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An NLRP3 inflammasome–triggered Th2-biased adaptive immune response promotes leishmaniasis

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Leishmaniasis is a major tropical disease that can present with cutaneous, mucocutaneous, or visceral manifestation and affects millions of individuals, causing substantial morbidity and mortality in third-world countries. The development of a Th1-adaptive immune response is associated with resistance to developing *Leishmania major* (*L. major*) infection. Inflammasomes are key components of the innate immune system that contribute to host defense against bacterial and viral pathogens; however, their role in regulating adaptive immunity during infection with protozoan parasites is less studied. Here, we demonstrated that the NLRP3 inflammasome balances Th1/Th2 responses during leishmaniasis. Mice lacking the inflammasome components NLRP3, ASC, or caspase 1 on a *Leishmania*-susceptible BALB/c background exhibited defective IL-1β and IL-18 production at the infection site and were resistant to cutaneous *L. major* infection. Moreover, we determined that production of IL-18 propagates disease in susceptible BALB/c mice by promoting the Th2 cytokine IL-4, and neutralization of IL-18 in these animals reduced *L. major* titers and footpad swelling. In conclusion, our results indicate that activation of the NLRP3 inflammasome is detrimental during leishmaniasis and suggest that IL-18 neutralization has potential as a therapeutic strategy to treat leishmaniasis patients.

Introduction

Leishmaniasis is a major health problem that affects more than 12 million people worldwide and is caused by over 20 different *Leishmania* species parasites that are endemic in tropical and subtropical regions of Asia, the Middle East, sub-Saharan Africa, and South America (1, 2). Cutaneous leishmaniasis comprises up to half of *Leishmania* species infections, representing 0.7–1.2 million new cases each year (2). The cutaneous disease is routinely associated with local inflammatory pathology that can occasionally spread to other sites to cause mucocutaneous and visceral leishmaniasis. Despite being the second highest cause of mortality and the fourth leading cause of morbidity among tropical diseases, little is known about the immunobiology of leishmaniasis (3).

Infection with *Leishmania major* (*L. major*) causes cutaneous leishmaniasis in humans and susceptible BALB/c mice. In the first hours after infection, resident macrophages, DCs, and neutrophils that are recruited to the infection site phagocytize *Leishmania* parasites and produce inflammatory mediators to contain the infection (4). However, these phagocytes also serve as obligate reservoirs for *Leishmania* replication (4). During chronic infection, T cell–mediated adaptive immunity is critical for controlling and clearing *Leishmania*. Indeed, *L. major* infection was the first infectious mouse model that established the importance of Th1 versus Th2 balance in determining disease outcome (5, 6). Th1 responses associated with the production of IFN-γ, IL-12, and TNF-α were associated with resistance, better prognosis, and parasite clearance. In contrast, Th2-adaptive immunity mediated by IL-4–, IL-5–, and IL-13–producing T cells increased disease susceptibility and persistence (5–7).

The innate immune system utilizes different sets of germline-encoded receptors to detect *L. major* infections. These include TLR family members that are expressed at the plasma membrane and in endosomal compartments and several intracellular receptors that are exclusively present in the cytoplasm (8, 9). The intracellular Nod-like receptor (NLR) family comprises 34 family members that sense pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively) in the cytoplasm (10, 11). A subset of these NLRs assembles inflammasomes, which are large multiprotein complexes that promote activation of inflammatory caspase 1. Active caspase 1 promotes the conversion of pro–IL-1β and pro–IL-18 into bioactive IL-1β and IL-18 (12). The NLRP3 inflammasome represents the best-characterized inflammasome, and it is minimally composed of the NLR protein NLRP3, the bipartite adaptor protein ASC, and caspase 1 (12–14). This inflammasome is activated in response to a variety of stimuli ranging from bacterial toxins, bacterial RNA, ATP, nigericin, uric acid, and silica crystals (15). The NLRP3 inflammasome has emerged as an essential component of the host's immune response against bacterial and viral pathogens (16–18), but its role during infection with protozoan parasites such as *L. major* is relatively unknown. Here, we show that the NLRP3 inflammasome is activated when susceptible BALB/c mice are infected with *L. major* and that the NLRP3 inflammasome promotes *L. major*–induced production

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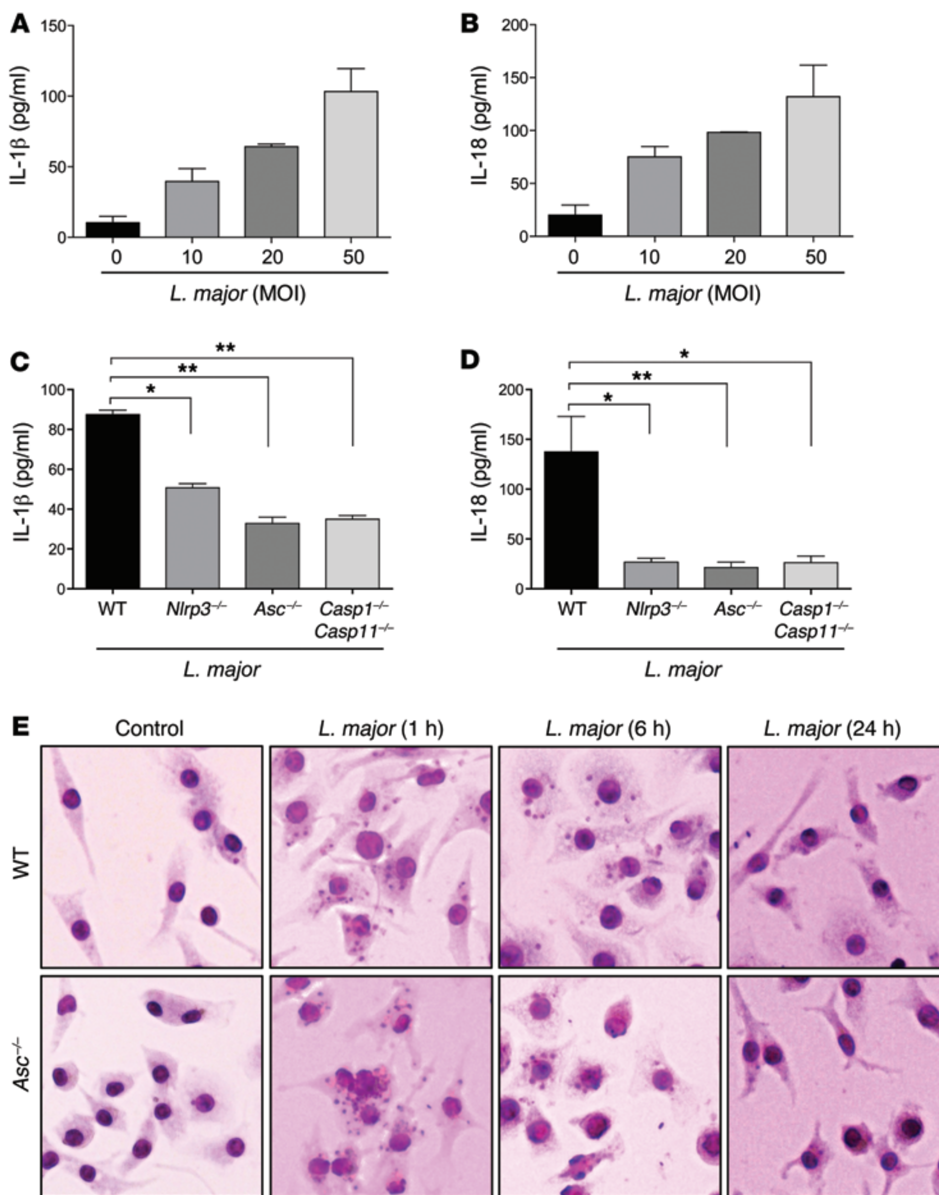

