RECENT ADVANCES IN THE IMMUNOLOGY OF HELMINTH INFECTION – PROTECTION, PATHOGENESIS AND PANACEAS

EDITED BY: Kara Filbey, Constance Finney, Paul Giacomin and Mark C. Siracusa
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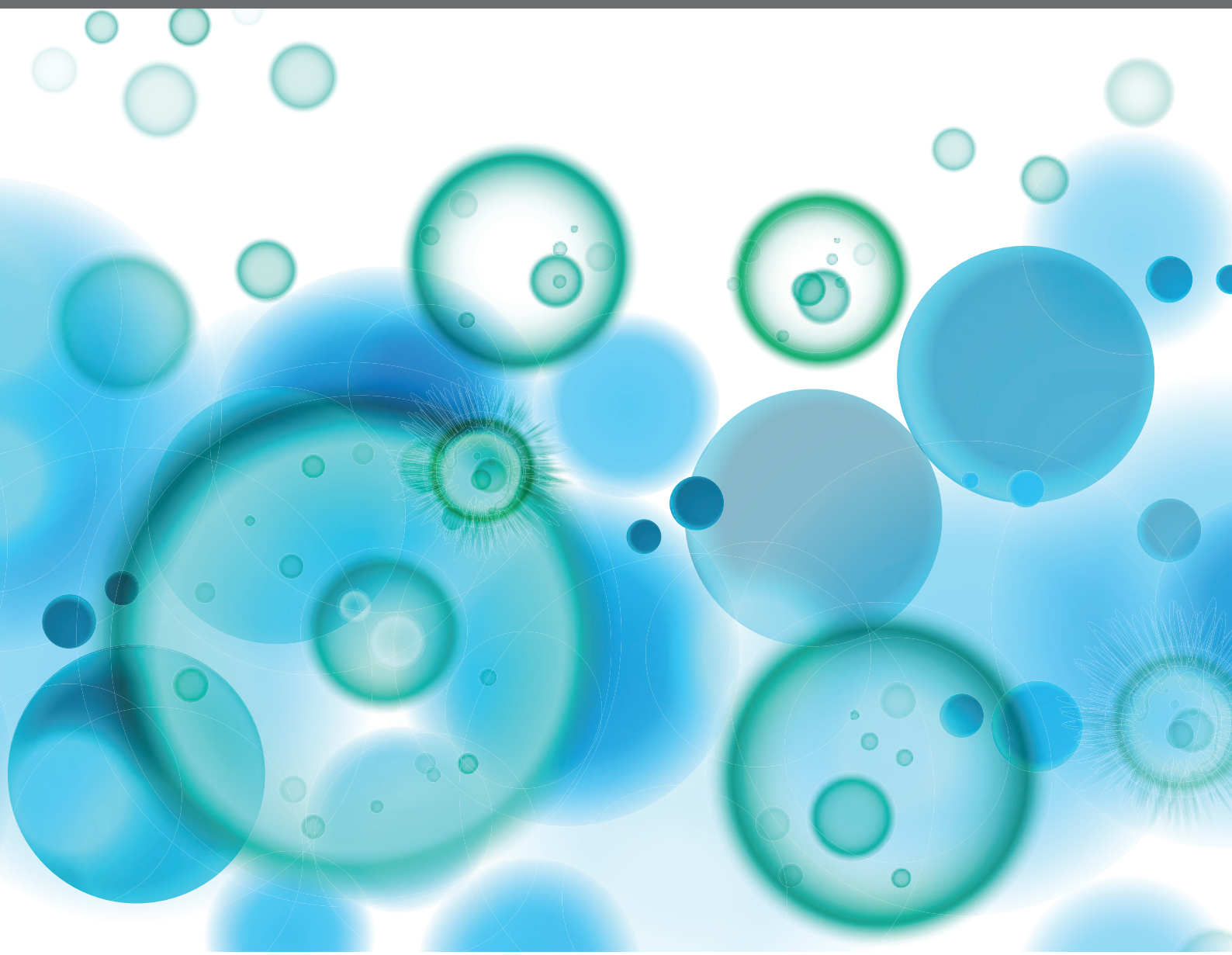

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RECENT ADVANCES IN THE IMMUNOLOGY OF HELMINTH INFECTION – PROTECTION, PATHOGENESIS AND PANACEAS

Topic Editors:
Kara Filbey, The University of Manchester, United Kingdom
Constance Finney, University of Calgary, Canada
Paul Giacomin, James Cook University, Australia
Mark C. Siracusa, Rutgers Biomedical and Health Sciences, United States

Dr. Paul Giacomin is a co-founder of Paragen Bio. Dr. Siracusa is the founder and president of Nemagen Discoveries. The other Topic Editors declare no competing interests with regard to the Research Topic subject.

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Table of Contents

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EDITORIAL
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Editorial: Recent Advances in the Immunology of Helminth Infection – Protection, Pathogenesis and Panaceas

Kara J. Filbey[1], Constance A. M. Finney[2], Paul R. Giacomin[3] and Mark C. Siracusa[4]*

[1] Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, United Kingdom, [2] Department of Biological Sciences, University of Calgary, Calgary, AB, Canada, [3] Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, QLD, Australia, [4] Department of Medicine, Rutgers New Jersey Medical School, Rutgers University, Newark, NJ, United States

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Editorial on the Research Topic

Recent Advances in the Immunology of Helminth Infection – Protection, Pathogenesis and Panaceas

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Edited and reviewed by:
Ian Marriot,
University of North Carolina at Charlotte, United States

****Correspondence:***
Kara J. Filbey
kara.filbey@manchester.ac.uk

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INTRODUCTION

Helminths (parasitic worms) are a diverse group of organisms that utilize a wide range of species as their intermediate and definitive hosts. The nematodes consist of the whipworms, roundworms, hookworms and filarial worms, and these sit alongside the platyhelminth flatworms (or blood flukes) and tapeworms - all of which have species that cause serious disease in humans. Some species have free living stages, others rely on insect vectors for transmission, while some can reproduce to release live larval stages within their mammalian host. The diversity of infection route, larval migration within the host and the location of the adult parasite have major implications for the pathology and immune responses elicited by each species.

Here, we briefly outline the contributions to the Research Topic Recent Advances in the Immunology of Helminth Infection – Protection, Pathogenesis and Panaceas.

PROTECTION

Many millions of people are infected with helminths world-wide (1), and there is, as yet, no effective vaccine against any helminth species. Indeed, the protective immune responses needed to kill and clear worms from the host are still to be fully elucidated. Zawawi and Else outline the challenges involved in helminth vaccine development, and review recent findings on the identification of suitable vaccine candidates as well as the results of recent pre-clinical and clinical trials in the context of soil-transmitted helminths. Silvane et al. investigate the protective immune responses induced by vaccination of mice with a peptide from a well-characterized protease inhibitor molecule made by the liver fluke *Fasciola hepatica*. Using a novel adjuvant formulation, FhKTM/CpG-ODN/

Coa-ASC16 induced enhanced type 1 and type 17 responses and reduced liver pathology after *F. hepatica* infection.

The lung and intestinal tract are the two tissues most often associated with helminth larval migration and adult dwelling respectively. They are both key sites of immune-mediated protection against helminth parasites and are inextricably linked *via* mucosal immune cross-talk (2). In this Research Topic, Weatherhead et al. review the critical interface of pulmonary immune responses and helminth infections, and Faniyi et al. outline the roles of chemosensory epithelial cells within the gastrointestinal tract in host protection against helminths. Illustrating further the complex nature of the intestinal immune system, original research by Mayer et al. shows that discrete areas of the intestine mount differing Th1 and Th2 responses to helminth antigens. The authors were able to demonstrate that antigens from *Schistosoma mansoni* eggs and *Heligmosomoides polygyrus* are transported from the gut to particular mesenteric lymph nodes by separate subsets of dendritic cells, inducing distinct responses, suggesting a highly antigen-dependent and site-specific response to helminth infections.

Type 2 innate lymphoid cells (ILC2s) have been of particular interest to helminth immunologists in recent years (3), and the review by Miller and Reinhardt focusses in detail on the inflammatory subset of ILC2s that arise in the lungs after infection with the model hookworm *Nippostrongylus brasiliensis*.

Of course, much of what we know regarding host immune responses to helminth infections has been gained from laboratory infection models. In their extensive review, Colombo and Grencis outline the models traditionally used to investigate soil-transmitted helminth infections in laboratory animals and highlight the major differences between these and natural infections of humans and wild rodents. They go on to discuss the potential benefits of using the 'trickle-infection' method for modelling more natural infections in the laboratory.

HELMINTH IMMUNOMODULATORS

An area of great interest in the field has been the immunomodulatory effects of helminths on their hosts, and their potential use as therapeutic agents in inflammatory diseases (4). Recent technological advances have hastened the discovery of individual molecules secreted by helminths, giving a deeper insight into just how helminth-driven immunomodulation may be occurring. Three broad-ranging reviews by Bobardt et al., Wiedemann and Voehringer and Ryan et al. take an in-depth look at the molecules made by a range of helminth species to impact the immune response against them, thus allowing them to successfully invade and persist in their hosts for long periods of time.

Two original research papers examined individual helminth-derived immunomodulatory molecules in precise detail. Firstly, Chauche et al. explain mechanistically how a truncated form of Alarmin Release Inhibitor from *H. polygyrus* (HpARI) binds to the cytokine interleukin (IL)-33 to prevent its degradation and enhance its actions *in vivo*. In contrast to the full-length form of HpARI which binds IL-33 and suppresses its actions, the truncated form HpARI_CCP1/2 amplifies IL-33-dependent responses to *N. brasiliensis* infection and fungal allergen administration. In a study by Jin et al. a protein from the excretory-secretory (ES) products of *Trichinella spiralis* - thioredoxin peroxidase-2 (TsTPX2) – is found to potently activate macrophages to a protective M2 phenotype, with the adoptive transfer of these antigen-activated macrophages limiting adult worm numbers significantly.

Most of the helminth-derived immunomodulatory molecules characterized to-date are proteins. However, recently the potential importance of micro(mi)RNAs released in extracellular vesicles secreted by helminths has been investigated (5) (and see below). In this vein, Ricafrente et al. use an innovative data-mining approach to identify potential gene targets for *F. hepatica* miRNAs within innate immune cells, thus providing information for the development of novel strategies for controlling liver fluke infection.

PANACEAS AND PATHOGENESIS

Moving from the identification of individual molecules to modulating inflammation and immunopathology in *in vivo* models of disease, Cleenewerk et al. outline the immunomodulatory effects of live infection with *S. mansoni* alongside its ES products, and discuss the potential for use in the clinic. Research by Yang et al. utilizes miRNA-containing extracellular vesicles from *T. spiralis* to reduce inflammatory immune responses and alleviate intestinal epithelial barrier damage in a model of colitis.

Two papers used the well-characterized gastrointestinal helminth *H. polygyrus* to ameliorate inflammation in diverse models of disease in mice. White et al. report that the potent skewing towards a type 2 immune response during helminth infection was protective in experimental autoimmune encephalitis (EAE). Importantly, this protection was ablated in mice lacking the interleukin-4/13 receptor alpha chain (IL-4Rα) and protection was not linked with regulatory T cell activity. Additionally, Filbey et al. show that infection significantly reduces skin inflammation in a model of contact hypersensitivity, likely *via* suppression of innate cell recruitment to the skin after allergen sensitization. Neutrophil-attracting chemokine levels, and neutrophil numbers in the skin of mice treated with the contact sensitizer dibutyl phthalate fluorescein isothiocynate (DBP-FITC), were significantly decreased by helminth infection.

Three reviews in this Research Topic expertly outline the impact of helminth infection on a diverse range of human health concerns. Firstly, Rajamanickam et al. focused on parameters associated with non-diabetic obesity and assessed outcomes in a human cohort before and 6 months after anthelminthic treatment. The predominant helminth species endemic to the Tamil Nadu region of southern India is the soil-transmitted GI helminth *Strongyloides stercoralis* which appeared to protect people from the inflammatory immune signature of non-diabetic obesity, a phenomenon that was reversed after worm clearance. An important review by Chetty et al. looks at the often overlooked field of female reproductive health, focusing on the impact of helminth infection on sexually-transmitted infections, female reproductive tract pathology and reproduction. Finally,

Fonte et al. take a timely look at the possible impacts of concomitant infection with helminths and SARS-CoV-2.

FINAL WORDS AND ACKNOWLEDGMENTS

An estimated quarter of the world's population is infected with helminths, which clearly have wide-ranging and potent effects on the immune systems of their hosts. Much is yet to be discovered regarding the responses elicited by and the exciting potential uses of helminths. As guest Topic Editors we hope that the Research Topic Recent Advances in the Immunology of Helminth Infection – Protection, Pathogenesis and Panaceas has offered an up-to-date insight into the diverse and collaborative field of helminth immunology. We would like to thank all of the contributing authors, editors, and especially all of the reviewers who dedicated their precious time in what has proved to be a most challenging year.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: PG is a co-founder of Paragen Bio, a biotech company that aims to translate and commercialise hookworm-derived proteins for inflammatory diseases. This has previously involved investment from pharma (Abbvie Inc) and venture capitalist firms (OneVentures, Brandon Capital).

MS is founder and president of the biotech company NemaGen Discoveries.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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The Gastrointestinal Helminth *Heligmosomoides bakeri* Suppresses Inflammation in a Model of Contact Hypersensitivity

*Kara J. Filbey‡, Palak H. Mehta†, Kimberley J. Meijlink†, Christophe Pellefigues, Alfonso J. Schmidt and Graham Le Gros**

Malaghan Institute of Medical Research, Wellington, New Zealand

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Edited by:
Henry J. McSorley, University of Edinburgh, United Kingdom

Reviewed by:
Sebastian Rausch, Freie Universität Berlin, Germany
Julia Esser-von Bieren, Helmholtz Zentrum München, Germany
Sheila Donnelly, University of Technology Sydney, Australia

***Correspondence:**
Graham Le Gros
glegros@malaghan.org.nz

†These authors have contributed equally to this work

‡Present address:
Kara J. Filbey, Lydia Becker Institute of Immunology and Inflammation Research, University of Manchester, Manchester, United Kingdom

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Helminths regulate host immune responses to ensure their own long-term survival. Numerous studies have demonstrated that these helminth-induced regulatory mechanisms can also limit host inflammatory responses in several disease models. We used the *Heligmosomoides bakeri (Hb)* infection model (also known as *H. polygyrus* or *H. polygyrus bakeri* in the literature) to test whether such immune regulation affects skin inflammatory responses induced by the model contact sensitiser dibutyl phthalate fluorescein isothiocynate (DBP-FITC). Skin lysates from DBP-FITC-sensitized, *Hb*-infected mice produced less neutrophil specific chemokines and had significantly reduced levels of skin thickening and cellular inflammatory responses in tissue and draining lymph nodes (LNs) compared to uninfected mice. *Hb*-induced suppression did not appear to be mediated by regulatory T cells, nor was it due to impaired dendritic cell (DC) activity. Mice cleared of infection remained unresponsive to DBP-FITC sensitization indicating that suppression was not via the secretion of *Hb*-derived short-lived regulatory molecules, although long-term effects on cells cannot be ruled out. Importantly, similar helminth-induced suppression of inflammation was also seen in the draining LN after intradermal injection of the ubiquitous allergen house dust mite (HDM). These findings demonstrate that *Hb* infection attenuates skin inflammatory responses by suppressing chemokine production and recruitment of innate cells. These findings further contribute to the growing body of evidence that helminth infection can modulate inflammatory and allergic responses via a number of mechanisms with potential to be exploited in therapeutic and preventative strategies in the future.

Keywords: helminth, immunoregulation, skin, infection, contact hypersensitivity (CHS)

INTRODUCTION

Various epidemiological studies have drawn a link between the incidence of helminth infection and reduction in allergic and inflammatory diseases (1–4). In seeking mechanisms by which this might occur, experimental models have linked helminth-induced regulatory T and B cells (Tregs and Bregs, respectively), to the dampening of responses to allergens (5, 6) such as HDM (7, 8), ovalbumin (OVA) (9, 10) and peanut (11), and to the amelioration of inflammation in a number of disease models including colitis (12–14), experimental autoimmune encephalitis (15), and diabetes (16, 17).

More recently, mechanisms other than the induction of regulatory lymphocytes have been uncovered that may play a role in protection by helminths. A protein made by *Hb*, a rodent gastrointestinal helminth, was found to actively block the release of IL-33 from necrotic airway epithelial cells after exposure to the fungal allergen *Alternaria,* thus dampening type 2 innate lymphoid cell (ILC2) and subsequent Th2 responses (18). Macrophages from *Trichinella spiralis*-infected donors, or those treated with *T. spiralis* excretory-secretory (ES) products *in vitro*, were found to be protective in models of colitis and OVA-induced allergic airway inflammation when transferred into recipient mice (19). A defense peptide from *Fasciola hepatica* can inhibit local osteoclast formation and limit bone destruction in a model of arthritis (20) and injection of *Schistosoma mansoni* eggs can limit allergic airway inflammation by reducing the influx of monocyte-derived dendritic cells (moDCs) into lung tissue (21). Also, chronic infection with *Litomosoides sigmodontis* protects mice from OVA-induced anaphylaxis by decreasing numbers and activity of mast cells (22).

These observations have given rise to the proposal that elimination of helminth infections from human communities might be linked to the global increase of allergic and atopic skin diseases (23–25). However, there are relatively few pre-clinical studies relating helminth infection in mice to immune responses in the skin, in particular in the context of allergy or contact hypersensitivity (CHS). Therefore, we undertook experiments to investigate the influence of infection with *Hb* on the outcome of a model of skin inflammation in mice. Chronic *Hb* infection is a well-characterized model where the parasite is confined to the small intestine throughout its lifecycle in the host. As well as stimulating a strong Th2 immune response, survival of adult *Hb* in the host intestine appears to be maintained by the expansion of both Tregs and Bregs, induced via various mechanisms including the expression of a TGF-beta molecular mimic in its ES products (26) and the induction of a subset of regulatory DCs that preferentially expand Tregs over Th2 cells (27).

To induce a relevant skin CHS reaction in mice we used a topically applied DBP-FITC prime-challenge model which stimulates a potent neutrophil and Th2 mediated inflammatory response (28–30). DBP is a phthalate ester commonly used as a plasticiser in many cosmetic and industrial products, which acts as an adjuvant to the hapten FITC in models of CHS (31). Inflammation in this model is dependent on type 2 CD4+ T cell responses (32) which are primed by specific subsets of DCs (29, 30, 33) in the local lymph node. The cytokine thymic stromal lymphopoietin (TSLP) has been shown to be key in the maturation and accumulation of these DCs and the subsequent initiation of a Th2 response to DBP-FITC (28, 29).

Strikingly, we found DBP-FITC-induced skin inflammation to be significantly reduced in helminth-infected mice, alongside attenuated accumulation of innate effector cells and a significant reduction in expression of inflammatory chemokines and cytokines in the skin during both the DBP-FITC sensitization and challenge phases of the inflammatory response.

Here, we expand the findings on the therapeutic potential of helminths in the context of models of allergic and inflammatory diseases of the skin and provide a basis for future experiments to investigate more in-depth mechanistic explanations for this phenomenon.

MATERIALS AND METHODS

Mice

6-10-week-old female C57BL/6 mice were used throughout and were bred and maintained by the MIMR Biomedical Research Unit. All procedures were approved by Victoria University of Wellington. Anaesthetisations involved intraperitoneal (i.p.) injection of ketamine/xylazine.

Parasites

Hb, previously identified as *H. polygyrus,* was maintained as previously described (34). *Hb* infection was cleared with two subcutaneous doses of ivermectin (200 μg) (Noromectin, Norbrook Laboratories Ltd, Newry, Northern Ireland) on days 13, 14 of infection. Clearance was confirmed by checking fecal samples for eggs 7 days later, before sensitization with DBP-FITC.

Contact Sensitization With DBP-FITC

For the sensitization, challenge model (**Figure 1A**), mice were sensitized on abdominal skin, that had been shaved a week prior, on days 0 and 2 by epicutaneous application of 40 μl 0.5% FITC (Sigma) in 1:1 mix of DBP (Aldrich) and acetone (Sharian). At day 6, a challenge dose of 20 μl DBP-FITC was applied to one ear pinna and 20 μl of vehicle (DBP only) to the other. For assessment of DC migration to the ear-draining LNs, mice were treated with 20 μl DBP-FITC on both ears at day 0 and LNs harvested on day 2. For the Treg depletion experiments, mice were treated twice with DBP-FITC, on days 0 and 2 (see **Figure 2A**), as was the case for assessment of cells in the ear tissue. For assessment of cytokines and chemokines in the ear tissue, mice were treated once with DBP-FITC on each ear and tissue was harvested at the timepoints indicated. Ear thickness was measured with a digital micrometer (Micromaster, Capa System) and is presented as fold-change from the starting measurement on day 6, measured separately for each group. Trans-epidermal water loss (TEWL) was measured with a TEWL open chamber unit (Dermalab). Naïve control mice were neither infected or treated with any compound.

Injection of HDM

Mice were anesthetized and intradermally injected with 200 μg crushed *Dermatophagoides pteronyssinus* (Greer) in 30 μl sterile PBS as previously described (35).

Treg Depletion

Mice were injected i.p. with 500 mg anti-CD25 antibody (PC61; BioXCell) or rat IgG1 isotype control (BioXCell) in sterile PBS, at the time points indicated in **Figure 2A**.

Tissue Digestion and Flow Cytometry

LNs were dissociated and passed through a 70 μm strainer before enumeration. For DC analysis LNs were teased apart followed by digestion in 100 μg/mL DNase I (Sigma) and

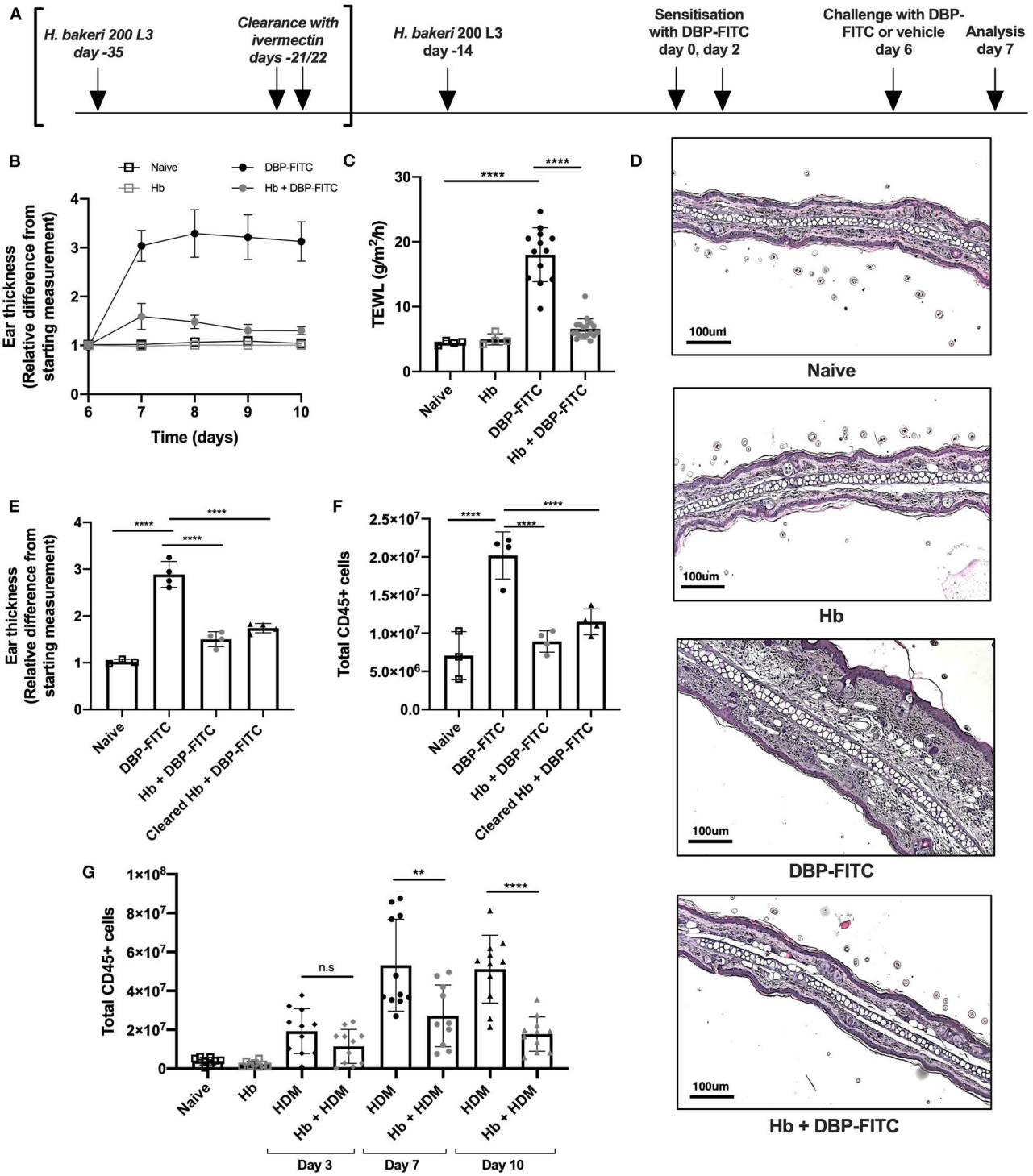

FIGURE 1 | DBP-FITC-induced skin inflammation is suppressed by *H. bakeri* infection. **(A)** Timeline of *Hb* infection and DBP-FITC topical sensitization and challenge model. Ivermectin treatment for *Hb* clearance experiments [results in **(E,F)**] is shown in brackets. **(B)** Relative difference in ear thickness compared to day 6 baseline measurements, in naïve mice (black lined clear squares), *Hb*-infected mice (gray lined clear squares) or in mice challenged DBP-FITC (gray circles) or without (black circles) prior *Hb* infection. **(C)** TEWL (g/m2/h) at site of DBP-FITC challenge measured at day 7, or in control naive mice. **(D)** Ear sections stained with H&E on day 7. Scale bars represent 100 um. **(E)** Relative difference in ear thickness in naïve mice, or at day 7 after DBP-FITC challenge in mice with *Hb* infection, or after clearance of the infection. **(F)** Total number of live CD45+ cells in ear dLN at day 7 in the same groups as **(E)**. **(G)** Total number of live CD45+ cells in ear dLN at days 3, 7, and 10 post-HDM intradermal injection into the ear, with or without prior *Hb* infection, or in naive mice. A one-way ANOVA with Tuckey's multiple comparisons test was used to test statistical significance. Graphs represent data combined from 2 experiments **(B,C,G)** or are representative of 2 repeat experiments **(D,E,F)** and show mean ± SD. n.s, not significant; $^{**}p < 0.01$, $^{****}p < 0.0001$.

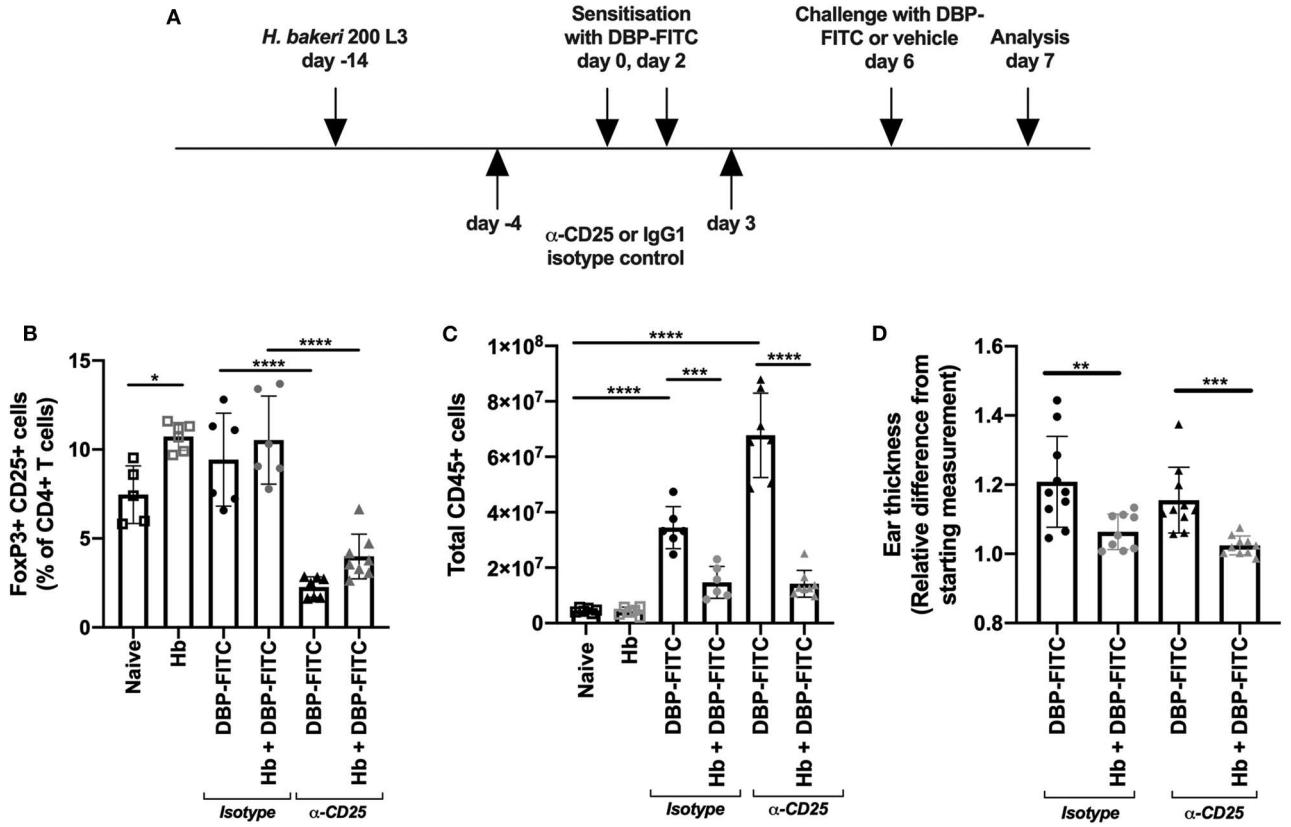

FIGURE 2 | Anti-CD25 depletion of *H. bakeri*- induced Tregs does not affect reduction of LN hyperplasia after DBP-FITC challenge. **(A)** Timeline of DBP-FITC topical application model including treatment with anti-CD25 or isotype control. **(B)** Frequency of FoxP3+ CD25+ cells within the CD4+ T cell compartment at day 7 in the ear dLN. **(C)** Total number of live CD45+ cells from the ear dLN at day 7. **(D)** Relative difference in ear thickness at day 7 after DBP-FITC challenge in mice with or without *Hb* infection, treated with anti-CD25 or an isotype control. A one-way ANOVA with Tuckey's multiple comparisons test was used to test statistical significance. Graphs represent data combined from 2 experiments and show mean ± SD. n.s, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

100 μg/mL Liberase TL (Roche, Germany) for 25 min at 37°C and passed through a 70 μm strainer. For skin cell preparations, ears were split into the dorsal and ventral layers, minced and digested for 30 min, shaking at 150 rpm in a 37°C incubator, in IMDM (Gibco) plus 5% FCS (Gibco) containing 2 mg/ml collagenase IV and 100 μg/mL DNase 1 (both Sigma). Digestion was stopped by adding 10% FBS plus 5 mM EDTA in HBSS and a single cell suspension was obtained by passing through a 70 μm strainer. Skin single cell suspensions and LN cells were stained for surface markers as previously described (36). Viability was assessed by staining with Live/Dead Fixable Blue Viability stain (Invitrogen), Zombie NIR (Biolegend) or DAPI (Sigma). Antibodies used were as follows: MHCII-Pacific Blue (M5/114.15.2), CD3-PE-Cy5 (145-2C11), CD11b-BV570 (M1/70), CD4-BV750 (GK1.5), CD64-PE-Dazzle594 (X54-5/7.1), Ly6C-AF700 (HK1.4), CD45-APC-Cy5.5 (104, all from Biolegend), B220-PE-CF594 (RA3-6B2), CD45-BUV395 (30.F11), CD3-BV786 (145-2C11), CD4-BV605 (RM4-5), CD8a-PE-Cy7 (53-6.7), CD25-BV421 (7D4), CD3-BV711 (145-2C11), CD11c-PE-Cy7 (HL3), CD11b-BUV395 (M1/70), CD326-BV711 (G8.8), CD86-PE (GL1), SiglecF-BV421 (E50-2440), Ly6G-BV711 (1A8, all from BD), and CD103-APC (E2E7 from eBioscience). Migratory DCs were characterized as $CD45^{+}$ $B220^{-}$ $CD11c^{var}$ and $MHCII^{high}$ as previously described (29)—see **Figure 3C**. For intra-nuclear staining the FoxP3-PE/Transcription Factor Staining Buffer set (eBioscience) was used as per manufacturer's instructions. Regulatory T cells were defined as $CD4^{+}$ $FoxP3^{+}$ $CD25^{+}$. All gating was based on fluorescence minus one (FMO) controls where applicable. Data were acquired using a BD LSRII or Cytek Aurora, and analyzed using FlowJo v10.

Sectioning, Staining, and Microscopy

For haematoxylin and eosin (H&E) staining, whole ears were fixed in 10% formalin (Sigma) for 24 h and dehydrated into xylene (Pronalys) overnight in a tissue processor (Thermo Fisher Citadel 200). Paraffin wax embedded tissue was sectioned on a microtome at 3–4 μm onto adhesive microscope slides (Trajan). Sections were rehydrated and stained with hematoxylin and eosin as per manufacturer's instructions (Thermo Fisher). Sections were observed using an Olympus BX51TF compound microscope using a 10x, N.A. 0.3 objective, and images taken in the middle of the ear section.

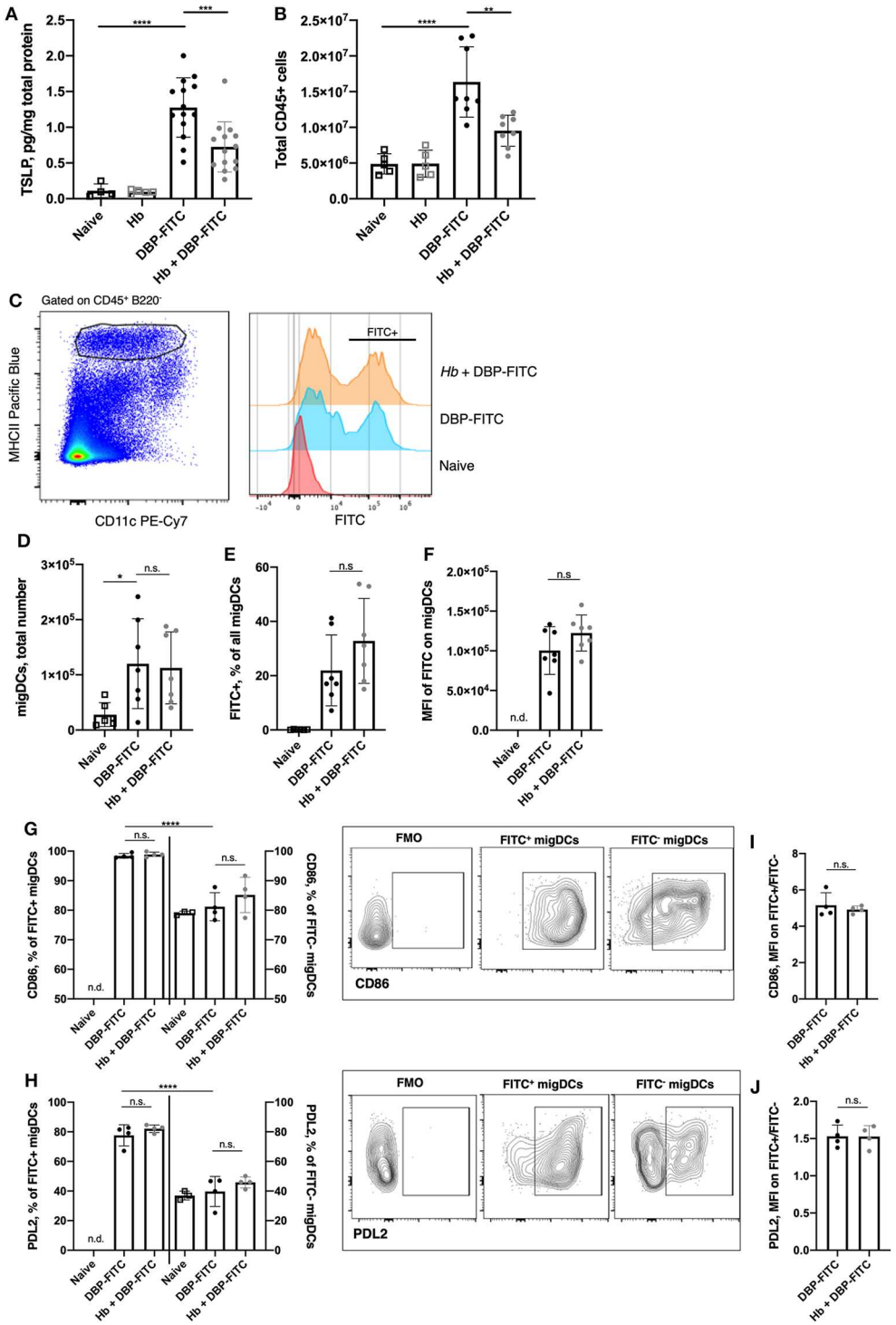

FIGURE 3 | *H. bakeri* infection does not affect DC uptake and transport of DBP-FITC to the skin draining LN after sensitization. **(A)** TSLP protein levels 24 h post-sensitization with DBP-FITC in tissue lysate measured by ELISA. **(B)** Total number of live CD45+ cells in the ear dLN at 2 days post DBP-FITC sensitization. **(C)** Representative plots of FITC expression by $CD45^+$ $B220^-$ $CD11c^{var}$ $MHCII^{high}$ migratory DCs (migDCs) in the ear dLN at day 2. **(D)** Total number of migratory DCs (migDCs) in the ear dLN at day 2. **(E)** Frequency of FITC+ migDCs in ear dLN at day 2. **(F)** Median fluorescence intensity (MFI) of FITC on migDCs in the ear dLN at day 2. **(G)** Frequency of $FITC^+$ and $FITC^-$ DCs expressing CD86 or **(H)** PDL2 in the ear dLN at d2, with plots showing representative gating. **(I)** Ratio of MFI of CD86 or **(J)** PDL2 expression in FITC+ vs. FITC- migDCs in the dLN at d2. A one-way ANOVA with Tuckey's multiple comparisons test was used to test statistical significance between multiple groups **(A–H)**, or an unpaired *t*-test between 2 groups **(I,J)**. FMO, fluorescence minus one. Graphs represent data combined from 2 experiments **(A–F)** or are representative of 2 repeat experiments **(G–J)**. Graphs show mean ± SD. n.s, not significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. n.d, not detectable.

For confocal microscopy, samples were processed and stained using a standard immunofluorescence protocol (37). Whole ears were incubated in 20% sucrose for 1 h and snap-frozen in OCT compound (Tissue-Tek) using a Stand-Alone Gentle Jane snap-freezing system (Leica Biosystems). Cryosections of 10 μm were fixed in cold acetone for 3 min and blocked with Fc Block (clone 2.4G2) for 1 h and stained with Ly6G-PE (clone 1A8, Pharmingen). For nuclear staining, sections were incubated with DAPI (2 mg/ml) for 10 min. Images were taken with an inverted IX 83 inverted microscope equipped with a FV1200 confocal head (Olympus) using a 20X, N.A. 0.75 objective. Images were acquired using the FV10-ASW software (v4.2b, Olympus) and processed with ImageJ (38).

Cytokine and Chemokine Quantification in Skin Tissue

Ear tissue was finely chopped into 1x Lysis Buffer (Cell Signaling Technology Inc.) with 1:100 phenylmethanesulfonyl fluoride solution (PMSF) (Sigma) and homogenized with 5 mm stainless steel beads (Qiagen) using a TissueLyserII (Qiagen). Samples were centrifuged and cytokines and chemokines measured in the supernatant with LEGENDplex multi-analyte assay kits (Biolegend). TSLP was measured with an ELISA DuoSet (R&D Systems). Results were normalized to total protein content in the sample measured by Bradford assay (Thermo Fisher).

Statistical Analysis

All statistical analyses were performed using Prism v8 (GraphPad). An unpaired *t*-test was used to compare two groups, and a one-way ANOVA with Tuckey's multiple comparisons test with adjusted *p*-values was used to compare several groups. Tests used are indicated in each figure legend. Significant results are indicated as follows: $^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$, $^{****} = p < 0.0001$. n.s. = not significant. All symbols represent individual samples and error bars represent mean ± standard deviation (SD).

RESULTS

DBP-FITC-Induced Skin Inflammation and LN Hyperplasia Are Suppressed by *H. bakeri* Infection

Topical application of DBP-FITC to the ear skin of mice induces Th2-dominated CHS inflammatory responses with similar characteristics to human atopic skin disease or atopic dermatitis (AD) (28, 32). We sought to determine the effect of chronic helminth infection on skin inflammatory responses by infecting mice with *Hb* 14 days prior to sensitization with DBP-FITC. At this time point in infection adult *Hb* are present in the lumen of the small intestine of most host mouse strains (including C57BL/6), and are known to stimulate a strongly polarized type 2 response (39). Both *Hb*-infected and control mice were sensitized to DBP-FITC on day 0 and day 2 with topical applications to the abdomen and then challenged with one DBP-FITC application to both sides of the ear at day 6. Analysis of ear tissues was completed 24 h after challenge corresponding to day 7 of the timeline (**Figure 1A**). Skin inflammation, as measured by swelling of the ear dermis, peaked 24 h after the day 6 challenge with DBP-FITC, and slowly reduced in size thereafter (**Figure 1B**). Trans-epidermal water loss (TEWL), a surrogate marker for the loss of skin barrier function at the site of DBP-FITC challenge (40), increased significantly 24 h after the day 6 challenge (**Figure 1C**). By comparison, *Hb*-infected mice had similar TEWL levels at the site of DBP-FITC challenge, compared to naive mice (**Figure 1C**). Haematoxylin and eosin (H&E) staining revealed a significant inflammatory infiltrate in the ear dermis following the challenge application of DBP-FITC, which was completely absent in *Hb*-infected mice (**Figure 1D**). To determine whether the effect of *Hb* infection on the DBP-FITC-induced inflammatory response was dependent on the presence of live worms secreting biologically active molecules, we used ivermectin to clear mice of adult worms and measured the subsequent DBP-FITC-induced skin inflammation and LN hyperplasia. Strikingly, mice cleared of *Hb* continued to benefit from reduced skin inflammatory responses (**Figure 1E**) and diminished LN hyperplasia (**Figure 1F**). Taken together, these data indicated that it was not the immediate action of short-lived regulatory molecules secreted by the live parasite that was diminishing the inflammatory responses to DBP-FITC.

Injection of house dust mite (HDM) intradermally into the ear results in a rapid and robust Th2 response in the draining auricular LN (35). In accordance with our findings with DBP-FITC, prior *Hb* infection also limits inflammation in the dLN after HDM injection at all time points measured (**Figure 1G**).

Anti-CD25 Depletion of *H. bakeri*-Induced Tregs Does Not Affect Reduction of Skin Inflammation

Hb is known to induce $CD4^{+}$ $FoxP3^{+}$ $CD25^{+}$ T regulatory (Treg) cells in local lymphoid organs, and these cells represent a key feature of the helminths ability to limit organ specific inflammation in a variety of disease models (41). We investigated whether the *Hb*-dependent suppression of DBP-FITC-induced inflammation and skin draining LN hyperplasia could be neutralized by a Treg specific monoclonal antibody depletion regime (**Figure 2A**). We found that helminth infection modestly increased the frequency of $FoxP3^{+}$ $CD25^{+}$ Tregs within the $CD4^{+}$ T cell compartment in the auricular LN draining the ear skin, and that 2 doses of anti-CD25 significantly reduced the frequency of Tregs in both uninfected and *Hb* infected mice (**Figure 2B**). Depletion of Tregs in DBP-FITC treated mice increased LN hyperplasia, illustrating that Tregs play some role in muting inflammatory responses even without the influence of a helminth infection. However, Treg depletion did not affect the *Hb*-induced suppression of the LN response to DBP-FITC (**Figure 2C**) or the reduction in skin thickness measured 24 h after challenge (**Figure 2D**). Taken together these experiments indicate that Tregs do not appear to play a major role in *Hb*-induced suppression of the DBP-FITC inflammatory response.

H. bakeri Infection Does Not Affect the DC-Mediated Uptake and Transport of DBP-FITC to the Skin Draining LN

The DBP-FITC model of CHS is dependent on the expression of TSLP, which acts on DCs to induce Th2 responses in the skin and local LN (28, 29, 42, 43). As expected, TSLP production in the skin was increased 24 h following DBP-FITC skin sensitization (**Figure 3A**). We found that prior *Hb* infection leads to a significantly decreased amount of TSLP being detected in the skin lysate.

We used the FITC signal detected on skin DCs to track allergen uptake, transport to the draining LN and expression of activation markers to determine whether *Hb* infection had any effect on DC function and immune responses in the draining auricular LN (29, 30). We found that 2 days after DBP-FITC application to the ear skin of naive mice there was significant LN hyperplasia detected (measured by quantifying live $CD45^+$ cells in the LN) and this was curtailed in *Hb*-infected mice (**Figure 3B**). The number of migratory DCs (defined as $CD45^+$ $B220^-$ $CD11c^{var}$ $MHCII^{high}$, **Figure 3C**) detected by flow cytometry in the skin dLN was not affected by *Hb* infection (**Figure 3D**). Neither was the proportion of these that were positive for FITC (**Figure 3E**), and the level of FITC uptake (illustrated by the median fluorescent intensity of the FITC detected) was the same in *Hb* and uninfected mice (**Figure 3F**). Furthermore, uptake of the FITC antigen leads to almost ubiquitous expression of CD86 compared to the lower proportion of $FITC^-$ DCs that express this activation marker (**Figure 3G**). Of note, *Hb* infection does not alter these proportions in either $FITC^+$ or $FITC^-$ DCs. The degree of CD86 expression by $FITC^+$ compared to $FITC^-$ DCs is around 5-fold higher (**Figure 3I**) and this ratio is not affected by *Hb* infection. Programmed death ligand-2 (PDL2), is a marker expressed on a subset of DCs that promote Th2 responses *in vitro* and *in vivo*, and are enriched in skin dLN (44). PDL2 was upregulated on $FITC^+$ compared to $FITC^-$ DCs in the LN after DBP-FITC sensitization, but again, *Hb* infection did not have an impact on the proportion of DCs expressing this marker (**Figure 3H**). Expression of PDL2 is around 1.5-fold higher on $FITC^+$ DCs and this is not altered by prior *Hb* infection (**Figure 3J**).

Collectively, these experiments show that although *Hb* infection reduced the levels of TSLP that could be detected in the skin, it did not appear to be enough of a reduction to translate into defects in migration to, or activation of DC in, the skin dLN.

Expression of Skin Derived Inflammatory Chemokines and Influx of Innate Cells Are Reduced in *H. bakeri* Infected Mice

We next considered whether *Hb* infection affected the local production of chemokines in the skin. There is a complex profile of chemokines expressed in skin after exposure to contact sensitisers, which orchestrates the influx of inflammatory cells throughout the course of the immune response (45). Skin tissue lysates were analyzed 12 h after DBP-FITC sensitization for a range of widely recognized inflammatory chemokines using a 13-plex bead array. Strikingly, the levels of the pro-inflammatory chemokines CXCL1 (or keratinocyte chemoattractant; KC) and CXCL5 (lipopolysaccharide-induced CXC chemokine; LIX) were found to be significantly reduced in the skin lysates of *Hb*-infected mice sensitized 12 h previously with DBP-FITC when compared to the skin lysates of uninfected mice sensitized with DBP-FITC (**Figures 4A,B**). Keratinocytes in the skin produce CXCL1 to recruit neutrophils in the initial stages of CHS (46), and CXCL5 is a neutrophil chemo-attractant in several skin inflammation settings (47, 48). Protein levels of CCL20 (macrophage inflammatory protein-3; MIP-3-alpha) and CCL22 (macrophage-derived chemokine; MDC) were also significantly reduced in skin from *Hb*-infected mice (**Figures 4C,D**). CCL20 acts to recruit immature DCs, effector and memory T cells, and to a lesser extent neutrophils, to sites of skin inflammation via interaction with its receptor CCR6 (49), and has been linked to inflammation of the skin in the context of psoriasis and AD in mice and humans (50–52). CCL22 plays a key role in the accumulation of $CD4^+$ T cells in the skin in AD and CHS (53, 54). Expression of the T cell recruiting chemokine CCL17 (thymus and activation regulated chemokine; TARC) was also reduced in *Hb*-infected mice, although this change did not reach statistical significance (**Figure 4E**).

CCL11 (eotaxin), the main chemoattractant for eosinophils, was detected in DBP-FITC treated skin but there was no difference in amounts detected between the lysates from control and *Hb*-infected mice (**Figure 4F**). None of the other chemokines measured were found to be downregulated by *Hb* infection (**Figure 4G**). Of note, skin left untreated in naïve mice produces very low levels of the inflammatory chemokines mentioned above, as application of the contact sensitiser is needed to elicit these early inflammatory events (45).

Since a number of key chemoattractant molecules were down-regulated in the skin of *Hb*-infected mice, we next determined whether the subsequent recruitment of innate and adaptive immune cell populations to the skin 24 h after DBP-FITC challenge was affected. Indeed, DBP-FITC challenge leads to a robust recruitment of $CD45^+$ cells to the ear skin tissue shown by both the absolute numbers and the proportions of live cells expressing CD45 (**Figure 5A**). In contrast, *Hb*-infected mice recruit significantly fewer $CD45^+$ cells when challenged with DBP-FITC. Even more strikingly, and corresponding with the decrease in production of the chemokines CXCL1 and CXCL5, proportions and total numbers of $Ly6G^+$ neutrophils within the $CD45^+$ cell compartment were significantly reduced in the ears of DBP-FITC-treated mice infected with *Hb* (**Figure 5B**). This finding was confirmed by immunofluorescent staining of ear sections which showed that after DBP-FITC challenge, a robust influx of neutrophils causes swelling and structural disruption under the skin surface (**Figure 5C**). Corroborating the flow cytometry results in **Figure 5B**, *Hb* infection almost completely abrogates this neutrophilic inflammation. Neutrophils are key innate effector cells in the initiation of CHS, and in their absence, CHS responses cannot be induced (46, 55).

There is a heterogenous population of macrophages, monocytes and moDCs in the skin (47, 48). Interestingly, we observed an increased proportion and number of skin $CD64^+$ macrophages in mice treated with DBP-FITC and

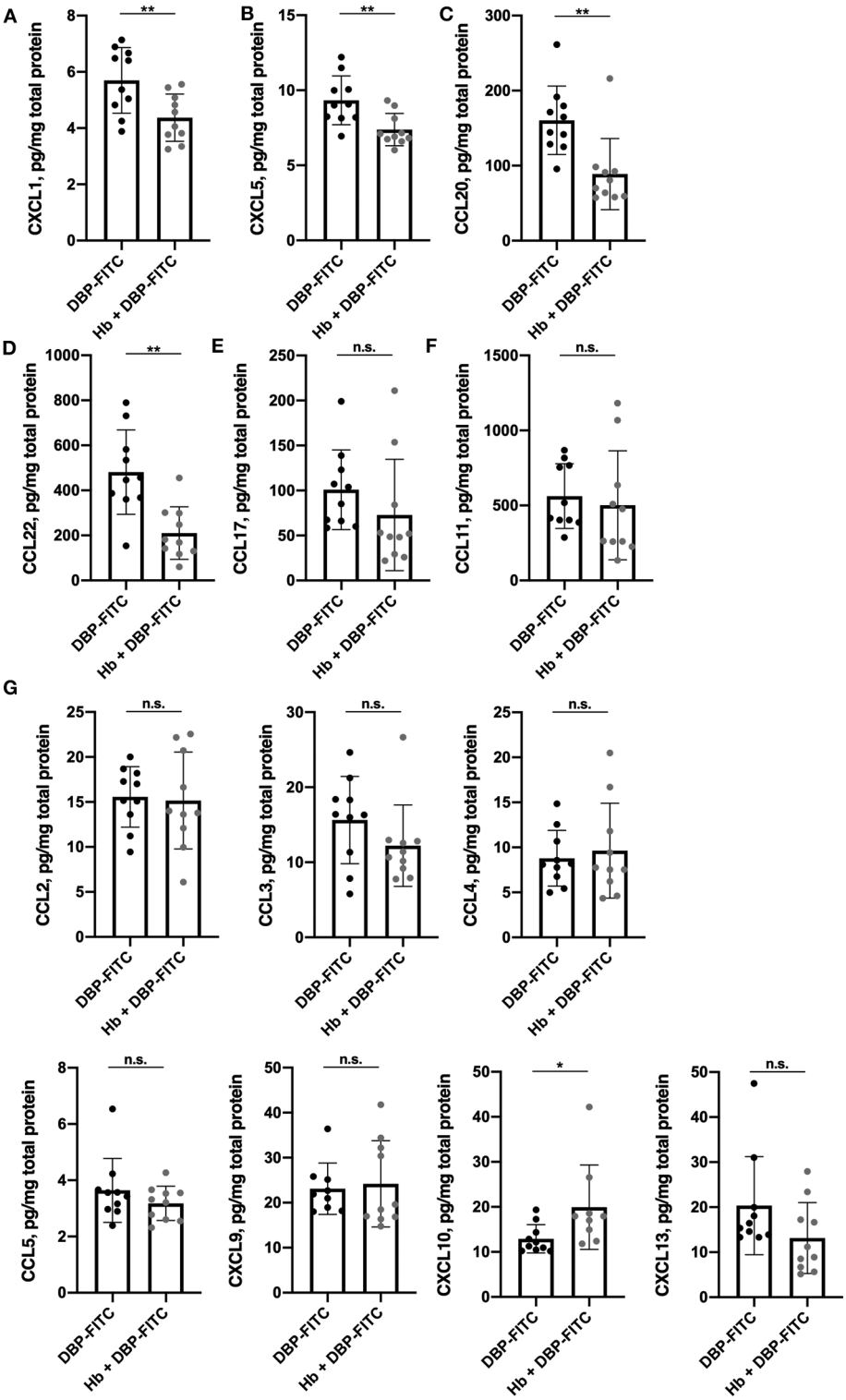

FIGURE 4 | Expression of a subset of skin-derived inflammatory chemokines is reduced in *H. bakeri*-infected mice after DBP-FITC sensitization. Chemokine protein expression in tissue lysate measured by LEGENDplex 12 h post- sensitization with DBP-FITC. **(A)** CXCL1, **(B)** CXCL5, **(C)** CCL20, **(D)** CCL22, **(E)** CCL17, **(F)** CCL11, **(G)** CCL2, 3, 4, and 5 and CXCL9, 10, and 13. Results are expressed as pg/mg total protein measured by Bradford assay. An unpaired *t*-test was used to assess statistical differences. Graphs represent data combined from 2 experiments and show mean ± SD. n.s., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$.

these were significantly reduced in mice infected with *Hb* (**Figure 5D**). A similar trend was observed in numbers of $Ly6C^+$ monocytes, although a less striking difference is seen in the proportions of these cells (**Figure 5E**). Eosinophils are a characteristic effector cell involved in allergic skin responses (32, 56) and were found in increased numbers in the skin after DBP-FITC treatment compared to in naïve mice (**Figure 5F**). Interestingly, *Hb* infection significantly increased the proportions of eosinophils within the $CD45^+$ compartment, although this did not translate into a change in numbers found in the skin between uninfected and infected mice treated with DBP-FITC (**Figure 5F**). This finding correlated with the similar levels of eosinophil chemoattractant CCL11 that were detected in the skin lysate in both uninfected and *Hb*-infected mice (**Figure 4F**). Skin inflammation in this DBP-FITC CHS model is $CD4^+$ T cell-dependent (32) and we found a significant increase in numbers of these cells in the skin after challenge compared to naïve mice which again, was decreased by prior helminth infection (**Figure 5G**). Of note, innate cells including neutrophils, macrophages, monocytes and eosinophils account for the majority of the inflammatory cellular milieu at the time point assessed.

Taken together, these results suggest that *Hb* infection limits the DBP-FITC-induced accumulation of some key innate inflammatory cell populations in the skin, in particular neutrophils, via a reduced production of the chemokines associated with their recruitment into skin tissues.

DISCUSSION

We have identified that mice infected with the intestinally confined helminth parasite *Hb* mount impaired inflammatory responses to skin allergens such as DBP-FITC and HDM when compared to uninfected mice. The suppression of skin and draining LN responses to DBP-FITC appeared to be due to a reduction in the release of chemokines involved in attracting neutrophils to sites of inflammation. Neutrophils are the first cells to respond to infection or damage in many tissues (57). Depletion of neutrophils, using either a regime of monoclonal antibody injection (46) or via genetic deficiency (55), has illustrated how important these cells are in both the sensitization and challenge phases of CHS. Helminth infection has previously been shown to limit skin inflammation via the downregulation of chemokines that attract neutrophils (58). However, other molecules such as the eicosanoid leukotriene B4 (59) and the transcription factor NF-E2–related factor-2 (Nrf2) (60) play critical roles in the recruitment of neutrophils to the skin during inflammation. Future work could assess whether molecules in the skin other than chemokines are affected by helminth infection, and whether depletion of neutrophils can indeed mimic the effects of *Hb* in our particular model of CHS.

Although we also saw a significant reduction in numbers of macrophages and monocytes in the skin of *Hb*-infected mice 24 h after DBP-FITC challenge, this did not correlate with reduced expression of chemokines normally associated with these cells earlier in the model (**Figure 4**). This may be due to the fact that chemokines are known to be sequentially expressed in skin during CHS (61) and so undertaking a more thorough examination of chemokine expression over a time-course of inflammation may reveal differences between infected and uninfected mice. Also, we did not stain for chemokine receptors on the surface of the cells from the skin, so it may also be that although chemokine levels themselves are similar in uninfected and *Hb*-infected mice, helminth infection could impact the cells' responsiveness, due to decreased receptor expression. *In vitro* experiments testing the ability of different cell types from uninfected and infected donors to migrate along a chemokine gradient, or *in vivo* cell tracking experiments may shed light on the mechanisms involved.

Proportions of eosinophils are increased in the skin in CHS as we (**Figure 5F**) and others have shown (28, 32). However, although *Hb* infection increased the proportions of $SiglecF^+$ cells within the $CD45^+$ compartment in the skin, this did not translate to absolute numbers of eosinophils counted (**Figure 5F**). Perhaps surprisingly, we saw no differences between infected and uninfected mice in early expression of the eosinophil chemoattractant CCL11 after DBP-FITC challenge, and so measurement of other molecules that recruit and activate eosinophils, such as IL-5 would be logical. Similar results have been noted in a previous study that found chronic *Litomosoides sigmodontis* infection protected against skin hypersensitivity (58). In this study, eosinophil numbers in the skin after allergen challenge were not affected by helminth infection, however, their production of the neutrophil chemoattractants CXCL1 and CXCL2 were, and this resulted in a greatly reduced neutrophil influx (58). Therefore, it is a possibility the functionality of eosinophils could also be affected by *Hb* infection in our model and further experiments would be needed to investigate this.

Several papers have demonstrated that mast cells are important for CHS responses and that they play a role in recruitment of neutrophils to the skin via production of chemokines (55, 62). Basophils have also been implicated in allergic skin responses, in particular in IgE-mediated allergic dermatitis models, where allergen-specific IgE mediates the activation and degranulation of basophils to release their inflammatory mediators via ligation of the high affinity IgE-receptor FcER1 on their surface and cross-linking by the allergen molecule (63–65). Interestingly, one paper investigating different types of allergic response found that contact hypersensitivity with a chemical hapten was not affected by depletion of basophils (64), whereas another found a significant attenuation of ear swelling after hapten application in basophil depleted mice (66). Although we did not assess mast cell or basophil recruitment in our current model, previous work from our group has shown, using the Basoph-8 reporter mice (67), that basophils are recruited to the skin after injection with the allergen HDM and after prolonged topical application of a vitamin D analog MC903 that induces AD-like skin inflammation (68), and these mice could be utilized in the future to further understand the role of basophils in the *Hb*/DBP-FITC model. Indeed, studies in both mice and humans have implicated that helminth infection can reduce the responsiveness of basophils

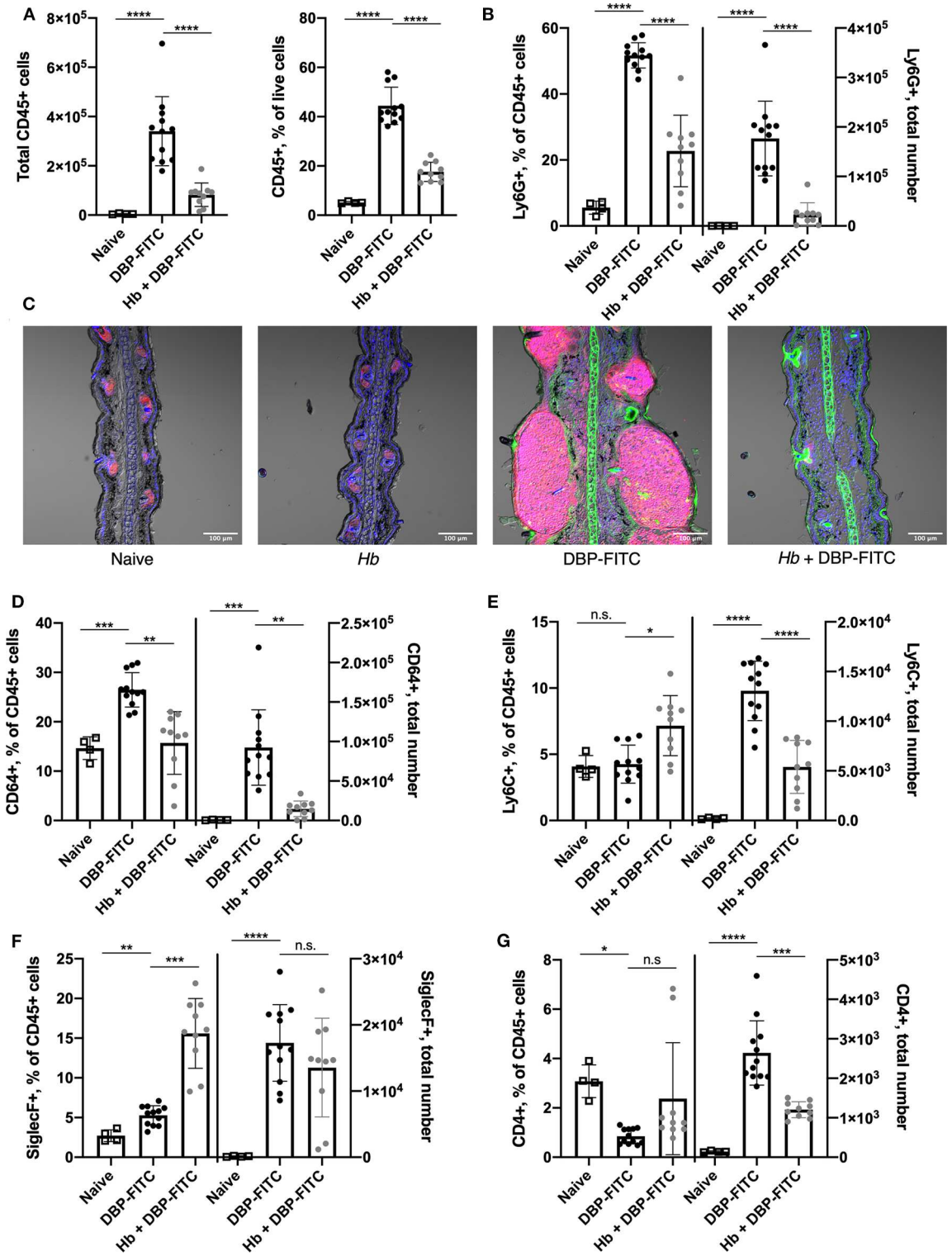

FIGURE 5 | Prior Hb infection limits influx of innate inflammatory cells after DBP-FITC challenge. **(A)** Total number of CD45+ cells and their frequency within all live cells in the skin tissue at day 7 (24 h after DBP-FITC challenge). **(B)** Frequency within CD45+ cells, and total number of neutrophils (Ly6G+) in the skin at day 7. **(C)**. Immunofluorescent staining of ear sections taken at day 7. Ly6G+ neutrophils (pink), FITC (green) and DAPI (blue). Scale bar represents 100 um. **(D)** Frequency within CD45+ cells and total number of macrophages (CD64+), **(E)** monocytes (Ly6C+), **(F)** eosinophils (SiglecF+), and **(G)** CD4+ T cells in the skin at day 7. A one-way ANOVA with Tuckey's multiple comparisons test was used to test statistical significance. Graphs represent data combined from 2 experiments and show mean ± SD. n.s, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

to IgE (69, 70). Measuring the production of allergen-specific antibodies, particularly IgE, would be an interesting next step in our model as well as assessment of basophil responsiveness to antibody.

DCs are vital for the initiation of the immune response to haptens in the skin and several studies have dissected the roles for the different DC subsets at this stage. Mice lacking epidermal Langerhan's cells or dermal $CD103^+$ DCs can still elicit Th2 responses in the dLN after DBP-FITC sensitization (30), with the highest number of $FITC^+$ DCs in the LN made up of $IRF4^+$ $CD11b^+$ and $CD11b^{lo}$ $CD103^{lo}$ $CD326^{lo}$ $CD301b^+$ dermal DCs which express high levels of MHCII and the activation marker CD86, and are involved in other Th2 skin responses (30, 71–73). Signaling through the TSLP receptor via STAT5 in DCs is necessary for the initiation of Th2 responses in the skin draining LN (74) and dermal DC subsets that are preferentially activated in the DBP-FITC CHS model are highly responsive to TSLP (28, 29). We found that expression of TSLP was reduced in the skin of *Hb*-infected mice after DBP-FITC treatment, although this did not appear to affect DC antigen uptake, activation or recruitment to the draining LN during the early stages of the inflammatory response. We did not see a difference in proportions of $CD4^+$ T cells in the skin after DBP-FITC challenge in uninfected and infected mice. However, assessment of their activation by DCs, in terms of cytokine output upon restimulation would be a logical experiment to perform in the future.

Although previous work has highlighted the importance of induced Tregs in helminth-mediated suppression of immune responses [reviewed recently in (75)], our work points to a Treg-independent mechanism, as Treg depletion had no discernible effect on *Hb*-induced suppression of DBP-FITC inflammation. However, as the anti-CD25 depletion regime is not 100% effective (our protocol resulted in a 60–75% depletion of $CD4^+$ $FoxP3^+$ Tregs in the skin draining LN), the effect of helminth-induced Tregs cannot be completely ruled out and more complete models of Treg deletion [such as diptheria toxin injection into FoxP3-iDTR (76) or DEREG mice (77)] could be used in the future to confirm this finding.

Individual molecules isolated from the *Hb* excretory-secretory milieu have been found to have therapeutic potential in a number of inflammatory disease settings (78). However, our results showing a continued suppression of DBP-FITC-induced inflammation in the ear after worm clearance suggest that the active secretion of molecules by live worms into the intestinal environment does not directly impact cellular infiltration into the skin and draining LN after DBP-FITC challenge. However, this does not preclude either a long-lasting effect on cells by helminth-derived molecules, or the secretion of molecules that have long half-lives and long-range effects. *Hb* excretory-secretory products (HES) contain a complex mix of proteins (79, 80) and only a few have been characterized fully (18, 79, 81–83). So far, the half-lives and pharmaco-kinetics of individual, or families of, molecules made by the helminth are yet to be determined. An effect of long-lived and long-range worm-derived molecules, present even after infection is cleared by drug therapy, cannot be ruled out and further experiments could be undertaken to further discriminate the role of HES in this model. Intriguingly, injection of *Schistosoma mansoni* egg-derived chemokine binding protein (SmCKBP) limits neutrophil infiltration in a model of CHS by neutralizing the activity of several neutrophil-specific chemokines (84) and the hookworm *Ancylostoma caninum* produces a glycoprotein that inhibits neutrophil migration *in vitro* (85). Furthermore, analogs of the immunomodulatory molecule ES-62 from *Acanthocheilonema viteae* can ameliorate oxazolone-induced skin inflammation (86). Interestingly, it has been shown that the main cellular targets for glucocorticoids, the widely used anti-inflammatory treatment for allergic skin conditions, are macrophages and neutrophils (the cell types we found to be most impacted in infected mice), via binding of the glucocorticoid receptor (GR) (87). It is possible that *Hb* secretes a molecule that binds to GR on these cells to directly modulate their function or activation and prevent their accumulation into the skin in infected mice after DBP-FITC challenge. Previous studies have shown that other helminths do produce steroid hormones (88) but it is not known whether *Hb* makes such molecules.

One other study utilizing *Hb* infection and skin allergic responses showed that *Hb* did not reduce inflammation after prolonged epicutaneous OVA application, although in concordance with our findings, there was evidence of decreased $CD4^+$ T cell infiltration into the skin in infected mice (89). The model used is technically very different from ours, using OVA applied for long periods of time (three, 1-week long applications on skin patches) with severity of disease measured by an observational clinical skin score and cells counted after immunohistological staining (89). Importantly, in this study, mice were infected with *Hb* larvae at the same time as systemic sensitization to the OVA allergen was started. In our model, the larvae had already had 2 weeks to undergo full maturation and were established in the small intestine as adults before the DBP-FITC model was initiated. This may point to a role for adult worms at the early stages of sensitization, or a change in systemic distribution or activation of immune cells by the presence of worm infection that alters the response in distal sites to an allergen upon first encounter. Supporting this, *Hb* has been shown to alter allergic responses in a number of tissues distal to the site of the infection in the intestine, and many of the experimental models use infection before the onset of allergic sensitization (7, 11). It would be interesting to see if infecting mice in between allergen sensitization and challenge in our DBP-FITC model would alter the magnitude of the response compared to in uninfected animals. A model of this kind would also mirror a scenario more likely to arise in human conditions whereby intervention to prevent allergic responses would likely occur after sensitization occurred rather than beforehand.

There is evidence from epidemiological studies showing an inverse correlation between infection with helminths and severity of allergic reactions in the skin in humans (1, 2, 90, 91). Our study points to the interesting possibility that controlled infections of humans with helminths of a similar nature to *Hb* could be used to prevent and potentially treat chronic skin diseases such as atopic dermatitis and psoriasis. The potential for therapeutic treatment with helminths for a number of inflammatory and allergic diseases is already

being explored with mixed results (92–95). Additionally, helminth-derived molecules have recently started to be tested in human subjects (96), and could yet be shown to have a role in limiting inflammation in the skin, perhaps upon topical application.

Importantly, we have preliminary evidence that a helminth-induced amelioration of skin responses is not limited to DBP-FITC-induced inflammation, but extends to other clinically relevant skin inflammation models, including the injection of the ubiquitous HDM (**Figure 1**) and the application of a vitamin D analog contact sensitizer MC903 (manuscript in preparation).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee, Victoria University of Wellington, New Zealand.

