechanisms that would not normally be activated.

Together, these findings provide a foothold for initiating investigations into novel functions of caspase-8 and contribute to our understanding of how human monocytes respond to infection with a parasite that causes a large disease burden and morbidity globally.

Supporting Information



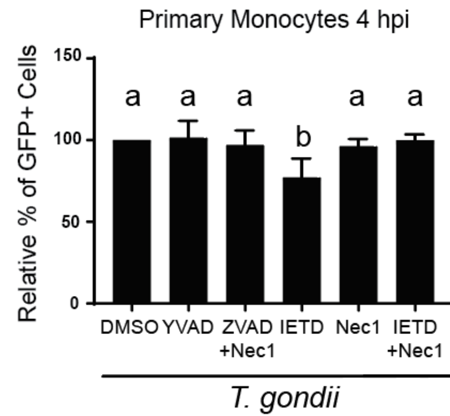


Fig. 3.S1. *T. gondii* infection efficiency in caspase KO and caspase-inhibited human monocytes (A) EV and caspase KO THP-1 cells were mock treated or infected with GFP-expressing *T. gondii* (MOI = 2) for 16 hours. Cells were harvested and the percentage of GFP^+ cells in each condition was quantified by flow cytometry. Results of 3 independent experiments are plotted. **(B)** Primary human monocytes were pretreated with 10 ng/mL Nec1 for 30 min before incubating the cells with 10 µM IETD, or vehicle control for an additional 30 min. Cells were then mock treated or infected with GFP-expressing *T. gondii* (MOI = 2) for 4 hours. Cells were harvested and the percentage of GFP^+ cells in each condition was quantified by flow cytometry. Results of 5-7 independent experiments are plotted. Values are expressed as the mean ± SD. Letters denote statistically significant differences between conditions not containing the same letter, with the cutoff for significance at $P < 0.05$ (one-way ANOVA followed by a Tukey post-test).

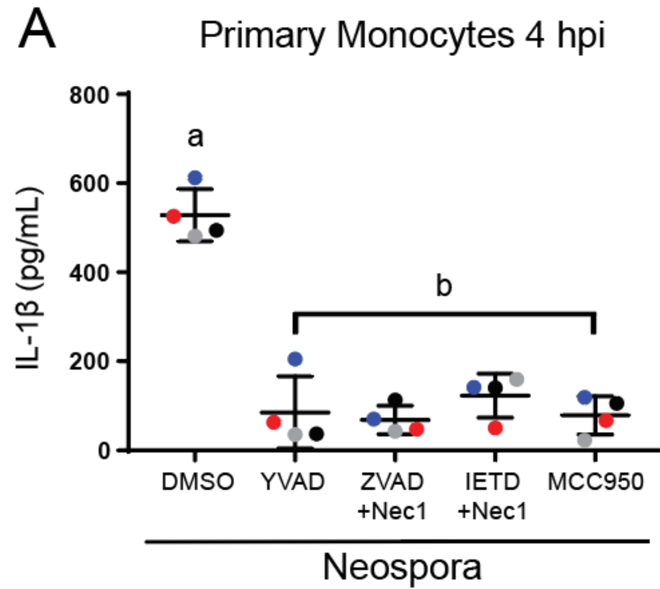


Fig. 3.S2. *Neospora caninum*-induced IL-1β release from primary human monocytes depends on caspase-1, caspase-8 and the NLRP3 inflammasome. (A) Primary human monocytes were pretreated with 10 ng/mL Nec1 or an equal volume of DMSO for 30 min before incubating the cells with 10 μM IETD, 5 μM ZVAD, 50 μM YVAD, 2 μM MCC950 or vehicle control for 30 min. Cells were then mock treated or infected with *N. caninum* (MOI = 2) for 4 hours. IL-1β in the supernatant was measured by ELISA. Each color-coded dot represents a separate human blood donor. The results of 4 independent experiments are plotted. Values are expressed as the mean ± SD. Letters denote statistically significant differences between conditions not containing the same letter, with the cutoff for significance at $P < 0.05$ (one-way ANOVA followed by a Tukey post-test).

Materials and Methods

Ethics Statement

Human whole blood was collected by the Institute for Clinical and Translational Science (ICTS) at the University of California, Irvine 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 (HS #2017–3753).

Mammalian, *T. gondii*, and *N. caninum* Culture

PBMCs were isolated from human whole blood by density gradient centrifugation using lymphocyte separation media (MP Biomedicals, Santa Ana, CA). Monocytes were enriched from PBMCs by counterflow elutriation, as previously described (285), and stained for purity after isolation. This protocol typically resulted in >90% pure monocyte cultures (ranging from 85–95%) based on $CD11b^{+}$ and $CD3^{-}$ $CD20^{-}$ $CD56^{-}$ staining **(Sup. 5)**. Freshly isolated monocytes were resuspended in RPMI 1640 (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and either 10% heat-inactivated FBS (Omega Scientific, Tarzana, CA) (R-10%) or no serum (R-0%). Monocytes were used immediately after isolation for experiments.

The human monocytic THP-1 cell line was cultured in R-10% (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The caspase-1, -4, -5, and -8 KO THP-1 cells were cultured in the same medium supplemented with 2 µg/ml puromycin.

Human foreskin fibroblasts (HFFs; from the lab of Dr. John Boothroyd, Stanford University School of Medicine) were cultured in D-10% medium: DMEM (HyClone) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. *T. gondii* (*Prugniaud*) and *N. caninum* (NC1) tachyzoites were maintained

by serial passage in confluent monolayers of HFFs. *T. gondii* constitutively expressing GFP were used (273).