Figure 1. *L. major* induces inflammasome-mediated IL-1β and IL-18 production by macrophages in vitro. (**A** and **B**) BALB/c WT BMDMs were primed with 500 ng/ml LPS for 6 hours followed by the indicated MOI of *L. major* for 48 hours. Supernatants from stimulated BMDMs were analyzed by ELISA for IL-1β (**A**) and IL-18 (**B**). (**C** and **D**) WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ BMDMs were stimulated with 500 ng/ml LPS for 6 hours followed by *L. major* infection (20 MOI) for 48 hours, and IL-1β (**C**) and IL-18 (**D**) in the supernatants were examined by ELISA. (**E**) WT and *Asc*$^{-/-}$ BMDMs were infected with 20 MOI of *L. major* for 1 hour. Infected BMDMs were washed to get rid of excess *L. major* and supplemented with fresh media. BMDMs were then immediately stained with Giemsa to determine phagocytosis of *L. major* (1 hour). Other groups of BMDMs were incubated for an additional 6 and 24 hours to determine clearance of phagocytosed *L. major* before Giemsa staining of the cells (6-hour and 24-hour time points). Giemsa-stained cells were examined under a light microscope to visualize *L. major* phagocytosis and clearance (original magnification, ×40). Data in **A** and **B** are the mean of duplicates and are representative of at least 3 independent experiments. Data in **C** and **D** are cumulative of 3 to 5 independent experiments with n = 3–7 in each group. Images in **E** are representative of 3 independent experiments. The Mann-Whitney U test was performed to evaluate significant differences between WT and KO BMDMs. ELISA data are shown as the mean ± SEM. *$P < 0.05$; **$P < 0.01$.

of IL-1β and IL-18 in vitro and in vivo. Remarkably, we found that IL-18 enhanced IL-4 secretion by increasing GATA3 and cMAF expression in activated T cells, thereby skewing adaptive immunity toward detrimental Th2 responses. Indeed, BALB/c mice lacking NLRP3, ASC, or caspase 1 produced less IL-18 and IL-4, which resulted in increased protection against leishmaniasis. In agreement, IL-18 neutralization protected susceptible BALB/c hosts against *L. major* infection. In conclusion, we demonstrate that NLRP3 inflammasome–induced bias of adaptive immunity toward Th2-type responses is detrimental during leishmaniasis.

Results

L. major induces inflammasome-dependent IL-1β and IL-18 secretion from infected macrophages. Inflammasomes promote the maturation and release of IL-1β and IL-18 (19). Although inflammasome activation in response to bacteria, viruses, and fungal infection is a vital component of protective immunity (20), the role that inflammasomes play in regulating immune responses to unicellular protozoan parasites remains poorly characterized. We infected BALB/c bone marrow–derived macrophages (BMDMs) with *L. major* to determine whether this promoted inflammasome-dependent cytokine secretion. We observed a dose-dependent increase in IL-1β and IL-18 release from infected BMDMs (Figure 1, A and B). To formally establish that IL-1β and IL-18 release was inflammasome dependent, we infected WT and *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ BMDMs with *L. major*. Notably, *L. major*–induced IL-1β and IL-18 secretion was markedly diminished in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ BMDMs (Figure 1, C and D). The role of inflammasome signaling in mediating secretion of IL-1β and IL-18 was specific, because extracellular levels of the inflammasome-independent cytokines IL-6, KC, and TNF-α were similar between *L. major*–infected WT and inflammasome-deficient BMDMs (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI79526DS1). Moreover, *L. major* uptake by WT and *Asc*$^{-/-}$ BMDMs was comparable (Figure 1E and Supplemental Figure 2), ruling out the possibility that reduced IL-1β

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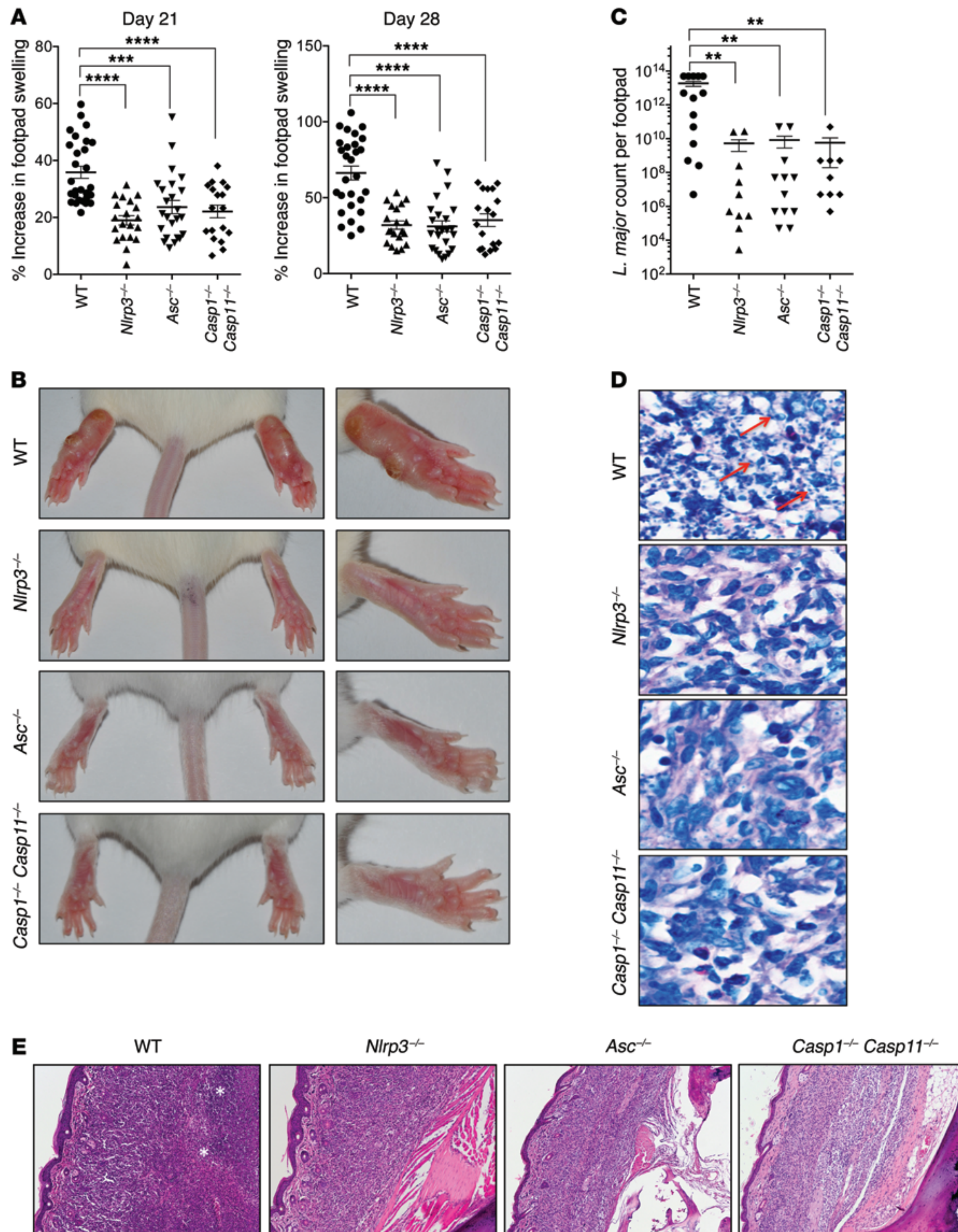