AUTHOR CONTRIBUTIONS

KF conceptualized the project, performed and supervised the experiments, analyzed and interpreted data, and wrote the manuscript. PM developed methodologies, performed experiments, and edited the manuscript. KM developed methodologies and performed experiments. CP provided expertise, developed methodologies, and performed experiments. AS provided expertise. GL supported design of the project and experiments, supported analysis of the data, and edited the manuscript.

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Extracellular Vesicles Derived From *Trichinella spiralis* Muscle Larvae Ameliorate TNBS-Induced Colitis in Mice

Yong Yang[1†], Lei Liu[1†], Xiaolei Liu[1†], YuanYuan Zhang[1], Haining Shi[2], Wanzhong Jia[3], HongFei Zhu[4], Hong Jia[4], Mingyuan Liu[1,5] and Xue Bai[1*]*

[1] Key Laboratory of Zoonosis Research, Ministry of Education, Institute of Zoonosis, College of Veterinary Medicine, Jilin University, Changchun, China, [2] Mucosal Immunology and Biology Research Center, Massachusetts General Hospital, Boston, MA, United States, [3] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China, [4] Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China, [5] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China

Helminths are masters at modulating the host immune response through a wide variety of versatile mechanisms. These complex strategies facilitate parasite survival in the host and can also be exploited to prevent chronic immune disorders by minimizing excessive inflammation. Extracellular vesicles (EVs) are small membrane-bound structures secreted by helminths which mediate immune evasion during parasite infection. The goal of this study was to investigate the immunoregulatory properties of *Trichinella spiralis* EVs (*Ts*-EVs) in a murine model of colitis. We found that *Ts*-EVs significantly ameliorated 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. *Ts*-EVs alleviated intestinal epithelium barrier damage, markedly reduced pro-inflammatory cytokine secretion and neutrophil infiltration, and upregulated immunoregulatory cytokine expression in colon tissue. *Ts*-EVs also modulated the adaptive immune response by influencing T-cell composition. The numbers of Th1 and Th17 cells in MLNs, as well as the expression levels of Th1/Th17-associated cytokines and transcription factors in colon were reduced. In contrast, Th2 and Treg cells were increased after *Ts*-EVs treatment. Furthermore, sequencing of EV-derived microRNAs (miRNAs) indicated that an array of miRNAs was involved in the regulation of the host immune response, including inflammation. These findings expand our knowledge of host-parasite interactions, and may help design novel and effective strategies to prevent parasite infections or to treat inflammatory diseases like IBD. Further studies are needed to identify the specific cargo molecules carried by *Ts*-EVs and to clarify their roles during *T. spiralis* infection.

Keywords: *Trichinella spiralis*, extracellular vesicles, experimental colitis, immunomodulation, parasite-host communication

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***Correspondence:**
Mingyuan Liu
liumy36@163.com
Xue Bai
namiya23@163.com

† These authors have contributed equally to this work

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Yang Y, Liu L, Liu X, Zhang Y, Shi H, Jia W, Zhu H, Jia H, Liu M and Bai X (2020) Extracellular Vesicles Derived From Trichinella spiralis Muscle Larvae Ameliorate TNBS-Induced Colitis in Mice. Front. Immunol. 11:1174. doi: 10.3389/fimmu.2020.01174

INTRODUCTION

Trichinellosis is one of the most serious food-borne parasitic diseases in the world. It is caused by the intracellular parasitic helminth *Trichinella spiralis*, which is both a public health problem and a challenge for the economic productivity of the pig industry. Accidental ingestion of raw or undercooked meat containing infectious larvae of *T. spiralis* results in human infections. Due to changing diets and cooking habits, trichinellosis is considered an emerging or re-emerging infectious disease in several regions of the world (1).

In order to successfully invade and establish a chronic infection in the host and to prevent host immune attack, helminths, including *T. spiralis*, have evolved a variety of sophisticated immunoregulatory mechanisms. Some of these strategies have been identified, including induction of immune cell apoptosis, disruption of pattern recognition receptors (PRRs) and downstream signaling pathways, inhibition of the complement system, regulation of dendritic cell (DC) and macrophage differentiation toward tolerogenic DC and M2 macrophage phenotypes, expansion of the number of Treg cells and induction of Th2 immune responses (2). The ability of helminths to manipulate the host immune response and create an anti-inflammatory environment not only facilitate long-term parasite survival, but also protect against inflammatory disorders.

In this regard, growing numbers of epidemiological investigations in different regions of the world have found an inverse relationship between the prevalence of autoimmune diseases like IBD and parasitic infections, giving rise to the so-called "hygiene hypothesis" (3). Based on these investigations and hypothesis, the therapeutic potential of parasitic helminths against autoimmune diseases has been investigated in animal models. This approach has showed promising results, protecting against or alleviating numerous inflammatory conditions, such as inflammatory bowel disease (IBD), allergic airway inflammation, type I diabetes and autoimmune encephalomyelitis (4). Therefore, immunomodulatory "helminth therapy" is an attractive autoimmune therapy approach.

Diverse biomolecules released from or expressed on the surface of parasites, such as active enzymes and inhibitors, play a pivotal role mediating host-parasite interactions (5). Identification of parasite effector molecules has focused mainly on active protein moieties, whereas other functional structures have been neglected. Extracellular vesicles (EVs) are small membrane-bound vesicles secreted by parasites which contain functional proteins, carbohydrates, lipids, mRNA and non-coding RNAs. Recent studies showed that delivery of bioactive molecules and functional microRNAs from helminths to different host cells via EVs results in a series of intracellular signaling events that modulate host-parasite interactions and help parasites establish long term infections in inhospitable environments (6). For example, it was shown that EVs released by the gastrointestinal nematode *Heligmosomoides polygyrus* reduced Th2-type innate immune responses and eosinophilia induced by the allergenic fungus Alternaria, indicating that EVs can modulate the host innate immune response during parasitic infection (7). EVs released by *Echinococcus* metacestodes interfered with the antigen presentation pathway in murine dendritic cells and regulated macrophage activation through the LPS/TLR4 signaling pathway (8, 9). Using animal models of inflammatory diseases, studies have shown that EVs from *Nippostrongylus brasiliensis* and *Fasciola hepatica* can protect against inflammatory bowel disease (IBD) by regulating host immune balance (10, 11).

A recent study showed that *T. spiralis* muscle larvae also release EVs with immunomodulatory potential, with effects similar to whole ES L1 in regulating PBMC activity (12). Despite intense interest, information on the role of *T. spiralis* EVs in parasite-host communication is still limited. The goal of this study was to characterize, in detail, the miRNA composition of EVs released by the muscle larval stage of *T. spiralis,* and to investigate their potential regulatory effects on the host immune response in a murine model of colitis. Our results offer novel insights on the interaction between *T. spiralis* and the immune system of the host, on how this parasite immunoregulates its environment during infection, and also suggests new therapeutic tools to treat inflammatory diseases.

MATERIALS AND METHODS

Animals and Ethics Statement

Female Balb/c mice aged 6–8 weeks (18–20 g) and female Wistar rats were purchased from the Experimental Animal Center of Jilin University. All animal experiments were in strict accordance with the National Institutes of Health guidelines (publication no. 85–23, revised 1996). Animals protocols were reviewed and approved by the Ethics Committee of Jilin University, affiliated with the Provincial Animal Health Committee, Jilin Province, China (Ethical Clearance number 201803022).

Isolation of *T. spiralis* Muscle Larvae Extracellular Vesicles (*Ts*-EVs)

Trichinella spiralis (ISS534) was maintained in Wistar rats. Muscle larvae (ML) were recovered from the muscles of infected rats at 35 days post infection (dpi) by pepsin-HCl digestion, followed by cultivation in RPMI-1640 at 37°C and 5% CO_2. After 18 h of culture, excretory/secretory products (ESPs) of ML were collected and centrifuged at 800 g/10 min and then at 5,000 g/20 min to remove parasite debris. The supernatant was filtered using low-protein binding 0.22 μm pore filters (Merck Millipore, USA) and then concentrated using a 10 kDa spin concentrator (Merck Millipore, USA) (13). For the isolation of *Ts*-EVs, concentrated ESPs were subjected to ultracentrifugation at 120,000 g for 2 h at 4°C using an Optima TL100 ultracentrifuge (Beckman Coulter, USA) with a TLA-55 rotor. They were washed with PBS and ultracentrifuged again at 120,000 g for 2 h at 4°C (14). The resulting pellets were resuspended in a small volume (200 μL) of PBS. *Ts*-EVs concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Beyotime, China) and then *Ts*-EVs were stored at 4°C until use.

Characterization of *Ts*-EVs by Transmission Electron Microscopy, NanoSight, and Western Blotting

Firstly, the morphology, structure and size of *Ts*-EVs were analyzed by negative staining transmission electron microscopy (TEM). Briefly, a 10 μL drop of *Ts*-EVs suspension was adsorbed onto a copper grid for 1 min and then immersed in 2% glutaraldehyde at room temperature. Then the grid was negatively stained using 2% phosphotungstic acid for 1 min, and air dried at room temperature. The *Ts*-EVs were examined using a HitachiH-7650 transmission electron microscope (Hitachi Limited, Japan) at 80 kV. Secondly, *Ts*-EVs particles were characterized in terms of their size and concentration using a NanoSight NS300 instrument (Malvern Instruments, United Kingdom).

Finally, to identify specific markers of *Ts*-EVs, equal amounts of samples (30 μg per well, *Ts*-EVs and ML crude proteins) were electrophoresed in a 12% SDS-PAGE gel and transferred to a PVDF membrane (Immobilon, Millipore, USA). The membrane was blocked with 5% bovine serum albumin (BSA) and incubated with the primary antibody, which included polyclonal rabbit anti-CD63 (1:1,000, Abcam, United Kingdom) and polyclonal goat anti-enolase (1:200, Abcam, United Kingdom). Subsequently, two different horseradish peroxidase (HRP)-conjugated secondary antibodies were used: goat anti-rabbit IgG (1:2,000, Cell Signaling, USA) and donkey anti-goat IgG (1:50,000, Jackson ImmunoResearch, USA). Peroxidase activity was visualized using the ECL Plus Western blotting detection system (GE Healthcare Buckinghamshire, UK).

TNBS-Induced Colitis and Treatment With *Ts*-EVs

Colitis was induced by intrarectal administration of 100 μL of 1.25 mg 2,4,6-trinitrobenzene sulfonic acid (TNBS) solution (Sigma, USA). A total number of 40 mice were randomly assigned to 4 different groups (10 mice/group): PBS as control group; TNBS group; TNBS+PBS group; and TNBS+*Ts*-EVs group. Briefly, mice deprived of food for 24 h were lightly anesthetized with sodium pentobarbital (50 mg/kg, ip). Next, colitis was induced by intrarectal administration of TNBS (1.25 mg in 100 μL of a 50% ethanol solution) through a flexible catheter inserted 3.5 cm into the rectum. Before the induction of colitis, mice in the TNBS+*Ts*-EVs group were injected intraperitoneally three times with *Ts*-EVs (50 μg/mice) resuspended in 100 μL PBS, and mice in the TNBS+PBS group were injected intraperitoneally (IP) three times with PBS as control group. All mice were sacrificed 3 days after intrarectal administration of TNBS.

Assessment of Colitis

Mice were observed daily and scored for disease severity by using a 6-point scale disease activity index (DAI). Scores depended on weight loss, stool shape, and presence of blood in the stool (**Table 1**). On day 3, all mice were killed and their colons were removed. We measured the length of the colon as an indirect indicator of inflammation and macroscopically assessed the degree of colonic damage. Three parameters were considered: degree of hyperemia, wall thickness, and degree of colonic ulceration. The total score ranged from 0 to 9 (**Table 2**). All scores were determined by observers blinded to the treatment groups.

Approximately 1 cm of colon was resected for histopathology examination, fixed in 4% neutral-buffered formalin, embedded in paraffin, sectioned at 5 μm thickness and stained separately with hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS). We determined the histological damage score to measure the severity of inflammation based on the following parameters (**Table 3**): extent of inflammation, inflammatory cell infiltration, extent of crypt damage, and loss of goblet cells. The total score ranged from 0 to 8.

MPO Activity Assay

Inflammatory cell (polymorphonuclear neutrophil) infiltration into colonic tissue was quantified by measuring MPO activity with an MPO assay kit (Nanjing Jiancheng Bio-engineering Institute, China), following the manufacturer's instructions. MPO activity was expressed as units per gram of total protein (U/g).

Intestinal Permeability Assay

Intestinal permeability was assessed by using fluorescein isothiocyanate-labeled dextran (FITC-D) with molecular weight of 4,000 Da (Sigma). Briefly, 2 days after induction of colitis by TNBS treatment, mice were orally administrated with 600 mg/kg of FITC-D. After 4 h administration, blood was collected from the eyes of the mice and centrifuged at 12,000 g for 5 min to separate

TABLE 1 | The criteria of Disease Activity Index (DAI) score.

Weight loss (%)	Stool shape	Stool bleeding	Score
<4%	Normal	None	0
4-10%	Loose stool	Slight bleeding	1
>10%	Diarrhea	Severe bleeding	2

TABLE 2 | The criteria of colonic macroscopic score.

Hyperemia	Wall thickening	Ulcer	Score
None	None	None ulcer	0
Focal	Slight	Slight ulcer	1
Multifocal	Moderate	Moderate ulcer	2
Diffuse	Severe	Severe ulcer	3

TABLE 3 | The criteria of colonic histopathological score.

Extent of inflammation	Inflammatory cell infiltration	Extent of crypt damage	Loss of goblet cells	Score
None	None	None	None	0
Mucous layer	Focal	Slight	Focal	1
Serous layer	Diffuse	Severe	Diffuse	2

serum. Fluorescence intensity in the serum was measured by a microplate reader with excitation and emission wavelengths of 490 and 525 nm, respectively. The concentrations of FITC-D were calculated by a standard curve generated by serial dilution of FITC-D. Each sample was measured in triplicate.

Immunofluorescence Analysis

Colonic tissue sections were deparaffinized and subjected to an antigen retrieval process. Briefly, sections were heated in sodium citrate buffer (pH 6.0) with a 500-watt microwave oven until the temperature reached 100°C. When the temperature of the sodium citrate buffer dropped below 60°C, tissue slices were treated with protease K. Non-specific binding was blocked by incubating with 5% normal goat serum for 30 min at room temperature. For occludin and zonula occludens-1 (ZO-1) staining, sections were separately incubated with rabbit monoclonal anti-occludin antibody (1:50 dilution; Abcam, USA) and rabbit polyclonal anti-ZO-1 antibody (1:200 dilution; Abcam, USA) overnight at 4°C. After wards, sections were washed with PBS and incubated with Alexa Fluor 555 goat anti-rabbit IgG antibody (1:400 dilution; Abcam, USA) in the dark for 45 min at room temperature. Sections were then washed with PBS, stained with Hoechst dye (1:2,000 dilution; Abcam, USA) for 5 min and washed again with PBS. Finally, images of the stained sections were analyzed by confocal microscopy.

Cytokine Assays

Colon tissue was homogenized in tissue protein extraction reagent (Thermo Scientific, USA). After completion of lysis, the homogenized tissue was centrifuged at 12,000 g for 10 min. The collected supernatant was stored at −80°C until analysis. Levels of Th1 cytokines (IL-1β, IFN-γ, and TNF-α), Th2 cytokines (IL-4 and IL-13), a Th17 cytokine (IL-17A), regulatory cytokines (IL-10 and TGF-β) and chemokines (MCP-1 and MIP-3α) were analyzed using the Meso Scale Discovery (MSD) electrochemiluminescence platform. All measurements were performed in triplicate. The average absorbance at 620 nm was determined for each sample and was used to calculate cytokine concentrations in picograms per milliliter (pg/mL).

RNA Extraction and Quantitative Real-Time PCR

Colon RNA was extracted, purified (Qiagen, Germany) and converted to cDNA (Stratagene, USA), following the manufacturer's instructions. Quantitative real-time PCR was performed using FastStart Universal SYBR Green Master (Rox) reagents (Roche Diagnostics, Indianapolis, IN) and a 7500 Real-Time PCR machine (Applied Biosystems, Foster City, CA). Primer sequences are listed in **Table 4**. The reaction conditions were: 95°C for 10 min; followed by 40 cycles of 95°C for 15 s, 56°C for 1 min and 72°C for 1 min; and concluding with a melting curve analysis. Fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Flow Cytometry Analysis

All antibodies and reagents were purchased from BD (BD Biosciences, USA). Mesenteric lymph node (MLN) cells were isolated from different groups of mice. For analysis of intracellular cytokine expression, MLN cells were stimulated with Leukocyte Activation Cocktail (2 μL of Cocktail for every 1 mL of cell culture; approximately 1×10^6 cells/mL) containing PMA, ionomycin and GolgiPlug™ (Brefeldin A) for 5 h at 37°C and 5% CO_2. Next, the cultured cells were collected and incubated with purified monoclonal rat anti-mouse CD16/CD32 (0.5 mg/mL) antibody for 10 min to block non-specific staining, and then with FITC-conjugated monoclonal rat anti-mouse CD4 (0.5 mg/mL) antibody for 30 min at 4°C. After fixation and permeabilization with Cytofix/Cytoperm solution, the cells were intracellularly stained with APC-conjugated monoclonal rat anti-mouse IFN-γ and IL-4 (0.2 mg/mL) antibody, PE-conjugated monoclonal rat anti-mouse IL-13 (eBioscience, USA) and IL-17A (0.2 mg/mL) antibody for 45 min at 4°C. In order to analyze $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, MLN cells were first incubated with FITC-conjugated monoclonal rat anti-mouse CD4 (0.5 mg/mL) antibody and APC-conjugated monoclonal rat anti-mouse CD25 (0.2 mg/mL) antibody for 30 min at 4°C. After washing, cells were fixed, permeabilized and stained with PE-conjugated monoclonal rat-anti mouse Foxp3 (0.2 mg/mL) antibody for 30 min at 4°C. The corresponding fluorochrome-labeled isotype control antibodies served as negative controls. Data were acquired on a FACS Aria II flow cytometer (BD Biosciences) and samples were analyzed using FlowJo software (TreeStar, USA).

TABLE 4 | Primers used for real-time PCR analysis.

Genes	Primer	Sequence(5′-3′)
T-bet	Forward primer	TCAACCAGCACCAGACAGAG
	Reverse primer	AACATCCTGTAATGGCTTGTG
RORγt	Forward primer	AGTGTAATGTGGCCTACTCCT
	Reverse primer	GCTGCTGTTGCAGTTGTTTCT
GAGT3	Forward primer	CTTATCAAGCCCAAGCGAAG
	Reverse primer	CCCATTAGCGTTCCTCCTC
Foxp-3	Forward primer	GGTATATGCTCCCGGCAACT
	Reverse primer	GATCATGGCTGGGTTGTC
IFN-γ	Forward primer	GCTCTGAGACAATGAACGCT
	Reverse primer	AAAGAGATAATCTGGCTCTGC
IL-17A	Forward primer	ATCCCTCAAAGCTCAGCGTGTC
	Reverse primer	GGGTCTTCATTGCGGTGGAGAG
IL-4	Forward primer	TTGTCATCCTGCTCTTCTTTCT
	Reverse primer	CTGTGGTGTTCTTCGTTGCT
IL-10	Forward primer	CCTCAGTTCCCATTCTATTTATTCACT
	Reverse primer	TTGAAAGGACACCATAGCAAAGG

Small RNA Library Construction and Bioinformatics Analysis

Biological replicates of *T. spiralis* EVs (*Ts*-EVs) isolated from three different batches of muscle larvae were analyzed. For the analysis of small RNAs, total RNA from *Ts*-EVs was extracted using Trizol according to the manufacturer's

instructions (Life Technologies). Purity and concentration of total RNA were analyzed with an Agilent 2100 system (Agilent Technologies, Santa Clara, CA, USA) to make sure that samples were of sufficiently high quality for sequencing. Small RNAs between 18 and 30 nt were isolated by 15% polyacrylamide gel electrophoresis (PAGE) and small RNA libraries were prepared using a TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA, USA), following the manufacturer's instructions. The generated libraries were sequenced using an Illumina®HiSeq 2000/2500 system (Beijing Genomics Institute, Shenzhen, China).

After filtering the raw data, the clean reads were mapped to the draft genome sequence of *T. spiralis* using SOAP software. For miRNA analysis, miRBase v. 22 and GenBank was used to identify known *T. spiralis* miRNAs from the clean reads. Finally, the unmatched small RNAs were analyzed using Mireap software (http://sourcefor-ge.net/projects/mireap) for novel miRNA prediction analysis based on the hairpin structure characteristics of the miRNA precursor. Only common overlapping miRNA sequences in all replicates were selected for further analyses. To determine the potential roles of specific miRNAs from *Ts*-EVs in host cells, miRanda and TargetScan were used to predict the mouse target genes of miRNAs. The predictions from the two tools were combined and the common target genes were the basis for further analysis. A clustering heatmap was drawn using the statistical programming language R package pheatmap (http://cran.r-project.org/web/packages/pheatmap/index.html) and the ggplot2 package was utilized to identify and annotate the target genes.

Statistical Analysis

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the GraphPad Prism 5 software for Windows. One-way analyses of variance (ANOVA) followed by Tukey's multiple-comparison test were used to compare significant differences between different conditions. Different p-values are shown as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

RESULTS

Characterization of *T. spiralis* Muscle Larvae Extracellular Vesicles (*Ts*-EVs)

In order to verify the quality of the *Ts*-EVs preparations, transmission electron microscopy (TEM) was used to evaluate their size and morphology. TEM revealed that most EVs were closed round vesicles with a diameter of 30–150 nm (**Figure 1A**). Western blot analysis showed that the specific markers of EVs, CD63 and enolase, were present in *Ts*-EVs (**Figure 1B**). Furthermore, NanoSight was used to study the size distribution of EVs. This analysis showed that the peak size of *Ts*-EVs was 133 nm and that most measured between 50 and 250 nm (**Figure 1C**). All these data indicate that these vesicles are *Ts*-EVs.

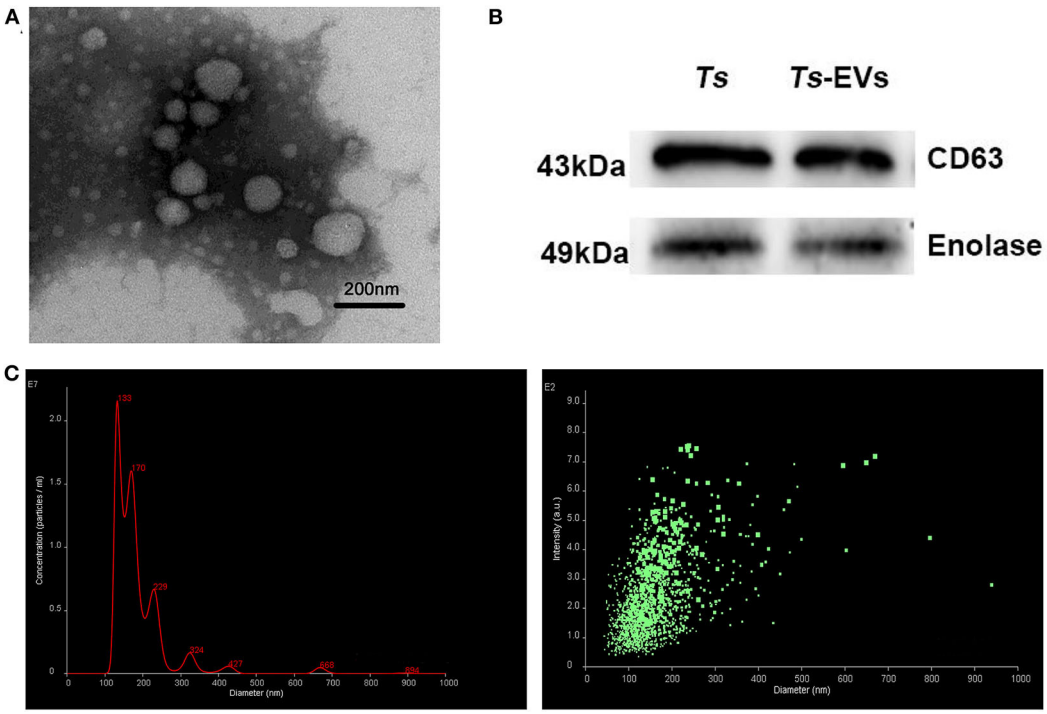

FIGURE 1 | Characterization of *T. spiralis* muscle larvae extracellular vesicles (*Ts*-EVs). **(A)** *Ts*-EVs ultrastructure was visualized by negative-staining TEM (magnification 40,000×; scale-bars = 200 nm). **(B)** Expression of *Ts*-EVs-specific markers CD63 and enolase was determined by Western blotting. **(C)** Size distribution profile of *Ts*-EVs was investigated using NTA. TEM: Transmission electron microscopy; NTA: Nano tracking analysis.

Ts-EVs Ameliorate the Severity of TNBS-Induced Colitis in Mice

To test whether *Ts*-EVs could ameliorate TNBS-induced colitis, Balb/c mice were intraperitoneally injected three times with *Ts*-EVs (50 μg/mice) every 3 days before colitis induction. As expected, intrarectal TNBS administration to mice induced obvious symptoms of colitis such as weight loss (13%), diarrhea and rectal bleeding, compared with the control mice. However,

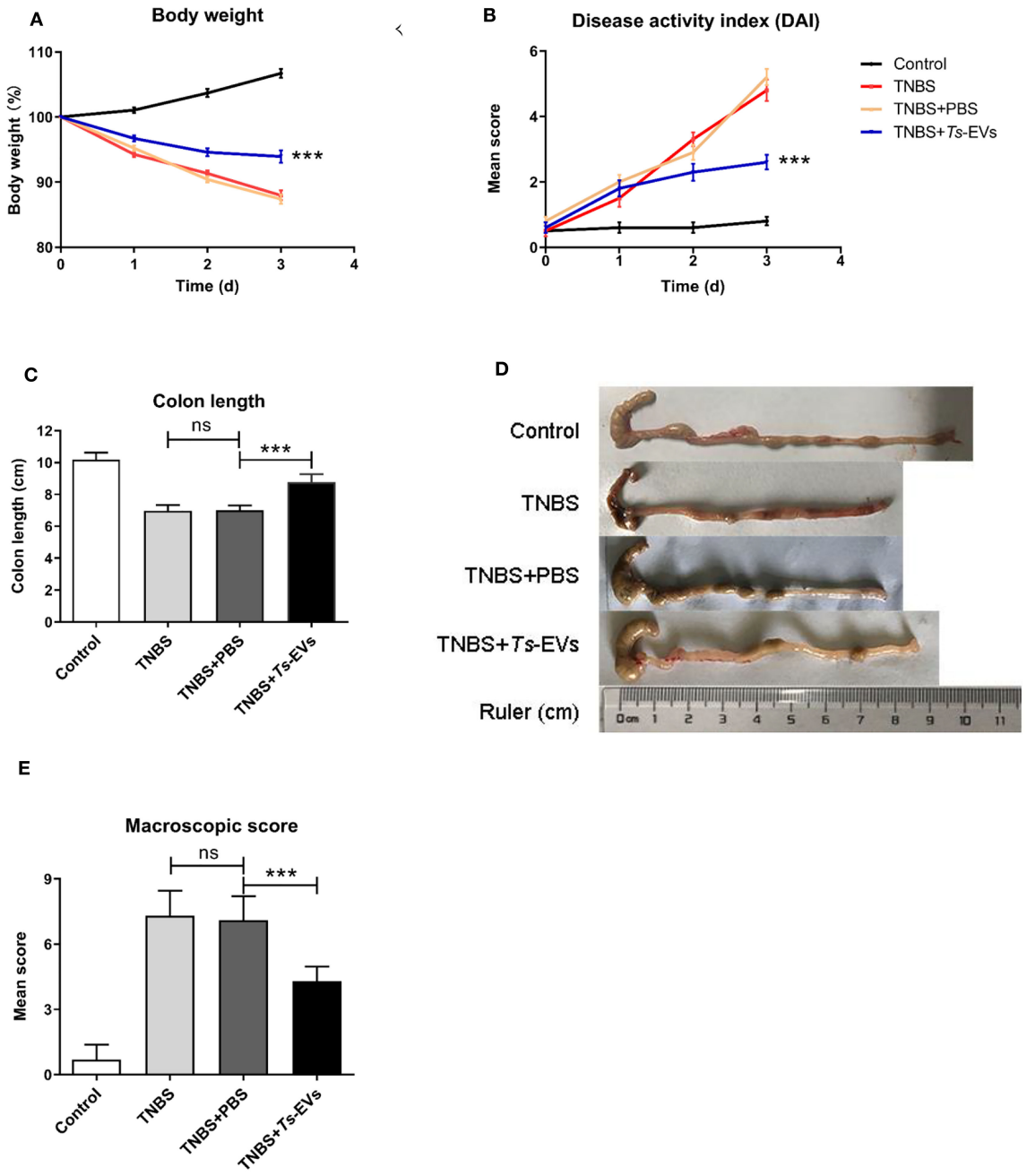

FIGURE 2 | *Ts*-EVs ameliorate clinical symptoms and preserve the normal macroscopic structure and length of the colon in TNBS-induced colitis. Mice received three intraperitoneal injections of *Ts*-EVs (50 μg) prior to intrarectal administration of TNBS. The protective effect against TNBS-induced colitis was determined in three independent experiments. One representative experiment is shown here. **(A)** Body weight was recorded daily in each group. **(B)** The disease activity index (DAI) was evaluated daily based on three parameters (weight loss, stool shape, and presence of blood in stool), as described in methods (day 3, $^{***}p < 0.001$, TNBS + PBS vs. TNBS + *Ts*-EVs). **(C,D)** After 3 days, colons were removed and their length was measured as an indirect marker of inflammation. **(E)** Mean macroscopic scores of colonic inflammation based on 3 parameters (hyperemia, wall thickening, and ulcers), as described in methods. Results are shown as mean ± standard deviations (SD) of each group ($n = 10$ per group). Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple-comparison test. $^{***}p < 0.001$.

the rate of body weight loss in the TNBS+*Ts*-EVs group was significantly reduced on the final day of the experiment (**Figure 2A**, $p < 0.001$). The DAI scores in the TNBS group, assessed according to **Table 1**, were dramatically increased (**Figure 2B**). Mice in the TNBS group typically showed reduced colon length, whereas the colon length in the TNBS+*Ts*-EVs

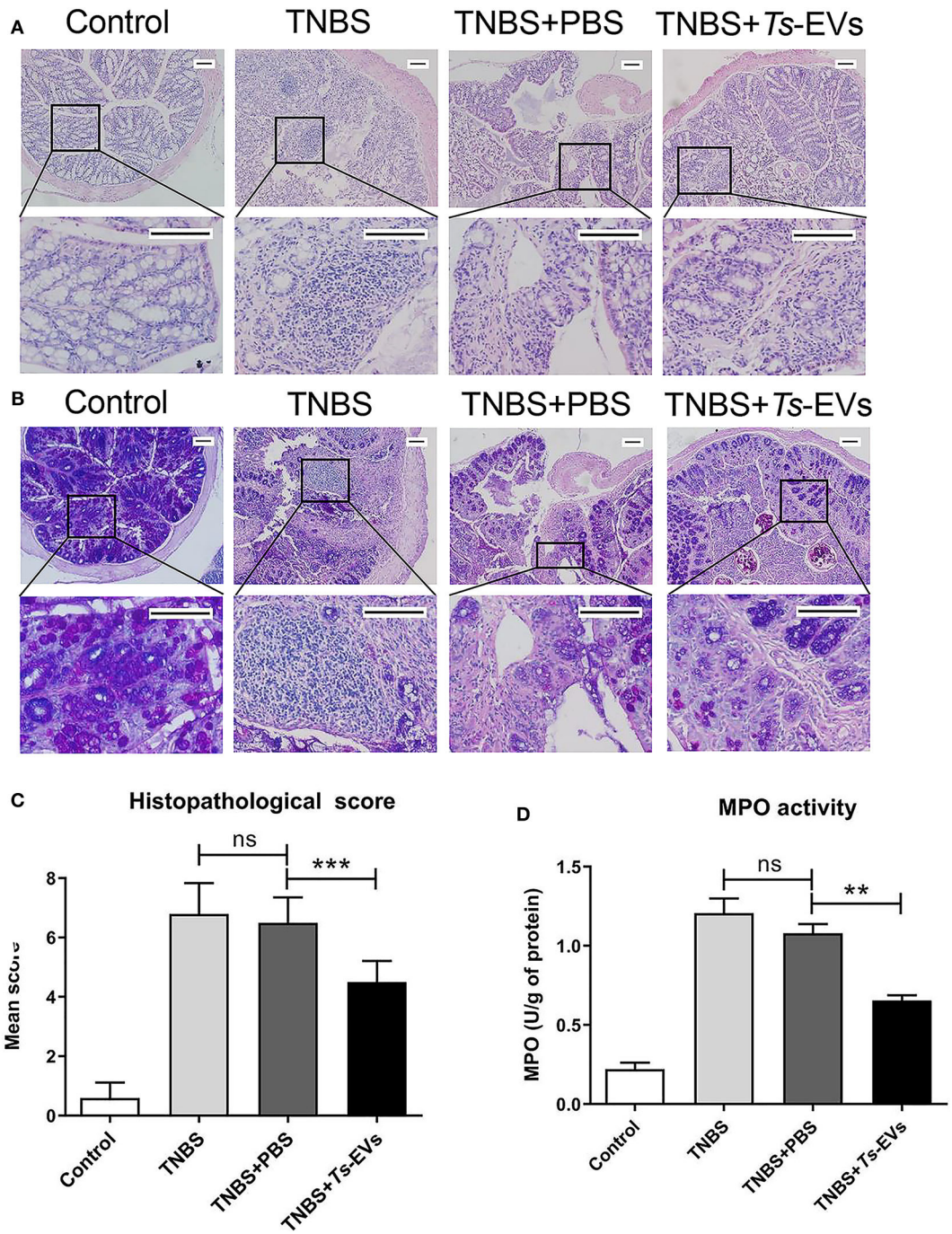

FIGURE 3 | *Ts*-EVs reduced histopathological signs of colon damage and myeloperoxidase (MPO) activity in TNBS-induced colitis. **(A)** Colon tissue samples were examined after hematoxylin and eosin (H&E) staining (magnification 100× and 400×, scale-bars = 100 μm). **(B)** Colon tissue samples were examined after periodic acid Schiff (PAS) staining (magnification 100× and 400×, scale-bars = 100 μm). **(C)** Histopathological scores based on the extent of inflammation, inflammatory cell infiltration, extent of crypt damage and loss of goblet cells, as described in methods. **(D)** Myeloperoxidase (MPO) activity was determined by a spectrophotometric method. Results are shown as mean ± SD of each group ($n = 10$). Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple-comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$.

group was significantly increased (**Figures 2C,D**, $p < 0.001$). In addition, colon macroscopic scores, evaluated based on **Table 2**, showed that mice treated with *Ts*-EVs prior to TNBS induction displayed markedly suppressed macroscopic signs of colonic involvement when compared with the TNBS group (**Figure 2E**, $p < 0.001$). There were no significant differences between the TNBS and TNBS+PBS groups.

We also analyzed histopathological changes in the colon by H&E and PAS staining. The colonic mucosa was clearly damaged in the TNBS group, showing epithelial destruction, intense inflammatory infiltration, significant crypt damage and loss of goblet cells. In contrast, administration of *Ts*-EVs prior to colitis induction prevented damage of epithelial structures and reduced inflammatory infiltration (**Figure 3A**). Interestingly, PAS staining of colon tissue showed widespread reduction of goblet cells in mice with TNBS-induced colitis, whereas the goblet cells in the TNBS+*Ts*-EVs group showed only focal areas of reduction (**Figure 3B**). This is consistent with previous H&E stainings examining goblet cells. We evaluated the H&E and PAS staining results based on the criteria listed in **Table 3** and concluded that *Ts*-EVs administration clearly reduced the degree of histological damage when compared with the TNBS group (**Figure 3C**, $p < 0.001$). MPO activity reflects the degree of infiltration of neutrophils into various tissues. Our results showed that *Ts*-EVs treatment significantly reduced colonic MPO activity when compared with the TNBS group (**Figure 3D**, $p < 0.001$). Altogether, these results demonstrate that *Ts*-EVs can ameliorate the severity of TNBS-induced colitis.

Ts-EVs Reduce the Permeability and Enhance the Expression Levels of Occludin and ZO-1 Tight Junction Protein in TNBS-Induced Colitis

To explore the effects of *Ts*-EVs on intestinal barrier integrity during TNBS-induced acute colitis, we assessed intestinal permeability using FITC-D. As shown in **Figure 4A**, TNBS administration significantly increased intestinal permeability compared with the control group. In contrast, preventive treatment with *Ts*-EVs effectively counteracted the TNBS-induced increase in intestinal permeability in mice. In addtion, we examined the expression levels of occludin and ZO-1 protein in colonic tissues by immunofluorescence. Occludin and ZO-1 are located on the membrane of colonic mucosal epithelial cells and play a crucial role protecting the integrity of the intestinal mucosal barrier and regulating intestinal

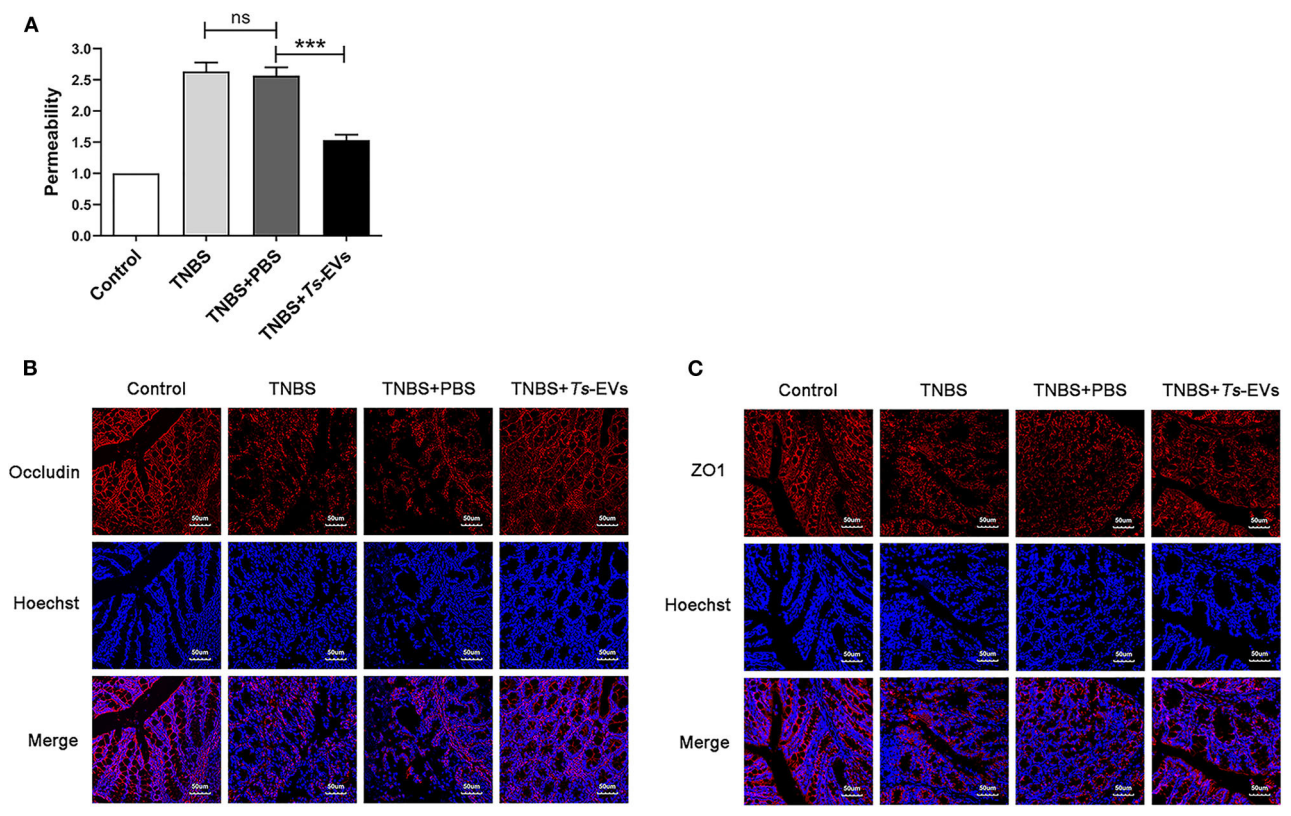

FIGURE 4 | *Ts*-EVs restored the impaired permeability and preserved tight junctions integrity inTNBS-induced colitis. **(A)** Intestinal permeability was measured by FITC-Dextran permeability assay. Relative permeability of TNBS and *Ts*-EV treatment in mice. Results are shown as mean ± SD of each group ($n = 3$). Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple-comparison test. ***$p < 0.001$. **(B,C)** Tight junction proteins were measured by using laser scanning confocal microscopy. **(B)** Colonic occludin (red), nuclei (blue). **(C)** Colonic ZO-1 (red), nuclei (blue). Magnification of the images was 400×, Scale-bars = 50 μm. These figures are representative of three independent experiments.

permeability. Immunofluorescence staining showed that the levels of expression of occludin in the TNBS+*Ts*-EVs group were significantly higher than in the TNBS group (**Figure 4B**). In addition, weaker ZO-1-specific fluorescence was observed in the TNBS group, whereas the expression of ZO-1 was significantly enhanced in the *Ts*-EVs-treated group (**Figure 4C**).

Ts-EVs Regulate the Expression Levels of Inflammatory Cytokines in TNBS-Induced Colitis

Inflammatory cytokines play a central role in the pathogenesis of IBD. To determine whether the protective effects of *Ts*-EVs against TNBS-induced colitis in mice were associated with inhibition of inflammatory cytokines, supernatants from homogenized colonic tissue were collected. The levels of Th1 or pro-inflammatory cytokines (IL-1β, IFN-γ, and TNF-α), Th2 cytokines (IL-4 and IL-13), Th17 cytokine (IL-17A), anti-inflammatory cytokines (IL-10 and TGF-β) and chemokines (MCP-1 and MIP-3α) were analyzed by MSD. As shown in **Figure 5**, TNBS administration induced the release of all the aforementioned inflammatory cytokines compared with the control group. In contrast, preventive treatment with *Ts*-EVs significantly decreased the expression levels of the pro-inflammatory cytokines IL-1β ($p < 0.001$), TNF-α ($p < 0.001$), IFN-γ ($p < 0.01$), and IL-17A ($p < 0.01$) when compared with the TNBS group (**Figure 5A**). Moreover, compared with the TNBS group, the expression levels of the anti-inflammatory cytokines IL-10 ($p < 0.001$) and TGF-β ($p < 0.001$), and of the Th2-related cytokines IL-4 ($p < 0.01$) and IL-13 ($p < 0.01$), were significantly higher in mice treated with *Ts*-EVs (**Figure 5B**). Interestingly, compared with the TNBS group, there was no significant change in chemokine MCP-1 expression after *Ts*-EVs treatment, but the levels of chemokine MIP-3α

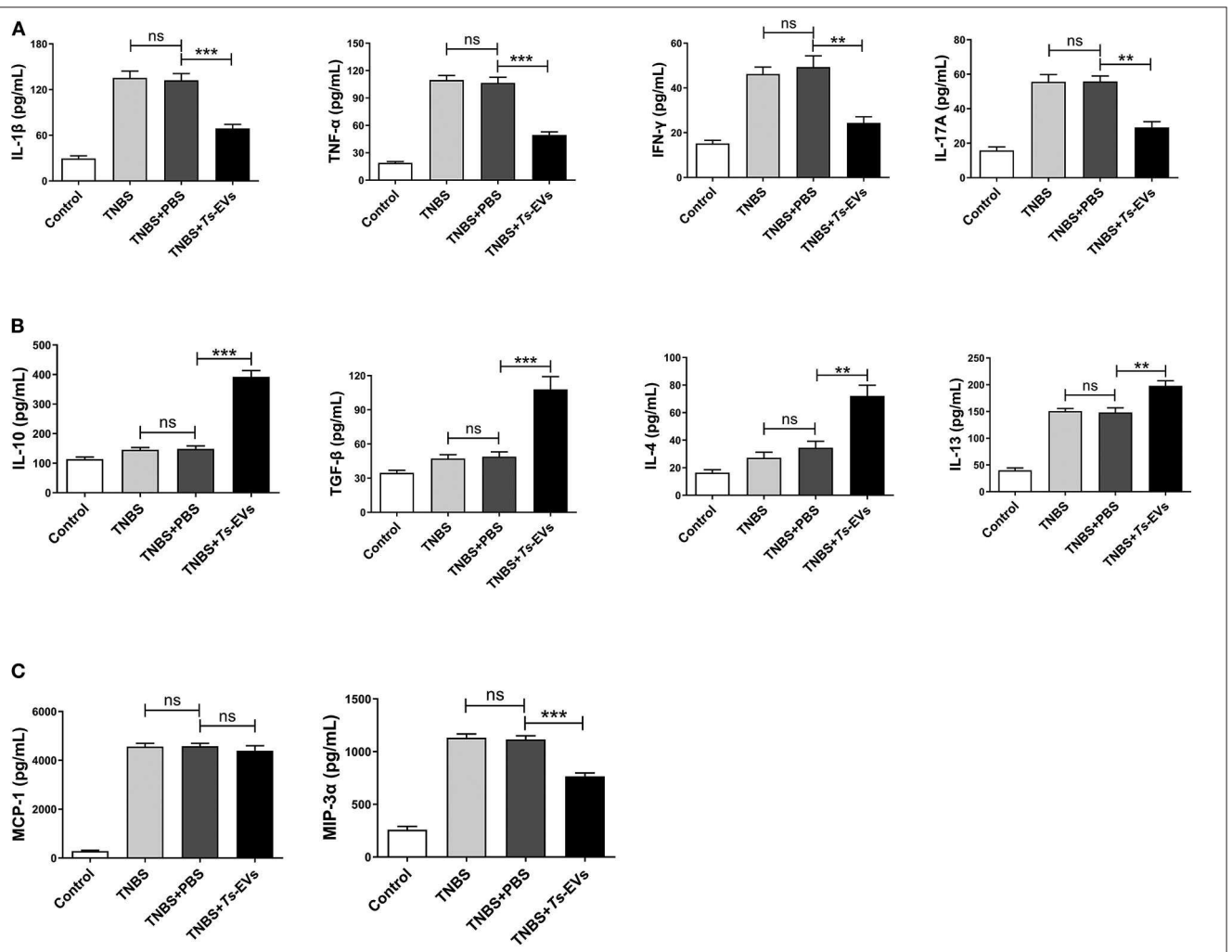

FIGURE 5 | Levels of cytokines and chemokines in colons of mice with TNBS-induced colitis pretreated or not with *Ts*-EVs. Cytokines levels in homogenized colon tissue supernatants were determined by MSD. **(A)** Expression levels of Th1 cytokines (IL-1β, IFN-γ, and TNF-α) and a Th17 cytokine (IL-17A). **(B)** Expression levels of Th2 cytokines (IL-4 and IL-13) and regulatory cytokines (IL-10 and TGF-β). **(C)** Expression levels of chemokines (MCP-1 and MIP-3α). Results are shown as mean ± SD of each group ($n = 3$). Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple-comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$.

($p < 0.001$) were significantly reduced (**Figure 5C**). Collectively, our findings indicate that *Ts*-EVs inhibit the production of pro-inflammatory cytokines and upregulate the levels of anti-inflammatory cytokines, contributing to the inhibition of colitis.

Ts-EVs Regulate T Cell Differentiation During TNBS-Induced Colitis

To explore the effects of *Ts*-EVs on the local T-cell immune response during TNBS-induced colitis, the levels of mRNA coding for T helper- and Treg-associated cytokines such as IFN-γ, IL-4, IL-17A, IL-10, as well as for the transcription factors T-bet, GATA-3, ROR-γt, Foxp3 were analyzed by qRT-PCR after extracting total mRNA from colon tissue. Compared with the TNBS group, the TNBS+*Ts*-EVs group showed significant inhibition of T-bet ($p < 0.001$) and RORγt ($p < 0.01$) mRNA expression, while the expression levels of GATA3 ($p < 0.01$) and Foxp3 ($p < 0.001$) mRNA were increased (**Figure 6A**). Consistent with the levels of mRNA coding for their respective transcription factors, the qRT-PCR analysis showed that IFN-γ ($p < 0.01$) and IL-17A mRNAs ($p < 0.001$) were significantly reduced, whereas IL-4 ($p < 0.01$) and IL-10 mRNA ($p < 0.001$) expression was upregulated by treatment with *Ts*-EVs (**Figure 6B**).

To further investigate the effects of *Ts*-EVs on Th cell differentiation, MLN cells were isolated from the different groups and analyzed by flow cytometry. This analysis showed upregulated populations of $CD4^+IFN\text{-}\gamma^+$ Th1 cells and $CD4^+IL\text{-}17A^+$ Th17 cells in the TNBS group when compared with the control group. In contrast, administration of *Ts*-EVs before colitis induction significantly decreased the populations of Th1 and Th17 cells when compared with the TNBS group (**Figures 7A,B**). Conversely, mice treated with *Ts*-EVs showed distinctly increased populations of $CD4^+IL\text{-}4^+$ Th2 cells and $CD4^+IL\text{-}13^+$ Th2 cells when compared with the TNBS group (**Figures 7C,D**). Thus, our results clearly indicate that the ability of *Ts*-EVs to ameliorate colitis is associated with an expansion of Th2 cells and a reduction of Th1 and Th17 cells in the MLNs.

$CD4^+CD25^+Foxp3^+$ Treg cells play a pivotal role in maintaining immune tolerance and reducing the intensity of inflammation. $Foxp3^+$ is one of the key transcription factors that control the development and function of Treg cells and plays a key role in regulating Treg activity. To measure changes in the levels of Tregs in mice with TNBS-induced colitis which had been pretreated with *Ts*-EVs, we isolated cells from the MLNs. As shown in **Figure 8**, we found a significant reduction in the percentage of $CD4^+CD25^+$ $Foxp3^+$ Treg cells in the TNBS group, when compared with the control group. In contrast, mice pretreated with *Ts*-EVs had a reproducible and marked increase in the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells (**Figure 8**). Hence, our data indicate that *Ts*-EVs exert a protective effect against TNBS-induced acute colitis by upregulating Treg cells.

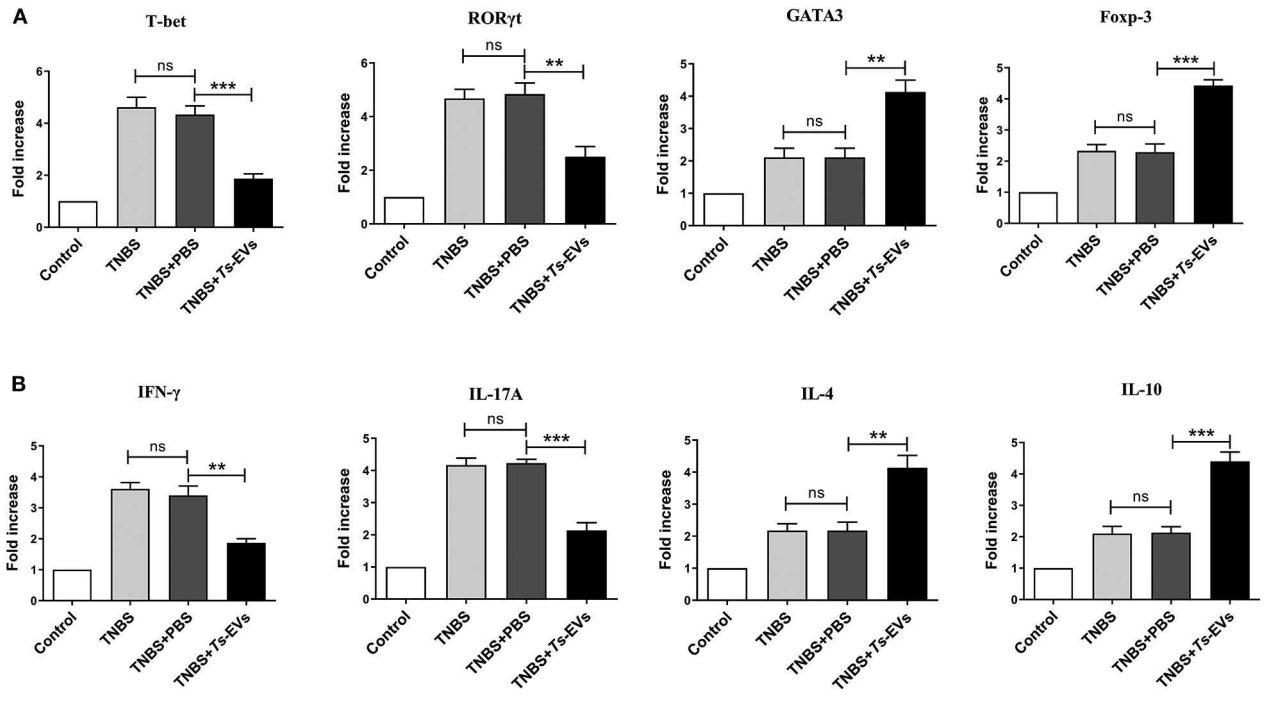

FIGURE 6 | qReal-time PCR analyses of gene expression levels in colons of mice with TNBS-induced colitis pretreated or not with *Ts*-EVs. **(A)** T-bet, GATA3, RORγt and Foxp-3 mRNA expression levels in the colons were measured by qRT-PCR. **(B)** IFN-γ, IL-17A, IL-4, and IL-10 mRNA expression levels. Results are shown as mean ± SD of each group ($n = 3$). Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple-comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$.

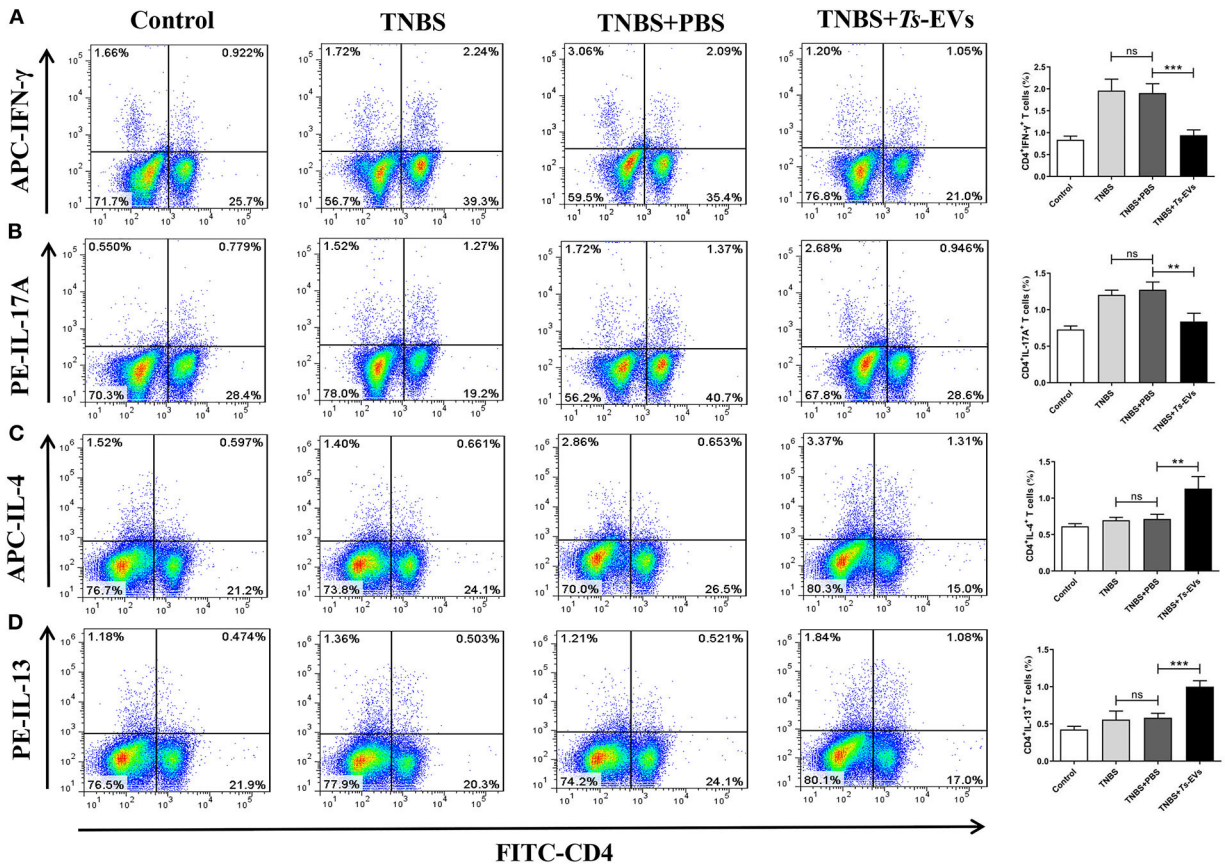

FIGURE 7 | Flow cytometry analysis of Th1, Th2 and Th17 cells in the MLNs of mice with TNBS-induced colitis pretreated or not with *Ts*-EVs. Mesenteric lymph node (MLNs) cells were isolated from the different groups of mice, stimulated with Leukocyte Activation Cocktail containing PMA, ionomycin and GolgiPlug™ (Brefeldin A), and stained with specific antibodies. **(A)** Th1 ($CD4^+IFN\text{-}\gamma^+$) cells. **(B)** Th17 ($CD4^+IL\text{-}17A^+$) cells. **(C,D)**, Th2 ($CD4^+IL\text{-}4^+$ and $IL\text{-}13^+$) cells. Results are shown as mean ± SD of each group ($n = 3$). Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple-comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$.

Ts-EVs Contain Helminth-Specific miRNAs With Immunomodulatory Potential

miRNAs contained in EVs play an important role in EV-mediated host-parasite communication. Therefore, the miRNA content of *Ts*-EVs was characterized using the Illumina HiSeq platform together with bioinformatics analysis. The high throughput sequencing results showed that the length distribution of the miRNAs was 18–30 nt, with 25 nt as the main length. Based on the abundance of transcripts per million (TPM), the top 50 miRNAs with predicted important regulatory roles in various biological processes are shown in **Figure 9**. Our results show that the murine host genes targeted by the identified miRNAs are related to the immune response, angiogenesis, apoptotic process, autophagy, cell communication, cell cycle, coagulation and other biological processes (**Figures 9A,B**). Interestingly, the immune response-associated target genes are mostly involved in antigen processing and presentation, pattern recognition receptor signaling pathways, cellular response to cytokines IL-1 and INF-γ, regulation of the adaptive immune response and leukocyte activation (**Figure 9C**).

DISCUSSION

The growth, development and long-term survival of *T. spiralis* in the host depend on biologically active, immunoregulatory compounds released by the parasite. These allow *T. spiralis* to create an immunologically friendly environment for successfully parasitism. In addition, they can also ameliorate autoimmune diseases caused by exacerbated immune responses, such as IBD (15). In recent years, extracellular vesicles (EVs) released by parasites have been the focus of extensive interest as "bridges" communicating parasites with the host during infection (16).

In this study, we isolated EVs from *T. spiralis* muscle larvae and evaluated their immunoregulatory potential in the TNBS-induced murine colitis model. First, we used TEM and NTA to identify the morphology and physical properties of the isolated EVs. The EVs were closed circular or elliptical vesicular structures ranging in size from 50 to 250 nm, with an average diameter of 178 nm. The size and characteristics of the EVs we isolated were similar to those described in a previous study and also to those released by other helminths, such as *Schistoso*ma mansoni and *Trichuris muris* (17, 18). The transmembrane

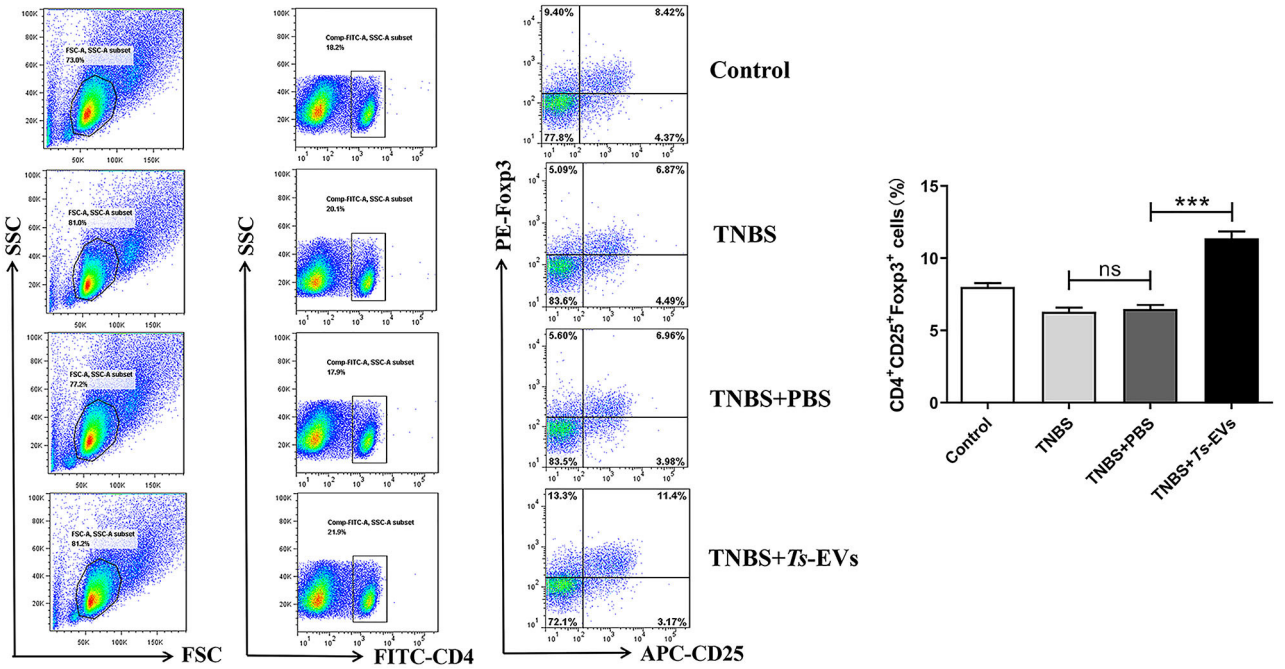

FIGURE 8 | Flow cytometry analysis of Treg cells in the MLNs of mice with TNBS-induced colitis pretreated or not with *Ts*-EVs. The percentages of $CD4^+CD25^+Foxp3^+$ Treg cells are shown. Results are shown as mean ± SD of each group ($n = 3$). Statistical analysis was performed with one-way ANOVA followed by Tukey's multiple-comparison test. $^{***}p < 0.001$.

protein CD63 and enolase are common surface markers of extracellular vesicles in many cell types and are usually used to identify EVs after isolation (19, 20). Our Western blot results showed high expression of CD63 and enolase in *T. spiralis* EVs. Collectively, our results confirmed the high quality of the *Ts*-EVs we obtained and established that the isolated EVs could be used in subsequent experiments.

The mechanisms by which parasite-secreted molecules restrain host immune responses has been investigated in various mouse models of disease, with roles ascribed to expansion of immunoregulatory cells like tolerogenic dendritic cells, alternatively activated macrophages and regulatory T and B cells, as well as suppression of inflammatory T helper (Th)-1 and−17 responses and induction of parasite-specific Th2 responses (21, 22). In this study, we evaluated whether *Ts*-EVs could ameliorate the development of TNBS-induced experimental colitis in mice by intraperitoneal injection of *Ts*-EVs, and explored the underlying mechanisms. Our results showed that pretreatment of mice with *Ts*-EVs reduced clinical signs of colitis as determined by the DAI score, inflammatory cell infiltration and intestinal histopathology. Different injection routes of EVs have been proved to influence their tissue distribution, and transperitoneal injections resulted in significantly higher EV accumulation in gastrointestinal tract (23). Helminth-derived EVs were also previously found to be capable of internalizing by murine small intestinal organoids, and transperitoneal injection of parasite-derived EVs could attenuate mucosal intestinal damage and prevent intestinal inflammation in experimental models (10). Accordingly, we supposed, the *Ts*-EVs could enrich in the gastrointestinal tract after IP injection, and then might be taken up by different host immune cells, and further modulated intestinal immunity through various mechanisms. It was quite unfortunate that, in our present study, the distribution of the *Ts*-EVs in the colon of mice and the mechanisms of EVs with protein and miRNA cargo internalization by host cells, such as macrophages, DC and intestinal epithelial cell were not explored, but will be further studied.

It is well-known that pro-inflammatory cytokines play a pivotal role in the initiation and amplification of inflammation by recruiting and activating leucocytes (24). Improvement of inflammatory disorders by infection with *T. spiralis* and other parasites has been ascribed to the anti-inflammatory properties of their secreted molecules (25, 26). We examined whether *Ts*-EVs could protect against IBD by exerting anti-inflammatory effects. We found that *Ts*-EVs dramatically reduced the expression of pro-inflammatory cytokines, whereas the levels of anti-inflammatory cytokines like IL-10 and TGF-β increased significantly in colon tissue of mice with TNBS-induced colitis. These results are similar to those reported in previous studies for EVs isolated from *Nippostrongylus brasiliensis* and *Fasciola hepatica,* which reduced the severity of inflammation in a murine colitis model (10). Our results indicate that *Ts*-EVs have the ability of modulating the balance between pro-inflammatory and anti-inflammatory cytokines, which helps improve the intestinal histopathology.

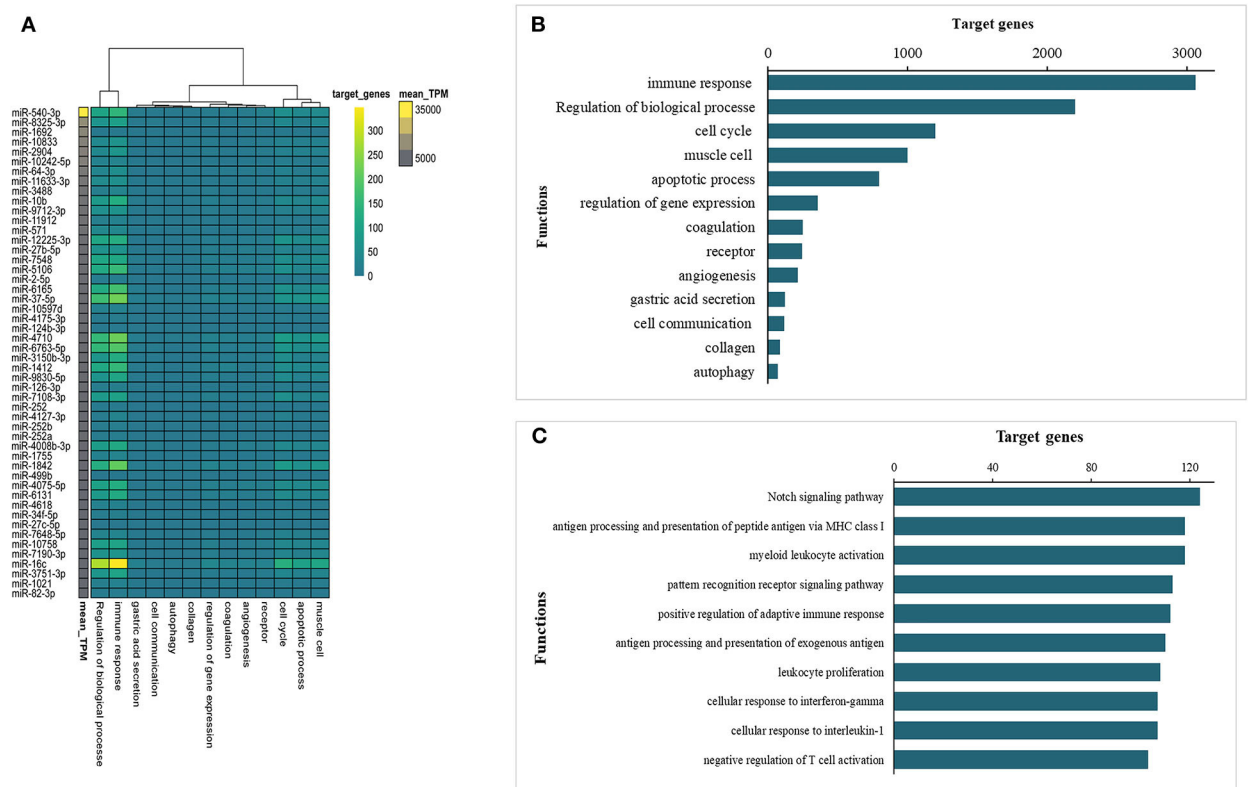

FIGURE 9 | Prediction of interactions between *Ts*-EV miRNAs and murine host genes. **(A)** Functional map of *Ts*-EVs miRNAs and murine host target genes involved in various biological processes (heat map corresponds to individual target genes in the murine host based on the 50 most abundant miRNAs). Data are available in **Table S1**. **(B)** Total number of targeted gene networks identified and classified based on different biological process. Data are available in **Table S1**. **(C)** Total number of targeted gene networks identified and classified as "immune response." Data are available in **Table S1**.

Chemokines are a family of structurally related cytokines which play a fundamental role in the regulation of leukocyte recruitment and activation in the colon (27). In the present study, we found that pretreatment with *Ts*-EV*s* decreased the expression of macrophage inflammatory protein-3α (MIP-3α) in the intestines of mice with colitis. MIP-3α induces $CD4^+$ T and immature dendritic cell transmigration and plays an important role in colonic adaptive immune responses. Therapy with anti-MIP-3α mAbs significantly ameliorated colonic injury and reduced intestinal inflammation induced by TNBS in mice (28). We hypothesize that the decreased levels of MIP-3α in the colons of mice treated with *Ts*-EVs reduce the influx of $CD4^+$T cells and dendritic cells, which in turn improves colonic inflammation. Further studies are needed to clarify what other chemokines are regulated by *Ts*-EVs.

MPO is mainly present in the cytoplasm of neutrophils, and its activity reflects the degree of infiltration by neutrophil inflammatory cells in damaged tissues. Therefore, MPO can be used to evaluate disease severity in colitis (29). Our results showed that MPO activity in the TNBS+*Ts*-EVs group was significantly reduced, indicating that inflammatory cell infiltration in colonic tissue was significantly diminished. Intestinal barrier dysfunction plays a pivotal role in the initiation and acceleration of mucosal inflammation, and is an important pathogenic factor in the development of IBD. Tight junction proteins play a key role in maintaining the intestinal barrier. Pro-inflammatory cytokines, such as IFN-γ and TNF-α, induce instability of epithelial TJ proteins involved in maintaining intestinal epithelial integrity, leading to epithelial barrier dysfunction (30). Anti-inflammatory agents can improve intestinal barrier function. Occludin and ZO-1 are key members of the tight junction protein family and play an important role in maintaining the integrity of the intestinal mucosal barrier (31). Interestingly, administration of *Ts*-EVs increased occludin and ZO-1 expression and protected against epithelial junction damage in the TNBS-induced colitis model, thereby contributing to maintain the integrity of the intestinal barrier. Moreover, we investigated the influence of *Ts*-EVs on intestinal permeability. The result showed that *Ts*-EVs restored the impaired permeability induced by TNBS in mice, which further indicated that *Ts*-EVs could improve the intestinal integrity damaged by inflammation. These results highly confirmed that *Ts*-EVs exert potent anti-inflammatory effects.

