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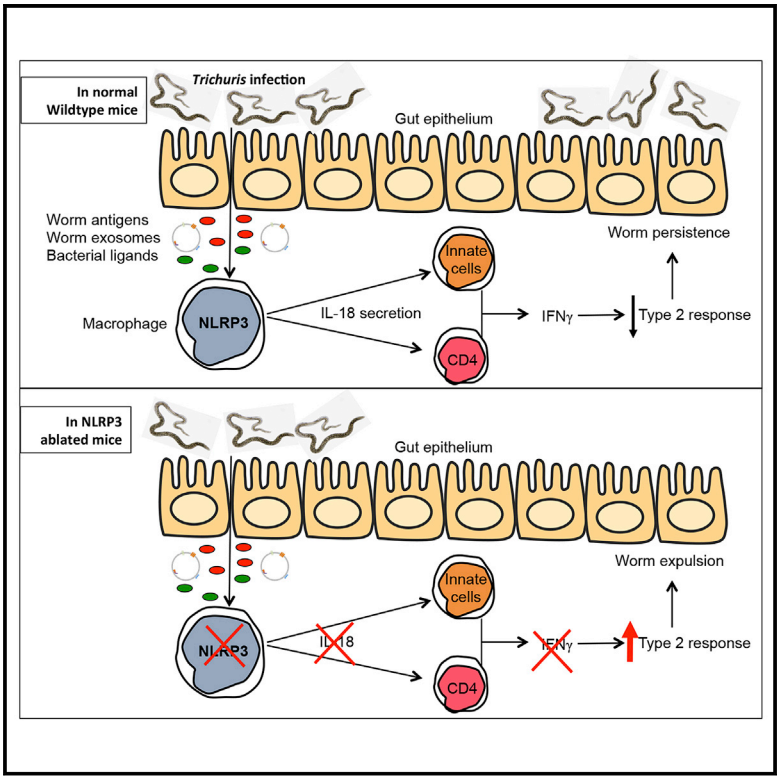
The NLRP3 Inflammasome Suppresses Protective Immunity to Gastrointestinal Helminth Infection

Graphical Abstract



Highlights

- Helminth infections result in elevated IL-18 secretion in mice and humans
- Worms secrete factors that promote NLRP3-dependent IL-18 secretion *in vitro*
- NLRP3 is an endogenous brake on protective type-2-mediated immunity to worms
- NLRP3 limits both innate and adaptive T cell-mediated anti-parasitic immunity

Authors

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In Brief

Inflammasomes can protect us against infections with bacteria and viruses, but Alhallaf et al. now find that these inflammasomes may also actually prevent our immune system from being able to fight parasitic worm infections.

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The NLRP3 Inflammasome Suppresses Protective Immunity to Gastrointestinal Helminth Infection

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SUMMARY

Inflammasomes promote immunity to microbial pathogens by regulating the function of IL-1-family cytokines such as IL-18 and IL-1β. However, the roles for inflammasomes during parasitic helminth infections remain unclear. We demonstrate that mice and humans infected with gastrointestinal nematodes display increased IL-18 secretion, which in *Trichuris*-infected or worm antigen-treated mice and in macrophages co-cultured with *Trichuris* antigens or exosome-like vesicles was dependent on the NLRP3 inflammasome. NLRP3-deficient mice displayed reduced pro-inflammatory type 1 cytokine responses and augmented protective type 2 immunity, which was reversed by IL-18 administration. NLRP3-dependent suppression of immunity partially required $CD4^+$ cells but was apparent even in $Rag1^{-/-}$ mice that lack adaptive immune cells, suggesting that NLRP3 influences both innate and adaptive immunity. These data highlight a role for NLRP3 in limiting protective immunity to helminths, suggesting that targeting the NLRP3 inflammasome may be an approach for limiting the disease burden associated with helminth infections.

INTRODUCTION

The interleukin-1 (IL-1) family of cytokines, in particular, IL-1α, IL-1β, and IL-18, are important for the initiation and amplification of innate and adaptive immune responses and resistance against microbial infections. These cytokines are produced by innate immune cells such as macrophages, dendritic cells, and monocytes, as well as non-hematopoietic cells such as epithelial or endothelial cells (Sims and Smith, 2010). Because of their potent and diverse functions, the biological activity of IL-1 family cytokines must be tightly controlled, since dysregulation of these responses is associated with the detrimental inflammation associated with autoimmune diseases (Dinarello, 2011). Regulation of the activity of many IL-1 cytokines is achieved by inflammasomes; multi-protein immune complexes that control the cleavage and subsequent activation of immature versions of these cytokines by the intracellular cysteine protease caspase-1 (Netea et al., 2015).

Inflammasomes play a critical role in immune defense against small, easily phagocytosed infectious pathogens by promoting type 1 immune responses via secretion of IL-1β and IL-18 (McIntire et al., 2009; Strowig et al., 2012; Arbore et al., 2016). The nod-like receptor (NLR) family, Pyrin Domain Containing 3 (NLRP3) inflammasome is the most well-characterized inflammasome and is important for immunity to diverse pathogens such as bacteria (Broz et al., 2010; Muruve et al., 2008), viruses (Allen et al., 2009; Kamada et al., 2014; Thomas et al., 2009), protozoan parasites (Gorfu et al., 2014; Silva et al., 2013), and fungal pathogens (Hise et al., 2009). Other inflammasomes, such as NLRP1, AIM2, and NLRC4, are similarly important for anti-microbial defense (Ewald et al., 2014; Rathinam et al., 2010; Tomalka et al., 2011). Inflammasomes are also implicated in the immunopathology of a number of inflammatory disorders, including diabetes (Lee et al., 2013), chronic kidney disease and diabetic nephropathy (Vilaysane et al., 2010), and inflammatory bowel diseases (IBDs) (Villani et al., 2009).

There is emerging evidence that, in addition to promoting pro-inflammatory type 1 or type 17 immune responses, inflammasomes can directly or indirectly influence type 2 immune responses. For example, activation of the NLRP3 inflammasome promotes allergic type 2 inflammation in some murine models (Besnard et al., 2011; Ritter et al., 2014), and NLRP3 promotes non-protective type 2 immune responses to infections with the protozoan parasite *Leishmania major* (Gurung et al., 2015). These observations are consistent with a report that NLRP3 can directly promote T-helper type 2 (Th2) cell differentiation by acting as a transcription factor (Bruchard et al., 2015). Other studies suggest that NLRP3 may suppress type 2 immune responses, for example, by inactivation of the alarmin IL-33

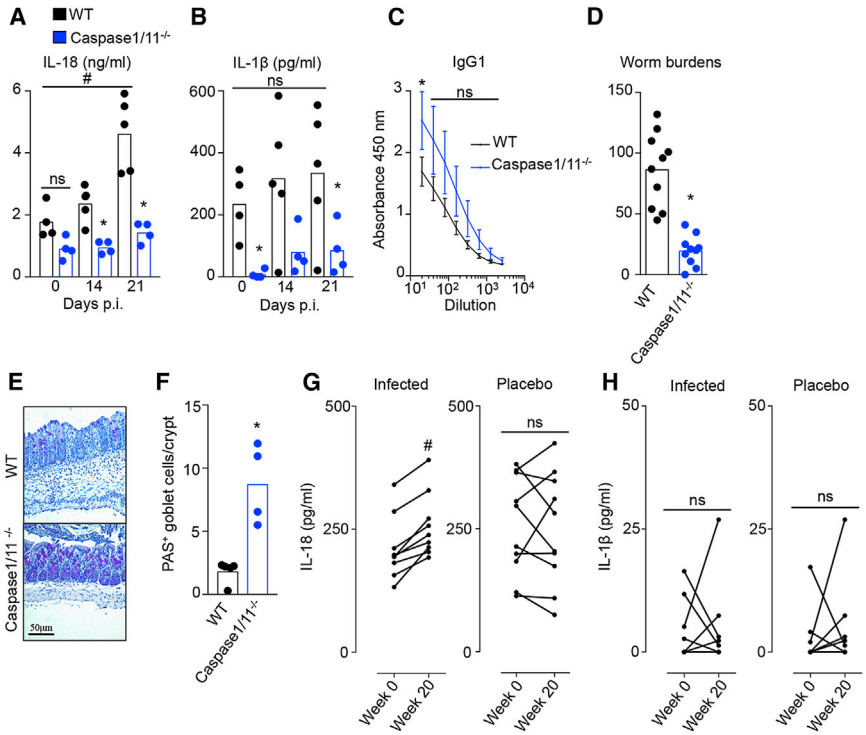

Figure 1. Helminth Infections Promote Caspase 1/11-Dependent IL-18 Expression that Limits Anti-parasitic Immunity

(A and B) C57BL/6 wild-type (WT) and caspase-1/11$^{-/-}$ mice were infected with *Trichuris* and sacrificed at day 14 or day 21 post-infection (p.i.). Serum IL-18 (A) and IL-1β (B) levels are shown.

(C) *Trichuris* antigen-specific IgG1 titers at day 21 p.i.

(D) Cecal worm burdens day 21 p.i., pooled from 2 experiments.

(E) Representative images of PAS/Alcian-blue-stained cecum at day 21 p.i. Scale bar, 50 μm.