Figure 2. Inflammasome signaling promotes susceptibility to *L. major* infection in vivo. Footpads of WT (n = 28), *Nlrp3*$^{-/-}$ (n = 20), *Asc*$^{-/-}$ (n = 24), and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ (n = 18) mice were infected with 10^6 *L. major* promastigotes. (**A**) Footpad swelling was measured on days 21 and 28 after infection. (**B**) Representative images of footpads of WT BALB/c, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice on day 28 after infection. (**C**) On day 28, mice were euthanized, and *L. major* titers in the footpads of infected WT (n = 14), *Nlrp3*$^{-/-}$ (n = 10), *Asc*$^{-/-}$ (n = 12), and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ (n = 9) mice were determined using a limiting dilution assay as described in the Methods. (**D**) On day 28 after infection, footpad sections were stained with Giemsa to determine *L. major* titers. Red arrows indicate *L. major* amastigotes in the cytoplasm of cells in WT footpad sections (original magnification, ×60). (**E**) H&E-stained sections of the footpads from WT, BALB/c, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice on day 28 after infection (original magnification, ×10). White asterisks in the H&E-stained section of the WT footpad show necrotic lesions in the inflamed footpad. Dunnett's multiple comparisons test was performed to evaluate significance between multiple groups. Data represent the mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

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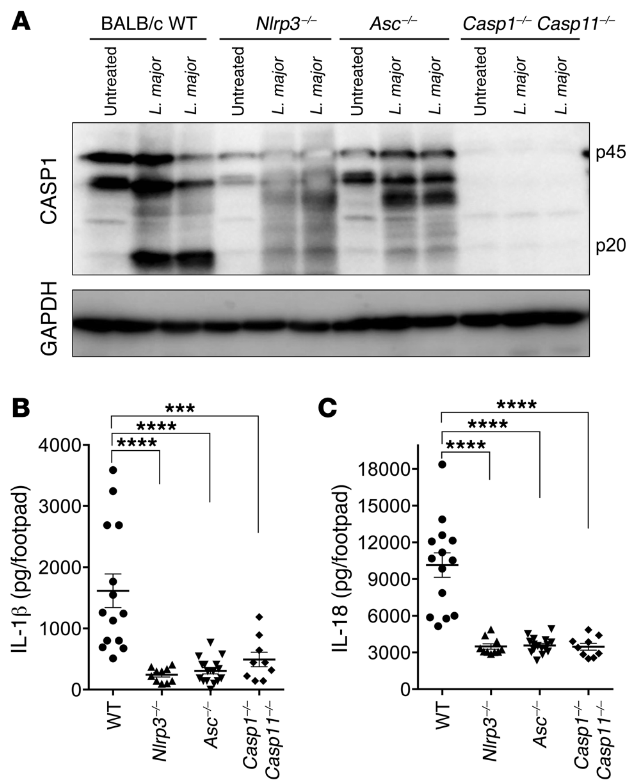

Figure 3. *L. major* induces NLRP3 inflammasome activation in vivo. WT (n = 14), *Nlrp3*$^{-/-}$ (n = 10), *Asc*$^{-/-}$ (n = 16), and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ (n = 9) mice were infected with 10^6 *L. major* promastigotes. Twenty-eight days later, the infected footpads were harvested and homogenized as detailed in the Methods. (**A**) Protein lysates from the representative footpads were run on an SDS-PAGE gel, and activation of caspase 1 was determined by Western blotting. GAPDH was used as a loading control. (**B** and **C**) IL-1β and IL-18 cytokine levels were determined by ELISA in the footpad lysates. Dunnett's multiple comparisons test was performed to evaluate significance between multiple groups. Data were combined from 2 independent experiments and represent the mean ± SEM. ***P < 0.001; ****P < 0.0001.

and IL-18 secretion was due to defective *L. major* phagocytosis and clearance. Collectively, these experiments demonstrate that *L. major* induces inflammasome-mediated IL-1β and IL-18 secretion.

NLRP3 inflammasome activation increases susceptibility to in vivo L. major infection. To address the role of inflammasome signaling in cutaneous leishmaniasis, footpads of WT BALB/c mice and mice lacking the inflammasome molecules NLRP3, ASC, or caspases 1 and 11 were cutaneously infected with *L. major*. All animals developed footpad swelling, but this was markedly attenuated in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice relative to that seen in WT controls (Figure 2, A and B). In agreement, we observed significantly reduced parasite burden in the footpads of *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice (Figure 2C), suggesting that defective NLRP3 inflammasome activation results in improved parasite clearance. Indeed, Giemsa staining showed numerous amastigotes in the footpads of WT mice, but not in those of infected *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, or *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice (Figure 2D). H&E-stained footpad sections of infected WT mice showed large necrotic lesions that were virtually absent in the footpads of infected *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice (Figure 2E). Taken together, these results demonstrate that NLRP3 inflammasome activation contributes to host susceptibility during *L. major* infection in susceptible BALB/c mice.