All mammalian 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

Generation of Knockout (KO) Cell Lines

Empty vector and knockout THP-1 cells were generated using the Lenti-CRISPR-Cas9 system (157). Guide RNAs (sgRNA) targeting human caspase-1, -4, -5, and -8 were cloned into the LentiCRISPR v2 plasmid (Feng Zhang, Addgene plasmid #52961). Virus was generated by transfecting the sgRNA plasmid constructs into HEK 293T cells with the psPAX2 packaging (Didier Trono, Addgene plasmid #12260) and pCMV-VSVG envelope (Bob Weinberg, Addgene plasmid #8454) plasmids. Viral supernatants collected at 48 hr post-transfection were used to infect THP-1 cells by spinfection at 1800 rpm for 1 hr. Single-cell knockout clones were generated by limiting dilution in 96-well plates under puromycin selection. Single-cell clones were sequenced after PCR amplification of a 500 bp region near the Cas9 binding site. Interference of CRISPR edits (ICE) analysis software (Synthego) was used to characterize the indel for each clone (286). A caspase-4 clone containing a 1 base pair (bp) deletion at the CARD binding domain, a caspase-5 clone containing a 20 bp deletion at the p20 subunit, a caspase-8 clone containing a 5 bp deletion in the death effector domain and a caspase-1 clone containing a 1 bp insertion in the substrate binding pocket were used in subsequent experiments. All the caspase KO clones were screened via Western blot for the presence of Cas9 and the absence of the gene targeted for deletion. The sequences for the guide RNAs were as follows: Caspase-1: TAATGAGAGCAAGACGTGTG, Caspase-4: GAGAAACAACCGCACACGCC, Caspase-5: TGGGGCTCACTATGACATCG, Caspase-8: GCCTGGACTACATTCCGCAA.

Infection and Stimulation of Monocytes

Primary human monocytes and monocytic THP-1 cells were resuspended in R-0% or R-10% medium directly after isolation and incubated with small molecule inhibitors or equal volumes of the vehicle control and incubated for 40 minutes at 37˚C. *T. gondii*-infected HFFs were washed with D-10% medium, scraped, and syringe lysed. Lysed tachyzoites were washed with medium, passed through a 5-μm filter (EMD Millipore, Billerica, MA), and washed with medium 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 2. All infections were performed with a GFP-expressing type II *Prugniaud* strain of *T. gondii*. "Mock" infections were samples in which an equivalent volume of culture medium without parasites was added to the cells.

Cells were stimulated with 100 ng/ml ultrapure *E. coli* LPS (List Biological Laboratories, Campbell, CA) and then 5 mM ATP (Sigma-Aldrich, St. Louis, MO) for the last 30 min of culture, as indicated. "Mock" treatment was the addition of the equivalent volume of media (without parasites or LPS) to cells. At the indicated time point, monocytes were pelleted by centrifugation at 500xG for 5 min. Collected cells were stained, fixed, or lysed accordingly, as described below.

Inhibitors

MCC950 (Adipogen, San Diego, CA) was resuspended in deionized water. IETD (R&D Systems, Minneapolis, MN), Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK or YVAD) (Cayman Chemical, Ann Arbor, MI), ZVAD (Selleckchem, Houston, TX) and Z-DEVD-FMK (Torcis Biosciences, Bristol, UK) were all resuspended in DMSO. Monocytes were treated with the inhibitors or with an equivalent volume of the appropriate vehicle, for 30 min at 37˚C and then infected or stimulated as described above. Nec1 (Cayman Chemical, Ann Arbor, MI) or an

equal volume of DMSO control was added to cells 30 minutes before YVAD or IETD treatment. MCC950, IETD, Nec1, YVAD, and ZVAD were all used at concentrations that did not induce cell death or reduce infection efficiency.

Flow Cytometry

To measure the efficiency of *T. gondii* infections, cells were harvested at the indicated time points and immediately analyzed by FACS to determine the percent of GFP^{+} (*T. gondii*-infected) cells. To measure cell viability, cells were harvested, washed and resuspended in FACS buffer (2% FBS in PBS) containing propidium iodide (eBioscience, San Diego, CA) and analyzed by flow cytometry without fixation.

Samples were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). 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 (qPCR)

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. cDNA was synthesized using the Superscript III First-Strand Synthesis kit (Life Technologies), according to the manufacturer's instructions, and subsequently used as template in quantitative real-time PCR (qPCR). qPCR was performed in triplicate using a Bio-Rad iCycler PCR system (Bio-Rad, Hercules, CA) and iTaq Universal SYBR Green Supermix (Bio-Rad). Previously published sequences for *pro-IL-1β* (287) and *GAPDH* (288)primers were used. 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). qPCR data were analyzed using

the threshold cycle method, as previously described (289), and gene expression data are shown normalized to that of the housekeeping gene *GAPDH*. In all qPCR assays, cDNA 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

Concentrations of human IL-1β, TNFα and IL-6 protein were quantified using ELISA MAX Deluxe kits (BioLegend, San Diego, CA), according to the manufacturer's instructions. Cytokines in the supernatant were diluted in blocking buffer and added directly to plates. Intracellular IL-1β was measured by lysing cells through 3 quick freeze-thaw cycles using liquid nitrogen and a 37-degree water bath followed by manual disruption of the cell pellet through pipetting. The resulting lysate was centrifuged for 10 minutes at 14,000xG to remove cellular debris and the lysate was diluted in blocking buffer before addition to the plate. Signal from ELISA plates was read with a Spectra Max Plus 384 plate reader (Molecular Devices, San Jose, CA) using SoftMax Pro Version 5 software (molecular Devices), and the threshold of detection was 0.5 pg/ ml.