(F) Quantification of goblet cell numbers per cecal crypt unit.

Data are expressed as mean with individual data points or mean ± SEM and are representative of 4 experiments (n = 4–5/group).

(G and H) Human clinical trial volunteers with celiac disease were infected with 15 *Necator americanus* larvae (n = 9) or a mock infection (placebo, n = 10). Serum IL-18 (G) and IL-1β (H) levels in individuals pre-infection (week 0) or 20 weeks p.i. *p < 0.05 compared to WT. #p < 0.05 compared to day 0 or week 0.

(Cayrol and Girard, 2009; Moulin et al., 2007), or indirectly via promotion of type 1 biased immune responses (Ritter et al., 2010). Hence, the role of inflammasomes in regulating type 2 immune responses remains incompletely defined.

Type 2 immune responses are associated with protective immunity to helminths, while type 1 responses promote chronic infections and immunopathology, although very little is known about whether inflammasomes control immunity to parasitic helminth infection. The blood fluke *Schistosoma mansoni* secretes proteins that directly activate the NLRP3 inflammasome and thereby promote IL-1β secretion (Ritter et al., 2010; Ferguson et al., 2015), and genetic deficiency of NLRP3 results in suppressed Th1, Th2, and Th17 immune responses and reduced liver pathology (Ritter et al., 2010). Similarly, secreted products from the intestinal nematode *Heligmosomoides polygyrus* activate the NLRP3 inflammasome *in vitro* and increase IL-1β secretion *in vitro* and *in vivo* (Zaiss et al., 2013); however, the *in vivo* role for inflammasomes in regulating anti-parasitic immunity was not determined. Hence, while the NLRP3 inflammasome is a critical determinant of immunity and immunopathology associated with diverse pathogens, a role for any inflammasome complex in controlling type 2 immunity during infections with large metazoan parasites like gastrointestinal helminths is unclear.

In the present study, we describe a role for the NLRP3 inflammasome in suppressing protective innate and adaptive immune responses to infection with the gastrointestinal nematode *Trichuris muris*. *Trichuris* infection resulted in elevated IL-18 secretion in mice, and similar results were seen in human subjects experimentally infected with gastrointestinal nematodes. *In vivo* and *in vitro* murine studies revealed that proteins and exosome-like extracellular vesicles secreted by *Trichuris* enhance NLRP3-dependent IL-18 and IL-1β secretion, in combination with microbial signals such as lipopolysaccharide (LPS). Targeted ablation of NLRP3 caused reduced type 1 cytokine responses, increased type 2 responses, accelerated worm expulsion, and lessened intestinal immunopathology, which was reversed by rIL-18 administration. While the presence of CD4$^+$ cells was important for the ability of NLRP3 to influence anti-parasitic immunity, NLRP3 could suppress protective immunity to *Trichuris* even in the absence of a fully functional adaptive immune system, highlighting a complex mechanism by which the NLRP3 inflammasome regulates inflammation and immunity to parasitic helminth infections.

RESULTS

Helminth Infections Promote Caspase-1-Dependent IL-18 Expression that Limits Anti-parasitic Immunity

To investigate whether helminth infection was associated with inflammasome activation, wild-type (WT) C57BL/6 mice were infected with *T. muris* and IL-18 and IL-1β levels were quantified in the serum at different time points post-infection (p.i.). Caspase-1/caspase-11 double-deficient mice were included as a control due to their impaired ability to be activated by most inflammasome complexes (Schroder and Tschopp, 2010). *Trichuris* infection in WT mice caused significant elevations in serum IL-18 levels at day 21 p.i. (Figure 1A), while IL-1β levels were not significantly increased at any time point (Figure 1B). Caspase-1/11-deficient mice had significantly reduced serum IL-18 and IL-1β

levels compared with WT mice, suggesting that *Trichuris* elicits caspase-1/11 dependent IL-18 expression (Figures 1A and 1B). Following infection, caspase-1/11-deficient mice had increased *Trichuris* antigen (Ag)-specific serum immunoglobulin G1 (IgG1) titers (Figure 1C) and significantly reduced worm burdens at day 21 p.i compared with WT mice (Figure 1D), suggesting improved anti-parasitic immunity. Consistent with an increased anti-parasitic response, caspase-1/11-deficient mice displayed significantly increased numbers of goblet cells in the cecum compared with infected WT mice (Figures 1E and 1F). Together, these data suggest that caspase-1/11-dependent inflammasome activation suppresses immunity to *Trichuris* infection.

We next aimed to determine whether gastrointestinal nematode infection of humans was also associated with inflammasome activation. Nineteen otherwise healthy subjects with celiac disease (on a gluten-free diet) from a non-helminth endemic area (Brisbane, Australia) were either infected with 15 *Necator americanus* (human hookworm) infective third-stage larvae (L3, n = 9) or were treated with a placebo (chili pepper solution, n = 10) and serum was collected at week 0 (day of treatment) and week 20 post-inoculation. Interestingly, helminth-infected humans, but not placebo-treated humans, displayed significant increases in serum IL-18 levels at week 20 p.i. (Figure 1G). IL-1β levels were typically below the level of detection of the assay and not impacted by helminth or placebo treatment (Figure 1H). These data suggest that gastrointestinal helminth infections in humans are also associated with inflammasome activation, specifically resulting in elevated IL-18 production.

Trichuris and Its Secreted Factors Promote NLRP3-Dependent *In Vivo* and *In Vitro* Secretion of IL-18 and IL-1β

Inflammasomes are multi-protein complexes that contain members of the NLR family of proteins (i.e., NLRP3, NLRP1, NLRC4) or non-NLR proteins such as AIM2, which give the various inflammasomes their distinct properties and effector functions (Davis et al., 2011). Since the ligands for NLRP1, NLRC4, and AIM2 have been defined and are not likely to be contained within *Trichuris* (other than DNA), the well-characterized NLRP3 inflammasome was a likely mediator. Hence, we examined the potential role for the NLRP3 inflammasome in *Trichuris*-mediated stimulation of IL-18 and IL-1β secretion. WT and NLRP3$^{-/-}$ mice were infected with *T. muris* and serum IL-18 and IL-1β levels were measured at day 21 p.i. While WT mice exhibited significant increases in serum IL-18 and a trend toward increased IL-1β levels compared to naive mice, NLRP3$^{-/-}$ mice displayed significantly reduced levels of these cytokines (Figure 2A), suggesting that NLRP3 is important for *in vivo* IL-18 and IL-1β secretion. Previous studies have demonstrated that factors excreted and secreted by worms can activate inflammasomes *in vitro* (Ritter et al., 2010; Zaiss et al., 2013; Ferguson et al., 2015). To examine whether products released by *Trichuris* similarly activate inflammasomes, we purified excretory/secretory (ES) products from *Trichuris* adult worms and injected WT mice or NLRP3$^{-/-}$ mice intraperitoneally (i.p.) daily for 6 days and measured circulating IL-18 and IL-1β levels at days 2 and 6. Injection of WT mice with ES products resulted in significantly increased levels of serum IL-18 and IL-1β at 2 days post-injection, with even higher levels at day 6 post-injection (Figure 2B). In contrast, NLRP3$^{-/-}$ mice exhibited no increases in these cytokines following ES injection, suggesting that factors excreted or secreted by *Trichuris* may promote *in vivo* NLRP3 inflammasome-mediated IL-18 and IL-1β secretion.

We next adopted *in vitro* experiments to determine which cell type may be responsive to *Trichuris* ES, and whether ES products are sufficient for NLRP3-dependent IL-18 and IL-1β secretion. Macrophages are key cells responsible for inflammasome activation in response to pathogens (Sims and Smith, 2010), including helminths (Ferguson et al., 2015). Therefore, we generated bone-marrow-derived macrophages from WT mice and cultured them in the presence and absence of *Trichuris* ES products, known inflammasome activators (LPS and nigericin toxin) or culture media alone. Culturing macrophages from WT mice with ES, LPS, or nigericin alone did not cause significant increases in IL-18 or IL-1β secretion compared to media control (Figure 2C). As expected, LPS and nigericin stimulation did significantly increase IL-18 or IL-1β secretion compared to media; however, these levels were increased substantially if *Trichuris* ES was also included in the cultures (Figure 2C). We next aimed to determine whether ES-mediated IL-18 or IL-1β secretion was dependent on NLRP3 and whether a specific fraction of *Trichuris* ES may exert these effects. Parasitic worm ES products are highly complex mixture of proteins, lipids, carbohydrates, microRNAs, and small molecules, and recent studies have highlighted that these can be contained within extracellular vesicles such as exosomes, which facilitate host-pathogen interactions and immunomodulation (Eichenberger et al., 2018). Similar to results from Figure 2C, ES products or purified exosomes alone or in combination with LPS did not significantly increase secretion of IL-18 or IL-1β compared to treatment with media or LPS only (Figure 2D). However, if WT macrophages were stimulated with ES or exosomes in the presence of known inflammasome activators (LPS and nigericin), the levels of both IL-18 and IL-1β rose significantly compared to stimulation with LPS + nigericin alone (Figure 2D). Critically, this synergistic stimulation of IL-18 or IL-1β secretion by *Trichuris* ES products and exosomes was not apparent when cells from NLRP3$^{-/-}$ mice were used, indicating that molecules and extracellular vesicles secreted by *Trichuris* augment NLRP3-dependent secretion of IL-18 or IL-1β, in concert with microbial signals and toxins.

