Internalization and TLR-dependent type I interferon production by monocytes in response to Toxoplasma gondii

Seong-Ji Han1,4, Heather J Melichar1, Janine L Coombes1,5, Shiao Wei Chan1, Anita A Koshy2,6, John C Boothroyd3, Gregory M Barton1 and Ellen A Robey1

The classic anti-viral cytokine interferon (IFN)-β can be induced during parasitic infection, but relatively little is known about the cell types and signaling pathways involved. Here we show that inflammatory monocytes (IMs), but not neutrophils, produce IFN-β in response to T. gondii infection. This difference correlated with the mode of parasite entry into host cells, with phagocytic uptake predominating in IMs and active invasion predominating in neutrophils. We also show that expression of IFN-β requires phagocytic uptake of the parasite by IMs, and signaling through Toll-like receptors (TLRs) and MyD88. Finally, we show that IMs are major producers of IFN-β in mesenteric lymph nodes following in vivo oral infection of mice, and mice lacking the receptor for type I IFN-1 show higher parasite loads and reduced survival. Our data reveal a TLR and internalization-dependent pathway in IMs for IFN-β induction to a non-viral pathogen.

Immunology and Cell Biology (2014) 92, 872–881; doi:10.1038/icb.2014.70; published online 26 August 2014

Infection with a wide variety of pathogens can trigger type I interferon (IFN-1) induction in mammalian hosts. Most of our information about the IFN-1 response, however, comes from viral infection models, and the mechanism of induction to non-viral infection remains relatively unexplored. The intracellular protozoan parasite, Toxoplasma gondii, provides an ideal pathogen to address this question, as it is an important opportunistic pathogen of humans with relevance to related protozoan parasites including Plasmodium and Cryptosporidium, and provides a tractable experimental pathogen that allows for physiological oral infection studies in one of its natural hosts, the mouse.

The induction of IFN-1, as well as pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-12, is initiated when pathogen-associated molecular patterns (PAMPs) are detected by innate immune sensors, including the Toll-like receptor (TLR) family. Multiple TLRs contribute to the host defense during T. gondii infection.1-3 Moreover, different TLRs acting in different host cell types can evoke distinct immune responses to T. gondii. For example, TLR11 and TLR12 have a key role in IL-12 production by conventional dendritic cells (DCs) and macrophages,3,5 TLR2 and TLR4 are required for TNF-α production by macrophages,6,7 and plasmacytoid DCs (pDCs) produce IFN-β through the activation of TLR12.3 Production of IFN-1 can require distinct intracellular signaling pathways, as exemplified by the requirement for endosomal trafficking for type I IFN-1 production, but not TNF-α production, following TLR4 triggering.3,8-11 The cell types, innate immune pattern recognition receptors and intracellular trafficking requirements for IFN-1 production during T. gondii infection require further study.

PAMPs can be detected at the cell surface, in vacuolar compartments and in the cytosol of host cells, and the subcellular location can have a profound impact on the nature of the host response.11 As T. gondii invades a cell, it directly injects proteins into the host cell, and invasion culminates in the formation of a specialized parasitophorous vacuole within which the parasite replicates and from which it also releases proteins into the host cell. In addition, during in vivo infection, material from parasites could also be released into the extracellular space or taken up by phagocytes. Thus, parasite PAMPs could potentially engage pattern recognition receptors at multiple cellular locations. In addition to providing a potential source of PAMPs, the proteins that T. gondii secretes into host cells can directly regulate host innate immunity.12-15 Understanding how and where

1Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, University of California-Berkeley, Berkeley, CA, USA; 2Department of Medicine (Infectious Disease) and Department of Neurology, Stanford University School of Medicine, Stanford, CA, USA and 3Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA.
4Current address: Program in Barrier Immunity and Repair, Mucosal Immunology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA.
5Current address: Institute of Infection and Global Health, Liverpool Science Park IC2, University of Liverpool, Liverpool, Merseyside L3 5RF, UK.
6Current address: Department of Neurology, Department of Immunobiology, BIOS Institute, University of Arizona College of Medicine, Tucson, AZ, USA. Correspondence: Professor EA Robey, Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, University of California-Berkeley, 142 Life Sciences Addition, #3200, Berkeley, CA 94720-3200, USA. E-mail: erobey@berkeley.edu
Received 19 May 2014; revised 25 June 2014; accepted 27 June 2014; published online 26 August 2014
parasites regulate the IFN-1 response could shed light on alternative modes of IFN-1 production by non-viral pathogens.

Here we show that inflammatory monocytes (IMs), but not neutrophils, produce IFN-β in response to *T. gondii* infection. This difference correlated with the mode of parasite entry into host cells, with phagocytic uptake predominating in IMs and active invasion predominating in neutrophils. We also show that the expression of IFN-β by IMs requires phagocytic uptake of parasites as well as signaling through TLR4 and MyD88. Finally, we show that IMs are the major producers of IFN-β in mesenteric lymph nodes (MLNs) following *in vivo* oral infection of mice. Our data reveal a TLR and internalization-dependent pathway in IMs for IFN-β induction to a non-viral pathway.