Caspase Activity Assay

Caspase-1 activity was quantified using a Caspase-Glo 1 Inflammasome Assay kit (Promega, Madison, WI). THP-1 cells were treated and harvested as described above. 50,000 cells resuspended in 100 µL of media or 100 µL of supernatant were added in triplicate to opaque 96-well plates. 100 µL of caspase-1 activity detection reagent was added to 2 of the 3 replicates for each sample while 100 µL of the caspase-1 activity reagent containing the caspase-1 inhibitor YVAD was added to the 3rd replicate. The plate was sealed, mix by shaking for 2 minutes and left to incubate at room temperature in the dark for 1 hour. Luminescence in the plate was then read with a SpectraMax i3x (Molecular Devices, San Jose, CA) and analyzed

using SoftMax Pro Version 5 software (Molecular Devices, San Jose, CA). Caspase-1 activity was quantified by subtracting the luminescence in the wells containing detection reagent plus YVAD from the average luminescence of the two wells containing only the caspase-1 activity reagent.

Western Blots

At the harvest time point, cells were lysed by addition of 2X Laemmli buffer containing 10% 2-ME or a TNE lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 3mM EDTA). Each Buffer also contained a protease inhibitor cocktail (pH 8.0 1mM Na3VO4, 5mM NaF, 10 mM Na4PO7). For experiments in which supernatant was analyzed, serum-free R-0% medium was used during the infection, and supernatant was concentrated using Amicon Ultra Centrifugal filters (EMD Millipore, Burlington, MA), according to the manufacturer's instructions. Concentrated supernatant was diluted with 2X Laemmli buffer containing 10% 2-ME. Samples were boiled at 100˚C for 10 to 15 min, and then centrifuged for 10 minutes at 14,000xG to remove cellular debris. Samples were separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) for immunoblotting. Membranes were blocked for 1 h at room temperature (RT) with blocking buffer: 5% non-fat milk or 5% bovine serum albumin (BSA) (Fisher Bioreagents). Membranes were then incubated with primary antibodies diluted in blocking buffer for 3 h at RT or overnight at 4˚C. Membranes were probed with antibodies against caspase-1 (ab179515; Abcam), caspase-4 (#4450; Cell Signaling Technology), caspase-8 (#9746; Cell Signaling Technology), GSDMD (NBP2-33422; Novus Biologicals), or β-actin (AC-15; Sigma-Aldrich). Membranes were blotted for pro-IL-1β and IL-1β (3ZD from the National Cancer Institute Biological Resources Branch) using the SNAP i.d. Protein Detection System (EMD Millipore), according to the manufacturer's instructions. Primary Abs were followed by HRP-conjugated secondary Abs (BioLegend), and membranes were developed with SuperSignal West Femto Maximum Sensitivity Substrate

(Thermo Fisher Scientific, Carlsbad, CA), Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, U.K.) substrate, Clarity Western ECL Substrate (Bio-Rad, Hercules, CA), ECL Prime Substrate (Thermo Fisher Scientific, Carlsbad, CA) or Clarity Western (Bio-Rad, Hercules, CA). Signal was detected using a Nikon camera, as previously described (290) or with x-ray film development with Amersham Hyperfilm ECL (Global Life Sciences, London, UK). Quantification analysis of blots was performed using ImageJ software.

Statistics

Statistical analyses were performed using GraphPad Instat software. Analysis of variance (ANOVA) followed by Tukey’s or Bonferroni’s test, as indicated, were used for comparison between means. Differences were considered significant when the P value was <0.05.

Acknowledgements

We would like to thank members of the Lodoen, Andrade, and Morrissette labs, as well as Dr. Eric Pearlman and Dr. David Fruman for helpful discussion on the project. Dr. Andrea Tenner and her lab were also a wonderful help and provided access to their flow cytometer and elutriator. Finally, we are grateful to the administration, nurses and blood donors of the UC Irvine Institute of Clinical and Translational Sciences (ICTS) for maintaining the Healthy Blood Donor program, without which these projects would not be possible. This work was supported by NIH R01AI120846 to (M.B.L.), American Cancer Society RSG-14-202-01-MPC (to M.B.L.), and NIH T32AI060573-12 (to W.J.P.)

Chapter 4

Concluding Remarks

Concluding Remarks

Myeloid cells perform a wide variety of functions including directing development, maintaining tissue homeostasis and controlling infections. Myeloid cells' first function in the life of an individual is often to assist with ancient development programs. The phagocytic and apoptotic capabilities of these cells help to shape the architecture of organs like the brain and to eliminate cells that are not needed later in development (3–5,291). Myeloid cells, and in particular neutrophils, monocytes and monocyte-derived macrophages and dendritic cells, are generally associated with the immune response to pathogens and injury. As these cells often serve as the first point of immune contact with pathogens and injury, they have developed a wide variety of receptors and sensors to recognize and respond to the broad spectrum insults they may encounter. Toll like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), complement receptors, Fcγ receptors, and scavenging receptors can all bind unique PAMPs and DAMPs and channel these signals into a well-tuned immune response that is most effective at addressing each type of pathogen or injury (3,4,14–16,27,57,68). The complexity and elegance of this system is astounding in its ability to recognize and respond to new or novel pathogens from all families and phyla of life.