NLRP3 Limits Protective Type 2 Immunity and Promotes Interferon-γ Responses following *Trichuris* Infection

Given the critical role for NLRP3 in *Trichuris*-mediated inflammasome activation and IL-18 secretion, we assessed the *in vivo* role for NLRP3 in protective immunity to *Trichuris* infection. Similar to results seen in caspase 1/11$^{-/-}$ mice (Figure 1), NLRP3$^{-/-}$ mice had significantly increased *Trichuris* Ag-specific serum IgG1 titers (Figure 3A) and reduced worm burdens at day 14, 21, and 35 p.i compared to WT mice (Figure 3B). NLRP3$^{-/-}$ mice also displayed significantly increased frequencies and total numbers of eosinophils in the mesenteric lymph node (mLN) compared to WT mice (Figures 3C and 3D), consistent with an elevated cellular type 2 immune response. Further analysis of

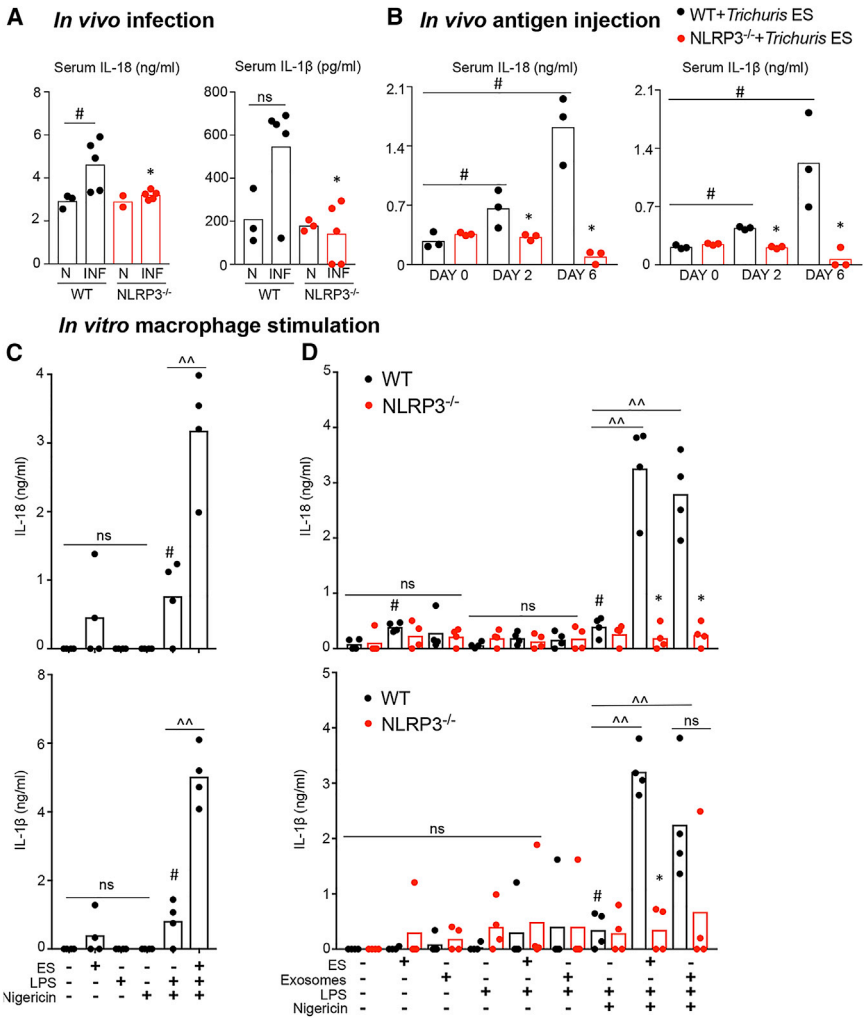

Figure 2. *Trichuris* and Its Secreted Factors Promote *In Vivo* and *In Vitro* NLRP3-Dependent Secretion of IL-18 and IL-1β

(A) WT and NLRP3$^{-/-}$ mice were infected (INF) with *T. muris*, and serum IL-18 and IL-1β levels were determined at day 21 p.i. (n = 3–5 mice/group). N, naive mice.

(B) WT and NLRP3$^{-/-}$ mice were injected intraperitoneally with 50 μg of *Trichuris* excretory/secretory (ES) products and were bled immediately (day 0) or 2 or 6 days post-injection, and serum levels of IL-18 and IL-1β levels were determined (n = 3/group).

(C) Bone-marrow-derived macrophages from WT mice were cultured with combinations of either 25 μg/mL of *Trichuris* ES, 1 μg/mL LPS, or 20 μM nigericin. Plus (+) symbols denote inclusion of reagent; minus (–) symbol denotes media control.

(D) Bone-marrow-derived macrophages from WT or NLRP3$^{-/-}$ mice were cultured with combinations of 25 μg/mL of *Trichuris* ES, 25 μg/mL purified *Trichuris* adult worm exosomes, 1 μg/mL LPS, or 20 μM nigericin (n = 4 wells/treatment). IL-18 and IL-1β were measured in supernatants or sera by ELISA. All results are representative of 3 experiments. *p < 0.05 compared to WT. #p < 0.05 compared to N, DAY 0, or media only control, ^^p < 0.05 compared to LPS + nigericin; ns, not significantly different.

cellular immune responses at the site of infection (cecum) revealed that *Trichuris*-infected NLRP3$^{-/-}$ mice displayed elevated eosinophil frequencies in the cecum compared to WT mice (Figure S1A). Frequencies of other cell types typically associated with type 2 immunity such as basophils (Figure S1B), mast cells (Figure S1C), and macrophages (Figure S1D) were not elevated in the ceca of infected NLRP3$^{-/-}$ mice. The degree of intestinal immunopathology was significantly decreased in NLRP3$^{-/-}$ mice compared to WT mice (Figures 3E and 3F), which corresponded with significantly increased goblet cell responses (Figures 3G and 3H). One of the most well-studied mechanisms by which the NLRP3 inflammasome is activated is by engagement of the P2X$_7$R by extracellular ATP (Riteau et al., 2010). However, mice deficient in P2X$_7$R do not display the same enhanced immunity phenotype as NLRP3$^{-/-}$ mice during *Trichuris* infection, suggesting that a different pathway to NLRP3 inflammasome activation may be more important (Figure S2). Together, these data suggest that the NLRP3 inflammasome inhibits protective type-2-associated immune responses and promotes immunopathology following *Trichuris* infections, likely via a P2X$_7$R-independent mechanism.

To investigate the immunological mechanisms by which NLRP3 controls immunity to *Trichuris* infections, we assessed antigen-specific cytokine responses in the mLN of WT and NLRP3$^{-/-}$ mice and cytokine responses in the colon. NLRP3$^{-/-}$ mice displayed increased IL-4 and IL-13 expression by Ag-stimulated mLN cells compared to WT mice (Figure 3I), consistent with an augmented type 2 immune response in the intestinal draining lymph nodes. This was associated with increased levels of IL-13 and thymic stromal lymphopoietin (TSLP) in the colon tissue (Figure S3A). Levels of the type 2-associated cytokines IL-25 and IL-33 in the colon were not significantly elevated in infected NLRP3$^{-/-}$ mice, nor were there increases in innate lymphoid cell type 2 (ILC2) responses in either the mLN or colonic lamina propria, since frequencies of CD3$^-$ CD19$^-$ CD90$^+$ GATA3$^+$ cells in these tissues were not significantly different to those in infected WT mice (Figure S3B). Together, these data suggest that NLRP3 limits type 2 cytokine responses to *Trichuris* infection. The augmented type-2-associated immune responses observed in NLRP3$^{-/-}$ mice was accompanied by a significant reduction in type 1 cytokine responses, measured by interferon (IFN)-γ expression (Figure 3I). Given that IFN-γ is associated with the development of chronic infections and suppression of protective type 2 immune responses to helminths, including *Trichuris* (Else et al., 1994; Coomes et al., 2015), we investigated the cellular sources of IFN-γ. While WT mice displayed significant increases in frequencies (Figure 3J) and total numbers (Figure 3K) of CD4$^+$