Aberrant activation and differentiation of Th1 cells which produce interferon-γ (IFN-γ) and Th17 cells which produce IL-17 is generally associated with autoimmune diseases,

especially IBD. Th2-type and regulatory T cell responses and production of Th2/immunoregulatory cytokines, e.g., IL-4, IL-13, IL-10, and TGF-β play a crucial role in protecting against Th1/Th17 immune-mediated diseases. TNBS-induced colitis is characterized by transmural inflammation, a process that is regulated by Th1/Th17 immune responses, along with overproduction of interferon IFN-γ and IL-17 in the colonic mucosa (32). Thus, TNBS-induced colitis is a valuable model to investigate the regulation of T cell activation and differentiation by parasites and their products. To investigate *Ts*-EVs immunoregulatory mechanisms, we evaluated their ability to modulate the balance between different T cell subpopulations in this model. A recent study showed that EVs released by *Fasciola hepatica* exerted a protective effect in DSS-induced colitis that was independent of adaptive immunity (11). In our study, mice treated with *Ts*-EVs showed decreased expression of the Th1 cytokine IFN-γ and the Th17 cytokine IL-17A, and of their transcriptional factors T-bet and RORγt as well. Consistently, intracellular staining showed that the percentages of IFN-γ-producing $CD4^+$ T cells and IL-17A-producing $CD4^+$ T cells in the MLNs of *Ts*-EVs-treated mice were significantly lower than in the TNBS group. Based on these results, we believe that *Ts*-EVs are vigorous immune regulators with the capacity to strongly modulate the Th1/Th17 axes in the context of intestinal damage.

In addition to reduced Th1/Th17 immune responses, we also observed increased expression of Th2 (IL-4, IL-13, GAGT3) and Treg (IL-10, TGF-β and Foxp3)-associated cytokines and transcription factors in mice pretreated with *Ts*-EVs when compared with the TNBS group, and these results were confirmed by flow cytometry. These data suggest that the capacity of *Ts*-EVs to induce Th2/Treg cell differentiation also explains their anti-inflammatory effects. Similar results have been reported in *T. spiralis,* since specific antigens of this parasite, like serine protease inhibitors, can promote Th2 or /and Treg immune responses (33). DCs are the key link between innate and adaptive immunity, and play a critical role in the activation and differentiation of T lymphocytes. Thus, they could be a key target for regulating the host immune response by parasites. DCs interact with ES products and parasite molecules which can induce a tolerogenic immune microenvironment by regulating the balance between T cell subgroups, ultimately impacting autoimmune disease progression in mouse models (34, 35). For example, mice with experimental autoimmune encephalomyelitis (EAE) injected with DCs treated with *Ts*-MLES showed significantly reduced EAE severity, an outcome associated with downregulation of Th1/Th17 responses, boosting of regulatory T cells and increased secretion of regulatory IL-10 and TGF-β cytokines (36). In our previous study, we found that adoptive transfer of *Ts*-MLES-DC alleviated disease activity in mice with TNBS-induced colitis by shifting the immune response from Th1 toward Th2 and regulatory (37). Recent studies indicated that extracellular vesicles from the *Echinococcus granulosus* larval stage were internalized by dendritic cells and interfered with their antigen presentation function by downregulating MHCII expression (8). It would be interesting to investigate the potential of *Ts*-EVs to regulate DC maturation and subsequent T cell polarization.

MicroRNAs (miRNAs) are small non-coding RNA molecules that are emerging as important post-transcriptional inhibitors. They negatively regulate target gene expression by degrading mRNA or repressing translation. A growing body of evidence indicates that miRNAs are involved in the regulation of numerous biological processes, such as cell development, differentiation, proliferation, apoptosis, and in various diseases (38). There are also numerous studies showing that miRNAs regulate a wide spectrum of immune system functions and shape both innate and adaptive immunity, including Th1 and Th2 polarization and inflammatory responses (39, 40). Some studies have also found that pathogen-derived microRNAs can regulate host immune responses via cross-species interactions, subverting the immune system during infection (41). Investigations of extracellular vesicles released by parasitic helminths showed they contain abundant miRNAs and that these exosome-derived miRNAs participate in parasite-driven immunoregulation. Recent studies found that *S. japonicum* miRNAs can be delivered to host macrophages and T helper cells, where they regulate macrophage proliferation and Th cells differentiation by altering target gene expression (42, 43). In line with these studies, prediction of the interactions between microRNAs from *Ts*-EVs and murine host genes indicated that these microRNAs may play a vital role in the modulation of host immune system functions, such as antigen presentation and immune cell activation. Further research is necessary to investigate whether miRNAs from *T. spiralis* can be transported into host cells via *Ts*-EVs as an important mechanism to regulate host genes associated with intestinal immunity and inflammation. However, in depth elucidation of how *Ts*-EVs miRNAs subvert the host immune defense system will provide novel insights into *T. spiralis*-host interactions.

In conclusion, *Ts*-EVs showed immunoregulatory effects in a mouse model of IBD. The protective effects of *Ts*-EVs against TNBS-induced colitis involved regulation of Th1/Th2 balance and induction of Treg differentiation. These effects partly reproduced the beneficial effects of *T. spiralis* self and ES products. miRNAs contained within the EVs could be assigned to target genes which regulate the host immune response. These findings expand our knowledge of the mechanisms by which *T. spiralis* evades the host immune system and suggest new therapeutic approaches for IBD and other autoimmune disorders. However, we did not identify the specific miRNAs or proteins in *Ts*-EVs which mediate the immunoregulatory effects. Further hotspot studies are needed to identify the specific cargo molecules carried by *Ts*-EVs and to clarify their function during *T. spiralis* infection.

DATA AVAILABILITY STATEMENT

The datasets generated for the study can be found in NCBI Sequence Read Archive (SRA) under accession code PRJNA631364.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Jilin University, affiliated with the Provincial Animal Health Committee, Jilin Province, China.

AUTHOR CONTRIBUTIONS

The study was conceived and designed by XB and ML. YY, LL, and YZ performed the experiments. YY, XB, and XL analyzed the data. YY, LL and XB wrote the manuscript. XL, HS, WJ, HZ, HJ, and ML improved the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01174/full#supplementary-material

Table S1 | Prediction of *Ts*-EV miRNA interactions with murine host target genes. Heat map corresponds to individual target genes in the murine host based on the 50 most abundant miRNAs by R package pheatmap. Functional map of *Ts*-EV miRNAs and murine host target genes categorized by ggplot2 package.

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Immunity to Soil-Transmitted Helminths: Evidence From the Field and Laboratory Models

Stefano A. P. Colombo[1] *and Richard K. Grencis*[2]*

[1] *Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom,* [2] *Division of Infection, Immunity and Respiratory Medicine, Wellcome Trust Centre for Cell Matrix Research, Lydia Becker Institute for Immunology and Inflammation, The University of Manchester, Manchester, United Kingdom*

Infection with soil-transmitted helminths (STH) remains a major burden on global health and agriculture. Our understanding of the immunological mechanisms that govern whether an individual is resistant or susceptible to infection is derived primarily from model infections in rodents. Typically, experimental infections employ an artificially high, single bolus of parasites that leads to rapid expulsion of the primary infection and robust immunity to subsequent challenges. However, immunity *in natura* is generated slowly, and is only partially effective, with individuals in endemic areas retaining low-level infections throughout their lives. Therefore, there is a gap between traditional model STH systems and observations in the field. Here, we review the immune response to traditional model STH infections in the laboratory. We compare these data to studies of natural infection in humans and rodents in endemic areas, highlighting crucial differences between experimental and natural infection. We then detail the literature to date on the use of "trickle" infections to experimentally model the kinetics of natural infection.

Keywords: *trichuris muris*, trickle infection, Th2 immunity, *Heligmosomoides bakeri*, mucosal immunology, helminths, parasitism

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Monash University, Australia

***Correspondence:**
Richard K. Grencis
richard.grencis@manchester.ac.uk

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INTRODUCTION

Soil-transmitted helminths (STH) are a highly diverse group of parasites present across the globe. Chronic life-long infection with at least one species of STH is common for most vertebrates (1). This includes humans and livestock in low to middle income countries (LMIC). The morbidity and reduced fitness associated with infection make STH helminthiases a major concern both for global health and for agriculture in endemic areas (2, 3). The infectious stages of these parasites are abundant in the environment and, due to their robustness against environmental insult, can persist there for long periods. The longevity of these parasites is compounded by their capacity to act as potent immunomodulators of their hosts (4).

A key determinant in the relationship between a host and STH parasite is the host's immune response. The host must balance an effective response to the parasite with limiting potentially detrimental immunopathology and exhausting vital resources (5). Similarly, the parasite must promote an immune response in the host that supports its own survival but that also protects the host from excessive pathology and infection by other potential pathogens. Given this, it is highly likely that anti-parasite immune responses have evolved to limit parasite burden and promote wound repair rather than to cause rapid and total parasite expulsion.

The majority of studies on immune responses to STHs are performed using rodent-specific STHs that have been adapted to the laboratory setting. These include the gastrointestinal (GI)

nematodes, *Trichuris muris, Heligmosomoides sp.* (Formally *Nematospiroides dubius.* Herein we refer to the laboratory strain as *H. bakeri* and those identified in wild rodents as *H. polygyrus.* It should be noted, however, in the literature to date these names have been used interchangeably for experimental infections), *Trichinella spiralis* and *Nippostrongylus brasiliensis*. Additionally, in some cases, human-specific species can be experimentally modeled in rodents, for example *Necator americanus* (6–9) and *Ancylostoma ceylanicum* (10, 11) albeit with limited success. Further, the larval migration that occurs during ascariasis, and hookworm infection, can be modeled in mice using the porcine STH, *Ascaris suum* (12).

Traditional experimental infections using well-established models typically rely on infecting mice with a single, artificially high dose of parasites. This is in contrast to the natural scenario in which frequent low-level exposures are likely to be more common. There is also a clear difference between the kinetics of traditional experimental infections and those seen in naturally infected populations. Thus, should we wish to fully understand the nuances of STH infection, there is a need to ensure that we are accurately modeling the natural situation.

INFECTION IN THE LABORATORY

Whilst there are species-specific responses based on the model STH used, many aspects of the immune response to experimental high-dose infections can be generalized. Upon invasion of the host, and often throughout infection, STHs cause considerable damage to tissue surrounding the site of infection. Migration of *N. brasiliensis* through the lung causes gross changes in tissue architecture and long term damage (13). Likewise, invasion of the gut epithelium and lamina propria, by *T. muris* and *H. bakeri*, respectively, causes considerable remodeling of the intestinal environment (14, 15).

Breaches by STHs at these barrier sites are associated with the release of alarmins, particularly interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) (16–19). These cytokines trigger innate responses and prime the induction of an adaptive type-2 (Th2) immune response (16, 20, 21). They have also been established as essential to protection against infection with a number of model STHs (22–25). Epithelial cells themselves are potent reservoirs of these cytokines (26–28). Of recent interest is the role tuft cells play in sensing and responding to STH infection. Tuft cells are an epithelial cell subset that exist at low frequency during homeostasis but rapidly proliferate following STH infection (28–30). They sense the presence of STHs and intestinal microbes via taste-chemosensory receptors such as TRPM5 (29, 31) and secrete IL-25 and cysteinyl leukotrienes (CysLTs) to support the establishment of a Th2 mucosal response (28, 32).

Among the first lymphoid responders are the type-2 innate lymphoid cells (ILC2s). ILC2s have been shown to expand during STH infection and act as early sources of IL-4, IL-5 and IL-13 (33–35). Their depletion results in the delayed induction of Th2 immunity (33), although a non-redundant role for these cells in parasite expulsion has only been demonstrated for *N. brasiliensis* (36, 37). A broad role for alternative activation of macrophages (M2) has also been shown in most model STHs. M2s are required for the trapping and killing of the larval stages of *H. bakeri* and *N. brasiliensis* (38–40), this function is dependent on the production of arginase-1 (Arg-1) (41) and can be regulated by the expression level of resistin-like molecule (RELM)-α (40). Whilst expansion of other innate cells – including neutrophils, eosinophils, basophils, and mastocytes—at sites of infection is well-documented (42–44) a functional role for these cells in parasite expulsion has been harder to define and in some cases may be species-specific. For example, depletion of basophils is sufficient to trigger susceptibility to *T. muris* infection (45) but has no impact on resistance to *H. bakeri* (46). Similarly, mast cells and eosinophils have been linked to resistance to *H. bakeri* and *T. spiralis* (46, 47) but are redundant for expulsion of *T. muris* (48). Further, neutrophilia has been linked with expulsion of *N. brasiliensis* and *H. bakeri* (41, 42), via the release of neutrophil extracellular traps (NETs) (49) and support of M2 polarization (50). However, in cases where ablation of a given cell type does not result in a failure to attenuate infection, these cells may instead function to repair tissue damage once the infection has been resolved (51), or to moderate ongoing responses (40, 52). Alternatively, they may act to prime distal mucosal sites against future infection with other STH species, for example ILC2s primed by *T. spiralis* infection in the gut migrate to the lung and contribute to protection against a subsequent *N. brasiliensis* infection (53). Similarly, infection with *H. bakeri* results in protection against *N. brasiliensis* infection via IL-33-dependent induction of IL-5$^+$CD4$^+$ T cells capable of recruiting activated eosinophils to the lung (54).

Central to the expulsion of STHs is the CD4$^+$ T cell. This can be inferred form studies of athymic nude mice which sustain long term high dose infections, compared to WT mice which readily expel parasites (55, 56). Depletion or ablation of CD4$^+$ cells is enough to induce to susceptibility to infection in otherwise resistant mouse strains (15, 57). Further, adoptive transfer of CD4$^+$ T cells to T and B cell deficient mice is sufficient to confer protection against infection (58). It is noteworthy that T cell deficient mouse strains such as athymic mice or recombinase 1 or 2 deficient mice still have a functional ILC2 compartment (36, 59, 60). A key function of CD4$^+$ T cells is to provide Th2 cytokines—over and above those produced by ILC2s—in particular IL-4 and IL-13 which signal through IL-4 Receptor α (IL-4Rα) (61). IL-4Rα signaling drives a broad array of down-stream responses that are essential for the expulsion of STHs. These include; hyperproliferation of goblet cells (62); increased expression and secretion of mucins and anti-parasitic peptides, such as Muc5ac and RELM-β (63–66); increased turnover of epithelial cells (67, 68); enhanced gut contractility (69); immunoglobulin (Ig) class-switching to generate parasite-specific IgG1 (46, 70); and polarization of macrophages to an M2 phenotype (41, 71). The CD4$^+$ T cell is also likely to be key to adaptive immune memory to STH infections. Under laboratory conditions, in immunocompetent mice, in response to a high-dose infection, these responses are robustly generated and lead to relatively rapid expulsion of the infection; although the kinetics differ based on genetic background of the host (39, 72–74).

In laboratory models of STH infection, as well as driving parasite expulsion, the immune response to a primary high-dose infection is sufficient to generate immunity to subsequent challenge infections (75–78). During secondary challenges the rate of expulsion is significantly accelerated. Depending on the species this may be a result of enhanced larval trapping mediated by parasite-specific IgG1 (46), priming of localized immune cells (50), or via expeditious induction of mucin secretion or mast cell activity. Regardless of mechanism, high-dose experimental infections produce robust sterilizing immunity to secondary infection.

NATURAL STH INFECTION IN MAMMALS

The artificially high doses given during experimental infections have proven a reliable system in which to investigate fundamental mechanisms of resistance to STH infection. However, this regime fails to reflect infection *in natura.* Not only is a single high-dose of parasites unrealistic in the wild, laboratory rodents are housed in pathogen free environments, with an abundance of resources, and a significant limit to stressors such as predators.

Experimental high-dose infections present a scenario in which primary STH infection is limited in duration, characterized by an immediate and potent Th2-polarized immune response, and generates sterilizing immunity to subsequent challenges. However, epidemiological evaluation of STH burden in human populations shows that in endemic areas, infected individuals suffer chronic parasitism throughout their lives (79–82). This holds true for non-human primates (83–85), livestock (86, 87), and wild rodent populations (88, 89). In humans, infection burden correlates strongly with age following one of two patterns: (i) parasite burden builds rapidly during early childhood but peaks shortly before adolescence, burden then declines and plateaus at a low level throughout adulthood e.g., *Trichuris trichiura,* and *Ascaris lumbricoides* (79, 90, 91); or (ii) STH burden builds consistently throughout childhood and early adolescence but plateaus at a moderate level prior to adulthood e.g., *Necator americanus* and *Ancylostoma duodenale* (82). Both patterns indicate that protective immunity to infection develops with age. However, they also suggest that this protection is incomplete and is preceded by a sustained period of susceptibility.

Our understanding of immune responses to STH infection *in natura* is limited. Much of what is known is founded on inferences drawn from blood samples taken from individuals living in endemic regions. As such, these data are caveated by an array of confounding factors. What is clear is that, in humans, up-regulation of the Th2 immune response is associated with STH infection. Importantly, Th2 associated markers, such as IgE and Th2 cytokines, show a clear negative correlation with worm burden (90, 91). Further, a strong IL-5 response in peripheral blood mononuclear cells (PBMC) isolated from infected individuals was shown to be predictive of resistance to reinfection following anthelminthic treatment (92, 93). A recent study utilizing mass cytometry to profile the Th2 and regulatory compartments before and after deworming in an Indonesian cohort confirmed a clear link between infection status and the expansion of ILC2s and Th2 cells, and reaffirmed the role of these cells in production of Th2 cytokines (94). Thus, a functional role for Th2 immunity in resistance to STH infection *in natura* is likely. What is interesting is that the development of this response is age-associated, with observed increases in anti-parasite IgE levels, and IL-13 and IL-4 production, in older individuals within the same cohort concurrent with a decrease in Th1-associated cytokines (90, 91, 95, 96). However, a complete polarization to Th2 immunity is rare, with most individuals maintaining a mixed Th1/Th2 response. The inability to generate a fully polarized protective response may be, in part, a consequence of STH-mediated immunosuppressive mechanisms. In one human cohort, deworming with the anthelmintic Albendazole resulted in an increase in STH-specific cytokine responses, and correlated with $CD4^+$ T cells decreasing expression of the inhibitory molecule cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (97). A role for STH-controlled immune suppression in humans is supported by evidence that peptides derived from human-infecting hookworms (*Necator americanus* and *Ancylostoma duodenale*) are able to induce IL-10 and TGF-β signaling, and suppress IL-13 secretion in rodent models of colitis and allergy (98–100).

Age-associated, slowly developed resistance to infection is not unique to humans. Both feral and domestic sheep show progressive decreases in STH infection prevalence and fecal egg burden with age (101–103), as do domestic cattle (104). Wood mice (*Apodemus sylvaticus*) show an age-associated plateau in infection intensity of *H. polygyrus* akin to that seen in human hookworm infection (88). Further, non-human primates demonstrate slow acquisition of immunity to STHs following long periods of susceptibility, with the infection intensity kinetics of *T. trichiura* and other STHs paralleling those seen in humans (84, 85). Unfortunately, whilst the kinetics of these infections are broadly characterized, there is little in the way of immunological data accompanying these parasitological findings. However, it has been observed in wood mice that *H. polygyrus*-specific IgG1 titers increase with age and that treatment with the anthelminthics Ivermectin and Pyrantel was more effective in older mice relative to younger animals (105). This is consistent with a role for IgG1 in host-protection against *Heligmosomoides* (70).

Of significant importance to the outcome of infection is the overlapping geographical distribution of these parasites; not only with one another but also with other pathogens. STH-STH co-infections are highly common, and have been shown in a number of human cohorts to occur more frequently than single STH infections (106–108). In cases of STH-STH co-infection, infected individuals exhibit higher levels of infection for each individual species relative to individuals with a single-species infection (107, 108). From field data it remains unclear as to whether this is a correlative effect—i.e., an individual susceptible to infection with one species is simply more likely to be susceptible to infection with other STHs—or if STHs act synergistically by activating mechanisms that increase host susceptibility to infection. Experimental co-infection with *H. bakeri* and *T. muris*/*T. spiralis* has demonstrated that mice normally resistant to infection with *T. muris* or *T. spiralis* are

rendered susceptible to infection when concurrently infected with *H. bakeri* (109, 110), although the mechanism through which this is mediated remains unresolved. Conversely, existing infection in the gut with *H. bakeri* or *T. spiralis* is protective against subsequent *N. brasiliensis* infection via priming of host-protective responses (53, 54). STH coinfection is also highly common with protist, bacterial and viral pathogens important to human health including malaria, tuberculosis and HIV. The primary focus of research into coinfections of this nature has been the effect STH coinfection has on the outcome of immune responses targeted the other pathogen. The overarching hypothesis being that as potent, and chronic, inducers of Th2/T-regulatory immunity STH infection will suppress the required Th1 immunity that targets single-cell/viral pathogens and thus increase susceptibility to infection and/or impact the efficacy of vaccines (111–113). However, it is also likely that infection with such pathogens will feedback onto the immune response against the STH. Therefore, it is important to bear in mind that *in natura* STH infections do not exist in isolation and have evolved in the context of a host immune system responding to a complex mix of co-infecting pathogens that elicit a diverse range of responses. Developing model systems with which to interrogate this reality presents exciting and challenging opportunities.

MODELING NATURAL INFECTION

Given the difference in lifestyles experienced by laboratory rodents and their wild counterparts, it is perhaps unsurprising that there are considerable differences in their immune systems. Wild mice appear to exist in a state of higher immune activation with a more diverse repertoire of effector/effector-memory cells (114, 115) likely due to greater antigenic exposure (116). This may, in part, explain differences in observations between laboratory experiments, and natural exposure to STHs.

Few studies have attempted to experimentally mimic a "natural" setting for STH infection. Co-housing different mouse strains in large in-door enclosures and allowing for "natural" infection of *H. bakeri* (through contact with larvae in the enclosure as opposed to controlled oral administration) removed strain-specific resistance to infection resulting in longer-lived infections in BALB/c mice (117). Given the time period in which this experiment was conducted, the means through which this change in immune response occurred was not investigated. It could be speculated that co-housing mice on different backgrounds resulted in a change in the composition of the microbiome rendering previously resistant mice more susceptible to infection. It has previously been shown that strain specific resistance to infection by the enteric bacterial pathogen *Citrobacter rodentium* can be imposed on normally susceptible mice via fecal transfer from a resistant mouse strain; this effect was mediated by induction of host innate responses including IL-22-stimulated production of antimicrobial peptides (118, 119). However, whether a similar effect can be achieved with model STHs has yet to be shown.

More recently, C57BL/6 mice housed in controlled outdoor enclosures (a process known as "rewilding") were shown to become susceptible to high-dose *T. muris* infection and exhibited impaired IL-13 production (120). Similar to observations in humans, higher worm burdens and biomass were correlated with reduced numbers of IL-13$^+$CD4$^+$ cells and increased frequency of IFNγ^+CD4$^+$ cells. The authors also found rewilding resulted in a marked increase in fecal microbial diversity. It will be of great interest to define the precise relationship between this increase in community diversity and the outcome of infection. In a subsequent analysis comparing uninfected mice housed in specific pathogen free (SPF) to those that were rewilded, it was shown that overall composition of blood and mesenteric lymph node immune cells was dramatically altered by the rewilding process including increases in central and effector memory T cells (121, 122). Interestingly, germ-free mice reconstituted with the caecal content of rewilded mice showed a significant increase in the proportion of granulocytes—in particular neutrophils—in the peripheral blood relative to mice reconstituted with caecal contents from SPF mice (121). Thus, rewilding has a profound and complex effect on immune cell composition, in part regulated by the microbiome, that may be responsible for impaired resistance to STH infection.

Together, these data suggest that inbred laboratory mice are not simply innately more resistant to STH infection than their wild counterparts, but that that environmental context is a major influence over the outcome of STH infection. Whilst studies that seek to recreate a more natural setting are valuable in bridging the gap between the laboratory and the field, they require an abundance of space and specialized facilities. They also re-introduce a myriad of confounding variables that reductionist laboratory model systems aim to nullify. Thus, the challenge is to develop an infection regime that is easily applicable to a traditional laboratory setting, recapitulates the dynamics observed in natural infections, and that limits the introduction of confounding factors.

TRICKLE INFECTION

One factor that is easy to manipulate in a controlled fashion is the dose of parasites administered. In the *T. muris* system altering single infection dose within a single inbred strain of mouse is sufficient to change both resistance phenotype and the polarization of the immune response (123) with a high dose infection generating a Th2 response and acute infection and a single low dose, chronicity through the generation of a Th1 response (76). However, a single low-dose *T. muris* infection, in which a chronic infection characterized by a regulated Th1 response is established, also does not recapitulate the dynamic shift from susceptibility to a partial resistance, the observation generally seen in the field. The concept of "threshold" and "subthreshold" levels of infection associated with resistance or susceptibility is not new [see review by Behnke (124)]. Indeed, several observations from both natural and experimental STH infections in ruminants suggest that for some parasites—such as *Ostertagia ostertagia* (125), *Nematodirus battus* (126) and *Trichostrongylus sp* (127, 128)—"lower levels" of infection are consistent with longer survival of parasite burdens.

Historically, there has been interest in so-called "trickle" infections. In principal the trickle infection seeks to mimic natural exposure to a parasite by infecting animals with frequent low-doses of a given STH rather than a single high-dose.

Early on it was observed that, in rats, daily doses of five, third larval stage (L3) *N. brasiliensis* over either a 12 or 16 week period resulted in steady increase in worm numbers and egg output during the observation periods. Adult worm numbers at the end of the experiments were $\leq$ 30% of the total number of L3 administered. This data is, therefore, suggestive of partial immunity developing particularly in the latter stages of infection with stunted female worms containing reduced eggs numbers and the presence of few pre-adult/larval stages present. Indeed, if a large (50–1000 L3) single dose challenge infection was then administered to "trickled" rats, clear resistance was evident. When the trickle infection regime was increased to 50 L3 per day over 16 weeks, a rapid increase in parasite burden was observed that peaked at 2 weeks post infection, followed by a steady decline in infection intensity and an increase in the proportion of "stunted" adult female worms for the duration of experiment (129). The kinetics of this infection regime suggested that, in contrast to a high-dose infection in which a robust immune response would drive rapid parasite expulsion, the immune response developed slowly with repeated exposure and was only partially effective i.e., it limited subsequent infections but did not prevent their establishment. Although levels of infection differed, similar observations were made by Ovington (130). The immune response during trickle infections of *N. brasiliensis* has received little attention. With regards to peripheral antibody responses to parasite surface antigens, there were few differences between single dose and trickle infections (131). Ferens et al. (132, 133) using a shorter trickle of 10 infections of 25 L3 over a 4 week period, followed by a single large challenge, observed that trickle infections primed for a much more robust lung inflammation during the migratory phase of the infection through the lungs, than a single large dose priming infection. Bronchiolar lavage showed that trickle infection generated a marked elevation in eosinophils and alveolar macrophages. This may be indicative that trickle can effectively prime for robust immune mechanisms operating against pre-intestinal larval stages.

In concert with these observations, twice weekly trickle infection of 10–50 *H. bakeri* L3 in mice (134) or 30–50 *Ancylostoma ceylaniucum* larvae in hamsters (135) showed similar increases in worm burden followed by a steady decline. This slow expulsion of infection for both species was inhibited by treatment with cortisone (134, 135) suggesting immune control and induction of at least partial immunity by trickle infections. Again, in both systems, a high dose challenge after trickle was largely expelled although some worms still remained in the intestine.

A short-term trickle infection using *T. muris* demonstrated that infecting C57BL/6 mice on alternate days over the first 35 days of infection resulted an accumulation of parasites. Cytokine and serological analysis at the experimental end-point suggested that the trickle infected mice had an immunophenotype that was intermediate between mice that were infected with a Th2 polarizing high-dose infection and mice that had received a single low-dose infection known to drive a Th1 response (76). This intermediate phenotype parallels the immune-status of individuals in endemic regions that show a mixed Th1/Th2 response as opposed to the strong Th2 polarized responses seen in traditional experimental STH infections in rodents. However, trickle infection of Balb/K mice, a strain that is markedly more resistant than C57BL/6 to a single high dose infection, indicated that although trickle does lead to maintenance of worms within the intestine, lower levels of trickle were required to achieve this and this strain generated stronger Th2 response to the infection.

Similarly, CBA mice susceptible to *H. bakeri* infection had a blunted immune response during trickle infection and failed to initiate parasite expulsion compared to resistant SWR mice which were able to reduce their parasite burdens (136). Thus, as is evident with single-dose infections, genetic background can influence the progression of trickle infections.

Experimental trickle infections of *Trichuris suis* have also been undertaken in pigs. Pedersen and Saeed (137) used a trickle regime of 250 eggs twice weekly for 4 weeks and showed that substantial numbers of worms could be found in the gut at week 4 post infection although numbers were considerably reduced by week 14 (137). Trickled animals challenged at this point were significantly immune to a single high dose challenge infection. Nejsum et al. (138) used a more intense trickle regime administering at least 100 eggs per day over a 4, 8, or 14-week period, i.e., cumulative infections of $\sim$ 4,000, 11,000, and 30,000 eggs, respectively. Significant numbers of worms (hundreds) were observed in the intestines at weeks 4 and 8, much lower than the numbest of eggs received. By week 14 few parasites were found in the intestine. Taken together, the data indicates immunity to *T. suis* can be built up after trickle infections over time and that the dynamics can be affected by the specific conditions of the trickle infection regime used.

We have recently reported a detailed characterization of a long-term trickle infection with *T. muris* (139). By performing weekly infections of 20 embryonated *T. muris* eggs in C57BL/6 mice we observed infection kinetics that closely mimicked those seen in human *T. trichiura* infection. Worm burden rose steadily with subsequent infections for 9 weeks, however, at 11 weeks we observed a decrease in worm burden and an absence of very early larval stages in the caeca of infected mice. This apparent acquisition of immunity correlated with an increase in Th2-associated immune responses including goblet cell hyperplasia, Muc5ac expression, and accelerated epithelial turnover (139). Importantly, depletion of $CD4^+$ cells during the period of expulsion after trickle, removes protection. Given that these responses have been linked to resistance in previous studies using high-dose infection (66, 67, 140) it is reasonable to conclude that the modes by which resistance to *T. muris* are mediated are similar if not identical between single-dose and trickle infection. What is different between these modes of infection is the environment in which the initial response develops. In single high-dose infection immunologically naïve mice are a blank slate in which Th2 immunity can be rapidly generated. However, during trickle infection a Th2 response must develop in the context of an on-going Th1 response. Given that these types of immunity are mutually antagonistic, understanding the

processes through which an on-going Th1 response transitions into a Th2-dominated state will be undoubtedly illuminating on fundamental mechanisms of immune regulation. Thus, a number of interesting questions present themselves.

Is There a Set Threshold of Worm Burden That Can Be Tolerated Before Mechanisms of Expulsion Are Generated?

During trickle infection a transition occurs between susceptibility to infection and subsequent resistance to future challenges. In the case of *T. muris* this transition is dependent upon the number of doses, with fewer doses being insufficient to generate a protective immune response given the same exposure time (139). This would suggest that it is parasite burden, not the length of exposure that is essential in driving a shift in immune response. A dose-dependency in response to STHs can clearly be seen when comparing the outcome of single high-dose (acute, Th2-dominated) and low-dose (chronic, Th1 dominated) *T. muris* infection (123). However, what drives this polarization remains unclear. It is possible that there is a genetically set threshold of STH burden, influenced by local environment, that may be tolerated by the host beyond which point the cost exerted by the parasite becomes too great and must be reduced. One explanation could be that there exists a balance between the host response and STH-mediated immune-regulation designed to suppress Th2 immunity (141–143) and the effect of tissue damage that necessitates Th2-dependent wound repair (144). Were this the case, when the damaged caused by the STH becomes greater than its ability to immune-modulate the host, protective immunity is induced. These processes would be dynamic and change as number of infection events alter and the host responds. Recent work using single-dose *T. muris* infections has implicated B cells as important regulators of the balance between Th1/Th2 immunity. In BALB/c mice, which produce a potent Th2 response to a single high dose *T. muris*, infection antibody depletion of B cells had no effect. However, in C57BL/6 mice, which initially show a mixed Th1/Th2 response following infection, depletion of B cells resulted in an increase in Th1 cytokines, enhanced IFNγ-associated gene expression, and susceptibility to infection (145). This effect was antibody-independent and places B cells as potential regulators of IFNγ signaling during mixed Th responses. It is exciting to speculate that B cells, whilst previously thought to be largely dispensable for protection against *T. muris* (56, 57, 146), may play a role in tuning the Th response during trickle infection.

With each subsequent dose of parasite, as parasite burden increases, the relative concentration of available antigen is likely to rise proportionally. The effect of antigen load on T cell receptor (TCR) activation in STH infection remains poorly understood. Based on *in vitro* studies, using recombinant peptides not derived from STHs, it is canonically thought that a high level of TCR signaling, stimulated by higher antigen concentration or "quality," favors Th1 differentiation whereas weaker signals allow for Th2 polarization (147). These observations were also mirrored *in vivo* (148) where it was speculated that paradoxically, large pathogens such as STH do not release amounts of antigen that readily gain access to antigen processing pathways, unlike rapidly dividing microbes. This may act in concert with immunomodulatory mechanisms employed by helminths. Antigen released from the eggs of the blood fluke *Schistosoma mansoni* actively reduces dendritic cell-T cell interactions lowering activation signal strength and directly biasing toward Th2 differentiation (149). This proactive induction of Th2 immunity by *S. mansoni* is thought to protect the host against severe pathology caused egg passage (150).

Is There a Role for Tissue Damage in the Induction of Th2 Responses During Trickle Infection?

There is a well-established link between tissue damage and the type-2 immune response. As large macroparasites, STHs cause considerable damage upon invasion of the host and during the course of infection. Indeed, it has been argued that the responses to tissue damage and STH infection co-evolved so that mechanisms that facilitate parasite expulsion also mediate wound repair (144). Tissue damage results in epithelial, mesenchymal and innate-derived cytokines, capable of inducing Th2 responses, being released; these include TSLP, IL-25 and IL-33 (**Figure 1**) (25, 28, 151). Subsequent expansion of ILC2s, eosinophils, basophils, M2s and Th2 cells promotes/regulates both parasite expulsion and wound healing. As these cytokines are often produced as a result of cell damage, their concentrations present during infection are likely to reflect the magnitude of damage caused. Indeed, a role for IL-25 has been posited in late-stage expulsion of *H. bakeri* functioning as a key inducer of effector responses against adult-stage parasites (152). Therefore, during a trickle infection the concentration of these alarmins may increase with each subsequent challenge until a threshold concentration is reached that is sufficient to drive a protective response. This notion is consistent with the slow but progressive increase in Th2-associated responses observed during *T. muris* trickle (139). Further, damage-inducing microparticles have been previously shown to act as potent adjuvants capable of driving innate and antigen-specific Th2 immune responses *in vivo* comparable in efficacy to Alum (153, 154), as does mechanical abrasion (155). Epithelial derived micro(mi)RNAs are also known to influence resistance to STHs. Epithelial specific deletion of *Dicer,* a key gene encoding an RNAase involved in miRNA action, can change resistance to *T. muris* to susceptibility. MiR-375 was identified as an important miRNA in epithelial cells and deletion of MiR-375 in mice phenocopies the *Dicer* null response to *T. muris* (156). Little is known of the miRNA response to *T. muris* after trickle infections. A combination of the repeated release of alarmins, miRNAs and the Th2-specific adjuvant effect of tissue damage resulting from regular repeat infection may facilitate protective immunity.

How Is an Effective Memory Response Generated During Trickle Infection?

Consistent with single-dose infection models there is an essential role for $CD4^+$ T cells in immunity during trickle infection (139). These cells likely act as the dominant source of IL-13

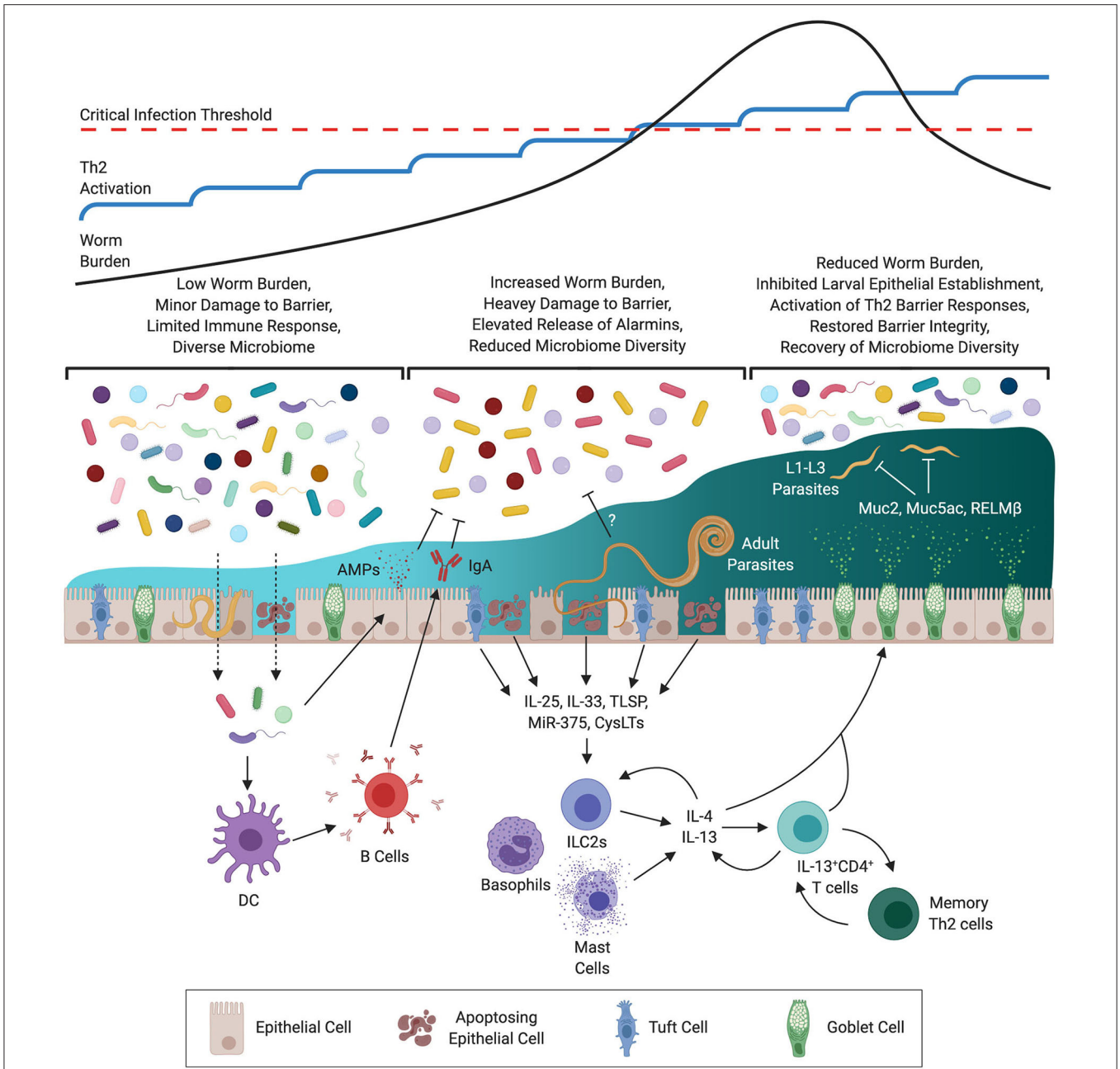

FIGURE 1 | Development of Immunity During *Trichuris muris* Trickle Infection. At the outset of infection the low level of worm burden results in minor damage to the epithelial barrier. Whilst insufficient to drive a protective Th2 response this minor damage may be sufficient to allow for opportunistic invasion by commensal bacteria triggering the release of antimicrobial peptides (AMPs) and IgA. With repeated infections the level of barrier damage is exacerbated resulting in increased release of alarmins, micro RNAs (MiRs), and cysteinyl leukotrienes (CysLTs) from epithelial, mesenchymal, and innate cells. During this time a decrease in diversity of the microbiome is observed, this may be a result of immune-mediated regulation to prevent invasion by opportunistic pathogenic bacteria, or via STH-mediated remodeling. Activation of innate cells by type-2 signals results in the release of type-2 cytokines (IL-4 & IL-13) resulting in polarization of $CD4^+$ T cells to a Th2 phenotype. Th2 cells then amplify the level of IL-4 & IL-13 signaling to activate host-protective responses at the epithelial barrier including goblet cell hyperproliferation, production of mucins such as Muc5ac, and heightened epithelial cell turnover. These responses operate primarily on early larval stages (L1–3) limiting the establishment of juvenile parasites within the epithelium. As a consequence barrier integrity is restored and intestinal microbial communities recover.

that drives anti-parasite effector mechanisms. During infection, homing of T cells to the site of infection is essential for effective parasite expulsion (58, 157, 158). Following single high-dose STH infection, the memory T cells generated persist in the mucosa long after parasite expulsion and are sufficient to facilitate protection against subsequent challenge (159–161).

However, the memory T cells generated following high-dose infection were primed in the context of a potent Th2 response, whereas memory cells generated during the early stages of a *T. muris* trickle infection will likely have been polarized to a Th1 phenotype. Moreover, the activity of the potentially pathogenic Th1 cells is regulated by IL-10 (162, 163) and thus regulation of ongoing responses also accompanies trickle infections. This scenario raises the question as to whether the $CD4^+$ cells required for protective immunity in *T. muris* trickle infection arise from early memory T cells whose phenotype is plastic and informed by *de novo* production of Th2 cytokines by innate cells/environment, or if new $CD4^+$ T cells are recruited later in infection? Repolarization of Th1 effector T cells into Th2 cells has previously been shown as a result of STH infection, OVA-specific Th1 cells transferred into naïve mice adopted a Th2 phenotype during *N. brasiliensis* infection (164), however, this was not in the context of an ongoing Th1 response. Instead, if naïve $CD4^+$ T cells are recruited that differentiate into Th2 cells it will be interesting to determine if their TCR repertoire differs from Th1 cells generated early in infection. Identification of the specific antigens recognized by $CD4^+$ T cells following the development of resistance under a trickle infection may provide a fertile avenue for the discovery of novel vaccine candidates.

What Effect Does the Microbiome Have on the Outcome of Trickle Infection?

There exists an evident relationship between the immune system and the microbiome, especially in the gut where it is required for both the development and maintenance of the mucosal barrier (165), with loss of community diversity associated with inflammatory bowel disease (IBD) (166, 167). Whilst there are relatively few field studies that have investigated the relationship between STH infection and the microbiome in humans, it does appear that infection can affect microbial composition (168, 169). This is consistent with laboratory studies of chronic STH infection that have consistently shown that STHs alter the microbiome (170–172) and that these changes in composition can be reversed following expulsion of the infection (173). Chronic STH infection has been associated with expansion of bacterial genera with the capacity to promote the T regulatory response such as *Lactobacillus* (174–176) which may contribute to chronicity by suppressing the induction of a Th2 response. During the Th1-dominated susceptible phase of *T. muris* trickle infection there is a strong reduction of microbial diversity and an expansion of genera associated with chronic STH infection. Interestingly, the reduction in microbial diversity during the susceptible phase leads to a reduced efficiency of egg hatching (177) which is heavily dependent upon the intestinal microbiota (178). It can be speculated that this would have the net effect of keeping successive infection levels low, reducing the induction of protective immunity. Coinciding with the development of resistance during *T. muris* trickle infection the microbiome appears to partially recover with an increase in diversity and recovery of genera that had been lost earlier in infection (139). The nature of this relationship requires further assessment as several possibilities present themselves: (i) the development of a Th2 response actively promotes a homeostatic microbiota making the recovery in diversity a direct consequence of acquired immunity to STHs; (ii) recovery of the microbiome following loss of diversity occurs independently of host-driven mechanisms, but subsequently facilitates resistance by directly promoting a Th2 response; (iii) STHs produce antimicrobial peptides that restructure the microbiome to suit their own physiology, and when their numbers are reduced this effect is lost and the microbiome recovers as an indirect consequence of host-protective immunity (**Figure 1**).

CONCLUDING REMARKS

Investigation of resistance and susceptibility to intestinal nematode parasites and their underlying immune mechanisms has not only informed on immunity to these particular infectious agents, but has identified novel and fundamental new information on how immunity works. This has no doubt arisen in part from the fact that infection by STHs present a particular set of challenges to the host immune system not seen in other pathogen infections.

The available evidence from the field and from experimental trickle infections of STH has led to a number of generally consistent core observations. Infection from exposure to a low number of infectious stages in any one infection event is more likely to lead to parasite patency than exposure to a "high" number of infectious stages in any one infection event (where the parasites are more often than not expelled, even if not completely). Thus, there appears to be a threshold for an infection event, below which the parasites do not get immunologically expelled and above which they do. This is not only influenced by host genetics, but also the local intestinal environment. It will also vary between different parasite species and the life cycle strategy that they have evolved. It is also clear that as long as the individual infection event remains below a certain level, increases in parasite load are tolerated up to a "critical point." Again, number of infection events and interval between them will influence the ultimate success of the infection and the speed with which the "critical" point is reached. Ultimately, host protective immunity does begin to operate, although it is generally only partial and not sterilizing immunity. Adult parasites often remain for extended periods, although parasite fecundity eventually drops and new juvenile stages do not appear to be able to complete their development effectively i.e., are expelled. Thus, trickle infections are exemplars of concomitant immunity (179, 180). Protective immunological memory does occur with resistance to both high and low dose infection events, although some level of existing infection generally persists.

The single/challenge high dose infection approach to study experimental immunity to STH has been and continues to be spectacularly informative. Nevertheless, bearing in mind the way in which infections are acquired naturally, the trickle infection approach is set to further inform and refine our understanding of how protective immunity is generated, how it is regulated and,

importantly, how it can be improved upon, especially for hosts that are naturally, chronically infected.

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A Truncated Form of HpARI Stabilizes IL-33, Amplifying Responses to the Cytokine

*Caroline Chauché[1,2], Francesco Vacca[1,3], Shin Li Chia[1], Josh Richards[1], William F. Gregory[1], Adefunke Ogunkanbi[4], Martin Wear[5] and Henry J. McSorley[1,4]**

[1] Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom, [2] Wellcome Centre for Integrative Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom, [3] Malaghan Institute of Medical Research, Wellington, New Zealand, [4] Division of Cell Signalling and Immunology, School of Life Sciences, University of Dundee, Dundee, United Kingdom, [5] The Edinburgh Protein Production Facility (EPPF), Wellcome Trust Centre for Cell Biology (WTCCB), University of Edinburgh, Edinburgh, United Kingdom

The murine intestinal nematode *Heligmosomoides polygyrus* releases the *H. polygyrus* Alarmin Release Inhibitor (HpARI) - a protein which binds to IL-33 and to DNA, effectively tethering the cytokine in the nucleus of necrotic cells. Previous work showed that a non-natural truncation consisting of the first 2 domains of HpARI (HpARI_CCP1/2) retains binding to both DNA and IL-33, and inhibited IL-33 release *in vivo*. Here, we show that the affinity of HpARI_CCP1/2 for IL-33 is significantly lower than that of the full-length protein, and that HpARI_CCP1/2 lacks the ability to prevent interaction of IL-33 with its receptor. When HpARI_CCP1/2 was applied *in vivo* it potently amplified IL-33-dependent immune responses to *Alternaria alternata* allergen, *Nippostrongylus brasiliensis* infection and recombinant IL-33 injection, in direct contrast to the IL-33-suppressive effects of full-length HpARI. Mechanistically, we found that HpARI_CCP1/2 is able to bind to and stabilize IL-33, preventing its degradation and maintaining the cytokine in its active form. This study highlights the importance of IL-33 inactivation, the potential for IL-33 stabilization *in vivo*, and describes a new tool for IL-33 research.

Keywords: *Heligmosomoides polygyrus*, IL-33, allergy, cytokine, ILC2

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***Correspondence:**
Henry J. McSorley
hmcsorley001@dundee.ac.uk

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INTRODUCTION

Heligmosomoides polygyrus is a parasitic nematode that infects the intestines of mice. It has a fecal/oral lifecycle, with infective L3 larvae being ingested, and then rapidly penetrating the epithelium of the proximal duodenum. There, the larvae develop to L4 stage and emerge as adults into the intestinal lumen at around day 10 of infection (1, 2). The transit of the parasite through the intestinal wall is likely to cause epithelial damage and cell death, resulting in the release of alarmins such as IL-33 from stromal cells or mast cells (3), in turn inducing an anti-parasite type 2 immune response (4). In order to negate this response, and allow persistence of the parasite in the host, *H. polygyrus* secretes multiple immunomodulatory factors, including Hp-TGM, a protein mimic of host TGF-β (5), and microRNA-containing extracellular vesicles (6) which modulate transcription of multiple host genes, including suppression of Suppression of Tumorigenicity 2 (ST2), the IL-33 receptor. Furthermore, our recent work shows that *H. polygyrus* secretes HpBARI, a protein which binds and blocks ST2 (7). We previously showed that the parasite also secretes the *H. polygyrus* Alarmin Release Inhibitor (HpARI), which blocks IL-33 responses (8).

IL-33 is an alarmin cytokine constitutively produced by epithelial cells. It is stored preformed in the nucleus and released on necrotic cell death, due to mechanical, protease-mediated or chemical damage to the epithelium (9). On necrotic cell death, proteases from the cell cytoplasm, or those secreted by recruited mast cells, neutrophils or those in allergens can then cleave the cytokine between the N-terminus chromatin-binding domain and the C-terminus receptor binding domain, potently increasing the activity of the cytokine (10–12). The IL-33 receptor-binding domain contains four free cysteine residues, which upon release from the reducing nuclear environment into the oxidizing extracellular environment rapidly form disulphide bonds, changing the cytokine's conformation, rendering it unable to bind to its receptor and effectively inactivating it (13). Proteases can also further degrade IL-33 to smaller, inactive forms (12). Thus, the active form of IL-33 has only a very short half-life, and by 1 h after release the vast majority of IL-33 is inactive or degraded.

HpARI binds to the active reduced form of IL-33 and to genomic DNA. This dual binding tethers IL-33 within the nucleus of necrotic cells, preventing its release, and inhibiting interaction of IL-33 with ST2. The HpARI protein consists of 3 Complement Control Protein domains (CCP1-3), and our previous data showed that HpARI binds IL-33 through the CCP2 domain, while DNA-binding was mediated by the CCP1 domain (8). Here, we further characterize the functions of the CCP domains of HpARI, finding that CCP3 stabilizes the interaction between HpARI and IL-33, increasing its affinity and being required for blockade of IL-33-ST2 interactions. Furthermore, we show that HpARI_CCP1/2 (the HpARI truncation lacking CCP3) is able to stabilize IL-33, increasing its half-life and amplifying its effects.

MATERIALS AND METHODS

Protein Expression and Purification

Constructs encoding HpARI, HpARI_CCP1/2 and HpARI_CCP2/3 (all with C-terminus myc and 6-His tags) were cloned into the pSecTAG2A expression vector as previously described (8). Purified plasmids were transfected into Expi293F^{TM} cells, and supernatants collected 5 days later. Expi293F^{TM} cells were maintained, and transfections carried out using the Expi293 Expression System according to manufacturer's instructions (ThermoFisher Scientific). Expressed protein in supernatants were purified over a HisTrap excel column (GE Healthcare) and eluted in 500 mM imidazole. Eluted protein was then dialysed to PBS, and repurified on a HiTRAP chelating HP column (GE Healthcare) charged with 0.1 M $NiSO_4$. Elution was performed using an imidazole gradient and fractions positive for the protein of interest were pooled, dialysed to PBS and filter-sterilized. Protein concentration was measured at A280 nM (Nanodrop, ThermoFisher Scientific), using calculated extinction coefficient.

Surface Plasmon Resonance (SPR)

SPR measurements were performed using a BIAcore T200 instrument (GE Healthcare). Ni^{2+}-nitrilotriacetic acid (NTA) sensor chips, 1-ethyl-3-(3-diaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine ($H_2N(CH_2)_2OH$) were purchased from GE Healthcare. HpARI, HpARI_CCP1/2 or HpARI_CCP2/3 were immobilized and covalently stabilized on an NTA sensor chip essentially as described (14) with the following modifications: following Ni^{2+} priming (30 sec injection of 500 μM $NiCl_2$ at 5 $\mu l \cdot min^{-1}$), dextran surface carboxylate groups were minimally activated by an injection of 0.2 M EDC; 50 mM NHS at 5 $\mu l \cdot min^{-1}$ for 240 sec. Respective proteins (at concentrations between 10 and 400 nM), in 10 mM NaH_2PO_4, pH 7.5; 150 mM NaCl; 50 μM EDTA; 0.05% surfactant P20, were captured *via* the hexa-His tag and simultaneously covalently stabilized to 400 RU, by varying the contact time. Immediately following the capture/stabilization a single 15 s injection of 350 mM EDTA and 50 mM Imidazole in 10 mM NaH_2PO_4, pH 7.5; 150 mM NaCl; 50 mM EDTA; 0.05% surfactant P20, at 30 $\mu l \cdot min^{-1}$, was used to remove non-covalently bound protein, followed by a 180 sec injection of 1 M $H_2N(CH_2)_2OH$, pH 8.5 at 5 $\mu l \cdot min^{-1}$. Prior to any experiments, the surface was further conditioned with a 600 s wash with 10 mM NaH_2PO_4, pH 7.5; 150 mM NaCl; 50 μM EDTA; 0.05% surfactant P20 at 100 $\mu l \cdot min^{-1}$.

SPR single-cycle kinetic titration binding experiments were performed at 25°C. Three-fold dilution series of mIL-33 (2.47 nM to 200 nM), were injected over the sensor surface, in 10 mM NaH_2PO_4, pH 7.5; 150 mM NaCl; 50 μM EDTA; 0.05% surfactant P20, at 30 $ml.min^{-1}$ for 30 s followed by a final 600 s dissociation phase. The on- (k_+) and off-rate (k_-) constants and the equilibrium dissociation constants were calculated from the double referenced sensorgrams by global fitting of a 1:1 binding model, with mass transport considerations, using analysis software (v2.02) provided with the Biacore T200 instrument.

Immunoprecipitation

Protein G dynabeads (ThermoFisher Scientific) were coated with 1 μg mouse ST2-Fc (Biolegend), and washed on a DynaMag-2 magnet with PBS 0.02% Tween 20. 100 ng recombinant murine IL-33 (Biolegend) was then mixed with 1 μg HpARI, HpARI_CCP1/2 or HpARI_CCP2/3, and incubated at room temperature for 15 min, prior to adding to ST2-Fc-coated protein G dynabeads. Beads were washed and bound IL-33 eluted with 50 mM glycine pH2.8, then ran on 4–12% SDS-PAGE gels (ThermoFisher Scientific) under reducing conditions, and transferred to nitrocellulose membranes for western blotting, probing with anti-IL-33 goat polyclonal antibody (R&D Systems AF3626), rabbit anti-goat IgG-HRP secondary antibody (ThermoFisher Scientific) and detected using WesternSure Premium reagent (Licor). Densitometry was carried out using ImageJ, and expressed as fold change from controls at each timepoint.

Animals

BALB/cAnNCrl and C57BL/6JCrl mice were purchased from Charles River, UK. Heterozygous IL-13eGFP$^{+/GFP}$ mice (15) were bred in-house. All mice were accommodated and procedures performed under UK Home Office licenses with institutional oversight performed by qualified veterinarians.

Alternaria Models

Alternaria alternata allergen was used *in vivo* as previously described (8, 16). *Alternaria* allergen (10 μg), OVA (20 μg), HpARI (10 μg) and HpARI_CCP1/2 (10 μg) were intranasally administered to BALB/c mice. Where indicated, the OVA-specific response was recalled by daily intranasal administration of 20 μg OVA protein on days 14, 15, and 16. Tissues were harvested 24 h or 17 days after initial *Alternaria* allergen administration. Lungs were flushed with 4 washes of 0.5 ml ice-cold PBS to collect bronchoalveolar lavage cells, followed by lung dissection for single cell preparation.

Nippostrongylus brasiliensis Infection

The life cycle of *N. brasiliensis* was maintained in Sprague-Dawley rats as previously described (17), and infective L3 larvae were prepared from 1 to 3 week rat fecal cultures. C57BL/6 mice were subcutaneously infected with 400 L3 *N. brasiliensis* larvae, and culled 3 or 6 days later.

Intraperitoneal IL-33 Treatment

Recombinant murine IL-33 (Biolegend) was injected intraperitoneally to C57BL/6 mice (100 ng/mouse). Mice were culled 3 h later and peritoneal lavage cells collected in 3 washes of 3 ml ice-cold RPMI.

Flow Cytometry

Cells were stained with Fixable Blue Live/Dead stain (ThermoFisher Scientific), then blocked with anti-mouse CD16/32 antibody and surface stained with CD3 (FITC, clone 145-2C11), CD5 (FITC, clone 53-7.3), CD11b (FITC, M1/70), CD19 (FITC, clone 6D5), GR1 (FITC, clone RB6-8C5), CD45 (AF700, clone 30-F11), ICOS (PCP, clone 15F9), CD4 (PE-Dazzle, cloneRM4.5), CD11c (AF647, clone N418), Ly6G (PerCP, clone 1A8), CD25 (BV650, clone PC61) (Biolegend); CD49b (FITC, clone DX5), ST2 (APC, clone RMST2-2) (ThermoFisher Scientific); Siglec-F (PE, clone ES22-10D8) (Miltenyi). The lineage stain consisted of CD3, CD5, CD11b, CD19, CD49b and GR1, all on FITC. Samples were acquired on an LSR Fortessa (BD Biosciences) and analyzed using FlowJo 10 (Treestar).

CMT-64 Cell Line

CMT-64 cells (ECACC 10032301) were maintained by serial passage in "complete" RPMI [RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml Penicillin and 100 μg/ml Streptomycin (ThermoFisher Scientific)] at 37°C, 5% CO_2. Cells were seeded into 24- or 96-well plates for Triton-X100 or freeze-thaw treatment, respectively. Cells were grown to 100% confluency prior to 2 washes with PBS. For Triton-X100 treatment, cells were then washed into RPMI 1640 containing 0.1% BSA with or without 0.1% Triton-X100, and incubated at 37°C as indicated, prior to collection of supernatants and measurement of IL-33 by ELISA and western blot. For freeze-thaw assays, cells were then washed into complete RPMI containing 10 μg/ml of HpARI or HpARI_CCP1/2, frozen on dry ice for at least 1 h, then thawed and incubated at 37°C as indicated, prior to collection of supernatants and application to bone marrow cell cultures.

Bone Marrow Cell Culture

Single cell suspensions of bone marrow cells were prepared from C57BL/6 mice, by flushing tibias and femurs with RPMI 1640 medium using a 21 g needle. Cells were resuspended in red blood cell lysis buffer (Sigma) for 5 min at room temperature, prior to resuspension in medium and passing through a 70 μm cell strainer. Cells were cultured in round-bottom 96-well-plates in a final 200 μl volume, containing 0.5×10^6 cells/well. IL-2 and IL-7 were added at 10 ng/ml final concentration, with 50 μl of CMT-64 freeze-thaw supernatant. Cells were then cultured at 37°C, 5% CO_2, for 5 days, prior to assessment of responses by cytokine ELISA and flow cytometry.

Cytokine Measurement

ELISAs were carried out to manufacturer's instructions for IL-5, IL-13 (Ready-SET-go, ThermoFisher Scientific) and IL-33 (Duoset, Biotechne). IL-33 was also measured in CMT-64 supernatants by western blot – supernatants were ran on 4–12% NuPAGE gels (ThermoFisher Scientific) under reducing conditions, before transferring to nitrocellulose membrane and probing with goat anti-mIL-33 (Biotechne), and rabbit anti-goat IgG HRP secondary antibody (Thermo Fisher), and detected using WesternSure Premium reagent (Licor).

Statistical Analysis

All data was analyzed using Prism (Graphpad Software Inc.). One-way ANOVA with Dunnet's multiple comparisons post-test was used to compare multiple independent groups, while two-way ANOVA and Tukey's multiple comparison's post-test was used to compare multiple timepoints or concentrations between independent groups. Where necessary, data was log-transformed to give a normal distribution and to equalize variances. $****p < 0.0001$, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$, N.S. = Not significant ($p > 0.05$).

RESULTS

HpARI CCP2 Binds IL-33, While HpARI CCP3 Is Required to Block IL-33-ST2 Interaction

Constructs encoding full-length HpARI, or truncations lacking CCP3 (HpARI_CCP1/2), or lacking CCP1 (HpARI_CCP2/3) were expressed in Expi293F™ mammalian cells, and purified on 6-His tags. These constructs were then tested for binding to IL-33 in surface plasmon resonance experiments, showing that the affinity for IL-33 of full-length HpARI and HpARI_CCP2/3 were similar (K_d of 1.1 +/− 0.44 nM and 1.4 +/− 0.14 nM, respectively), while HpARI_CCP1/2 had approximately a 10-fold lower affinity for the cytokine (K_d = 9.8 +/−6.7 nM). This difference in affinity was largely due to an approximately 20-fold faster off-rate for HpARI_CCP1/2 (K_- of 30×10^{-4} s^{-1} vs. 1.5×10^{-4} s^{-1} for HpARI) (**Figure 1A**).

The CCP3 domain also appears important for preventing IL-33-ST2 interactions. While full-length HpARI and HpARI_CCP2/3 were able to prevent IL-33 immunoprecipitation by ST2-Fc, HpARI_CCP1/2 could not (**Figure 1B**).

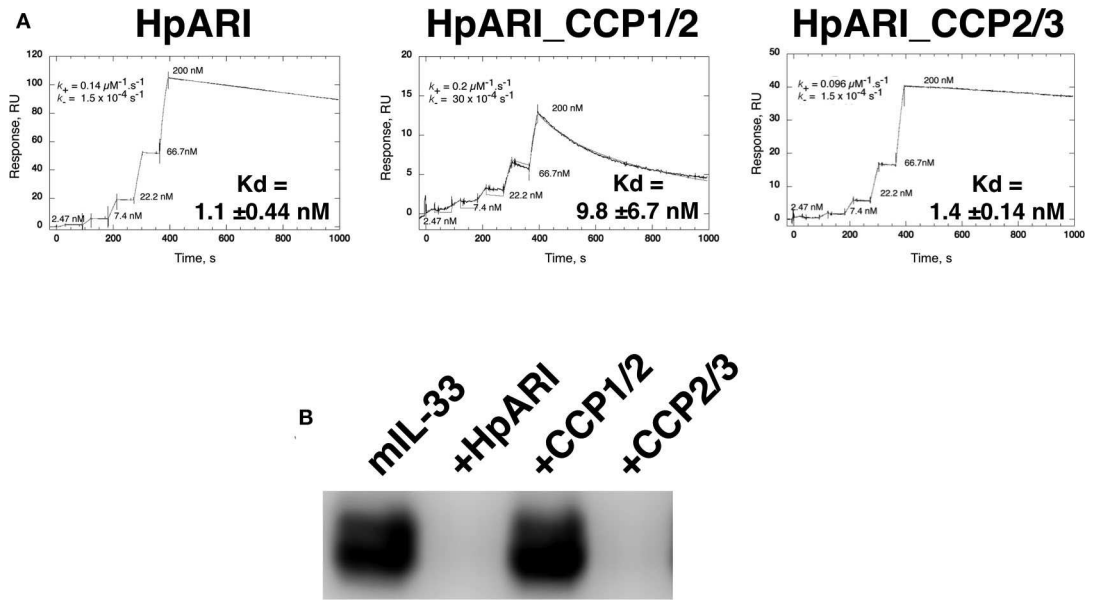

FIGURE 1 | (A) Surface plasmon resonance measurements of IL-33 binding to chip-bound HpARI, HpARI_CCP1/2 and HpARI_CCP2/3. Kd values calculated from 3 replicate experiments, and indicates mean and SD. **(B)** ST2-Fc was bound to protein G-coated magnetic beads and used to immunoprecipitate murine IL-33 (mIL-33). IL-33 western blot of eluted material shown. Image representative of two independent experiments.

HpARI_CCP1/2 Increases Responses to IL-33

We previously showed that HpARI_CCP1/2 was capable of suppressing the release of IL-33 *in vivo*, 15 min after *Alternaria alternata* administration (8). To assess whether HpARI_CCP1/2 could replicate the inhibition of IL-33-dependent responses seen with full-length HpARI, we administered HpARI or HpARI_CCP1/2 together with *Alternaria* allergen and OVA protein and assessed type 2 immune responses after OVA challenge 2 weeks later (**Figure 2A**). While HpARI suppressed allergic reactivity in this model (as shown previously (8)), HpARI_CCP1/2 had the opposite effect, increasing BAL and lung eosinophil, and lung ILC2 and $ICOS^+ST2^+$ Th2 cell numbers (18) (**Figure 2A** and **Supplementary Figure 1**).

When the innate *Alternaria*-induced immune response was assessed 24 h after initial administration of the allergen to naïve mice, we found that although HpARI_CCP1/2 did not change the eosinophil response compared to *Alternaria* alone, HpARI_CCP1/2 increased BAL neutrophil numbers. At this timepoint, no ILC2 proliferation has yet occurred, as previously described (19), so total lung ILC2 cell numbers were similar in all groups (data not shown). However, allergen-activated ILC2s showed strong upregulation of CD25 expression, as described previously during activation of ILC2s in this model (20), which was further increased by HpARI_CCP1/2 (**Figure 2B**).

To exclude the possibility that HpARI_CCP1/2 is interfering with the *Alternaria* allergen directly, exacerbating the response to it, we used a second model of IL-33-dependent responses (21–23), infecting mice with *Nippostrongylus brasiliensis* and administering HpARI or HpARI_CCP1/2 to the lungs during the first 3 days of infection. During *N. brasiliensis* infection, L3 larvae migrate through the lung at days 1–4, enter the intestines as L4 larvae and develop to adults at days 4–10 post-infection (21). Mice were culled at days 3 and 6 post-infection, when parasites were present in the lung and gut, respectively, and the type 2 immune response in the lung was assessed at both timepoints. Again, HpARI suppressed type 2 immune responses as shown previously (8), while HpARI_CCP1/2 increased BAL eosinophilia, IL-5 and IL-13 production (**Figure 2C**). Neither HpARI nor HpARI_CCP1/2 had any effect on BAL neutrophilia at these timepoints (data not shown), implying that neutrophil recruitment in *N. brasiliensis* is not IL-33 dependent. Similarly, in *Strongyloides venezuelensis* lung-stage infection, neutrophil recruitment is IL-33-independent (24).

Finally, we utilized a model of recombinant IL-33 intraperitoneal injection, which induces a mast cell-dependent neutrophilia (25, 26), in contrast to the ILC2-dependent, largely eosinophilic response seen on IL-33 release in the lung. Again, here we found that while HpARI suppressed IL-33 induced neutrophilia, HpARI_CCP1/2 exacerbated it (**Figure 2D**).

In conclusion, HpARI_CCP1/2 amplifies IL-33-dependent responses *in vivo*. We hypothesized that this activity was due to stabilization of the cytokine, increasing its effective half-life. To test this hypothesis, we developed an *in vitro* model of IL-33 release and IL-33 responses.

HpARI_CCP1/2 Maintains IL-33 in Its Active Form

The CMT-64 cell line constitutively produces IL-33, which is released on cellular necrosis (12). Confluent CMT-64 cells were washed into PBS+0.1% BSA, and necrosis induced by addition of 0.1% Triton-X100, in the presence or absence of HpARI

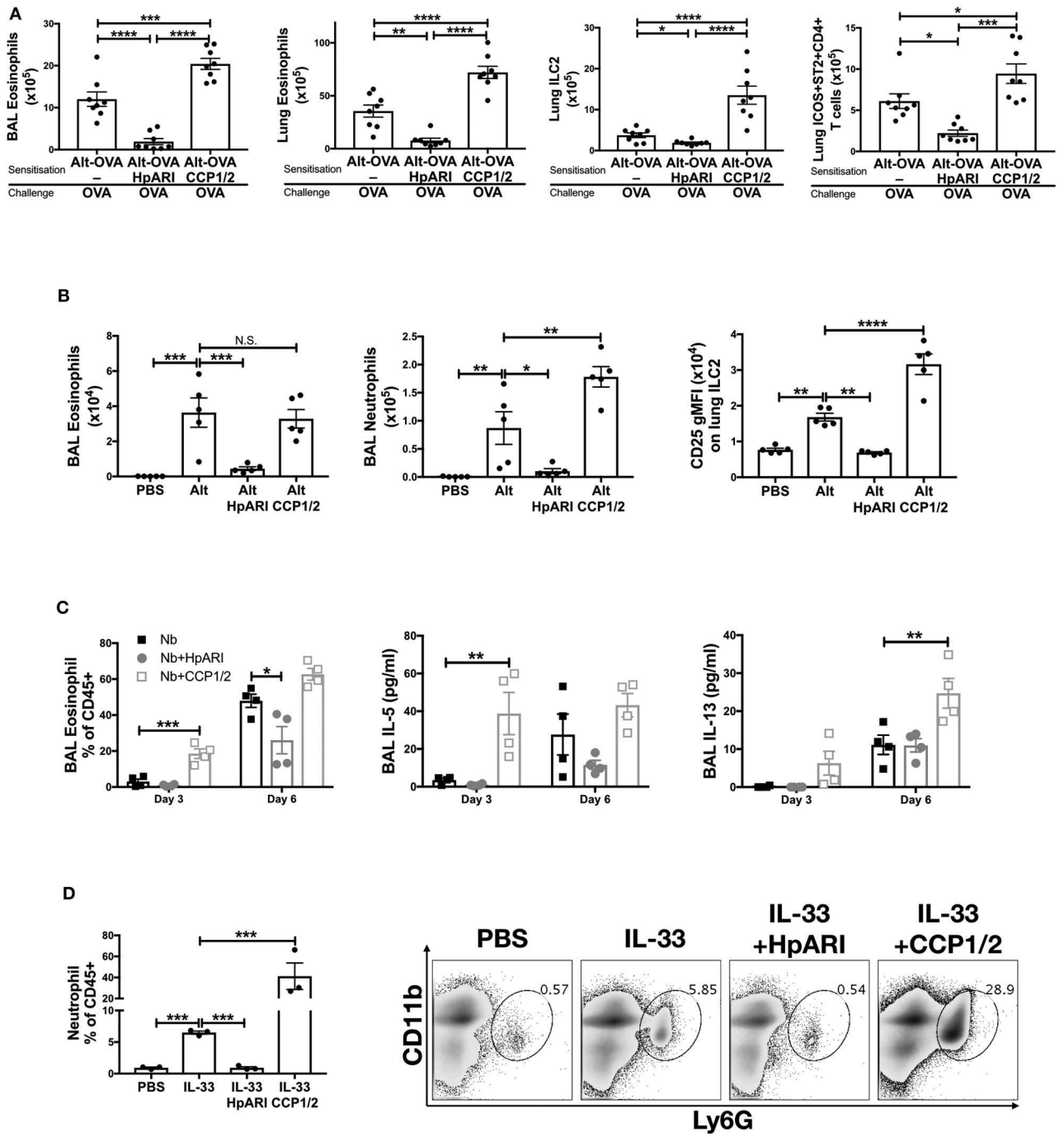

FIGURE 2 | (A) HpARI or HpARI_CCP1/2 (CCP1/2) were co-administered with *Alternaria* allergen and OVA by the intranasal route, then the OVA-specific response recalled 2 weeks later. BAL and lung eosinophil (Siglecf+CD11c–CD45+), and lung ILC2 (ICOS+lineage–CD45+) and Th2 (ICOS+ST2+CD4+lineage+CD45+) cell numbers shown. Data pooled from 2 repeat experiments each containing 4 mice per group. **(B)** HpARI_CCP1/2 (CCP1/2) was coadministered with *Alternaria* allergen by the intranasal route. After 24 h, BAL eosinophil (Siglecf+CD11c–CD45+) and neutrophil (Ly6G+CD11b+Siglecf–CD11c–CD45+) cell numbers, and lung ILC2 CD25 geometric mean fluorescent intensity were assessed by flow cytometry. Data representative of 2 repeat experiments each containing 3–5 mice per group. **(C)** HpARI or HpARI_CCP1/2 were intranasally administered on days 0, 1, and 2 after infection with *Nippostrongylus brasiliensis*. BAL eosinophil (Siglecf+CD11c–)% of CD45+ cells, and BAL IL-5 and IL-13 were measured on days 3 and 6 post-infection. Data representative of 3 repeat experiments, each with 4 mice per group. **(D)** Recombinant IL-33 was intraperitoneally injected with HpARI or HpARI_CCP1/2, and proportions of Ly6G+CD11b+ neutrophils in the CD45+ peritoneal lavage population assessed 3 h post-injection. Representative FACS plots shown of CD45+ live cells. Data representative of 2 repeat experiments, each with 3 mice per group. Error bars show SEM. N.S. = Not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

or HpARI_CCP1/2. Over a 24 h timecourse following Triton-X100 addition, we assessed IL-33 release by ELISA and western blot. IL-33 ELISA showed that Triton-X100 caused rapid IL-33 release, with high concentrations of the cytokine detected in culture supernatants within 15 min of addition of the detergent in control wells. IL-33 levels then gradually decreased at later timepoints, presumably as the protein was degraded (**Figure 3A**) (12). HpARI addition ablated the IL-33 signal seen in the ELISA, as shown in our previous study (8): as well as retarding the release of the cytokine, HpARI binding also out-competes the ELISA antibodies, abolishing detection of IL-33. HpARI_CCP1/2 did not abolish detection of IL-33 in the ELISA, but did reduce the IL-33 signal at early timepoints. Moreover, in the presence of HpARI_CCP1/2, IL-33 accumulated over the timecourse and maintained high levels at later timepoints.