Myeloid cells are also adept at eliminating both extracellular and intracellular pathogens they come across. They use methods, such as phagocytosis followed by fusion of the phagosome with lysosomes, the production of reactive oxygen and nitrogen species, starvation through amino acid sequestration, self-destruction through inflammatory cell death, the formation of granulomas, trogocytosis, and the release of cytotoxic granules and proteins to mediate the elimination of pathogens (1,3,14,18,55,292). Simultaneously, the recognition of pathogens through the aforementioned receptors will also trigger fine-tuned cell signaling that results in the production and release of pro-inflammatory cytokines and chemokines like IL-6, TNF-α, CCL2, fractalkine, IL-12 and IL-1β, which signal to other immune cells and the

vasculature (1,3,12,14,19,41,50,71,167). These signals can function to recruit more cells to sites of infection and to induce priming, proliferation, differentiation and polarization of specific immune cells that will be required to respond to the insult.

While myeloid cells are adept at inducing this initial inflammatory response to injury or infection, they must also be equally capable of turning off this response and assisting with tissue healing and regeneration by releasing cytokines like IL-10, IL-4, VEGF and TGFβ (4,5,9,19,20,35). When the balance of pro-inflammatory and anti-inflammatory signaling goes awry, and the balance is tipped too far to the side of inflammation, the development of pro-inflammatory autoimmune diseases can arise (7,14,19,27,46,167,251,293). Although the role of T cells and other leukocytes in autoimmune diseases has been well examined, myeloid cells can drive these diseases as well. In particular, monocytes and IL-1β have been implicated in the development of atherosclerosis, rheumatoid arthritis, CAPS, Type II diabetes, and Alzheimer's disease (7,27,31,46,74,204,250,251,293,294). Given the morbidity and mortality, and the enormous economic impact related to each of these conditions, there is a strong push to learn more about how these diseases progress and how IL-1β production and release are regulated by myeloid cells like monocytes.

To date, therapies that target IL-1 family cytokines have been used in the treatment of many inflammatory diseases and have shown relative success, but there are also some serious shortcomings with these therapies (181,190,196,203,208,295). First, by inhibiting IL-1 activity they can leave a patient relatively immunocompromised and susceptible to other infections (50,159,165). Ideally, improved therapies would allow for normal production of IL-1 family cytokines during stimulation of cells with a pathogen, but selectively inhibit its aberrant and pathological release. Secondly, the current anti-IL-1 therapies do not stop the aberrant cytokine production. Instead they function by trying to neutralize the activities of the cytokine in the extracellular space (181,190,203,206). However, these drugs cannot be everywhere at all times,

so they cannot completely eliminate IL-1 signaling. Moreover, such therapeutics do not treat diseases at their root cause, which is the pathogenic production and release of IL-1. Therefore, the goal of our research is to investigate the mechanisms that drive IL-1β production and release in myeloid cells.

IL-1β production and release are tightly regulated at multiple steps, likely because IL-1β is such a potent pyrogen. Research from other labs has revealed that release of IL-1β and other IL-1 family cytokines depends on the activation of a multiprotein complex called an inflammasome (166,171,217). Several inflammasomes, all consisting of different NLR or pyrin-domain containing proteins, have been described. The best described inflammasome, which induces IL-1β release in response to the widest variety of signals, is the NLRP3 inflammasome. The NLRP3 inflammasome consists of the Nod-like receptor protein 3, the adaptor protein ASC which contains pyrin and CARD domains, and a caspase, such as caspase-1. Formation of the inflammasome serves to nucleate and activate caspase-1 through auto-proteolytic cleavage (166,171,277).

Inflammasome activity requires both a priming signal and an activating signal (160,166,171). Priming of the inflammasome refers to the production of pro-IL-1β and NLR transcripts and protein. This is achieved through activation and translocation of the transcription factor NF-κB. Signaling through various TLRs, CLRs and other receptors can all lead to activation of NF-κB, so the inflammasome can be primed in response to a wide variety of signals (166,167,171,252). After the NLR and pro-IL-1β protein are produced, pro-IL-1β still requires catalytic processing of its pro-domain by a caspase like caspase-1 (274). This processing converts pro-IL-1β to mature-IL-1β, which can now bind its receptor (193,195). Caspase-1 is a zymogen, so it also requires processing to carry out its protease activities. As mentioned above, the activation of caspase-1, and thus the maturation of IL-1β, is performed by the inflammasome (167,171,217,230,252). Inflammasome activation requires conformational

changes in the shape of its NLR or pyrin-containing sensor (168,171,204). Anthrax lethal toxin activates the NLRP1 inflammasome in humans, the Type 3 secretion system activates the NLRC4 inflammasome, impairment of RhoA GTPases activates the Pyrin inflammasome and double-stranded DNA activates the AIM2 inflammasome (160,168,171,204). The NLRP3 inflammasome and NLRP3 are different in that it does not bind and respond to one specific stimulus. Instead PAMPs or DAMPs that trigger potassium efflux appear to cause conformational changes in the NEK7 protein, which binds NLRP3 in an inactive form (160,161,168,170,171,204). NEK7, after sensing potassium efflux, changes its structure and then allows for the oligomerization of NLRP3 into an active inflammasome and the activation of caspase-1 and IL-1β (170).