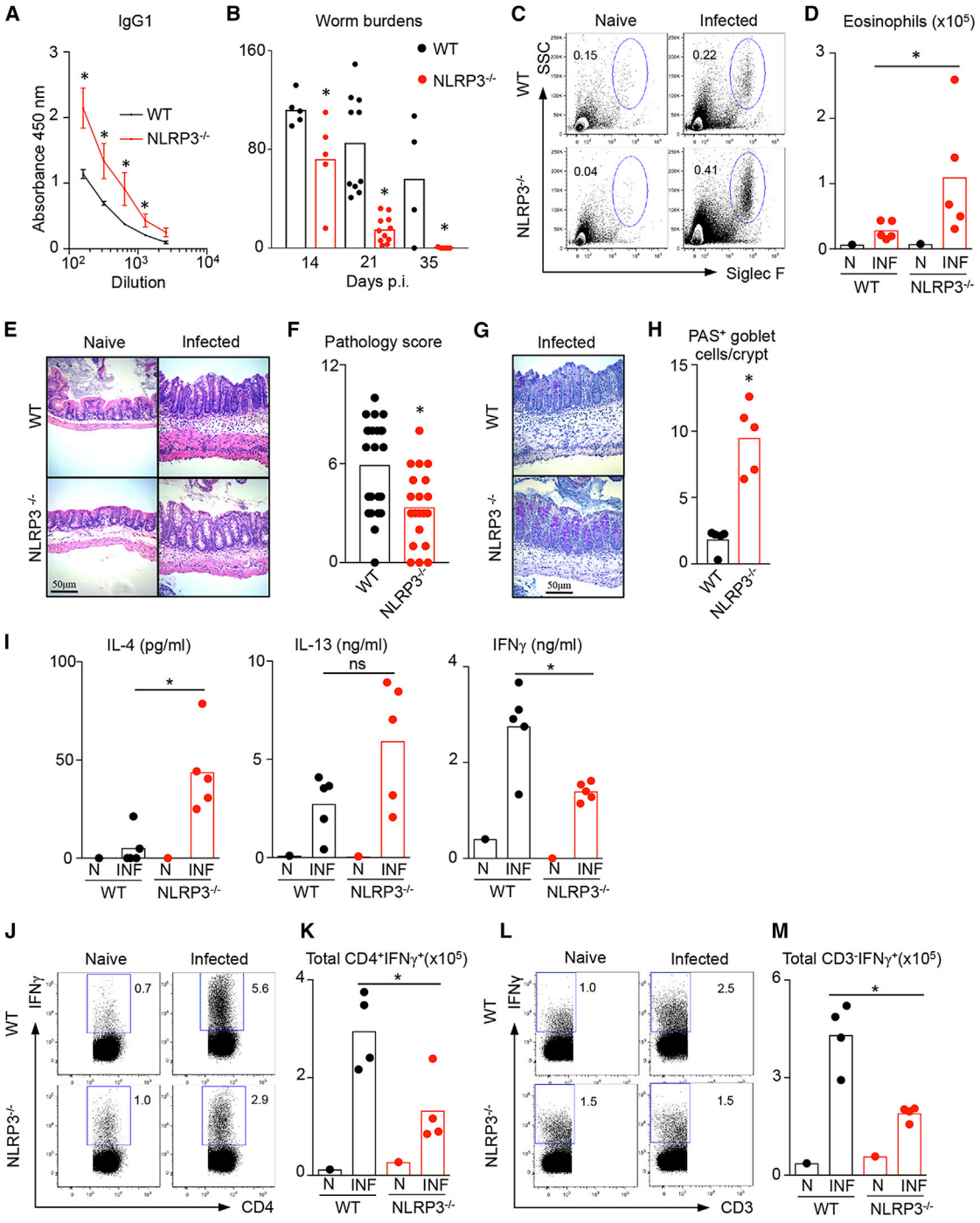

Figure 3. NLRP3 Limits Protective Type 2 Immunity and Promotes IFN-γ Responses following *Trichuris* Infection

WT and NLRP3$^{-/-}$ mice were infected with *T. muris* and sacrificed at day 14, day 21, or day 35 p.i. N, naive mice.

(A) *Trichuris* antigen-specific IgG1 titers at day 21 p.i.

(B) Cecal worm burdens at day 14, day 21, and day 35 p.i. pooled from 2 experiments.

(C) Representative plots displaying Siglec F^{+} eosinophils frequencies in the mesenteric lymph nodes (mLN) at day 21 p.i.

(D) Total mLN eosinophils.

(E) Representative images of H&E-stained cecum at day 21 p.i. Scale bar, 50 μm.

(F) Blinded scores of cecal pathology, pooled from 4 experiments.

(G) Representative images of PAS/Alcian-blue-stained cecum tissue at day 21 p.i.

(H) Quantification of goblet cell numbers per cecal crypt unit.

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T cells that express IFN-γ, these responses were significantly diminished in NLRP3$^{-/-}$ mice. Similarly, IFN-γ responses by non-T cells (CD3^{-} cells) were also significantly reduced in the absence of NLRP3 (Figures 3L and 3M), together suggesting that NLRP3 promotes innate and adaptive IFN-γ responses following helminth infection, potentially representing a mechanism by which inflammasomes limit protective type 2 immunity to helminths.

Therapeutic NLRP3 Inhibition Suppresses Type 1 Immune Responses and Promotes Resistance to *Trichuris* Infection

To confirm the role for NLRP3 in regulating immunity to *Trichuris* infection, and to avoid potential impacts of developmental abnormalities in gene knockout mice, we next targeted the NLRP3 inflammasome therapeutically with a selective chemical inhibitor MCC950 (Coll et al., 2015). C57BL/6 WT mice were i.p. treated daily with either PBS vehicle control or 20 mg/kg/day of MCC950 starting on the initial day of *Trichuris* infection. Treatment with MCC950 lowered serum IL-18 and IL-1β levels in *Trichuris*-infected mice, but these reductions were not statistically significant (Figures 4A and 4B). MCC950 treatment significantly increased serum titers of *Trichuris* Ag-specific IgG1 (Figure 4C), significantly lowered worm burdens at day 21 p.i. (Figure 4D) and resulted in a non-significant trend toward increased mLN eosinophil numbers compared to the PBS control-treated mice (Figure 4E), consistent with enhanced anti-parasitic responses. *Trichuris*-infected mice treated with MCC950 displayed evidence of decreased intestinal inflammation compared to PBS control mice; however, blinded pathology scores were not significantly reduced (Figures 4F and 4G). MCC950 treatment resulted in elevated *Trichuris* Ag-specific type 2 cytokine responses in the mLN and concurrent significant reductions in IFN-γ responses (Figure 4H). Analysis of the cellular sources of IFN-γ revealed that IFN-γ responses by CD4^{+} CD3^{+} T cells and non-T cells (CD3^{-} cells) were both significantly reduced by MCC950 treatment (Figures 4I and 4J). Together, these data suggest that targeting the NLRP3 inflammasome therapeutically with a chemical inhibitor has a similar phenotypic effect to genetic NLRP3 deficiency.

Exogenous Delivery of rIL-18 Reverses the Enhanced Anti-parasitic Immunity of NLRP3-Deficient Mice

Activation of the NLRP3 inflammasome can have multiple downstream effects, including the release of mature IL-18, IL-1β, and regulation of IL-33 secretion (Strowig et al., 2012). Data thus far have indicated that *Trichuris* infection increases IL-18 expression, and ablation of NLRP3 diminishes this response, suggesting that NLRP3 may control immunity or susceptibility to this parasite by regulating IL-18. To more precisely define the molecular mechanism of how NLRP3 controls immunity to *Trichuris*, we examined whether delivery of exogenous IL-18 to NLRP3-deficient mice could reverse the robust immunity of NLRP3-deficient mice. NLRP3$^{-/-}$ mice were either treated every 3 days with PBS vehicle or recombinant murine IL-18 (200 ng/mouse i.p.) from days 7–19 post-*Trichuris* infection. C57BL/6 WT mice were included as a control and received PBS only. Results demonstrated that, as expected, the levels of IL-18 in the serum were increased in NLRP3$^{-/-}$ mice treated with rIL-18 (Figure 5A). Furthermore, while NLRP3$^{-/-}$ mice treated with PBS exhibited a trend toward increases in *Trichuris* Ag-specific serum IgG1 titers (Figure 5B) and significantly increased eosinophil numbers in the mLN (Figure 5C), rIL-18 treatment of NLRP3$^{-/-}$ mice normalized these responses to WT + PBS levels (Figures 5B and 5C). Similarly, rIL-18 treatment of NLRP3$^{-/-}$ mice resulted in significantly higher worm burdens (Figure 5D) and increased CD4^{+} IFN-γ responses (Figure 5E) and a trend toward increased CD3^{-} IFN-γ responses (Figure 5F) compared to NLRP3$^{-/-}$ mice treated with PBS. Last, treatment of NLRP3$^{-/-}$ mice with rIL-18 led to increased inflammation in the cecum (Figure 5G) and a significant increase in pathology score compared to PBS-treated mice (Figure 5H). Together, these data suggest that NLRP3 may limit immunity to *Trichuris* by enhancing IL-18 production, which promotes type 1 cytokine responses and resultant immunopathology associated with *Trichuris* infections.