NLRP3 mediates local caspase 1 activation and inflammasome-associated cytokine production at the site of infection. Having established that defective NLRP3 inflammasome activation conferred protection against *L. major* pathogenesis (Figure 2), we sought to determine whether this resulted from local inflammasome activation in the footpads of cutaneously infected mice. To this end, we evaluated *L. major*–induced caspase 1 processing in footpad homogenates of infected WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice. *L. major* infection resulted in robust caspase 1 maturation in the footpads of infected WT mice. In contrast, the appearance of the caspase 1 p20 subunit was abrogated in homogenates of *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ footpad samples (Figure 3A). As expected, immunoreactive bands were not detected in the homogenates of infected *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice, demonstrating specificity of these results (Figure 3A). In agreement, we detected high levels of IL-1β and IL-18 in the footpad homogenates of *L. major*–infected WT mice that were significantly blunted in the absence of NLRP3, ASC, and caspase 1 (Figure 3, B and C). These results demonstrate that *L. major* induces local NLRP3 inflammasome activation and IL-1β and IL-18 production in the footpads of infected mice.

Increased resistance to L. major infection in NLRP3 inflammasome–deficient mice is associated with reduced Th2 cytokines and increased Th1 cytokines. Based on our collective observations, we hypothesized that increased IL-1β and IL-18 production in *L. major* –infected WT mice promoted disease pathogenesis by skewing adaptive immunity against the parasite. BALB/c mice are known to mount Th2-dominated adaptive immune responses during *L. major* infection that are characterized by significant secretion of IL-4, IL-5, and IL-13 (5, 6). These Th2 cytokines inhibit macrophage activation and dampen their ability to resolve *L. major* infection (21–23). To elucidate how NLRP3 inflammasome signaling influences susceptibility to *L. major* infection, we investigated local levels of these Th2 cytokines in the footpads of infected WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice. Notably, amounts of IL-4 and IL-5 were significantly reduced in the footpads of *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice relative to the levels detected in WT controls (Figure 4, A and B). Recent studies have shown that IL-17 promotes cutaneous leishmaniasis in susceptible BALB/c mice (24). Given that IL-1 promotes Th17 cell differentiation and IL-17 production (25), we analyzed IL-17 in the footpads of these mice. Compared with WT mice, IL-17 levels were significantly reduced in the footpads of *L. major*–infected *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice (Supplemental Figure 3). Concurrently, we detected markedly increased levels of Th1-associated IFN-γ in the footpads of *L. major*–infected *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice compared with those in WT controls (Figure 4C). In contrast, IL-10 levels were not differentially modulated in WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, or *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice (Figure 4D). Together, these results suggest that the NLRP3 inflammasome controls host susceptibility to cutaneous *L. major* infection by modulating the balance between Th1 and Th2 responses.

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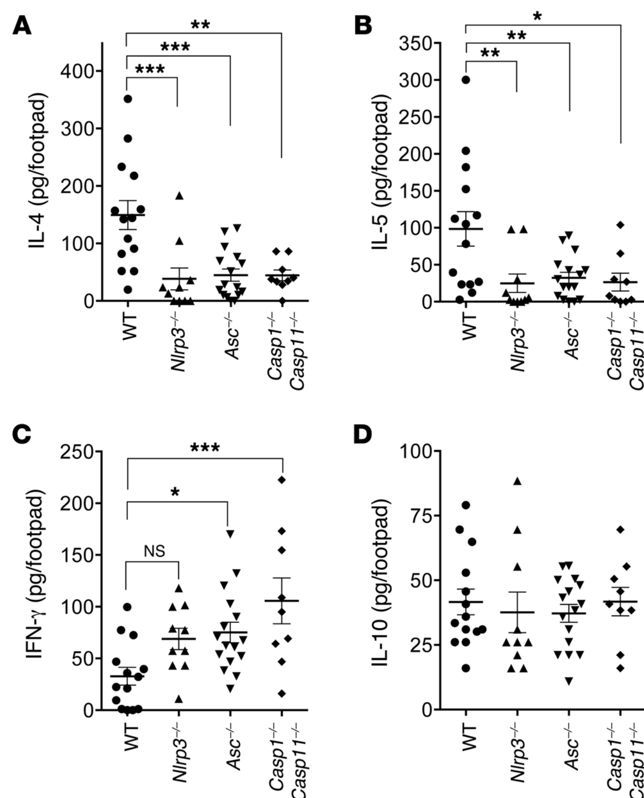

Figure 4. NLRP3 inflammasome signaling induces a Th2-biased cytokine profile in the footpads of *L. major*–infected mice. (**A**–**D**) Supernatants from homogenized footpads of *L. major*–infected WT (n = 14), *Nlrp3*$^{-/-}$ (n = 10), *Asc*$^{-/-}$ (n = 16), and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ (n = 9) mice on day 28 after infection were analyzed by ELISA for the presence of IL-4 (**A**), IL-5 (**B**), IFN-γ (**C**), and IL-10 (**D**). Data were combined from 2 independent experiments and represent the mean ± SEM. Significance was determined using Dunnett's multiple comparisons test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

NLRP3 inflammasome deficiency results in enhanced T cell–mediated IFN-γ production during L. major infection. In addition to increasing Th2-associated cytokines, our results showed that the NLRP3 inflammasome dampens local IFN-γ production in the footpads of infected mice. IFN-γ is associated with resistance to *L. major* infection, and T cells have been identified as the major source of IFN-γ during *L. major* infection (6, 26). We therefore investigated whether increased IFN-γ levels in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice were associated with greater production of IFN-γ by T cells. We detected similar frequencies of CD4$^+$ and CD8$^+$ T cells in the lymph nodes of WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice that were infected with *L. major* (Supplemental Figure 4). In contrast, the frequency of IFN-γ–producing CD4$^+$ T cells was significantly increased in the absence of NLRP3, ASC, and caspase 1 (Figure 5, A and B). In addition, gated analysis of CD4$^+$IFN-γ$^+$ T cells showed increased IFN-γ production by CD4$^+$ T cells from *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice on a per-cell basis (Figure 5, C and D). Analysis of the CD8$^+$ T cell population in the draining popliteal lymph nodes showed similarly increased frequencies of IFN-γ–producing T cells in *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice. Moreover, we also noted that these CD8$^+$IFN-γ$^+$ T cells produced greater amounts of IFN-γ on a per-cell basis (Figure 5, E–H).

TNF is another Th1 cytokine produced by activated T cells and confers protection against *L. major* infection (27–29). Notably, we observed significantly increased frequencies of TNF-α–producing CD4$^+$ and CD8$^+$ T cells in *L. major*–infected *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice relative to those detected in BALB/c WT controls (Supplemental Figure 5), further supporting a key role for the NLRP3 inflammasome in biasing adaptive immune responses toward a Th2 profile during *L. major* infection.