**RESULTS**

**TLR-dependent IFN-β induction after infection of IMs *in vitro***

To investigate the mechanism of IFN-β induction in response to *T. gondii* infection, we infected freshly isolated bone marrow cells with parasites, and examined IFN-β mRNA levels by quantitative reverse transcription PCR (qRT–PCR) at different times after infection. For these experiments, we used irradiated parasites, which can invade host cells but cannot replicate, in order to avoid host cell death at later time points. We observed an increase in IFN-β mRNA that peaked around 8 h after infection (Figure 1a). Thus, *in vitro* infection of bone marrow cells provides a convenient assay to study IFN-β induction in response to *T. gondii* infection.

Separate flow cytometric analysis of bone marrow cells infected *in vitro* with red fluorescent protein (RFP)-expressing parasites revealed that the RFP signal was preferentially found within Ly6C<sup>hi</sup>CD11b<sup>+</sup> cells as well as Ly6G<sup>+</sup>CD11b<sup>+</sup> cells (neutrophils; Figure 2a). Ly6C<sup>hi</sup>CD11b<sup>+</sup> cells are rapidly released from the bone marrow in response to infection and give rise to IMs found at sites of infection.20 For brevity, we refer herein to Ly6C<sup>hi</sup>CD11b<sup>+</sup> cells from both the resting bone marrow and from infected mice as IMs. IMs have been previously implicated in IFN-1 production in response to certain viruses and bacteria.17–20 To determine whether IMs or neutrophils contribute significantly to the IFN-β response to *T. gondii*, we enriched bone marrow cells for different cell types before infection. Because no single marker distinguishes IMs from other cells, we enriched for IMs and neutrophils using Ly6-B2, or enriched for neutrophils only using Ly6G (Figures 1b and c). Ly6-B2 and Ly6G have been used in combination to distinguish IMs from neutrophils in tissue sections.21 Both enrichment procedures effectively removed pDCs (Figure 1b), which are known to be the major producers of IFN-β following viral infection and have also been reported to express IFN-β *in vitro* in response to *T. gondii*.22,23 We observe significant induction of IFN-β from Ly6-B2-enriched fractions, but not from neutrophil only (Ly6G-enriched) fractions (Figure 1c). The level of IFN-β produced by Ly6-B2-enriched bone marrow cells in response to parasites is comparable in magnitude to the response to the TLR3 agonist, polyIC (Figure 1c). It is noteworthy, however, that the response of unfractonated bone marrow to parasites is considerably higher than the response of Ly6-B2-enriched cells to parasites, indicating that the majority of the response of bone marrow cells to parasites is found in the Ly6-B2-negative population (Figure 1c). We chose to focus on IFN-β production from the IM population because they represent an important population during *in vivo* Toxoplasma infection,24 and because our preliminary *in vivo* experiments implicated IMs, rather than pDCs in IFN-β production after oral infection (data not shown).

Multiple TLRs have been previously implicated in sensing *T. gondii*, including the sensing of parasite profilin via TLR11 and 12 and parasite glycosylphosphatidylinositol (GPI) 4-anchored proteins via TLR2 and 4.6 To examine the role of TLRs in IFN-β production by IMs, we examined responses in bone marrow cells isolated from various mutant mice. Ly6-B2-enriched bone marrow cells from MyD88 knockout (KO) mice failed to express IFN-β in response to *T. gondii* infection, consistent with a role for TLR signaling (Figure 1d). Moreover, production of IFN-β by Ly6-B2<sup>+</sup> cells isolated from TLR4 KO or from TLR2<sup>−</sup>4<sup>−</sup> double KO mice was reduced to levels similar to the uninfected control samples, whereas loss of TLR2 led to a more modest reduction that did not reach statistical significance (Figure 1e). These data suggest that TLR4 is the predominant TLR through which IMs trigger IFN-β production in response to *T. gondii*.

**Uptake of parasite material is necessary for IFN-β production by IMs**

IFN-1 is induced by intracellular infection and typically involves intracellular detection of PAMPs.20 Flow cytometry analysis of whole bone marrow infected with RFP-labeled parasites revealed that a large proportion of IMs contained parasite fluorescence (25–50%; Figure 1a), suggesting that IFN-β production by IMs may involve intracellular detection of parasite PAMPs. To further investigate this possibility, we infected bone marrow cells with RFP-expressing parasites, fractionated Ly6-B2<sup>+</sup> cells into parasite containing (RFP<sup>+</sup>) and non-containing (RFP<sup>−</sup>) populations and compared IFN-β expression by qRT-PCR analysis. IFN-β expression was limited to the RFP<sup>+</sup> Ly6-B2<sup>+</sup> cells (Figures 2b and c), suggesting that the production of IFN-β by IMs occurs via intracellular recognition of *T. gondii* PAMPs.

IMs containing fluorescent parasites may have taken up parasites by phagocytosis or may have been actively invaded by the parasites. To examine the mode of parasite internalization by IMs, we infected bone marrow cells, sorted IMs (Ly6-B2<sup>−</sup>Ly6G<sup>+</sup>) by fluorescence-activated cell sorting (FACS) and performed immunofluorescence microscopy using antibodies to a parasite protein, GRA6, which accumulates in the parasitophorous vacuole of invaded cells. We find that ~70% (282 out of 413) of RFP<sup>+</sup> IMs show GRA6 staining adjacent to RFP parasites, suggesting productive invasion by the parasite. The remaining ~30% (131 out of 413) showed no detectable GRA6 staining and a more amorphous RFP signal suggestive of phagocytosis or parasite destruction by the host cell (Figure 3a). Thus, the RFP signal in IMs likely reflects both active invasion by the parasites and phagocytic uptake of parasites by the IMs.