NLRP3 Partially Requires CD4^{+} T Cells to Limit Protective Immunity to *T. muris* Infection

Inflammasomes are critical mediators of innate immunity to infections (Strowig et al., 2012); however, they can also influence the development of adaptive immunity via regulation of T cell responses (Ciraci et al., 2012). Further, CD4^{+} T cells can also express NLRP3 inflammasomes, which can promote Th1 cell differentiation (Arbore et al., 2016). Immunity to *Trichuris* is dependent on the generation of adaptive CD4^{+} T cell responses, hence, to assess whether NLRP3-dependent regulation of immunity to *Trichuris* is dependent on CD4^{+} T cells, WT or NLRP3$^{-/-}$ mice were treated every 3 days with either 0.5 mg of a neutralizing anti-CD4 mAb or a control rat IgG following *Trichuris* infection. As expected, treatment with anti-CD4 mAb was able to effectively deplete CD3^{+} CD4^{+} cells in infected mice (Figure 6A). While NLRP3$^{-/-}$ mice treated with control Ig exhibited characteristic significant increases in total mLN eosinophils compared to WT mice (Figure 6B), these responses were significantly diminished when CD4^{+} cells were ablated. Anti-CD4 mAb treatment caused a significant increase in worm burdens in NLRP3$^{-/-}$ mice at day 21 p.i. compared to control IgG-treated mice (Figure 6C), which corresponded with significantly increased cecal pathology (Figures 6D and 6F) and significantly reduced presence of goblet cells (Figures 6E and 6G). Together, these data suggest that CD4^{+} cells are partially required for the ability of NLRP3 to suppress immunity to *Trichuris* infection.

(I) *Trichuris* antigen-specific IL-4, IL-13, and IFN-γ production by restimulated mLN cells.
(J) Frequencies of CD3^{+} CD4^{+} IFN-γ^{+} cells in the mLN following *ex vivo* stimulation.
(K) Total mLN CD4^{+} IFN-γ^{+} cells.
(L) Frequencies of CD3^{-} IFN-γ^{+} cells in the mLN.
(M) Total mLN CD3 IFN-γ^{+} cells. Data are expressed as mean with individual data points, or mean ± SEM, and are representative of 7 experiments where n = 4–5/group. *p < 0.05 compared to WT; ns, not significantly different.

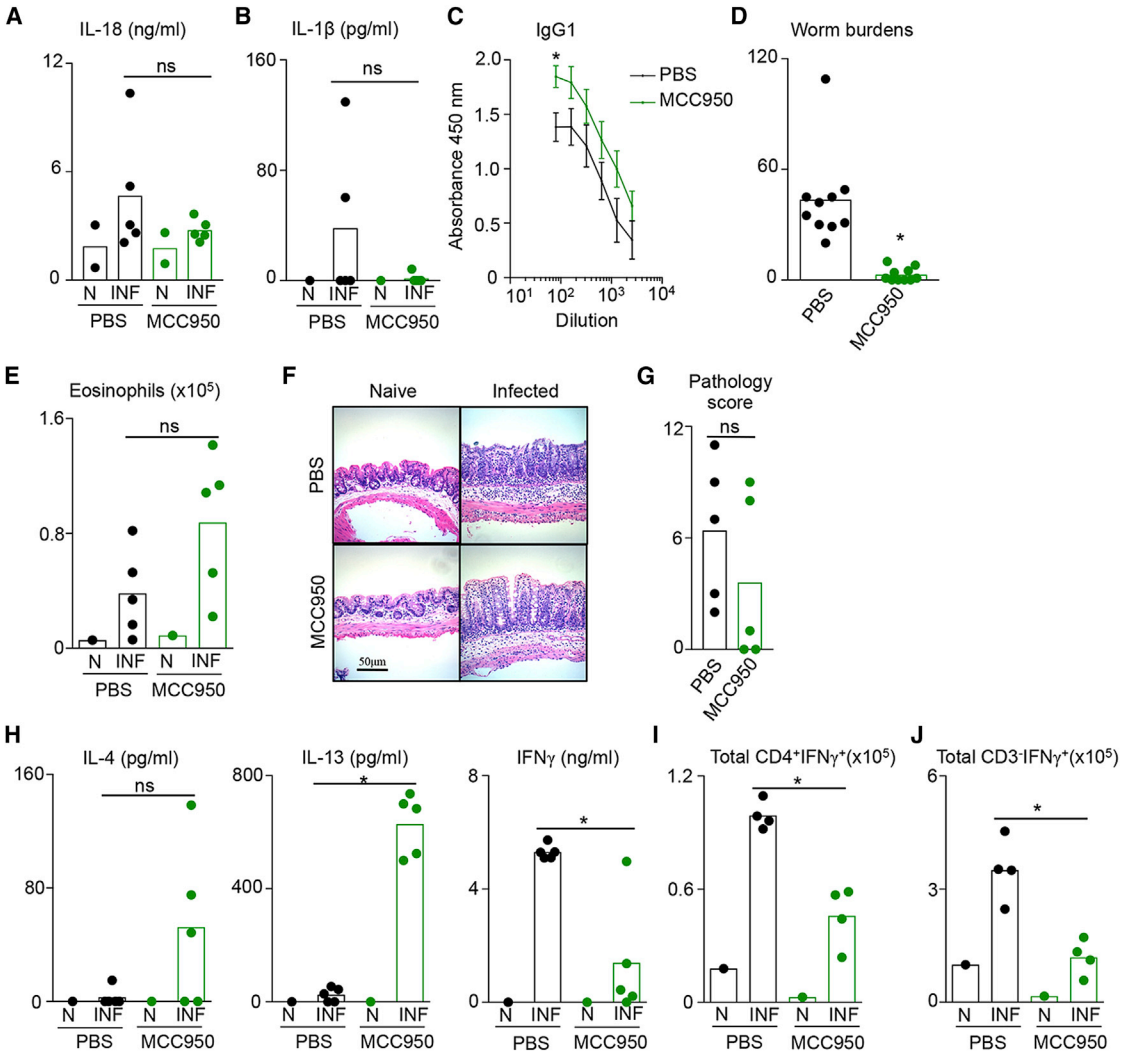

Figure 4. Therapeutic NLRP3 Inhibition Suppresses Type 1 Immune Responses and Promotes Resistance to *Trichuris* Infection

(A–C) C57BL/6 WT mice were treated intraperitoneally (i.p.) daily with either PBS or 20 mg/kg/day of MCC950 following *T. muris* infection (INF) and sacrificed at day 21 p.i. Serum IL-18 (A) and IL-1β (B) levels and *Trichuris* antigen-specific serum IgG1 titers (C) are shown. N, naive mice.

(D) Worm burdens, pooled from 2 experiments.

(E) Total mesenteric lymph node (mLN) eosinophils.

(F) Representative images of H&E-stained cecum tissue. Scale bar, 50 μm.

(G) Blinded scores of cecal pathology.

(H) *Trichuris* antigen-specific IL-4, IL-13, and IFN-γ secretion by restimulated mLN cells.

(I) Total mLN $CD4^+$ IFN-γ^+ cells.

(J) Total mLN $CD3^-$ IFN-γ^+ cells. Data are expressed as mean with individual data points or mean ± SEM and are representative of 4 experiments, n = 4–5 infected or 1–2 naive mice/group. *$p < 0.05$ compared to PBS control; ns, not significantly different.

NLRP3 Can Regulate Immunity to Trichuris Independent of the Adaptive Immune System

To further define the cellular mechanisms of NLRP3-dependent regulation of immunity to infection, we aimed to assess whether NLRP3 could influence innate immunity to *Trichuris*. C57BL/6 $Rag1^{-/-}$ mice (which do not possess a functional adaptive immune system) were infected with *Trichuris* treated daily with either PBS or 20 mg/kg/day i.p. of MCC950. C57BL/6 WT mice were included as a control for assessment of worm burdens. Critically, MCC950 treatment was able to significantly reduce worm burdens in $Rag1^{-/-}$ mice at day 21 p.i. compared to PBS-treated mice (Figure 7A), which corresponded with significantly increased eosinophil numbers in the mLN (Figure 7B). Analysis of cytokine responses in the mLN revealed that while $Rag1^{-/-}$ mice treated with PBS displayed increased IFN-γ responses within cells that express CD90 following infection, mice that received the MCC950 had a reduced IFN-γ response in these cells (Figure 7C). Further analysis of this