After pro-IL-1β is cleaved, it still must be released from the cell. However, IL-1β is not released through Golgi-mediated exocytosis (221). IL-1β and other IL-1β family cytokines are instead thought to be released primarily through an inflammatory form of cell death called pyroptosis (222). Pyroptosis occurs when active caspase-1 cleaves gasdermin D into two C- and N-terminal fragments. The N-terminal fragment of gasdermin D then forms pores in the cell membrane. These pores are large enough to allow IL-1β to pass through them, but they also lead to osmosis-driven cell swelling and lysis where intracellular contents like mature-IL-1β are released from the dead cell (217,226,228,293).

Most of these pathways have been worked out in mouse and human macrophages. However, we now know that human monocytes can induce IL-1β release in ways that mouse monocytes and human macrophages cannot. For example, human monocytes can use a single priming signal like LPS binding to TLR4 or *Toxoplasma gondii* invasion of the host cell to mediate IL-1β production and release from cells (66,171,178,275). In response to these same stimuli, macrophages will not release IL-1β. Furthermore, caspases other than caspase-1 have been shown to contribute to IL-1β release from human monocytes. The inflammatory caspases-

4 and -5 in humans and caspase-11 in mice have all been implicated in the activity of the noncanonical NLRP3 inflammasome (163,175,179,275). Similarly, human monocyte-like cells can activate an alternative NLRP3 inflammasome that requires caspase-8 activity (178). Caspase-8 has been implicated in directing activity of the NLRP3 inflammasome in many different systems (210,213,225,234–236,238,276). Finally, IL-1β has also been suggested to be released independent of pyroptosis or pore formation from human monocytes and other myeloid cells (157,178,219,221,240,244). Considering that human monocytes are major producers of IL-1β in the blood and implicated in the development of many autoimmune disorders (31,34,46,166,204,209,250,296), we wanted to know more about how these cells, in particular, regulate IL-1β release.

In this pursuit, we have chosen to examine the regulation of IL-1β in the context of *Toxoplasma gondii* infection of human monocytes. *T. gondii* is an obligate intracellular parasite that is estimated to infect approximately 30% of the global population (86,96,104). It is life-threatening in immunocompromised individuals but is relatively well controlled by individuals who have an intact immune response (85,88,91,108,110,111,133,246,297). *T. gondii* efficiently infects monocytes, and upon infection, human monocytes will produce and release IL-1β (66,134,157). Multiple studies have shown that human monocytes and IL-1β contribute to successful immune defense against this parasite (52,81,119,121,188,279). Therefore *T. gondii* infection of human monocytes provides an excellent model in which to study how a medically relevant, complex, live, and prevalent pathogen induces an IL-1β response that is protective for the host.

One unresolved question in the field of *T. gondii* biology is how *T. gondii* initially activates innate immune signaling in human cells. DAMPs from *T. gondii*-infected cells which have lysed can trigger activation of the RAGE receptor on neighboring innate immune cells and initiate an immune response to the parasite (67), but human innate immune recognition of the

infection before cell lysis is poorly understood. *T. gondii* is not known to directly induce activation of any known human innate immune receptor, yet the parasite induces human monocytes to activate NF-κB translocation during infection prior to cell lysis events (129,153,157,160,293,298). Prior research by our lab built on the finding that the parasite protein GRA15 induced NF-κB translocation, and we found that GRA15 was responsible for *pro-IL-1β* transcript induction during *T. gondii* infection of human monocytes (134). However, infection of primary human monocytes with Δ*gra15* parasites only reduced IL-1β release by about 50% (66), suggesting the possible existence of additional pathways for NF-κB activation during *T. gondii* infection. The innate immune receptor for the parasite and how they activate the signaling pathways that lead to IL-1β production were unknown. Similarly, in this system it was still unclear how the inflammasome was being activated, how IL-1β was being released, and if caspases other than caspase-1 were also involved in this response. Our work presented in this dissertation attempts to answer each of these questions, and in doing so, reveals new information about the regulation of inflammasomes and IL-1β release in human cells and proposes new possible avenues for future investigation.

We began our studies with the goal of identifying host signaling pathways that lead to NF-κB translocation during *T. gondii* infection of human monocytes. During this pursuit we found that spleen tyrosine kinase (Syk), which had been implicated in NLRP3 inflammasome priming during fungal and bacterial infections by signaling through CLRs, was activated within 15 minutes of infection. Syk then induced a PKCδ-CARD9-MALT1 signaling pathway that led to inflammasome priming through NF-κB activation and transcription of *pro-IL-1β* and *NLRP3*. Each of these signaling components was required for IL-1β production and release from *T. gondii*-infected monocytes. Considering that GRA15 also induces NF-κB translocation, it is an intriguing possibility that Syk and GRA15 may influence each other's activity. Syk KO THP-1 cells or Syk-inhibited primary human monocytes showed almost a complete loss in *pro-IL-1β*