Inflammasome-mediated IL-18 production, rather than IL-1β, regulates Th2 responses. We showed that defective NLRP3 inflammasome–induced IL-1β and IL-18 production is associated with increased resistance to *L. major* infection. This was associated with reduced Th2 responses (IL-4 and IL-5 production) and a concomitant increase in Th1 responses (IFN-γ and TNF production). To determine the relative contributions of IL-1β and IL-18 to balancing Th1- and Th2-adaptive immunity, splenocytes from WT BALB/c mice were stimulated for 72 hours in vitro with anti-CD3 monoclonal antibodies in the presence or absence of IL-1β and IL-18. Following stimulation, production of the hallmark Th1 (IFN-γ) and Th2 (IL-4) cytokines was analyzed in culture supernatants. As reported, IL-1β and IL-18 triggered T cells to produce IFN-γ, but not IL-4, in the absence of anti-CD3 stimulation (Supplemental Figure 6). Interestingly, we found that IL-18 significantly reduced the production of IFN-γ by anti-CD3–stimulated splenocytes (Figure 6A). Furthermore, IL-18 significantly enhanced the ability of anti-CD3–stimulated splenocytes to produce IL-4 (Figure 6B). These IL-18–induced responses were specific, because IL-18–binding protein (IL-18BP) reduced IL-18–dependent IL-4 production to the levels observed with anti-CD3 stimulation alone (Figure 6C). Unlike IL-18, recombinant IL-1β did not significantly modulate IFN-γ and IL-4 production by anti-CD3–activated T cells (Figure 6, A–C), nor did it act synergistically with IL-18 to dampen IFN-γ and increase IL-4 production (Supplemental Figure 6). These results demonstrate that IL-18 has a direct role in skewing the Th1 and Th2 profiles of activated T cells and suggest that NLRP3 inflammasome–driven IL-18 enhances IL-4 production, which increases *L. major* pathogenesis. To directly analyze the importance of IL-18 in cutaneous leishmaniasis, IL-18 was neutralized in infected mice by treating them with IL-18BP in vivo. Remarkably, footpad swelling and local *L. major* titers in the footpads and lymph nodes of infected BALB/c mice were significantly reduced with IL-18BP treatment compared with PBS-treated controls (Figure 6, D and E, and Supplemental Figure 7A). As expected, IL-4 levels in the footpads were significantly reduced in the IL-18BP–treated groups (Figure 6F).

To evaluate the mechanistic details of how IL-18 promotes IL-4 responses, we treated anti-CD3–stimulated splenocytes with IL-18 or IL-1β and measured the expression of the IL-4–promoting transcription factors GATA3 and cMAF in CD4$^+$ T cells (30, 31). IL-4 signaling is a known regulator of GATA3 expression (32, 33). As expected, the addition of IL-4 increased GATA3 expression, while neutralization of IL-4 (anti–IL-4 mAb) reduced GATA3 expression on CD4$^+$ T cells (Supplemental Figure 7B).

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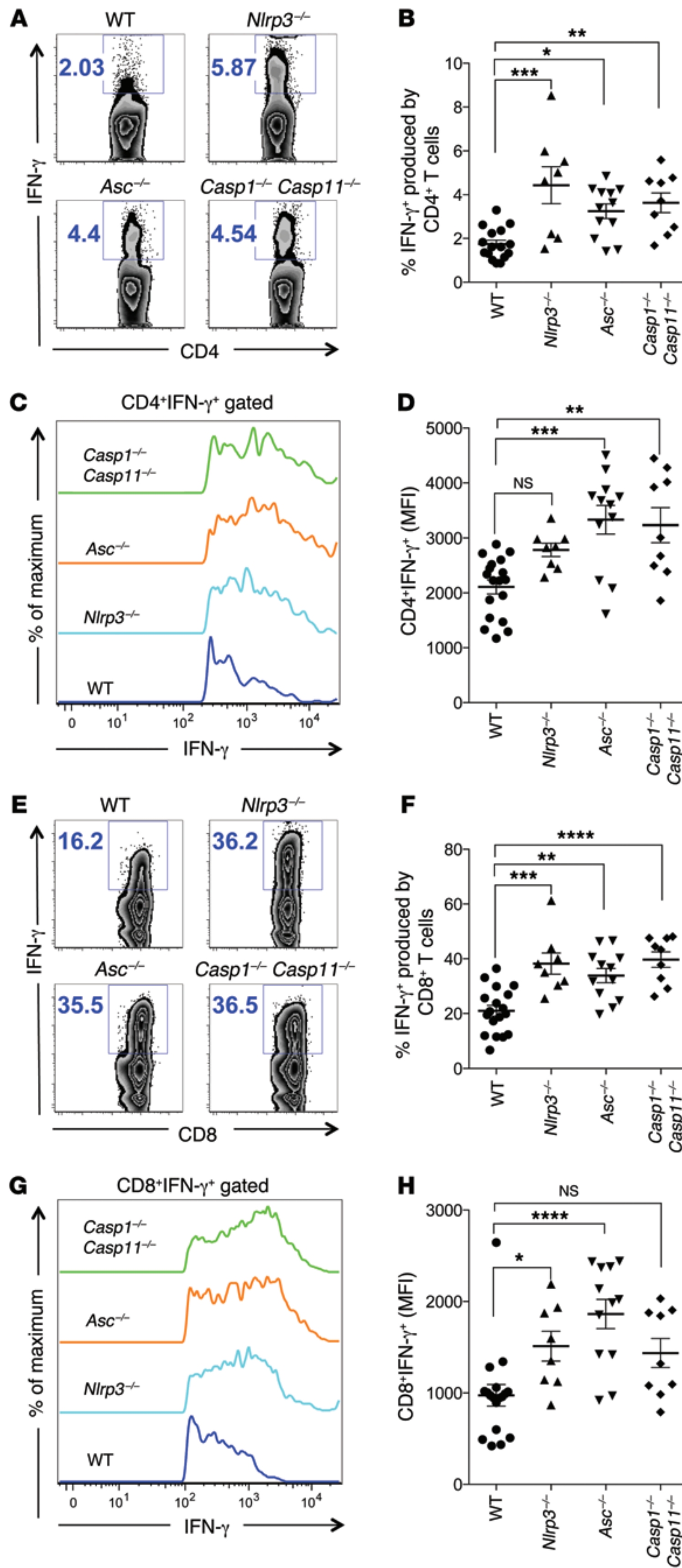

Figure 5. NLRP3 inflammasome negatively regulates T cell–mediated IFN-γ production. WT (n = 18), *Nlrp3*$^{-/-}$ (n = 8), *Asc*$^{-/-}$ (n = 12), and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ (n = 9) mice were infected with 10^6 *L. major* promastigotes. Infected mice were euthanized 28 days later, and draining popliteal lymph nodes were harvested. Single-cell suspensions of popliteal lymph node cells were stimulated with anti-CD3 and anti-CD28 antibodies for 4 hours in the presence of monesin. T cells and cytokine production were evaluated by flow cytometry. Representative flow plots of CD4$^+$ (**A**) and CD8$^+$ T cells (**E**) that produced IFN-γ. Cumulative bar scatter plots represent the frequencies of CD4$^+$ (**B**) and CD8$^+$ T cells (**F**) that produced IFN-γ. Representative flow plot (**C**) and scatter plot (**D**) show the mean fluorescence intensity (MFI) of IFN-γ produced by CD4$^+$ T cells. Representative flow plot (**G**) and scatter plot (**H**) show the MFI of IFN-γ produced by CD8$^+$ T cells. Dunnett's multiple comparisons test was used to evaluate significance between groups. Data represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

In agreement with our previous data, IL-18, but not IL-1β, increased GATA3 expression on CD4$^+$ T cells (Figure 6, G and H). Similar to GATA3, IL-18 increased the expression of cMAF in anti-CD3–stimulated CD4$^+$ T cells (Supplemental Figure 7C). Collectively, our results establish IL-18 production by the NLRP3 inflammasome as a central regulator of adaptive immunity that promotes Th2 responses and increases *L. major* pathogenesis in susceptible hosts (Figure 7).