To separate IMs that were invaded from those that phagocytosed parasites, we took advantage of a genetically engineered RFP-labeled parasite strain that injects Cre protein into host cells during active invasion, driving expression of green fluorescent protein (GFP) in cells from Cre-regulated GFP reporter mice.26,27 Host cells harboring parasites because of active invasion (‘successfully infected’) can be identified by co-expression of GFP and RFP. Host cells that have taken up parasites by phagocytosis, which does not allow Cre access to the host nucleus, will be contained within the RFP only fraction (Figure 3b). In addition, as it takes several hours for GFP expression to be detectable following Cre injection into host cells, recently invaded cells will also be RFP<sup>−</sup>GFP<sup>−</sup>. Host cells that were injected with parasite effectors but not infected, as well as those that were invaded but subsequently killed and degraded the parasites (‘infected/uninfected’), express GFP but not RFP. Eight hours after infection, Ly6-B2<sup>+</sup> cells were FACS-sorted based on RFP and GFP levels, and IFN-β expression was measured by qRT-PCR (Figure 3c). IFN-β was
Figure 1 TLR-dependent production of IFN-β by inflammatory monocytes after T. gondii infection in vitro. (a) Bone marrow cells isolated from WT mice were infected in vitro with irradiated parasites (multiplicity of infection (MOI) = 1) and relative IFN-β expression levels at indicated time points were measured by qRT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample. This experiment was performed twice with similar results and compiled data from both experiments are shown. (b) Bone marrow cells were isolated from WT mice and enriched for Ly6G* (neutrophils) and Ly6-B2+ (neutrophils and IMs) cells. Starting population and enriched populations were infected in vitro for 8 h with irradiated parasites. (c) Bone marrow cells isolated from WT mice were enriched for Ly6G* (neutrophils) and Ly6-B2+ (neutrophils and IMs) cells. Enriched Ly6-B2+ cells were infected in vitro for 8 h with irradiated parasites. (d) Plots show proportion of Ly6G*Ly6-B2+ and Ly6G-Ly6-B2+ cells (top), Ly6C*CD11b* (middle) and siglecH*B220+ (bottom) in non-enriched and enriched samples. (e) Bone marrow cells were isolated from WT and the indicated KO mice and enriched for Ly6G* (neutrophils) and Ly6-B2+ (neutrophils and IMs) cells. Enriched Ly6-B2+ cells were infected in vitro for 8 h with irradiated parasites. Relative IFN-β expression was measured by qRT-PCR and normalized to GAPDH. IFN-β expression in enriched populations is shown relative to starting population (value = 1). The experiment was performed seven times with similar results and compiled data from all experiments are shown. Three of the experimental replicates also included a polyIC-treated neutrophil + IM-enriched sample. Each dot represents value from an individual experiment (P = 7.19 x 10^-9, F = 30.32). (d) Combined data of four independent experiments are shown, and each dot represents an individual mouse (P = 3.34 x 10^-6, F = 34.85). (e) Combined data from six independent experiments are shown, and each dot represents an individual mouse (P = 5.72 x 10^-6, F = 10.78). Asterisks indicate pairs of values that are statistically different based on a post-hoc Tukey’s test.

predominantly produced by GFP*RFP+ (phagocytosis) cells, with only background IFN-β levels in the GFP*RFP+ (successfully infected) population, indicating that active invasion of IMs does not trigger IFN-β induction. Interestingly, the injected/uninfected subpopulation (GFP*RFP+) had IFN-β levels below uninfected, suggesting the ability of parasite effectors to downmodulate IFN-β expression, as had been previously suggested.28 It is also noteworthy that the ratio of GFP*RFP+ (successfully infected) to GFP*RFP+ (phagocytosed)
IMs was ~0.7, in contrast to neutrophils where the ratio was ~1.7 (Figures 3d and e). This indicates that neutrophils are a better target for successful invasion compared with IMs, which may in part account for their failure to produce IFN-β in response to *T. gondii* (Figure 3e). To further explore the impact of invasion versus phagocytosis on IFN-β induction, we examined responses to heat-killed parasites, which can be phagocytosed but cannot invade. Interestingly, the response to heat-killed parasites was greater than the response to live parasites, particularly at higher ratios of parasites to host cells, whereas increasing numbers of live parasites appeared to inhibit IFN-β induction (Figure 3f). These data, together with an earlier report, indicate that active invasion does not trigger IFN-β induction, but rather inhibits the response.

To further test whether phagocytosis is required for IFN-β induction, we treated cells with Latrunculin A, a drug that prevents phagocytosis through actin depolymerization. Treatment with Latrunculin A led to reduced uptake of parasites, as indicated by a drop in RFP fluorescence, particularly in IMs (Figure 3g). Latrunculin A treatment also led to a reduction in IFN-β expression to background levels (Figure 3g). To provide a control for cell viability, we stimulated drug-treated cells with lipopolysaccharide (LPS; TLR4 ligand) and Pam3 (TLR2 ligand), which are known to induce TNF-α through the activation of TLRs on the cell surface. We confirmed that TNF-α production in response to heat-killed parasites was also inhibited by Latrunculin A, suggesting that induction of TNF-α also requires parasite internalization (Figure 3h).