transcript production during infection with WT *T. gondii*, whereas Δ*gra15* parasites reduced transcripts by about 50%. These data suggest that GRA15 may require Syk for its effects on NF-κB activation, and therefore could be acting in conjunction or just upstream of Syk. GRA15 has recently been demonstrated to recruit and bind tumor necrosis factor receptor associated factors (TRAFs) like TRAF2 and TRAF6 at the PV during IFN-γ stimulation (135,299). TRAF6 is an important signaling molecule downstream of TLR4 and MyD88 as it binds with IRAK1/IRAK4 (300). Therefore, it is possible that GRA15 or other GRA proteins may influence Syk activity by bringing some of these signaling molecules together at the PV. It is also possible that GRA15 could bind and enhance the activation of other signaling molecules or receptors which activate Syk. These data suggest that immunoprecipitations or microscopy experiments looking at the interactions of Syk, GRA15 and their associated proteins, could provide fruitful data. Considering that GRA15 is responsible for about 50% of the IL-1β released from *T. gondii*-infected primary monocytes (66), we could also perform infections with Δ*gra15* parasites in primary monocytes where Syk activity is inhibited. By comparing IL-1β release and *pro-IL-1β* transcripts from these cells to IL-1β release and transcript levels in monocytes infected with WT parasites with Syk inhibition or Δ*gra15* parasites without Syk inhibition, we could determine if Syk and GRA15 synergize to promote inflammasome priming. Ideally, these experiments could help describe a mechanism by which GRA15 and Syk influence each other's activity.

and lead to a potential discovery of an early innate immune receptor for *Toxoplasma gondii*.

During our investigations of how the NLRP3 inflammasome is activated during *T. gondii* infection we queried whether caspases could be involved in this process. In addition to caspase-1, we observed a reliance on caspase-8, but not caspases-3, -4 or -5 for IL-1β release from *T. gondii*-infected monocytes. Surprisingly, caspase-8 did not appear to contribute to NLRP3 inflammasome priming or activation, but instead caspase-8 influenced the release of IL-1β, as

mature IL-1β accumulated in the cytoplasm of infected caspase-8 KO cells. Finally, we have also observed that IL-1β is released from *T. gondii*-infected monocytes independent of cell death, pore formation in the plasma membrane or activity of gasdermin D and gasdermin E, and the mechanism by which caspase-8 influences IL-1β release from viable cells is still unclear. Other research has suggested that IL-1β can be released from viable cells through release of exosomes or secretory autophagy (220,240,244,301), and we are currently investigating these possibilities.

Together the data that we have collected represent some important and novel findings in the field. These data were the first to describe a parasite activating the Syk signaling pathway for inflammasome activation. They also suggest a novel role for caspase-8 in the release of IL-1β from viable cells, and add to the mounting evidence that there are multiple mechanisms besides pyroptosis which contribute to IL-1β release. What remains unclear is how Syk is activated by *T. gondii* and what sensor may identify infection of human cells and lead to Syk activation. It is also unclear what the activating signal for potassium efflux and NLRP3 inflammasome activation may be during infection, and how caspase-8 influences IL-1β release from viable cells.

Although the mechanism by which Syk is activated during *T. gondii* infection remains unclear, our investigations have given us some insights. Based on current data in the field, Syk is exclusively recruited to and activated by transmembrane receptor and adaptor proteins that contain an immune-tyrosine activation motif (ITAM) (58,265,266,302,303). Therefore, since Syk is activated within 15 minutes of infection, it is likely that some ITAM-bearing receptor or adaptor protein is involved in early innate immune recognition of *T. gondii* or a determinant of the parasite. Currently, we have compiled a list of 36 different ITAM bearing proteins that are expressed in human monocytes. Our goal is to use a CRISPR library to knock out or knock down each of these proteins in THP-1 cells and screen the KO cells for IL-1β release during *T.*

gondii infection. Currently we have a system working where we can use electroporation to deliver synthetic CRISPR peptides to THP-1 cells. We still need to optimize this process and decide the target genes we would like to KO. Some of our highest priority candidates include several Fcγ receptors, ITAM adaptor proteins and CTLs. Unpublished nanostring data shows that Fcγ receptor 1-3 transcripts are increased during infection of human monocytes. Also, phagocytosis often requires engagement of Fcγ receptors (53,304), and phagocytosis of the parasite does occur in our infection cultures. So, it is possible that Syk could be activated via Fcγ engagement of the parasite during phagocytosis. We also know that in cultures where phagocytosis does not occur, invasion of the parasite still induces IL-1β release. Therefore, we are also interested ITAM-bearing proteins that may be activated during the invasion process. DAP12, is an ITAM bearing adaptor protein that can associate with many different receptors (305), so it is another one of our leading candidates for an ITAM-bearing protein that is required for Syk activation. Finally, transcripts of several SLAM family proteins and CTLs like CLEC9 are also increased during infection and may be responsible for Syk activation. As discussed above, GRA15 may even be influencing the activation of these receptors as well.

Once we have generated a pool of ITAM-bearing protein KO THP-1 cells using the synthetic peptide CRISPR library, we plan to use combinations of mycalolide b treatments of monocytes and parasites as well as heat killed parasites to generate conditions where monocytes will either be only infected by the parasite, only phagocytose the parasite, allow for only attachment of the parasite or allow both phagocytosis and invasion of the parasite to occur. The supernatants from these cells could then be collected and used for ELISA assays to measure IL-1β release. This would serve as a screen to identify ITAM-bearing proteins that are responsible for IL-1β release during either infection or invasion of the parasite. Cells that are KOs for one of these ITAM-bearing proteins could be sequenced to determine which ITAM-bearing protein was knocked out and the location of the mutation. Then these cells could also

be tested for Syk phosphorylation during *T. gondii* invasion, phagocytosis or attachment. If an ITAM-bearing protein was discovered to be essential for this phosphorylation, then further experiments demonstrating how it is activated and potentially what parasite factors it may interact with will be conducted and could lead to the first description of an early innate immune receptor that responds to *T. gondii*.