Discussion

Leishmaniasis is an infectious disease caused by *Leishmania* species and represents a major health problem in equatorial developing countries. Visceral and cutaneous leishmaniasis are the 2 prevalent forms of the disease (34, 35). Despite substantial efforts, the immunobiology of leishmaniasis is not well understood. Here, we made use of the cutaneous *L. major* infection model to study the role of inflammasome signaling in leishmaniasis (36). To this end, we backcrossed mice with targeted deletions in key inflammasome molecules with mice on an *L. major*–susceptible BALB/c genetic background. We found *L. major* to activate the NLRP3 inflammasome in infected macrophages, which resulted in significant secretion of IL-1β and IL-18. Of note, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ mice were resistant to cutaneous *L. major* infection. Further analyses revealed that NLRP3 inflammasome–induced IL-18 production promoted Th2 responses and inhibited Th1-adaptive immunity. IL-18–mediated skewing of adaptive immune responses was characterized by reduced IFN-γ secretion by activated T cells and increased production of IL-4, a cytokine that exacerbates leishmaniasis pathology. Concurrently, neutralization of IL-18 reduced IL-4 production and inhibited *L. major*–induced disease pathogenesis in BALB/c mice (Figure 7).

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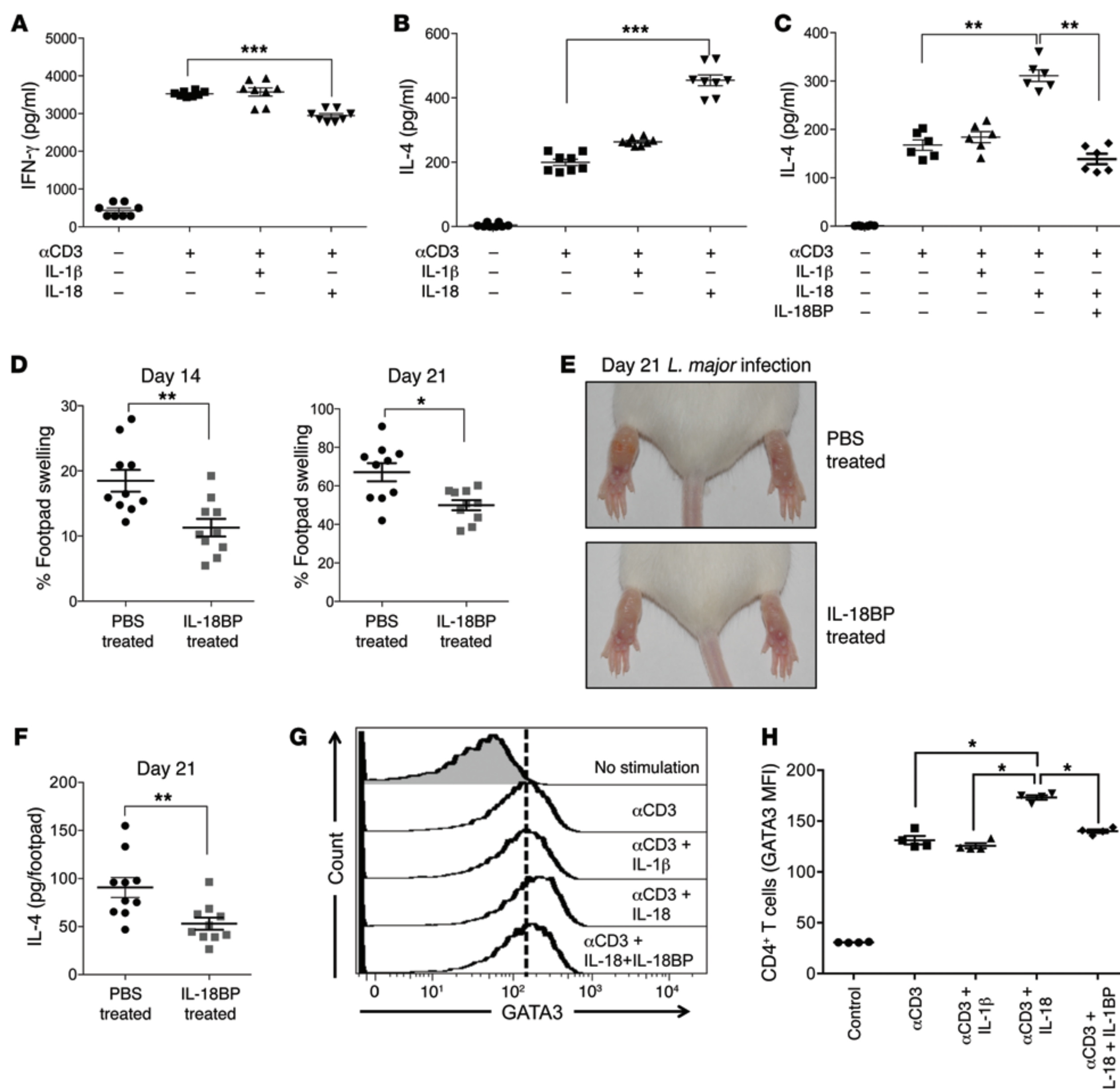

Figure 6. IL-18 promotes Th2 responses during T cell activation in BALB/c mice. Single-cell suspensions of splenocytes from WT BALB/c mice were stimulated for 72 hours with anti-CD3 Ab in the presence of IL-1β and IL-18. IFN-γ (**A**) and IL-4 (**B**) cytokine levels in the supernatants were determined by ELISA. (**C**) WT BALB/c splenocytes were stimulated for 72 hours with anti-CD3 in the presence of IL-18 and IL-18BP, and IL-4 levels in the supernatants were determined by ELISA. (**D**–**F**) WT BALB/c mice were treated with PBS (n = 10) or IL-18BP (n = 10) on days –1, 0, 1, 3, 7, and 14. Mice were infected with 10^6 *L. major* promastigotes, and footpad swelling was monitored on days 14 and 21 (**D** and **E**). (**F**) IL-4 cytokine levels in the footpads of infected mice were determined on day 21 after infection. (**G** and **H**) Single-cell suspensions of splenocytes from WT BALB/c mice were stimulated for 48 hours with anti-CD3 Ab in the presence of IL-1β and IL-18, and expression of GATA3 was determined by flow cytometry. (**G**) Representative histogram plots of GATA3 expression. (**H**) MFI of GATA3 in CD4+ T cells. Data in **A**–**C** are presented as duplicates from 3 to 4 WT mice and are representative of at least 5 independent experiments. Data in **G** and **H** are presented as duplicates from 2 WT mice and are representative of 2 independent experiments. Data represent the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 by Mann-Whitney U test.