**IMs produce IFN-β after oral *T. gondii* infection.**

We next sought to extend our *in vitro* observations to an *in vivo* oral infection setting. We orally infected mice with Toxoplasma cysts, harvested the gut-draining MLNs at various times post-infection, and analyzed IFN-β expression by qRT-PCR. Expression of IFN-β was consistently elevated at days 4–7 post-infection, although there was considerable mouse-to-mouse variation in the level of induction.

---

**Figure 2** Intracellular detection of parasites triggers IFN-β production by inflammatory monocytes. (a) Bone marrow cells from wild-type mice were infected with live RFP+ parasites for 8 h and then analyzed by flow cytometry. Graph shows proportion of indicated cell population that contains RFP. Experiment was performed twice with similar results and data from a representative experiment is shown. Average values of experimental triplicates are shown. (b, c) Bone marrow cells were infected *in vitro* for 8 h with live RFP+ parasites, Ly6-B2+ cells were sorted for RFP+ and RFP− using fluorescence-activated cell sorting (FACS), and IFN-β expression was examined by qRT-PCR. Representative flow cytometry plots are shown in b, and IFN-β expression is shown in c. IFN-β expression relative to the IFN-β expression of uninfected control sample is shown. Combined data from four independent experiments (P=2.309×10−3, F=12.84). Asterisks indicate pairs of values that are statistically different based on a post-hoc Tukey’s test.
(Figure 4a). We then asked whether IMs contributed significantly to IFN-β induction in vivo. We used a cell depletion approach to address this question, which provided a more robust assay with limited cell numbers. We compared samples depleted of Ly6-B2+ cells (IMs and neutrophils, ~3% of starting MLN cells) or Ly6G+ cells (neutrophils only, ~0.25% of starting MLN cells). As a control, we also analyzed the starting samples and samples depleted of T cells using antibodies to CD3 (~40% of starting MLN cells). Notably, depletion of both

![Image](https://example.com/image1.png)

**Figure 4a:** (a) Normalized relative IFN-β expression in indicated cell population. (b) Ratio of GFP+/RFP+ in infected population. (c) Normalized relative IFN-β expression in uninfected and infected population. (d) IMs and neutrophils ratio of GFP+/RFP+ in indicated cell population. (e) Normalized relative IFN-β expression in heat-killed and live population. (f) Normalized relative IFN-β expression in indicated cell population. (g) Gated on Ly6C<sup>high</sup>CD11b<sup>+</sup> (IM) and untreated Latrunculin A. (h) TNF-α (%) in indicated cell population.

**Immunology and Cell Biology**
neutrophils and IMs led to a twofold decrease of IFN-β, whereas depletion of neutrophils alone had no significant effect (Figures 4b–d). It is also noteworthy that depletion of T cells, which led to a proportion increase of IMs in the population, led to a 2.5-fold increase of IFN-β expression. Together, these data suggest that IMs contribute significantly to the IFN-β expression by MLN cells after oral T. gondii infection. We also found that mice lacking the TLR adaptor MyD88 displayed significantly reduced IFN-β induction in MLNs 5 days following oral infection (Figure 4e). The lack of requirement for TLR7 or TLR9 (Figure 4f) is consistent with a lack of involvement of pDCs in this response. Taken together, these data require the participation of TLR7 or TLR9 in the IFN-β induction in response to viral infection, and thus we examined the data separately for female and male mice. Interestingly, the increase in parasite load in INFAR KO mice was more pronounced when only male mice were considered (Figure 5b). These results suggest that IFN-1 induced upon oral infection by T. gondii has a modest protective role, resulting in decreased parasite load and increased survival in WT compared with INFAR KO mice.

**DISCUSSION**

IFN-1 induction in response to viral infection has been extensively studied, but the mechanism of induction during parasitic infection is less well understood. For T. gondii, we lack key information about the host innate immune sensors, the specialized cell types and intracellular signaling compartments involved. Here we show that IMs, but not neutrophils, produce IFN-β in response to T. gondii. We also show that IFN-β production requires phagocytic uptake of the parasite by IMs and is reduced in IMs lacking TLR4 or MyD88. Finally, we show that IMs are the major source of IFN-β in the gut-draining MLNs after oral infection. Our data identify TLR and phagocytosis-dependent detection of parasite PAMPs by IMs and shed light on the mechanism of IFN-β production during infection by a non-viral pathogen.

* T. gondii can stimulate innate immunity via multiple TLRs, including TLR11 and 12 detection of the parasite protein profilin.34–36 and TLR2 and 4 detection of parasite GPIs.6 Profilin can induce IL-12 expression by DCs via TLR11 and TLR12, and IFN-1 production by pDCs in vitro.37–39 In addition, in vitro exposure of macrophages to parasite GPIs triggers TNF-α via TLR2 and TLR4.6 Here we show that a distinct cell type, the IM, produces IFN-β in a TLR4- and MyD88-dependent manner. Altogether, these results highlight how specificity in innate immune responses can be generated by different TLRs acting in specialized cell types, thus allowing for distinct host responses to be triggered by a single pathogen.

Interestingly, production of IFN-β by IMs requires uptake of the parasite into the host cell, reminiscent of the requirement for TLR2 internalization for IFN-β induction by certain viruses.31 Internalization may be necessary to deliver TLR2 and TLR4 to an intracellular compartment that is permissive for IFN-1 induction. This would be analogous to the requirement for endosomal trafficking of TLR4 by CD14 and LPS,32 and the AP3-dependent transport of TLR9 and CpG ligands to a LAMP2+ lysosome-related organelle.8 Thus, our data fit with an emerging theme in which the initial encounter between TLR and ligand is sufficient to trigger inflammatory cytokine production, whereas an additional ligand-dependent transport step is required for IFN-1 production.