Our data may also provide some clues about the signals that lead to potassium efflux and NLRP3 inflammasome activation during *T. gondii* infection. We and other labs have observed that caspase-1 is cleaved to its active form in unstimulated resting human monocytes (66,181). Using a caspase activity assay, we also observed low levels of active caspase-1 in unstimulated monocytes. Although the levels of caspase-1 activity did increase during *T. gondii* infection, these data support the idea that monocytes have low levels of caspase-1 activity at baseline, at least *in vitro*. This may account for why monocytes, but not human macrophages can release IL-1β in response to just a priming signal (181). How caspase-1 is activated in resting monocytes is unknown, but this activation likely still depends on activity of an inflammasome. Since inhibiting potassium efflux and the NLRP3 inflammasome inhibited IL-1β release from *T. gondii*-infected human monocytes, it is possible that this low level of caspase-1 activity in resting monocytes is also driven by the NLRP3 inflammasome.

Much remains unclear about how the NLRP3 inflammasome is activated during *T. gondii* infection. One possibility is that the inflammasome is already active at a low level in resting monocytes as described above. In this scenario there would be no additional signaling required to induce inflammasome activation during infection. However, given that caspase-1 activity increased during infection compared to the resting state, this suggests that infection does induce NLRP3 inflammasome activation. One possibility is that Syk, in addition to priming the inflammasome, can also activate the NLRP3 inflammasome during infection. Other investigators have seen that Syk can phosphorylate the NLRP3 inflammasome adaptor protein ASC, and this

phosphorylation contributes to inflammasome activation (172,268). However, because Syk was required for inflammasome priming, we were unable to untangle any role it has in inflammasome activation from its contributions to priming. In the future it could be possible to generate or use THP-1 cells that have inducible NF-κB activity to investigate a possible role for Syk in NLRP3 inflammasome activation during *T. gondii* infection. In these cells Syk could be inhibited before T. gondii infection occurs and NF-κB activity could be turned on despite the Syk inhibition. Then we could look at Syk's role in inflammasome activation by using Western blots and microscopy to look at ASC oligomerization as well as using caspase-1 activity assays.

Our data may also suggest that there could be different activation states of the NLRP3 inflammasome. The amount of IL-1β released from monocytes stimulated with a single priming signal like LPS pales in comparison to the IL-1β released from monocytes where a secondary inflammasome activating signal like extracellular ATP is added as well. These data suggest that the amount of IL-1β released from cells is primarily driven by the extent to which the NLRP3 inflammasome is activated. Therefore, if the activation state of the inflammasome lies on a spectrum instead of being binary on or off, there may be different activation states of the inflammasome that lie on different points of that spectrum which have not yet been described. For example, even though infected monocytes release bioactive IL-1β we have not been able to observe the formation of a nucleated inflammasome and ASC spec in *T. gondii* infected monocytes. It is possible that we have still not developed the correct techniques to observe these specs, or it could be possible that the NLRP3 inflammasome can still be active, albeit at lower levels, without completely nucleating and forming these large specs. Perhaps monocytes can contain functional NLRP3 in dimers, small fibers, or other structures that determine the level of caspase-1 activity and what substrates it may cleave. There is plenty that we do not understand regarding how caspase-1 can be active within a cell, yet not cleave gasdermin D and lead to pyroptosis as we observe in *T. gondii*-infected monocytes. It is possible that there

are parasite virulence factors that may inhibit pyroptosis, and what we are observing is a backup mechanism for IL-1β release without cell death. These findings would also be exciting, as they would show redundancy in the IL-1β release process that may be relevant in other infections and disease states, as well as identifying either new *T. gondii* virulence factors or new roles for old ones. These observations could warrant further investigations into describing these hypothetical activation states of the NLRP3 inflammasome.

We hypothesize that the role of caspase-8 in IL-1β release and the mechanism by which IL-1β is released from *T. gondii* infected monocytes are likely linked. It was surprising to observe that in caspase-8 KO or inhibited monocytes there was little to no reduction in priming, inflammasome activation, or cleavage of IL-1β. Rather, the principal effect of caspase-8 deficiency was the reduced release of IL-1β after infection. The simplest explanation for our findings is that caspase-8 contributes to the release mechanism for IL-1β. To our knowledge, caspase-8 has not previously been associated with the release of IL-1β in a mechanism that is independent of gasdermin family proteins or cell death.

One possibility that we need to consider is that most or all of the IL-1β released from infected cells may come from a small population of dead or dying cells going through apoptosis that we have not been able to detect. While still possible, we find this scenario unlikely. First, primary monocytes in culture are still largely viable for up to 48 hours after isolation. Remembering that we see active caspase-1 in these unstimulated cells, this suggests that caspase-1 can be active at low levels without triggering pyroptosis in monocytes, otherwise we would see a steady drop off in the viability of these cells over time as they undergo pyroptosis. Therefore, we think there is likely a threshold of caspase-1 activity that these monocytes need to reach to cleave gasdermin D and other family members to induce pyroptosis, and we still don't understand all of the players that regulate that threshold. We also find it unlikely that only a small, basically undetectable, number of cells would die after infection even though routinely

about 60-70% of cells in culture are infected during experiments. What these data really suggest is that there is plenty that we still do not understand about NLRP3 inflammasome regulation and its connections with cell death.