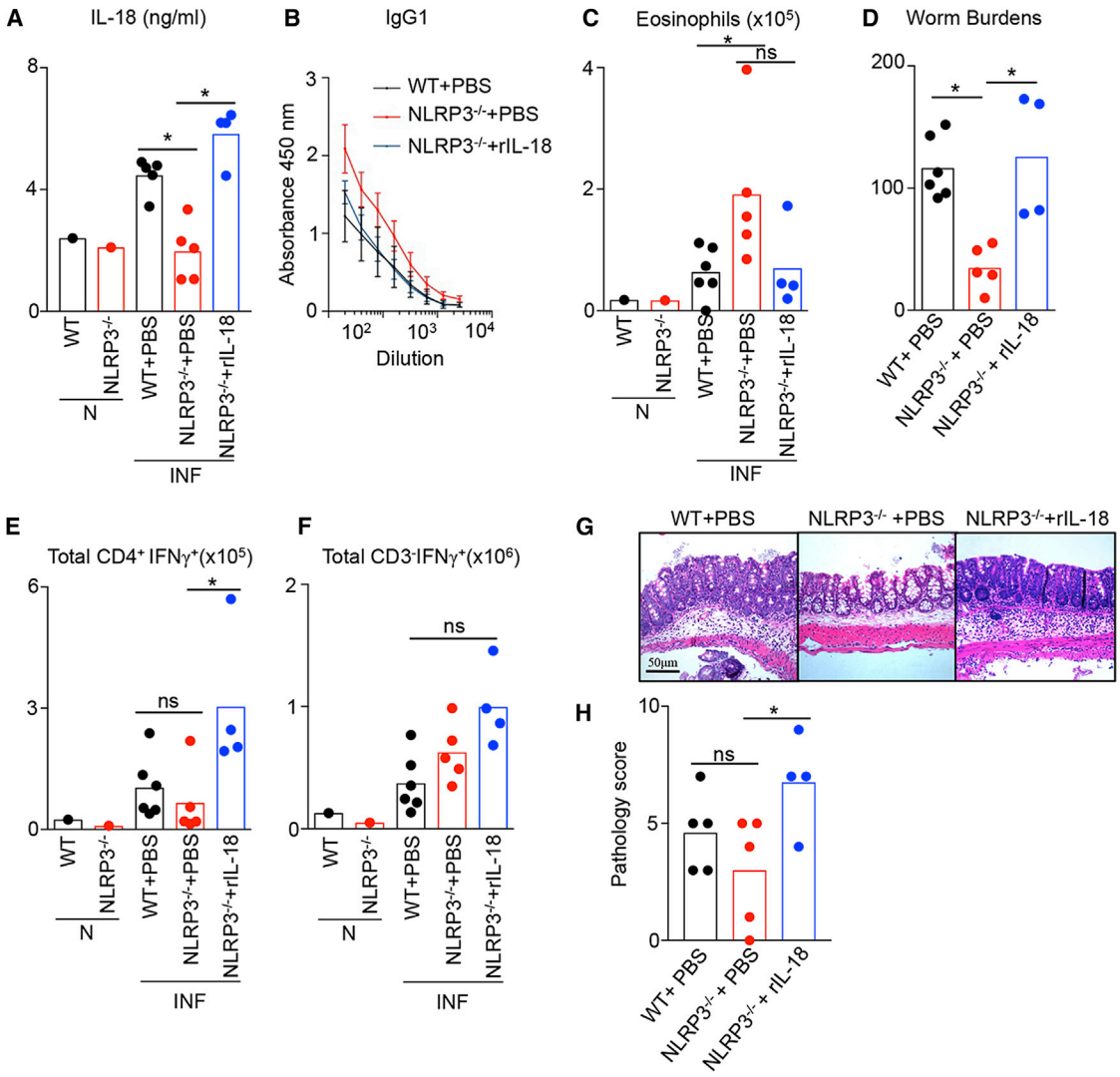

Figure 5. Exogenous Delivery of rIL-18 Reverses the Enhanced Anti-parasitic Immunity of NLRP3-Deficient Mice

NLRP3$^{-/-}$ mice were either daily treated with PBS vehicle or recombinant murine IL-18 (200 ng/mouse i.p.) from days 7–19 post-*Trichuris* infection (INF) and were sacrificed at day 21 p.i. WT control mice received PBS only.

(A) Serum IL-18 levels.

(B) *Trichuris* antigen-specific serum IgG1 titers.

(C) Total mLN eosinophils. N, naive mice.

(D) Worm burdens.

(E) Total CD3$^+$ CD4$^+$ IFN-γ^+ cells in the mesenteric lymph node (mLN).

(F) Total mLN CD3$^-$ IFN-γ^+ cells.

(G) Representative images of H&E-stained cecum tissue from infected mice. Scale bar, 50 μm.

(H) Blinded scores of cecal pathology. Data are expressed as mean with individual data points or mean ± SEM and are representative of 3 experiments, n = 4–6 mice/group. *p < 0.05; ns, not significantly different.

cell population demonstrated that IFN-γ-expressing CD90$^+$ cells following *Trichuris* infection co-expressed the ILC markers Sca-1 and CD127 as well as T-bet (Figure 7D), a phenotype consistent with ILC type 1 cells (ILC1). Histological analyses demonstrated that, while MCC950 treatment did not result in significant reductions in immunopathology in the cecum (Figures 7E and 7G), there were significant increases in goblet cell responses (Figures 7F and 7H). These data suggest that the NLRP3 inflammasome can influence innate immunity to *Trichuris* infection, potentially acting via regulation of the function of innate cells such as ILC1s, and, when taken into consideration with results that CD4$^+$ T cells are also important for NLRP3-dependent immune regulation (Figure 6), imply that the NLRP3 inflammasome can influence elements of both the innate and adaptive immune system to limit protective immunity to helminths.

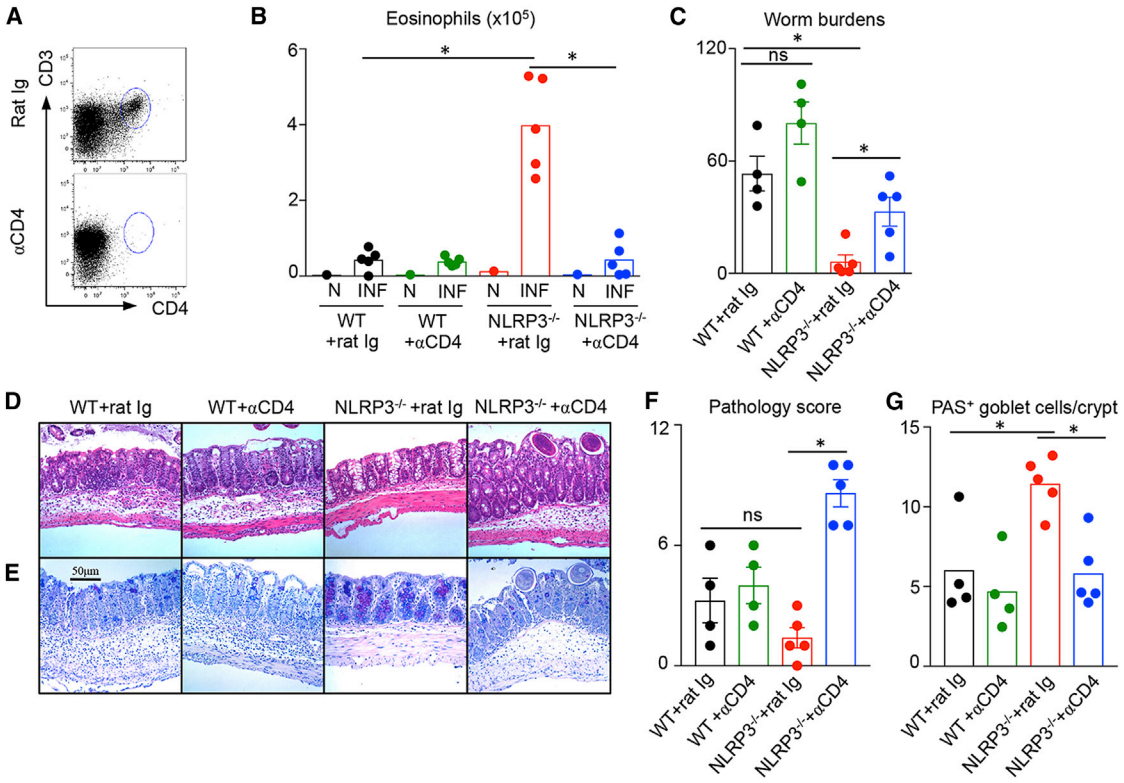

Figure 6. NLRP3 Partially Requires CD4⁺ T Cells to Limit Protective Immunity to *T. muris* Infection

WT or NLRP3$^{-/-}$ mice were treated every 3 days with either 0.5 mg of a neutralizing anti-CD4 mAb or a control rat IgG following *T. muris* infection and were sacrificed at day 21 p.i.

(A) Representative plots demonstrating successful depletion of CD3⁺ CD4⁺ cells in mesenteric lymph nodes (mLN) of infected mice following anti-CD4 treatment.

(B) Total mLN eosinophils.

(C) Worm burdens.

(D) Representative images of H&E-stained cecum tissue.

(E) Representative images of PAS/Alcian-blue-stained cecum tissue.

(F) Blinded scores of cecal pathology.

(G) Blinded quantification of goblet cell numbers per cecal crypt unit. Scale bar, 50 μm. Data are expressed as mean with individual data points and are representative of 2 experiments, n = 4–5 mice/group. *p < 0.05; ns, not significantly different.

DISCUSSION

Parasitic helminth infections are among the most common and debilitating causes of chronic disease in humans, infecting over a billion people worldwide. However, there are no vaccines that can protect humans against infection, owing in part to the complexity of helminth life cycles, the immune-suppressive capacity of helminth antigens, and our relative lack of understanding of how protective type 2 immune responses are initiated and regulated. Inflammasomes are critical regulators of immunity to pathogens, yet their roles in immunity to parasitic helminths have been largely unexplored. The present study has identified a role for the NLRP3 inflammasome in limiting both innate and adaptive immunity of mice to a gastrointestinal helminth, via regulation of IL-18 expression. We demonstrate that chronic helminth infections of humans are similarly associated with elevated IL-18 expression. Critically, targeting this pathway therapeutically with a chemical inhibitor was able to accelerate type 2 immune-mediated clearance of the parasite, shedding light on how innate immune receptors control immunity to helminths and potentially identifying a strategy for limiting the disease burden in helminth-infected animal or human populations.