In contrast, when IL-33 in the same samples was assessed by western blot, a very strong signal was seen at all timepoints at a size consistent with full-length IL-33 protein (∼30 kDa), while a weaker signal was seen at around 18–20 kDa, consistent with processed mature IL-33 (**Figure 3B** and **Supplementary Figures 2A,B**). While a strong full-length IL-33 band was seen across all timepoints and treatments, the density of the mature bands were dynamically altered by the presence of each treatment. In control wells, mature IL-33 was present early after Triton-X100 treatment and was degraded at later timepoints. In contrast, in the presence of HpARI_CCP1/2, the mature form was present at lower intensities than in control wells at early timepoints, but accumulated over the timecourse and was strongest at 24 h post Triton-X100 treatment, reflecting ELISA data (**Figure 3A**). HpARI treatment had a similar effect to HpARI_CCP1/2 when IL-33 was assessed by western blot. Quantification of band intensities by densitometry reflected this increase of mature IL-33 signal in the presence of HpARI or HpARI_CCP1/2 (**Supplementary Figure 2C**). The difference in IL-33 signal strength between ELISA and western blot in the presence of HpARI was seen in a previous study (8), and is thought to be due to interference with antibody binding to the endogenous IL-33-HpARI complex in ELISA, but in a denaturing western blot proteins from this complex are dissociated and available for antibody detection. Together, this data suggests that binding of IL-33 by HpARI or HpARI_CCP1/2 stabilizes the mature cytokine, protecting it from degradation.

To assess the activity of the cytokine released, we induced necrosis of CMT-64 cells via freeze-thaw treatment. This treatment could be carried out in complete culture medium (without toxic additives such as Triton-X100), allowing downstream assessment of cellular responses to the released cytokine. On thaw, necrotic CMT-64 cells were incubated for up to 48 h at 37°C, and IL-33 levels in supernatants assessed by ELISA. Similarly to Triton-X100-mediated necrosis, we found high levels of IL-33 released rapidly after freeze-thaw necrosis, which gradually decreased over the 48 h timecourse in control wells, while IL-33 levels increased over the timecourse in the presence of HpARI_CCP1/2 (**Figure 3C**). These supernatants were applied to total bone marrow cells from IL-13eGFP$^{+/GFP}$ reporter mice (15) cultured in the presence of IL-2 and IL-7 (to support ILC2 differentiation), and cytokine responses were assessed 5 days later. As shown in **Figure 3D**, control freeze-thaw CMT-64 supernatants could only induce bone marrow cell IL-5 and IL-13 production at early timepoints post-thaw, implying that after ∼6 h post-thaw, all IL-33 present in the culture medium was inactive. This response appeared IL-33-dependent as HpARI entirely inhibited IL-5 and IL-13 release. In contrast, supernatants from cells freeze-thawed in the presence of HpARI_CCP1/2 were able to maintain high levels of IL-5 and IL-13 stimulation (∼10-fold higher than the peak production seen in control wells) and this stimulation was maintained even when supernatants had been incubated for 48 h post-thaw. To specifically assess the ILC2 response within these total bone marrow cell cultures, we used flow cytometry for IL-13eGFP reporter or CD25 expression on ICOS^{+}lineage^{-}CD45^{+} ILC2s to confirm that these cells were activated by supernatants from medium of freeze-thaw control wells at early (45 min post-thaw), but not late (48 h post-thaw) timepoints, while wells containing HpARI_CCP1/2 remained highly activated throughout the timecourse (**Figure 3E** and **Supplementary Figure 3**).

DISCUSSION

HpARI blocks IL-33 responses and is secreted by *H. polygyrus*, as part of a suite of immunomodulatory effector molecules which act to prevent immune-mediated ejection of the parasite (27). HpARI acts by binding to IL-33 through the HpARI CCP2 domain and to genomic DNA in necrotic cells through the HpARI CCP1 domain, tethering the cytokine within the necrotic cell nucleus and preventing its release (8). Here, we further characterize these interactions, showing that a synthetic, non-natural construct lacking the CCP3 domain (HpARI_CCP1/2) binds IL-33 with an approximately 10-fold lower affinity than the full-length HpARI protein, and lacks the blocking activity of HpARI against IL-33-ST2 interactions. Furthermore, HpARI_CCP1/2 had the surprising effect of stabilizing and amplifying IL-33 responses *in vitro* and *in vivo*.

As opposed to HpARI_CCP1/2, HpARI_CCP2/3 showed high affinity binding to IL-33, and prevented ligation of ST2 by IL-33, replicating the IL-33-blocking effects of full-length HpARI. In a previous study (8), we showed that HpARI_CCP2/3 lacked the DNA-binding activity of full-length HpARI and HpARI_CCP1/2, implying that this activity is mediated by the CCP1 domain. We previously also showed that HpARI_CCP2/3 increased, rather than decreased IL-33 levels in the bronchoalveolar lavage of mice 15 min after *Alternaria* allergen treatment. Our work here supports the hypothesis that this increase in IL-33 is due to HpARI_CCP2/3 preventing the rapid uptake and degradation of bound IL-33 by ST2-expressing immune cells (13, 28, 29), while lacking the DNA-binding (and hence tethering function) of HpARI or HpARI_CCP1/2. Thus, all IL-33 released is retained in the bronchoalveolar lavage, leading to increased IL-33 levels compared to controls.

IL-33 is known to mediate parasite expulsion in a type-2 dependent-manner (22). The HpARI_CCP1/2 truncated protein maintains the activity of IL-33, potentially amplifying its anti-parasitic effects. It is worthwhile emphasizing that this truncated construct is not a protein naturally secreted by the parasite, but rather a synthetic product with an unexpected activity.

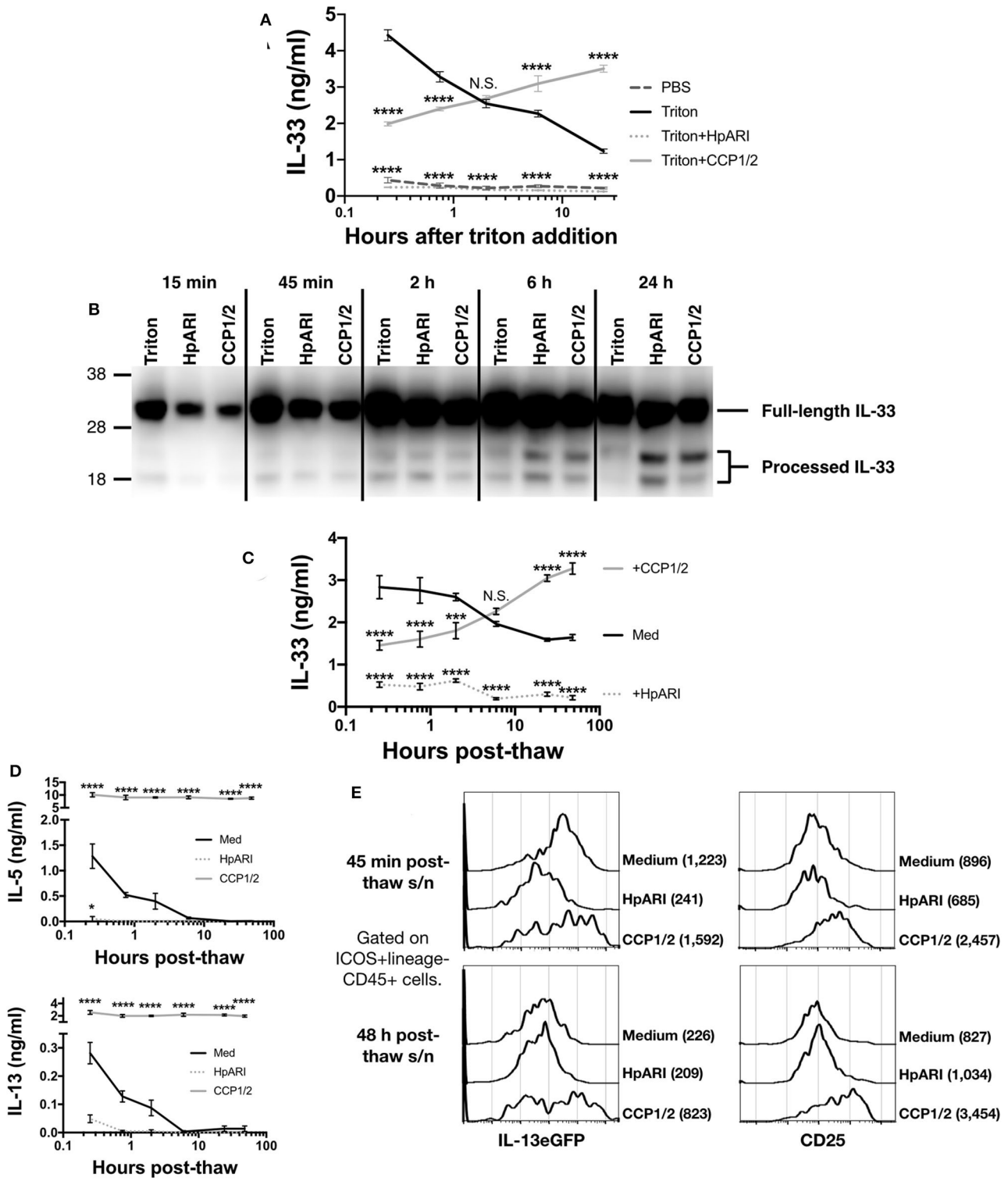

FIGURE 3 | (A) CMT-64 cells were cultured to confluency and treated with 0.1% Triton-X100+0.1% BSA alone, or in the presence of HpARI or HpARI_CCP1/2 (CCP1/2). Supernatants were harvested over a timecourse and IL-33 levels assessed by ELISA. Each measurement contains 4 technical replicates and is representative of 3 repeat experiments. **(B)** IL-33 western blot of pooled samples from **(A)**. Representative of 3 repeat experiments. **(C)** CMT-64 cells were cultured to confluency in RPMI+10% FCS, and freeze-thawed in the presence of complete medium (Med), HpARI or HpARI_CCP1/2. After thaw, cultures of necrotic cells were incubated at 37°C, and supernatants taken over a timecourse, and assessed for IL-33 levels by ELISA. Each timepoint shows 4 technical replicates. **(D)** Supernatants

(Continued)

FIGURE 3 | from **(C)** were applied to IL-13eGFP$^{+/GFP}$ bone marrow cells in the presence of IL-2 and IL-7 and cultured for 5 days. Levels of IL-5 (upper panel) and IL-13 (lower panel) in supernatants were assessed by ELISA. Each timepoint shows 4 technical replicates. **(E)** Bone marrow cells from **(D)** after 5 days of culture were pooled, stained, and gated on ICOS+lineage–CD45+ ILC2s, and assessed for IL-13eGFP and CD25 expression. Numbers in parentheses indicate geometric mean fluorescent intensity for each condition. All data from **(C–E)** is representative of 3 repeat experiments. Error bars show SEM. N.S. = Not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

As the IL-33 pathway is strongly implicated in human asthma, HpARI, with its unique mechanism of action and strong binding to IL-33, is a potential therapeutic agent. IL-33 is a potently inflammatory cytokine which is kept tightly regulated. Once released, IL-33 undergoes rapid oxidation and degradation, confining its effects to a short time after release (12, 13). Addition of HpARI or HpARI_CCP1/2 prevented degradation of the cytokine and maintained it in its active form, possibly due to steric hinderance of proteases. As HpARI also blocked the interaction of IL-33 with its receptor there was no cellular response to IL-33 in the presence of HpARI, while HpARI_CCP1/2, which lacks this IL-33-ST2 blocking activity, was unable to inhibit responses to IL-33. Furthermore, most surprisingly, HpARI_CCP1/2 was able to maintain the effects of IL-33 over a long timecourse, potently exacerbating IL-33-dependent responses *in vivo* and *in vitro*.

The effects of HpARI_CCP1/2 may not be confined to extending the half-life of IL-33 by preventing its degradation, but may prevent the much more rapid oxidation of the cytokine. Partial oxidation of IL-33 occurs *in vivo* within 15 min of release (13), therefore the activity of released IL-33 *in vivo* may be less than that of fully active IL-33. Indeed, when a purified wild-type or an oxidation-resistant mutant of human IL-33 were tested *in vitro*, the mutant form of IL-33 was found to be 30-fold more potent than WT IL-33 (13). In this study, we were not able to measure the difference between reduced and oxidized IL-33, therefore we cannot make definitive statements about this activity of HpARI_CCP1/2. However, inhibition of IL-33 inactivation, either through prevention of oxidation or proteolytic degradation, could be a potent method for amplifying IL-33-dependent responses.

Although IL-33 is strongly implicated in inducing eosinophilic inflammation in anti-parasite or allergic type 2 immune responses (21, 30), the cytokine has also shown protective effects in models of colitis (31), graft-vs.-host disease (32), autoimmunity (33), obesity (34), wound healing and tissue restoration (35, 36). Therefore, treatments which amplify endogenous IL-33 responses could have clinical potential in a range of treatments.

HpARI_CCP1/2 could also be a useful tool for IL-33 research. Modulating IL-33 responses by using HpARI and HpARI_CCP1/2 in parallel allows assessment of the role of IL-33 in a system in the absence of potentially confounding effects of recombinant cytokine administration or genetic manipulation. In addition, the strategy of IL-33 stabilization by HpARI_CCP1/2 may be able to be replicated using a monoclonal antibody-based therapy, with low-affinity or non-blocking antibodies potentially able to amplify IL-33 responses. As anti-IL-33 treatments enter clinical trials (37), this is an important consideration, as sub-optimal antibodies could result in amplification rather than suppression of IL-33 responses.

This study sheds further light on the mechanism of binding of HpARI to IL-33, the function of the domains of HpARI, and the effects of IL-33 degradation and inactivation. Further structural characterization of HpARI–IL-33 binding will be useful in characterizing this interaction and could allow guided design of more effective IL-33-blocking or IL-33-amplifying therapeutic agents.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

All mice were accommodated and procedures performed under UK Home Office licenses with institutional oversight performed by qualified veterinarians. UK Home Office project license number 70/8733.

AUTHOR CONTRIBUTIONS

CC, FV, MW, and HM designed and planned experiments. CC, FV, SC, JR, WG, AO, MW, and HM undertook experiments. MW provided guidance on the design of the SPR experiments and carried these out. HM supervised the work and wrote the first version of the paper. CC, FV, and AO were involved in reviewing and revising the paper. All authors have approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01363/full#supplementary-material

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REVIEW
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The Heterogeneity, Origins, and Impact of Migratory iILC2 Cells in Anti-helminth Immunity

Mindy M. Miller[1] *and R. Lee Reinhardt*[1,2]*

[1] Department of Biomedical Research, National Jewish Health, Denver, CO, United States, [2] Department of Immunology and Microbiology, University of Colorado-Anschutz Medical, Aurora, CO, United States

Soil-transmitted helminths represent a major global health burden with infections and infection-related comorbidities causing significant reductions in the quality of life for individuals living in endemic areas. Repeated infections and chronic colonization by these large extracellular worms in mammals led to the evolution of type-2 immunity characterized by the production of the type-2 cytokines interleukin (IL)-4, IL-5, and IL-13. Although a number of adaptive and innate immune cells produce type-2 cytokines, a key cellular source in the context of helminth infection is group 2 innate lymphoid cells (ILC2s). ILC2s promote mucosal barrier homeostasis, integrity, and repair by rapidly responding to epithelial cues in mucosal tissues. Though tissue-resident ILC2s (nILC2s) have been studied in detail over the last decade, considerably less is known with regard to a subset of inflammatory ILC2s (iILC2s) that migrate to the lungs of mice early after *Nippostrongylus brasiliensis* infection and are potent early producers of type-2 cytokines. This review will discuss the relationship and differences between nILC2s and iILC2s that establish their unique roles in anti-helminth immunity. We have placed particular emphasis on studies investigating iILC2 origin, function, and their potential long-term contribution to tissue-resident ILC2 reservoirs in settings of helminth infection.

Keywords: iILC2, nILC2, ILC2, helminth, *Nippostrongylus brasiliensis*, IL-4, IL-13, type-2 immunity

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***Correspondence:**
R. Lee Reinhardt
reinhardtl@NJHealth.org

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INTRODUCTION

Global Health Burden of Soil-Transmitted Helminths

Soil-transmitted helminths include roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Necator americanus; Ancylostoma duodenale*) together accounting for infections in at least 1.4 billion people currently, with half of the world's population remaining at risk (1–3). Hookworm transmission is initiated when fertilized eggs are excreted in the feces of infected hosts and hatch in the environment to release infectious larvae. When bare skin is exposed to fecal-contaminated soil, hookworm larvae penetrate the skin, enter the circulation of the host, and migrate to the lungs. Here they enter the parenchyma via pulmonary alveolar capillaries, differentiate and move into pulmonary airspaces, ascend the pharynx, and are swallowed. Larvae eventually take residence in the lumen of the small intestine where they mature into adults and lay eggs. The exception to this lifecycle is the hookworm *Anclostoma duodenale*, which can be transmitted through the direct ingestion of larvae instead of epithelial penetration.

While mortality due to STH is concerning, the massive clinical burden associated with helminths manifests from the comorbidities related to anemia, abdominal pain, diarrhea, dehydration, and physical and cognitive growth retardation (4–6). It is estimated that combined STH infection contributes to 5–14 million disability-adjusted life years (DALYs) which disproportionately affects low-income economies already stressed with unmet health care needs (3, 7, 8). However, even this is likely an underestimation of the overall burden associated with STH infections (9). One DALY equates to the loss of 1 year of "healthy" life over the lifetime of an individual. Although reports of infection rates and DALYs vary, hookworms alone are estimated to infect 400 million individuals and are responsible for 3.2 million DALYs (1, 7). This is concerning in particular for school-aged children as hookworm infection has long been associated with reduced cognitive function and academic performance (10–13). Moreover, mothers infected with helminths even a single time during pregnancy may give birth to infants with impaired cognitive and gross motor function (14). Unfortunately, this early-life impairment could lead individuals on a trajectory toward lower productivity and earning capacity when reaching adulthood (15). These comorbidities are compounded by nutrition deficits associated with high helminth burdens. Chronic and repeated helminth colonization has been described as the "world's most important nutrition problem" (16).

Preventative measures have proven highly successful in limiting helminth infections among industrialized nations. This may be best demonstrated by the reduction in the incidence in *Necator americanus* infections within the southern United States following the Rockefeller Sanitary Commissions' influence in implementing better hygiene practices and access to anthelmintics starting in the early 1900s (17). As a result of these practices, hookworm infection in the United States was effectively eliminated (18). A similar effect was observed during the industrialization of Japan (18). However, in developing nations and more rural regions of the world where basic sanitary needs—such as running water and sewage treatment plants—are lacking and where access to drugs is limited, such preventative measures have been less successful (3, 19, 20). This is despite the continued goal of the World Health Organization to eliminate STH infections as a public health concern (21). The majority of the WHO's approach has been based on mass chemotherapeutic approaches that would provide regular treatment to 75% of school age children living in regions endemic to helminth infections. In support of such an approach, several pharmaceutical agents exist to eliminate STH infections, the most common being benzimidazoles which kill the parasite through preventing microtubule polymerization. Although anthelmintic drugs have proven effective at eliminating current infections, they are not preventative and must be re-administered for each subsequent infection (6). As such, repeated treatments have been deemed ineffective in many of the low-socioeconomic communities due to costs associated with frequent clinic visits and the logistics of providing routine access to drugs within key endemic populations. For example, 39 countries do not yet meet the 75% treatment goal, and when preschool-aged children are included in the target demographic, <50% of children are receiving the expected chemotherapy regimen (3, 22). Even among children receiving treatment, the high rates of reinfection and inconsistent access to drugs has made the goal of helminth eradication by this approach difficult to achieve (5, 23–25). Furthermore, there is increasing concern that sporadic chemotherapeutic interventions will increase the incidence of drug-resistance, particularly in nematodes (20, 26, 27). Additional concern lies in side effects associated with these drugs. Even though most anthelmintics are well-tolerated, side effects include gastrointestinal discomfort and the high doses required to treat echinococcal liver cysts have been associated with hair loss, bone marrow suppression, and hepatic injury (28). Thus, while advances in anthelmintic drugs are likely to continue to make a positive impact on the global burden of helminth-related morbidity, there is a continued need for additional pharmaceutical agents and/or vaccines tailored toward the safe prevention/elimination of infections. Novel approaches to limit infection and worm burden would benefit from a more complete understanding of the immune response to helminths.

Type-2 Inflammation and Anti-helminth Immunity

The majority of animal studies assessing immunity to soil-transmitted helminths utilize either the murine hookworm *Nippostrongylus brasiliensis,* which mimics the lifecycle of the human pathogen *Necator americanus,* or the fecal-oral, intestinal roundworm model of *Heligomosomoides polygyrus*. Whether assessing the lung or the intestine in these models, a characteristic wound healing or tissue repair response is observed and is referred to as type-2 immunity (29). Although type-2 immune responses are often observed in the context of allergic asthma, this form of immunity likely evolved to protect the host from comorbidities associated with chronic or repeated helminth exposure and is ideally suited to promoting parasite clearance and tissue repair (30–32). While the relationship between anti-helminth immunity and allergic disease is complex, evidence suggests that despite invoking similar type-2 inflammatory processes, helminth infection does not always exacerbate allergic inflammation (33). In fact, recent literature shows that there is likely an early-life window that can be exploited to influence an individual's susceptibility to chronic diseases including asthma in later life (34). Such helminth-mediated suppression of allergic immunity would support the observation that individuals living in rural areas endemic to helminth infection develop allergic disease in a smaller percentage of the population than do individuals living in more industrialized communities that are devoid of parasitic helminths (35–38). Empirical studies also support this conclusion (39–42). Mice infected with various parasitic helminths, including *Heligmosomoides polygyrus, Nippostrongylys brasiliensis,* and *Litomosoides sigmodontis*, show reduced allergic lung disease when sensitized and challenged with allergens. Together, the evidence suggests that mammals evolved suppressive mechanisms that work in concert with type-2 inflammation to tolerate and/or clear helminth infection. These mechanisms

are likely advantageous to individuals by limiting the damage induced by repeated worm infection and colonization. As such, allergy and asthma may be more recent manifestations of type-2 inflammation in hosts that lack the natural exposure to these parasitic worms (43, 44). In this case, the "poised" type-2 immunity designed to tolerate helminths now responds inappropriately to innocuous allergens. This represents an important variation of the hygiene hypothesis (45–47).

Type-2 immunity is orchestrated through the production of the type-2 cytokines interleukin (IL)-4, IL-5, and IL-13 (48). These three cytokines have both unique and redundant roles in anti-helminth immunity. IL-4 produced by follicular helper T cells in the context of helminth infection is critical for the production of immunoglobulin (Ig) E and high-affinity IgG1 (49–51). Although the crosslinking of IgE receptors on basophils and mast cells appears limited in primary *N. brasiliensis* infection, IgE-mediated activation of basophils is likely more extensive during secondary helminth responses (52, 53). In addition, IL-4 from basophils and eosinophils also promotes type-2 inflammation at the site of infection/colonization (54–56). Although IL-13 likely plays a role within pathogenic IgE response to allergens, it does not appear to affect the production of IgE during acute helminth infection (57, 58). Instead, IL-13 primarily acts on the epithelium at mucosal barriers. Specifically, IL-13 can enhance goblet and tuft cell hyperplasia, increase mucus production, accelerate epithelial cell turnover, and aid in smooth muscle contractility in settings of type-2 inflammation (57, 59–62). IL-13 appears to be dominant to IL-4 in these processes primarily due to two factors. First, while the IL-4 receptor found on goblet cells and smooth muscle cells can bind both IL-4 and IL13, it has higher affinity for IL-13 (63, 64). Second, while IL-4 is the dominant cytokine produced in lymphoid tissues during helminth infection, IL-13 appears to be more restricted to immune cells residing in mucosal tissues (50, 57). This increases the availability of IL-13 to modulate goblet cell hyperplasia and smooth muscle contractility. Furthermore, type-2 cytokines can induce the production of downstream epithelial cytokines that are equally important in pathogen clearance. For example. IL-4/IL-13-induced goblet cells produce the cytokine RELM-beta which can directly impair helminth fecundity and survival (65, 66). Unlike IL-4 and IL-13—which can compensate to promote characteristic type-2 hallmarks (67, 68)—IL-5 appears to have a more specific role in type-2 responses confined primarily to the mobilization of eosinophils from the bone marrow to enhance wound healing and further increase IL-4 levels as they are the most prevalent IL-4-competent population at the peak of the *N. brasiliensis* response (56). Moreover, eosinophils likely play a role in direct killing of infectious larvae, particularly after reinfection (69).

The biologic importance of IL-4, IL-5, and IL-13 in anti-helminth immunity is best evidenced by changes in helminth worm clearance among cytokine-deficient mice. In support of IL-13 being the dominant type-2 cytokine in promotion of worm expulsion, IL-13-deficient mice displayed delayed helminth clearance compared to IL-4-deficient animals (70, 71). However, mice lacking both IL-4 and IL-13 exhibited a greater deficit in worm clearance than that observed in IL-13 single knockouts, indicating synergy of these type-2 cytokines in the context of *N. brasiliensis* infection (72). This result was phenocopied in mice where IL-13-producing cells were deleted upon diphtheria toxin administration (57). Importantly, IL-4/IL-5/IL-13-triple knockout mice show exceptionally delayed *N. brasiliensis* clearance (73). Together these cytokines perform three key functions important to anti-helminth immunity. First, they promote the mobilization of innate and adaptive type-2 immune cells to sites of helminth infection and mucosal barrier damage. Second, they work synergistically and independently to induce the weep (mucus production) and sweep (smooth muscle contractility) response to mechanically expel the worms. Lastly, these cytokines are involved in the repair of damaged epithelium that results during helminth migration and colonization. Although IL-4, IL-5, and IL-13 are responsible for the majority of type-2 immune hallmarks observed during helminth infection, IL-9 has been described to have additional effects on worm clearance (73, 74).

A systematic assessment of the immune response throughout *N. brasiliensis* infection highlights the lung as an essential location of immune orchestration (**Figure 1**). While the first encounter of the pathogen occurs at the epithelium when larvae penetrate the skin and enter circulation, this event is relatively quick (0–6 h). Nonetheless, there will be some local irritation and epithelial damage that recruits neutrophils and eosinophils to the tissue. The most extensive tissue damage occurs after the parasite has traveled through the vasculature and invades the lung parenchyma via alveolar capillaries in the airspace between 18 and 72 h after infection (31). This leads to hemorrhage and acute lung injury, partly mediated through neutrophil accumulation and activation (31). Epithelial cell-derived trefoil factor 2 controls helminth induced hemorrhagic lung injury and is necessary for IL-33 production (75). Resulting epithelial injury releases IL-25 and IL-33, both of which prompt type-2 cytokine production first from type-2 innate lymphoid cells (ILC2s) and, later, Th2 cells. After entry into the lung parenchyma, L3 larvae undergo maturation to the L4 stage and migrate to the airways where they travel up the pharynx and are swallowed. Upon entering the upper small intestine, L4 larvae latch onto the epithelium to feed, mature into L5 larvae, and produce eggs which are excreted in feces. Similar to the lung, damaged intestinal epithelium produces IL-25 and IL-33 further promoting the weep and sweep response. In mice with intact immune systems, the helminth is cleared within 8–10 days after infection. This is also when adaptive Th2 cells and eosinophils predominate as the major type-2 producing immune populations in the lung (56). Of note, the pathology observed in the lung after day 7 of infection by *N. brasiliensis* strongly resembles that of human asthmatic airways (32, 76).

As discussed above, tissue alarmins IL-25 and IL-33 are essential to ILC2 function in anti-helminth immunity. While damaged/dying epithelium represent important sources of these alarmins during the initial stages of helminth infection, many additional cellular sources have been described which likely impact ILC2 cells later in the response. For example, IL-33 is produced by adventitial stromal cells during helminth infection whereas type II pneumocytes and white adipose tissue-resident

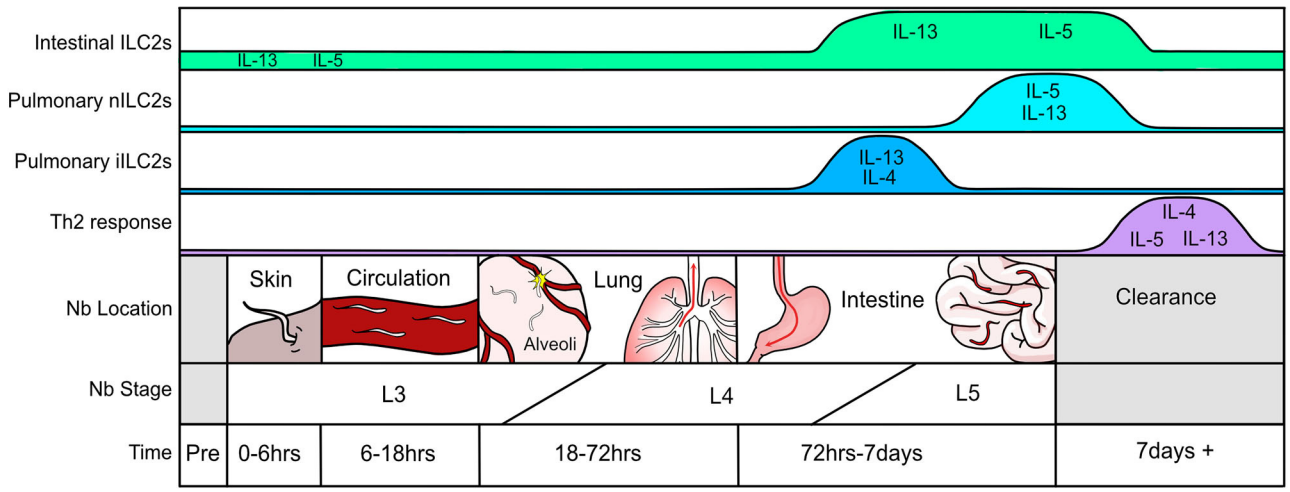


FIGURE 1 | Timeline of the immune response to *Nippostrongylus brasiliensis* infection.

stromal stem cells produce IL-33 in other settings of type-2 inflammation (77–80). Similarly, IL-25 has been shown to be produced by many type-2 immune cells including Th2 cells, mast cells, alveolar macrophages, eosinophils and basophils—all of which may contribute to ILC2 activation (81–84). These cellular sources are in addition to more recently described IL-25-producing intestinal tuft cells and chemosensory brush cells found in the lung which play key roles in ILC2 homeostasis and expansion in settings of type-2 inflammation (85–89).

As initial responders of tissue damage caused by helminth infection, ILC2s serve a unique role in orchestrating the type-2 response that is required for worm clearance and epithelial repair. A detailed understanding of the immune response, especially that of ILC2s, is requisite to address the public health needs of treating helminth infections and preventing associated comorbidities.

GROUP 2 INNATE LYMPHOID CELLS

ILC2s and Anti-helminth Immunity

Discovery of ILC2s

In 2001, a rare non-B/non-T (NBNT) cell population that responded to IL-25 and produced type-2 cytokines was described in settings of allergic inflammation (81). These cells resembled $CD4^+$ T cells in many ways but lacked a known antigen receptor. It was proposed that this population worked in concert with Th2 cells to promote type-2 immune hallmarks. This IL-25-responsive NBNT population was later observed in the context of helminth infection where it was described as a prominent producer of type-2 cytokines during infection (90). However, it was not until almost a decade after their initial discovery that this NBNT population became part of the collective consciousness of researchers studying type-2 immunity (91–93). These studies showed that this innate lymphoid population rapidly produces IL-13 in response to the tissue alarmins IL-33 and IL-25. It was later shown that innate lymphoid cells could respond to the tissue alarmin TSLP to produce IL-5 and IL-13, but this appears to occur mainly in the skin leaving the impact of TSLP in other stages of helminth infection less clear (94, 95). While early studies used different names to describe innate lymphoid cells, consensus was reached in 2013 to identify them as group 2 innate lymphoid cells (ILC2s) based on their production of type-2 cytokines, distinguishing them from other innate lymphocytes classified as group 1 and group 3 ILCs (96). Over the last 10 years, significant advances have been made in elucidating the unique biological roles for all three subsets of ILCs, but ILC2s appear uniquely suited to respond to helminth infections and aid in the promotion of a protective type-2 immune response (97).

While wild-type mice can clear *N. brasiliensis* within 8–10 days of infection, mice lacking T cells and B cells remain colonized with worms after several weeks of infection (59). Although this highlights the essential nature of Th2 cells in productive worm clearance, additional importance of ILC2s in anti-helminth immunity is 2-fold. Not only do these cells contribute to parasite expulsion by orchestrating early cytokine production via their sensing of tissue alarmins, but ILC2s are also involved in mucosal barrier homeostasis and the reparative response after helminth-mediated tissue damage. The potential contribution of ILC2s in parasite clearance can first be seen in a study from 2006 prior to the discovery of ILC2s where IL-25-deficient mice which exhibited impaired worm clearance relative to wild-type mice, despite the presence of T cells (90). Looking back at this study, ILC2s can be directly implicated in this process as RAG-deficient mice (lacking B and T cells) given recombinant IL-25 cleared *N. brasiliensis* within 5 days of infection (90). These results were replicated in the first studies defining ILC2s where it was shown that IL-33 could also induce rapid helminth clearance, linking both IL-25 and IL-33 as early activators of ILC2s (92, 93). Indeed, transfer of ILC2s back into mice deficient in both T cells and ILCs ($Rag2^{-/-}Il2r\gamma^{-/-}$ mice) was sufficient to promote worm clearance (91, 93). It is important to note that this rapid

clearance following alarmin administration was dependent on type-2 cytokines and particularly IL-13-expressing ILC2 cells (57, 90, 92, 93). In addition to their role in primary infection, ILC2 cells in concert with memory Th2 cells effectively limit worm burden and larval-induced lung damage upon secondary infection implicating a broader yet less defined role for ILC2 cells after repeated helminth exposure (98).

ILC2s in Barrier Homeostasis

In addition to their role in helminth expulsion, ILC2s in the small intestine serve an important function in barrier homeostasis via recognition of IL-25 made by chemosensory tuft cells residing in the epithelium (85–87). At steady state, dietary polysaccharides and metabolites bind directly to receptors on epithelial tuft cells and regulate their production of IL-25 (86, 99, 100). ILC2s in the intestinal lamina propria sense the tuft cell-derived IL-25. This maintains a low level of IL-13 production by intestinal resident ILC2s which then cues stem cells to differentiate into relatively low numbers of goblet and tuft cells (101). Upon colonization of the intestine by helminths such as *N. brasiliensis* and *H. polygyrus*, tuft cell expansion is increased (85–87, 102). Tuft cells act as early sentinels of intestinal infection by recognizing metabolites generated by pathogens that breech the mucosal barrier and produce substantially more IL-25 leading to increased ILC2-derived IL-13. This feed-forward circuit leads to the characteristic goblet and tuft cell hyperplasia observed in settings of type-2 inflammation. The increase in tuft cell-derived IL-25 as well as other alarmins generated as a result of tissue damage and cell death work in concert to promote type-2 immunity.

ILC2 Function

As discussed above, the lifecycle of the hookworm in the host causes a substantial amount of tissue damage and ILC2s provide an essential function in wound healing. This is particularly evident at two stages of infection in the lung: first when the infectious L3 larvae enter the lung parenchyma via the capillary endothelium, and then during the second stage of damage as L4 larvae migrate in search of the alveolar airspace. This is grossly observed in the lungs of mice 3 days after *N. brasiliensis* infection as punctate foci of damage and diffuse pulmonary hemorrhage. As sensors of epithelial damage, ILC2s are poised to respond quickly to such injury. It is clear that one of the major roles of tissue-resident ILC2s is to promote the mobilization of eosinophils in the bone marrow, which is enacted via their capacity to produce large amounts of IL-5 on a per cell basis (103). This corresponds to the characteristic eosinophilia that peaks around 9–12 days post-infection (73, 104). By day 12 in the response to *N. brasiliensis*, ILC2s are making IL-9 which is thought to autocrine amplify and be essential for tissue repair and restoration of lung function (105). Although IL-9 has a much lesser role in helminth immunity as compared to IL-4 and IL-13, ILC2-derived IL-9 likely enhances the production of IL-5 and IL-13, making this cytokine a potential modulator of the severity of type-2 inflammation (73, 74, 106). Also contributing to tissue repair is ILC2-derived epidermal growth factor (EGF)-like molecule amphiregulin (107). Though less is known about ILC2-generated amphiregulin in tissue repair within helminth infection, it is likely to be similar to that of other models with mucosal tissue damage. Such examples include the role of amphiregulin in modulation of dextran sulfate induced intestinal inflammation and influenza infection where ILC2-derived amphiregulin was critical in restoring barrier integrity and mediating airway remodeling (108, 109). A role for amphiregulin produced by ILC2 cells has also been observed in atopic dermatitis (110). In these models IL-33 induces the expression of amphiregulin in ILC2 cells, thereby promoting tissue repair. While the activity of ILC2-derived amphiregulin in tissue repair has not been studied in helminth models, it is likely to play a role as its expression is increased 12 days after *N. brasiliensis* infection (105). More recently, it was suggested that amphiregulin-producing ILC2s are a subset distinct from IL-5/IL-13-producing ILC2s (111). This study assessed pulmonary ILC2s at steady state in neonatal mice and used gene expression profiles to separate ILC2s into either *Klrg1/Il5/Il13*- or *Icos/amphiregulin*-expressing subsets. Future studies that identify and explore tissue-resident ILC2 heterogeneity may be able to assign differing roles for these subsets in anti-helminth immunity and other tissue-damaging infections or diseases.

Inflammatory iILC2s and Anti-helminth Immunity

Phenotype

Until recently, ILC2s were largely considered to be a homogenous population. This was in contrast to ILC1s and ILC3s which consisted of distinct populations within each subset. However, evidence began to emerge that suggested heterogeneity within ILC2s. Early experiments using *Il1rl1*- (IL-33 receptor) and *Il17rb*- (IL-25 receptor) deficient mice revealed that IL-33 and IL-25 were not equal in their ability to promote type-2 hallmarks or helminth clearance (92). A theme became apparent in which IL-33 was the dominant alarmin associated with ILC2 activation, and IL-25 mediated a more selective role (112–114). Transcriptomics analyses later revealed distinct gene signatures in both mouse and human ILC2s further supporting that this innate lymphoid population was more diverse than previously believed (115–117).

Despite the suggested heterogeneity among ILC2s, the majority of studies to date were unable to assign distinct roles to different ILC2 subsets. The inability to reliably identify unique ILC2 subsets *in vivo* likely masked their separate but important contributions in anti-helminth immunity. However, this changed when a subset of ILC2s that could be distinguished by their differential responsiveness to an epithelial alarmin was characterized in the context of *N. brasiliensis* infection (118). This study identified a population of IL-25-responsive ILC2s that accumulated in the lungs of mice 5 days post-*N. brasiliensis* infection or in mice that were given intraperitoneal IL-25. These cells, which were coined inflammatory ILC2s or iILC2s, were not found in the lung at steady state and disappeared within 12 days after *N. brasiliensis* infection. This is in contrast to the ever-present, tissue-resident, IL-33-responsive ILC2 subset, termed natural ILC2s or nILC2s. This study also demonstrated that

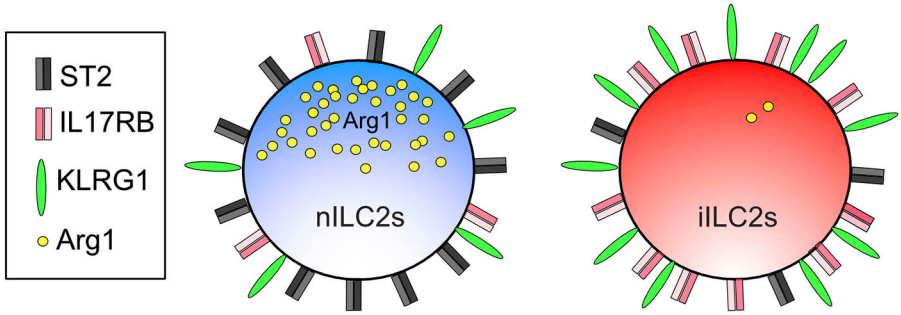
iILC2s phenotypically express high KLRG1 and low CD90 levels, whereas nILC2s have low KLRG1 but high CD90 expression. In some ways, iILC2s appear similar to IL-25-elicited MPP^{type2} cells as both populations are Lin^{-} $c\text{-}Kit^{+}$ and respond to the same alarmin. However, MPP^{type2} cells are characterized by the lack of CD90 and IL-7Rα expression which distinguishes them from iILC2s (114, 118, 119).

As their functional determinants imply, iILC2s which preferentially respond to IL-25 express more IL-25 receptor, whereas IL-33-responsive nILC2s display more IL-33 receptor on their surface (118, 120). Together, these markers allowed the consistent delineation between iILC2 and nILC2 cells (**Figure 2**). In addition to these cell surface markers, arginase 1 (Arg1) was later identified as a robust discriminator of the two subsets. It was previously thought that all ILC2s and their progenitors express this enzyme (121–123). However, while nILC2s display high Arg1 reporter expression using $Arg1^{YFP}$ reporter mice, iILC2s express very little allowing for distinction between subsets (120). Interestingly, Arg1 expression in human pulmonary ILC2s obtained from individuals with chronic lung disease also appears to define two separate subsets. Human IL-33 $receptor^{+}$ ILC2s displayed high Arg1 levels but not $CRTH2^{+}$ ILC2s (123). Whether these two subsets in humans are related to nILC2s and iILC2s in mice is not yet known. Future investigations that delineate these ILC2 subsets using distinct phenotypic markers are likely to provide a more comprehensive understanding of the specific contributions allotted to these and potentially other ILC2 subsets.

iILC2 Function in Helminth Infection

The impact of iILC2 cells in anti-helminth immunity is demonstrated in mice lacking *Il25* or the IL-25 receptor *Il17rb* —which fail to specifically activate iILC2s—and display impaired *N. brasiliensis* expulsion (90, 92). This is complemented by experiments showing exogenous IL-25 administration is able to mediate *N. brasiliensis* clearance in mice lacking T cells but not in those lacking both T cells and ILCs (90, 93). Moreover, mice deficient for the AP-1 transcription factor BATF fail to generate pulmonary iILC2s and display impaired IL-25-mediated helminth clearance (40). Importantly, BATF deficiency had no impact on IL-33-mediated worm clearance or nILC2 numbers. Together, these data indicate that the IL-25-responsive iILC2 subset aids in mobilizing the immune response for rapid pathogen clearance and acts distinctly from tissue-resident IL-33-responsive subsets particularly in settings where IL-25 dominates the early alarmin response to infection.

It is logical that differences in cytokine production would exist between the ILC2 subsets. Tissue-resident pulmonary nILC2s, which are active at the peak of the response (days 8–10) to *N. brasiliensis,* are highly skewed toward IL-13 and IL-5 production



	nILC2	iILC2
ST2	+++	+
IL17RB	+	+++
KLRG1	++	+++
CD90	+++	++
Arg1	+++	very low/-

FIGURE 2 | Phenotype of nILC2 vs. iILC2.

with minimal IL-4 production (56, 57). Similarly, in response to intraperitoneal IL-25, there is a reported preference among iILC2s to produce IL-13 and little IL-4 as determined in 4C13R mice, which express AmCyan and DsRed-DR under *Il4* and *Il13* regulatory elements, respectively (118). However, in the context of *N. brasiliensis* infection, iILC2s make IL-4 in addition to IL-13 at 5 days post-infection (120). This study utilized the $IL4^{4get}$, $IL4^{KN2}$, $IL13^{Yetcre13}$, and $IL13^{Smart13}$ reporter mice which can be used as a readout of mRNA ($IL4^{4get}$ and $IL13^{Yetcre13}$) or protein ($IL4^{KN2}$ and $IL13^{Smart13}$) production (57, 93, 124, 125). Further differences between cytokine capabilities can be parsed out by assessing the temporal response to infection. At day 5 post-*N. brasiliensis* infection, cytokine production by ILC2s demonstrates that only the iILC2 compartment, and not nILC2s, are making type-2 cytokines (120). This is relatively early in the response and precedes cytokine production by tissue-resident IL-33-responsive nILC2s (126, 127).

While IL-13 is the major ILC2-derived cytokine in the intestine, IL-4 can promote goblet and tuft cell differentiation in organoid cultures similar to that observed after administration of recombinant IL-13, suggesting there may be a role for ILC2-derived IL-4 in maintaining intestinal barrier homeostasis (85–87, 128). Indeed, IL-4 production by intestinal ILC2s has been reported in response to *Heligmosomoides polygyrus* (129). Although this is an indication that ILC2-derived IL-4 in the intestine after *H. polygyrus* infection may play a role similar to that of early, migratory iILC2 cells in the lung after *N. brasiliensis* infection, some care should be noted. This study used IL-4-transcript reporter mice ($IL4^{4get}$), which have been shown to mark ILC2 populations that are not readily producing IL-4 protein (56, 57, 93). Further complicating this conclusion, the prior study assessed cytokine protein only *ex vivo* after restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, a stimulus that drives translation of all cytokine-competent loci and may not reflect true *in vivo* cytokine production (56). Thus, it remains somewhat unclear if these intestinal ILC2 cells are actively producing IL-4 protein *in vivo*. If IL-4 protein production is confirmed, it is of interest to assess whether this IL-4 production reflects either: tissue-specific differences among nILC2 cells in the lung and intestine; helminth-specific differences related to infection by *N. brasiliensis* or *H. polygyrus*; or reflects an intestinal iILC2 population that maintains IL-4 protein production throughout the course of *H. polygyrus* infection.

Polyfunctionality and potential plasticity of iILC2s has also been described. Intraperitoneal IL-25 administration generated iILC2s that express elevated levels of Rorγt compared to nILC2s, though not as high as that of ILC3s from the small intestine (118). Furthermore, iILC2s were capable of producing IL-17 when stimulated with PMA and ionomycin, indicating potential plasticity between iILC2s and ILC3-like cells (118, 130). The polyfunctionality of iILC2s to produce IL-17 along with type-2 cytokines also sets them apart from nILC2s. This may indicate critical functional differences between the two subsets as iILC2s contribute to protection from the IL-17-sensitive pathogen *Candida albicans* (118). It may also reflect an inherent plasticity of iILC2 cells compared to nILC2 cells. Indeed, nILC2 cells require tissue specific signals in order to undergo their ultimate maturation (131). In support of ILC2/ILC3 plasticity, the lysine methyltransferase G9a could act as a switch to promote ILC3 and repress ILC2 commitment (132). Whether this epigenetic switch is active specifically in iILC2s or their progenitors relative to nILC2 cells is not clear. There is also some evidence to support ILC2/ILC3 plasticity in human ILC2s. Studies have shown two separate populations of human ILC2s delineated on c-Kit expression, as opposed to alarmin-receptor expression observed in mice. In these analyses, the c-Kit-positive subset exhibits similarities to ILC3s and is able to produce IL-17 (133, 134). The extent that ILC2 or ILC2 progenitors can regulate ILC3 responses will be an important area of future research as we try to better understand the importance of ILCs in barrier defense and repair. Future studies investigating the relative contribution of iILC2 compared to ILC3 populations would be of great interest toward defining their role in IL-17-mediated immunity.

Fate of iILC2s

The fate of iILC2s remains unclear. As iILC2s are only transiently found in the lung, it could be argued that they are relatively unimportant in the grand scheme of pulmonary immunity. However, the rapid disappearance of these cells has been attributed to their ability to convert to either the IL-33-responsive nILC2 population or IL-17-producing ILC3s (118). This study demonstrated that sorted iILC2s cultured under various conditions increased IL-33 receptor expression, a phenotype similar to that of nILC2s. *In vivo*, iILC2s transferred into congenic hosts upregulated the IL-33 receptor, again indicating a potential conversion to nILC2s (118). Furthermore, the fold increase of nILC2 cells after helminth infection was significantly reduced in $Il17rb^{-/-}$ mice, which lack iILC2s, indicating a strong contribution of iILC2s to the nILC2 pool (118). However, studies using a tamoxifen-inducible fate mapping system that labels ILC2s concluded that repopulation and maintenance of the lung nILC2 pool after helminth infection was the result of self-renewal by tissue-resident nILC2s and not the addition of "*de novo*" generated ILC2s, which would include iILC2s (135, 136). Specifically, this system used an $Arg1^{RFP-CreERT2}$ *R26R-YFP* mouse. In this mouse, all Arg1-expressing cells, including ILC2 precursors, were labeled with RFP and CreERT2. Administration of tamoxifen allowed Cre-mediated excision of the stop codon in the Rosa locus allowing permanent expression of YFP to fate map ILC2 populations throughout development. The majority of cells appeared to be self-renewed (i.e., retained YFP expression), which is consistent with prior literature suggesting self-renewal for both ILC1 and nILC2 populations (137, 138). Despite this, the fate mapping system identified that 12 days after a single *N. brasiliensis* infection, roughly 10–15% of nILC2s consisted of non-labeled cells (135, 136). This suggests that up to 15% of the tissue-resident pool may have been generated from converted iILC2s after barrier damage. Based on the knowledge that iILC2s are absent at 12 days post-*N. brasiliensis* infection, this time point is consistent with when iILC2s would have already converted to nILC2s. Thus, it is possible that although self-renewal is the predominant source of nILC2 homeostasis at rest, converting

iILC2 cells may contribute substantially to the overall tissue-resident ILC2 pool over a lifetime of infections. Whether iILC2s are truly multipotent progenitors is not yet established, but these initial findings may indicate more plasticity among ILCs than previously appreciated as was recently reviewed (139).

Regarding experiments to assess conversion, it should be noted that while delineation of iILC2 and nILC2 populations based on KLRG1 expression works well for *N. brasiliensis* infection, there are other situations which lead nILC2s to increase KLRG1 levels. In particular, daily IL-33-administration for 4 days, as well as repopulation of irradiated mice with bone marrow, leads nILC2s to upregulate KLRG1 levels and drop CD90 slightly so that their phenotype resembles an intermediate between nILC2s and iILC2s (120). Moreover, these changes may be strain- and context-specific as *Alternaria alternata* exposure or IL-33 administration over 2 weeks lead to upregulation of CD90 on ILC2s isolated from bronchoalveolar lavage fluid in C57BL/6 but not BALB/c mice (140). In contrast, ILC2s isolated from lung tissue displayed downregulated CD90 only in BALB/c mice. Thus, using CD90 or KLRG1 expression levels to assess conversion may be confounded by inherent changes of these markers due to time, activation status, and tissue-specific signals. Further studies using more stringent markers of ILC2 subsets, such as Arg1, to determine conversion would strengthen the argument that iILC2s contribute substantially to the immune landscape of the lung.

Origin of iILC2s

It is not currently well understood where pulmonary iILC2s originate. As discussed, ILC2s have been considered tissue-resident cells that are replenished by self-renewal within their tissue of residence (131, 137). Using the fate mapping system described above where ILC2s are permanently labeled at various timepoints throughout development, it was demonstrated that the majority of lung-resident ILC2s in adulthood were derived during the postnatal period with some contribution of prenatal-derived cells and little influx of cells seeded during adulthood (135). This data explains the prior evidence that pulmonary type 2 immune cells—including ILC2s—arise in the lung shortly after birth (141–143). After *N. brasiliensis* infection, there was a statistically significant increase in non-fate mapped pulmonary ILC2s indicating that ILC2s do not arise solely from self-renewal during inflammation but may be recruited to the lung from other sites (135, 136). This is supported by studies where the appearance of iILC2s in the lung depends on S1P-dependent migration (120, 136, 144, 145). In these studies, administration of the S1P-receptor agonist FTY720 prevented the appearance of iILC2s in the lung after IL-25 administration or helminth infection, indicating the requirement for iILC2s to egress from lymphoid organs into circulation. Mucosal sites such as the small intestine and lung as well as sites of hematopoiesis like the bone marrow are all candidate reservoirs (**Figure 3**). Furthermore, evidence that c-Kit$^+$ ILC precursors are also found in human blood, tonsils, and lung and can give rise to all ILC subsets indicates the potential for multiple sites of origin (146).

The most convincing source of migratory iILC2s in the lung has been the small intestine (136, 144, 145). In a comprehensive experiment to determine the source of iILC2s, total ILC2s were isolated from the lung, small intestine, and precursors from the bone marrow. When these cells were transferred into congenic hosts, pulmonary iILC2s arose in mice that received small intestine ILC2s (144). However, it should be noted that mice given ILC2 precursors from the bone marrow—but not the lung—also gave rise to pulmonary iILC2s, although to a lesser extent than those from intestine. It has been further demonstrated that IL-25-responsive iILC2s in the lung resemble small intestine ILC2s by transcriptional profile and phenotype (136, 144, 145, 147). Recently, a population of ILC2s resembling pulmonary iILC2s were found in mesenteric lymph nodes after *N. brasiliensis* infection which were absent in IL-33-deficient mice (147). Although migration of intestinal ILC2s to the mesenteric lymph nodes has been described previously (144), this study suggests that IL-33 binds to ST2$^+$ nILC2s in the small intestine and through induction of the gene *Tph1*, generates ST2$^-$ iILC2s. This mechanism would be consistent with the idea that pulmonary iILC2 cells originating in the intestine lose their nILC2 phenotype prior to their arrival in the lung. We previously proposed that such a conversion from an nILC2 phenotype toward an iILC2 phenotype must occur if pulmonary iILC2s originate in the intestine based on the uniform expression of the nILC2 marker arginase-1 within intestinal ILC2 cells and its absence among pulmonary iILC2 cells (120). However, because pulmonary and mesenteric iILC2 populations have not been directly compared, care should be taken in overinterpreting these conclusions at least as they relate to an intestinal origin for pulmonary iILC2 populations. In this regard, a few outstanding questions remain unresolved. First, addition of IL-25 and not IL-33 drives the appearance of pulmonary iILC2 cells suggesting that IL-33 may differentially impact mesenteric iILC2 and pulmonary

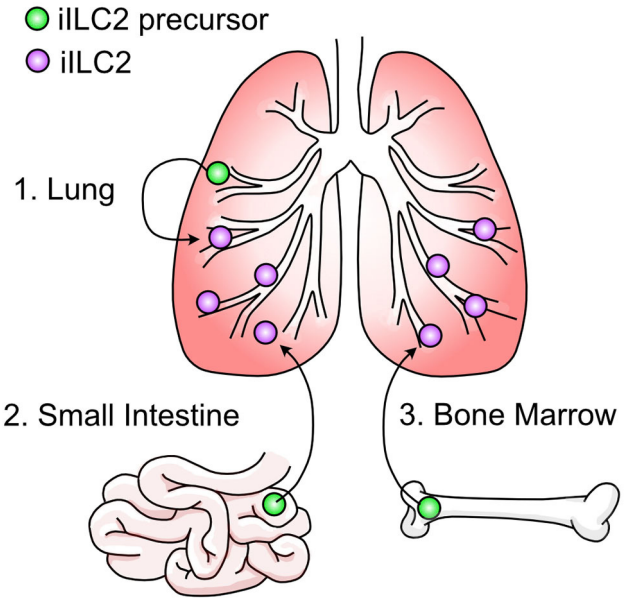


FIGURE 3 | Three potential origins of pulmonary iILC2s.

iILC2 (118, 147). Second, while ST2 expression is abundant on nILC2 cells residing in the lung, few intestinal nILC2 cells express the IL-33 receptor at steady state (except in conditions where IL-33 expression in the intestine is forced by a transgene) (120, 147). How IL-33 is modulating intestinal ILC2s or whether specific $ST2^+$ subsets are responsible for the generation of mesenteric iILC2s will be of interest. Third, while the mesenteric lymph node iILC2 population is observed 7 days post *N. brasiliensis* infection, pulmonary iILC2s are more transient and largely absent by this timepoint in the lung (118). Fourth, while iILC2s and nILC2s in the mesenteric lymph node or intestine express similar levels of KLRG1, iILC2s in the lung express noticeably more KLRG1 than pulmonary nILC2s. This discrepancy, along with that of arginase-1 expression as noted above, suggests that phenotypes of ILC2s likely differ depending on their tissue of residence making extrapolations based on phenotype with regard to cell origin difficult (118, 120, 136, 144, 145, 147). That said, there is evidence supporting that an intestinal origin for pulmonary iILC2 extends beyond studies with *N. brasiliensis*. Mice infected with the parasitic nematode *Trichinella spiralis*, which infects only the intestinal tract, demonstrated mobilization of iILC2s to the lung (145). These mice also displayed increased pulmonary mucin production due to iILC2-derived IL-13, indicating that an intestinal infection alone can mobilize iILC2s to the lung and drive mucosal immunity at distal sites. At present, conclusive demonstration of an intestinal origin for pulmonary iILC2s and its impact on iILC2 biology in the lung as well as investigation of whether other tissue sources outside the intestine contribute to pulmonary iILC2 cells await further experimentation.

In order for pulmonary iILC2s to originate from a lung source and undergo S1P-mediated migration, these cells would have to egress from the lung into circulation and then return back to the lung. This does not seem outside the realm of possibility, given the high degree of nILC2 motility and migration to perivascular spaces upon IL-33 treatment (148). In support, pulmonary ILC2s reside in adventitial niches allowing these cells the proximity to readily sample the vasculature (78). This scenario would also require iILC2s to be generated from nILC2s or a lung-resident precursor. Although *in vivo* support is limited, one study describes the requirement of Notch signaling for the generation of iILC2s, and when nILC2s were cultured on Notch ligand-expressing cells they adopted an iILC2 phenotype and gained the ability to produce IL-17 (130). Additionally, $IL\text{-}18R\alpha^+$ ILC precursors have recently been reported in the murine lung that are capable of giving rise to nILC2s (111). It would be of interest to assess whether this lung tissue-resident precursor subset can also differentiate into iILC2s during helminth infection.

As the primary site of hematopoiesis in adults, the bone marrow also serves as a reservoir for ILC2 precursors (ILC2Ps). ILC2Ps have been defined as $Lin^-Sca1^{hi}GATA3^{hi}CD90^+CD127^+Id2^+IL2r\alpha^+$ (149). However, common ILC precursors and ILC2Ps in the bone marrow express gut homing molecules such as $\alpha4\beta7$ and CCR9 making it difficult to acertain if such markers observed on lung iILC2 reflect an intestinal or bone marrow migrant (150). Further, even though lung ILC2s are seeded into their tissues of residence during pre- and postnatal development (135), there is evidence that IL-33 drives the egress of ILC2s from bone marrow progenitors (151). It is possible that other inflammatory signals may release iILC2 cells from a bone marrow progenitor, although this has not yet been assessed. Technical advances such as the generation of polychromic transcription factor reporter mice have been used to identify both uncommited ILC precursors and ILC2-specific precursors in the bone marrow as well as ILC2 precursors in the small intestine lamina propria (152). This would serve as an interesting model to identify the progenitor population as well as anatomical location that can give rise to iILC2s.

DISCUSSION

Current Gaps in Knowledge and Future Directions

While the role of iILC2s in helminth infection is clear, there have been few studies demonstrating their appearance in other infection or disease models. As early mediators of type-2 immunity, it is interesting to speculate that iILC2s are involved in allergic responses. However, two recent studies have failed to demonstrate their recruitment to the lung using the house dust mite (HDM) model of allergic inflammation (120, 140). It is unknown whether IL-25 production is the main driver in iILC2 lung accumulation in the HDM setting or perhaps there is insufficient IL-25 production in the HDM model—and other models of type-2 immunity—to promote the expansion and migration of iILC2s to the lung. Disparities in pulmonary iILC2 accumulation between allergic and helminth models could also reflect differential involvement and location of IL-25-producing intestinal tuft cells and pulmonary brush cells. With this in mind, if iILC2s are first induced by intraperitoneal injections of IL-25 and then artificially transferred into T cell- and ILC2-deficient mice, they are able to mediate allergic responses upon HDM challenge (130). This data may indicate that while certain allergic responses do not normally recruit these cells to the lung, if iILC2 cells are already present in the lung (i.e., due to a helminth infection) at the time of allergen exposure, these cells may yet exacerbate type-2 inflammation. Whether such a mechanism explains why some individuals relocating to the United States from helminth-endemic regions show increased type-2 inflammation upon allergen exposure is not known (153). This is interesting to consider in context of mounting evidence that suggests helminth colonization suppresses the onset and severity of allergic disease (40, 154, 155). Although helminths are the focus here, other infections or insults that generate IL-25 and recruit iILC2s to the lung may similarly exacerbate allergic outcomes upon allergen exposure. Such models need to be explored to indicate iILC2 involvement.

While the use of reporter mouse systems have greatly impacted our overall understanding of ILC2 biology and provided unique opportunities to track the fate and function of these rare cells *in vivo*, it is necessary to acknowledge the inherent pitfalls associated with such systems. Reporter systems are often designed to enhance detection. This leads to

alterations in gene regulation as well as mRNA and protein stability that may not reflect true biology of the wildtype gene. Aside from reporter "leakiness" where fluorescence or surface molecules may falsely indicate gene expression, there may also be contextual nuances that are missed. Gene expression and regulation is complex and sensitive to cell type, location, immune environment, and many other factors, each of which may lead to over-interpretation of results if not rigorously evaluated. As such, while innovative reporter models are likely to continue to be an important part of an investigators tool kit, complementation with unbiased approaches is becoming standard. An excellent example of this is the increasing use of single cell RNA sequencing to explore ILC2 biology. We expect platforms designed to interrogate the transcriptomics, genomics, proteomics, and metabolomics of ILC2 cells at the single cell level, when paired with traditional methods, will be critical to our collective understanding of ILC2s in settings of helminth infection.

Most importantly, how iILC2s contribute to the short- and long-term pulmonary landscape will be of great clinical interest. Eventually it will be necessary to detect pulmonary iILC2s in helminth-infected humans. If iILC2s convert to nILC2s and are retained in the lung, there would be significant contribution of these former iILC2s to the lung-resident population over the course of a lifetime as each infection could potentially contribute to the pulmonary ILC2 pool. In this scenario, *de novo* ILC2 populations like iILC2 cells, rather than self-renewing, tissue-resident populations, would likely become the predominant ILC2 population in mucosal sites. Such studies would expand our understanding of basic lung immunity, while continuing to inform the development of novel therapeutic strategies aimed at reducing the global health burden of STHs.

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MM and RR wrote the review. Both authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Helminth Sensing at the Intestinal Epithelial Barrier—A Taste of Things to Come

Aduragbemi A. Faniyi[1,2], *Kevin J. Wijanarko*[1,3], *James Tollitt*[1] *and John J. Worthington*[1]*

[1] *Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, United Kingdom,* [2] *Institute of Inflammation and Ageing, University of Birmingham, Birmingham, United Kingdom,* [3] *Department of Biomedical Sciences, City University of Hong Kong, Kowloon, Hong Kong*

Human intestinal helminth infection affects more than 1 billion people often in the world's most deprived communities. These parasites are one of the most prevalent neglected tropical diseases worldwide bringing huge morbidities to the host population. Effective treatments and vaccines for helminths are currently limited, and therefore, it is essential to understand the molecular sensors that the intestinal epithelium utilizes in detecting helminths and how the responding factors produced act as modulators of immunity. Defining the cellular and molecular mechanisms that enable helminth detection and expulsion will be critical in identifying potential therapeutic targets to alleviate disease. However, despite decades of research, we have only recently been able to identify the tuft cell as a key helminth sensor at the epithelial barrier. In this review, we will highlight the key intestinal epithelial chemosensory roles associated with the detection of intestinal helminths, summarizing the recent advances in tuft cell initiation of protective type 2 immunity. We will discuss other potential sensory roles of epithelial subsets and introduce enteroendocrine cells as potential key sensors of the microbial alterations that a helminth infection produces, which, given their direct communication to the nervous system *via* the recently described neuropod, have the potential to transfer the epithelial immune interface systemically.

Keywords: tuft cells, enteroendocrine cell (EEC), microbiome, epithelium, helminth, G protein-coupled receptor (GPCR)

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Kara Filbey,
University of Manchester,
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Jakob Von Moltke,
University of Washington,
United States
Danielle Smyth,
University of Dundee, United Kingdom

***Correspondence:**
John J. Worthington
j.j.worthington@lancaster.ac.uk

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INTRODUCTION

Soil-transmitted helminths (STHs) affect >1 billion people in the world's most deprived communities (1). These parasites are one of the most prevalent neglected tropical diseases worldwide bringing huge morbidities to the host population. Sub-Saharan Africa alone is estimated to lose 2.3 million disability-adjusted life-years annually (2). Constant advances have been made in identifying type 2 immune responses as key to helminth control and expulsion (3–8), with the cytokine interleukin (IL)-13 being crucial in driving the characteristic "allergic" immune response (3). CD4+ T-cells and more recently type 2 innate lymphoid cells (ILC2s) are key producers of these cytokines, with ILC2 believed to be the major initiators of type 2 immunity (9, 10) driven by the release of the epithelial alarmins IL-33, thymic stromal lymphopoietin (TSLP), and IL-25 (11).

Following the identification that tuft cells of the rat epithelium (12) possessed alpha-gustducin, the G-protein subunit of numerous taste receptors, it had been postulated that these cells could act as solitary chemosensory apparatus within tissues. The identification of the tuft cell-specific marker doublecortin like kinase 1 (DCLK1) (13–17) and the discovery of the downstream master transcription factors, Pou domain, class 2, transcription factor 3 (*Pou2f3*) and growth factor-independent 1b (*GFI1b*) (15, 18–20), allowed further elucidation of this tuft cell chemosensory hypothesis. In 2016, three key papers cemented the importance of tuft cells in sensing parasites and brought tuft cell biology to the forefront of helminth immunity (21). Through examination of Pou2f3 null mice during a small intestinal helminth infection, Gerbe et al. (19) defined that IL-13 acted downstream of the tuft cell lineage, suggesting a tuft cell initiated IL-25-driven positive feed-forward loop resulting in ILC2 expansion and IL-13-driven tuft and goblet cell hyperplasia (**Figure 1**), essential for helminth expulsion (22, 23). In parallel to these studies, von Moltke et al. (24) confirmed tuft cells as IL-25 expressers that, following small intestinal helminthiasis, underwent hyperplasia *via* an ILC2 derived IL-13 interaction with the IL-4Rα in a feed-forward loop, presumably *via* stem cell niche signaling (25, 26). Finally, mice null for the G-protein subunit gustducin or the transient receptor potential cation channel, subfamily M, member 5 (TRMP5), a cation channel known to be important in the signaling cascade of chemosensory cells in the gut, mirrored the delayed tuft and goblet cell hyperplasia following a small intestinal helminth infection, giving the first indication of the chemosensory mechanisms of initial parasite detection (27). This minireview will focus on recent advancements in tuft cell biology as well as examining the potential for other epithelial chemosensory responses to helminths themselves and the microbial dysbiosis infection induces.

CURRENT TUFT CELL ADVANCEMENTS

Single-cell analysis of the intestinal epithelium now suggests that tuft cells are a heterogenous population, with two proposed distinct subsets—tuft-1 and tuft-2—with differing cytokine profiles (28, 29). In response to undefined helminth antigens, small intestinal tuft cells produce TSLP as well as IL-25, which are crucial for the initiation of the anthelmintic mucosal response (28, 30). Only tuft-2 cells produce TSLP, although a functional role for TSLP from tuft cells has yet to be demonstrated (28). Schneider et al. (31) showed that genetic deletion of tumor necrosis factor alpha-induced protein 3 (*Tnfaip3*), a negative regulator of IL-25 signaling in ILC2, caused tuft cell and goblet cell hyperplasia, as well as small intestinal lengthening. Tissue remodeling that mimics the histological features of a helminth infection (31), reinforcing the importance of IL-25 signaling in type 2 immunity.

The initial identification of the potential taste receptor signaling pathways involved in parasite recognition at the epithelial barrier by Howitt et al. (27) has since been expanded upon. Luo et al. (32) further elucidated the *Trpm5*-dependent sensory pathway by showing that stimulation of intestinal organoids using larvae and antigens of the small intestinal helminth *Trichinella spiralis* stimulates increased intracellular calcium levels, resulting in tuft cell depolarization. They further observed that IL-13 administration promotes tuft cell hyperplasia as well as upregulation of genes including the *Tas2r* family of bitter taste G-protein coupled receptors (GPCRs) and the succinate receptor *Sucnr1*, indicating an adaptive ability of these chemosensory cells during *T. spiralis* infection. The importance of *Tas2r* in tuft cell recognition of helminths was demonstrated when pretreatment of small intestinal villi with allyl isothiocyanate, an inhibitor of bitter taste receptors, abolished *T. spiralis*-induced tuft cell-derived IL-25. Conversely, increased tuft cell production of IL-25 was seen after the administration of salicin, a *Tas2r* agonist (32). Two groups have also demonstrated the importance of *Sucnr1* with Lei et al. (33) demonstrating that tuft cells are the sole epithelial expressers of this receptor in the small intestine. They, in parallel with Schneider et al. (31) demonstrated that dietary succinate increases small intestinal tuft cell secretion of IL-25 and promotes hyperplasia. Interestingly, examination of *Sucnr1* null mice demonstrated a prevention of succinate-induced tuft and goblet cell hyperplasia (33). Nadjsombati et al. (34) demonstrated that succinate metabolites are produced by the small intestinal helminth *Nippostrongylus brasiliensis in vitro*; yet, immunity against *N. brasiliensis* is not abrogated in *Sucnr1*$^{-/-}$ mice, suggesting no requirement or at least redundancy in this potential helminth recognition pathway.