Interestingly, while using LPS stimulation as a positive control for inflammasome priming, we also demonstrated how one cell can use different mechanisms, depending on the stimulus, to reach the same result. Both LPS and *T. gondii* infection induced Syk activation, and Syk contributed to IL-1β release during both stimulations. However, although Syk was required for inflammasome priming during *T. gondii* infection, it did not seem to contribute to inflammasome priming to the same extent in LPS-stimulated cells. This is likely due to extracellular LPS binding TLR4 and signaling through MyD88 for NF-κB activation, which does not depend on Syk activity. Instead it is likely that Syk contributes more substantially to inflammasome activation in LPS-stimulated cells. These data demonstrate the importance of using multiple stimuli to study mechanisms of immune activation, as each stimulation may reveal different pathways that could be targets for drug development for different diseases.

As described earlier, there are 3 major subsets of monocytes in humans based on their expression of CD14 and CD16 (25,34,306). Our initial investigations into each of these subsets have shown that the classical inflammatory subset is preferentially infected by the parasite and produces the most IL-1β during infection. Dissecting the differences between these monocyte subsets could help to determine how a parasite may “choose” which cell to invade and potentially reveal new factors that can influence the amount of IL-1β that is released from monocytes during stimulation.

We are also interested in the differences between macrophages and monocytes that allow monocytes to release IL-1β after *T. gondii* infection, whereas macrophages can only make the protein but not release it (164,166,171,178,275). Examining the differences in transcript production between these two cells during resting and infected states may give us some clues

as to how the inflammasome is activated in resting monocytes or monocytes given only an inflammasome priming signal. Previous work from the lab showed that when monocytes differentiate to macrophages they downregulate the amount of *NLRP3* and *IL-1β* transcripts produced compared to monocytes during *T. gondii* infection (66). This reduction in available NLRP3 during infection could help explain why the inflammasome is more difficult to activate in macrophages, but what drives these differences between cell types and any other factors that could contribute to these differences could still use further investigation.

Our last big remaining question is how IL-1β is released from these cells and how caspase-8 may be involved in that process. We want to investigate what signals set this process in motion, what caspase-8 may interact with, and how the cell makes the decision to use this release mechanism compared to others. Our preliminary data also suggests that, as others have seen, caspase-1 may negatively regulate caspase-8 activity. When other labs have described roles for caspase-8 in inflammasome activity it has often been seen in cells where caspase-1 is knocked out or inactive (210,215,234,237). In these cells, caspase-8 seems to be functioning as a backup, less efficient, executioner of IL-1β release. Furthermore, when others have shown that caspase-1 can negatively regulate caspase-8, this has been through the activity of cleaved gasdermin D (175). Given that we do not observe cleaved gasdermin D in *T. gondii*-infected monocytes it seems unlikely that caspase-1 would be regulating caspase-8 activity in this way. It is possible that future investigations into this regulation could also reveal novel mechanism of caspase regulation. Again, we are curious if a *T. gondii* virulence factor is somehow dampening caspase-1 activity or the activity of its substrates. We could begin to investigate the mechanism that caspase-8 utilizes to influence IL-1β release from infected monocytes by first performing some microscopy and immunoprecipitation experiments where both caspase-8 and IL-1β are pulled down. These experiments could give us insights into binding partners of these proteins, where they may be located within the cell and inform a

hypothesis regarding how IL-1β is being released from infected cells. It is possible that IL-1β is released from viable monocytes through secretory autophagy (241,242,244). Therefore, we could also inhibit autophagy and observe if this inhibition reduces IL-1β release. We could also use microscopy and immunopercepitations to observe if IL-1β associates and colocalizes with secretory autophagy proteins.

Interestingly, our descriptions of the activity of the NLRP3 inflammasome during *T. gondii* infection of human monocytes does not fit well with any previously described NLRP3 inflammasome. Unlike the canonical NLRP3 inflammasome, *T. gondii* infection induces release of IL-1β during stimulation with a single priming signal and independent of gasdermin D and pyroptosis (165,171,186). Unlike the noncanonical NLRP3 inflammasome, infection does not require the activity of caspases -4 or -5 for IL-1β release (179,183,184). While the alternative NLRP3 inflammasome also requires caspase-8 for IL-1β release from viable cells in response to a single priming signal, this release has been suggested to be independent of potassium efflux, and caspase -8 was suggested to contribute to inflammasome activation and not the IL-1β release mechanism (178). Considering that we also do not observe the formation of ASC specs in infected monocytes we believe we may be describing a different type of NLRP3 inflammasome and further discussion of how we categorize and describe different NLRP3 inflammasomes is warranted.

In closing, the work presented here represents a significant advancement in our understanding of monocytes' ability to produce and release IL-1β during *T. gondii* infection. More broadly, we have shown for the first time that a parasite can induce priming of the NLRP3 inflammasome activity through activation of Syk and have suggested a novel role for caspase-8 in IL-1β release from viable cells. Specifically, this work contributes to our understanding of IL-1β release mechanisms that are independent of cell death, could potentially lead to the discovery of an innate immune sensor of *T. gondii* infection, and possibly expand upon the

known roles of caspases in inflammation. Considering how critical IL-1β can be in the development of many autoimmune diseases, and the morbidity and mortality of *T. gondii* infections around the globe, we hope this work may also contribute to the development of both better anti-inflammatory and anti-parasitic drugs. Importantly, in addition to improving our knowledge of how human monocytes regulate IL-1β during *T. gondii* infection, the work presented here can also help inform future investigations in the lab in the broader fields of myeloid and IL-1β biology.

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