NLRP3 has been implicated in promoting type 1 immune-mediated protection against pathogens and detrimental inflammation in some disease settings (Silva et al., 2013; Thomas et al., 2009). In addition, recent studies have highlighted that NLRP3 can have important roles in promoting type 2 immune responses (Bruchard et al., 2015; Gurung et al., 2015). Our findings herein are consistent with a role for NLRP3 in promoting non-protective type 1 immune responses to *Trichuris*, potentially by eliciting IL-18 secretion, a cytokine also known as "IFN-γ-inducing factor" (Micallef et al., 1996). IL-18 is a pleiotropic cytokine that can promote either a type 1 or type 2 response, depending on the nature of the cytokine milieu, infectious stimuli, and genetic background of the host (Xu et al., 2000). For instance, IL-18 can induce type 1 immune responses and resistance to one species of the *Leishmania* parasite (Li et al., 2004) but induces non-protective type 2 responses to another species

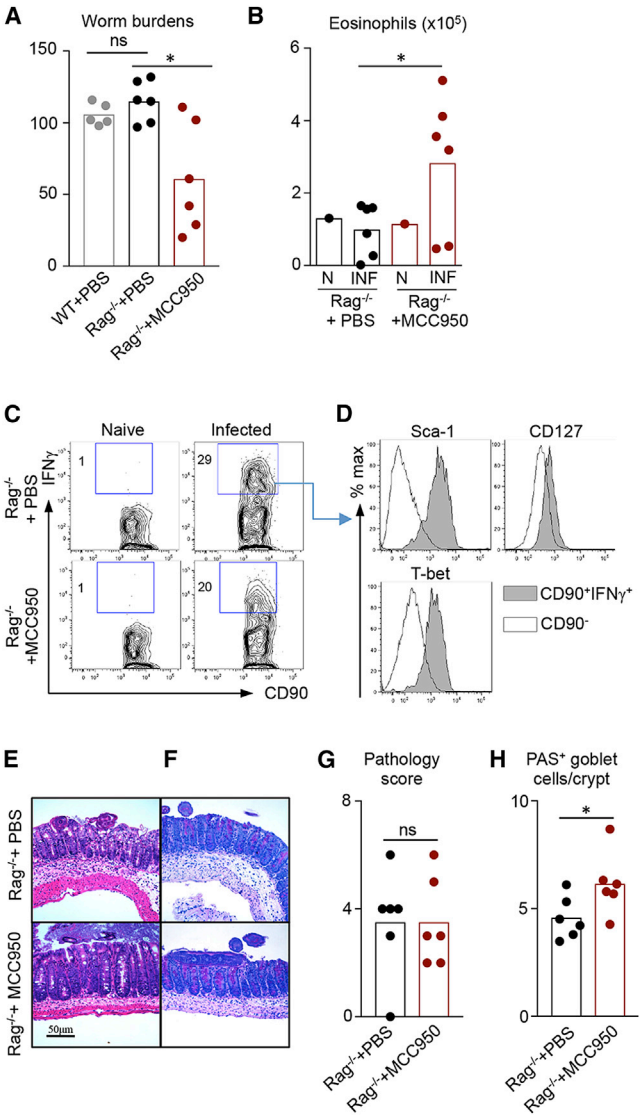

Figure 7. NLRP3 Can Regulate Immunity to *T. muris* Independent of the Adaptive Immune System

C57BL/6 Rag1$^{-/-}$ mice were treated daily with either PBS or 20 mg/kg/day i.p. of the NLRP3 inhibitor, MCC950 following Trichuris infection and were sacrificed at day 21 p.i. C57BL/6 WT mice received PBS only and were only used as a control for worm burden.

(A) Worm burdens.

(B) Total mLN eosinophils.

(C) Frequencies of CD90$^+$ IFN-γ^+ cells in the mLN.

(D) IFN-γ-expressing CD90$^+$ cells (shaded histograms) were gated and analyzed for co-expression of ILC markers Sca-1, CD127, and T-bet. Clear histograms represent gated CD90$^-$ cells from the same sample.

(E) Representative images of H&E-stained cecum tissue.

(F) Representative images of PAS/Alcian-blue-stained cecum tissue.

(G) Blinded scores of cecal pathology.

(H) Blinded quantification of goblet cell numbers per cecal crypt unit. Scale bar, 50 μm. Data are expressed as mean with individual data points and are representative of 3 experiments, n = 5–6 mice/group. *$p < 0.05$ compared to NLRP3$^{-/-}$ + rat Ig. ns, not significantly different.

(Bryson et al., 2008). Previous studies have demonstrated that IL-18 is a critical regulator of immunity to *Trichuris*, with differences in the relative role depending on the inflammatory context. While one report highlights how IL-18 promotes type 2 responses when IFN-γ is absent (Liu et al., 2006), another report implicates IL-18 in suppressing type 2 cytokine responses to *Trichuris* (Helmby et al., 2001), which is more consistent with our findings in NLRP3$^{-/-}$ mice. Hence it is likely that, in the context of the present study, lowered IL-18 expression in the absence of NLRP3 may contribute to the elevated type 2 responses and diminished type 1 responses following *Trichuris* infection. However, it remains unclear whether elevations in type 2 immunity were a consequence, or a cause, of reductions in Th1 cell or ILC1 responses observed in NLRP3$^{-/-}$ mice (i.e., cross-regulation of type 1 versus type 2 responses), or whether this was a direct effect of IL-18 or other factors.

Our data are supportive for a central role for NLRP3-dependent IL-18 in regulating immunity and inflammation following *Trichuris* infection, but we have also demonstrated that IL-1β levels are similarly regulated by NLRP3, MCC950, and caspase-1/11, raising the question as to the role for IL-1β in this system. While IL-1β can have a role in suppressing protective immunity to other helminth species (Zaiss et al., 2013), this contrasts with what is observed during *Trichuris* infections, where IL-1/IL-1R interactions promote type 2 immunity (Helmby and Grencis, 2004; Humphreys and Grencis, 2009). IL-1β also has an emerging role in stimulating ILC2 responses (Ohne et al., 2016) suggesting that, at least in the context of *Trichuris* infection, defective IL-1β production in NLRP3$^{-/-}$ mice would be unlikely to be responsible for the increased type 2 immune responses we observed in the present study.

The NLRP3 inflammasome can be activated by a wide variety of stimuli, ranging from pathogen-associated molecular patterns, danger signals (ATP, ADP, DNA), toxins, and crystals (Schroder and Tschopp, 2010). We found that *Trichuris* adult worms release factors that can support NLRP3-dependent IL-18 and IL-1β secretion *in vivo* and in cultured macrophages. Interestingly, worm secreted factors do not appear to induce NLRP3-dependent IL-1β and IL-18 secretion alone, since signals from known inflammasome activators (e.g., LPS and nigericin) were also required. It is therefore unclear whether worm-derived molecules are operating as a "first signal" to promote pro-IL-1β/pro-IL-18 generation or as a "second signal" to activate the NLRP3 inflammasome and promote cytokine maturation and release, or both. We also cannot definitively say that *Trichuris* and its secretions definitively elicit *in vivo* IL-18 secretion, since it remains possible that IL-18 secretion is diminished in the absence of NLRP3 due to an inability to respond to the microbiota. Regardless, our results are consistent with the premise that worm-derived factors may be able to augment NLRP3 activation, provided that signals from commensal microbes are also present.

While our study has not pinpointed the precise worm-derived molecule(s) responsible for enhancing NLRP3-dependent IL-1β and IL-18 secretion, as has been demonstrated for schistosomes (Ritter et al., 2010), we found that purified exosome-like extracellular vesicles released from the worm can mediate these effects. Helminth-derived exosomes have been recently

implicated in immune modulation (Buck et al., 2014) and pathogenesis (Chaiyadet et al., 2015) by parasitic helminths. *T. muris* exosomes contain hundreds of proteins, micro-RNAs, and mRNAs that are postulated to mediate host-pathogen interactions (Eichenberger et al., 2018), and the present study suggests that some of these factors may regulate activation of inflammasomes by host cells such as macrophages. Given that activation of this pathway is associated with greater parasite persistence in the intestine, it is possible that parasites such as *Trichuris* may have evolved strategies to selectively target inflammasomes to regulate localized inflammation and enhance their survival within a host (Hewitson et al., 2009). In addition to worm-secreted factors and signals from commensal microbes, there are likely other mechanisms by which a worm infection could elicit inflammasome activation. For instance, helminth infections can induce substantial tissue damage during colonization and feeding that can release danger signals such as ATP, which is a well-described activator of the NLRP3 inflammasome (Riteau et al., 2010). However, our finding that the ATP-$P2X_7R$ pathway does not appear to control *Trichuris* infections in the same way as NLRP3 suggests that other activation pathways are likely to play more substantial roles. Other danger signals such as ADP can also activate inflammasomes (Baron et al., 2015) and can promote immunity to helminths by interacting with A2B receptors (Patel et al., 2014).