Schneider et al. (31) also reported that small intestinal tuft cells on *Tritrichomonas*-colonized mice highly express not only *Sucnr1* but also GPCRs for short-chain fatty acids *Ffar3*. This result is also corroborated by other groups, who reported that *Ffar3* is highly expressed by intestinal tuft-2 cells, but not intestinal tuft-1 cells or tracheal tuft cells (28, 34). Although the discovery of *Ffar3* expression on small intestinal tuft cells is an interesting find, little is known at the moment on how the receptor impacts anthelmintic immunity. Interestingly, in a murine model of allergic airway inflammation, *Ffar3* knockout abrogates *Heligmosomoides polygyrus*-induced alleviation of airway inflammation but did not affect worm burden in the small intestinal niche of this helminth (35). Murine small and large intestinal tuft cells also express choline acetyltransferase, which catalyzes the production of acetylcholine (36). Although the close interaction between airway tuft cells and cholinergic neurons has been previously demonstrated (37), with recent demonstrations of tuft cell acetylcholine driving ciliary beating in a Trpm5-dependent fashion (38), their role in cholinergic neuron signaling in the intestine is less clear. There is evidence that during genetic and antagonist muscarinic receptor blockade, small intestinal tuft cells arise with an enteroendocrine-like phenotype to sustain the murine intestinal epithelial cholinergic niche (39). Moreover, as acetylcholine receptors are also expressed on diverse cell types, including goblet cells (where acetylcholine promotes mucus secretion), dendritic cells, macrophages, as well as B and T cells (40, 41), there is the unexplored possibility that tuft cells may also play a larger role in anthelmintic immunity *via* their production of acetylcholine.

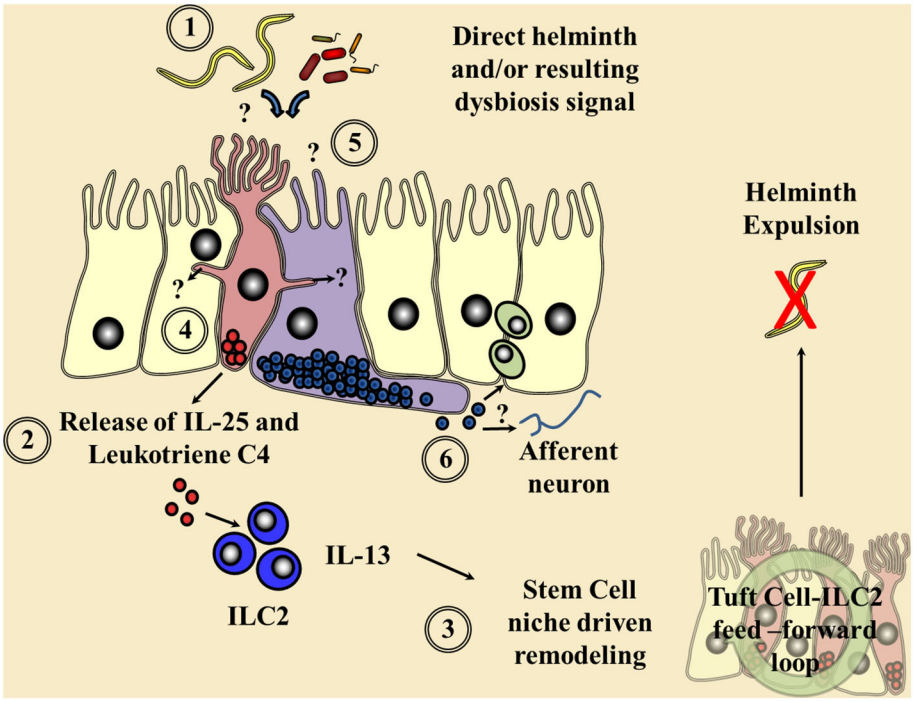

FIGURE 1 | Current understanding of chemosensory detection of helminths at the epithelial barrier and a flavor of possible future perspectives. (1) Helminths are detected by tuft cells (red) through an as yet undefined receptor and ligand, although microbial dysbiosis produced *via* helminth colonization may be a potential candidate. (2) Gustducin-α and the transient receptor potential cation channel, subfamily M, member 5 (TRMP5) are required for the signaling cascade and Ca2+ flux, allowing the secretion of the alarmin interleukin (IL)-25 and leukotriene C4 in an arachidonate 5-lipoxygenase (ALOX5)-dependent mechanism signaling to resident type 2 innate lymphoid cells (ILC2s). (3) These factors in turn increase ILC2 numbers and their secretion of the cytokine IL-13, driving a feed-forward loop *via* the stem cell niche resulting in helminth expulsion. Tuft cell-derived acetylcholine could also possibly alter this epithelial stem cell niche and local immune responses. (4) Potential cross communication of tuft cells *via* cytospinules and the relay of helminth-derived signals to coordinate surrounding epithelial response. (5) The potential of enteroendocrine cells (purple), which host an array of chemosensory apparatus, to directly sense a helminth infection or infection-induced microbial dysbiosis. (6) The release of enteroendocrine peptide hormones signaling to the surrounding immune system either directly or *via* neuronal communication is proposed.

Recent findings have also begun to elucidate the initial on switch of the tuft cell/ILC2 feed-forward loop, which, given the production of IL-25 in the naive state (24), was likely to be another messenger or danger signal. Tuft cells had previously been shown to produce leukotriene C4 (13), but McGinty et al. (42) have demonstrated that following small intestinal helminth infection, tuft cells secrete leukotriene C4, in an *Alox5*-dependent manner, that could signal to surrounding ILC2s *via* their expression of leukotriene receptors CYSLTR1 and 2 (**Figure 1**). Given that several immune cells can produce leukotrienes and the long-lived nature of tuft cells (43), bone marrow transfer experiments were superseded by targeted cell-specific null models demonstrating that it was indeed tuft cell-derived leukotriene that was key in driving ILC2 expansion early during small intestinal *N. brasiliensis* infection (42). However, the precise chemoreceptor or the helminth products they detect remain unknown. Parallel studies by Ualiyeva et al. (44) have also demonstrated that tuft cells located in the lung can release leukotrienes in response to aeroallergens *via* the P2Y2 receptor, indicating systemic potential for helminth detection. Interestingly, although tuft cells also produce IL-25 in response to protist-derived succinate *via* SUCNR1, McGinty et al. (42) demonstrated that stimulation of tuft cells with succinate, although driving IL-25, resulted in no leukotriene production but importantly no defect in ILC2-driven responses. Furthermore, TAS1R3 also expressed on tuft cells responds to *Tritrichomonas muris* and succinate, but not to a helminth infection (45), indicating an ability of tuft cells to selectively respond to different parasites.

This variety and flexibility of the cellular secretome of tuft cells further mirror the responses of enteroendocrine cells (EECs), key chemosensory cells of nutrient detection, in being able to orchestrate an array of digestive requirements to the numerous nutrients detected. Therefore, it is likely that these pathways have been utilized by the innate immune system in evolution to allow the "tasting" of parasites and allow an equally diverse response in immunity as digestion. Furthermore, EECs demonstrate heterogeneity spatially to respond to the nutrients in the likely locations they would appear (46–49); so it is likely that chemosensory cells detecting and responding to parasites would also differentiate in a spatiotemporal fashion to specific parasite niches along the intestinal tract and beyond, as indicated in spatial studies of

tuft cells (28, 29, 36, 50). Tuft cells also possess cytospinules which project into the nuclei of neighboring cells, providing them with a unique ability to communicate cellular cargo to the surrounding epithelium (51). Moreover, tuft cells are often associated with EECs (52), while both cell types can act as reserve stem cell niche in the small intestine upon Paneth cell ablation (53), indicating potential overlap and collective function (**Figure 1**).

ENTEROENDOCRINE CELLS—KEY CHEMOSENSORY CELLS OF THE EPITHELIUM

EECs are specialized trans-epithelial signal transduction conduits which respond to luminal nutrients by secreting peptide hormones to control gastrointestinal enzyme secretion, motility, and appetite regulation (54, 55). Despite constituting only 1% of the total epithelium, these cells span from the entire length of the gastrointestinal tract and collectively form the largest endocrine system of the body (56). Peptide-secreting intestinal epithelial cells described as having a high degree of amine precursor uptake were reported as early as the 1960s (57). Initially thought to arise from neural crest cells due to their production of neuropeptides such as serotonin, lineage tracing on avian embryos proved that these cells do not arise from the ectoderm (58, 59). Like other intestinal epithelial cells, EECs originate from *Lgr5*$^{+}$ intestinal stem cells within the intestinal crypt, integrating Wnt, Notch, and mitogen-activated protein kinase-dependent signaling (60), and require the expression of the secretory cell lineage transcription factor atonal bHLH transcription factor 1 (61–65), finally forming EECs *via* the expression of the transcription factors neurogenin3 and neurogenic differentiation 1 (NeuroD1) (64, 66, 67). Neurogenin3^{+} EEC progenitor cells will further differentiate to give rise to multiple mature EEC types, traditionally identified with a one-cell one-peptide dogma. This historic classification included glucagon-like-peptide-1 (GLP-1)-producing L-cells, cholecystokinin (CCK)-producing I-cells, gastrin-producing G-cells, gastric inhibitory peptide-producing K-cells, somatostatin-secreting D-cells, secretin-producing S-cells, and serotonin-producing enterochromaffin cells. However, it is now known that there is considerable secretome overlap and plasticity between the different EEC lineages. Using transgenic reporter mice, multiple groups have shown that CCK, GLP-1, and secretin are coexpressed by a large subset of EECs (68, 69). A recent single-cell transcriptional analysis using Neurog3 reporter mice showed that hormonal co-secretion differs by cell lineage, with a large proportion of EECs secreting multiple hormones (70). Furthermore, EECs also show hormonal plasticity in response to various extracellular cues, such as the upregulation of secretin production in response to bone morphogenic protein as well as their physical location within the crypt/villi dictating their secretome (70, 71).

Although still incompletely understood, recent evidence has shown that EECs have a huge potential to interact with the immune system, with a strong potential for playing a role in the chemosensory sensing of helminths and orchestrating immunity (56). Indeed, helminth infections in particular can drive hyperplasia of EECs in a variety of animal species, often thought to be the driving force to alterations in feeding that accompany a helminth infection in the upper small intestine (72–77). These alterations in EEC hyperplasia, like tuft cells, are also driven by type 2 cytokines in both small intestinal (*T. spiralis*) and large intestinal (*Trichuris muris*) helminth infections (78–81), with the EEC peptide CCK shown to influence the resulting immune response *via* driving weight loss in a feed-forward loop (81). Moreover, EECs can secrete peptide hormones as well as cytokines in response to pathogen-associated molecules (82), and given that intestinal immune cells potentially express peptide hormone receptors (83–85), there is the intriguing possibility that EECs are critical and novel modulators of barrier immunity to helminths (**Figure 1**).

Interestingly, EECs are the chief epithelial expressers of the receptors that sense bacterial metabolites, such as Ffar3/2 (86, 87), and therefore have the unique ability to relay dysbiosis into physiological adaptation (88). It is now well-established that microbial dysbiosis occurs during an intestinal helminth infection (89, 90), and these changes are transient following helminth expulsion (91, 92), meaning microbial alterations may provide a clear signal to the epithelium of a helminth infection. Indeed, the microbiota is a well-established essential signal for repair during intestinal inflammation (93) and antibiotic-induced microbial dysbiosis alters succinate levels altering tuft cell numbers in the absence of a helminth infection (33). Microbial load increases greatly in the cecum and large intestine, but small intestinal dysbiosis does occur during a large intestinal helminth infection (94). Although these microbial changes are not as instantaneous as detecting the helminths themselves, they can occur within days of infection (95). Moreover, helminth-driven dysbiosis may actually strengthen existing innate barrier responses, as during large intestinal *Trichuris suis* infection, the addition of the dietary supplement inulin heightens the microbial changes *T. suis* initiates (96), resulting in tuft cell hyperplasia (97).

EECs have a heightened ability to potentially sense helminths and/or the microbial dysbiosis they produce *via* the huge array of chemosensory apparatus they possess. Classically, the peptide hormones secreted by EECs signal to the brain in a paracrine fashion *via* local vagal afferents to mediate digestion and satiety. Recently, Bohórquez et al. (98) demonstrated that CCK-expressing EECs possess basal axon-like cytoplasmic processes, termed neuropods, which transpose nutritional and microbial intestinal signals directly to the brain (99). Neuropods are rich in mitochondria, dense secretory vesicles, presynaptic proteins, and neurofilaments and lie in close contact to enteric glia (100). Neuropods are present in both ileal and colonic EECs (98) and have the capacity to respond to and transmit glucose stimuli to vagal neurons in milliseconds (99). The EEC neuropod therefore has the exciting potential to communicate intestinal chemosensory information directly to the brain and, given the novel neurological control of ILCs (101), presents an exciting immunological addition to the gut–brain axis.

DISCUSSION

Given that RNA-seq analysis of bitter taste receptor-expressing cells in multiple barrier tissues is strongly linked to innate immune transcripts (102), it is clear that we are only at the beginning of fully elucidating the complex interactions of chemosensory, immune, and neuronal cellular interactions during infection. It still remains imperative to define the helminth products that initiate these epithelial cascades which drive immunity. Although tuft cells are reported to respond almost instantly to a helminth infection (42), it remains a possibility that helminth-derived microbial alterations could be a potential slower innate trigger, particularly in the large intestine where reports of helminth-induced tuft cell alterations have so far been absent. Alternatively, chemosensing may fall to EECs in the large intestinal niche and tuft cells may even act in concert with EECs utilizing microspinule communication to harness neighboring EECs neuropod signaling to help drive the systemic immunity often seen during a helminth infection. In summary, the initial fascinating epithelial chemosensory discoveries discussed above could simply be a taste of things to come.

AUTHOR CONTRIBUTIONS

JW contributed to the conceptualization. AF, KW, JT, and JW contributed to writing the original draft. JW contributed to the writing, review, and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Clinical Use of *Schistosoma mansoni* Antigens as Novel Immunotherapies for Autoimmune Disorders

L. Cleenewerk[1], *Johan Garssen*[1,2] *and Astrid Hogenkamp*[1]*

[1] *Division of Pharmacology, Department of Pharmaceutical Sciences, Faculty of Beta Sciences, Utrecht University, Utrecht, Netherlands,* [2] *Division of Immunology, Danone Nutricia Research B.V., Utrecht, Netherlands*

The hygiene hypothesis states that improved hygiene and the resulting disappearance of once endemic diseases is at the origin of the enormous increase in immune related disorders such as autoimmune diseases seen in the industrialized world. Helminths, such as *Schistosoma mansoni,* are thought to provide protection against the development of autoimmune diseases by regulating the host's immune response. This modulation primarily involves induction of regulatory immune responses, such as generation of tolerogenic dendritic cells and alternatively activated macrophages. This points toward the potential of employing helminths or their products/metabolites as therapeutics for autoimmune diseases that are characterized by an excessive inflammatory state, such as multiple sclerosis (MS), type I diabetes (T1D) and inflammatory bowel disease (IBD). In this review, we examine the known mechanisms of immune modulation by *S. mansoni*, explore preclinical and clinical studies that investigated the use of an array helminthic products in these diseases, and propose that helminthic therapy opens opportunities in the treatment of chronic inflammatory disorders.

Keywords: *Schistosoma mansoni*, helminths, immune modulation, autoimmune diseases, hygiene hypothesis, M2 macrophages, tolerogenic dendritic cells

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***Correspondence:**
Astrid Hogenkamp
A.Hogenkamp@uu.nl

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INTRODUCTION: THE HYGIENE HYPOTHESIS

The incidence of autoimmune diseases, such as inflammatory bowel disease (IBD), multiple sclerosis (MS) and type1 diabetes (T1D) in industrialized countries has continuously increased over the past 50 years, and continues to rise steadily (1, 2). The exact cause of these immune disorders remains unknown, but they are thought to arise as a result of a complex interplay between genetic and environmental factors, leading to immune dysregulation (1). Since genetic changes occur at a slow rate, it is unlikely that the higher incidence of immune disorders over this relatively short period of time is related to a genetic drift. However, there have been substantial changes in environmental conditions (the exposome), including dietary changes, increased pollution, and hygiene that are thought to contribute to the surge in autoimmune disorders (1, 3).

The observation that increasing hygiene in industrialized countries and the resulting low incidence of infectious diseases correlates with an increasing prevalence of allergic and autoimmune diseases, led to the postulation of the hygiene hypothesis in 1989 (4). The hygiene hypothesis states that reduced exposure to pathogens leads to a more reactive immune system, which can result in autoimmunity (1, 2, 4).

Bacteria, viruses and parasites have all been implied as players in the hygiene hypothesis (1, 2). These so-called "Old Friends" have co-evolved with humans since the early days of humanity and have been beneficial to our species through their immunoregulatory properties (5). However, with the development of a modern lifestyle, urbanization and increased hygiene, most of these "Old Friends" have been largely removed from our environment. Of particular importance are microbes and parasites that infect humans and induce an asymptomatic carrier state, by inhibiting an inflammatory response (1). In 1970, Greenwood first demonstrated that immunomodulatory properties of parasites can indeed prevent the development of autoimmune diseases in mice by infecting them with *Plasmodium berghei* (6). Parasites such as helminths are particularly well-known for this property (1). Helminths have likely co-evolved with humans, developing the potent ability to induce a state of tolerance in the human body, and fine tuning the immune response to prevent both elimination of the parasite from the body and death of the host from the infection (1). Depending on the species, various mechanisms can induce such a regulatory profile of the immune system, making helminths interesting candidates for new immunomodulatory therapeutics in autoimmune diseases. Despite species-specific differences in the life cycles, tissue tropism and clinical presentation, they are all known to modulate the human host's immune system (7). *Schistosoma (S.) mansoni* is one of the most commonly encountered helminth infections and its immunomodulatory properties (outlined in **Figure 1**) have been studied extensively (8). We therefore chose *S. mansoni* as a representative member of helminths to explore their mechanisms of immune regulation in autoimmunity. Furthermore, this review provides an overview of the current state of knowledge regarding the use of helminths as a treatment for inflammatory bowel disease, Type I diabetes and multiple sclerosis. To our knowledge, *S. mansoni* has not been used in clinical trials relating to these autoimmune disorders, whereas the use of various other helminthic species (e.g., *Trichuris (T.) suis*) has already been shown to have promising effects. This review therefore also aims to highlight potential mechanistic differences between helminth species, which may provide further insight into the therapeutic potential of *S. mansoni* in helminth-based immunotherapy.

SCHISTOSOMA MANSONI

History and Epidemiology

Parasites are defined as eukaryotes that use another organism as their habitat (9). Due to the human history of migration, domestication and globalization, which has allowed us to encounter many different parasites, humans are host to over 300 parasite species (9). Parasites have been known to humanity from the beginning of civilization: The Ancient Egyptians were among the first to describe intestinal worms in humans (9). Egypt is also where Theodor Bilharz first identified the helminth *S. haematobium* in 1851 (10). In 1902, Manson discovered another species, *S. mansoni*, the causing agent of intestinal schistosomiasis. *S. mansoni* is responsible for the majority of schistosomiasis cases and accounts for around 300,000 deaths per year (11, 12). Due to under- and misdiagnosis, the number of *S. mansoni* infected individuals likely ranges between 391 and 587 million (13). Because transmission occurs via contaminated water sources, *S. mansoni* is most prominently found throughout the African continent in areas with poor sanitation, with the highest risk of infection in the southern and sub-Saharan Africa and the Nile River valley in Sudan and Egypt. It is also found in several areas in South America, including Brazil, Suriname and Venezuela, and the Caribbean (14).

Clinical Course of *S. mansoni* Infection

Infection of a human host is part of the highly complex life cycle of the schistosoma parasites, which is illustrated in **Figure 2**. This includes sexual reproduction of the adult worms in the human vascular system, an asexual phase in the intermediate snail hosts, followed by a return to a human host after exposure to contaminated water (11, 15). In an infected human host, adult male and female worms copulate in the mesenteric vein. The female worm produces up to 300 eggs daily, approximately half of which are expulsed through the intestinal wall and subsequently excreted with the feces (11, 16, 17). If the excreted eggs reach freshwater and are exposed to suitable environmental conditions, the larvae hatch (18). At this stage, they are termed miracidia, and actively swim using ciliary movements until they encounter the snail intermediate host (11). Once they penetrate the soft tissues of the snail host, the miracidia develop into mature sporocysts (18). Next, the sporocysts reproduce asexually through production of thousands of germinal cells which develop into daughter sporocysts (18, 19), which mature into cercariae that are eventually released from the snail (11). Once the cercariae encounter a human, they penetrate the new host and transform into schistosomules that circulate through the human tissues, lymphatics and venules until they reach the hepatic portal system, where they mature into adult female and male worms, and the reproduction cycle continues (11).

Primary infection usually occurs at a very young age, when children are exposed to contaminated water while bathing or playing. However, acute schistosomiasis is rarely observed in children, most likely due to B and T cell imprinting of children born to infected mothers (11). Therefore, acute infection is most often observed in travelers from non-endemic areas. Since schistosomiasis begins with cercaria entering the skin, the first reaction to infection occurs there, usually within 24–48 h after invasion (20). Dying cercariae in the skin trigger an innate immune response, which leads to a hypersensitivity response and the resulting cercarial dermatitis which presents as urticaria or angioedema (20). Cercarial dermatitis is the result of an inflammatory reaction to a variety of excretory/secretory (ES) proteins that facilitate skin penetration (21).

Late chronic infection causes intestinal disease and hepato-splenic schistosomiasis (15). Chronic infection is established once the mature worms start producing eggs that are then secreted in the stool by the human host. Adult worms do not induce an inflammatory response and therefore do not cause any direct symptoms (15, 22). They are equipped with a variety of strategies that allow them to evade an immune response. In contrast, the eggs are well-capable of inducing an

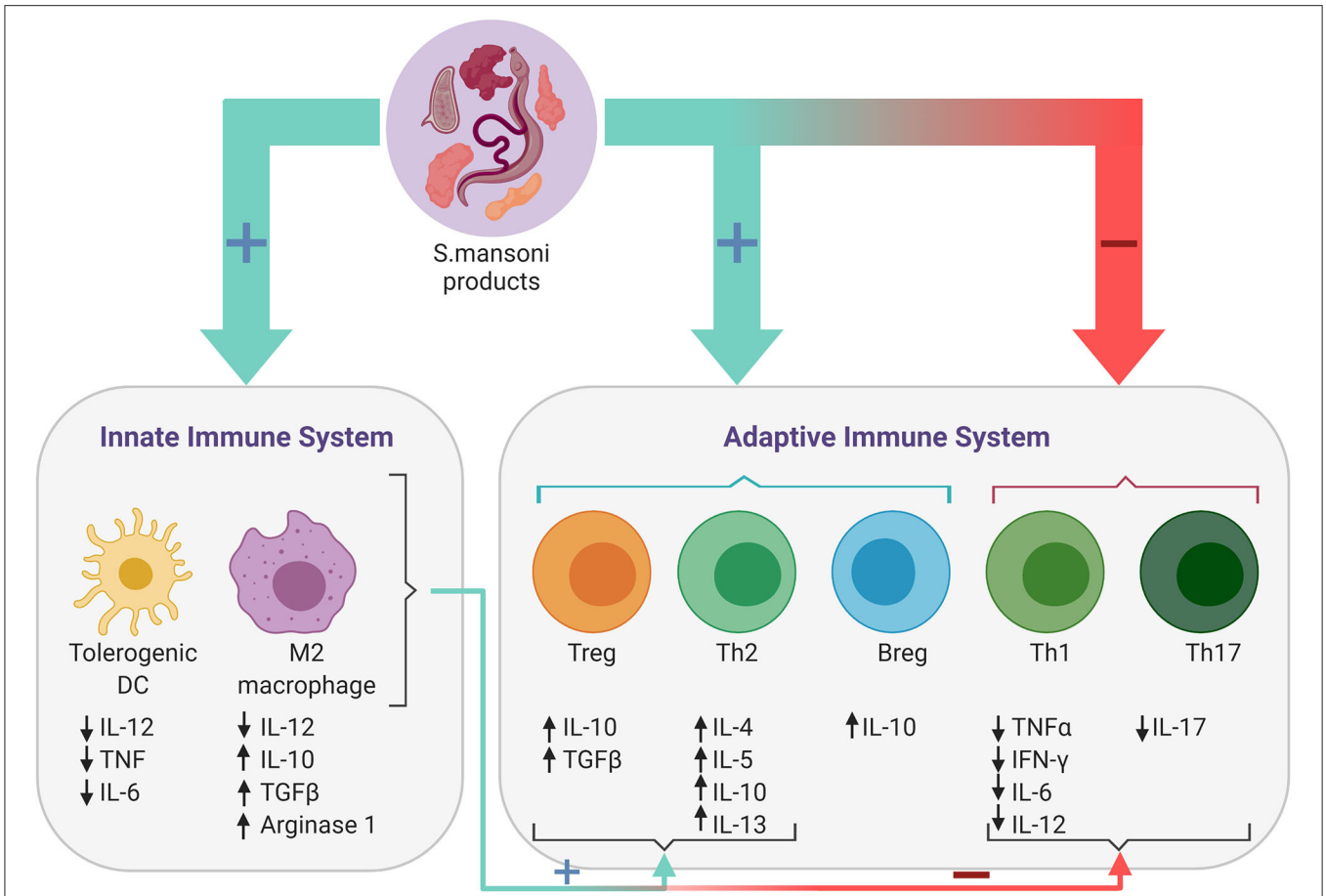

FIGURE 1 | Immune responses induced by *Schistosoma mansoni*. Antigens present on the surface of or secreted by the parasitic worm or its eggs, regulate the host's immune response by modulating both the adaptive and the innate immune response. *S. mansoni* products downregulate Th1 and Th17 responses and reduce levels of the associated pro-inflammatory cytokines, while promoting Th2 and regulatory B- (Bregs) and T-cell (Tregs) responses. Furthermore, *S. mansoni* products also promote the differentiation of tolerogenic dendritic cells (DCs) and alternatively activated (M2) macrophages, which in turn induce Breg and Th2-mediated responses, while simultaneously inhibiting the proinflammatory response of Th1 and Th17 cells.

inflammatory response on which they rely to pass from blood vessels into the lumen of the gut so they can be excreted and continue the life cycle (15, 22). However, approximately half of the eggs become trapped in the tissues and attract inflammatory cells, leading to the formation of granulomas and fibrosis. Resulting complications of the chronic infection include organ obstruction, portal hypertension and hepatosplenomegaly with potential gastrointestinal bleeding (11).

Development of Resistance

Resistance to *S. mansoni* infection is associated with Th2 responses which are characterized by antigen-specific Immunoglobulin (Ig) E, IL-4, and IL-5 production (15). Although it plays an important role in allergic disease, IgE originally developed in response to parasitic infections and provides protection against reinfection with helminths, such as schistosomes (11). While young children mainly produce blocking antibodies, such as IgM, IgG and IgG4, older children and adults predominantly produce the protective IgE, and thus are largely resistant to reinfection (11). The switch from Th1 to Th2 is crucial for survival of the host, indicated by the findings that patients with severe hepatosplenic schistosomiasis have high levels of Th1-associated cytokines [Tumor necrosis factor α (TNFα), IFNγ], while asymptomatic patients exhibit high levels of Th2 associated cytokines (IL-4, IL-5, IL-13) and IgE (23).

The development of resistance against infection with schistosomiasis is slow, and generally takes 10–15 years (15). Children that are regularly exposed to the parasite only show limited resistance between ages 5 and 11. Since the worms do not replicate within the human host, multiple reinfections with schistosomes will eventually lead to a higher worm and egg load. Once the infected individual reaches teenage years, the egg load and the intensity of infection gradually decrease (11).

Immune Modulation by *S. mansoni*

It is well-known that parasites can induce an immunosuppressive environment to evade the immune system. This also benefits the host, as a reduced inflammatory response limits tissue

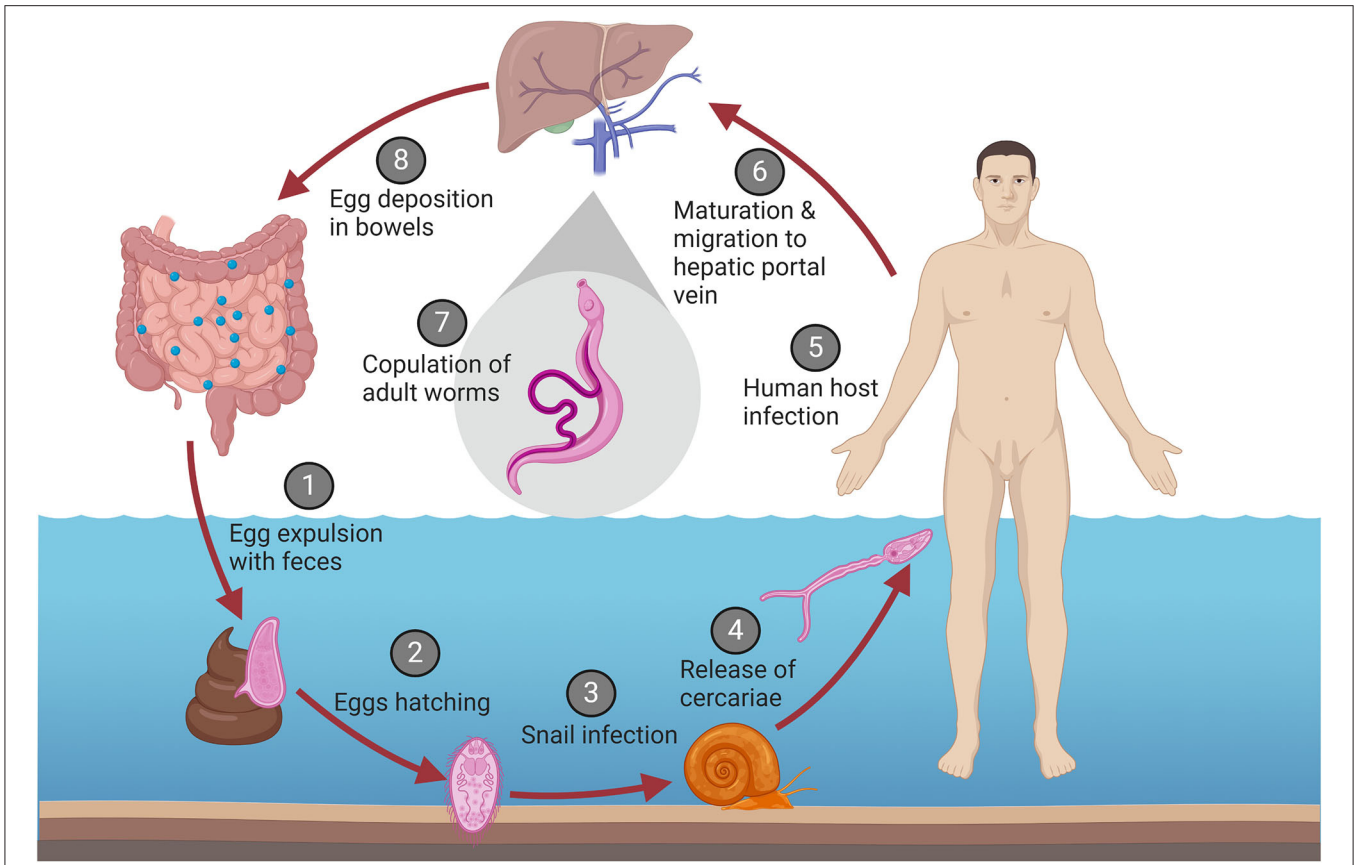

FIGURE 2 | Life cycle of *S. mansoni*. (1) Eggs are excreted with the feces of an infected human host. (2) Under the right conditions in a freshwater environment, the eggs hatch and release the larvae (termed miracidia). (3) The miracidia infect the intermediate snail host, where they develop into sporocysts, asexually replicate, and mature into cercariae. (4) The cercariae are released back into the water. (5) Once the cercariae encounter a human host, they penetrate the skin. (6) After penetration, the cercariae transform into schistosomules and migrate to the hepatic portal vein. (7) The schistosomules mature into adult male and female worms and copulate. (8) The female worm migrates to the mesenteric venules of the bowels and begins egg deposition, that are secreted with the feces and reiterate the cycle.

damage (12). Murine studies have shown that repeated infections with *S. mansoni* lead to the suppression of the immune response, promoting survival of the adult worms (24). The following sections will discuss the early immune response induced by helminthic proteases, and explain the role of dendritic cells, macrophages, and different T-cell subsets herein.

Early Immune Responses Induced by Proteases

Proteases are crucial for the survival of parasites. A range of different proteases assist in invasion, nutrient uptake, hatching, evasion of the immune system, and modulation of the host's physiology (25). *S. mansoni* proteases have been shown to regulate vascular functions (25), causing vasodilation, which allows the relatively large adult worms to move more freely to the narrow blood vessels and deposit their eggs there (26). The most well-known *S. mansoni* proteases are cysteine and aspartic proteases, as well as the serine proteases (SP)s (25). The best studied protease, the 28kDa *S. mansoni* cercarial elastase (SmCE) is largely responsible for skin penetration (27, 28). Once cercariae come into contact with the human host, they can enter the skin within 1.5 min, with the help of SmCE (11, 25, 29). SmCE is capable of degrading a large variety of human skin macromolecules (21, 30). Importantly, this protease is also able to elicit an immune response in the host. SmCE induces the production of anti-elastase IgG2a antibodies, which induce macrophage-mediated cytotoxicity against schistosomula and cercariae, resulting in effective killing of the parasite at this stage (31). In addition, both the alternative and classical complement-mediated pathways contribute to the clearance of the parasite during early infection (32, 33).

Although SmCE plays a key role in eliciting this response, it is also involved in resistance against complement-mediated killing (34, 35). During the transformation of cercariae into schistosomula, SmCE assists in remodeling the outer layer of the tegument (i.e., the outer surface of schistosomula and adult worms) and shedding of the glycocalyx which is a potent inducer of the complement system (30, 32, 36).

Next to shedding the glycocalyx, the transforming cercariae remodel the single membrane surface into a complex bi-layer membrane structure, incorporating different host molecules, multi-layered vesicles and glucose transporters (28, 37). Interestingly, the outer surface of the bi-membrane structure

can adsorb human blood molecules, therefore masking it from recognition of the immune cells (37). However, several tegument proteins are targeted by the immune system, as can be shown by the production of specific IgE against members of the Tegumental allergen-like (TAL) family (38).

Although the early antigens discussed in this section are crucial for evading initial immune responses during and after invasion, they do not actively modulate the immune response. These proteins are attractive candidates for vaccine development, but are not suitable for immunomodulatory therapy. The processes that lead to immunosuppression by *S. mansoni* will be covered in more detail in the following sections, highlighting the role of dendritic cells and macrophages as these cells largely determine whether a Th1 or Th2 dominant immune response will be initiated (1).

Dendritic Cells

Dendritic cells (DCs) are crucial for connecting the adaptive and innate immune responses (1). Depending on the stimuli DCs receive, they can adopt either a tolerogenic or an immunogenic activation state, which in turn affects the differentiation of T-cells (1). The maturation of dendritic cells begins with the uptake of an antigen via pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C type lectin receptors (CLRs). PRRs recognize the so-called pathogen-associated molecular patterns (PAMPs) on infectious agents, which leads to internalization of the pathogen (1, 39). Whereas, immunogenic DCs develop in response to "danger" signals in the form of PAMPs, cytokines or other signals from activated T-cells (39), tolerogenic DCs usually arise in response to apoptotic cells or commensal bacteria, in the absence of "danger" signals. These DCs do not exhibit markers of activation such as MHC and CD86 upregulation. The tolerogenic DC induce Th2 and Treg responses, as seen in helminthic infections (1). Importantly, tolerogenic DCs have been shown to prevent the development of autoimmunity (1). Therefore, helminthic products that promote the development of tolerogenic DCs have therapeutic potential for treating autoimmune disorders.

Indeed, certain helminthic products have been found to direct naïve DCs toward the tolerogenic profile by binding to TLRs or CLRs (such as DC-SIGN) (1). In particular, soluble components secreted by *S. mansoni* eggs, called soluble egg antigen (SEA), and egg-derived dsRNA have shown immunoregulatory properties through induction of tolerogenic antigens. SEA comprises all soluble components of the *S. mansoni* eggs, of which only few have been identified and characterized (40). Studies with murine bone-marrow derived DCs have found that the presence *in vitro* of SEA prevents TLR-dependent conventional activation of DCs. The tolerogenic profile of SEA-exposed DCs was confirmed by minimal upregulation of MHC, absence of CD80/CD86 upregulation and lack of Th1 and Th17-type cytokine production, such as IL-6, TNF and IL-12 (41), and maintain their ability to endocytose, which is lost during conventional maturation of DCs (42). To confirm that these unconventional DCs effectively drive a Th2 response, SEA-treated DCs were transferred to mice. Indeed, when murine SEA-treated DCs were transferred into live animals, they induced the differentiation of naïve T-cells into Th2 cells and the production of IL-4, IL-5, and IL-10 (41, 43). Furthermore, the induction of a tolerogenic DC profile by SEA has been found to be dependent on CD40. Although SEA does not upregulate CD40, absence of CD40 leads to failure to develop Th2 responses by SEA-exposed DCs (44).

On a molecular level, SEA has been found to inhibit pro-inflammatory responses by interacting with the nuclear factor κ B (NFκB) family member B-cell lymphoma 3-encoded protein (Bcl3) (40). Klaver et al. showed that the glycosylation of SEA is essential for the Th2-driving of DCs by suppressing lipopolysaccharide (LPS)-induced, TLR-mediated production of pro-inflammatory cytokines. It is still unclear how exactly DCs drive Th2 differentiation after activation by SEA, but it is known that CD40, OX40L and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB1) expression are required (44–46). However, it is certain that unconventional activation profiles of DC induced by SEA can actively promote Th2 response development (39).

Non-SEA components have also been found to induce tolerogenic DCs. One of the tegumental antigens, Schistosoma mansoni protein 29 (Sm29), has been shown to induce tolerogenic DCs *in vitro* (17, 47). Sm29 is located in the tegument of adult *S. mansoni* and constantly exposed to the immune system, which would explain its immunosuppressive characteristics (47). DCs treated with Sm29 exhibited several characteristics of a tolerogenic profile: higher expression of HLA-DR, CD83, CD80, and CD86 as well as of IL-10 and IL-10R, and increased the frequency of CD4+ T-cells expressing the regulatory molecules CTLA4 and CD25 (47). Taken together these findings suggest Sm29 contributes to the differentiation of naïve T-cells into Treg, unlike the SEA that is a potent Th2 inducer (48).

Macrophages

Schistosoma mansoni also affects macrophage activity. Macrophages can either be activated via the classical pathway (M1) or the alternative pathway (M2). M1 activation occurs in response to TLR ligands or IFNγ, M2 activation occurs in response to IL-4/IL-13. M2 macrophages, in contrary to M1 macrophages, have low expression of IL-12, but high expression of IL-10, TGFβ and arginase 1 (49). M2 macrophages are present in granulomas and have been found to play a key role in the immunomodulation during schistosomiasis. They have anti-inflammatory functions and play a direct role in modulating fibrosis and survival of the host by downregulating inflammation (23, 50). Interleukin-4-inducing principle from Schistosoma mansoni eggs (IPSE/alpha-1), a major component of SEA, is the main driver of M2 differentiation (49). IPSE/alpha-1 binds to immunoglobulins, with a high affinity for IgE (51). Once it binds to IgE bound to FceRI receptors on the surface of basophils, it triggers the release of IL-4 and IL-13 (51), which directly induces the differentiation of monocytes into alternatively-activated-macrophage-like phenotype and inhibits the secretion of pro-inflammatory cytokines by LPS-stimulated monocytes (51).

Next to SEA, the lipid lysophosphatidylcoline (LPC) can also induce macrophage differentiation into an M2 phenotype (50). LPC is excreted by the worm as a degradation product. It has anti-inflammatory properties such as increasing the immunosuppressive function of Treg cells, promoting eosinophil recruitment and stimulating Th2 polarization through Toll-like receptor 2 (TLR2) dependent mechanisms (50). LPC activates peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor required for M2 polarization, which in turn increases Major Histocompatibility Complex, Class I-Related (MR1), chitinase 3-like 3 (Ym1), IL-10 and TGFβ, but not Nitric Oxide Synthase 2 (NOS2), gene expression, which is characteristic for M2 macrophages (50). Additionally, LPC induces IL-10 production by macrophages (50). Thus, SEA and LPC, amongst others, drive M2 differentiation of macrophages, which contributes to the immune shift to Th2 response observed in schistosomiasis.

B-Cells and IgE

The modulation of dendritic cells and macrophages by *S. mansoni* is important as these cells are directly responsible for the switch of a Th1 to a Th2 mediated immune response. However, *S. mansoni* also employs evasion strategies that directly target the adaptive immune system, in particular B cells and IgE. Because IgE does not play a central role in autoimmune disorders, mechanisms used to evade B cell-mediated immunity will only be briefly discussed.

As the source of the IgE which protects the host, B-cells play an important part in the immune response against *S. mansoni* (52). In *S. mansoni* infected individuals, T helper 2 (Th2) responses and their associated characteristics (IL-4, IL-5 cytokines, eosinophilia, and specific IgE secretion) have been associated with resistance against re-infection (15, 53). *S mansoni* has developed mechanisms to interfere with IgE signaling to evade the immune system. *S. mansoni* antigens are able to cleave the surface-bound low-affinity IgE receptor CD23 (54), which could potentially interfere with T-cell activation. Additionally, *S. mansoni* was found to secrete a homologue of soluble CD23 acting as a decoy receptor by binding IgE and inhibiting activation of the high-affinity IgE receptor FcεRI. This in turn prevents degranulation of basophils and mast cells, inhibiting the release of cytotoxic molecules and inflammatory mediators, which usually contribute to killing of the parasite (54). In addition to conventional B cells, a small subset of B-cells, B regulatory cells (Bregs), are also involved in the response to *S. mansoni* (55). Bregs have recently been identified, as a subset of B-cells capable of producing IL-10 (1), which induces Treg differentiation *in vitro* (55). Furthermore, Bregs can downregulate immune responses through direct interaction with effector T-cells (1). IPSE/alpha-1 can induce production of IL-10 in naïve B cells (55) and stimulate the differentiation of Breg cells (56) Additionally, B cells bind SEA and internalize it, leading to a 3-fold upregulation in the production of IL-10 (55). Bregs have been implicated as important players in autoimmunity (57), and helminth infections have been shown to affect their function in autoimmunity, potentially altering the course of disease (58). Therefore, stimulation of these cells by *S. mansoni* products warrants further investigation.

HELMINTH SECRETIONS AS A THERAPY IN AUTOIMMUNE DISORDERS: SHOULD THE USE OF *S. MANSONI* BE PROMOTED?

The immunosuppressive capacity of *S. mansoni* has become of interest in the context of autoimmune disorders (1). Since autoimmune disease are characterized by an overactive immune system and predominant Th1 and/or Th17 responses, it seems plausible that infection with these immunosuppressive helminths could potentially be beneficial in preventing or treating autoimmune inflammatory disorders. However, despite the extensive amount of *in vitro* and *in vivo* studies investigating the effects of *S. mansoni* in autoimmunity, there are no clinical trial reporting use of *S. mansoni* products in autoimmune disorders so far. A recombinant protein of the closely related *S. haematobium* has recently entered clinical trials for the use in IBD (59). Based on the data regarding its effects—discussed in the previous and in the following sections—it could be argued that *S. mansoni* is an eligible candidate for future clinical trials.

In addition, immunomodulation is not a characteristic unique to *S. mansoni* (7); other helminths have already been successfully employed in clinical trials, which—in part—paves the way for future studies investigating the potential benefits of *S. mansoni* in autoimmune disorders. Although species-specific differences are evident, the immunomodulatory mechanisms described in the previous sections largely apply to many helminths such as *T. suis*. *T. suis* has been used repeatedly in clinical trials, especially in IBD and MS (60). This soil-transmitted swine helminth species only transiently infects humans in a self-limiting fashion, while still promoting Th2 immune responses. These characteristics are desirable for human trials.

An extensive study of its excretory/secretory (E/S) proteins has shown that *T. suis* proteins skew DC and macrophage polarization toward a tolerogenic and M2 profile, respectively (60). It has been shown that E/S proteins of *T. suis* inhibit classical activation of DCs, and these DCs skew T cell activation toward a Th2 profile (61). In addition E/S proteins were found to elicit specific Th2 responses, as characterized by the production of IL-4, IL-5, IL-13, and IgE (62). Furthermore, the first transcriptome analysis of *T. suis* uncovered over one hundred potential immunomodulatory proteins (63). Some serine protease inhibitors (serpins) produced by *T. suis* (TsTCI, TsCEI) have been shown to modulate immune responses by inhibiting host proteases, such as chymotrypsin and neutrophil elastase (64). In addition, three novel immunomodulatory proteins (Tsui7583957, Tsui7234544, and Tsui7304731) were identified, but their individual effects have not yet been elucidated (62). However, although several attempts have been made to elucidate the mechanisms of the E/S proteins (65–67), the nature of these components and their exact mechanisms of action are not yet known. The lack of such a large knowledge-gap makes *S. mansoni* an attractive candidate for future studies. Although the nature of the E/S proteins of *T. suis* may be different

from *S. mansoni,* the general concepts remain the same (i.e., serine proteases, tolerogenic DC induction). In the following sections, a selection of studies using different helminthic species and/or their products as a therapeutic for IBD, MS and T1D will be discussed, considering them as proof-of-concept studies for the use of *S. mansoni* and its products in the clinic. A summary of these findings is shown in **Figure 3**.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) encompasses both Crohn's disease (CD) and ulcerative colitis (UC). IBD is characterized by chronic intestinal inflammation leading to irreversible damage of the bowel. While understanding of IBD is still limited, it is believed that CD is mediated largely by Th1 responses, although Th17 responses are emerging more and more as an important contributor to the disease development and progression (68). Furthermore, macrophages and DCs are increasingly recognized as key players in IBD pathogenesis as abnormal activation of these cells leads to inflammatory reactions which contribute to the chronic inflammation. Given their key role in initiating and maintaining inflammation, they are an attractive target for therapeutic agents (69–74). In the developed countries, the prevalence and incidence of CD has risen dramatically since 1940, but this disease is rare in areas where parasitic infections are endemic (75). Taken together, this suggests that helminthic products pose an attractive therapeutic approach.

Preclinical Studies

Elliott et al. investigated whether freeze-thaw-killed *S. mansoni* eggs could protect mice from developing trinitrobenzesulfonic acid (TNBS-induced colitis by inhibiting Th1 dependent immune responses (75). Inflammation in TNBS-induced colitis manifests with infiltrating CD4+ T-cells expressing high levels of proinflammatory cytokines, such as IFNγ (75). Histological assessment of the colons showed that mice treated with eggs prior to the TNBS challenge had significantly attenuated colitis and decreased mortality compared to untreated TNBS-exposed mice (75). In mice receiving *S. mansoni* treatment, mesenteric lymph nodes and splenic T-cells produced lower levels of IFNγ and higher levels of IL-4. Furthermore, levels of IL-10 mRNA in the colon were increased in egg-treated mice (75). These findings suggest that treatment with *S. mansoni* eggs inhibits Th1-related inflammatory responses in colitis by inducing regulatory and Th2 responses, which leads to a reduction in symptom severity and mortality (75).

Smith et al. conducted a similar study in which they aimed to determine whether *S. mansoni* infection could protect mice against dextran sodium sulfate (DSS)-induced colitis (76). To determine whether potential protection against colitis was caused by worm or egg antigens, they infected mice either with male worms, resulting in no egg production, or both sexes, causing the release of eggs by female worms. Mice infected with male *S. mansoni* cercariae had a significantly lower disease activity index (DAI), which combines scores for weight loss, bloody feces and stool consistency (76). Additionally, DSS-treated mice presented a shortened colon, while parasite-infected mice had normal-length colons (76). In contrast, schistosome eggs did not provide protection against DSS-induced colitis. Instead, administration of male and female cercariae, which leads to egg production, exacerbated the colitis compared to uninfected mice (76). This observation is likely linked to the inflammation induced by the eggs in the colon to facilitate their passage into the lumen (76). Further results of this study also showed that while IL-10 and TGF-β play protective roles in DSS-induced colitis, the protection provided by male *S. mansoni* cercariae is independent of these cytokines, since treatment with anti-IL-10 and anti-TGFβ did not result in increased symptom scores (76).

Conversely, depletion of macrophages abolished the protective effects of *S. mansoni* and rendered the mice fully susceptible to colitis (76). However, contrary to previous findings, it was demonstrated that the protective effects were not determined by alternatively activated macrophages but instead were mediated by F4/80+ macrophages infiltrating the colon. These macrophages, isolated from schistosome-infected mice, provided protection against DSS-induced colitis if transferred to naive mice prior to DSS exposure (76). Taken together, these results provide evidence that helminthic infections, in particular *S. mansoni* infections, protect against the development of DSS-induced colitis. Additionally, these findings indicate that the protection against colitis is not dependent on the Th1/Th2 axis or Treg cells, but is instead mediated by macrophages, which are not alternatively activated (76).

This latter conclusion is in contrast to the findings by Moreels et al. who suggest that the beneficial effects of *S. mansoni* infection observed in their study were related to an attenuated Th1 response to TNBS, mediated by a shift from Th1 to Th2 profiles In their rat model for TNBS-induced colitis, they demonstrated that disease symptoms and gut inflammation were attenuated in animals with a concomitant *S. mansoni* infection (77). IL-2 secretion in spleens of infected rats was decreased, implying an attenuated Th1 response to TNBS. Moreover, IL-4 levels in the spleen of infected rats were transiently increased, although not significantly (77). Furthermore, the duration of inflammation in response to TNBS stimulation was shorter, and less intense, which correlated with a reduction in inflammatory infiltrates in the colon and faster regeneration of the damaged mucosal layer (77).

The conflicting findings from these studies may be explained by differences in experimental setup. Elliott et al. (75) used dead eggs that were injected, while Smith et al. (76) infected the mice with live worm pairs to induce egg-laying. It is plausible that dead and live eggs would induce different immune responses, and the presence of adult worms in combination with the eggs—in contrast to eggs alone—could also affect the immune response. Mice react differently to *S. mansoni* infections than rats, hence the results obtained by Moreels et al. cannot easily be compared to the outcomes of the murine studies. However, since *S. mansoni* infection attenuated symptoms in all models (75–77), helminth therapy with *S. mansoni* presents itself as a promising approach to preventing onset of IBD. Furthermore, it is likely that both macrophages and Th2 responses are involved in mediating the

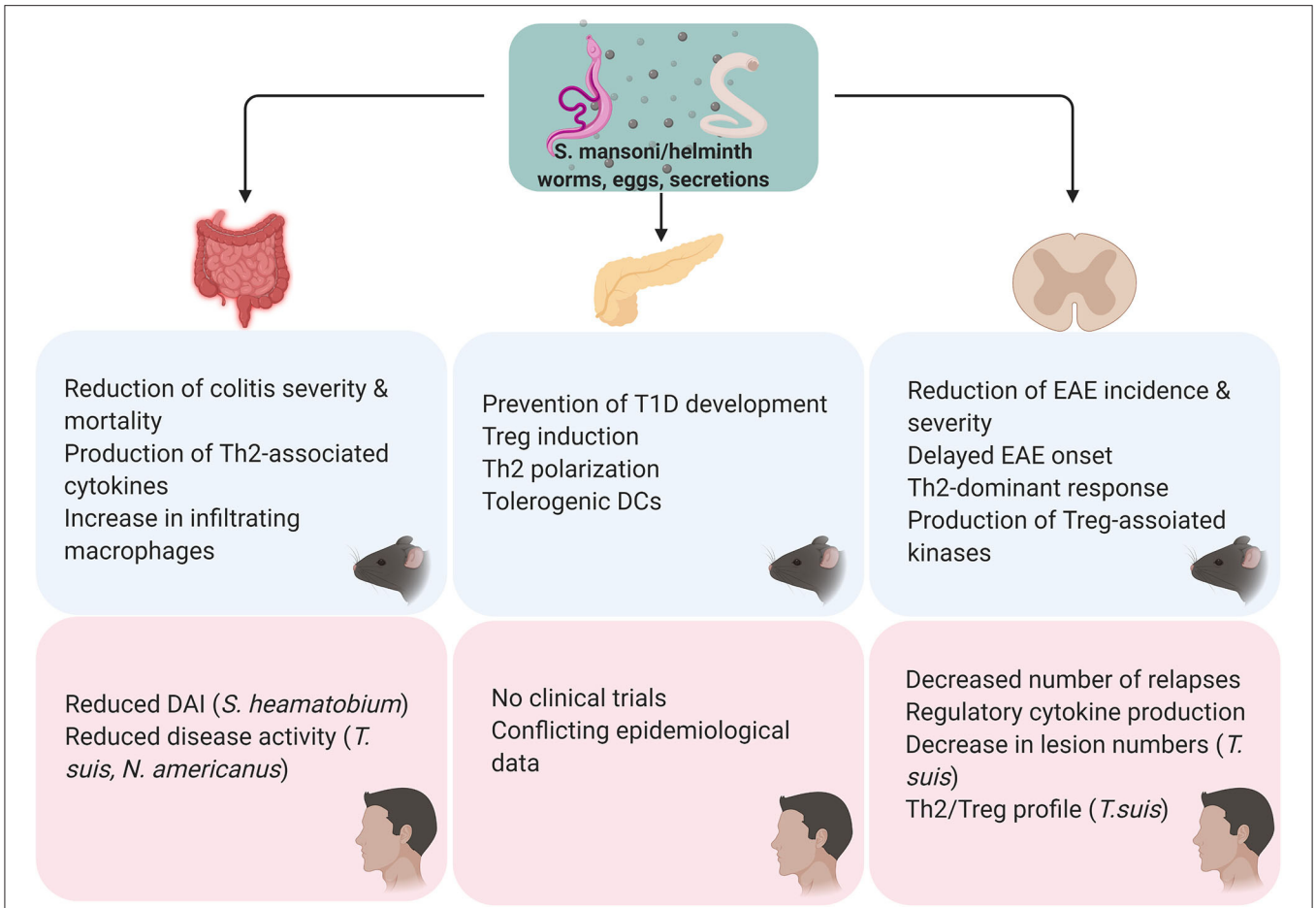

FIGURE 3 | Overview of the findings of S. mansoni/helminth therapy in pre-clinical and in clinical studies. All the *in vivo* studies were performed using *S. mansoni* antigens. For all studies, either cercariae, adult worms, eggs or secretions were used as a therapeutic agent. Preclinical studies in colitis models have found *S. mansoni* therapy to reduce the severity of the disease course, its mortality and modulate the immunological profile by shifting the immune response from a Th1 to a Th2/regulatory profile. Clinical trials using *S. haematobium*, *T. suis*, and Necator americanus found that helminth therapy effectively improves disease activity. Regarding T1D, preclinical studies in NOD mice showed that helminth therapy inhibits the development of diabetes through the induction of a Th2/regulatory profile and tolerogenic DCs. Clinical studies investigating helminth therapy in T1D have not been conducted and epidemiological data on this subject is conflicting. S. mansoni therapy has been shown to prevent onset and delay the severity of EAE *in vivo*, through a shift from a Th1 to a Th27regulatory profile. Similarly, clinical studies have observed that helminth therapy might be able to reduce relapse frequency and improve lesions via induction of a Th2/regulatory profile.

protective effects of *S. mansoni* although the exact mechanism still needs to be identified.

In a murine model of TNBS-induced colitis, treatment with protein 28 Kd glutathione S Transferase (P28GST) isolated from *S. heamatobium* reduced both clinical and histological scores (78). Levels of pro-inflammatory cytokines (IL-6, TNF, IL-1ß) were significantly downregulated and expression of Th1 and Th17 markers T-bet and ROR-γ, was inhibited. On contrary, an increase in the levels of Arg/iNOS mRNA levels suggested M2 activation of macrophages (78). In a similar study, recombinant schistosome P28GST improved colitis symptoms in rats, which has been related to a Th2 shift of the immune response (79). *S. heamatobium* is closely related to *S. mansoni*, and the P28GST protein expressed by *S. mansoni* has been described to protect against inflammation (80, 81). Thus, proteins with known immunomodulatory properties derived from *S. mansoni* or its close relatives have the potential of being used in the clinic to treat inflammatory disorders.

Human Studies

Although the preclinical data suggests that *S. mansoni* protects against the development of IBD, no clinical trials using this particular helminth have been performed so far. However, administration of the recombinant *S. heamatobium* P28GST has been shown safe and effective after administration to 8 CD patients with mild disease in a small recent open label 2a clinical trial (59). Patients received monthly subcutaneous injections of the protein over the course of 3 months and were monitored for 9 months following the treatment. At 3 months after the first injection, disease activity scores decreased by 30% compared to baseline. Furthermore, side effects occurred mostly at the injection site or were possibly related to CD manifestations (59).

These promising data suggest P28GST may be used to treat CD, and it would be interesting to assess whether similar results can be achieved with P28GST of *S. mansoni* origin.

Several other helminth species have been used in clinical trials involving IBD-patients. In a clinical trial where *T. suis* eggs were administered to Crohn's patients unresponsive to conventional treatments, it was observed that parasitic treatment might hold therapeutic potential in Crohn's disease (82). A total of 29 patients were enrolled in this 24-week open label study aiming to determine the safety and clinical effects of *T. suis* eggs in Crohn's disease. The patients did not experience any side effects attributable to the infection with the parasite, such as diarrhea, nausea, or abdominal pain. After 24 weeks, close to 80% of patients experienced an improvement in clinical activity (82). Due to the study being open label and the absence of a control group, a placebo effect cannot be excluded. Additionally, the study did not score any other immune parameters such as cytokine levels or immune cell populations, so there is no biological evidence that the results are related to immune modulation. Nevertheless, the results of this study support the hypothesis that administration of helminthic products could benefit patients with IBD, especially if they are non-responsive to conventional treatment (82).

The same group of researchers also conducted a similar clinical trial in patients with active ulcerative colitis (83). In this randomized clinical trial, patients with ongoing, treatment resistant ulcerative colitis ingested *T. suis* eggs every 2 weeks and were assessed for their disease activity scores for 12 weeks following the treatment. A significant improvement in disease activity was seen in 43.3% of the *T. suis* treated patients compared to 16.7% in the placebo group. The treatment was deemed safe, due to the reported side-effects being attributable to other conditions and absence of parasitic reproduction in the human host (83). Although helminthic therapy only induced significant improvement in less than half of the patients, and no data are available as to how the *T. suis* eggs improved symptoms in these patients, results from this study show that it may be beneficial for patients with severe, treatment resistant ulcerative colitis (83). Still, in following studies, it is recommended to measure cytokine levels and other markers of inflammation and disease progression to determine whether the reduction in symptoms is indeed due to the proposed mechanism of action of helminths, i.e., by shifting the immune response to a Th2 profile and thereby reducing the inflammation in the gut.

Intriguingly, ulcerative colitis is believed to be a Th2-mediated disease, which would imply that the Th2 shift induced by parasites exacerbates the disease (84). However, studies have shown that infection with parasites can prevent the development of asthma, which is also Th2-mediated. This effect is believed to be due to helminths regulating the immune system by inducing the secretion of immunoregulatory cytokines, such as IL-10 (85). Even though the exact mechanism is not understood, an improvement in symptoms was observed in patients that were otherwise unresponsive to treatment, without causing any adverse events. Therefore, further investigating the possibility of using helminths as a therapy could lead to the development of a therapeutic agent for some treatment-resistant patients (83).

Apart from *T. suis,* the hookworm *Necator (N.) Americanus* has also been administered to IBD patients in an attempt to reduce the inflammatory response. Unlike *T. suis,* which only transiently infects humans and would therefore require repeated administration, *N. americanus* establishes long-lasting infection (86). In a proof-of-concept study by Croese et al. administration of the hookworm *N. americanus* to five Crohn's patients with long standing but mostly inactive disease resulted in an improvement of disease activity 20 weeks after infection with the helminth (87). However, the attempt to reduce the dose of immunosuppressive drugs following symptom improvement resulted in symptom exacerbation in two patients (87). Similar to the studies mentioned above, these results indicate that helminthic therapy might be a powerful therapy to improve IBD symptoms, at least in combination with conventional immunosuppressive drugs. In the study by Croese et al. the attempt to reduce the dose of immunosuppressive drugs caused the symptoms to exacerbate (87). High doses of immunosuppressants and their side effects might cause problems in combination with parasitic infection. Furthermore, although *N. americanus* does not usually cause pathology, and symptoms are mostly limited to easily treated anemia and itch on the skin penetration site, it can cause an enteropathy (87). Enteropathies were observed in the inoculated patients and although the enteropathy resolved in all five patients in this study (87), it may not be recommended to administer an agent that can cause inflammation in the gut to patients with chronic gut inflammation. The risk/benefit ratio needs to be carefully evaluated in order to provide the best treatment option to every individual patient.

Hookworms and their excretory/secretory products have been studied extensively and their therapeutic properties are reviewed elsewhere (88). *S. mansoni* excretory/secretory products have also been extensively studied, and the immunological effects of these products has been investigated repeatedly. In summary, the promising results of hookworm clinical studies and the knowledge of schistosome immunomodulation provide a steppingstone for further trials using *S. mansoni* and its products as a treatment option in treatment-unresponsive patients.

Type 1 Diabetes (T1D)

Type 1 diabetes results from an autoimmune response in which CD4+ and CD8+ cells induce destruction of the pancreatic insulin-producing β-cells, leading to insulin deficiency (89, 90). Several studies have provided results that identify T1D as a largely Th1 mediated disease. For example, Katz et al. have shown that Th1 cells that express an autoreactive receptor induced T1D in NOD mice, whereas Th2 cells with the same receptor did not (91). Although the pathophysiology of T1D is not solely dependent on Th1-mediated immune responses, immune modulation toward a more protective immunologic profile could provide protection against development and/or progression of the disease. In respect to this, helminthic products have been shown to modulate the immune response *in vivo* by suppressing Th1-associated immune processes (92, 93). Macrophages and dendritic cells are also key players in the development and pathophysiology of T1D. Their contributions to T1D have

extensively been described elsewhere (94–98). In brief, classically activated macrophages and immunogenic DCs contribute to the inflammatory landscape in T1D, whereas M2 macrophages and tolerogenic DCs attenuate the inflammatory response. Thus, modulating the activity of macrophages and DCs by using *S. mansoni* or other helminthic products emerges as a promising therapeutic approach for T1D.

Preclinical Studies

Non-obese diabetic (NOD) mice spontaneously develop diabetes (99), which is accompanied by expansion of an autoreactive CD4+ cell population that behaves in a Th1-like manner, infiltration of B-cells, dendritic cells and macrophages into the islets of the pancreas before the development of diabetes-like symptoms (93). Cooke et al. have shown that infection with *S. mansoni* cercariae significantly inhibits the development of diabetes in NOD mice (100). In infected mice, the incidence of diabetes is considerably reduced to 10–15%, compared with 70% of the control mice (100). Furthermore, blood glucose levels in infected mice were demonstrated to be below the cut-off value of 20 mmol/l, which is considered diabetic (100).

Several further studies have shown that helminthic infections and/or products can inhibit the development of diabetes in NOD mice through various mechanisms, such as Th2 polarization, induction of Treg cells and an increase in TGFβ (100–102). Zaccone et al. showed that SEA induces phenotypic changes in murine primary splenic DCs *in vitro*. These phenotypic changes mainly include increased expression of CLRs, such as galectins 1 and 3, and SIGN-R1 (99). Since galectins recognize schistosome antigens and are crucial for the adaptive immune responses induced by the parasite, these results suggest that SEA induces tolerogenic DCs that can inhibit a Th1 response (99). Moreover, SEA was shown to dramatically increase IL-4 and TGFβ mRNA expression in peritoneal exudate cells in mice injected with SEA, compared to controls (99). Furthermore, SEA treatment induced the expression of high levels of IL-4, IL-10 and IFNγ in pancreatic CD4+ T-cells (99). Furthermore, analysis of costimulatory molecules on peritoneal cells and surface markers on peritoneal macrophages suggested the presence of M2 macrophages. Taken together, this demonstrates that SEA-dependent Th2/Treg responses and phenotypic changes in macrophages and DCs may have protective roles in T1D (99). In another study, Zaccone et al. showed that omega-1, one of the main glycoproteins in SEA, drives the differentiation of naïve T cells into Treg cells in DC/T cell cocultures (103).

Altogether, these results show that *S. mansoni* can prevent diabetes in NOD mice (100) and that this protective effect is likely mediated by SEA through modulation of the macrophage and DC activation, leading to regulatory T-cell profiles rather than inflammatory responses (99, 103).

Human Studies

Studies aimed at investigating the role of helminths in the human situation are scarce. To our knowledge, the use of *S. mansoni* in the prevention or treatment of T1D has not been studied to date. Epidemiological studies determining the correlation between the incidence of parasitic infections (in general) and T1D are largely lacking, and the ones that have been performed found contradictory results (104). For instance, a study in Southern India found that the prevalence of lymphatic filariasis, a parasitic disease affecting the lymphatic vessels, in patients with T1D was 0%, compared to 2.6% in non-diabetic people, suggesting that parasitic infections protect against T1D (105). However, a large population-based study in Denmark found no correlation between infection with the helminth *Enterobius vermicularis* and the incidence of T1D (106). Evidently, these are entirely different parasites which are likely to induce distinct immune responses. Moreover, parasitic diseases are more prevalent in developing countries such as India (105), which could lead to patients being infected with multiple parasites at once, thus affecting the results of the study. In summary, the role of helminths in T1D remains largely unknown. However, as there are numerous *in vivo* studies that have found strong evidence that helminthic products protect against the development of T1D, it would be worth investigating the potential benefits of treating T1D patients.

Multiple Sclerosis (MS)

MS is a progressive neurodegenerative disease characterized by gradual loss of mobility, vision and coordination. Worldwide, two million people are affected by this debilitating disease that cannot be cured (107, 108). In MS, chronic inflammation of the central nervous system leads to demyelination and neurodegeneration (109). Similar to T1D, MS is mainly controlled by a Th1-dominated immune response, although other T-cell subsets, such as Th17 cells, and other lymphocytes are also involved (107). Similarly to T1D and IBD, macrophages and DCs play a crucial role in driving the inflammatory process in MS. Skewing of macrophages to a M2 profile and inducing tolerogenic DCs have been proposed as therapeutic interventions (110–114). MS has a higher incidence in industrialized countries than in developing countries, and there is an inverse relationship between helminth infections and MS incidence (108). Thus, treatment with helminthic products presents a promising alternative to conventional treatments.

Preclinical Studies

The experimental autoimmune encephalomyelitis (EAE) model is used as the murine equivalent of MS to study the disease. In order to induce EAE, the animals are immunized with a neuroantigen and subsequently develop demyelination and paralysis (108). Similar to MS, EAE is characterized by a strong proinflammatory, Th1-mediated immune response (107). IL-12 appears to be the inducer of the immune disorder in EAE, by activating macrophages and triggering the production of nitric oxide (NO), which is associated with axonal damage and demyelination. High levels of TNFα and TNF-β have also been shown to exacerbate symptoms during relapse in EAE and MS (107). Moreover, an upregulation of Th2-associated cytokines has been associated with recovery from EAE, and adoptive transfer of Th2 cells specific for a neuroantigen has not induced the disease (107).

Infection with live *S. mansoni* cercariae 6 weeks prior to EAE induction significantly decreased the incidence and severity of EAE and delayed its onset (107). Furthermore, the production

of the proinflammatory mediators IFNγ, TNFα, and NO was significantly reduced (107). In the spinal cord, levels of IL-12 were significantly reduced in mice infected with *S. mansoni*, suggesting attenuated Th1 induction (107). Additionally, *S. mansoni* infection reduced T cell, $F4/80^+$ macrophage, and $B220^+$ cell infiltration into the spinal cord (107). Altogether, these results indicate that *S. mansoni* infection protects against the development and progression of EAE, by modulating the immune response, particularly the infiltration of certain inflammatory cell subsets into the CNS (107).

Similarly to cercariae, egg immunization prior to EAE induction resulted in improved clinical scores and delayed onset of the disease (115). Egg immunization 2 days after EAE induction also resulted in delayed onset and decreased clinical scores, while there was no improvement in clinical scores or disease progression if immunization occurs 7–10 days after EAE induction (115). The observed improvements in symptoms and the delay of onset were attributed to reduced CNS infiltration by inflammatory cells and up- and downregulation of IL-4 and IFNγ, respectively (115). IL-10 and IL-5 levels were also elevated in the spleen cells of *S. mansoni* egg immunized mice, which suggested increased Th2 and regulatory responses in these mice (115).

Apart from *S. mansoni*, treatment with helminthic products of S. japonicum (116), *Trichuris suis* and *Trichuris spiralis*, all showed reduction in symptom severity and disease progression related to induction of differential immune activation, including Th2-associated cytokine production and alternative DC activation (14, 116).

In summary, administration of live helminths or helminthic products in EAE animal models appears to reduce the incidence and progression of EAE if administered before the active phase of the disease (61, 107, 115, 116). However, treatment during the active, clinical phase of the disease is not effective. This is likely due to disease progression becoming a self-driving process as a result of extensive inflammation around the lesions, and irreversible tissue damage (108).

Human Studies

To our knowledge, no human studies investigating the role of *S. mansoni* in MS have been conducted. However, the potential benefits of helminth infections have been studied. Correale and Farez conducted an observational cohort study during which they followed 12 MS patients with relapsing-remitting MS and concomitant infection with intestinal parasites for over 4 years to investigate whether natural infection with intestinal parasites reduced the number and intensity of symptom exacerbations and changed the immune reactivity (58). MS patients with a parasitic infection were observed to have significantly less relapses; during the study period of 55 months, 3 relapses were observed in the infected group, in contrast to 56 relapses in the uninfected group (58). Moreover, cytokine levels in infected and uninfected patients were measured to determine the effect of the infection on the inflammatory response. Collection and analysis of peripheral blood mononuclear cells revealed an increased amount of IL-10 and TGF-ß secreting cells, and a decreased amount of IL-12 and IFNγ secreting cells in parasite infected patients. There was no difference in IL-4 levels (58). These results indicate that parasitic infections downregulate the inflammatory response in MS.

In a follow-up study involving the same 12 patients, anti-parasitic treatment was demonstrated to change the immunological profile in helminth-infected MS patients and cause symptom exacerbations (117). Anti-parasitic treatment was required in 4 patients that experienced severe symptoms of the parasite infection. and resulted in decreased egg load and reduced levels of IgE, implying successful resolution of the parasitic infections (117). However, the number of exacerbations and the disability score increased significantly in all treated patients (117). Furthermore, anti-parasitic treatment increased the number of new or enlarging lesions in the brain, compared to untreated, parasite-infected controls who presented with a stable number and size of lesions (117). These observations are likely related to the reversal of the previously observed immunomodulation by the parasite. FoxP3+ cells, IL-10, and TGF-β levels decreased significantly, while levels of IL-12 and IFNγ increased after treatment (117). These findings further support the hypothesis that parasites inhibit the progression of MS by inducing a regulatory state of the immune system. Resolution of the infection leads to symptom exacerbation, which is likely due to the removal of the immunomodulatory activities of the parasites (117).