In addition to allowing for intracellular triggering of TLRs, uptake of parasites by host IMs may also be necessary to process parasite PAMPs for recognition by TLRs. In fact, it has been previously shown that TLR2 can be triggered by the lipid and glycocalyx moiety of parasite GPIs, but not intact GPI, leading to the suggestion that processing of GPI by macrophage phospholipase occurs before TLR recognition.33 A requirement for intracellular processing of parasite PAMPs could also help to explain our observation that endocytosis is required for both TNF-α and IFN-β production by IMs in response to heat-killed parasites.

IMs have a key role in host protection against oral infection by *T. gondii*,24 although the precise mechanism of protection is unknown. Our data suggest that part of the protective function of IMs may lie in...
their ability to detect the parasites early during infection and to elaborate cytokines, such as IFN-β, to orchestrate subsequent immune responses. *Toxoplasma* is highly adapted to grow within its mammalian host and can actively manipulate host immune responses via a variety of secreted effectors. In this regard, it is intriguing that live parasites,
which can actively invade host cells, triggered a weaker IFN-β response compared with heat-killed parasites, as reported here and in an independent study. Moreover, neutrophils, which preferentially harbor live parasites, failed to produce IFN-β in response to T. gondii. These observations suggest that live Toxoplasma parasites may actually suppress host IFN-β production consistent with a previous report. Along these same lines, while TNF-α induction by parasite PAMPs can be detected in some studies, other studies reveal the inhibition of TNF-α by active parasite invasion. Thus, during Toxoplasma infection, the overall innate immune response of the mammalian host triggered by detection of parasite PAMPs by innate immune sensors is counterbalanced by active suppression by the parasites. In this regard, it is interesting to consider that the ratio of live to dead parasites encountered by host cells in different infection settings could be a crucial factor in determining whether parasite suppression of host immunity wins out over parasite detection by the host immune system.

METHODS

Mice

C57Bl/6, MyD88 KO and Cre-regulated GFP reporter (Rosa26-Cre) mice were purchased from Jackson Laboratories (Sacramento, CA, USA) or were bred in-house and maintained under specific pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved Life Science Addition animal facility at the University of California, Berkeley. In brief, the Cre-reporter mice have been engineered with a green fluorescent protein gene (ZsGreen) downstream of a transcriptional stop codon that is flanked by IoxP sites knocked into the Rosa26 locus. Thus, only after Cre-mediated recombination, cells will express GFP. All experiments were approved by the Animal Care and Use Committee at UC Berkeley. IFNAR KO mice were a gift from Dr D Portnoy (UC Berkeley). The LysM-GFP reporter mice were a gift from Dr T Graf (Albert Einstein College of Medicine, Bronx, NY) and have been previously described. In experiments using TLR2/4 KO mice, both the mutant and C57Bl/6 control mice carried a functional nramp1 allele (G169). For the majority of in vivo experiments, KO mice were co-housed with WT control mice. As some antibiotics can inhibit T. gondii growth, we placed all mice on drinking water without antibiotics at least 1 week before oral infection.

T. gondii infections

Type II Prugniaud parasites engineered to express RFP and ovalbumin were maintained, purified and used for oral infection as described previously. Mice were infected with 30–50 cysts by oral gavage. For the in vitro experiments, parasites were irradiated with 14,000 rad or heat-killed for 10 min in a 56 °C waterbath. Parasite cultures were regularly screened for

![Figure 5 Impact of type I interferon following oral T. gondii infection. (a) WT and IFNAR KO mice were orally infected with 40–50 cysts and mesenteric lymph nodes were analyzed 3–8 days post infection. Each dot represents one mouse. Infected cells were enumerated by flow cytometry of mesenteric lymph nodes 5 days post infection. Graph shows combined data from at least five independent experiments, and is normalized to the average of infected WT mice on the day of analysis. The left graph shows combined data using male and female mice (left; P<0.0002). The same data are shown using male mice (middle; P=0.0274) and female mice (right; P<0.0001). The statistics were calculated using the unpaired t-test. (b) WT and IFNAR KO mice were orally infected with 40–50 T. gondii cysts and monitored for survival over 18 days. The left graph shows combined data of five independent survival experiments using male and female mice. The middle and right graphs show the survival of male and female mice from the same experiments. Presented are combined data of five independent experiments. The P-value was calculated using the Mantel–Cox log-rank test.