The role of inflammasomes in *Leishmania* species infection is largely unclear. A recent report suggested that the NLRP3 inflammasome confers protection against *Leishmania amazonensis* (*L. amazonensis*) infection in resistant C57BL/6 mice (37). Similar to our results presented here, the cited report showed that *Leishmania* species induces NLRP3 inflammasome activation and significant IL-1β production from infected macrophages (37). This report further proposed that NLRP3 inflammasome deficiency increases susceptibility to *L. amazonensis* infection on a resistant C57BL/6 background (37). In contrast, we demonstrate here that defective NLRP3 inflammasome activation renders mice on a susceptible BALB/c genetic background resistant to *L. major* infection. Differences in results can be due to the use of different *Leishmania* species and/or the genetic background of inflammasome-

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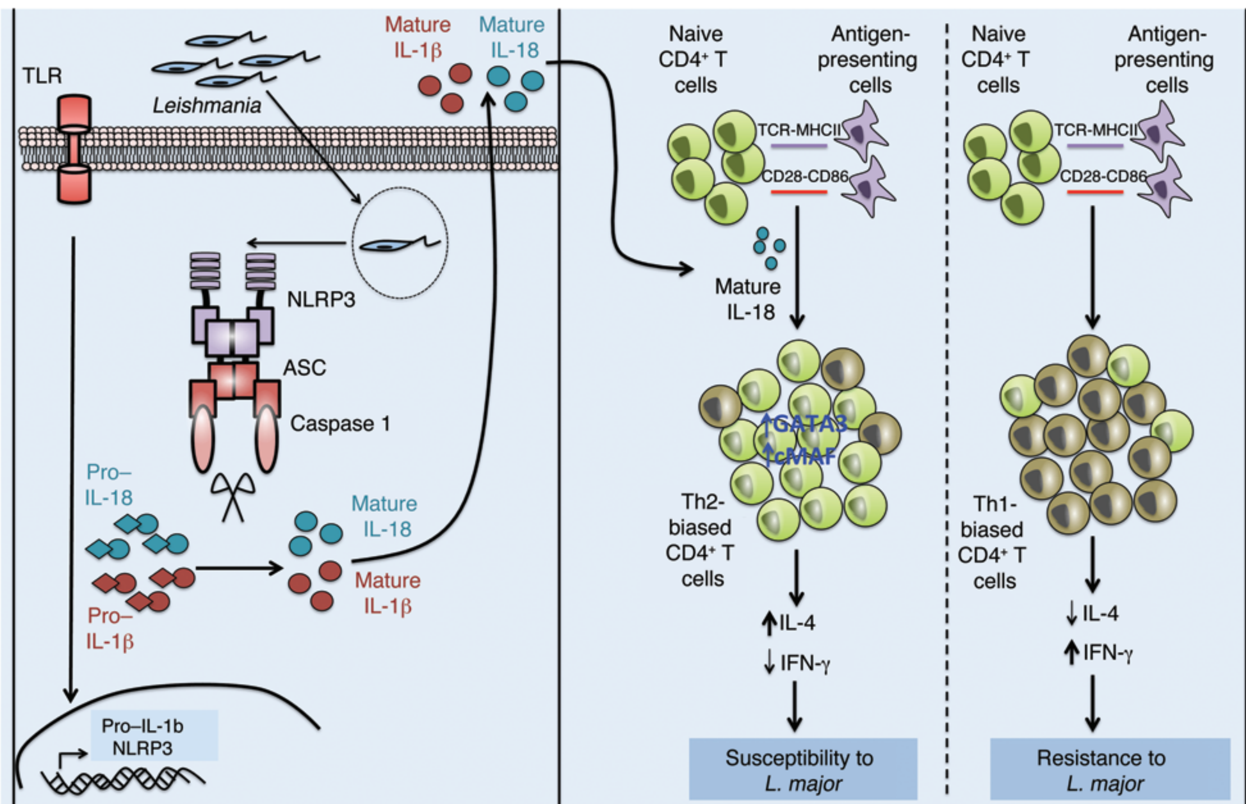

Figure 7. Model for *L. major*–induced NLPR3 inflammasome activation and IL-18–induced skewing of T cell responses. *L. major* infection of BALB/c mice activates NLRP3 inflammasomes and production of IL-1β and IL-18. IL-18 promotes a Th2-biased adaptive immune response and enhances IL-4 production by $CD4^+$ T cells.

deficient mice. In agreement, the NLRP3 inflammasome provides protection against *L. amazonensis*, but not *L. major*, in C57BL/6 mice (37). Indeed, C57BL/6 and BALB/c mice are known to mount different immune responses to *Leishmania* species infection (7, 38–40). C57BL/6 mice are relatively resistant, while BALB/c mice are highly susceptible to *Leishmania* species infection. These differences highlight the need to closely evaluate immune responses during *Leishmania* species infections, since the responses can vary dramatically based on the genetic background of the host. Furthermore, *Leishmania* species themselves vary in the immune responses that they induce in the course of disease pathogenesis (34). For instance, *Leishmania donovani* (*L. donovani*) causes a highly lethal visceral infection, whereas *L. major* and *L. amazonensis* trigger less severe, localized cutaneous infections (34).

IL-1β and IL-18 are inflammasome-dependent cytokines that contribute critically to immune responses during infections and autoimmune diseases. Our study shows that both IL-1β and IL-18 are produced during *L. major* infection in vitro and in vivo. Interestingly, our studies suggest IL-18 to be a central cytokine that modulates T cell responses during *L. major* infection. However, the role for IL-18 during *Leishmania* species infection is less clear in the literature. While some studies show a positive role for IL-18 in promoting Th1 responses and resistance during *Leishmania* species infection (41), other studies have shown that IL-18 might enhance Th2-biased responses and promote susceptibility (42). Indeed, IL-18 can induce either a Th1 or Th2 response, depending on the cytokine milieu and genetic background of the host (43, 44). Thus, future studies looking at more in-depth, side-by-side analyses of *Leishmania* species infection in different genetic backgrounds are critically needed to discern the discrepancies between the various studies. Furthermore, our observation that IL-17 is significantly reduced in inflammasome-deficient mice during *L. major* infection warrants further investigation. Given the role for IL-17 in potentiating leishmaniasis (24), it is quite possible that both IL-17 and IL-4 are playing a synergistic role in disease progression. Future studies will elucidate the contribution of IL-1β and IL-18 in promoting IL-17 responses during *L. major* infection in mice. Nonetheless, our studies clearly show that in a susceptible BALB/c host, IL-18 promotes a Th2-biased response, as determined by increased IL-4 production and reduced IFN-γ response. Given that IL-18 was first discovered as an IFN-γ–inducing factor (45–47), we believe our studies uncover a novel role for IL-18 in certain settings, in which IL-18 promotes Th2 responses.