It must be noted that the patients were infected with different parasites, not one specific type of parasite. Even though almost all parasitic infections modulate the immune system and skew the immune response toward a Th2/Treg profile, slight differences in the mechanisms exist (58). Nevertheless, these studies clearly show benefits of parasitic infections on disease progression (58, 117) and support the hypothesis that parasitic infections are in part responsible for the lower incidence of MS in endemic areas (117). Moreover, since the infections with parasites occurred after onset of MS, these studies show that parasitic infections may also be of therapeutic use in patients with ongoing disease, in addition to a prophylactic potential. Unfortunately, if the exacerbation of parasite-related symptoms calls for anti-parasitic treatments, the MS symptoms worsen (117). It is therefore necessary to identify the mechanisms and the antigens involved in the observed immunoregulation, so that treatments that do not require live parasites can be developed.

In contrast to the previous studies, which do not focus on a single parasitic species, Fleming et al. conducted a phase I study in 2011 to determine the safety and potential benefits of administration of *T. suis* eggs to remitting-relapsing MS patients (118). Contrary to a similar study performed in Denmark, which found no clinical efficacy of *T. suis* eggs in MS patients (119), Fleming et al. observed a slight increase in Treg cells, IL-10 and IL-4 in *T. suis* treated patients, and a reduction of the lesions visible in the MRI scans indicating that administration of *T. suis* could have potential beneficial effects for patients with MS (118). Furthermore, oral administration of *T. suis* eggs appeared to be safe, as it resulted in only minor gastrointestinal troubles that spontaneously resolved after a few days (118). This phase I trial was conducted with only a very limited number of patients ($n = 5$), making it difficult to draw any definite conclusions. However, in a recent phase II study following up

on the study by Fleming et al. administration of *T. suis* eggs resulted in a decrease of brain lesions, which was accompanied by a decrease in active CD4+ and CD8+ cells, and an increase in the levels of Treg cells and IL-4 expressing cells, implying a shift of the immune response from Th1 to Th2 mediated activity (120). The results of this study are very promising, and will hopefully provide the steppingstone for a phase III trial and the potential to use helminths as therapeutics in the near future.

DISCUSSION

The increasing incidence of chronic inflammatory disorders such as IBD, T1D, and MS in the industrialized countries is a concerning development, and treatment options are still limited. Helminth therapy has a lot of potential, as it could permanently reprogram the immune system without affecting the response to common infections. Intriguingly, despite its high global prevalence, extensively studied immunomodulatory properties, strong *in vitro* and *in vivo* evidence indicating beneficial effects in a range of autoimmune disorders, *S. mansoni* has not extensively been studied in corresponding clinical trials. To our knowledge, the study reporting on P28GST, derived from the closely related *S. haematobium* describes the only clinical trial involving treatment of an autoimmune disorder with schistosoma (59).

The feasibility of using *S. mansoni* to treat autoimmune disorders is further supported by the observations from clinical trials using other helminth species. These tend to show promising effect, and have generally been found to be safe, although some studies failed to meet primary endpoints or have been terminated for unknown reasons (121). It is important to realize, however, that the immune modulatory properties of helminths vary with species. For example, while *T. trichuria* has been shown to promote the development of inflammatory bowel disease by corrupting the gut epithelial barrier and promoting Th1 responses, *S. mansoni* has a protective effect on inflammation in the gut through promoting the production of IL-10 (2). Obviously, helminth treatment will not be a "one size fits all" therapy. The characteristics of each helminth species need to be carefully studied to provide safe and effective treatments adjusted to the pathophysiology of the disease it would be intended to treat. Only a handful of parasites have been studied in detail, and much of the intricate processes fine tuning the immune response to avoid detection by the host remain unknown. However, although the helminths used in the clinical studies discussed in this review all employ different immunomodulatory strategies, reprogramming of macrophages and DCs is a common feature which supports our hypothesis that *S. mansoni* is a promising candidate for immunotherapy in autoimmune disorders.

Helminth therapies will obviously also have limitations. Despite the promising results, some doubts have been raised concerning the safety and effectiveness of helminth therapy. In this context, the unknown nature of most of the soluble helminthic proteins is a potential drawback. For example, SEA includes a large variety of different proteins, and it is not yet known which (long-term) effects these might have (40). Furthermore, certain helminths have been shown to promote the development of inflammatory disorders (2). Additionally, it has been argued that the decreased incidence of chronic inflammatory disorders such as allergies and autoimmune disease in developing countries is due to under diagnosis, instead of the higher prevalence of helminthic infections (2). However, results from a large number of epidemiological studies investigating the incidence of such disorders in parasite endemic regions, clearly indicate that helminth infections protect against the development of autoimmune, chronic inflammatory disorders. Furthermore, *in vitro* and *in vivo* studies, such as the ones presented in the current review, provide clear evidence that helminthic products modulate the immune system and can induce a state of tolerance, desirable in chronic autoimmune inflammatory disorders.

Another limiting factor in the interpretation of the observed beneficial effects arises from the fact that animal models are not always a reliable representation of the human situation, exemplified by the different responses by mice and rats to *S. mansoni* infections (75, 77). Additionally, even though EAE models have assisted in the development of several effective, now marketed treatments, others that have been effective in animal models have failed in the human situation (108). In particular, a hurdle to the approval of helminth therapy in MS could be the fact that in animal models, EAE could only be prevented, and not treated or cured after onset of the disease (61, 115, 116). This implies that the use of these parasitic products in patients with established disease would not be beneficial. However, a small number of clinical trials in MS patients with established disease did yield positive results, indicating that helminthic infections can protect against disease progression and symptom exacerbation after disease onset (108, 119, 120). Furthermore, the preventive properties could be of interest when people with a genetic predisposition for developing chronic inflammatory disorders are considered.

Despite these limitations, extensive *in vivo* studies using murine models of IBD, T1D, and MS and have found largely beneficial effects of helminthic species on disease incidence and progression, and several clinical trials have given a first glance at the benefits of parasitic treatment for MS and IBD-patients (58, 82, 87, 117, 118, 120). Compared to administering live worms causing chronic infections, protein therapy would be transient and avoid the risk of developing chronic schistosomiasis. Furthermore, in areas with poor sanitation, live helminth administration may lead to transmission of the parasite. Using biologically active and well-defined proteins would abrogate these risks, and circumvents the leniency to undergo helminth treatment as the idea of live worm or egg administration may appear repulsive to patients.

CONCLUSION

The idea of using our "Old Friends" as a treatment, or even prophylaxis, opens up a whole new array of potential

therapeutics and has the potential to revolutionize the way we treat chronic inflammatory disorders. Based on the studies described in this review, we strongly suggest further study of helminths, especially *S. mansoni*, as an immunomodulatory agent in autoimmune diseases such as T1D, IBD, and MS. Administration of *S. mansoni* proteins in clinical studies could result in the development of new therapeutics without the potential risks of parasite-induced adverse events.

AUTHOR CONTRIBUTIONS

LC wrote the manuscript and prepared the figures. AH and JG revised the manuscript. All authors read and approved the final manuscript.

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MINI REVIEW
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Fasciola hepatica-Derived Molecules as Regulators of the Host Immune Response

*Sinéad Ryan[1], Jenna Shiels[1], Clifford C. Taggart[1], John P. Dalton[2] and Sinéad Weldon[1]**

[1] Airway Innate Immunity Research (AiiR) Group, Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, United Kingdom, [2] Centre of One Health (COH), Ryan Institute, School of Natural Sciences, National University of Ireland Galway, Galway, United Kingdom

Helminths (worms) are one of the most successful organisms in nature given their ability to infect millions of humans and animals worldwide. Their success can be attributed to their ability to modulate the host immune response for their own benefit by releasing excretory-secretory (ES) products. Accordingly, ES products have been lauded as a potential source of immunomodulators/biotherapeutics for an array of inflammatory diseases. However, there is a significant lack of knowledge regarding the specific interactions between these products and cells of the immune response. Many different compounds have been identified within the helminth "secretome," including antioxidants, proteases, mucin-like peptides, as well as helminth defense molecules (HDMs), each with unique influences on the host inflammatory response. HDMs are a conserved group of proteins initially discovered in the secretome of the liver fluke, *Fasciola hepatica*. HDMs interact with cell membranes without cytotoxic effects and do not exert antimicrobial activity, suggesting that these peptides evolved specifically for immunomodulatory purposes. A peptide generated from the HDM sequence, termed FhHDM-1, has shown extensive anti-inflammatory abilities in clinically relevant models of diseases such as diabetes, multiple sclerosis, asthma, and acute lung injury, offering hope for the development of a new class of therapeutics. In this review, the current knowledge of host immunomodulation by a range of *F. hepatica* ES products, particularly FhHDM-1, will be discussed. Immune regulators, including HDMs, have been identified from other helminths and will also be outlined to broaden our understanding of the variety of effects these potent molecules exert on immune cells.

Keywords: parasite, helminth, helminth defense molecule, *Fasciola*, FhHDM-1, immunomodulation

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Universidad de la República, Uruguay
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***Correspondence:**
Sinéad Weldon
s.weldon@qub.ac.uk

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INTRODUCTION

Helminths are parasitic worms classified as flukes, tapeworms or roundworms according to their appearance and the organ in which they reside during infection (1). Diseases caused by helminths constitute the majority of Neglected Tropical Diseases (NTDs) as classified by the World Health Organization (WHO). Helminths are one of the most successful infectious agents in nature as infection is highly prevalent and, as a result, over one billion people are affected worldwide (2, 3). One of the most prevalent zoonotic helminth diseases is fascioliasis caused by *Fasciola hepatica* and

the larger *Fasciola gigantica*. This is a major foodborne disease that is currently thought to impact approximately two million people in over 70 countries, with developing countries more severely affected (4, 5).

The clinical manifestations of helminth infections are diverse; some infections elicit acute symptoms aligned with pathology caused by worm migration through host tissues, while others may be asymptomatic (6, 7). Co-evolution of humans and helminths may have shaped the human immune system as helminths developed sophisticated mechanisms to induce tolerance and evade expulsion by the host enabling them to become successful chronic pathogens (7–9). A range of genomic, transcriptomic, immunomic, glycomic, and proteomic approaches alongside database mining has provided further perspective on host-parasite interactions and led to the identification of various helminth molecules including those within excretory-secretory (ES) products that influence the host inflammatory response (10–15). These molecules have garnered much attention with the ultimate aim of exploiting their immunoregulatory mechanisms for the treatment of human diseases (16, 17). A number of molecules from *F. hepatica* and other worms are currently under investigation for immunotherapeutic potential and are the main focus of this review.

FASCIOLA HEPATICA

F. hepatica infection of humans and livestock occurs primarily through the consumption of encysted metacercariae. After ingestion, the metacercariae excyst and become newly excysted juveniles (NEJs) within the duodenum. What follows is the highly pathogenic and infectious migratory stage of *F. hepatica* infection where NEJs cross the intestinal wall to the liver via the peritoneum (18, 19). This phase is characterized by inflammation and damage until the NEJs reach the liver bile ducts where they mature into egg-producing adults. Different T cell responses and cytokine profiles observed in cells from the mesenteric (more IL-5) and hepatic lymph (more IL-4) nodes of mice infected with *F. hepatica* suggest that NEJ and hepatic-stage parasites produce different antigens that alter host responses (20). Despite initial inflammation, up to 50% of infected humans are asymptomatic (21). This is an extraordinary feat for any infectious agent as it indicates the ability to subvert the host immune response which is typically armed to expulse a pathogen. An increased abundance of IgG4 antibodies reactive to antigens (e.g., cathepsin L1) suggests a Th2-driven response is mounted (22); however, much of our knowledge of the immunology of fascioliasis is derived from ruminant animal infection and experimental models using rodents.

Immunology of Fascioliasis

Helminth infestations often exist as chronic infections as a consequence of a Th2/regulatory response in the host that can support the survival and integrity of host tissue and the parasite (23, 24). The immune response mounted during the early stages of fascioliasis is generally regarded as a mixed Th1/Th2 response where cytokines such as IFNγ, IL-4, IL-10, and TGF-β are elevated. As the infection progresses, a Th2 response is amplified in conjunction with suppression of Th1 inflammation, thus allowing a prolonged infection that may be dependent on IL-4 (20). In the early stages of bovine *F. hepatica* infection, both IFNγ and IL-10 are increased, corroborating the idea that the initial immune response is mixed (25). Stimulation of peripheral blood mononuclear cells from cattle and sheep with *F. hepatica* ES products showed similar profiles (26, 27). In addition, TGF-β and IL-10 may modulate IL-4 and IFNγ in acute and chronic infection, respectively (28).

A cellular source of IL-10 was revealed in murine *F. hepatica* infection where, among increased macrophages and dendritic cells (DCs) in the peritoneal cavity, there was a significant population of $CD25^{+}Foxp3^{+}$ Treg and inducible Treg cells with the propensity to secrete IL-10 (29). Infection of IL-$10^{-/-}$ mice showed that IL-4 and IFNγ responses were hindered by IL-10 (29). As IL-4 is a critical cytokine observed throughout the pathogenesis of *F. hepatica* infection, the appearance of an abundant population of alternatively activated macrophage (AAM) as early as 7 days after infection of mice in unsurprising (30). AAMs remain in the peritoneum for up to 3 weeks after oral infection with *F. hepatica* metacercariae, highlighting their key role in helminth disease (29). Eosinophilia in the peritoneum is evident in murine liver fluke infection (29) and bovine *F. hepatica* disease (31), and eosinophils contribute to tissue pathology, particularly in the liver (32). However, in sheep, eosinophils undergo apoptosis suggesting a mechanism by which *F. hepatica* evades the host response (33).

FASCIOLA HEPATICA EXCRETORY-SECRETORY PRODUCTS

As parasites release ES products during host infiltration, it was deduced that they function as effector molecules capable of modulating the host immune system, enabling parasite survival. Various immunomodulatory molecules have been identified in the ES products of *F. hepatica* (**Table 1**) (34). Many of these molecules are advantageous to the helminth and, through manipulation of host immune processes, they facilitate prolonged parasitic infection. Anti-inflammatory effects (**Figure 1**) have been reported in rodent models of infection and inflammatory disease suggesting the potential for ES product development as therapeutics. However, many of the products discussed below are unique to certain life stages of the liver fluke leaving it difficult to define mechanisms without analysis of their purified or recombinantly produced forms.

Antioxidants

The antioxidant enzymes thioredoxin peroxidase/peroxiredoxin (TPx/Prx) in *F. hepatica* ES products detoxify reactive metabolites produced by the host (35, 36). *F. hepatica* ES products induced AAMs and TPx did so without traditional Th2 signaling, i.e., IL-4 or IL-13 (30, 37). Administration of purified TPx to BALB/c mice induced a Th2 response as well as expression of Ym-1, TGF-β, and IL-10, and release of

TABLE 1 | *F. hepatica*-derived immunomodulatory molecules.

Molecule	Abbreviation	Actions	References
Fatty acid binding protein	FaBP, Fh12, Fh15	Reduction of pro-inflammatory cytokines in LPS-induced models of sepsis	(44–46)
Helminth defense molecule	FhHDM-1	Inhibits lysosomal acidification and prevents macrophage antigen presentation Inhibits formation of the NLRP3 inflammasome and thus release of IL-1β Reduces inflammation in models of multiple sclerosis, type 1 diabetes, and allergic asthma	(53, 67, 69, 96)
Mucin	Fhmuc	Increases $CD11b^{+}MHCII^{+}$ macrophage during LPS stimulation and TLR4 expression is increased in DCs alluding to an increased Th1-type inflammatory response	(58, 59)
TGF-like molecule	FhTLM	Inhibits SMAD2/3 signaling and induces a regulatory phenotype in bovine macrophages	(64)
Kunitz-type molecule	FhKTM	Decreased inflammatory cytokine secretions in DCs	(56)
Glutathione S-transferases	FhGSTs	Suppress NF-κB pathway stimulation in macrophages and mice with endotoxemic shock have improved survival in the presence of GST treatment	(41, 42)
Thioredoxin Peroxidase/Peroxiredoxin	TPx/Prx	Induces Ym-1 expression and arginase activity in murine macrophages Antagonizes actions of ROS and induces AAM phenotype	(30, 35, 37)

prostaglandin E_2 (PGE_2) from murine macrophages (30, 37). Glutathione S-transferases (GSTs) constitute up to 4% of the total protein in *F. hepatica* ES products and protect the helminth from free radicals that arise from the host response mounted to expulse the worm (38–40). In DCs, recombinant Sigma-class GST (rFhGST-si) interacts with TLR4 to stimulate IL-6 and MIP-2 production and CD40 expression via mitogen-activated protein kinase (MAPK) and NF-κB activity (41). Crucially, rFhGST-si inhibited development of Th17 cells without any interaction with the Th2-type response (41). Although recombinant Mu-class GST isoforms (rFhGST-mu) had no effect on DC activation (41), anti-inflammatory properties of native FhGST-mu (nFhGST-mu) were recently identified in monocytic cells stimulated with a range of TLR agonists and bacteria such as *Klebsiella pneumonia,* and treatment protected mice from endotoxemia (41, 42). In addition, nFhGST-mu suppressed the NF-κB pathway possibly via JAK/STAT signaling proteins and thus it was proposed that nFhGST may be a key antigen utilized by *F. hepatica* to suppress Th1 responses (42).

Fatty Acid Binding Proteins

F. hepatica fatty acid binding proteins (FaBPs) are a group of chaperones that mediate lipid responses within the cell and are closely linked with inflammation and metabolism (43). Four FaBPs identified in *F. hepatica* are known antioxidants with a nutritive role for the parasite (44). Investigations into their anti-inflammatory properties demonstrated that the 12 kDa Fh12 product reduced pro-inflammatory cytokine production in the LPS-induced model of murine sepsis (45). Similarly, the 14.5 kDa Fh15 molecule attenuated production of IL-1β and TNF-α in human THP-1 macrophages (46). In a murine model of sepsis, Fh15 treatment was associated with a significant decrease in circulating cytokines (46).

Cysteine Proteases

Cysteine proteases constitute approximately 80% of the ES products from *F. hepatica* and they play major roles throughout infection (47). Five clades of *F. hepatica* cathepsin L (FhCL) have been identified; three associated with mature adult worms (FhCL1, FhCL2, and FhCL5) and two specific to infective juvenile stage (FhCL3 and FhCL4). Increased secretion of FhCL3 during the initial stages of infection aid the immature NEJ by preventing attachment of host eosinophils (48). Conversely, once the fluke has reached the liver, FhCL1/2 secretions elicit anti-coagulant effects that allow blood feeding for the parasite (49). FhCL1 dampened the Th1 response elicited by administration of the *Bordetella pertussis* vaccine in mice (50). The decrease in the IFNγ response concurs with previous evidence that concurrent *F. hepatica* and *B. pertussis* infection had a decreased Th1-centric response (51). Interestingly, the effects of FhCL1 translated into decreased inflammatory mediators and protective effects in LPS-induced septic shock (52). Although recombinant FhCL1 partially activated DCs via TLR4, these DCs suppressed the development of Th17 cells and did not induce the differentiation of Th2 cells (41). Hypo-responsiveness in peritoneal macrophages stimulated with LPS and FhCL1 indicated that MyD88-independent/TRIF-dependent signaling through cleavage of TLR3 in the endosome was inhibited (52). However, in murine models of type 1 diabetes (T1D) and multiple sclerosis, FhCL1 treatment showed no benefit (53).

Protease Inhibitors

Kunitz serine protease inhibitors have been identified in the total extract and tegument of *F. hepatica* (54). Interestingly, *F. hepatica* Kunitz type molecule (FhKTM) has an unique specificity for cysteine proteases (13) and was shown to associate with cathepsin L (55). FhKTM induced a regulatory IL-27-dependent phenotype in LPS-stimulated DCs that impaired Th1 and Th17 responses (56).

Mucin-Like Peptides

Analysis of the NEJ stage of *F. hepatica* infection led to the discovery of proteins with similarities to mucins (57, 58). A synthetic mucin-derived peptide (Fhmuc) increased peritoneal $CD11b^{+}MHCII^{+}$ cells in mice exposed to LPS (59). In contrast to other *F. hepatica* ES products discussed here, but similar

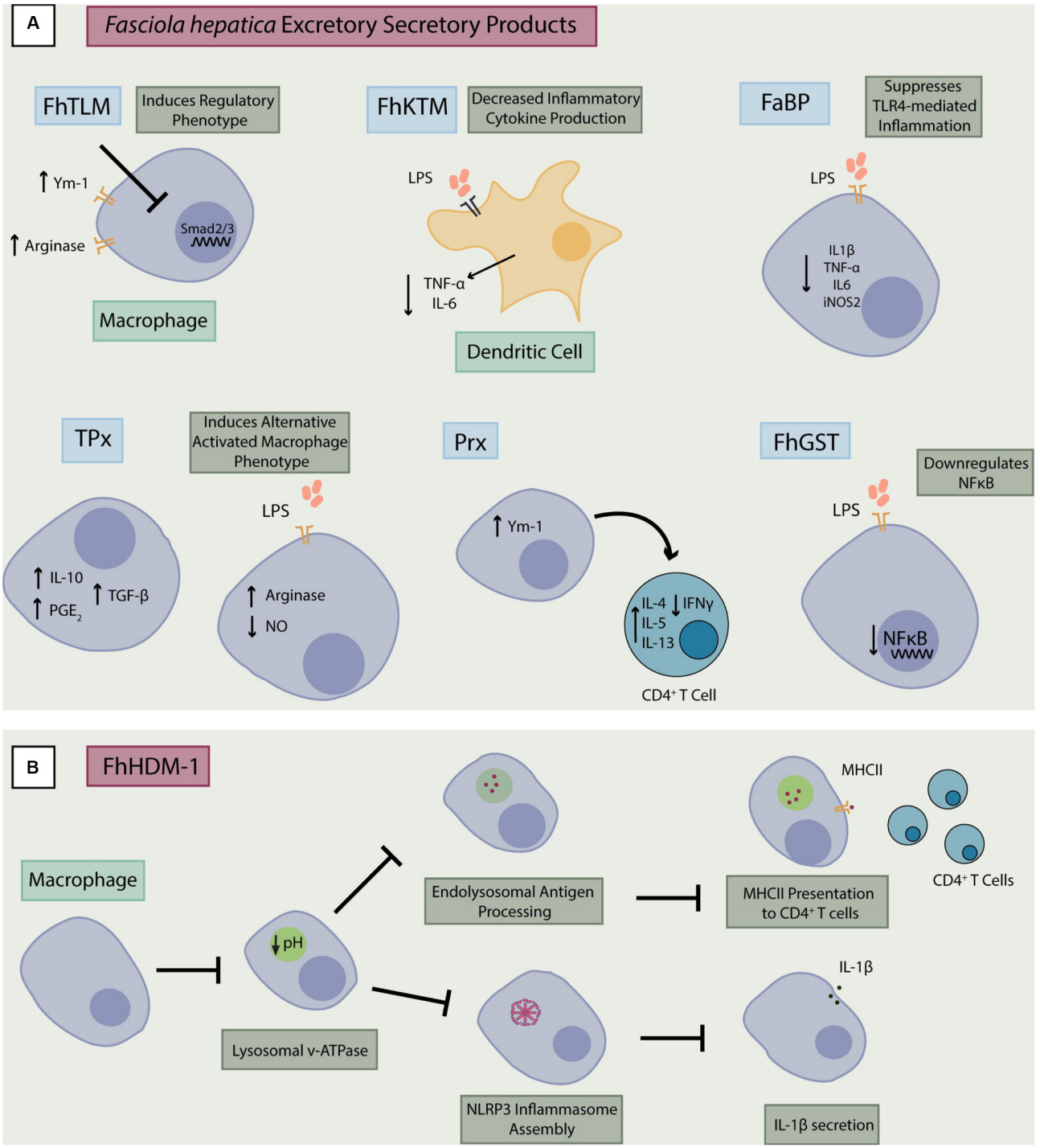

FIGURE 1 | Immunomodulation by *F. hepatica* excretory-secretory products. **(A)** Excretory-secretory products from *F. hepatica* can modulate inflammatory responses in macrophages and dendritic cells in several ways. They may inhibit activity of NF-κB and the subsequent release of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β). They may also induce a regulatory phenotype through increased expression of factors such as Ym-1 or Smad2/3 which results in production of regulatory factors (IL-10 and TGF-β), augmentation of Th2 responses and suppression of Th1 inflammation. **(B)** FhHDM-1 interacts with macrophages and prevents lysosomal acidification which is necessary for antigen processing and major histocompatibility complex II (MHCII) presentation to T cells (68, 69). This action on the lysosome by FhHDM-1 also prevents assembly of the NLRP3 inflammasome, inhibiting release of IL-1β from the macrophage. FhTLM, *F. hepatica* TGF-like molecule; FhKTM, *F. hepatica* Kunitz-type molecule; FaBP, fatty acid binding protein; TPx, Thioredoxin peroxidase; Px, Peroxiredoxin, FhGST, *F. hepatica* Glutathione S-transferase; FhHDM-1, *F. hepatica* helminth defense molecule-1.

to FhCL1 and FhGST-si (41), Fhmuc elicits pro-inflammatory properties, with increased LPS-induced TLR4 expression in DCs and polarization of the T cell response (59). This ability of *F. hepatica* to modulate the host immune response may have potential implications for vaccination strategies (59). As *F. hepatica* ES products contain significant levels of glycans, it is probable that native *F. hepatica* mucin-like peptides undergo glycosylation, which is not represented with the synthetic peptide. Thus, the immunomodulatory effects of native Fhmuc might be different to those described for the synthetic peptide. Indeed, Rodríguez et al. have shown that glycans from *F. hepatica* modulate DC function to induce a Th2 response and suppress Th1 inflammation (60–62).

TGF-β Mimics

Three distinct TGF-β homologs were identified in *F. hepatica* through bioinformatic approaches (63). *F. hepatica* activin/TGF-like molecule (FhTLM) is highly conserved with other TGF-β homologs from nematode parasites and has a limited temporal expression pattern across parasite development (63). Recombinant FhTLM supported NEJ viability and development (63). FhTLM may be less potent than mammalian TGF-β, however, SMAD-2/3 signaling characteristic of the regulatory phenotype was observed in bovine macrophages as well as alternative activation (64).

Helminth Defense Molecules (HDMs)

During helminth infiltration, increased bacterial infection is common as a consequence of the characteristic tissue damage, although the host inflammatory response remains stable. In schistosomiasis, enteric bacteria are displaced during destruction of the gut; however, symptoms of infection or sepsis are not apparent suggesting the host may be immunosuppressed by the parasite (65). Thus, it was hypothesized that parasites secrete antimicrobial peptides (AMPs) similar to those released by the host as a protective mechanism during infection. Investigation of *F. hepatica* ES products discovered an 8 kDa protein constitutively expressed at all stages of the life-cycle and throughout infection (14). BLAST analyses indicated the sequence was conserved throughout trematode helminth species including *Paragonimus westermani*, *Schistosoma mansoni,* and *Schistosoma japonicum,* and thus was named the helminth defense molecule (HDM). HDMs are classified into three clades: Schistosome HDMs, Fasciola/Asian fluke HDMs and Sm16-like molecules. All clade members have a predicted N-terminal peptide and α-helical structure as well as a highly conserved, largely hydrophobic C-terminal sequence of approximately 35 residues (14).

F. HEPATICA HELMINTH DEFENSE MOLECULE-1 (FhHDM-1)

The first discovered HDM was from *F. hepatica* (FhHDM-1, **Figure 1**) (14). FhHDM-1 is predicted to have a predominantly α-helical secondary structure with a C-terminal amphipathic helix bearing structural and biochemical resemblance to mammalian cathelicidins. Moreover, the 34 residue C-terminal sequence from FhHDM-1 has striking similarity to the human cathelicidin, LL-37 (14, 66, 67). Like LL-37, FhHDM-1 and its conserved C-terminal fragment neutralize LPS preventing TLR4 activation on target cells such as macrophages (14, 68). However, in a murine model of intratracheal LPS-induced acute lung injury, intraperitoneal administration of FhHDM-1 decreased neutrophilic lung inflammation, suggesting mechanisms beyond LPS neutralization may be involved (69). Perhaps unexpectedly, FhHDM and other HDMs did not elicit any antimicrobial activity against different bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (70). However, in contrast to the mammalian AMPs, they did not induce pore formation in macrophages or the release of lactate dehydrogenase indicating that HDMs do not elicit cytotoxic effects.

The immunomodulatory role of FhHDM-1 was investigated in models of inflammatory disease where ES products showed promise as anti-inflammatories. *F. hepatica* ES products reduced inflammation in the non-obese diabetic (NOD) T1D mouse model that correlated with an increase in M2 macrophages and $Foxp3^+$ Tregs (71). Administration of a synthetic FhHDM-1, but not FhCL1, in NOD mice had a comparable effect with a decreased disease burden characterized by improved survivor function and fewer mice developing diabetes (53). While destruction of pancreatic β cells is mediated mainly by autoreactive T cells (72), inhibition of macrophage activity by FhHDM-1 may elicit positive effects on clinical measurements (53). *F. hepatica* total extract, ES products and FhHDM-1 have proven beneficial in other autoimmune diseases, for example in the murine experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (53, 73, 74). A predominant theory behind the activity of helminth ES products is their ability to induce a regulatory Th2 response that is often characterized by a M2 (AAM) phenotype in macrophages. While FhHDM-1 reduced TNF-α and IL-6 secretion in LPS-stimulated macrophages (53), there were no significant alterations in surface receptor expression or release of Th1 suppressing cytokines, such as TGF-β and IL-10. Furthermore, the auto-antigen specific T cell response remained unchanged in EAE mice that received FhHDM-1 despite showing reduced disease severity (53). This implies that mechanisms other than an induced Th2 response may be responsible for decreased Th1/Th17-mediated pro-inflammatory activity.

FhHDM-1 associates with lipid rafts in the macrophage plasma membrane and is endocytosed (67). Within the macrophage, FhHDM-1 is cleaved by lysosomal cathepsin L to release a C-terminal peptide that can form an amphipathic helix, and this peptide prevented acidification of the lysosomes through inhibition of vacuolar ATPase (vATPase) activity (67). Macrophage process antigens and present them to MHC-II on $CD4^+$ T cells; therefore, impairment of this process via vATPase inhibition would prevent initiation of the adaptive immune response (67). To further define FhHDM-1 mechanisms, Donnelly and colleagues hypothesized that inhibition of lysosomal activity in the macrophage (75)

would impact on inflammasome activity and release of pro-inflammatory IL-1β (76). Indeed, it was observed that FhHDM-1 reduced IL-1β release in macrophages stimulated with the NLRP3 activator NanoSiO$_2$ and alum. The cysteine protease cathepsin B is a pH-dependent lysosomal protease involved in activation of the NLRP3 inflammasome (76). As FhHDM-1 inhibits lysosomal acidification, it would inhibit cathepsin B activity and the NLRP3 inflammasome. Replicates of these experiments carried out using the 34 residue C-terminal sequence of FhHDM-1 indicate that these effects on the inflammasome are unique to FhHDM-1 (76). Gene expression analysis of macrophages stimulated with LPS predicted that signaling associated with high-mobility group box-1 (HMGB-1) and IL-17 were attenuated by FhHDM-1 (69). This posed the question of whether FhHDM-1 nay be effective in preventing allergic inflammation, which was subsequently tested in a rodent model of house dust mite-induced asthma (69). In this model, FhHDM-1 treatment reduced neutrophil and eosinophil cell counts, inflammatory markers and airway mucus content (69).

WHAT HAVE WE LEARNED FROM OTHER HELMINTH EXCRETORY-SECRETORY PRODUCTS?

Immunomodulatory functions and modes of action have been outlined for ES products from several parasites. The filarial worm *Acanthocheilonema viteae* releases a glycoprotein called ES-62 that can interfere with DC TLR4 expression by inducing autophagosomal degradation (77). By inhibiting mast cell responses, ES-62 and its small molecule analogs prevented the excessive inflammatory response in murine models of asthma (78, 79). However, ES-62 also controls the Th1 response by suppressing NF-κB-mediated inflammation in DCs (80). More recently, ES-62 was found to inhibit IL-33/ST2/MyD88 signaling and modulate the pro-inflammatory responses resulting from crosstalk between ST2, FcεRI, and TLR4, which may contribute to reported protective effects of ES-62 in chronic models of asthma (81).

Mice with a gastrointestinal infiltration of *Heligmosomoides polygyrus* have characteristic increases in the number of Treg ($CD4^+CD25^+$) cells. ES products from this parasite potentiate the expression of Foxp3 in $CD4^+$ T cells *in vitro* through mimicry of TGF-β (82). These findings led to the discovery of *H. polygyrus* TGF-β mimic (Hp-TGM) that operates through traditional TGF-β signaling pathways leading to polarization of $CD4^+$ T cells with potent suppressive abilities (83). The recently identified *H. polygyrus* alarmin release inhibitor (HpARI) binds to active IL-33 preventing its interaction with ST2 both in murine and human models, and could provide novel therapeutic options in Th2 dominated disease, such as asthma (84).

S. mansoni ES products have pleiotropic effects on the immune response. Factors released from the parasite and its eggs can modulate both Th1 and Th2 responses. For example, IPSE (IL-4-producing principle from schistosome eggs) expanded the population of regulatory B cells, which in turn activated Tregs via IL-10 (85) and *Schistosoma haematobium* IPSE showed therapeutic efficacy in a murine model of hemorrhage in the bladder (86). *S. mansoni* chemokine binding protein (SmCKBP) is secreted from live eggs and can bind and neutralize the neutrophil chemoattractant CXCL-8 (87). In an experimental granulomatous inflammation model, blocking of live egg smCKBP increased recruitment of neutrophils, macrophage and eosinophils and the size of the egg granuloma, suggesting this ES product may limit leukocyte recruitment to protect the egg (87).

CONCLUDING REMARKS

The success of *F. hepatica* infection stems from the worm's ability to modify and manipulate the host immune response. While many years of research have uncovered effective mechanisms by which the parasite can establish a long-term infection, there is much more to be revealed. Investigations in various models of inflammatory disease indicated potential therapeutic benefit of helminths and ES products, however, to-date the majority of human clinical trials have not replicated these findings (11, 88–91). While small animal models of inflammatory disease provide valuable insights, they often fail to recapitulate the various complex processes at play in human conditions (91). There are a number of factors that need to be considered such as differences in metabolism and the impact of microbiota, particularly on the immune response (92), that may affect efficacy of helminths and their products. Nonetheless, clinical trial outcomes have highlighted the need for a greater understanding of the complexity of changes to the immune response induced by helminths during infection.

A typical helminth genome contains around 50,000 genes, which is much greater than the human genome (approximately 20,000), and it has been proposed that each parasite has undergone specific adaptations for their particular niche (93). Furthermore, as helminths have a multistage life cycle with distinct developmental stages through select tissues and organ systems, they may release distinct molecules within a particular niche (e.g., the intestine or lung) or migratory stages that exert more localized immunomodulatory effects (94), which may be of relevance for targeting tissue-specific inflammation. As recently reviewed by Cortés et al. (95) and van der Zande et al. (9), a number of studies have identified potential roles for helminth-host microbiome interaction in the pathophysiology of helminth disease and in parasite-mediated suppression of host inflammation, which may be relevant for the targeting of gut and lung inflammation and (immuno)metabolic dysfunction. In addition to the protein molecules outlined herein, there are a number of other families of helminth immunomodulators, which include various carbohydrate, nucleotide and lipid mediators as well as extracellular vesicles that require further investigation (16). Better understanding of the individual components of helminth ES products and in-depth characterization of their functional roles using defined products may help shed further

light on their potential efficacy as therapeutic or prophylactic agents for human disease. The discovery and characterization of HDMs from *F. hepatica* and other trematodes may provide one such avenue for novel therapeutics for autoimmune and inflammatory conditions. While further work is needed to better define these molecules, their host targets and their functional effects, there is an expectation that this work will spark the development of novel biotherapeutics for an array of inflammatory diseases.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Impact of Helminth Infection on Metabolic and Immune Homeostasis in Non-diabetic Obesity

Anuradha Rajamanickam[1], Saravanan Munisankar[1], Kannan Thiruvengadam[2], Pradeep A. Menon[2], Chandrakumar Dolla[2], Thomas B. Nutman[3] and Subash Babu[1,3,4]*

[1] National Institute of Health-National Institute for Research in Tuberculosis (NIRT)-International Center for Excellence in Research, Chennai, India, [2] Department of Epidemiology, National Institute for Research in Tuberculosis, Chennai, India, [3] Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, United States, [4] Frederick National Laboratory for Cancer Research Sponsored by the National Cancer Institute, Frederick, MD, United States

Several epidemiological and immunological studies indicate a reciprocal association between obesity/metabolic syndrome and helminth infections. Numerous studies demonstrated that obesity is concomitant with chronic low-grade inflammation, which is marked by vital changes in cellular composition and function of adipose tissue. However, the effect of helminth infection on the homeostatic milieu in obesity is not well-understood. To determine the relationship between *Strongyloides stercoralis (Ss)* infection and obesity, we examined an array of parameters linked with obesity both before and at 6 months following anthelmintic treatment. To this end, we measured serum levels of pancreatic hormones, incretins, adipokines and Type-1, Type-2, Type-17, and other proinflammatory cytokines in those with non-diabetic obesity with (INF) or without *Ss* infection (UN). In INF individuals, we evaluated the levels of these parameters at 6 months following anthelmintic treatment. INF individuals revealed significantly lower levels of insulin, glucagon, C-peptide, and GLP-1 and significantly elevated levels of GIP compared to UN individuals. INF individuals also showed significantly lower levels of Type-1, Type-17 and other pro-inflammatory cytokines and significantly increased levels of Type-2 and regulatory cytokines in comparison to UN individuals. Most of these changes were significantly reversed following anthelmintic treatment. *Ss* infection is associated with a significant alteration of pancreatic hormones, incretins, adipokines, and cytokines in obese individuals and its partial reversal following anthelmintic treatment. Our data offer a possible biological mechanism for the protective effect of *Ss* infection on obesity.

Keywords: strongyloides stercoralis infection, obesity, adipocytokines, pancreatic hormones, cytokines

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****Correspondence:***
Anuradha Rajamanickam
anuradha@nirt.res.in

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INTRODUCTION

Obesity and metabolic disorders are major public health problems because of their high prevalence worldwide. In 2016, ∼1.9 billion adults were overweight and of these 650 million were obese (1). In India, more than 135 million people are suffering from obesity (2). Obesity is described as unequal body weight for height with an extra deposit of adipose tissue along with low-grade chronic and systemic inflammation (3). Obesity induced inflammation can lead to the development of type

2 diabetes, cardiovascular disease, liver disease, certain type of cancers and other pathological conditions (4, 5).

Obesity is linked with an abnormal expansion in adipose tissue mass and adiposity, as well as poorly regulated levels of adipokines and dysregulation of Type-1 and Type-2 cytokines (6). Adipose tissue aids in energy storage and secretes adipokines—adiponectin, leptin, tumor necrosis factor-α (TNF-α), resistin, and plasminogen-activator type 1 (PAI-1) (7). Obese adipose tissue primarily releases proinflammatory cytokines such as TNF-α, IL-6, leptin, visfatin, resistin, and plasminogen activator inhibitor-1. Obesity activates an immune response which incorporates a systemic elevation of inflammatory cytokines, the recruitment of immune cells to inflamed tissues, activation of leukocytes, and the generation of repair tissue responses (8).

Helminth infections affect approximately one-quarter of the world's population and are widespread in lower to middle-income countries (9). The occurrence of obesity is commonly predominant in urbanized countries where most helminth infections have been eliminated (10, 11). Recent data in both animal and human studies showed a reciprocal association between helminth infection and metabolic disorders, type-2 diabetes, insulin resistance and obesity (12–16), suggesting that helminths may have role in the prevention or delay of these diseases. In previous studies, we have shown that glycemic, hormonal, and cytokine factors in T2DM individuals are modulated by *Strongyloides stercoralis* infection and these changes are partially reversed following anthelmintic therapy (17). Additionally, we have also shown that T2DM with *Ss* infected individuals exhibited significantly lower systemic levels of cytokines and chemokines and dampens the pro-inflammatory milieu, an effect which is then reversed upon anthelmintic treatment (18). However, the mechanisms of how helminth infections mediate protection against non-diabetic obesity are unknown.

Therefore, in the current study, we wanted to examine the association among *Ss* infection and non-diabetic obesity and assessed the influence of *Ss* infection on factors essential in adipose tissue homeostasis. To this end, we estimated systemic levels of pancreatic hormones (insulin, glucagon and C-peptide), incretins (Ghrelin, GIP, GLP-1), adipokines (adiponectin, adipsin, resistin, leptin, visfatin and PAI-1) and a variety of cytokines, including Type-1 (IFN-γ, IL-2, and TNF-α), Type-17 (IL-17A and IL-22), Type-2/regulatory cytokines (IL-4, IL-5, IL-13, and IL-10) and other pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12, and GM-CSF) in those with obesity with or without concomitant *Ss* infection. We also examined the consequence of anthelmintic therapy on the above-mentioned factors in *Ss*-infected individuals.

METHODS

Ethics Statement

The study protocol was (12-I-073) permitted by Institutional Review Boards of the National Institute of Allergy and Infectious Diseases (USA) and the National Institute for Research in Tuberculosis (India) (approval no. NCT00375583 and NCT00001230). All individuals were screened as part of a natural history study protocol, and informed written consent was acquired from all individuals.

Study Population

We enrolled 115 study participants comprising of 58 clinically asymptomatic *Ss*-infected study participants with obesity (hereafter INF), and 57 study participants with obesity and negative for *Ss* infection (hereafter UN) in Kanchipuram District, Tamil Nadu, South India (**Table 1**). None had previous anthelmintic treatment, a history of helminth infections or of HIV. Individuals with diabetes or prediabetes (HbA1c above 5.7%) were excluded. These individuals were different from the individuals recruited in our previous studies (17, 18).

Measurement of Anthropometric and Biochemical Parameters

Anthropometric measurements, including height, weight, waist circumference and biochemical parameters, including plasma glucose, lipid profiles and HbA1c were obtained using standardized techniques as detailed elsewhere (19).

Parasitological Examination and Anthelmintic Treatment

The recombinant NIE antigen-ELISA detects IgG antibodies and was performed for the identification of *Ss* infection, as described previously (20, 21). Single stool specimens were collected at baseline from all screened individuals before anthelmintic therapy. Other intestinal helminth infections were identified by Stool microscopy and expelled from the study. Circulating filarial antigen tests were used for the diagnosis of filarial infection and those who were positive were excluded from the study. A single dose of ivermectin (12 mg) and albendazole (400 mg) were given to the INF study participants. Following 6 months of anthelmintic treatment, stool and blood samples were collected from the treated individuals to check the IgG antibody levels and to identify the presence of helminth infection. All INF individuals

TABLE 1 | Demographic and biochemical parameters.

	Ss+ $n = 58$	Ss- $n = 57$	*p*-values
M/F	30/28	28/29	0.8528
Age	36 (20–64)	39 (24–64)	0.1975
BMI (kg/m^2)	31 (30–36)	34 (30–40)	0.8073
RBG (mg/dl)	96 (70–168)	99 (69–159)	0.4700
HbA1c (%)	5.2 (5.1–5.5)	5.3 (5.2–5.6)	0.3774
Urea (mg/dl)	19.8 (8–40)	22.1 (13–42)	0.2972
Creatinine (mg/dl)	0.72 (0.3–1.2)	0.75 (0.4–1.3)	0.4448
ALT (U/L)	19.5 (10–116)	20.1 (5–87)	0.9766
AST (U/L)	22.4 (14–58)	23.8 (14–130)	0.4609

INF *refers to individuals with Ss-infection with obesity,* ***UN*** *refers to individuals with obesity without Ss infection. For age and gender p-value calculated using chi square (and Fisher's exact) test. Other parameter's p-values calculated using Mann-Whitney U-test.*

exhibited reductions in IgG titers at this time point and no helminths were detected by microscopy.

Determination of Obesity

BMI more than or equal to 30 kg/m^2 is described as obesity based on The American Association of Clinical Endocrinologists/American College of Endocrinology algorithm and American Diabetes Association guidelines. Height and body weight were measured using a digital scale.

Measurement of Serum Adipocytokines and Cytokine Levels

Plasma levels of pancreatic hormones (insulin, glucagon, and C-peptide), incretins (Ghrelin, GIP, and GLP-1), adipokines (adiponectin, adipsin, resistin, leptin, visfatin, and PAI-1) and the levels of Type-1 cytokines: IFN-γ, IL-2, TNF-α, Type-17 cytokines: IL-17A, IL-22, Type-2 cytokines: IL-4, IL-5, IL-13, regulatory cytokine: IL-10 and the pro-inflammatory cytokines: IL-1α, IL-1β, IL-6, IL-12, and GM-CSF were measured using a Human Magnetic Luminex Assay kit (R&D Systems, USA) on the Bio-Rad Luminex platform (Luminex, USA), according to the manufacturer's instructions.

Statistical Analysis

Central tendency was measured by using Geometric means (GM). Comparison between INF and UN were performed using Mann-Whitney *U*-tests with Holm's correction for multiple comparisons and the before and after treatment parameters were analyzed using Wilcoxon signed rank test. Backward stepwise methods in multiple logistic regression analysis was performed to identify factors that were influenced by *Ss* infection. Analyses were performed using Graph-Pad PRISM Version 8.0 (GraphPad, San Diego, CA) and Stata 15 (College Station, TX). JMP14 software was used to plot Principle Component Analysis (PCA).

RESULTS

Study Population Characteristics

The baseline demographic characteristics and biochemical parameters are shown in **Table 1**. There were no significant differences in age, sex, BMI or other biochemical parameters between the two groups.

Lower Systemic Levels of Pancreatic Hormones and Altered Levels of Incretins and Adipokines in INF Individuals

To estimate the effect of *Ss* infection on pancreatic hormones (C-peptide, Insulin, and Glucagon), incretins (Ghrelin, GIP, and GLP-1) and adipokines (adiponectin, adipsin, resistin, leptin, visfatin, and PAI-1) in obesity, we assessed the levels of aforesaid parameters in INF and UN study participants. As illustrated in **Figure 1A**, the levels of insulin (GM of 17.09 pg/ml in INF compared to 30.45 pg/ml in UN; $p = 0.0012$), glucagon (GM of 178.7 pg/ml in INF compared to 225.4 pg/ml in UN; $p = 0.0011$), C-peptide (GM of 84.17 pg/ml in INF compared to 139.8 pg/ml

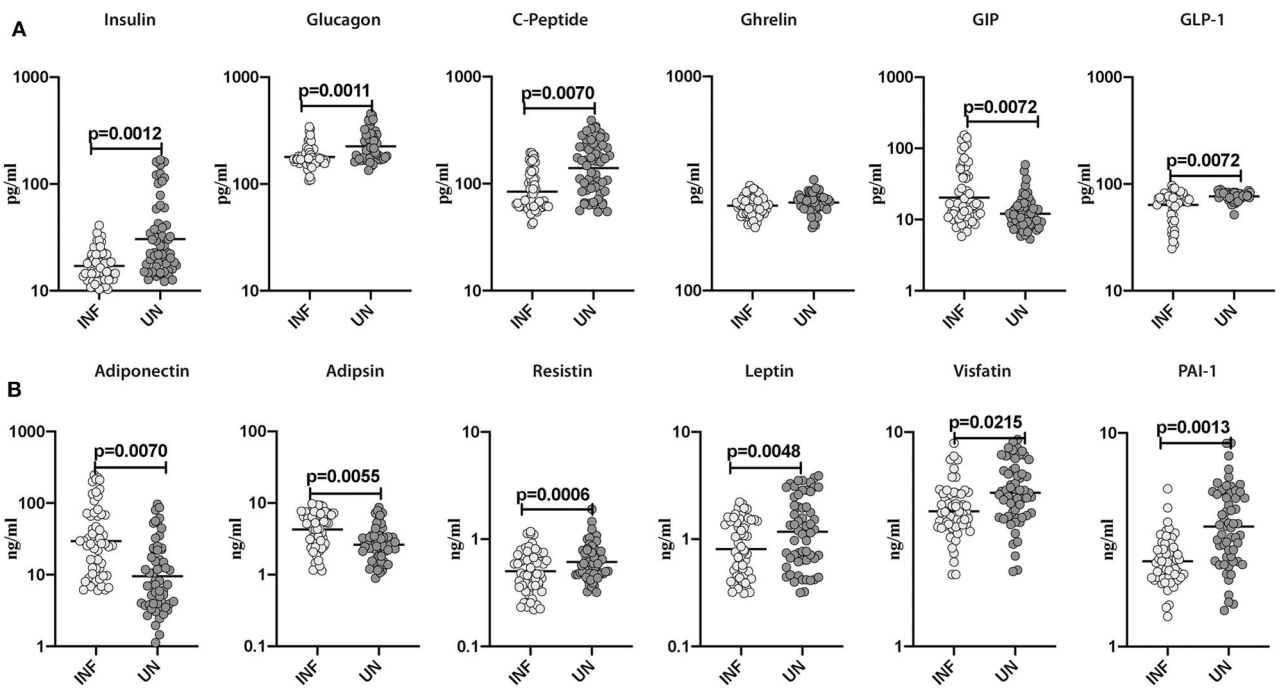

FIGURE 1 | Lower systemic levels of pancreatic hormones and altered levels of incretins and adipokines in INF individuals. **(A)** Plasma levels of insulin, glucagon, C-peptide, ghrelin, GIP and GLP-1 in INF and UN individuals were measured. **(B)** Plasma levels of adiponectin, adipsin, resistin, leptin, visfatin and PAI-1 in INF and UN individuals were measured. Each dot is an individual subject with the bar representing the geometric mean (GM). Mann– Whitney *U*-test with Holms correction for multiple comparisons were done to calculate *p*-values.

in UN; $p = 0.0070$) and GLP-1 (GM of 63.69 pg/ml in INF compared to 76.71 pg/ml in UN; $p = 0.0072$) were significantly lower in INF than UN individuals. In contrast, GIP (GM of 20.39 pg/ml in INF compared to 12.07 pg/ml in UN; $p = 0.0072$) levels were significantly higher in INF compared to UN individuals. As shown in **Figure 1B**, resistin (GM of 0.503 ng/ml in INF compared to 0.613 ng/ml in UN; $p = 0.0006$), leptin (GM of 0.808 ng/ml in INF compared to 1.171 ng/ml in UN; $p = 0.0048$), visfatin (GM of 4.267 ng/ml in INF compared to 5.217 pg/ml in UN; $p = 0.0215$) and PAI-1 (GM of 2.51 ng/ml in INF compared to 3.66 ng/ml in UN; $p = 0.0013$) were significantly lower in INF than UN individuals. In contrast, adiponectin (GM of 29.41 ng/ml in INF compared to 9.54 ng/ml in UN; $p = 0.0070$) and adipsin (GM of 4.27 ng/ml in INF compared to 2.63 ng/ml in UN; $p = 0.0055$) were significantly higher in INF than UN individuals. Therefore, *Ss* infection is characterized by lower systemic levels of the pancreatic hormones, incretins and altered levels of adipokines in *Ss*-infected individuals with obesity.

Lower Systemic Levels of Type-1, Type-17, and Other Pro-inflammatory Cytokines and Elevated Levels of Type-2 Cytokines in INF Individuals

Subsequently, we sought to examine the influence of Ss infection on the systemic levels of Type-1 (IFN-γ, TNF-α, and IL-2), Type-17 (IL-17A and IL-22), Type-2 (IL-4, IL-5, and IL-13),

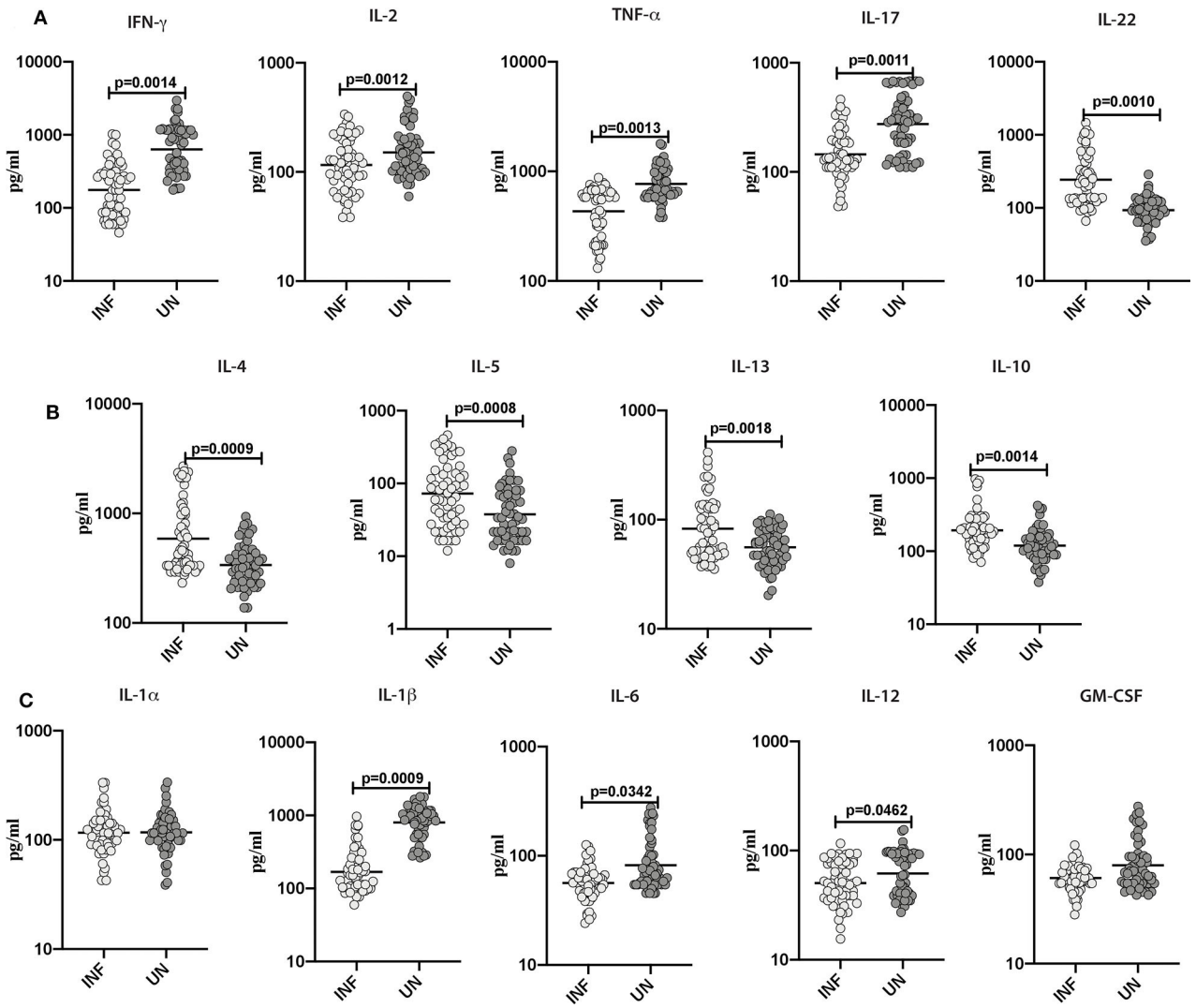

FIGURE 2 | Lower systemic levels of Type-1 and Type-17 and other pro-inflammatory cytokines and higher levels of Type-2 cytokines in INF individuals. **(A)** Plasma levels of Type-1 (IFNγ, TNFα and IL-2)-, Type-17 (IL-17A and IL-22)- cytokines in INF and UN. **(B)** Plasma levels of Type-2 (IL-4, IL-5, and IL-13)- and regulatory (IL-10) cytokine in INF and UN individuals were measured. **(C)** Plasma levels of other pro-inflammatory (IL-1α, IL-1β, IL-6, IL-12, and GM-CSF) in INF and UN individuals were measured. Each dot is an individual subject with the bar representing the geometric mean (GM). Mann–Whitney *U*-test with Holms correction for multiple comparisons were done to calculate *p*-values.

regulatory (IL-10) and pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-12, and GM-CSF) in INF and UN individuals. As illustrated in **Figure 2A**, INF individuals exhibited significantly lower levels of IFN-γ (GM of 176.2 pg/ml in INF compared to 631.6 pg/ml in UN; $p = 0.0014$), IL-2 (GM of 115.8 pg/ml compared to 150.9 pg/ml; $p = 0.0013$), TNF-α (GM of 432.2 pg/ml in INF compared to 768.8 pg/ml in UN; $p = 0.0012$), IL-17A (GM of 144.7 pg/ml in INF compared to 275.1 pg/ml in UN; $p = 0.0087$) in comparison with UN individuals. In contrast, INF individuals exhibited significantly higher levels of IL-22 (GM of 234.4 pg/ml in INF compared to 93.14 pg/ml in UN; $p = 0.0010$) when compared to UN individuals. As illustrated in **Figure 2B**, IL-4 (GM of 588.4 pg/ml in INF compared to 338.8 pg/ml in UN; $p = 0.0009$), IL-5 (GM of 72.71 pg/ml in INF compared to 37.79 pg/ml in UN; $p = 0.0008$), IL-13 (GM of 82.68 pg/ml in INF compared to 56 pg/ml in UN; $p = 0.0006$), and IL-10 (GM of 193.4 pg/ml in INF compared to 119.4 pg/ml in UN; $p = 0.0007$) levels were significantly lower in INF compared to UN individuals. As illustrated in **Figure 2C**, IL-1β (GM of 168.7 pg/ml in INF compared to 800 pg/ml in UN; $p = 0.0009$), IL-6 (GM of 56.35 pg/ml in INF compared to 82.19 pg/ml in UN; $p = 0.0009$) and IL-12 (GM of 50.39 pg/ml in INF compared to 61.89 pg/ml in UN; $p = 0.0009$) levels were significantly lower in INF than UN individuals. Thus, *Ss* infection with obesity is characterized by lower systemic levels of IL-17, Type-1 associated cytokines and pro-inflammatory cytokines and higher systemic levels of IL-22 and Type-2 associated cytokines.

Anthelmintic Treatment Alters Plasma Levels of Pancreatic Hormones, Incretins, and Adipokines in INF Individuals

Subsequently, we sought to examine the effect of anthelmintic therapy on the levels of pancreatic hormones, incretins and adipokines in INF individuals. For this purpose, we assessed the plasma levels of pancreatic hormones, incretins and adipokines in INF individuals following 6 months of anthelmintic therapy. As illustrated in **Figure 3A**, the post-treatment levels of insulin (percentage increase of 9%; $p = 0.0009$), C-peptide (percentage increase of 6%; $p = 0.0012$), GIP (percentage increase of 13%; $p = 0.0011$) and GLP-1 (percentage increase of 16%; $p = 0.0183$) were significantly increased than pre-treatment levels. As illustrated in **Figure 3B**, the post-treatment levels of adiponectin (percentage decrease of 11%; $p = 0.0005$) and adipsin (percentage decrease of 25%; $p = 0.0003$) were significantly decreased than the pre-treatment levels. In contrast, the post-treatment levels of resistin (percentage increase of 14%; $p = 0.0006$), leptin (percentage increase of 13%; $p = 0.0004$), and PAI-1 (percentage increase of 21%; $p = 0.0055$) were significantly increased than the pre-treatment levels. As illustrated in **Supplementary Figure 1**, the levels of pancreatic hormones, incretins and adipokines were not significantly different between the uninfected and post-treated individuals. Therefore, anthelmintic therapy is associated with significant alteration of pancreatic hormones, incretins and adipokines in obese individuals.

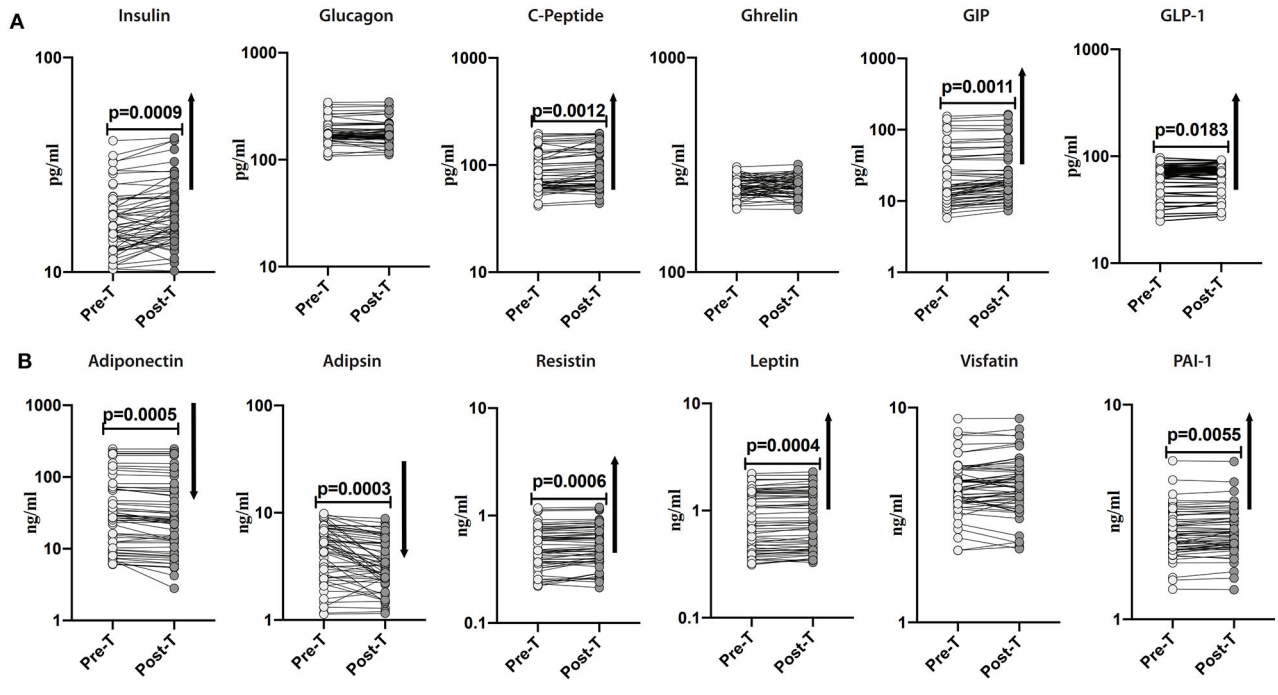

FIGURE 3 | Anthelmintic treatment alters systemic levels of pancreatic hormones, incretins, and adipokines in INF individuals. **(A)** Plasma levels of insulin, glucagon, C-peptide, ghrelin, GIP, and GLP-1 in INF individuals pre-treatment [Pre-T] and 6 months following treatment [post-T] were measured. **(B)** Plasma levels of adiponectin, adipsin, resistin, leptin, visfatin, and PAI-1 in INF individuals pre-treatment [Pre-T] and 6 months following treatment [post-T] were measured. *p*-values were calculated using the Wilcoxon matched pair test.

Anthelmintic Treatment Results in Significantly Increased Levels of Type-1, Type-17 and Pro-inflammatory Cytokines and Decreased Levels of Type-2 Cytokines and IL-10 in INF Individuals

To examine the impact of anthelmintic therapy on systemic levels of Type-1 (IFN-γ, TNF-α, and IL-2)- and Type-17 (IL-17A and IL-22)- associated cytokines, we assessed the cytokines in INF individuals at baseline and following 6 months of anthelmintic therapy. As illustrated in **Figure 4A**, following anthelmintic treatment, the levels of IFN-γ (percentage increase of 8.3%; $p = 0.0014$), IL-2 (percentage increase of 7.9%; $p = 0.0012$), TNF-α (percentage increase of 4.6%; $p = 0.0013$), IL-17A (percentage increase of 6.4%; $p = 0.0011$) were increased, whereas IL-22 (percentage decrease of 1.2%; $p = 0.0010$) levels were decreased.

Next, to examine the impact of anthelmintic therapy on Type-2- and regulatory cytokines, we assessed the cytokines in INF individuals and baseline and following 6 months of anthelmintic therapy. As illustrated in **Figure 4B**, IL-4 (percentage decrease of 6.8%; $p = 0.0009$), IL-5 (percentage decease of 14.5%; $p = 0.0008$), IL-13 (percentage decrease of 12.7%; $p = 0.0006$) and IL-10 (percentage decrease of 8%; $p = 0.0007$) levels were decreased when compared to their pre-treatment levels.

Further, to examine the impact of anthelmintic treatment on pro-inflammatory cytokines, we measured the pro-inflammatory cytokines in INF individuals at 6 months following anthelmintic treatment. As shown in **Figure 4C**, IL-6 (percentage increase of 5.3%; $p = 0.0008$) levels were increased when compared to pre-treatment levels. Other pro-inflammatory cytokines did not show any significant difference when compared to their pre-treatment levels.

As shown in **Supplementary Figure 2**, the levels of Type-1, Type-17, and pro-inflammatory cytokines and Type-2 cytokines were not significantly different between the uninfected and post-treated individuals. Thus, anthelmintic treatment is associated with increased Type-1 and Type-17 associated cytokines, pro-inflammatory cytokine, IL-6, and decreased Type-2 associated cytokines in obese individuals.

Principle Component Analysis Reveals Tendencies in Pancreatic Hormones, Incretins, Adipokines, and Cytokine Milieu in Helminth-Obese Individuals

PCA was used to visualize differences between the groups based on the entire data set. To determine the clustering pattern of pancreatic hormones, incretins, adipokines, and cytokines between INF (red circle) and UN (blue circle) individuals, we strategized PCA with diverse inputs. As illustrated in **Figure 5A**, PCA analysis exhibited that pancreatic hormones, incretins, adipokines cluster differently between INF and UN individuals. The score plot of the first two components revealed 23.2 and 16.7% of overall variance, correspondingly. As shown in **Figure 5B**, PCA analysis of cytokines exhibited two different clusters between INF and UN individuals. The score plot of the first two components revealed 25.5 and 16.9% of overall variance, correspondingly. Thus, PCA analysis reveals the overall influence of pancreatic hormones, incretins, adipokines, and cytokine of *Ss* infection on obesity.

Multivariate Regression Analysis of Helminth-Obesity Interaction

The influence of confounding variables on obese individuals with different analytes was evaluated in this study using multivariate regression analysis. As illustrated in **Table 2**, even after correcting for the influence of the age and sex, the levels of biochemical parameters such as AST, ALT and urea, RBG, HbA1c, diabetic parameters such as insulin, glucagon, C-peptide, GIP, GLP-1, adiponectin, adipsin, resistin, leptin, visfatin, and PAI-1; cytokines like IFN-γ, IL-2, TNF-α, IL-17, IL-22, IL-4, IL-5, IL-13, IL-10, IL-1β, IL-6, and IL-12 were all significantly manipulated by *Ss* infection. Therefore, our data corroborate that *Ss* infection has a great impact on numerous significant factors in obese individuals, including blood glucose levels, and the levels of the adipocytokines and the more conventional cytokines.

DISCUSSION

Helminth infections are known to limit harmful inflammatory responses and assist local and systemic metabolic homeostasis (22). Previously published reports revealed that helminths could limit the progression of metabolic diseases (23, 24), probably by altering host inflammatory responses (24). Thus, it has been proposed that a decrease in helminth infections could influence the incidence of inflammatory diseases, T2DM, obesity, insulin resistance and metabolic syndrome in many of the high income countries (25).

An earlier study showed that insulin and C-peptide levels were increased in obese individuals compared to lean controls and suggested obese subjects have an impaired glucose homeostasis and exhibit prediabetic factors, including hyperinsulinemia, and insulin resistance (26–29). Glucagon levels were elevated in obese individuals (26). Likewise, the present study has demonstrated that *Ss* infection was associated with lower systemic levels of insulin, glucagon and C-peptide levels when compared with obese without *Ss* infection and reversal following anthelmintic treatment. Recently, we have shown that *Ss* infection has a protective role on diabetes-related parameters and that *Ss* infected T2DM individuals showed decreased levels of insulin and glucagon that increased following therapy (17). Recent data also revealed that adiponectin reduces IFNγ and IL-17 responses by T cells in obese mice (30). Previously we have shown that adiponectin and adipsin levels were lowered in *Ss* infection with T2DM in comparison with *Ss* uninfected with T2DM individuals. In the present study, both adiponectin and adipsin are exhibited at elevated levels in the systemic circulation in INF individuals. These levels were significantly decreased after 6 months of anthelmintic therapy, indicating that *Ss* infection could modify

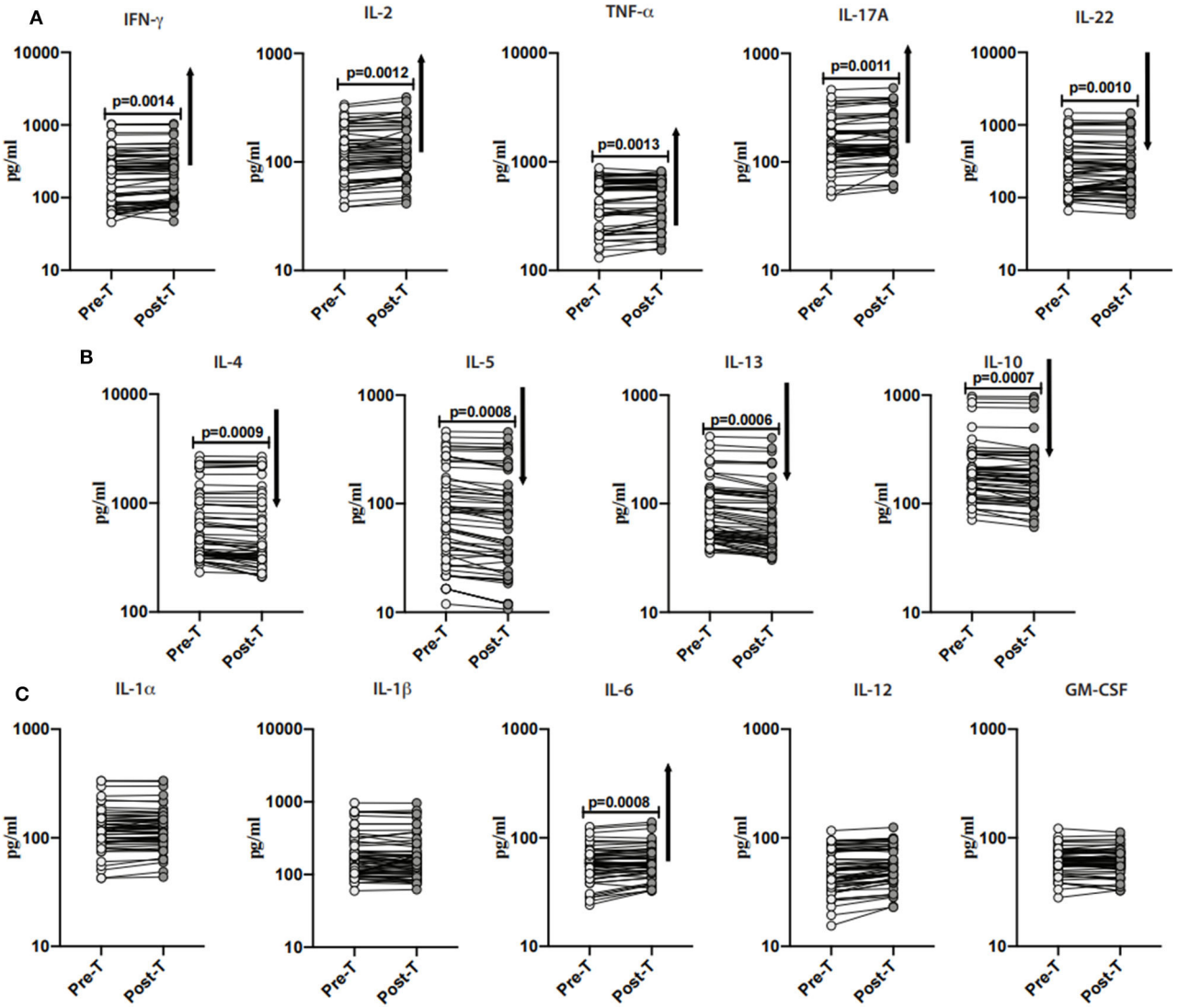

FIGURE 4 | Anthelmintic treatment alters systemic levels of Type-1, Type-17, Type-2- cytokines, and IL-10 in INF individuals. **(A)** Plasma levels of Type-1 (IFNγ, TNFα, and IL-2)- and Type-17 (IL-17A and IL-22)- cytokines in INF individuals pre-treatment [Pre-T] and 6 months following treatment [post-T] were measured. **(B)** Plasma levels of Type-2 (IL-4, IL-5, and IL-13)- and regulatory (IL-10) cytokine in INF individuals pre-treatment [Pre-T] and 6 months following treatment [post-T] were measured. **(C)** Plasma levels of other pro-inflammatory (IL-1α, IL-1β, IL-6, IL-12, and GM-CSF) in INF individuals pre-treatment [Pre-T] and 6 months following treatment [post-T] were measured. *p*-values were calculated using the Wilcoxon matched pair test.

the adipocytokine levels in INF individuals. Leptin induces pro-inflammatory immune responses and limits the proliferation of regulatory T cells, while adiponectin promotes the production of anti-inflammatory cytokines (31). The difference between the levels of these two adipokines has been shown to be linked with pro-inflammatory conditions and insulin resistance. A recent study revealed that leptin to adiponectin ratios in STH-infected individuals was increased following anthelmintic therapy which may in small part, lead to the moderate rise in insulin resistance (32). In our study, resistin, leptin, visfatin, PAI-1 levels were significantly lower in the INF group and increased following anthelmintic therapy. Helminths may have an impact on glucose homeostasis and insulin resistance in obesity through alternative mechanisms such as modulating the levels of adipocytokines (33, 34). This implies that adipokines have a key role in the facilitation of helminth-associated beneficial influence on insulin resistance.