Figure 4 Inflammatory monocytes produce of IFN-β after T. gondii in vivo infection. (a) Total RNA from WT MLNs were isolated at different time points (day 3–8) after oral infection, and expression of IFN-β was determined by qRT-PCR. IFN-β expression was normalized to GAPDH in each sample. (a) Experiment was performed twice and compiled data from both experiments are shown. Each dot represents an individual mouse (P=0.017966, F=3.15). (b–d) WT and IFNAR KO mice were orally infected and MLNs were harvested 5 days post infection. Single-cell suspensions were stained with anti-Ly6G, Ly6-B2 or CD3 antibody, and the indicated populations were depleted. IFN-β expression of depleted and non-depleted cell suspensions was determined by qRT-PCR normalized to GAPDH in each sample. The relative expression of IFN-β normalized to the average IFN-β expression of non-depleted sample on the day of analysis is shown. (b) Schematic of experimental setup. (c) Representative flow cytometry plots of non-depleted control sample and samples depleted of each subset as indicated. The proportion of CD3+ cells in depleted and non-depleted samples (top). The proportion of Ly6G+CD11b+ cells in depleted and non-depleted samples (middle). Bottom panel, Ly6c+CD11b+ cells in depleted and non-depleted samples. (d) Relative IFN-β expression in non-depleted samples or depleted samples is shown. Experiments were performed six times with two mice each (n=12). Values are means±s.e.m. of all experiments (P=7.88×10−10, F=40.84). (e and f) MLNs were harvested from WT and indicated KO mice, and 5 days post infection, IFN-β expression was determined by qRT-PCR and normalized to GAPDH in each mouse. The expression of IFN-β is shown relative to the average IFN-β expression of infected WT mice on the day of analysis. Values are means±s.e.m. of combined experiments (at least three experiments per mutant mouse strain; for e: P=0.0065, F=4.72; for f: P=1.1×10−12, F=45.14). Asterisks indicate pairs of values that are statistically different based on a post-hoc Tukey's test.
mycoplasma contamination and confirmed to be negative. To identify actively invaded cells, bone marrow cells from mice bearing a Cre-regulated GFP reporter were infected with parasites engineered to secrete Cre upon invasion of host cells. To generate this parasite strain, the above described parental Type II Prugniaud parasites engineered to express RFP and Ova and which lack the endogenous gene for hypoxanthine xanthine guanine phosphoribosyl transferase (HPT) were electroporated with a previously described linearized vector (ptoxoxifin-Cre), which expresses the selectable HPT marker and the epitope-tagged rhptry protein toxofilin fused to Cre recombinase. As previously described, the parasites were then subjected to several rounds of selection for the expression of HPT using media containing 25 µg ml⁻¹ mycophenolic acid and 50 mg ml⁻¹ xanthine before being cloned by limiting dilution. To verify secretion of a functional toxoCre fusion protein, single clones that were HPT⁺ were then tested for efficacy in causing Cre-mediated recombination in a Cre-reporter cell line.

**Tissue digestion and flow cytometry**

All infected and uninfected tissues were dissociated by collagenase digestion as described previously. Cell suspensions were stained and analyzed by flow cytometry. The following antibodies were used for cell surface staining: anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-CD3 (145-2C11), anti-Ly6C (HK1.4), anti-MHCII (M5/11.15.2), anti-CD19 (eBio1D3), anti-NK1.1 (PK136), anti-siglech (eBio440), anti-B220 (RA3-6B2), anti-CD4 (GK.5), anti-CD8 (53-6.7) and anti-CD69 (H.1F.23). Cells were purchased from eBioscience (San Diego, CA, USA), anti-Ly6G (1A8) was obtained from BD Pharmingen (San Jose, CA, USA), anti-Ly6-B2 antibody (7/4) from AbD Serotec (Raleigh, NC, USA) and CCR2 (475301) from R&D Systems (Minneapolis, MN, USA). For intracellular staining: anti-iNOS antibody was purchased from Millipore (Bedford, MA, USA) and anti-TNF-α (MP6-XT22) was purchased from BD Pharmingen. Cell suspensions from collagenase-digested tissue were stained for cell surface markers and permeabilized using the BD Cytofix/Cytoperm Kit (BD Pharmingen). For detection of intracellular TNF-α, cells were also treated ex vivo with GolgiPlug (BD Pharmingen) for 6 h at 37 °C in complete RPMI medium before cell surface staining and analyzed according to the manufacturer's instructions. Acquisitions were performed with a BD LSRII, and data were analyzed with the FlowJo software (FlowJo, Ashland, OR, USA).

**Real-time PCR**

Cell suspensions from collagenase-digested tissue were lysed in Trizol (Invitrogen, Grand Island, NY, USA), and mRNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen). cDNA was amplified using 2x SYBR green mix (AB Applied Biosystems, Grand Island, NY, USA) and GAPDH (ΔΔCT) analysis was used to determine relative expression among samples. The following primers were used: IFN-β forward 5′-GGTACACTGCGTTTGGCAT CCAA-3′ and reverse 5′-ACTGTCTGTTGGAGTTCATCC-3′; GAPDH: forward 5′-TGGGAAAGTGGAGTTGTCGCC-3′ and reverse 5′-AAGTGG TGATGCGCTTCCCG-3′. For some experiments, we present IFN-β expression in KO mice normalized to the average of IFN-β expression in infected WT mice analyzed on the same day. We occasionally performed experiments in which the *in vitro* response of all cell populations, including WT Ly6-B2-enriched bone marrow cells, was weak (<2.5-fold). We suspect that this was due to variation in the state of irradiated parasites, perhaps related to the ability of the parasites to be phagocytosed versus their ability to invade the host cells. To facilitate comparisons of WT versus mutant or manipulated samples, we excluded whole experiments in which no Toxoplasma-treated samples showed induction of 2.5-fold or greater compared with uninfected. As an alternative to real-time PCR, we attempted to use the previously described fluorescent IFN-β reporter mice. However, overlap from the RFP signal of the parasites complicated the detection of this already weak fluorescent signal, rendering this reporter unsuitable for this study.