While inflammasomes are critical regulators of early innate responses to infection, by permitting rapid maturation and release of inflammatory cytokines, there is substantial evidence that inflammasomes are involved in shaping adaptive immunity (Ciraci et al., 2012). Our results are consistent with multiple roles for the NLRP3 inflammasome in controlling immunity to gastrointestinal helminths. $NLRP3^{-/-}$ mice display enhanced parasite antigen-specific humoral and type 2 cytokine responses and the presence of $CD4^+$ T cells is important for the optimal ability of NLRP3 to suppress protective immunity to *Trichuris*, demonstrating that NLRP3 regulates adaptive immunity. Our results could not distinguish a potential role for $CD4^+$ T cell-intrinsic NLRP3 activation in regulating immunity to *Trichuris*, as has been shown in other models of infection and inflammation (Arbore et al., 2016); hence it remains possible that CD4 cells could either be an upstream mediator (inflammasome activation) or downstream mediator (Th2-driven inflammation) of immunity. However, the fact that NLRP3 could suppress immunity to *Trichuris* in the absence of functional T cells and B cells (in $Rag1^{-/-}$ mice) highlights the importance of NLRP3 in innate immunity. The precise mechanisms by which NLRP3 suppresses the innate response to *Trichuris* was not defined, but the selective elevation in eosinophil numbers in lymph nodes and intestinal tissues, increased goblet cell responses, and the modulation of ILC1 function that arose following NLRP3 inhibition suggests that there may be multiple mechanisms involved. Previous reports have shown that NLRP3-dependent IL-1β negatively regulates immunity to different helminth species by suppressing IL-25 and IL-33 (Zaiss et al., 2013), factors that are known to promote ILC2 responses. However, none of these factors were elevated in $NLRP3^{-/-}$ mice in the present study, suggesting that other mechanisms may promote type 2 immunity in the context of *Trichuris* infection. Interestingly, TSLP expression was elevated in infected $NLRP3^{-/-}$ mice, and TSLP is strongly associated with type 2 immune responses and protective immunity to helminths, including *Trichuris* (Taylor et al., 2009), via associations with a range of accessory cells including dendritic cells, basophils, ILCs, and mast cells. It is also possible that the absence of NLRP3-dependent cell death or pyroptosis in NLRP3-deficient mice may result in increased survival of innate immune cells (McIntire et al., 2009), which could provide increased immunity to parasitic helminth infection. Further, NLRP3 inhibition may influence the functions of non-hematopoietic cells in the intestine that promote worm expulsion, including epithelial cell turnover, goblet cell expansion, and mucus production and smooth muscle hyper-contractility.

Our observations that humans infected with a gastrointestinal nematode exhibit elevated serum IL-18 levels are consistent with activation of inflammasomes. Hence, it is conceivable that targeting the NLRP3 pathway in helminth-infected humans using MCC950 may be a rational approach for boosting type 2 immunity to helminths, lowering worm burdens and limiting the health problems these infections cause in the developing world. However, people living in helminth endemic regions are often exposed to co-infections with bacterial, viral, and parasitic pathogens such as tuberculosis, HIV, and malaria, and previous studies have demonstrated a protective role for NLRP3 in these infection models (Dorhoi et al., 2012; Shio et al., 2009). Hence, caution would be warranted to prevent such therapeutic strategies from having unwanted effects of reduced ability to fight other infections. Nevertheless, administering therapies that enhance a patient's natural ability to mount protective type 2 immune responses would have benefits over the use of anthelmintic drugs alone; for example, NLRP3 inhibition could promote the development of more robust parasite-specific memory Th2 cells and IgG1 responses that could protect an individual from reinfection. In conclusion, we have provided information on how the NLRP3 inflammasome regulates immunity and inflammation in helminth infections. Further work is necessary to define the role of this pathway in immunity to other helminth species and the roles for other inflammasomes (e.g., NLRP1, AIM2, NLRP6, NLRC4) in regulation of type 2 immune responses.

EXPERIMENTAL PROCEDURES

Mice and Treatments

Caspase-1/11 double-deficient, NLRP3-deficient, $P2X_7R$-deficient, and Rag-1-deficient mice, all on C57BL/6 genetic background, and C57BL/6 WT mice were bred and maintained at James Cook University (JCU) Cairns Campus. Male and female mice between 6 and 10 weeks of age were used. Experimental protocols were approved by the JCU Animal Ethics Committee (approval A2213). Mice were injected intraperitoneally (i.p.) with 50 μg of *Trichuris muris* adult worm ES antigens in PBS. Mice were i.p. treated with 20 mg/kg/day of MCC950 (Coll et al., 2015) or PBS vehicle, daily starting on day 0 of *Trichuris* infection. Mice were treated with neutralizing antibodies against CD4 (GK1.5, Bioxcell, 0.5 mg/mouse) starting on day 0 and continuing every 3 days. Recombinant murine IL-18 (R&D Systems) or PBS vehicle were injected i.p. at a dose of 200 ng/mouse daily from days 7–19 post-*Trichuris* infection.

Human Ethics and Experimental Procedures

The design and clinical results of the placebo-controlled clinical trial using *N. americanus* to treat celiac disease have been described elsewhere

(Daveson et al., 2011). The trial was registered at https://clinicaltrials.gov/ as NCT00671138. Briefly, otherwise healthy people with HLA-DQ2^{+} celiac disease on a gluten-free diet were recruited, randomized into two groups, and either infected percutaneously with 15 infective larvae of *N. americanus* or given placebo treatment with topical chili pepper solution. Blood was collected by venepuncture and serum was collected and stored at −80°C.

Parasitological Techniques

T. muris was maintained in genetically susceptible mouse strains and *T. muris* ES antigens were obtained as described previously (Artis et al., 1999). Mice were infected by oral gavage with approximately 200 embryonated *T. muris* eggs. *Trichuris*-specific serum IgG1 titers were assayed by ELISA on plates with coated with *T. muris* antigen (5 μg/mL). Maintenance of the life cycle of *Necator americanus* and experimental infections of human subjects were performed as described previously (Daveson et al., 2011).

Bone Marrow Macrophage Assays

Bone marrow was isolated from hind legs of C57BL/6 or NLRP3$^{-/-}$ mice by flushing bones with DMEM, and cells were cultured at 1 × 10^{6} cells/mL in DMEM supplemented with 10% fetal bovine serum (FBS), 5% horse serum (Invitrogen), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 10 ng/mL macrophage colony stimulating factor (M-CSF). On day 6, macrophages were harvested and plated overnight at a concentration of 2 × 10^{5} cells/mL. The following day, cells were treated with media alone or LPS (1 μg/mL) for 4 hr, 20 μM nigericin for 2 hr and 25 μg/mL of *Trichuris* adult worm ES products or 25 μg/mL worm exosomes for 6 hr, and cell-free supernatants were stored at −80°C.

Mouse Tissue Collection, Processing, and Pathology Scoring

Blood was collected by cardiac puncture for terminal procedures, or submandibular bleeding for non-terminal procedures, and serum was stored at −80°C. Single-cell suspensions of mLN or cecal patch tissue were prepared by passing through 70-μm nylon mesh filters. Colonic lamina propria lymphocytes (LPLs) were isolated as described previously (Zaph et al., 2007). For colon tissue homogenates, 1 cm of tissue was homogenized mechanically in 0.5 mL of PBS using a TissueLyzer (QIAGEN), and supernatants were stored at −80°C. Cecal tissue was fixed in 4% paraformaldehyde and embedded in paraffin, and 5-μM sections were stained with H&E or periodic acid-Schiff (PAS)/Alcian blue stains. Scoring of pathology was performed from H&E-stained slides by an individual blinded to the experimental group. Tissue sections were scored on a scale of 0–5 for the following parameters: (1) epithelial pathology (crypt elongation, hyperplasia, erosion), (2) mural inflammation, and (3) edema for a maximal score of 15. Goblet cell responses were assessed by counting PAS-positive cell number per crypt unit, with a minimum of 20 crypt units assessed for each tissue sample. For determination of worm burdens, ceca were collected, and worms were counted using a dissecting microscope.

Statistical Analyses

Statistical analyses for murine *in vitro* and *in vivo* studies were performed using unpaired Mann-Whitney U tests. Human cytokine data were compared using paired Mann-Whitney U tests. *In vivo* mouse experiments included n = 1–3 naive control mice and a minimum of n = 4–6 infected mice and were repeated at least twice to demonstrate reproducibility. Results are expressed as mean ± SEM or as individual data points and the differences were considered significant at a p value of <0.05.

DATA AND SOFTWARE AVAILABILITY

The flow cytometry data reported in this paper is available on Mendeley Data at https://doi:10.17632/pdmccttvwb.1

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.03.097.

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AUTHOR CONTRIBUTIONS

R.A. and P.R.G. conceived the project, designed the experiments, and performed data analysis, R.A., Z.A., C.M.M., J.S., A.K., and P.R.G. performed the experiments, A.A.B.R., M.A.C., and S.L.M. provided resources, mouse tools, and intellectual input into experimental design, J.C. and A.L. conduced the human trial and provided serum samples, N.C.S., A.K., and A.L. provided supervision, oversight, and leadership for the project, R.A. and P.R.G. wrote original drafts of the manuscript; all authors contributed to review and editing of the manuscript.

DECLARATION OF INTERESTS

M.A.C. currently holds a fractional Professorial Research Fellow appointment at the University of Queensland with his remaining time as CEO of Inflazome Ltd., a company headquartered in Dublin, Ireland that is developing drugs to address clinical unmet needs in inflammatory disease by targeting the inflammasome.

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