In conclusion, we demonstrate that defective NLRP3 inflammasome activation increases resistance to *L. major* infection in a susceptible BALB/c genetic background. The increased resistance in NLRP3 inflammasome–deficient mice is associated with reduced production of the hallmark Th2 cytokines IL-4 and IL-5, concomitant with increased production of the Th1-associated cytokine IFN-γ. We further show that IL-18 is responsible for biasing Th1- and Th2-adaptive immune responses during

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L. major infection. Furthermore, our data suggest that IL-18 promotes IL-4 production, while limiting IFN-γ production by activated T cells, further expanding the mechanisms by which IL-18 modulates adaptive immune responses. Mechanistically, IL-18 induces greater expression of GATA3 and cMAF by activated CD4$^+$ T cells to influence Th1 and Th2 biasing. Finally, we proved that the IL-18BP treatment that neutralized IL-18 protects BALB/c mice from *L. major*–induced footpad pathogenesis. Together, our results not only establish a detrimental role for the NLRP3 inflammasome during *L. major* infection, but also expose IL-18 as a potential therapeutic target in leishmaniasis.

Methods

Mice. *Nlrp3*$^{-/-}$ (14), *Asc*$^{-/-}$ (48), and *Casp1*$^{-/-}$ *Casp11*$^{-/-}$ (49) mice, all described previously (17), were backcrossed with BALB/c mice for more than 10 generations and maintained in our colony. The BALB/c WT mice were bred at St. Jude Children's Research Hospital.

L. major infection. The *L. major* strain WHOM/IR/-173 was grown in vitro in complete M199 media supplemented with 5% HEPES, 10% FBS, and 1% penicillin-streptomycin at 27°C. Metacyclic promastigotes of *L. major* were purified using Lectin Agarose (Sigma-Aldrich) as previously described (50). Mice were infected with 10^6 *L. major* promastigotes per footpad in a volume of 50 μl. Footpad measurements were taken weekly. Footpad sections were also analyzed for inflammation and necrosis by H&E staining. *L. major* MOI in the footpads were determined by directly staining the footpad sections using Giemsa stain. For quantification of *L. major* promastigotes, footpads were homogenized, and serial limiting dilutions of the homogenates were plated in 96-well flat-bottomed plates in complete M199 media. Five to 6 days after culture, each well was analyzed under a microscope for the presence or absence of *L. major* to determine the titers. For in vitro experiments, BMDMs were infected with 10, 20, or 50 MOI of *L. major* for 48 hours.

Macrophage differentiation and stimulation. BMDMs were prepared as described previously (17). Briefly, BM cells were grown in L cell–conditioned Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS, 1% nonessential amino acid, and 1% penicillin-streptomycin for 5 days to differentiate into macrophages. BMDMs were counted and seeded at 10^6 cells per well in 12-well cell culture plates on day 5. The next day, BMDMs were primed with LPS (500 ng/ml) for 4 hours, followed by infection with *L. major* for the next 48 hours. In other experiments, BMDMs were infected with the indicated MOI of *L. major*. One hour later, *L. major* was washed with PBS, and the clearance of *L. major* was followed over time using Giemsa staining (48900; Sigma-Aldrich).

Western blot analysis. Samples for immunoblotting were prepared from footpad lysates made in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. Samples were denatured in loading buffer containing SDS and 100 mM DTT and boiled for 5 minutes. SDS-PAGE–separated proteins were transferred to PVDF membranes and immunoblotted with primary antibodies against caspase 1 (AG-20B-0042; Adipogen) and GAPDH (D16H11; Cell Signaling Technology), followed by secondary anti-mouse and anti-rabbit HRP antibodies (Jackson ImmunoResearch Laboratories), as previously described (14).

Cytokine analysis. Footpads were homogenized in 1 ml PBS supplemented with protease inhibitors. The footpad supernatants were then analyzed for the indicated cytokine and chemokine (Figures 3 and 4). For in vitro experiments, cell supernatants were collected at the end of the experiment and analyzed for the indicated cytokines and chemokine. Concentrations of cytokines and chemokines were determined by multiplex ELISA for KC, IL-6, and TNF-α (EMD Millipore) and by classical ELISA for IL-1β (eBioscience) and IL-18 (MBL International).

In vitro restimulation and flow cytometry. Draining popliteal lymph nodes were harvested from mice, and single-cell suspensions were made. Cells were then stimulated with anti-CD3 and anti-CD28 for 4 hours in the presence of monesin. The lymph node cells were then stained with CD4, CD8, IFN-γ, and TNF-α antibodies (eBioscience) and analyzed using the LSR-II flow cytometer (BD Biosciences) and FlowJo software.

In vitro stimulation of splenocytes. Spleens were harvested from naive BALB/c mice, and a single-cell suspension was made. Red blood cells were lysed using ammonium-chloride-potassium lysis buffer. Splenocytes were counted and plated at 2×10^5 cells in a volume of 200 μl in 96-well round-bottomed plates. These splenocytes were stimulated with 1 μg/ml anti-CD3 in the presence or absence of IL-1β (10 ng/ml) and IL-18 (10 ng/ml). In some experiments, IL-18BP (1 μg/ml) was added to neutralize the effects of IL-18. Splenocytes were stimulated for 72 hours, and supernatants were collected and analyzed by ELISA for IL-4 and IFN-γ. For experiments involving transcription factors, splenocytes were stained with CD4 (eBioscience), GATA3 (BioLegend), and cMAF (eBioscience) and analyzed by flow cytometry.

IL-18 neutralization. To neutralize IL-18 in vivo, mice were administered 2 μg IL-18BP (RKQ9Z0M9; Reprokine) i.p. on day –1, day 0, day 1, day 3, day 7, and once a week thereafter.

Statistics. GraphPad Prism 5.0 software was used for data analysis. Data are represented as the mean ± SEM. Statistical significance was determined by the Mann-Whitney *U* test for comparison of 2 groups for in vitro and in vivo analysis and by Dunnett's multiple comparisons test for comparison of multiple groups in vivo. A *P* value of less than 0.05 was considered statistically significant.

Study approval. Animal studies were conducted under protocols approved by the IACUC of St. Jude Children's Research Hospital.

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