**Ex vivo depletion of cell populations**

MLNs from orally infected WT mice were collagenase digested and cell populations were depleted as indicated using EasySep Biotin Positive Selection Kit (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions for cell depletion.

**Preparation and enrichment of cell populations and *in vitro* infection**

Bone marrow cells were isolated from adult mouse leg bones by flushing the interior with a syringe filled with media, and filtering through nylon mesh to generate a single-cell suspension. Ly6G⁺ and Ly6-B2⁺ cells were enriched from bone marrow cells of WT and the variously engineered mice using the EasySep Biotin Positive Selection Kit according to the manufacturer's instructions (Stemcell Technologies). Pre-, Ly6G⁺- and Ly6-B2⁺-enriched cells (2.5×10⁶ cells per well in a 12-well tissue culture plate) were infected for 8 h with irradiated parasites (MOI = 1–2). To ensure that there was no fibroblast carryover from the parasite maintenance cultures, fibroblast cultures were harvested under parasite preparation conditions and incubated with uninfected control cells. In the figures, this condition was referred to as the uninfected starting population.

**Latrunculin A treatment**

Pre-, Ly6G⁺- and Ly6-B2⁺-enriched cells (2.5×10⁶) were treated with 0.1 µg ml⁻¹ Latrunculin A for 30–45 min at 37 °C. Treated and untreated cells were stimulated for 8 h with heat-killed parasites at a ratio of two parasites per cell. For intracellular TNF-α detection, bone marrow cells were treated with 0.1 µg ml⁻¹ Latrunculin A (Invitrogen) for 30–45 min before stimulation with heat-killed parasites as above, LPS (1 µg ml⁻¹) or Pam3 (1 µg ml⁻¹). Thirty minutes after stimulation, GolgiPlug was added for an additional 7 h, and intracellular TNF-α was detected as described above.

**FACS**

WT bone marrow cells were infected (MOI = 1–2) with RFP-expressing live parasites for 8 h and stained with anti-CD11b, Ly6G and Ly6-B2 antibodies. RFP-positive and RFP-negative Ly6-B2⁺CD11b⁺ cells were sorted using a MoFlo cell sorter.

**Immunofluorescence microscopy**

WT bone marrow cells were infected with live parasites for 8 h and 2×10⁵ Ly6-B2⁺Ly6-G⁻ bone marrow cells were sorted using FACS. FACS-sorted cells were spun onto slides and stained for GRA6 as described previously. Images were taken using a Nikon TE2000 inverted microscope with 40X/1.3 Nikon oil objectives (Nikon, Melville, NY, USA). Images were acquired using NIS Elements AR software (Nikon) and processed using Imaris software (Bitplane, South Windsor, CT, USA).

**Statistical analysis**

Values are expressed as mean ± standard error (s.e.m.). Statistical significance was calculated using a one-way analysis of variance and Tukey post-hoc test unless otherwise indicated. Differences were considered significant at P < 0.05. To calculate the statistics for Figures 4d–f, the values were first transformed using the natural logarithms because of the differences in the standard deviation of each group.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

We thank Hector Nolla and Alma Valeros for cell sorting (UC Berkeley CRL Flow Cytometry Facility), Boris Striepen for the engineered RFP-expressing parasites, members of the Robey, Barton, and Boothroyd labs for helpful suggestions and reagents. This work was funded by NIH grants R01 AI065337 and R01 AI099312 (to EAR), PO1 AI065831 (to EAR), RO1 AI21423 (to JCB), and KO8 NS 065116 (to AAK), and Wellcome Trust grant WT085494 (to JLC).
1 Scanga CA, Aliaberti J, Jankovic D, Tilloy F, Bennouna S, Denkers EY et al. Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. J Immunol 2002; 168: 5997–6001.

2 Sukhumwasi W, Egan CE, Warren AL, Taylor GA, Fox BA, Bzik DJ et al. TLR adaptor MyD88 is essential for pathogen control during oral toxoplasma gondii infection but not adaptive immunity induced by a vaccine strain of the parasite. J Immunol 2008; 181: 3464–3473.

3 Kibbalsky AA, Jankovic D, Oh H, Heiny S, Sungsik W, Mathur R et al. Recognition of profilin by Toll-like receptor 12 is critical for host resistance to Toxoplasma gondii. Immunity 2013; 38: 119–130.

4 Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS. Monocyte recruitment during infection and in autoimmune diseases. J Exp Med 2009; 207: 229–259.

5 Shi C, Pamer EG. Monocyte recruitment during infection and in autoimmune diseases. J Exp Med 2009; 207: 229–259.

6 Debiere-Grockiego F, Campos MA, Azzouz N, Schmidt J, Bieker J, Resende MG et al. Activation of TLR2 and TLR4 by glycosylphosphatidylinositol-derived from Toxoplasma gondii. J Immunol 2007; 179: 1129–1137.

7 Debiere-Grockiego F, Azzouz N, Schmidt J, Dubrenets JF, Geyer H, Geyer R et al. Roles of glycosylphosphatidylinositol-phospholipids of Toxoplasma gondii. Induction of tumor necrosis factor-alpha production in macrophages. J Biol Chem 2003; 278: 32987–32993.

8 Sasai M, Linehan MM, Iwasaki A. Bifurcation of Toll-like receptor 9 signaling by adaptin protein 3. Science 2005; 308: 1626–1629.

9 Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 2010; 463: 772–777.