Helminth infections have the ability to modulate immune responses (35). Previously, we have reported that *Ss*-infected individuals exhibited significantly decreased systemic levels of the pro-inflammatory cytokines and significantly heightened levels of the Type-2-related and regulatory cytokines (36). Type-1 related cytokines IL-1, IL-6, IL-8, IFN-γ, and MCP-1 are highly expressed in obese individuals (37). Another study showed that accumulation of inflammatory cells and adipocytes results in increased secretion of cytokines like tumor

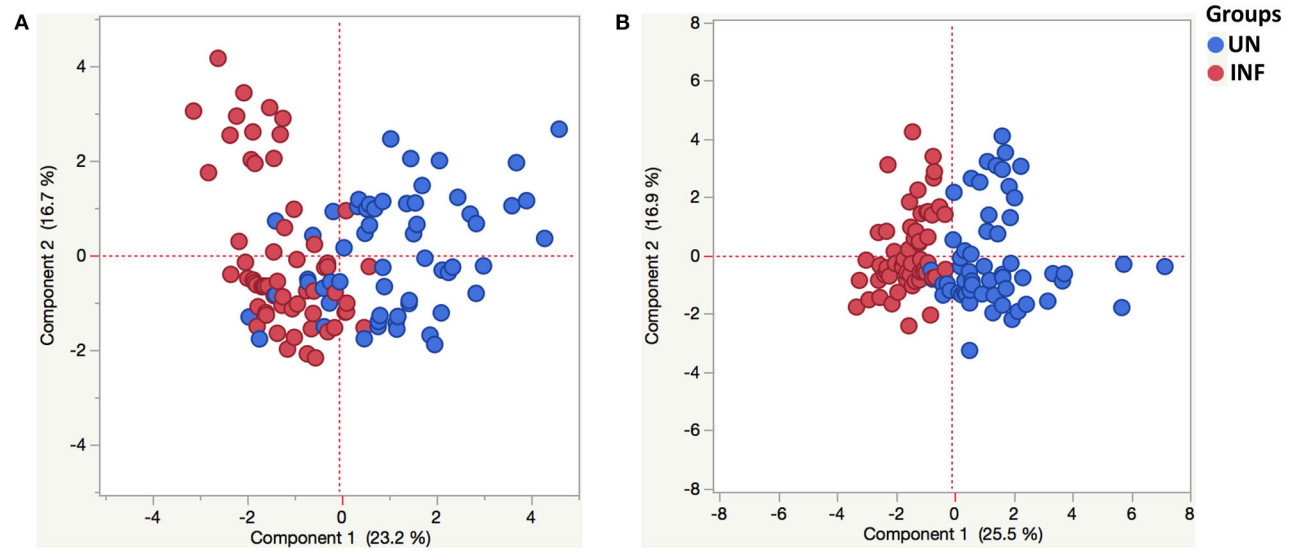

FIGURE 5 | Principle component analysis reveals tendencies in pancreatic hormones, incretins, adipokines and cytokine milieu in helminth-obese individuals. Principal component analysis (PCA) was performed to show the distribution of data from the combination of two groups INF (red circles) and UN (blue circles). The PCA represents the two principal components of variation. **(A)** PCA for insulin, glucagon, C-peptide, ghrelin, GIP and GLP-1, adiponectin, adipsin, resistin, leptin, visfatin, and PAI-1in INF and UN individuals. **(B)** PCA for Type-1 (IFNγ, TNFα, and IL-2), Type-17 (IL-17A and IL-22), and other pro-inflammatory (IL-1α, IL-1β, IL-6, IL-12, and GM-CSF) cytokines of INF and UN individuals.

TABLE 2 | Multiple logistic regression analysis on effect of *Ss* infection on obesity.

Factors	Crude OR (95% CI)	*p*-value	Adjusted OR (95% CI)	*p*-value
Insulin	0.268 (0.135–0.531)	<0.001	0.272 (0.138–0.537)	<0.001
Glucagon	0.114 (0.039–0.341)	<0.001	0.116 (0.039–0.345)	<0.001
C-peptide	0.279 (0.159–0.491)	<0.001	0.287 (0.164–0.500)	<0.001
Ghrelin	0.130 (0.011–1.652)	0.116	0.14 (0.011–1.822)	0.133
GIP	2.241 (1.394–3.602)	<0.001	2.213 (1.380–3.550)	<0.001
GLP-1	0.036 (0.004–0.384)	0.006	0.040 (0.004–0.402)	0.006
Adiponectin	1.827 (1.396–2.392)	<0.001	1.808 (1.389–2.354)	<0.001
Adipsin	2.545 (1.568–4.132)	<0.001	2.537 (1.566–4.111)	<0.001
Resistin	0.445 (0.235–0.843)	0.013	0.414 (0.214–0.802)	0.009
Leptin	0.615 (0.423–0.895)	0.011	0.619 (0.426–0.899)	0.012
Visfatin	0.257 (0.107–0.617)	0.002	0.254 (0.105–0.614)	0.002
PAI-1	0.123 (0.049–0.310)	<0.001	0.126 (0.051–0.316)	<0.001
IFNγ	0.275 (0.172–0.441)	<0.001	0.263 (0.162–0.429)	<0.001
IL-2	0.531 (0.322–0.877)	0.013	0.528 (0.320–0.871)	0.012
TNFα	0.092 (0.032–0.268)	<0.001	0.098 (0.034–0.282)	<0.001
IL-17	0.254 (0.145–0.447)	<0.001	0.259 (0.148–0.452)	<0.001
IL-22	12.497 (4.272–36.563)	<0.001	11.598 (3.984–33.766)	<0.001
IL-4	2.992 (1.717–5.213)	<0.001	3.108 (1.741–5.546)	<0.001
IL-5	1.640 (1.233–2.181)	<0.001	1.613 (1.212–2.145)	0.001
IL-13	2.671 (1.491–4.786)	<0.001	2.608 (1.459–4.664)	0.001
IL-10	3.202 (1.773–5.782)	<0.001	3.209 (1.770–5.819)	<0.001
IL-1α	1.005 (0.558–1.811)	0.989	1.032 (0.572–1.864)	0.918
IL1-β	0.137 (0.072–0.262)	<0.001	0.132 (0.067–0.261)	<0.001
IL-6	0.243 (0.112–0.529)	<0.001	0.239 (0.110–0.523)	<0.001
IL-12	0.481 (0.269–0.859)	0.013	0.493 (0.276–0.879)	0.016
GM-CSF	0.283 (0.130–0.616)	0.001	0.291 (0.133–0.635)	0.002

necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) and lead to the progression of metabolic syndrome, consequently worsening the outcome of obesity (38). Previously, we have shown that *Ss* infection in T2DM showed significantly lower levels of Type-1 and Type-17 associated cytokines and pro-inflammatory cytokines with an increase following anthelmintic treatment (17, 18). In line with this in our study, Type-1 (IFN-γ, TNF-α, and IL-2)- and Type-17 (IL-17A)- cytokines were significantly decreased in INF individuals when compared to those without *Ss* infection. Hence, our data implies that helminth infection is characterized by the alteration of Type-1- and Type-17- cytokine responses. In our current study, IL-22 levels were increased in *Ss*-infected obese individuals and decreased following treatment. Since IL-22 is known to play a crucial role in modulating lipid metabolism and the IL-22 pathway is vital for preserving epithelial integrity, lowering chronic inflammation, and improving metabolic syndromes (39, 40), our data implies that IL-22 modulation might have a potential impact on lipid metabolism in obese individuals.

IL-4 and IL-13 are critically associated with the regulation of adipose tissue homeostasis, indicating that helminths may have an impact on metabolic status by altering adipose tissue function (41). An earlier study on *Ss* infection with T2DM showed significantly increased levels of Type-2 related cytokines (17). In our current study, an increased expression of the prototypical Type-2-associated cytokines IL-4, IL-5, IL-13, and IL-10 in *Ss*-infected obese individuals was observed. IL-10 has been shown to be able to improve inflammation in obese adipose tissue and insulin resistance induced by proinflammatory cytokines, TNF-α and IL-6 (1, 42). Various studies have determined that helminth infection and helminth- derived molecules, *S.mansoni* secreted SEA (43), *Schistosoma mansoni* egg-derived ω1 recombinant SEA (44), *L. sigmodontis* antigen (15) play an important role in metabolic disorders by stimulating a T helper 2 (Th2) response and increasing insulin sensitivity. Our data suggest that *Ss* infection could limit the inflammatory process (lower levels of pancreatic hormones, Type-I, Type-17, pro-inflammatory cytokines) by producing type-2 cytokines. This needs to be studied further mechanistically.

Taken together, our data clearly show the positive impacts of helminth infection on obesity related metabolic dysfunction. Our study has certain limitations including not determining insulin resistance directly, not having a non-obese control group and not having a placebo control group. Nevertheless, our study reveals that *Ss* infection could play a defensive role opposing the deleterious consequences of obesity by altering hormones, adipokines and the associated cytokine milieu. Certain parameters such as glucagon, visfatin and cytokines remain not significantly altered following treatment, which could perhaps reflect different kinetics in the alteration of these parameters or a lack of alteration. Our data suggest a significant association between helminth infection and the alteration of the homeostatic milieu in obesity and provide better knowledge of helminth-driven immune- and non-immune–mediated modulation of host metabolism. Our data also reinforce the possibility for helminths as a new class of biologics in alleviating inflammatory diseases and metabolic disorders. A more detailed characterization of the definitive immunomodulatory components of helminths could promote a more precise treatment approach against obesity and other inflammatory diseases.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by All participants were examined as part of a natural history study protocol (12-I-073) approved by Institutional Review Boards of the National Institute of Allergy and Infectious Diseases (USA) and the National Institute for Research in Tuberculosis (India) (approval no. NCT00375583 and NCT00001230), and informed written consent was obtained from all participants. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SB and AR conceived and planned the experiments. AR and SM executed the experiments. AR, SB, and KT analyzed the data. PM and CD provided patient samples. TN contributed reagents, materials, and analysis tools. AR, SB, and TN wrote, reviewed, and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02195/full#supplementary-material

Supplementary Figure 1 | No significant differences in the systemic levels of pancreatic hormones, incretins, and adipokines between UN and Post treated individuals. **(A)** Plasma levels of insulin, glucagon, C-peptide, ghrelin, GIP and GLP-1 in UN, and Post-T individuals were measured. **(B)** Plasma levels of adiponectin, adipsin, resistin, leptin, visfatin and PAI-1 in UN and Post-T individuals were measured. Each dot is an individual subject with the bar representing the geometric mean (GM). Mann– Whitney *U*-test were done to calculate *p*-values.

Supplementary Figure 2 | No significant differences in the systemic levels of Type-1 and Type-17 and other pro-inflammatory cytokines and Type-2 cytokines between UN and Post treated individuals. **(A)** Plasma levels of Type-1 (IFNγ, TNFα, and IL-2)-, Type-17 (IL-17A and IL-22)- cytokines in UN and Post-T individuals were measured. **(B)** Plasma levels of Type-2 (IL-4, IL-5, and IL-13)- and regulatory (IL-10) cytokine in UN and Post-T individuals were measured. **(C)** Plasma levels of other pro-inflammatory (IL-1α, IL-1β, IL-6, IL-12, and GM-CSF) in UN and Post-T individuals were measured. Each dot is an individual subject with the bar representing the geometric mean (GM). Mann– Whitney *U*-test were done to calculate *p*-values.

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REVIEW
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Immunomodulation and Immune Escape Strategies of Gastrointestinal Helminths and Schistosomes

*Marie Wiedemann and David Voehringer**

Department of Infection Biology, University Hospital Erlangen and Friedrich-Alexander University Erlangen-Nuremberg (FAU), Erlangen, Germany

Parasitic worms (helminths) developed various immunoregulatory mechanisms to counteract the immune system of their host. The increasing identification and characterization of helminth-derived factors with strong immune modulatory activity provides novel insights into immune escape strategies of helminths. Such factors might be good targets to enhance anti-helminthic immune responses. In addition, immunosuppressive helminth-derived factors could be useful to develop new therapeutic strategies for treatment of chronic inflammatory conditions. This review will take an in depth look at the effects of immunomodulatory molecules produced by different helminths with a focus on schistosomes and mouse models of hookworm infections.

Keywords: nematode, trematode, immunomodulation, type 2 immunity, parasites, schistosomes, Helminths

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James Cook University, Australia

****Correspondence:***
David Voehringer
david.voehringer@uk-erlangen.de

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INTRODUCTION

Helminths are the most commonly found group of parasites in humans. Especially in regions with poor hygiene standards and insufficient access to medical care, helminths are spread easily and, in some cases, cause severe damage to the infected subjects. They are transmitted in various ways, (for e.g., via contaminated food, water or soil, or by close contact to animals). Most helminth infections are easily treatable with common anti-helminthic drugs, however, untreated infections can endanger especially young children or elderly people. Helminths are relatively large multicellular organisms that can cause tissue damage when larval stages migrate through different organs or adult worms feed on host tissue. Yet, the co-evolution of helminths with their hosts established mechanisms that prevent excessive immune responses so that the parasites can persist and complete their life cycles. Helminths produce a range of different, often highly specialized molecules, changing the general microenvironment around them, altering the density of tissue, or influencing certain types of immune cells. The parasites produce various kinds of these immunomodulatory molecules simultaneously in order to support the individual needs of the parasites in their different life stages (1). Many helminths evolved rather complex life cycles, sometimes requiring intermediate hosts to further develop before infecting their targeted final host. Helminths constitute a genetically very diverse group of organisms and can be divided in two major phyla: nematoda and platyhelminthes both of which include species with potent immunomodulatory function that will be discussed below (2).

LIFE CYCLE OF SCHISTOSOMES

Schistosomes, also known as blood flukes, belong to the group of trematodes within the phylum Platyhelminthes. Adult schistosomes show a distinct sexual dimorphism, the female worm being much smaller than the male. The male will surround the female, keeping her in his *gynacophoric canal* for the entirety of their lives. The adult schistosomes can live for up to 10 years inside the blood stream, where they will proceed to lay eggs inside of mesenteric veins in the bowel or rectum. From here, the eggs translocate to the liver and are excreted via feces or they reach the bladder and leave the body with the urine, depending on the type of schistosome. In water, secreted eggs hatch into miracidia larvae, which will proceed to enter a snail serving as the intermediate host. Next, they develop successive generations of sporocysts before being released as swimming cercariae larvae. These will now penetrate the skin of humans or other mammals and become schistosomula, entering the blood stream. Then, they migrate to the lung and liver and mature to fully grown adults, eventually finding a mate, and starting the reproductive cycle again.

Infections with schistosomes can cause schistosomiasis, a disease affecting about 200 million people world-wide. The disease is caused by eggs trapped in tissues where they are surrounded by granulomas composed of various cell types to prevent tissue damage by egg-derived enzymes and pro-inflammatory factors. Excessive granuloma formation can lead to tissue fibrosis and organ failure (3).

MOUSE MODELS OF HOOKWORM INFECTIONS

Parasitic nematodes, or roundworms, in humans include filaria, ascarids, trichurids, and hookworms. Many effects of nematodes on their hosts are studied in mice, using for example the rodent parasites *Nippostrongylus brasiliensis* or *Heligmosomoides bakeri* (formerly named *H. polygyrus*). The life cycle of *N. brasiliensis* is very similar to that of *Necator americanus* and *Ancylostoma duodenale*, the two main hookworm species infecting humans. Although *N. brasiliensis* is mainly found in rat populations and considered a "rat hookworm" that can be used to infect mice under laboratory conditions, a recent study showed that this helminth can also be isolated from wild *Mus musculus* in Korea (4). Eggs of *N. brasiliensis* can be found in the soil, where they will hatch to worms and molt twice before they become infective larvae. They will burrow through the skin of their host and enter the venous system. Then, they are transported to the lung, where they reside in the capillaries, molt again, rupture the capillaries, and enter the alveoli, are coughed up and swallowed. In the lumen of the small intestine, the worms molt for the last time to become fully mature. After mating, the female worm starts to lay eggs, which are secreted via the feces. Immunocompetent mice usually expel the adult worms by day 10 after infection by a process that requires IL-4- or IL-13-mediated activation of STAT6 in intestinal epithelial cells and smooth muscle cells to induce goblet cell hyperplasia, mucus secretion, and increased intestinal peristalsis causing a "weep and sweep" mechanism of worm expulsion (5, 6).

In contrast to the *N. brasiliensis* infection model, *H. bakeri* causes a chronic infection of the small intestine. In this model eggs in the soil hatch and develop into L3 stage larvae that are then taken up orally. The larvae are directly transported into the intestine where they burrow in the submucosa of the gut, molt once again and penetrate the muscular layer of the gut, where they develop into adult worms within 7 days. Male and female worms will then enter the intestinal lumen, mate and release eggs, which are excreted with the feces. Depending on the genetic background of mice and the strength of the immune response the adult worms can live for several weeks and continue to produce eggs (5, 6).

RESPONSE OF THE IMMUNE SYSTEM TO HELMINTH INFECTIONS

Generally, type 2 immune responses with high IgE levels, increased numbers and/or activity of Th2 cells, type 2 innate lymphoid cells (ILC2), eosinophils, basophils, mast cells, and alternatively activated macrophages (AAMs) are major characteristics of helminth infections (**Figure 1**). However, since different helminths inhabit distinct niches in the host's body, use different ways of entering the host and show disparate migration within the body, immune responses may vary depending on the infecting helminth species.

Compared to other pathogens, helminths are relatively large and very motile. Tissue damage caused during infection can be sensed by mucosal epithelial cells and keratinocytes. In response, these stromal cells will produce alarmins like interleukin 25 (IL-25), thymic stromal lymphopoietin (TSLP), and IL-33. Next, alarmins induce activation and differentiation of type 2 immune cells which then release several other cytokines like IL-4, IL-5, IL-9, and IL-13. IL-4 and IL-13 activate goblet cells to produce mucus while also triggering smooth muscle cell contractions, the recruitment of eosinophils and the differentiation of AAMs (7). IL-25 is mainly produced by tuft cells in the gut epithelium upon infection with *N. brasiliensis*, leading to activation of Th2 cells and ILC2s, which will start producing IL-13 in response. IL-13 will then lead to extensive differentiation of tuft and goblet cells and therefore, promotes an effective anti-helminthic mechanism (8–10). Later, the Th2 response also drives immunoglobulin class switch recombination in B cells to produce IgE and IgG4 in humans, or IgE and IgG1 in mice, directed mainly by IL-4R/STAT6 signaling and direct T-B cell interaction (11). The IgE antibodies can activate basophils involved in protective immunity during secondary helminth infections (12). Helminths

Abbreviations: AAM, alternatively activated macrophages; AChE, Acetylcholine esterase; CCP, complement control protein; CKBP, chemokine-binding protein; CPI, Cysteine protease inhibitor; HpARI, Heligmosomoides polygyrus alarmin release inhibitor; HpTGM, Heligmosomoides polygyrus TGF beta mimic protein; ISPE, IL-4-inducing principle from schistosoma eggs; PAS-1, Protein 1 from Ascaris suum; SEA, Schistosoma egg antigen; TSLP, thymic stromal lymphopoetin.

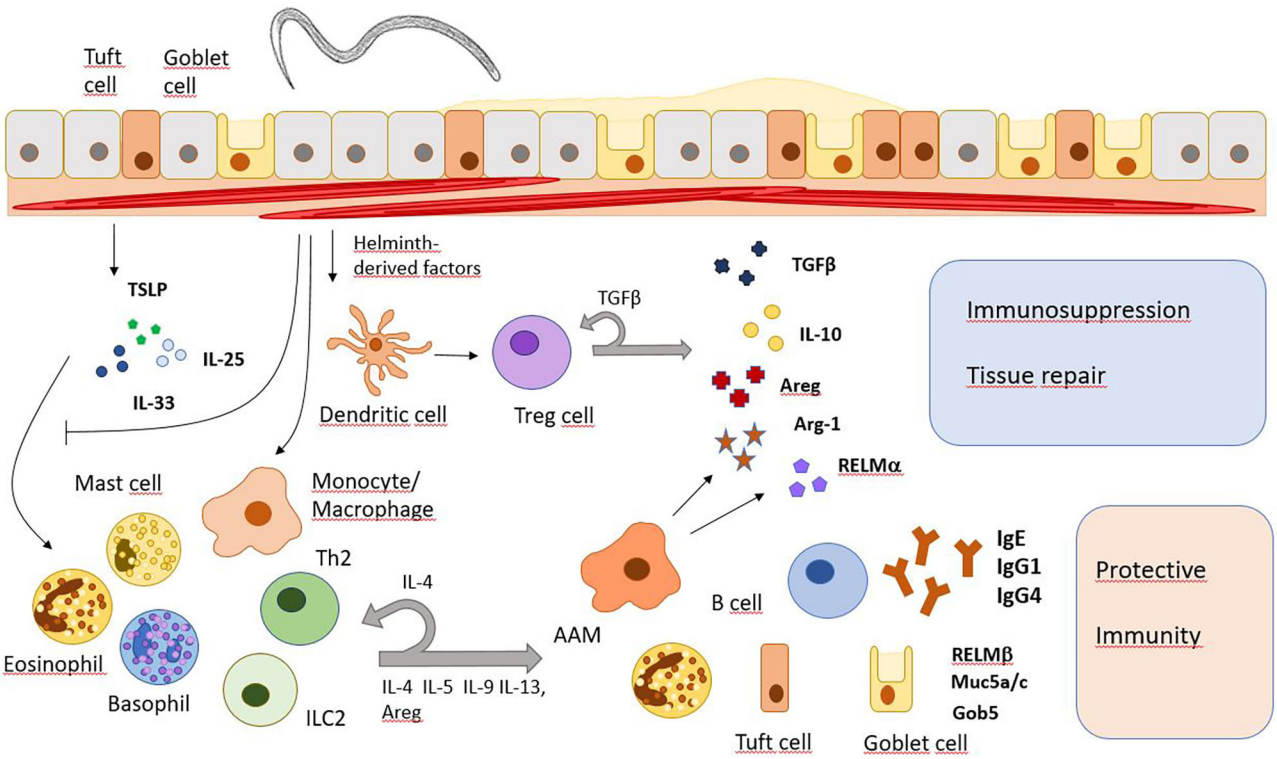

FIGURE 1 | Major immune response pathways after helminth infection. Helminth infections induce the release of alarmins (IL-25, IL-33, TSLP) which subsequently promote immune responses that promote worm expulsion or granuloma formation but also tissue repair and immunosuppression. However, they also inhibit the IL-33 signaling pathway and modulate gene expression in monocytes/macrophages and T cells. Areg, amphiregulin; Arg-1, arginase 1; Gob5, a Calcium-activated chloride channel in goblet cells; Muc5a/c, mucins 5a and c; RELM, resistin-like molecule.

residing in the peripheral blood of their host like *Schistosoma mansoni* also trigger a type 2 immune response during tissue migration and after the release of eggs. Granuloma formation around schistosome eggs is dependent on T cell-derived IL-4/IL-13 secretion and AAMs are critical to prevent a fatal infection in mice (13, 14). Helminth infection further induces the expansion of regulatory T cells (Tregs) and immunoregulatory monocytes along with higher levels of immunosuppressive cytokines IL-10 and TGFβ (15). Therefore, long term helminth infection shows similarities to other chronic infections where downregulation of the immune system prevents extensive harm to the body.

Helminths use several mechanisms of these immunoregulatory functions in order to ensure their own survival and minimize harm to the host caused by tissue damage when migrating through organs. Excreted proteins, metabolites, and extracellular vesicles are used to modulate the infected host's immune response by induction or suppression of immune cells, interfering with signaling pathways and the tissue reparative response to remodel their environmental niche in the host toward their own favor (**Table 1**). In this review, a selection of potent immunoregulatory factors of certain nematodes as well as schistosomes are described in detail. We will not discuss mechanisms of immunomodulation by helminth-derived exosomes and small RNAs which are covered by other recent reviews (56–58).

INTERFERENCE WITH CYTOKINE RESPONSES BY SECRETED FACTORS FROM *H. BAKERI* AND OTHER NEMATODES

Many helminth infections elicit rapid release of IL-33, a member of the IL-1 family. IL-33 has a short-lived role in the early immune response, being released by necrotic epithelial and endothelial cells. It is then quickly oxidized, leaving it inactive (59). It can function as an alarmin to alert the immune system of recent tissue damage or stress. The receptor for IL-33, composed of ST2 (IL-1RL1) and IL-1 receptor accessory protein (IL-1RAcP) is mainly expressed by innate immune cells and Th2 cells, driving the Th2 cell immune response and inducing a strong cytokine production (60). To counteract this response *H. bakeri* releases H. polygyrus alarmin release inhibitor (HpARI). It consists of three complement control protein (CCP) modules, one of which, the N-terminal CCP module pair (CCP1/2) binds to nuclear DNA. The remaining module binds active IL-33, attaching it to the DNA, hindering its release from necrotic

TABLE 1 | List of helminth-derived factors and their described functions.

Immunomodulating molecule		Parasite/s	Effects	References
HpARI		*H. bakeri*	Hinders release of IL-33	(16, 17)
HpBARI		*H. bakeri*	Blocks ST2	(18)
HpBARI_Hom2				
Unknown factor		*H. bakeri*	Stimulates IL-1β production in immune cells	(19)
HpTGM		*H. bakeri*	Ligand for TGFβ receptor	(20–22)
Unknown factor		*H. bakeri*	Decreases Smad7 in FoxP3$^-$ IL-10$^-$ CD4$^+$ T cells; Promotes Treg cell differentiation	(23)
GDH		*H. bakeri*	Induces an anti-inflammatory eicosanoid shift in macrophages	(24)
p43		*T. muris*	Binds IL-13	(25)
PAS-1		*A. suum*	Decreases eosinophilia; Lowers Th2 cytokines; Diminishes IgE; et al.	(26–29)
CPIs	HpCPI	*H. bakeri*	Immunomodulation of DCs	(30)
	AlCPI	*A. lumbricoides*	Affects perivascular infiltrating cells; Influences eosinophils, neutrophils and goblet cells in the lung; Reduces Th2 cytokines; Shift to IgG; Increases Tregs in spleen; Immunomodulation of HmoDCs	(31, 32)
	Nippocystatin	*N. brasiliensis*	Inhibition of T cell proliferation & cytokine production; Decreases IgE level; Inhibits processing by lysosomal cysteine proteases	(33)
	AvCystatin	*A. viteae*	Reduce APC efficiency, T cell response and allergy; Induce regulatory macrophages	(1)
	Onchocystatin	*O. volvulus*		(1)
	SjCystatin	*S. japonicum.*		(1)
AChE		*S. mansoni, S. haematobium, S. bovis, S. japonicum, N. brasiliensis, F. hepatica, D. caninum, et al.*	Motoneuronal function; Alters macrophage response; Influences cytokine production	(34, 35)
Na-ASP2		*Necator americanus*	Involved in tissue migration process; Induces neutrophil and monocyte influx; Supresses B cell receptor signaling	(36–38)
Nb-DNase	II	*N. brasiliensis*	Cuts NETs from neutrophils	(39)
smCKBP		*S. mansoni, S. japonicum, S. haematobium*	Influences recruitment of immune cells and granuloma size; Binds certain chemokines	(40, 41)
IPSE/α1		*S. mansoni, S. haematobium, et al.*	Induces IL-4 and IL-13 release from basophils; Induces IL-10 in B cells; Binds IgE	(14, 42–48)
Omega-1		*S. mansoni*	Th2 cell polarization; Drives DCs to promote Th2 cells; Downregulates DC maturation, function, and cytokine production; Enhances IL-1β production in peritoneal macrophages	(49–51)
Unknown factor		*S. mansoni*	Initiates DC driven Th2 cell polarization	(52)
SmSP2		*S. mansoni*	Hinders blood clot formation; Promotes migration, host invasion & immune evasion mechanisms; Processing of nutrients	(47, 53)
Calpain		*S. mansoni and S. mekongi*	Cuts fibronectin	(54, 55)

cells and therefore, preventing interaction with its receptors on immune cells. Studies have shown that HpARI interferes with the mode of action of both human and mouse IL-33. It was proven that HpARI successfully reduced the eosinophilic response following *N. brasiliensis* infection and increased the worm burden significantly. It was also shown to weaken the reaction of ILC2s in an allergy related model (16, 17). *H. bakeri* further secretes two factors named HpBARI and HpBARI_Hom2 which also contain CCP modules and directly block the IL-33 receptor ST2 (18). However, this is not the only way *H. bakeri* is able to diminish the IL-33 as well as the IL-25 response. It is also capable of inducing the production of IL-1β, mainly in regions with inflammatory cells in the peritoneum and the intestine (19). Here, levels are especially high in areas with a high parasite burden. The source of IL-1β are multiple cell types, nonetheless, CD11b$^+$ macrophages seem to be the main generators in an infection with helminths. Yet to be identified *H. bakeri* products stimulate IL-1β production in immune cells via the NLRP3 inflammasome pathway and NFκB activation in an inflammatory environment (19). A receptor for IL-1β, IL-1R1, is predominantly

expressed on intestinal epithelial cells. It was proven that IL-1β signaling in a parasitic context leads to a diminished expression of IL-33 and IL-25. Therefore, this mechanism of *H. bakeri* seems to be a very effective self-protective response (19).

Another immunomodulatory mechanism of *H. bakeri* is the activation of the TGFβ receptor on T cells in order to induce the production of Treg cells and various other immunoregulatory immune cells. TGFβ shows a large variety of anti-inflammatory functions. Made for example by dendritic cells (DCs) and Treg cells, it regulates a variety of different immune cells. It inhibits antigen presentation on DCs and macrophages, downregulates the effector functions in macrophages and NK cells, but also increases chemotaxis in eosinophils, mast cells and macrophages. Regarding the adaptive immune response, TGFβ decreases the cytotoxicity in $CD8^+$ effector T cells, as well as IFNγ expression and migration of resident memory T cells, while upregulating CD103 integrin. It also promotes the development of Treg cells, Th17 cells, Th9 cells, and IgA producing plasma cells, whereas it inhibits development of Th1 cells, Th2 cells, Th22 cells, and cytotoxic T lymphocytes (CTLs) (61). While some effects of TGFβ can have hostile activity against parasites, the immunoregulatory functions are outweighing the stimulatory effects. Interestingly, *H. bakeri* produces a TGFβ mimic protein (HpTGM) which has no structural homology to TGFβ, but still acts as a ligand for the TGFβ receptor triggering mouse and human FoxP3 expression in T cells (20). HpTGM is made up of five domains distantly related to CCP, and a family of homologs of HpTGM have been found in secretions of *H. bakeri*. However, TGFβ receptor ligand functions have only been proven in some of them (21). Other effects similar to TGFβ function, like promoting production of IL-17 and no induction of Th1 or Th2 cell mechanisms were also shown in HpTGMs (22).

Apart from secreting these TGFβ mimic proteins, *H. bakeri* also promotes TGFβ and IL-10 production by host cells in the gut and decreases Smad7 in $FoxP3^-$ $IL\text{-}10^-$ $CD4^+$ T cells. Smad7 inhibits TGFβ signaling by blocking phosphorylation of Smad2/3, the start of the receptor signaling cascade. When Smad7 is downregulated in the $CD4^+$ T cells, they respond to TGFβ by differentiation into $FoxpP3^+$ and/or $IL\text{-}10^+$ Treg cells (23). Providing these different mechanisms to enhance TGFβ signaling further promotes the survival of *H. bakeri*.

H. bakeri further induces an anti-inflammatory eicosanoid shift in macrophages by secretion of glutamate dehydrogenase (GDH) (24). Macrophages respond to GDH by induced expression of cyclooxygenases and production of prostaglandin E2 (PGE2) which displays some anti-inflammatory functions. This effect appears to be dependent on p38 MAPK activity and requires expression of hypoxia-inducible factor-1 alpha (HIF-1α).

The most abundant secreted protein p43 of the murine whipworm *Trichuris muris* was recently crystallized and identified as IL-13-binding protein which can inhibit IL-13-induced differentiation of alternatively activated macrophages (25).

Ascaris suum, the giant roundworm which inhabits the gut of pigs, secretes another protein with strong immunomodulatory function for the host cytokine system. This so-called Protein 1 from *Ascaris suum* (PAS-1) displays anti-inflammatory attributes in the lung and can diminish eosinophilia, decrease Th2 cytokines and lower IgE levels (26, 27). PAS-1 is secreted by larval and adult *A. suum* and apart from reducing eosinophils, it also impairs eosinophil peroxidase activity, and reduces levels of IL-4, IL-5, IL-13, and eotaxin. Additionally, it lowers expression of TNFα, IL-1β, and IL-6 in peritoneal macrophage cultures, increases IL-10 and TGFβ and suppresses influx of neutrophils. The partial induction of regulatory cytokines in macrophages could be one of the main reasons why PAS-1 is decreasing inflammation. Moreover, the anti-inflammatory traits of PAS-1 seemingly depend on the presence of IFNγ and IL-10, suggesting that these factors are necessary for the mode of action of PAS-1 (28). It also diminishes both the level of IgE and IgG1 in the serum of its host (29). Despite these various effects, the molecular mechanism of PAS-1-regulated immune responses remains to be identified.

HELMINTH-DERIVED CYSTEINE PROTEASE INHIBITORS AFFECT ANTIGEN PRESENTATION

Other main modulators of the immune system produced by nematodes are cystatins, also called cysteine protease inhibitors (CPIs). CPIs effectively inhibit proteases, which are responsible for many immune functions. Antigen recognition by the immune system is dependent on cleavage of foreign proteins to be displayed as peptide antigens in MHC II on the surface of antigen presenting cells (APCs). By inhibiting the responsible proteases for this process, parasites gain another possibility of modulating the immune response. CPIs are detected in many different parasites and show a wide variety of effects on many immune cells. For example, *H. bakeri* CPI (HpCPI) displays a strong immunomodulatory response in DCs. Bone marrow-derived $CD11c^+$ DCs (BMDCs), when treated with HpCPI and stimulated with CpG, a Toll-like receptor 9 ligand, have a lowered expression of CD40, CD86, and MHC class II, while also producing less IL-6 and TNFα (30). Further, CPI treated BMDCs are unable to effectively induce proliferation and IFNγ production in $CD4^+$ T cells. Likewise, it was shown that they lead to a weaker antigen-specific antibody response as an untreated control (30). In a mouse model displaying allergic airway inflammation responding to house dust mite, it was shown that CPI from *Ascaris lumbricoides* (AlCPI), one of the most common parasitic worms found in humans, especially affects perivascular infiltrating cells, eosinophils, neutrophils and goblet cells in the lung and reduces Th2 cytokines (31). Also, a distinct shift from IgE antibodies to IgG, mostly IgG2a, was reported. Furthermore, AlCPI leads to an increase in Treg cells in the spleen. *In vitro* tests with human monocyte derived DCs (HmoDCs) treated with AlCPI lead to an immunomodulatory effect, reducing HLA-DR, CD83, and CD86 and inducing IL-10 and IL-6 levels. It also resulted in a stop in HmoDC maturation (32). In a similar fashion, the nematode *N. brasiliensis* expresses the CPI nippocystatin. Tested *in vivo* in an ovalbumin (OVA) immunized

mouse model, it proved to inhibit proliferation of OVA-specific T cells and production of cytokines. IgE levels also significantly decreased, whereas IgG1 and IgG2 levels did not decline. *In vitro*, it was proven that processing of OVA by lysosomal cysteine proteases was inhibited by nippocystatin (33). A multitude of other helminths have also evolved to expressing different cystatins, examples are AvCystatin from *Acanthocheilonema viteae*, Onchocystatin from *Onchocerca volvulus*, or SjCystatin from *Schistosoma japonicum*. They exhibit many similarities in their function, like reducing APC efficiency, T cell response and allergic reaction or inducing regulatory macrophages (1). Therefore, expression of cystatins indeed appears as successful parasitic immune escape strategy.

HELMINTH-DERIVED ACETYLCHOLINESTERASES MODULATE THE IMMUNE RESPONSE

Acetylcholinesterase (AChE) represents a helminth-derived factor which directly interferes with intracellular signaling and gene expression. AChE is expressed in a variety of helminths, where it has been shown to have a broad range of functions. On one side, it is needed to ensure motility, for example in flatworms. Here, AChE controls the communication between acetylcholine (ACh) and nicotinic acetylcholine receptors (nAChR). ACh regulates muscular contraction by membrane depolarization due to ion influx in cells, as its receptor acts as an ion channel. To inhibit this interaction and prevent overstimulation, AChE cleaves ACh to choline and acetate. Because of its important motoneuronal function, AChE is seen as a possible target for drugs against schistosomiasis, because many schistosomes, such as *S. mans*oni, *S. haematobium*, *S. bovis*, and *S. japonicum* do express AChE. However, a drug targeting AChE, Metrifonate, was not approved due to high toxicity in the host. Further research might validate AChE as a possible vaccine target. AChE was also detected in other helminths besides schistosomes, such as in *N. brasiliensis*, *Fasciola hepatica*, and *Dipylidium caninum* (34).

Since several types of nAChRs are also present in mammals, executing diverse neurological and muscular signaling functions (62), helminths are also secreting AChE to influence host cell behavior. Transgenic *Trypanosoma musculi*, when expressing an AChE secreted by *N. brasiliensis*, showed a reduced infectivity and lead to splenocytes producing increased amounts of IFNγ and TNFα, while expressing less IL-4, IL-5, and IL-13 (35). This altered cytokine response could explain the observed enhanced macrophage M1 response with diminished arginase-1 activity and increased nitric oxide production. It was suggested that this shift to an M1 immune response might inhibit the induction of AAMs and interfere with expulsion of helminths. It was further shown that macrophages have a cholinergic anti-inflammatory pathway, where signaling with acetylcholine inhibits TNFα release in macrophages (63). The increased TNFα response due to helminth-derived AChE could also reinforce the M1 driven immune response. In mammalian immune cells, AChE is reduced when they encounter large amounts of LPS. A reduced expression results in an increase of ACh and less release of TNFα and several other cytokines. This anti-inflammatory response might protect the body from inflammatory overstimulation (64). Therefore, helminths seem to use the AChE, a factor they already expressed to regulate their muscular functions, and secrete it in order to direct their host's innate immune response away from an anti-helminthic activity.

CHEMOKINE MIMICS AND CHEMOKINE BINDING PROTEINS

Ancylostoma secreted protein 2 (ASP2) is produced by different hookworms, including Ancylostoma caninum, Ancylostoma ceylanicum, and Necator americanus. The latter secretes ASP2 (Na-ASP2) during its L3 larval stage. The protein has been considered as a promising target for anti-helminthic vaccines (36). It became of special interest as it is significantly involved in tissue dwelling processes and proteins of this nature are seen as good targets for vaccine strategies. It was proven that Na-ASP2 plays an important role in entry into the host tissue and migration before arriving in the intestine. Specifically, it induces leukocyte influx, mainly composed of neutrophils and monocytes. It is most likely able to act as a chemokine mimic for these cells as it shows similarities in structure and charge to CC-chemokines (37). It might be of question, why a helminth would willingly attract neutrophils, as they are known to trap pathogens. Indeed, skin-penetrating larvae of hookworms cause rapid recruitment of neutrophils which release neutrophil extracellular traps (NETs) trying to immobilize the invaders in the skin. However, the larvae then secrete a deoxyribonuclease (Nb-DNase II) to destroy the NETs enabling them to migrate further to the lung (39). Hookworm larvae may further benefit from edema and local inflammation caused by neutrophils as it increases the permeability of tissues and therefore, enable larval migration with ease through dense tissue of the host. Additionally, an increased amount of neutrophils could decrease contact of the helminth to other immune cells like NK cells or eosinophils, which could cause even more harm to the parasite (37).

However, neutrophil recruitment is not the only effect of Na-ASP2. In a human proteome microarray test, it was seen that Na-ASP2 binds to CD79A, a crucial part of the B cell antigen receptor complex. In human B cells, Na-ASP-2 is able to downregulate the transcription of about 1,000 messenger RNAs (mRNAs) and upregulate around 100 mRNAs. This subsequently leads to a different expression of a multitude of proteins derived from B cells, which notably affect transendothelial migration and suppresses B cell receptor signaling (38).

While parasite larvae require a way to actively migrate through tissue, schistosoma eggs need a possibility to translocate in order to be released from the host's body. Therefore, the blood fluke developed a secretion factor to ensure efficient expulsion of eggs from the host. Surrounded by granulomatous inflammation, many schistosome eggs are trapped in organ tissue, hence, *S. mansoni* eggs have been found to secrete the *S. mansoni* chemokine binding protein (smCKBP), which influences recruitment of immune cells and the size of the surrounding granuloma (**Figure 2**). Furthermore, smCKBP was also found to be expressed in *S. japonicum* and *S. haematobium*,

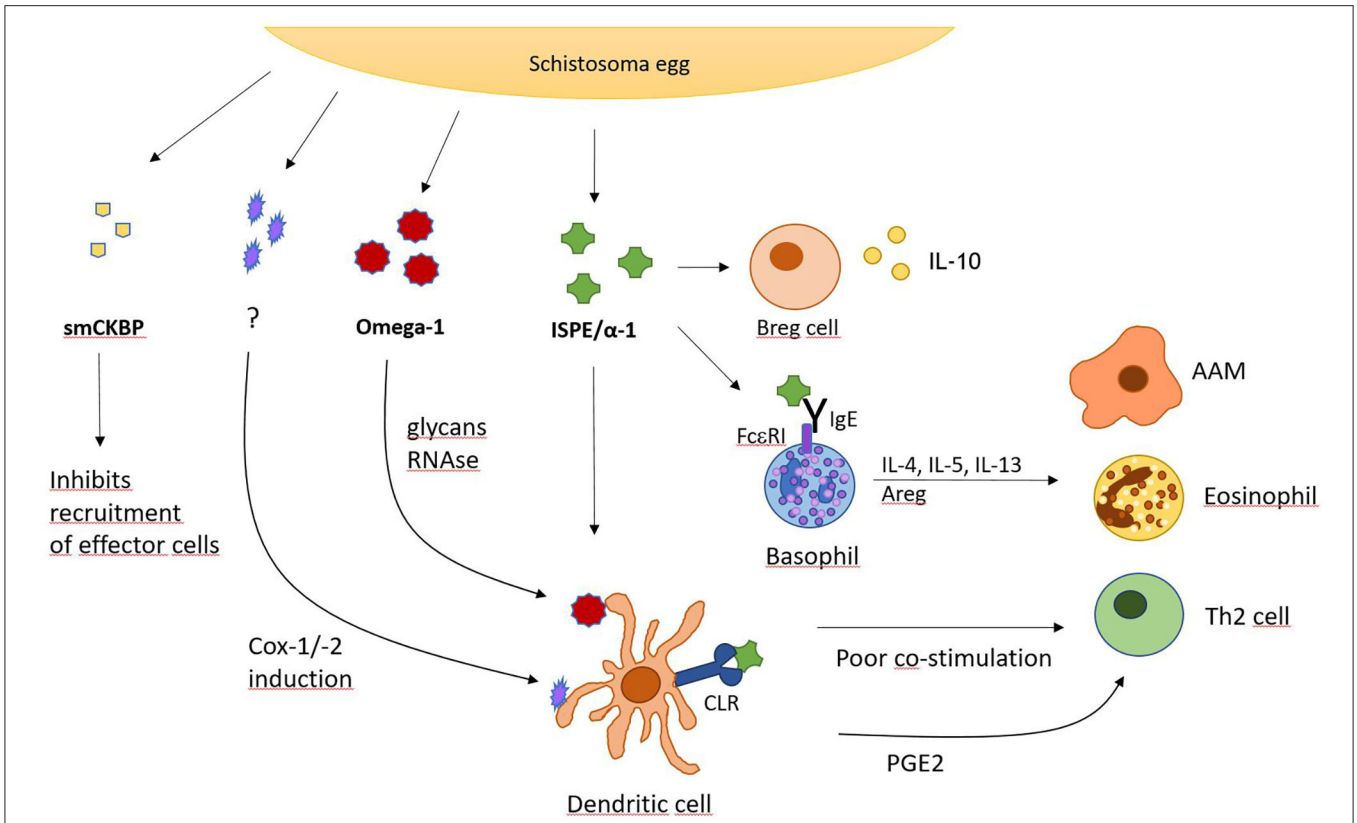

FIGURE 2 | *S. mansoni* egg-derived factors with immunomodulatory activity. Overview of secreted factors from *S. mansoni* eggs and their action on indicated cell types. CLR, C-type lectin receptor; Cox, cyclooxygenase; PGE2, prostaglandin E2.

however, only schistosoma eggs seem to express smCKBP, but none of the other life cycle stages. The smCKBP does interact with a variety of chemokines, including CXCL8, CX3CL1, CCL2, CCL3, and CCL5. By binding to these chemokines, smCKBP prevents them from interaction with their receptors and by this, inhibits chemokine receptor-mediated migration or activation of cells (40, 41). CXCL8, or IL-8, particularly promotes chemotaxis and degranulation of neutrophils (65), but also influences macrophages and mast cells. CX3CL1 does have a chemoattracting effect on monocytes, NK cells and T cells (66). CCL2 is mainly driving chemotaxis of monocytes, but also other cell types, and can regulate further mechanisms, like cytokine secretion or cell adhesion (67). Since CCL3 and CCL5 do have chemotactic properties as well, it is clear that smCKBP targets mainly chemotaxis of immune cells which are for example involved in building the granuloma. Therefore, the protein weakens the surrounding structure, making expulsion of the egg possible. Even though smCKBP has the ability to bind to chemokines, its primary structure has no similarity to proteins from mammals or viral CKBPs. Another interesting finding about smCKBP is that if mice are immunocompromised and for example do not possess $CD4^+$ T cells, *S. mansoni* eggs are not excreted as efficiently. Further, smCKBP does not interfere with CCL11 (also named eotaxin-1), and therefore, does not stop eosinophil infiltration (41).

OTHER IMMUNOMODULATORY FACTORS OF SCHISTOSOMES

IPSE/α1

Besides smCKBP, schistosomes express other potent immunomodulatory factors such as Interleukin-4-inducing principle from schistosome eggs (IPSE), also named α1. IPSE/α1 has been detected in different schistosome species, including *S. mansoni* and *S. haematobium*, which express homologs of IPSE/α1, showing some dissimilarities for example in immunomodulation and expression in life cycles (42). IPSE/α1 from *S. mansoni* eggs, which has been described quite extensively, strongly induces IL-4 as well as IL-13 in basophils and promotes an antibody response. IPSE/α1 is exclusively expressed in the egg stage and released from the subshell area of the egg where it comes into close proximity with various immune cells. Because it induces IL-4, it is presumably involved in activating the Th2 response that is created by *S. mansoni* infections (43). Also, the IL-4 and IL-13 production from basophils induced by IPSE/α1 diminishes inflammatory cytokine responses. This correlates with the fact, that IL-4 and IL-13 also induce AAMs. When stimulated with LPS and IPSE/α1, a stronger IL-4/IL-13 production is generated in basophils and a reduction of inflammatory cytokines in human monocytes is seen. These monocytes also develop an AAM-like phenotype with elevated

CD206 and CD209 (44). If the host is not able to produce IL-4 alone or IL-4 with IL-13, the mortality rate increases as the eggs are not excreted sufficiently, causing endotoxemia. Mice with impaired IL-4 production from T cells also exhibit lower granuloma formation and suffer from cachexia (14).

Additionally, it was seen that IPSE/α1 induces IL-10 in naïve B cells. For recombinant IPSE/α1 produced in tobacco plants, it was also shown to be able to induce IL-10 production in human $CD1d^+$ B cells. Consistent with these findings, IPSE/α1 is capable of promoting human and murine Breg cells and induce IL-10 production here as well, which results in a reduction of inflammation. The increased level of IL-10 promotes Treg cell development, shifting the host's immune response to a downregulated phenotype. It was also proven in this context, that the development of Breg cells in the marginal zone was not dependent on macrophages, however, it was increased via CD40 ligation (45).

Furthermore, IPSE/α1 binds to IgE via an antigen-unspecific mechanism. This has led to the assumption that it is activating basophils by cross-linking IgE bound to the high-affinity IgE receptor on the cell surface, resulting in release of histamine and activation of cytokine production in Th2 cells (46). However, it was also proposed that IPSE/α1 instead acts via a cross-linking independent mechanism (47). More research might be required to answer these pending questions. Nevertheless, IPSE/α1 does not only act as an extracellular signaling factor but is internalized by certain cell types. It contains a nuclear localization signal (NLS) at its C terminus, which ensures that IPSE/α1 is translocated into the nucleus. When expressed with an enhanced green fluorescent protein (eGFP), extracellular IPSE/α1-eGFP was taken up by CHO cells and transported to the nucleus. This process depends on the presence of the NLS. Human primary monocyte-derived DCs are able to internalize glycosylated IPSE/α1 as well. This mechanism appeared to be dependent on calcium and temperature levels. IPSE/α1 might also directly bind to DNA, regulate gene expression in immune cells and therefore, alter the immune response to the parasite's favor.

A likely way of the host cells to take up IPSE/α1 could be via C-type lectin receptors. These receptors bind to carbohydrates and are expressed on many cell types, including immune cells. IPSE/α1 is glycosylated with two N-glycan sites which are expressing Lewis X motifs (48). Given these circumstances, it is reasonable to speculate that IPSE/α1 is internalized by DCs or macrophages via the C-type lectin receptor pathway but not by basophils which express less C-type lectin receptors (68). Alternatively, DCs and macrophages may take up IPSE/α1 by Fc receptors when antibodies have bound to IPSE/α1. Basophils rather get activated via an IgE-dependent mechanism. In conclusion, IPSE/α1 from *S. mansoni* eggs is a perfect example of how variable a single immunomodulatory protein can be in ways of cell entry and effects on immune cells. However, more research is required to fully understand the impact of this molecule on the host's immune response.

Omega-1

Another important immunomodulatory glycoprotein produced by *S. mansoni* eggs is Omega-1. This molecule is one of the most abundant proteins in *S. mansoni* egg antigen (SEA) preparations with powerful influence on its host. It is able to drive DCs to a Th2 cell polarization phenotype and hence, beginning of egg production by adult flukes is associated with onset of Th2 development. Th2 cell polarization is usually supported by many different cell types like mast cells, basophils, and eosinophils, which produce IL-4 and other cytokines to induce and promote differentiation and survival of Th2 cells. Apart from these innate immune cells, APCs, especially DCs are also potent drivers of the Th2 response. They capture antigens shed from *S. mansoni* eggs and omega-1 was shown to instruct DCs to prime naive CD4+ T cells to develop into Th2 cells. It can alter and downregulate maturation and function of DCs and cytokine production. This is also shown in DCs treated with LPS, a glycolipid that drives maturation of DCs, as the presence of omega-1 impaired LPS-induced upregulation of CD83 and CD86 on the cell surface (49). Both CD83 and CD86 are important co-stimulatory molecules required for efficient T cell activation.

Furthermore, omega-1 not only stimulates maturation of Th2 cells, but acts as an initiating factor in Th2 differentiation as it promotes acute production of IL-4 *in vivo*. However, it was seen that the omega-1-induced Th2 response does not depend on IL-4 signaling, despite it being a potent Th2 cell driving cytokine (49). Moreover, omega-1 shows RNase activity as well as glycosylation (50). It was shown by site-directed mutagenesis that both do play an important role in Th2 cell polarization via DCs stimulated by omega-1. If either the RNase function or the glycosylation of omega-1 are impaired, DCs are not effectively conditioned to prime a Th2 cell response (50). The DCs take up omega-1 by binding to its glycans via mannose receptors on the cell surface making this internalization process highly dependent on the glycosylation pattern of omega-1. Once the protein is taken up, it degrades ribosomal and messenger RNA and therefore disrupts protein synthesis, conditioning DCs to prime Th2 reactions. In addition, Lewis-X glycan motifs were described on omega-1 which could also polarize Th2 cells (50).

Omega-1 also enhances the production of IL-1β in peritoneal macrophages when stimulated with TLR2 ligands (51). This does not happen with splenic macrophages or DCs. IL-1β is produced when the macrophage receives a signal, for example via TLR2, which triggers NFκB activation and transcription of pro-IL-1β. It requires a second signal, for example by Dectin-1 to form the inflammasome composed of caspase-8 and the ASC inflammasome adaptor protein (also named Pycard). The inflammasome will cleave pro-IL-1β and it will be released from the macrophage. It was shown that omega-1 is able to upregulate inflammasome activity and therefore, increase IL-1β production. The upregulation of IL-1β via inflammasome requires the presence of C-type lectin receptor Dectin-1, ASC, and caspase-8 (51). This showcases the ability of omega-1 to influence multiple pattern recognition receptor pathways and clearly demonstrates once again the multifarious ways a single factor from helminths can influence the immune system.

However, recent research has also discovered an omega-1-independent mechanism on how SEA drives DCs to prime Th2 cells through Dectin-1 and−2 signaling (52). While the exact factor responsible for this second mechanism remains unknown, it was seen that SEA promoted prostaglandin E2 (PGE2) production upon activating Dectin-1 and−2. Activation is followed by a signaling cascade including spleen tyrosine kinase (Syk), extracellular signal-regulated kinase (Erk), cytosolic phospholipase A2 (cPLA2) and cyclooxygenases 1 and 2 (Cox-1, Cox-2). Production of PGE2 leads to expression of OX40 ligand and this subsequently allows DCs to induce Th2 responses (52). Therefore, omega-1 is not the only schistosome-derived factor able to initiate DC-regulated Th2 activation.

SmSP2

Blood flukes not only need to influence the behavior of immune cells and ensure egg expulsion. Adult schistosomes are of large size and should hence alter normal blood flow and damage endothelial cells around them. This would normally lead to platelet activation and ultimately to the formation of blood clots. Despite this, blood coagulation is hardly ever observed around schistosomes residing in the host's blood vessels. Several mechanisms of schistosomes to hinder blood clot formation have been proposed and one of them is the production of serine protease SmSP2 (69). It consists of three domains: a serine protease domain, a thrombospondin type 1 repeat (TSR-1) and a histidine stretch. SmSP2 orthologs are also found in larval cestodes, *Echinococcus granulosus*, and *Taenia solium*. In adult *S. mansoni* SmSP2 can be found in the tegument and in the excretory/secretory products. It is present at the interface between host and parasite, promotes many different mechanisms in the parasite's migration, invasion of the host and immune evasion, while also being involved in the processing of nutrients. This omnipresence could render it as a potent target for anti-helminthic drugs.

To hinder blood coagulation in its host which would cause not only harm to the parasite itself, rendering it immobile, but also to the host, SmSP2 is especially present in the secreted products. Here, it shows different effects on proteins involved in blood clotting and even regulates the vascular tone. For example, it inactivates vasopressin, a hormone responsible for vasoconstriction leading to increased blood pressure. It also promotes fibrinolysis by activating plasminogen to plasmin, a host protein cleaving fibrin in coagulated blood. It further enhances the production of bradykinin from the host's endothelium, a protein that leads to release of tissue plasminogen activator (tPA). tPA cuts plasminogen to plasmin as well, further reducing blood clots. Additionally, it splits tPA into its more active double chain form, causing even more increase of plasmin. Furthermore, SmSP2 degrades fibronectin in blood clots and the TSR-1 domain in SmSP2 is capable of controlling cell adhesion, which basically allows interaction with other proteins, binding of glycosaminoglycans and inhibits angiogenesis near the schistosome (69). SmSP2 is therefore another clear example of how various the effects of one produced immunomodulatory factor can be. It is also especially interesting to see how diverse the expression pattern of modulatory molecules is between schistosome eggs and adults and that other parasites use the same molecule in different life stages. However, further research is needed on the effect of SmSP2 orthologs in other parasites. Additionally, there are other helminthic proteins showing similarities to SmSP2 in the way they affect the host. In this way, schistosomes possess several other mechanisms to hinder blood clot formation. For example, *S. mansoni* and *S. mekongi* express a tegumental calpain which is capable of cutting fibronectin similar to SmSP2 (54, 55).

Many serine proteases produced by helminths are quite well-described and showcase multiple modes of action. Besides influencing blood coagulation, they also influence the intra- and extracellular metabolism, regulate development and even digestion. As already shown with a few examples in this review, they can influence the composition of the host's tissue, alter cell invasion and help evade the immune system. These characteristics make them probably the most versatile immunomodulatory factors helminths present in the fight against the hosts immune system (53). Many serine proteases would be worth taking a closer look at but describing them would go beyond the scope of this review.

DISCUSSION

The co-evolution of helminths and their hosts led to an extremely broad range of mechanisms by which helminths influence the immune system of their hosts. They modulate migration, activity, cytokine production and differentiation of innate and adaptive immune cells. The mechanisms to influence the cells are just as diverse as the effects on the immune system in general. The parasites produce proteases to cleave or otherwise influence ligands to hinder activation, secrete adaptors that bind to receptors on the cell surface and influence intracellular signaling cascades. They induce mucus production, promote epithelial cell turnover in mucosal tissues and alter cellular responses. They secrete proteins similar to cytokines or chemokines, inhibit a variety of host factors and can influence the host's RNAs, lowering their expression or cutting them to render them useless for the host.

Consistent with the general notion that most helminths elicit type 2 immune responses, many studies mentioned in this review showed that helminths promote the activation and proliferation of Th2 cells and AAMs. These cell types can be involved in an anti-inflammatory response to infections or tissue damage. Nonetheless, Th2 cells are also described as immune effector cells that promote worm expulsion. Therefore, it might seem strange for helminths to strongly enhance the Th2 cell response. However, it was shown that a short initial Th2 response can contribute to worm expulsion, while long-term activation of type 2 immunity may lead to a shift of the T cell pool to increase the number of Treg cells (70). This is a natural reaction of the host's body to hinder overstimulation of the immune system and therefore, excessive collateral tissue damage in chronic inflammation. The helminths seem to use this response to lower inflammatory reactions and protect themselves from the immune

cells, but in the same way also protect the host from tissue damage, even leading to a tissue repairing phenotype. This might be especially useful to the host when the parasite is migrating through tissue to reach different organs.

Distinct macrophage responses also seem to be favored by helminths. This can be explained particularly by AAMs producing anti-inflammatory molecules and helping with clearance of cell debris to avoid a disproportionate recruitment of immune cells. Especially when parasites are damaging tissue due to their large size or when migrating, destroyed cells release alarmins that will attract inflammatory immune cells. Clearance of these alarmins by AAMs helps contain the inflammation but also protects the worm and the host from unnecessary tissue damage. Most sources are consistent with these explanations, but nevertheless, there have also been reports stating that for example the AAM response is unsafe for helminths and instead, the parasite seeks to inhibit AAM production and accepts the production of classically activated macrophages (71). In fact, it has been shown that AAMs protect mice from secondary infections with *H. bakeri* (72) and *N. brasiliensis* (73). Nonetheless, more research is required to understand how AAMs regulate anti-helminth immunity and restore tissue integrity.

The immunosuppressive activity of some helminths can also affect other immune reactions and, for example, ameliorate allergic responses. Hookworm infections can have protective properties against asthma and alleviate atopy caused by some allergens (74). Helminth infections also lead to decreased severity in skin prick tests. On the other side, some helminths, like *A. lumbricoides*, can significantly increase the risk for developing asthma (75). In some instances, helminth infection also worsened allergic responses in the clinical outcome and increased prevalence on aeroallergen-specific IgE. This response could be due to helminth-elicited IgE with cross-reactivity to allergens. Interestingly, many allergens share similarities to secretory products from helminths (76). Epidemiological studies have been performed in countries with poor sanitary conditions and high prevalence of helminth infections. Overall, the results are very inconsistent. Decreases, increases and no change in allergic outcome were observed in association with helminth infections (77). Many other reasons have to be taken into consideration when trying to explain the rise of allergies in developing countries over the past several years, like modernization of life style and a shift to consumption of more highly processed food items. Taken together, helminths seem to be partially able to ameliorate allergic reactions, but numerous studies also show a negative effect on allergic outcome. Because the effects are unpredictable and complex, more investigation on the connection between helminth infections and allergy should be done in order to potentially develop new anti-allergic therapeutics.

Given this immunomodulatory capacity of helminths, it is reasonable to assume that they may interfere with efficient vaccinations against pathogenic bacteria and viruses. Most vaccination strategies rely on the activation of a strong Th1 response along with proliferation of vaccine antigen-specific antibody-producing plasma cells. Since helminths interfere with the host's immune response, some vaccinations seem to be less efficient when administered to a helminth infected patient. When helminth infected mice are administered an influenza virus vaccine, protection against challenge infection, which would normally be provided by the vaccine, is not given anymore. This loss of protection is even seen after the helminth infection is cleared from the mice (78). A similar effect was seen in *S. mansoni* infected persons, where vaccination against tetanus toxoid showed altered immune responses in stimulated peripheral blood mononuclear cells (79). Therefore, preventing helminth infections may help to improve vaccination efficiencies. This could be achieved by raising the hygiene standards and by developing anti-helminthic vaccines which are not yet available for any of the human helminth infections. However, there is an ongoing search for potent vaccine candidates and vaccination strategies (80, 81). Administered at a young age, anti-helminthic vaccines would prevent infections and all negative consequences caused by the infection. Although there are many anti-helminthic drugs available, they have to be administered regularly, as the patients are very likely to become reinfected with parasites once treatment is completed. Thus, a vaccine would be the go-to strategy to ensure long-term protection. A recombinant vaccine against cestodes in livestock has shown efficacy and more vaccines are being developed and tested constantly (82). Despite these accomplishments, further research must be done in order to create an effective, easily producible vaccine approved for use in humans.

CONCLUSIONS

Helminths in general show many similarities in the way they influence the immune system or the way the immune system reacts to them. The most prominent responses are the activation of Th2 cells and AAMs, followed by activation of regulatory T cells and the induction of IgE and IgG4. However, the mechanisms used to influence the immune response are surprisingly diverse. While some helminths seem to have developed modulatory molecules directly against immune functions during thousands of years of co-evolution, others might just have had a role in the metabolism or in the motor function of the parasite. Nevertheless, helminths secrete an extremely broad spectrum of immunoregulatory molecules in order to ensure survival inside their host. The influence of these molecules is not only restricted to the immediate environment of the helminth. It can have consequences in the entire body, influencing for example allergic reactions and the efficacy of vaccinations. Clearly, more research is required in order to completely understand the fascinating world of molecular mechanisms used by parasites and the true impact they have on their hosts.

AUTHOR CONTRIBUTIONS

MW and DV wrote the manuscript and designed the figures. All authors contributed to the article and approved the submitted version.

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Trichinella spiralis Thioredoxin Peroxidase 2 Regulates Protective Th2 Immune Response in Mice by Directly Inducing Alternatively Activated Macrophages

Qi-Wang Jin[1,2†], Nian-Zhang Zhang[1†], Wen-Hui Li[1], Hong-Tao Qin[1], Yin-Ju Liu[1], John Asekhaen Ohiolei[1], Dong-Yu Niu[1], Hong-Bin Yan[1], Li Li[1], Wan-Zhong Jia[1], Ming-Xin Song[2] and Bao-Quan Fu[1,3]**

[1] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China, [2] College of Veterinary Medicine, Northeast Agricultural University, Harbin, China, [3] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China

Trichinella infection can induce macrophages into the alternatively activated phenotype, which is primarily associated with the development of a polarized Th2 immune response. In the present study, we examined the immunomodulatory effect of *T. spiralis* thioredoxin peroxidase-2 (TsTPX2), a protein derived from *T. spiralis* ES products, in the regulation of Th2 response through direct activation of macrophages. The location of TsTPX2 was detected by immunohistochemistry and immunofluorescence analyses. The immune response *in vivo* induced by rTsTPX2 was characterized by analyzing the Th2 cytokines and Th1 cytokines in the peripheral blood. The rTsTPX2-activated macrophages ($M_{rTsTPX2}$) were tested for polarization, their ability to evoke naïve $CD4^+$ T cells, and resistance to the larval infection after adoptive transfer in BALB/c mice. The immunolocalization analysis showed TsTPX2 in cuticles and stichosome of *T. spiralis* ML. The immunostaining was detected in cuticles and stichosome of *T. spiralis* Ad3 and ML, as well as in tissue-dwellings around ML after the intestines and muscle tissues of infected mice were incubated with anti-rTsTPX2 antibody. Immunization of BALB/c mice with rTsTPX2 could induce a Th1-suppressing mixed immune response given the increased levels of Th2 cytokines (IL-4 and IL-10) production along with the decreased levels of Th1 cytokines (IFN-γ, IL-12, and TNF-α). *In vitro* studies showed that rTsTPX2 could directly drive RAW264.7 and peritoneal macrophages to the M2 phenotype. Moreover, $M_{rTsTPX2}$ could promote $CD4^+$ T cells polarized into Th2 type *in vitro*. Adoptive transfer of $M_{rTsTPX2}$ into mice suppressed Th1 responses by enhancing Th2 responses and exhibited a 44.7% reduction in adult worm burden following challenge with *T. spiralis* infective larval, suggesting that the TsTPX2 is a potential vaccine candidate against

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Poom Adisakwattana, Mahidol University, Thailand

***Correspondence:**
Bao-Quan Fu
fubaoquan@caas.cn
Ming-Xin Song
songmx@neau.edu.cn

† These authors have contributed equally to this work

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trichinosis. Our study showed that TsTPX2 would be at least one of the molecules to switch macrophages into the M2 phenotype during *T. spiralis* infection, which provides a new therapeutic approach to various inflammatory disorders like allergies or autoimmune diseases.

Keywords: *Trichinella spiralis*, thioredoxin peroxidase-2, Th2 immune responses, macrophage, alternative activation

INTRODUCTION

Trichinella spiralis is a significant worldwide parasitic nematode that infects humans and other mammalian species, leading to trichinosis (1). Its life cycle involves three main stages-adults (Ad), newborn larvae (NBL), and muscle larvae (ML) (2). During the initial intestinal phase, *T. spiralis* elicits a Th1 type immune response (3). The Th2 type response is activated once the worm enters the enterocyte and becomes a well-characterized phenotype during the long-lasting infection of the muscles (4). The Th2 response to helminths is orchestrated by $CD4^+$ T cells and depends mainly on highly elevated type 2 cytokines (interleukin-4, IL-5, etc.). The Th2 immune response accelerates the formation of niche or cystica, which prevents the immune system from killing the *T. spiralis*. On the other hand, it may dislodge the parasite and repair damage, thus protecting the host from excessive harm (5).

A macrophage is an important factor that regulates the immune system and has a central role in turning innate immune responses to adaptive responses (6). The macrophages display different functions based on the model of activation. Classically activated macrophages (CAMacs, also known as M1) are typically instructed by lipopolysaccharide (LPS) and interferon-γ (IFN-γ). In mice, M1 can produce intracellular-killing nitric oxide (iNO) and instigate Th1 biased responses in the host (7–9). In contrast, alternatively activated macrophages (AAMacs, also known as M2) are induced by IL-4 and IL-13. M2 macrophages abundantly express mannose receptor (MRC-1), arginase-1 (Arg-1), and chitinase-like protein (Chil3, Ym1) in mice, which activate the Th2 responses in the host (10–12). Throughout the life cycle of *T. spiralis,* excretory/secretory (ES) products are considered crucial compounds, which modulate macrophage function toward the alternative phenotype *in vitro* or *in vivo* (13, 14). However, it remains unclear whether these components derived from *T. spiralis* ES products regulate Th2 immune responses or whether these components are induced through direct action on macrophage or other immune cells.

Thioredoxin peroxidases (TPX) belong to a family of antioxidant enzymes characterized by 2-cys residues, which protect helminths from host reactive oxygen species (ROS) (15, 16). Our previous study revealed that three TPXs were expressed in three main stages of *T. spiralis* (17); the TPX2 gene expression levels were the highest in Ad3, and lowest in NBL, which matched well with the time of switching from Th1 to Th2 immune responses (17). The recombinant proteins were showed to remove exogenous H_2O_2 *in vitro* (17). TPXs from *Fasciola hepatica* can drive Th2 responses through a mechanism involving AAMacs (18, 19). Nevertheless, limited information is available on the ability of *T. spiralis* TPXs in regulating immune responses.

In the present study, we determined the polarization of macrophages that are modulated by the purified recombinant TsTPX2 (rTsTPX2) and the regulation of immune responses induced by the activated macrophages. After adoptive transfer of rTsTPX2-activated macrophages into BALB/c mice, we also evaluated the immune responses against *T. spiralis* infective muscle larval infection. Identification of the role of TsTPX2 in the regulation of immune responses during *T. spiralis* infection might help understand immunomodulatory mechanisms exploited by this parasite to create an environment suitable for its survival in the host organism.

MATERIALS AND METHODS

Animals

Specific-pathogen-free (SPF) female Kunming mice and BALB/c mice, 6–8 weeks old, were purchased from Lanzhou Veterinary Research Institute Animal Center (Lanzhou, China). All animals were housed in an environment with a temperature of 22 ± 1°C, a relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 h. All animal studies (including mice euthanasia procedure) were carried out in compliance with the regulations and guidelines of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval No. LVRIAEC2019-012), institutional animal care and conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Institute of Animal Care and Use Committee (IACUC) guidelines.

Parasites

The Chinese *T. spiralis* Henan strain (ISS534) was maintained in Kunming mice. The ML were isolated from infected mice *via* the conventional artificial digestion method, as previously described (20). The Ad3 were isolated from the small intestine of infected mice 3 days post-infection with ML *via* the standard approach, as previously shown (20).