10 Blasius AL, Beutler B. Intracellular toll-like receptors. Immunity 2010; 32: 305–315.

11 Barton GM, Kagan J. A cell biological view of Toll-like receptor function: regulation through compartmentalization. Nat Rev Immunol 2009; 9: 535–542.

12 Rosowsi EE, Lu D, Andersen JF, Rodden MW, Serhan CN, Hayden MS. Monocyte recruitment during infection and autoimmune diseases. J Exp Med 2009; 202: 195–212.

13 Yamamoto M, Standley DM, Takashima S, Saiga H, Oukawaya M, Kayama H et al. A single polymorphic amino acid on Toxoplasma gondii kinase ROP16 determines the direct and strain-specific activity of STAT3. J Exp Med 2009; 206: 2747–2760.

14 Saei JP, Coller S, Boyle JP, Jacques MP, Boothroyd JC. Toxoplasma gondii tachyzoites are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. Immunity 2011; 35: 219–229.

15 Kang SJ, Liang HE, Reizis B, Locksley RM. Regulation of hierarchical clustering and activation of innate immune cells by dendritic cells. Immunity 2008; 29: 819–833.

16 Pepper M, Dzierzinski F, Wilson E, Tait E, Fang S, Yarovinsky F et al. Plasmodium dendritic cells are activated by Toxoplasma gondii to present antigen and produce cytokines. J Immunol 2008; 180: 6229–6236.

17 Gilleit M, Cao W, Liu YJ. Plasmodacytid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 2008; 8: 594–606.

18 Duny IR, Damatta RA, Fox B, Presti R, Greco S, Colonna M et al. G1(+) inflammatory monocytes are required for mucosal resistance to the pathogenic Toxoplasma gondii. Immunity 2008; 29: 306–317.

19 Takeuchi O, Akira S. Innate immunity to virus infection. Immunol Rev 2009; 227: 75–86.

20 Koshy AA, Fouts AE, Lodeon MS, Alkan O, Blau HM, Boothroyd JC. Toxoplasma gondii can recombine for analysis of host-parasite interactions. Nat Methods 2010; 7: 307–309.

21 Madisen L, Zwilling MA, Sunkin SM, Oh SW, Zarwala GA, Hut J et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci 2010; 13: 133–140.

22 Beltin DP, Peixoto L, Akopyants NS, Beverley SM, Wherry EJ, Christian DA et al. Differential induction of TLR3-dependent innate immune signaling by closely related parasite species. PLoS One 2014; 9: e88398.

23 Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS. Monocyte recruitment during infection and autoimmune diseases. J Exp Med 2009; 207: 229–259.

24 Combined action of nucleic acid-sensing Toll-like receptors and IRF1/IRF2 heterodimers imparts resistance to Toxoplasma gondii in mice. Cell Host Microbe 2013; 13: 42–53.

25 Baribal R, Lau L, Locksley RM, Barton GM. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol 2009; 10: 1200–1207.

26 Zanoni I, Olsuni R, Marek LR, Barresi S, Baribal R, Barton GM et al. CD14 controls the lps-induced endocytosis of toll-like receptor 4. Cell 2011; 147: 868–880.

27 Debiere-Grockiego F, Niehus S, Coddeville B, Eliss E, Poirier F, Weingart R et al. Binding of Toxoplasma gondii glycosylphosphatidylinositols to galectin-3 is required for their recognition by macrophages. J Biol Chem 2010; 285: 32744–32750.

28 Lim DC, Cooke BM, Dering C, Toxoplasma Saei JP, and Plasmodium protein kinases: roles in invasion and host cell remodelling. Int J Parasitol 2012; 42: 21–32.

29 Denkers EY, Butcher BA, Del Rio L, Kim L. Manipulation of mitogen-activated protein kinase/cellular factor-kappaB signaling cascades during intracellular Toxoplasma gondii infection. Immunol Rev 2004; 201: 191–205.

30 Faust N, Varas F, Kelly LM, Beck S, Graf T. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. Blood 2000; 96: 719–726.

31 Arpaia N, Godec J, Lau L, Sivick KE, McLachlin LM, Jones MB et al. TLR signaling is required for Salmonella typhimurium virulence. Cell 2011; 144: 675–688.

32 Chitgote T, Schaeffer M, Han SJ, van Dooren GG, Nollmann M, Herzmark P et al. Dynamics of neutrophil migration in lymph nodes during infection. Immunology and Cell Biology 2008; 86: 497–498.

33 Donald RG, Carter D, Ullman B, Ross DS. In situ tagging, cloning, and expression of the Toxoplasma gondii hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. Use as a selectable marker for stable transformation. J Biol Chem 1996; 271: 14010–14019.

34 Coombs JL, Han S-J, van Rooijen N, Raslet DH, Robey EA. Induction-injected regulation of natural killer cells by macrophages and collagen at the lymph node subcapsular sinus. Cell Rep 2012; 2: 124–135.

35 Scheu S, Dressing P, Locksley RM. Visualization of INFbeta production by plasmodacytid versus conventional dendritic cells under specific stimulation conditions in vivo. Proc Natl Acad Sci USA 2008; 105: 20416–20421.

36 Chitgote T, Han SJ, Schaeffer M, van Dooren GG, Herzmark P, Strenio B et al. Dynamics of T cell, antigen-presenting cell, and pathogen interactions during recall responses in the lymph node. Immunology 2009; 31: 342–355.