Recombinant Protein Preparation and Anti-rTsTPX2 Polyclonal Antibody Production

The rTsTPX2 was expressed through the prokaryotic expression system *E. coli* BL-21(DE3)/pET-30a (+) and purified as previously described (17). The endotoxin in the purified rTsTPX2 protein was removed by Pierce High-Capacity Endotoxin Removal Resin

(Thermo Fisher Scientific, United States) according to the manufacturer's protocol. The purified rTsTPX2 was used for functional analysis and immunization for preparing polyclonal antibody. Briefly, a 6-month-old rabbit was given a hypodermic injection of 500 μg of rTsTPX2 followed by three boosts immunization with 300 μg of rTsTPX2 at a 14-day interval. Seven days after the final boost, blood was collected by heart puncture, and sera were purified with affinity chromatography method.

Immunofluorescence and Immunohistochemistry

T. spiralis ML harvested at 35 days post-infection were fixed by ice-cold methyl alcohol and incubated in Triton-100 overnight. Worms were then incubated with anti-rTsTPX2 antibodies and Alexa Fluor® 488 labeled secondary antibodies. Stained *T. spiralis* ML were imaged with a confocal laser scanning microscope (Leica TCS SP8).

Small intestines harvested at 3 days post-infection and diaphragms harvested at 35 days post-infection from *T. spiralis* infected BALB/c mice were fixed in 4% paraformaldehyde. Thin sections of the embedded tissues were first stained with anti-rTsTPX2 antibodies and then incubated with Alexa Fluor® 488 labeled secondary antibodies or DAB Quanto (Thermo Fisher Scientific). Confocal images of stained tissues were obtained with a confocal laser scanning microscope (Leica TCS SP8).

Immunization of BALB/c Mice

Eight BALB/c mice per group were subcutaneously immunized with rTsTPX2 (50 μg per mouse) and boosted in the second week. Mice that received phosphate-buffered saline (PBS) and BSA were used as controls. The serum samples were collected at 3 weeks post the first injection. Anticoagulant blood samples were collected at 3 weeks post the first injection for Flow Cytometry analysis.

Cell Isolation and Culture

The RAW 264.7 murine macrophages were obtained from the China Center for Type Culture Collection and were maintained in our laboratory. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% FCS (GIBCO), 2 mM L-glutamine (GIBCO) and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO) at 37°C under 5% atmospheric CO_2.

Resident peritoneal macrophages were obtained from peritoneal lavage, as previously reported (21, 22). Briefly, BALB/c mice were euthanized and sterilized. Mice were then placed in 75% ethanol for 5 s, after which, the peritoneum was exposed without injuring the peritoneal membrane. Six milliliters of ice-cold PBS were then injected into the peritoneal cavity. The mouse was shacked for 20 s to detach and suspend the macrophages. Consequently, the cell suspension was collected with a 21G needle in a 10 mL syringe. After repeating the process of cell collection, the cell suspension was transferred into a 50 mL tube containing 20 mL of ice-cold PBS and placed on ice. The cell suspension was then centrifuged and re-suspended in RPMI-1640 with 5% FCS (GIBCO), 2 mM L-glutamine (GIBCO), and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO), and finally incubated in a 12-well cell-culture plate at 37°C for adherence. The non-adherences were removed every 2 h three times. The adherent cells were incubated at 37°C under 5% atmospheric CO_2 overnight for further study.

Splenocytes were isolated from BALB/c mice, as previously described (23, 24). Mice were killed by exsanguination and placed in 75% ethanol for 5 s, after which the spleen was harvested and connective tissues were removed. The fragments of spleen were then gently pressed against the strainer with a syringe plunger. The cells were flushed through the strainer with 5 mL Mouse 1 × Lymphocyte Medium and transferred to a 15 mL tube. The cell suspension was then gently mixed with 1 mL RPMI 1640 medium and centrifuged at 800 × *g* for 30 min at room temperature. Consequently, the lymphocyte layer was collected, and cells were washed with RPMI1640 medium two times. Finally, the lymphocyte layer was re-suspended with RPMI1640 medium for further use.

$CD4^+$ T cells were isolated from these splenocytes using anti-CD4 magnetic beads (Miltenyi Biotech) according to the manufacturer's instructions. There were approximately 95% harvested $CD4^+$ T cells after the FACS analysis.

Activation of *in vitro* Macrophages

RAW264.7 or resident peritoneal macrophages were induced in the presence of 50 μg/ml of rTsTPX2 (RAW_{tpx} and $M_{rTsTPX2}$), 10 ng/ml of IL-4 (RAW_{IL-4} and M_{IL-4}) as M2 positive control, 10 ng/ml of IFN-γ ($RAW_{IFN-\gamma}$ and $M_{IFN-\gamma}$) as M1 positive control, 50 μg/ml of BSA as mimic control or PBS as no-treatment control (NTC). After 24 h cultivation at 37°C, cells were washed with PBS and then stored at −80°C for further study.

Co-culture of Activated Macrophages and $CD4^+$ T Cells

The activated macrophages were adjusted to 1×10^6 cells/ml after washing with RPMI1640. $CD4^+$ T cells collected from healthy BALB/c mice were re-suspended at the concentration of 5×10^6 cells/ml. A total of 50 μl of activated macrophages was added to the $CD4^+$ T cells with the same volume in 96-well plates at a humidified atmosphere of 5% CO_2 at 37°C for 72 h. The culture supernatant was harvested for ELISA analysis of IL-4 and IFN-γ cytokines. The proliferation of $CD4^+$ T cells was measured using the MTs kit (Promega), and the stimulation index was calculated according to the following formula: $\text{Proliferation index} = \left[\left(OD_{protein} - OD_{1640}\right) - \left(OD_{PBS} - OD_{1640}\right)\right] \div \left(OD_{PBS} - OD_{1640}\right)$.

Adoptive Transfer of rTsTPX2-Activated Macrophages and Larvae Challenge in BALB/c Mice

A total of 5×10^5 $M_{rTsTPX2}$ re-suspended in 200 μl PBS was intraperitoneally injected into BALB/c mice. The peritoneal macrophages treated with IL-4 (M_{IL-4}), IFN-γ ($M_{IFN-\gamma}$), BSA (M_{BSA}), or PBS (M_{PBS}) were transferred into mice as controls. The serum and anticoagulated blood samples were

collected at 3 weeks post-injection. At 3 weeks post-injection, mice from each group were orally challenged with 500 infective *T. spiralis* ML. The adults were determined at 3 days (Ad3) after infection. The reduction rates of Ad3 burden in activated-macrophages-transfer mice were evaluated according to the following formula: Worm reduction% = (1-mean number of worm in activated-macrophages-transfer mice/mean number of worm in unactivated-macrophages-transfer mice) × 100%.

Cytokines Analysis

The levels of cytokines produced in the supernatant of activated macrophages co-cultured with $CD4^+$ T cells (IL-4 and IFN-γ) and in mice serum samples (IL-4, IL-10, IL-12p70, TNF-α and IFN-γ) were measured by LEGEND MAX™ Mouse ELISA kits (Biolegend) according to the manufacturer's protocol. The sensitivity of detection was 0.5 pg/mL for IL-4, 2.7 pg/mL for IL-10, 0.5 pg/mL for IL-12p70, 1.5 pg/mL for TNF-α and 8 pg/mL for IFN-γ .

Flow Cytometry Analysis of T Cells

The anticoagulated blood sample (100 μl) from each group was blocked with 0.4 μg anti-mouse CD16/32 antibody (Biolegend) for 15 min at room temperature (RT), followed by incubation with 0.2 μg fluorescent-labeled detection antibodies for 2 h at 4°C in the dark. T cells were stained with the following antibodies: PerCP-Cy5.5 Hamster anti-mouse CD3e (BD Biosciences), FITC Rat anti-mouse CD4 (BD Biosciences), PE Rat anti-mouse CD8a (BD Biosciences) and Isotype control antibodies (BD Biosciences). Erythrocytes in blood samples were lysed with 500 μl Red Blood Cell Lysis Buffer (Tiangen) on ice in the dark. The treated samples were then centrifuged at 500 × *g* for 10 min. Then, the pellet was re-suspended in 400 μl PBS. The suspensions were analyzed using a BD Accuri™ C6 Plus flow cytometer (BD Biosciences). All data sets were analyzed with Flowjo software (TreeStar, Ashland, OR, United States).

Quantitative Real-Time PCR Assays

Total RNA from macrophages was reverse-transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology). The qRT-PCR experiments were performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TaKaRa Biotechnology) on CFX96 Touch Real-Time PCR System (Bio-Rad). The qPCR primers were designed using Primer 3.0 online software[1] for the following targets: GAPDH: 5′-AGGTCGGTGTGAACGGATTTG-3′ and 5′-TGTAGACCATGTAGTTGAGGTCA-3′; Arg-1: 5′-CTCCAAGCCAAAGTCCTTAGAG-3′ and 5′-AGGAGCTGTCATTAGGGACATC-3′; MRC-1: 5′-CTCTGTTCAGCTATTGGACGC-3′ and 5′-CGGAATTTCTGGGATTCAGCTTC-3′; iNOS-1: 5′-ACATTCAGATCCCGAAACGC-3′ and R: 5′-GACAATCCACAACTCGCTCC-3′; CCL22: 5′-AGGTCCCTATGGTGCCAATGT-3′ and R: 5′-CGGCAGGATTTTGAGGTCC-3′. The relative mRNA expression of target genes was calculated using the comparative Ct method, with the formula $2^{-\Delta\Delta CT}$ (25).

[1]http://bioinfo.ut.ee/primer3-0.4.0/primer3/

Western Blotting

Macrophage pellets were re-suspended in 30 μl of RIPA buffer (Beyotime, China). After incubation on ice for 1 h, the lysates were centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was mixed with sample buffer (Genscript, China) and incubated in boiling water for 10 min. The total cellular protein (30 μg/well) was separated by 10% SDS-PAGE. The separated proteins were transferred onto polyvinylidene difluoride membranes (PVDF, Millipore), which were then blocked overnight with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). The membranes were respectively incubated with primary antibodies to detect Arg-1 (1: 1000 Cell Signaling Technology) and MRC-1 (1: 1000 Proteintech). The housekeeping gene encoding beta-actin (1: 5000 Thermo Fisher Scientific) was used as an internal control. Finally, the target protein bands were visualized by ECL substrate (Advansta, China), and images were collected by the ChemiDoc XRS^+ system (Bio-Rad, Inc.).

Statistical Analysis

Statistical analysis including student's *t*-test or one-way ANOVA was performed by IBM SPSS Statistics 19 software (IBM, Inc.). All experiments were run in triplicate. Data are expressed as the mean ± SD from each experiment. A $P < 0.05$ was considered statistically significant.

RESULTS

T. spiralis TPX2 Is Distributed in Tissue-Dwellings and Worm-Organs

To determine the distribution of TPX2 in *T. spiralis*, immunofluorescence and immunohistochemistry tests were performed. The results of immunofluorescence on *T. spiralis* ML indicated that TPX2 appeared in cuticles and was sporadically expressed in the stichosome of ML (**Figure 1A**). The TPX2 was heavily expressed in cuticles and stichosome of Ad3 and was rarely presented in the surrounding (**Figure 1B**). In contrast, the immunohistochemistry results on the ML embedded diaphragm indicated that TPX2 was dense on both ML and the surroundings (**Figure 1C**). In our previous research, we found that the TPX2 gene was highly expressed in Ad3, while the expression levels were lowest in NBL (17) which matched well with the time of switching from Th1 to Th2 immune responses. Furthermore, our Western blot results showed that TsTPX2 is a significant component of *T. spiralis* ML ES product, which could react with sera from pigs infected by *T. spiralis* or from rabbit to produce anti-rTsTPX2 polyclonal antibody (**Supplementary Figures 1**, **2**) (17). These data implied that TPX2 may play an essential role in switching Th1 to Th2 immune responses during *T. spiralis* infection.

rTsTPX2 Induced a Th1-Suppressing Mixed Immune Response *in vivo*

To determine the immunomodulatory effects of rTsTPX2, mice were subcutaneously immunized with the protein. At 3 weeks

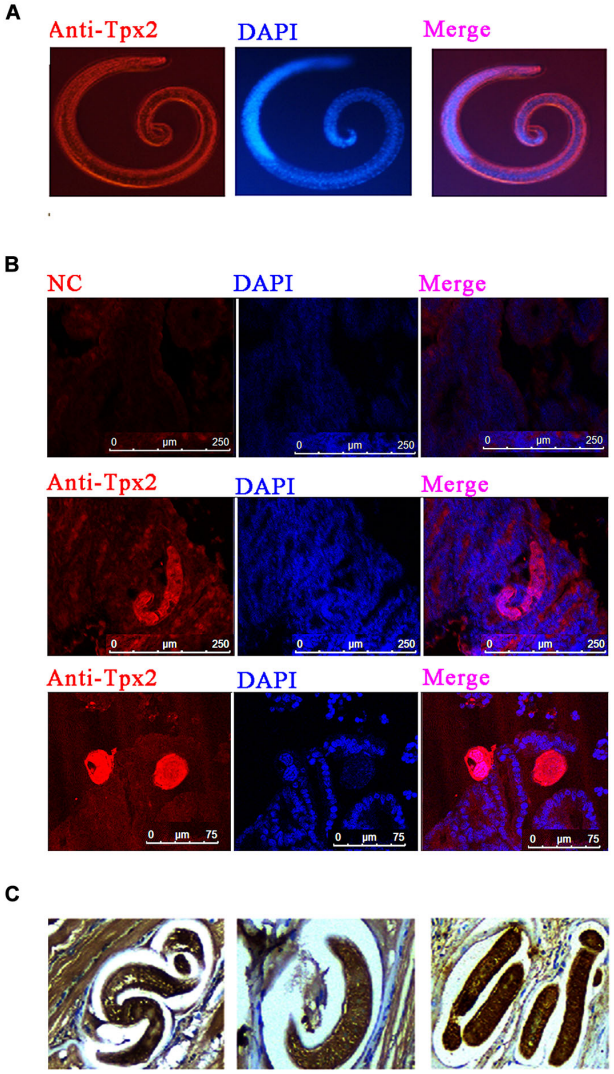

FIGURE 1 | Immunolocalization of TPX2 at different *T. spiralis* phases. **(A)** Immunofluorescence of TsTPX2 in muscle larvae (ML) at 35 dpi. **(B)** Immunofluorescence of TPX2 in adult worms at 3 days. **(C)** Immunohistochemistry of TsTPX2 in ML at 35 dpi.

post-injection, the cytokines were examined in serum samples from mice by ELISA. The level of Th2 cytokines (IL-4 and IL-10) from mice immunized with rTsTPX2 was significantly higher than that in mice treated with BSA (P = 0.018 for IL-4 and P = 0.017 for IL-10) and PBS (P = 0.004 for IL-4 and P = 0.014 for IL-10) (**Figure 2A**). On the contrary, mice immunized with rTsTPX2 had a significantly lower level of Th1 cytokines (IFN-γ and TNF-α) compared to the control groups PBS (P = 0.018 for IFN-γ) and BSA (P = 0.044 for TNF-α). Moreover, no significant changes in the Th1 cytokine IL-12p70 were observed between mice immunized with rTsTPX2 and control groups (P = 0.137 for PBS and P = 0.073 for BSA), although a distinct trend of suppression was visible (**Figure 2B**). These results indicate that direct injection of rTsTPX2 could orchestrate the Th1 and Th2 programs in mice by promoting Th2 cytokines, IL-4 and IL-10, and simultaneously suppressing Th1 cytokines, IFN-γ, IL-12p70 and TNF-α. The results suggested that this protein could inhibit a type 1 immune response and is flexible in its ability to elicit a mixed Th1/Th2 response, which may be responsible for preventing excessive inflammation to create an environment suitable for *T. spiralis* survival in the host (26).

The anticoagulated blood samples were harvested to determine the cellular immune response by flow cytometry. Mice injected with rTsTPX2 showed more $CD4^+$ T cells and fewer $CD8^+$ T cells compared to controls (**Figures 2C–E**).

rTsTPX2 Converts Macrophages to M2 Phenotype *in vitro*

Given the presence of rTsTPX2 could suppress the Th1 response *in vivo*, we sought to exploit whether the protein triggers the regulation of host immune response by driving macrophages into the M2 phenotype (27). The peritoneal macrophages from BALB/c mice and commercial RAW264.7 were respectively stimulated with rTsTPX2 for 24 h *in vitro*. Arg-1 and MRC-1 gene expressions were examined by both qRT-PCR and Western blotting. The results of qRT-PCR showed that the expression of both Arg-1 and MRC-1 genes was significantly higher in RAW264.7 macrophages stimulated with rTsTPX2 than in those stimulated with IFN-γ (P = 0.0158 and 0.0008) or those stimulated with BSA (P = 0.0202 and 0.004), but was lower than those stimulated with IL-4 (P = 0.0245 and 0.0006) (**Figure 3A**). The stimulus of peritoneal macrophages from BALB/c mice was consistent with the results of RAW264.7 macrophages using qRT-PCR detection (**Figure 3C**). For the IFN-γ stimulus, the expression of Arg-1 and MRC-1 in peritoneal macrophages was slightly up-regulated when compared with the mock groups (PBS and BSA stimulus), but the difference was not significant ($P > 0.05$).

The Western blotting further indicated consistent results with qRT-PCR; Arg-1 and MRC-1 genes were significantly up-regulated in both RAW264.7 macrophages (**Figure 3D**), and peritoneal macrophages from BALB/c mice (**Figure 3E**) stimulated with rTsTPX2. These results above indicated that rTsTPX2 could drive macrophages to M2 phenotype *in vitro*.

Then the identification was further confirmed by detection of iNOS-1 and CCL22 gene expression in RAW264.7 cells using qRT-PCR. The results showed that there were no significant differences between RAW_{tpx} and RAW_{IL-4} groups for the expression of iNOS-1 (M1) and CCL22 (M2), which indicated that rTsTPX2 can induce macrophages to an M2 phenotype (**Figure 3B**).

$M_{rTsTPX2}$ Promote the Production of Th2 Cytokines From Naïve $CD4^+$ T Cells *in vitro*

The observations that Th2 cytokines (IL-4 and IL-10) were significantly elevated upon administration of rTsTPX2 (**Figure 2A**) and that TPX could directly and alternatively activate macrophages (18), led us to evaluate whether the Th2 type immune response could be driven by $M_{rTsTPX2}$. To

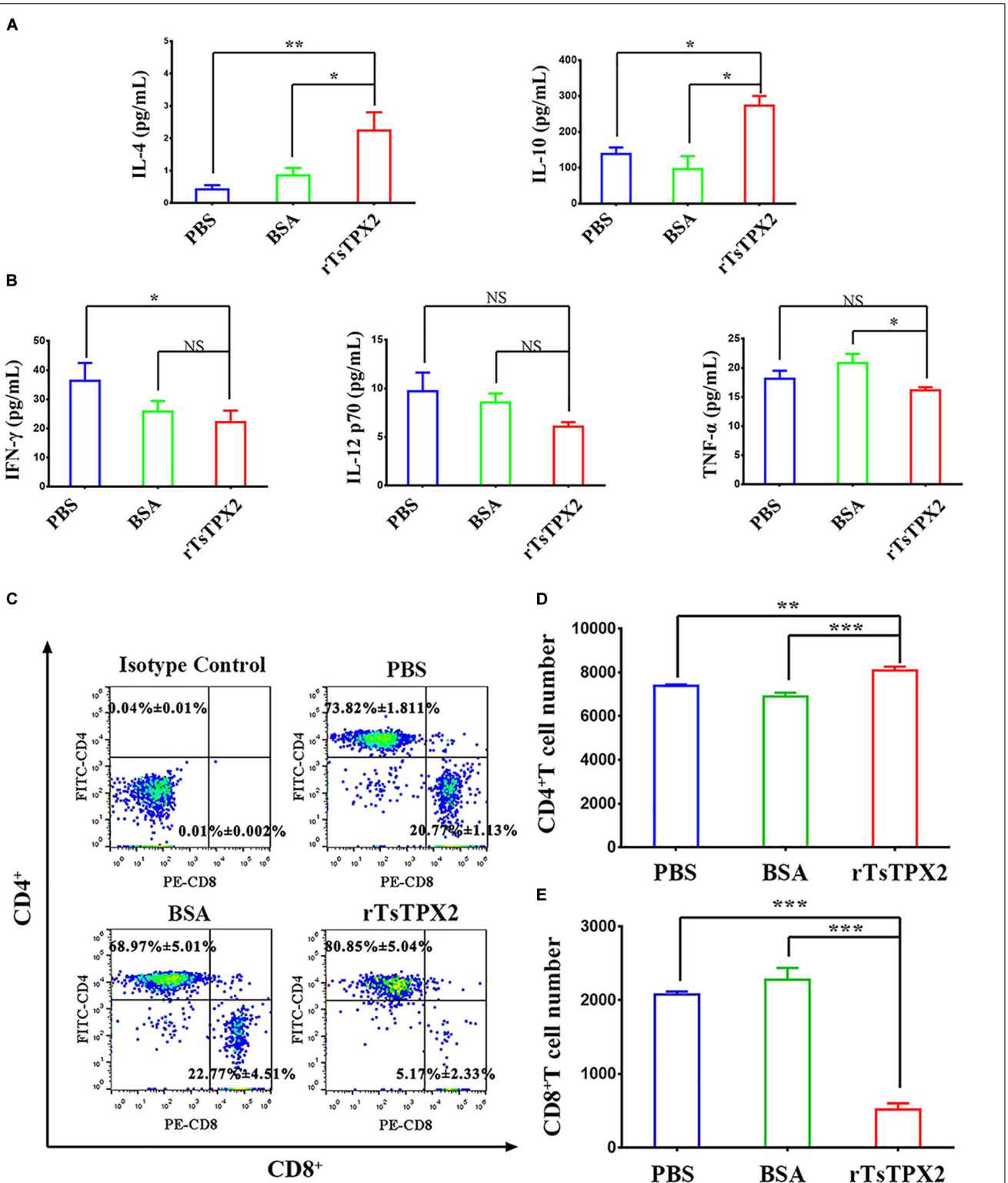

FIGURE 2 | rTsTPX2 inhibits Th1 responses in mice. **(A)** The levels of Th2 cytokines in sera from mice detected by ELISA. **(B)** The levels of Th1 cytokines in sera from mice detected by ELISA. **(C)** Analysis of $CD3^+CD4^+CD8^-$ and $CD3^+CD8^+CD4^-$ T lymphocytes. The number on the representative contour plots showing the ratio of each subset out of peripheral lymphocytes. **(D)** The total number of $CD3^+CD4^+CD8^-$ T cells in blood from mice. **(E)** The total number of $CD3^+CD8^+CD4^-$ T cells in blood from mice. Experimental groups include mice challenged with PBS as a negative control, BSA as mock control, or rTsTPX2. Statistical analysis was performed with Student's *t*-test, and data are mean ± SDs (representative of three experiments). $^{***}P < 0.001$, $^{**}0.001 < P < 0.01$, $^{*}0.01 < P < 0.05$. $n >= 5$ mice per group.

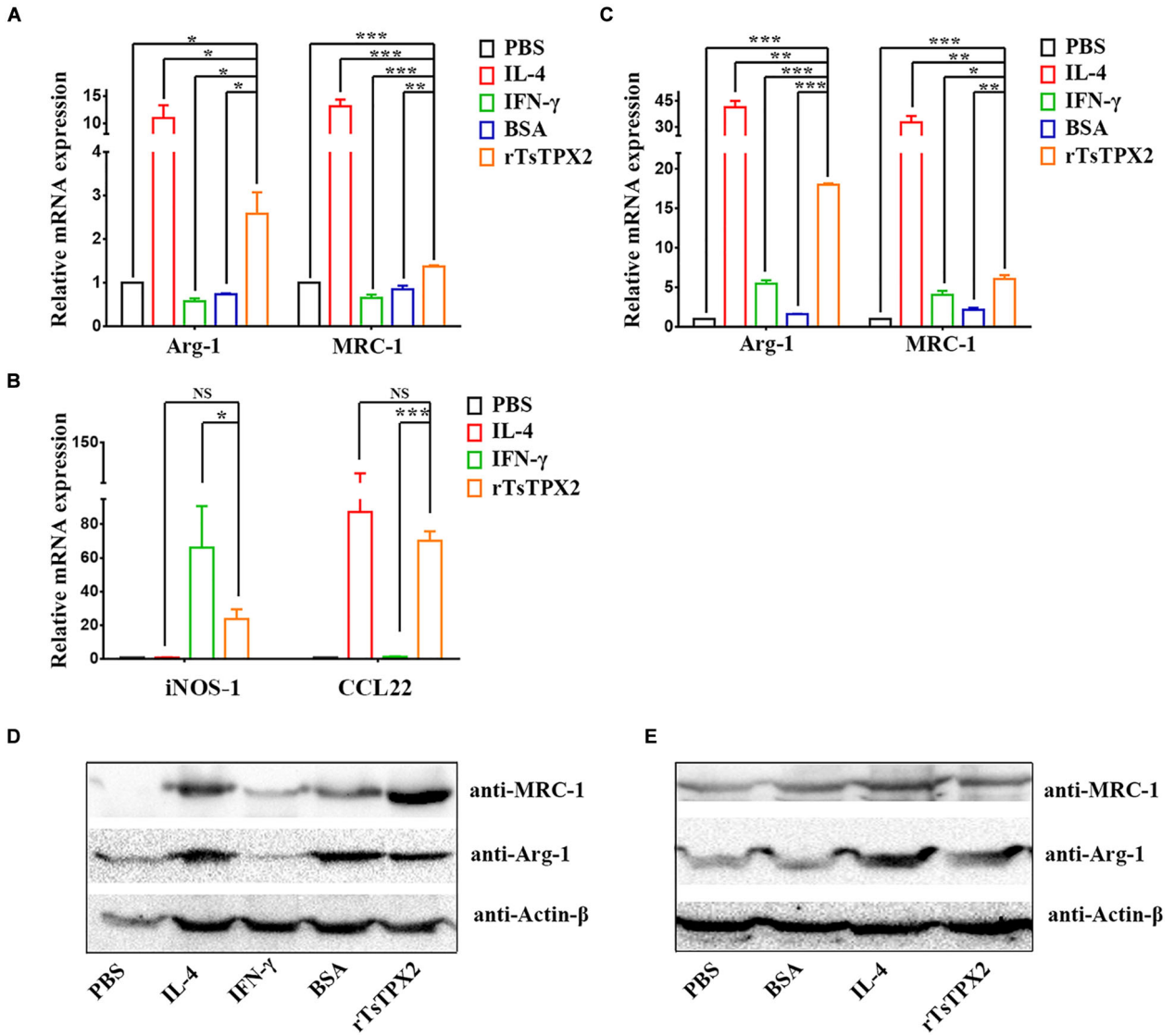

FIGURE 3 | The phenotype of macrophages induced *in vitro*. **(A–E)** The expressions of M1 and M2 markers were analyzed by both qRT-PCR **(A–C)** and Western Blotting **(D,E)**. **(A,B)** Analysis of the expression of Arg-1 MRC-1, iNOS-1 and CCL22 genes in RAW264.7 co-cultured with various stimuli by qPCR. **(C)** Analysis of the expression of Arg-1 and MRC-1 genes in peritoneal macrophages isolated from healthy BALB/c mice after co-cultured with various stimuli by qPCR. **(D,E)** Analysis of the expression of Arg-1 and MRC-1 genes in RAW264.7 and peritoneal macrophages after co-cultured with various stimuli by Western Blotting. Experimental groups include macrophages stimulated with PBS as blank control, IL-4 as M2 positive control, INF-γ as M1 positive control, BSA as mock control or rTsTPX2. Statistical analysis was performed with Student's *t*-test, and data are presented as mean ± SDs (representative of three experiments). ***$P < 0.001$, **$0.001 < P < 0.01$, *$0.01 < P < 0.05$. $n >= 5$ mice per group.

determine the relationship between $M_{rTsTPX2}$ and Th2 type immune response, we first examined the ability of $M_{rTsTPX2}$ to induce proliferation of $CD4^+$ T cells *in vitro*. As shown in **Figure 4A**, significantly higher proliferation of $CD4^+$ T cells was observed after co-incubation with $M_{rTsTPX2}$ than with $M_{IFN-\gamma}$ ($P = 0.015$), M_{BSA} ($P = 0.0305$) or M_{PBS} ($P = 0.0007$); yet, no significant difference was found compared with co-inoculation with M_{IL-4} ($P = 0.328$).

ELISA assay was then used to analyze the cytokine production from the supernatant of the co-culture medium. When cultured with $M_{rTsTPX2}$, the $CD4^+$ T cells produced a compound of Th2 (IL-4) and Th1 (IFN-γ) cytokines compared to controls. A significantly higher production of IL-4 was detected in the supernatant of $CD4^+$ T cells co-cultured with $M_{rTsTPX2}$ compared to cells co-cultured with $M_{IFN-\gamma}$ ($P = 0.013$), M_{BSA} ($P = 0.05$) or PBS ($P = 0.01$) (**Figure 4B**). Whereas the production of IFN-γ was reduced in the supernatant of $CD4^+$ T cells co-cultured with $M_{rTsTPX2}$ compared to those co-cultured with $M_{IFN-\gamma}$ ($P = 0.003$) but was increased compared to cells co-cultured with M_{PBS} ($P = 0.045$) or PBS ($P = 0.0356$) (**Figure 4C**). The results revealed that $M_{rTsTPX2}$ could elicit $CD4^+$ T cell proliferation to promote a higher level of IL-4 production,

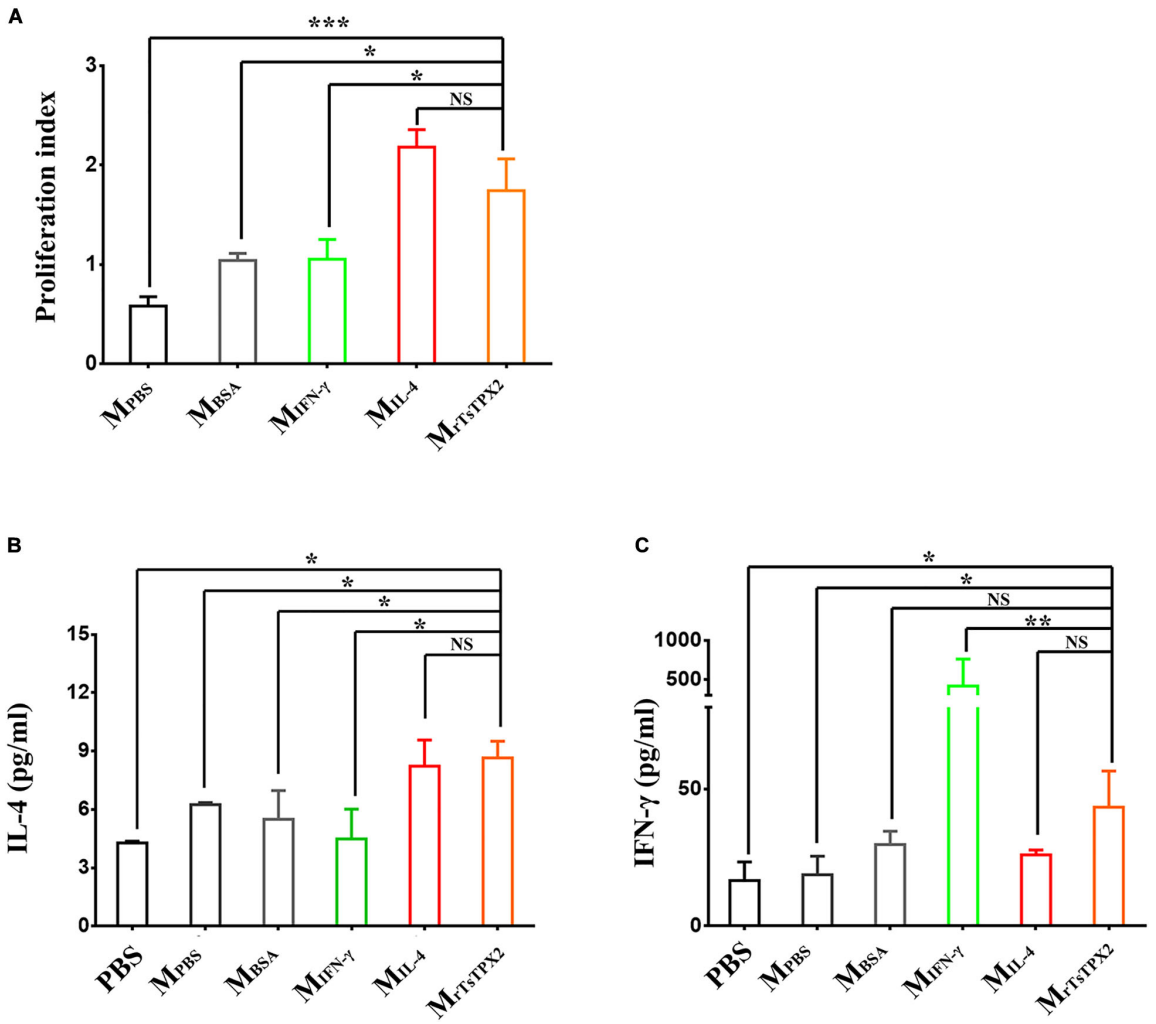

FIGURE 4 | The differentiation and proliferation of $CD4^+$ T cells induced by macrophages. **(A)** The proliferation index of $CD4^+$ T cells induced by macrophages at 72 h after co-culture, measured by MTs kit. **(B)** Levels of IL-4 production in supernatant of $CD4^+$ T cells co-cultured with macrophages. **(C)** Levels of IFN-γ production in supernatant of $CD4^+$ T cells co-cultured with macrophages. $CD4^+$ T cells isolated from healthy BALB/c mice co-cultured with macrophages induced by various stimuli, including PBS as blank control (M_{PBS}), IL-4 as M2 positive control (M_{IL-4}), INF-γ as M1 positive control ($M_{INF-\gamma}$), BSA as mock control (M_{BSA}) or rTsTPX2 ($M_{rTsTPX2}$). The experimental group PBS representative $CD4^+$ T cells dealing with PBS. Statistical analysis was performed with Student's *t*-test, and data are presented as mean ± SDs (representative of three experiments). ***$P < 0.001$, **$0.001 < P < 0.01$, *$0.01 < P < 0.05$, NS, not significant. n >= 3 mice per group.

indicating that a Th2 phenotype of $CD4^+$ T cells were directly driven by the $M_{rTsTPX2}$.

Adoptive Transfer of $M_{rTsTPX2}$ Weakens the Th1 Responses and Enhances the Th2 Immune Response in BALB/c Mice

Whether $M_{rTsTPX2}$ drives the Th2 type immune response was further evaluated by adoptively transferring $M_{rTsTPX2}$ into healthy BALB/c mice through intraperitoneal injection. At 3 weeks after injection, we examined the cytokines and T subclasses in the peripheral blood, **Figure 5A** shows the experimental protocol of macrophage adoptive transfer. The level of IL-4 cytokine in mice transferred with $M_{rTsTPX2}$ was significantly higher compared with those treated with PBS ($P = 0.009$), M_{BSA} ($P = 0.015$), or $M_{IFN-\gamma}$ ($P = 0.05$). However, no significant difference in IL-4 production was detected in mice transferred with $M_{rTsTPX2}$ and M_{IL-4} ($P = 0.325$) (**Figure 5B**). Regarding IFN-γ analysis, mice transferred with $M_{rTsTPX2}$ were significantly lower than those injected with PBS ($P = 0.035$), M_{BSA} ($P = 0.05$) or $M_{IFN-\gamma}$ ($P = 0.01$), and the difference between $M_{rTsTPX2}$ and M_{IL-4} transferred mice was not significant ($P = 0.6$) (**Figure 5C**). An injection of $M_{rTsTPX2}$ into mice induced an increased IL-4 and a decreased IFN-γ, which is similar to results observed in mice transferred with M_{IL-4}. These results demonstrated that the rTsTPX2-activated macrophages could elicit a Th2 immune response in mice.

Mice transferred with $M_{rTsTPX2}$ had elevated $CD4^+$ T cell percentage and reduced $CD8^+$ T cell percentage compared to the control groups (**Figure 5D**). The count of $CD4^+$ T cells in mice

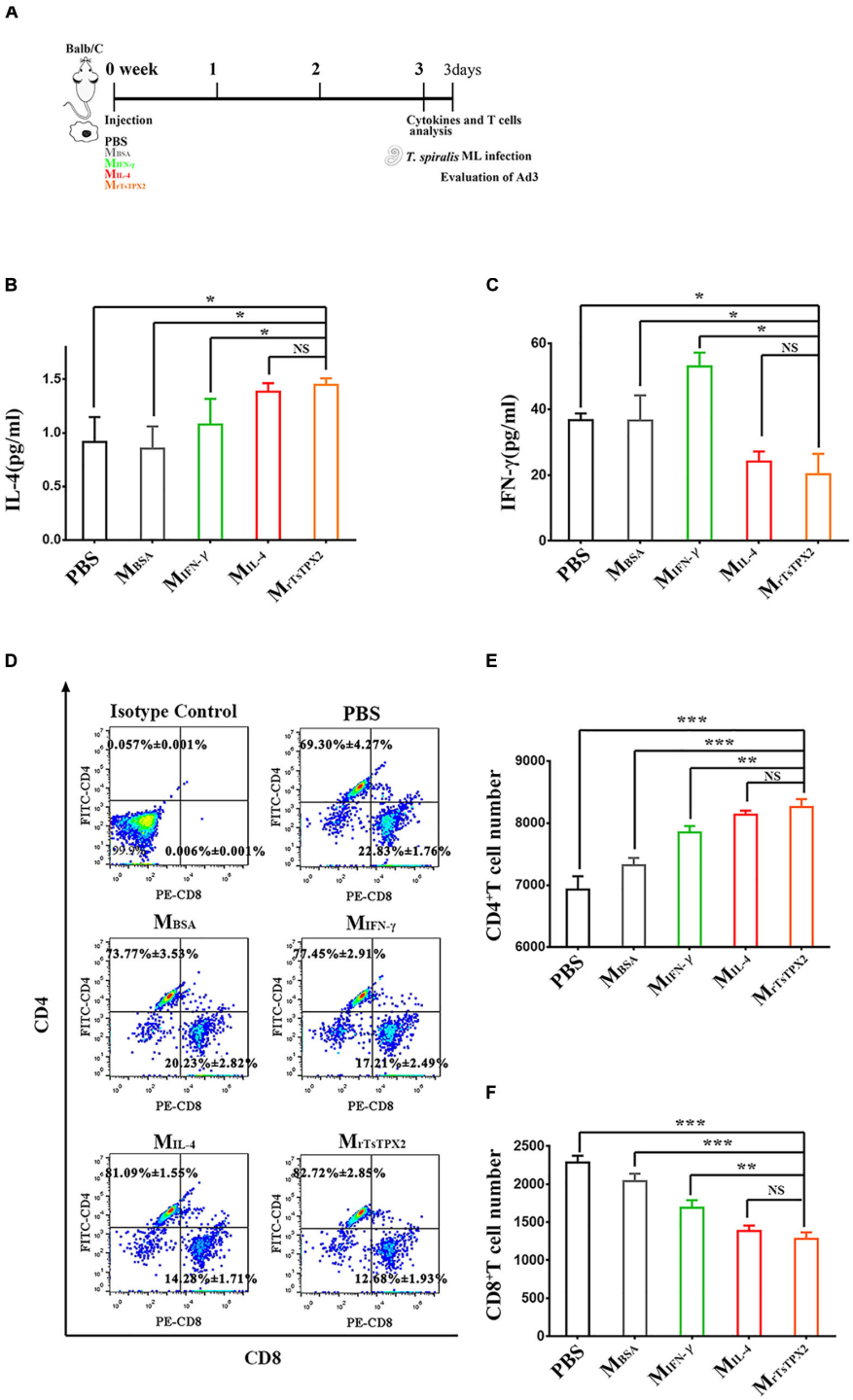

FIGURE 5 | The type of immune responses in mice adoptively transferred with macrophages. **(A)** Schematics of immune responses in mice adoptively transferred with one of the macrophage types. Three weeks after the first injection, the immunological status was analyzed, and mice were experimentally infected with 500 *T. spiralis* ML. Analysis of adult load at 3 days after infection. **(B)** Levels of IL-4 production in sera from mice transferred with various macrophage types. **(C)** Levels of IFN-γ production in sera from mice transferred with various macrophage types. **(D)** Analysis of $CD3^+CD4^+CD8^-$ and $CD3^+CD8^+CD4^-$ T lymphocytes. The number on the representative contour plots showing the ratio of each subset out of peripheral lymphocytes. **(E)** The total number of $CD3^+CD4^+CD8^-$ T cells in blood from mice. **(F)** The total number of $CD3^+CD8^+CD4^-$ T cells in blood from mice. Experimental groups include mice transferred with macrophages activation by IL-4 as M2 positive control (M_{IL-4}), INF-γ as M1 positive control ($M_{INF-\gamma}$), BSA as mock control (M_{BSA}) or rTsTPX2 ($M_{rTsTPX2}$). The experimental group, PBS representative mice, injected with PBS. Statistical analysis was performed with Student's *t*-test, and data are presented mean ± SDs (representative of three experiments). ***$P < 0.001$, **$0.001 < P < 0.01$, *$0.01 < P < 0.05$, NS, not significant. n >= 3 mice per group.

transferred with $M_{rTsTPX2}$ were significantly higher compared to the PBS ($P = 0.0004$), M_{BSA} ($P = 0.0002$) and $M_{IFN-\gamma}$ ($P = 0.0270$) groups (**Figure 5E**). No significant difference in $CD4^+$ T cells was detected in mice transferred with $M_{rTsTPX2}$ and M_{IL-4} ($P = 0.3790$) (**Figure 5E**). In contrast, the $CD8^+$ T cell numbers in mice transferred with $M_{rTsTPX2}$ were significantly lower than those injected with PBS ($P < 0.0001$), M_{BSA} ($P = 0.00013$) and $M_{IFN-\gamma}$ ($P = 0.0105$). The difference of $CD8^+$ T cell numbers between mice transferred with $M_{rTsTPX2}$ and M_{IL-4} was not statistically significant ($P = 0.3720$) (**Figure 5F**). The increased $CD4^+$ T cells along with the decreased $CD8^+$ T cells in mice transferred with $M_{rTsTPX2}$ implied that the rTsTPX2 induced macrophages could provide protection as the Th2 cells mediated the expulsion of worms from the mice.

Adoptive Transfer of $M_{rTsTPX2}$ Induced Protective Immunity

Macrophages activated by alternative pathways are considered to be involved in helminth containment. To determine whether $M_{rTsTPX2}$ can protect mice from *T. spiralis* infection, mice were orally challenged with 500 infective larvae, followed by the adoptive transfer of $M_{rTsTPX2}$. The adult worm burdens were evaluated at 3 days after infection. As shown in **Figure 6**, mice transferred with $M_{rTsTPX2}$ had significantly lower numbers of Ad3 than those from M_{BSA} ($P < 0.0001$) and $M_{IFN-\gamma}$ ($P < 0.0001$) groups. The Ad3 number in mice injected with $M_{rTsTPX2}$ had a reduction of 44.7% compared to the M_{BSA} group. However, there was no significant difference of Ad3 loadings in mice injected with $M_{rTsTPX2}$ and M_{IL-4} ($P = 0.1240$).

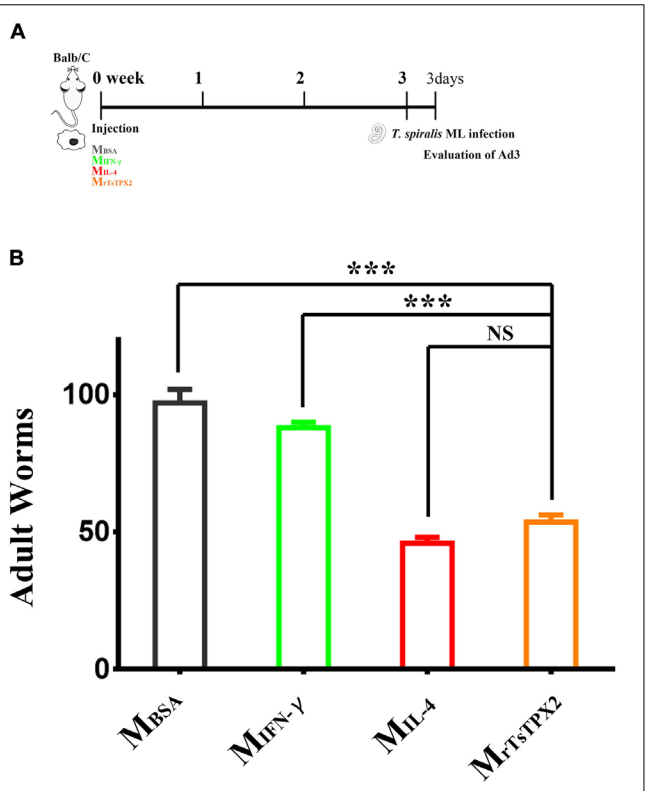

FIGURE 6 | Numbers of 3-day adults from the macrophages transferred mice. **(A)** Schematics of immune responses in mice adoptively transferred with one of the macrophage types. Three weeks after the first injection, the immunological status was analyzed, and mice were experimentally infected with 500 *T. spiralis* ML. Analysis of adult load at 3 days after infection. **(B)** Adult load in the intestine of mice. Experimental groups include mice transferred with macrophages activation by IL-4 as M2 positive control (M_{IL-4}), INF-γ as M1 positive control ($M_{INF-\gamma}$), BSA as mock control (M_{BSA}) or rTsTPX2 ($M_{rTsTPX2}$). Statistical analysis was performed with Student's *t*-test, and data presented are mean ± SDs (representative of three experiments). ***$P < 0.001$, NS: not significant. n >= 3 mice per group.

DISCUSSION

During the enteric phase, *T. spiralis* infection induces a Th1/Th2 mixed response, while in the systemic phase, the response is Th2-biased (28). During the transformation of immune responses, excretory-secretory (ES) products from the *T. spiralis* have been reported to play a crucial role in inhibiting inflammation (29) which can protect the parasite and the host at the same time (18, 19, 30). So far, only a structural molecule, chitin, has been identified to modulate the macrophage to the alternative phenotype (4, 31). The molecules identified in *T. spiralis* ES products that function in regulating the types of immune responses are still poorly understood when compared to other medical and veterinary important helminth species. In our previous study, the expression of TsTPX2 was up-regulated in both Ad3 and ML stages compared to NBL, almost matching the transformation of Th2 immune responses during *T. spiralis* infection (17). In addition, the TsTPX2 was detected in ML ES products by Western blotting analysis (**Supplementary Figure 2**) and in tissue-dwellings by immunohistochemistry and immunofluorescence techniques (**Figure 1**). Based on these results, we speculated that TsTPX2, as one of the ES products, might participate in the regulation of responses during *T. spiralis* infection. We found that rTsTPX2 could suppress Th1 immune responses by promoting Th2 cytokines, IL-4 and IL-10 and simultaneously demoting Th1 cytokines, IFN-γ, IL-12p70 and TNF-α. The ability of rTsTPX2 to inhibit a type 1 immune response is consistent with observations made in thioredoxin peroxidases derived from *Fasciola hepatica* and *Schistosoma mansoni* (18, 19).

Macrophages orchestrate Th1/Th2 responses by responding to different environmental signals (6). Exposure to IL-4 and IL-13 polarizes macrophages into the M2 phenotype and drives the host to the Th2 response (10–12, 32, 33). Here, we showed that rTsTPX2 could directly convert macrophages to an M2 phenotype *in vitro* (rTsTPX2 up-regulated Arg-1, CCL22 and MRC-1, as shown in **Figure 3**). Whereas, the transcription of Arg-1 and MRC-1 genes was also slightly up-regulated in IFN-γ stimulated cells (**Figure 3C**). The seeming paradox was eliminated through a sharp increase in expression of CAMs marker (iNOS-1 gene) in macrophages stimulated with IFN-γ compared to the M_{IL-4}, $M_{rTsTPX2}$, and mock control groups (**Figure 3B** and **Supplementary Figure 4**). The conversion of

macrophages into an M2 phenotype by rTsTPX2 was further determined through proliferating naïve $CD4^+$ T cells to secrete Th2 cytokine IL-4.

Naïve $CD4^+$ T cells, the precursors of Th cells, undergo clonal expansion and differentiation into distinct effector Th cell subsets, and these Th cell subsets directly promote the control of pathogens by producing signature cytokines (5, 34, 35). Many studies have shown that $CD4^+$ Th2 cells have a vital role in anti-helminth responses. The $CD4^+$ T-cell-depleted mice failed to mount protective immune responses against *S. mansoni* after vaccination (35). Moreover, the *Nippostrongylus brasiliensis* expelling rate was significantly decreased in $CD4^+$ T-cell-exhausted mice compared to normal controls (36); this capacity was recovered once $CD4^+$ T cells were injected in the depleted mice (37). In this study, we found that $CD4^+$ T cell proliferation occurred through rTsTPX2-activated macrophages *in vivo* and *in vitro*, in turn inducing the IL-4 production. Also, after co-culturing $M_{rTsTPX2}$ with $CD4^+$ T cells isolated from *T. spiralis* infected mice, the proliferation sharply increased (**Supplementary Figure 3**) suggesting that the rTsTPX2-activated macrophages could induce the memory of Th2 cells proliferation under the same situation.

The Th2 immune response has a critical role in anti-helminth immunity. The Th2 response is beneficial for the host not only to expel *T. spiralis* adults out of the intestines but also to repair or prevent muscle tissue damage (38, 39). Adoptive transfer of $M_{rTsTPX2}$ into mice evoked an increase in IL-4 production along with decreased levels of IFN-γ indicating that the Th2 immune response was altered which is consistent with the observations from mice adoptively transferred with TsES-activated macrophage (30). The Th2 immune response in mice transferred with $M_{rTsTPX2}$ was able to induce protection against *T. spiralis* demonstrating a 44.7% reduction in adult worms compared to the mimic control. The adult worms of *T. spiralis* release offspring to induce systemic infection and the reduction of adult worm reflects the effect of rTsTPX2 induced protective immunity against the worm infection (40).

During *T. spiralis* infection, the host's immune response switched from Th1 to Th2 response. Our data indicated that regarding TsTPX2, at least one molecule has a significant role in modulating these immune transformations by inducing macrophages to an M2 phenotype. These immunomodulatory mechanisms were exploited by this parasite to create an environment suitable for its survival in the host organism. On the other hand, these mechanisms are important in establishing new therapeutic approaches for various inflammatory disorders like allergies or autoimmune diseases.

The humoral immunity also exhibits an important role against the helminth infection. IgG1 isotype shows potent anti-inflammatory activity. Meanwhile, the IgG production driven by worms can also participate in restricting extreme inflammatory responses during the chronic infection process (41–43). However, in the present study, we only evaluated the protective Th2 responses in cytokines secretion instead of antibody production. The efficacy of rTsTPX2 in inducing the humoral response should be further warranted in the next study.

CONCLUSION

Our data suggested that TsTPX2 may directly induce macrophages to an M2 phenotype *in vitro* and *in vivo*. Immunization of mice with rTsTPX2 or $M_{rTsTPX2}$ could increase the number of CD4 + T cells and protect against *T. spiralis* infection by mediating worms expulsion from the host. Importantly, understanding the ability of TsTPX2 in the regulation of macrophages into the M2 phenotype can not only provide us a new insight into immunomodulatory mechanisms exploited by *T. spiralis* to create an environment proper for its survival in the hosts, but also establish novel therapeutic methods to various inflammatory disorders like allergies or autoimmune diseases.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

AUTHOR CONTRIBUTIONS

B-QF, M-XS, and N-ZZ conceived the project, designed the experiments, and critically revised the manuscript. Q-WJ, W-HL, Y-JL, D-YN, and H-TQ performed the experiments and analyzed the data. Q-WJ, N-ZZ, and JO drafted and revised the manuscript. H-BY, LL, and W-ZJ helped in the implementation of the study. All authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02015/full#supplementary-material

Supplementary Figure 1 | Expression and purification of recombinant TsTPX2 in *E. coli*. **(A)** SDS-PAGE analysis the expression of recombinant TsTPX2. Lane 1:

IPTG-induced inclusion body; Lane 2: IPTG-induced supernatant; Lane 3: renatured inclusion body; Lane 4: Purified renatured protein. Lane M: protein molecular weight marker. **(B)** Detection of rTsTPX2 by Western Blotting: Lane 1: rTsTPX2 reacted with anti-rTsTPX2 rabbit sera (1: 3200); Lane M: Protein molecular weight markers.

Supplementary Figure 2 | Detection TsTPX2 from ML ES products. **(A)** SDS-PAGE analysis the ML ES products. **(B)** Detection of TsTPX2 from ML ES production by Western Blotting with an anti-rTsTPX2 polyclonal antibody. Lane 1: ML ES products; Lane M: Protein molecular weight markers. The ML ES samples were harvested from *T. spiralis* strain (ISS534) maintained in Kunming mice at 35 days after infection. The ES protein from ML (6 μg/well) was separated by 10% SDS-PAGE. The separated proteins were stained with CBB (Coomassie brilliant blue) reagents **(A)** or transferred onto polyvinylidene difluoride membranes (PVDF, Millipore), which were then blocked overnight with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). The membranes were respectively incubated with primary antibody (anti-rTsTPX2 rabbit sera, 1:3200) and secondary antibody (goat anti-rabbit IgG (H + L) proteintech, 1: 5000).

Supplementary Figure 3 | The proliferation of activated $CD4^+$ T cells induced by macrophages. The $CD4^+$ T cells were isolated from *T. spiralis* infected mice at 35 days after ML infection. The proliferation index of $CD4^+$ T cells induced by macrophages at 72 h after co-culture measured by MTs kit. Statistical analysis was performed with Student's *t*-test, and data are expressed as mean ± SDs (representative of three experiments). $^{***}P < 0.001$, $^{**}0.001 < P < 0.01$, $^{*}0.01 < P < 0.05$, NS, not significant. $n >= 3$ mice per group.

Supplementary Figure 4 | The phenotype of macrophages induced *in vitro* by flow cytometry. The expressions of MRC-1 **(A)** and iNOS-1 **(B)**. Genes were analyzed by flow cytometry; the left panel was determined by Flowjo software (TreeStar, Ashland, OR, United States), while the right panel shows the mean of fluorescence intensity experimental group. RAW264.7 macrophages were incubated with respective proteins for 48 h. The PBS was used as non-treated control; the recombined mouse Cyclic GMP-AMP synthase (rmcGAS) expressed in the same system with rTsTPX2 was used as irrelevant control; the recombined IL-4 as M2 positive control and IFN-γ as M1 positive control. The following fluorescence conjunct antibodies, Alexa Fluor® 647 rat anti-mouse CD206 (MRC-1), FITC mouse anti-iNOS-1, PE rat anti-mouse F4/80, and the Isotype control antibodies were purchased from BD Biosciences (United States). After staining, the macrophages were firstly gated with F4/80-positive cells, then the iNOS-1 and CD206 (MRC-1) positive cells analyzed with mean of fluorescence intensity (MFI).

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The Helminth Parasite *Heligmosomoides polygyrus* Attenuates EAE in an IL-4Rα-Dependent Manner

Madeleine P. J. White[1†], Chris J. C. Johnston[2†], John R. Grainger[3], Joanne E. Konkel[3], Richard A. O'Connor[4], Stephen M. Anderton[4] and Rick M. Maizels[1]*

[1] Wellcome Centre for Molecular Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, United Kingdom, [2] Clinical Surgery, Royal Infirmary of Edinburgh and University of Edinburgh, Edinburgh, United Kingdom, [3] Faculty of Biology, Medicine and Health, Lydia Becker Institute of Immunology and Inflammation, University of Manchester, Manchester, United Kingdom, [4] MRC Centre for Inflammation Research, Centre for Multiple Sclerosis Research, Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, United Kingdom

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University of Technology Sydney, Australia

***Correspondence:**
Rick M. Maizels
rick.maizels@glasgow.ac.uk

[†] These authors have contributed equally to this work

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Helminth parasites are effective in biasing Th2 immunity and inducing regulatory pathways that minimize excessive inflammation within their hosts, thus allowing chronic infection to occur whilst also suppressing bystander atopic or autoimmune diseases. Multiple sclerosis (MS) is a severe autoimmune disease characterized by inflammatory lesions within the central nervous system; there are very limited therapeutic options for the progressive forms of the disease and none are curative. Here, we used the experimental autoimmune encephalomyelitis (EAE) model to examine if the intestinal helminth *Heligmosomoides polygyrus* and its excretory/secretory products (HES) are able to suppress inflammatory disease. Mice infected with *H. polygyrus* at the time of immunization with the peptide used to induce EAE (myelin-oligodendrocyte glycoprotein, pMOG), showed a delay in the onset and peak severity of EAE disease, however, treatment with HES only showed a marginal delay in disease onset. Mice that received *H. polygyrus* 4 weeks prior to EAE induction were also not significantly protected. *H. polygyrus* secretes a known TGF-β mimic (*Hp*-TGM) and simultaneous *H. polygyrus* infection with pMOG immunization led to a significant expansion of Tregs; however, administering the recombinant *Hp*-TGM to EAE mice failed to replicate the EAE protection seen during infection, indicating that this may not be central to the disease protecting mechanism. Mice infected with *H. polygyrus* also showed a systemic Th2 biasing, and restimulating splenocytes with pMOG showed release of pMOG-specific IL-4 as well as suppression of inflammatory IL-17A. Notably, a Th2-skewed response was found only in mice infected with *H. polygyrus* at the time of EAE induction and not those with a chronic infection. Furthermore, *H. polygyrus* failed to protect against disease in IL-4Rα$^{-/-}$ mice. Together these results indicate that the EAE disease protective mechanism of *H. polygyrus* is likely to be predominantly Th2 deviation, and further highlights Th2-biasing as a future therapeutic strategy for MS.

Keywords: autoimmunity, intestinal nematode, multiple sclerosis (MS), Th2 (type-2) immune responses, cytokine

INTRODUCTION

Over one-fourth of the global population are infected with helminth parasites, with the majority of these infections located within resource-poor tropical countries (1); however before sanitation improvements and widespread industrialization occurred within the last century, the prevalence of helminths was likely to be high across the globe. Helminth parasites are widely known to induce a state of host immune hyporesponsiveness that has been associated with a decreased incidence of inflammatory diseases, a concept that is consistent with the original "hygiene hypothesis," which suggested that increased prevalence of allergy and asthma in industrialized countries is at least in part due to the reduction in parasitic burden (2, 3).

Multiple sclerosis (MS) is a severe degenerative autoimmune disease characterized by lesions in the brain resulting in a range of symptoms including paralysis, loss of vision and co-ordination (4). MS is believed to be driven by autoreactive T helper cells, particularly the Th1 and Th17 subsets, which enter and are reactivated in the central nervous system (CNS) resulting in the recruitment of additional T cells and macrophages to establish inflammatory lesions. These lesions result in loss of myelin, oligodendrocyte destruction and axonal damage and can account for the broad range of symptoms seen in patients with MS (5). While $CD4^+$ T helper cells are well-established as important for initiating MS disease; B cells, $CD8^+$ T cells, and natural killer cells have also been implicated as drivers of disease pathogenesis (6).

The incidence of MS in developed countries is increasing and a number of reasons have been suggested for this, including a loss of co-evolved helminth infections (7, 8). Striking evidence for helminth-induced protection in MS came from an Argentinian patient cohort that unintentionally acquired gastrointestinal helminth infection with a variety of species. Those infected showed a significantly lower frequency of disease exacerbations and fewer visible lesions on sequential interval MRI scans in comparison to an uninfected MS patient cohort (matched for age, sex, and time since diagnosis of MS) over the same period of time (9). In addition, after 63 months of follow up, a subset of patients within the helminth-infected cohort were given anti-parasitic treatment which lead to an increase in clinical and radiological disease indicating helminth induced regulatory pathways are playing a role in disease reduction (10). Following on from these observational studies several clinical trials were initiated using *Trichiruis suis* and *Necator americanus,* which were chosen due to their favorable infection safety profile. However, while these studies confirmed safety, the efficacy of these trials were mixed, indicating that the context and dose required may differ from patient to patient, and helminth to helminth (11, 12). Ultimately, understanding how helminths modulate the immune system during infection in MS patients will be required if helminth therapy is to be used more effectively in the future.

To further understand the mechanisms by which helminths are able to suppress inflammatory diseases, we look to animal models of infection and disease. Experimental autoimmune encephalomyelitis (EAE) is a mouse model of MS that is induced by priming with myelin peptides and/or protein resulting in CNS-specific pro-inflammatory Th1 and Th17 cells that drive neurodegeneration (13, 14). In this model, disease is dependent on Th1 cytokines such as IL-12, and more so on the Th17-driving cytokine IL-23 (15), as well as GM-CSF and IL-1 (13). In contrast, Th2 cytokines and regulatory cells (Tregs) are known to reduce disease severity (16). Helminths are known to strongly induce Th2 responses and some are associated with increased Treg numbers, therefore it is unsurprising that mice subjected to EAE that are simultaneously infected with helminths show a reduced EAE disease severity compared to non-infected mice, as comprehensively reviewed by other authors (12, 17).

Helminths employ a range of immune suppressive mechanisms to enhance their survival within the host such as expansion of Tregs and myeloid derived suppressor cells or by releasing factors that can suppress anti-helminthic Th2 immunity. These pathways are also capable of suppressing bystander inflammation; for example infection of EAE mice with *Schistosoma mansoni* induced regulatory macrophages capable of modulating CNS inflammation (18), whereas immunizing with egg antigens from either *S. mansoni* or *S. japonicum* suppressed EAE progression by inducing Th2-deviation and IL-4 production, resulting in reduced MOG-specific Th1 and Th17 cytokines (19, 20). In the case of *Fasciola hepatica,* a reduction of EAE disease was attributed to TGF-β-induced suppression of Th1 and Th17 responses and an expansion of Tregs (21). Infection with a native mouse intestinal helminth parasite, *Heligmosomoides polygyrus,* is also associated with an increase in the number of regulatory T cells as well as strong Th2 responses. In part *H. polygyrus* drives Tregs by secreting a protein named *Hp*-TGM that mimics the activity of mammalian TGF-β and is known to induce Tregs *in vitro* (22). This protein is just one of many identified from *H. polygyrus* excretory/secretory products (HES), from which a number of exciting immune modulating proteins have been found.

Previous work has identified that infection with *H. polygyrus* suppresses EAE disease severity when infection begins after onset, however the mechanisms by which this suppression is mediated are not fully defined (23–25). Therefore, this study aimed to identify the role *H. polygyrus* and its excretory/secretory products play in EAE disease suppression, and whether this protective effect is mediated through Th2 immune-deviation or induction of Tregs. We determined that *H. polygyrus* is able to ameliorate EAE disease severity in an IL-4Rα-dependent manner and that protection requires live helminth infection as HES itself is unable to induce a significant amount of disease protection. Furthermore, disease protection is associated with increased Tregs, $GATA3^+$ and $ST2^+$ cells, reduced $ROR\gamma t^+$ and IL-17A cell responses, and a lower level of myeloid cell infiltration into the CNS.

METHODS

Animals

Female inbred C57BL/6, IL-$4R\alpha^{-/-}$ (26) and Foxp3-GFP C57BL/6 reporter (27) mice were used for experiments aged between 6-14 weeks old. All mice were either bred in-house or sourced from the University of Edinburgh and housed in the University of Glasgow animal facility. All experiments were performed under UK Home Office licence and approved by the

University of Glasgow and/or University of Edinburgh Ethical Review boards.

EAE Immunization

Mice were induced for EAE using previously published protocols (28). In short, an emulsion of MOG_{35-55} (Genscript, USA) was prepared in Complete Freund's Adjuvant (Sigma, USA) and passed through a 19G needle (BD Biosciences, USA) with glass syringe until homogenous and opaque. Mice received 100 µl subcutaneously in each hind limb, followed by 200 ng of pertussis toxin (Sigma) intra-peritoneally (i.p) in 200 µl of pertussis toxin buffer (Triton-X 0.017%, Tris pH 7.4 15 mM, Sodium chloride 0.5 M). On day 2, mice received a repeat dose 200 ng of pertussis toxin i.p. Mice were monitored closely and weighed daily from disease onset, EAE mice were scored as previously published (28), 0 = unaffected; 0.5 = loss of tonicity in the distal region of the tail; 1 = half-tail paralysis; 2 = full tail paralysis; 3 = one hind limb paralysis or severe weakness in both hind limbs; 4 = full hind limb paralysis; and 5 = moribund. If any group in one experiment reached the humane endpoint of severity, all groups were terminated for analysis, between days 19 and 25 post-EAE induction.

Treatment Regimens

The parasite *Heligmosomoides polygyrus* life cycle was maintained through serial passage of CBA x C57BL/6 F1 mice as previously described (29), and experimental EAE mice received 200 L3 larvae via oral gavage on either the same day as EAE induction or 4 weeks beforehand, as indicated by the Figure legends. *Hp*-TGM was prepared as previously described (22) and 1 µg/mouse was injected intraperitoneally either on days −1, 1, 3, 5 or days 10, 12, 14 as indicated in the Figure legend. Continuous infusion of HES or PBS via ALZET osmotic minipump (Charles River, UK) using 100 µl capacity (model 1004, 28 days). The minipumps were primed by incubation with HES or PBS overnight at 37°C 2 days before surgical insertion as previously described, and shown to be effective at immune system modulation over a 14-day period of release (22).

Parasite Counts

To assess the parasite burden, intestinal adult worms were counted macroscopically and feces were collected from the mice from day 14 post-parasite infection. The feces were weighed and left to soak in 1 ml of water for at least 1 h at 37°C, or overnight at 4°C, until soft and then mixed with 1 ml of saturated salt solution (0.27 g NaCl per ml of water). After agitation, samples were transferred to McMaster chambers and eggs were counted using a dissecting microscope (Leica, Germany). Fecal counts are expressed as the number of eggs per mg of feces.

Tissue Isolation and Single Cell Suspension Preparation

After mice were terminated by CO_2 asphyxiation, spleen and inguinal lymph nodes (iLN) were removed and processed into a single cell suspension by passing the tissues through a 70 µm cell strainer (Greiner Bio-One, Austria), then resuspended in complete culture medium containing Dulbecco's minimal essential medium, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutatmine, 10 mM Hepes and 10% heat-inactivated fetal calf serum (FCS) (all from Gibco, USA). Where needed, red blood cells (RBC) were lysed from 2 min at room temperature with 2 ml of RBC lysis buffer (Sigma, USA). Cell viability was calculated using Trypan Blue exclusion (Sigma) and then resuspended at a concentration of 5×10^6 or 1×10^7 for downstream flow cytometry or MOG-restimulation, respectively.

Spinal cords were also harvested following perfusion of the mouse with 20 ml of PBS (Gibco). The tissue was then finely chopped using a scalpel and resuspended in 2.4 mg/ml collagenase type II (Gibco) and left for 30 min in a shaking 37°C incubator. After incubation, breaking up the clumps by repeat pipetting with a P1000 pipet and passing through a 70 µm cell strainer (Greiner Bio-One) then washing the cells and resuspending in a 33% Percoll solution [33% Percoll (GE healthcare, USA), dPBS (Gibco) and HCl to neutralize] and centrifugation at 760 g for 30 min with no brake. The top layer containing myelin was discarded and the cell pellet resuspended in a low volume of PBS before flow cytometric analysis.

Flow Cytometry Analysis

Single cell suspensions of spleens, inguinal lymph nodes and spinal cords were stained using live/dead dye and stained with fluorescent antibodies before being run on a Celesta or LSRII flow cytometer (BD Biosciences, USA). Fcγ receptors were blocked by the addition of purified anti-CD16/CD32 (eBioscience, USA) as well as the blocking of non-specific antibody binding by the addition of polyclonal rat IgG (Sigma, USA), before staining with antibodies to stain extracellular surface markers including: rat anti-CD4-PerCP/Cy5.5 (clone GK1.5), rat anti-CD45- PE/cy7 (clone 30-F11), rat anti-CD3-BV711 (clone 17A2), rat anti-PD-1-PE (clone 29F.1A12, Biolegend, USA) and rat anti-ST2-AF488 (clone RMST2-2) (eBioscience). After extracellular staining was completed, the cells were fixed and permeabilised using the Foxp3 transcription factor buffer kit (eBioscience) as per manufacturer's guidelines. Antibodies used to stain transcription factors included: rat anti-RORγt-PE (clone AFKJS-9), rat anti-Foxp3-eF450 (clone FJK-16s, eBioscience) and rat anti-GATA3-AF488 (clone 16E10A23, Biolegend). After acquisition on the flow cytometer, data was analyzed using FlowJo (BD Biosciences, USA).

ELISA

Splenocytes were resuspended in complete tissue culture media at a concentration of 1×10^6 cells per well in a 96 well plate (Corning, USA) in the presence of MOG_{35-55} peptide (Genscript, USA) at concentrations ranging between 0.3 and 30 µg/ml for 72 h at 37°C in 5% CO_2. The plates were centrifuged at 400 g for 5 min to pellet the cells and supernatants removed and immediately stored at−80°C until ELISA analysis. Interleukin (IL-) 10, IL-4, IL-5, IL-17A and interferon-γ (IFN-γ) sandwich ELISAs were performed as per manufacturer's instructions (eBioscience, USA).

Statistical Analysis

All graphs and data analysis were performed using Prism (GraphPad, USA).

RESULTS

Infection With *H. polygyrus* at Day 0 Reduces EAE Disease Severity

Previous works have shown that infection with 200 L3 *H. polygyrus* larvae during the chronic phase of EAE is able to suppress clinical disease within 3–6 days post infection, indicating that the L4 larvae stage is able to attenuate disease symptoms and pathology (23, 24). To investigate further the impact of adult *H. polygyrus* on EAE, mice were infected on the same day as the EAE immunization protocol, which results in adult worms being present in the small intestine during the onset of EAE disease (days 10–14). Mice infected with *H. polygyrus* showed a significant amelioration in disease severity, and a 4-day delay in weight loss (**Figures 1A,B**). Additionally, the reduction in disease symptoms in *H. polygyrus*-infected mice correlated with reduced $CD4^+$ T cell (**Figure 1C**, **Supplementary Figure 1A**) and macrophage (**Figures 1D,E**, **Supplementary Figures 1B and 2**) infiltration into the spinal tissue, confirming that parasite infection impedes EAE disease progression in the CNS.

Infection with *H. polygyrus* was also found to alter the T cell immune profile in the periphery, resulting in localized alterations in major subpopulations. In the (hind limb-draining) inguinal lymph nodes, there was a significant increase in T regulatory cell (Treg) frequency (**Figure 2A**), and while no change was seen in cells expressing the disease-driving Th17 cell marker RORγt (**Figure 2B**), cells expressing the Th2 marker GATA3 trended toward an increase (**Figure 2C**). In the spleen, despite no increase in Tregs (**Figure 2D**), there was a marked effector T cell skewing with reduced RORγt Th17 (**Figure 2E**) and expanded $GATA3^+$ Th2 (**Figure 2F**, **Supplementary Figure 3**).

The shift in effector response mode was even more marked in autoantigen-specific assays, as upon restimulation with the immunizing peptide $pMOG_{35-55}$, the splenocytes from *H. polygyrus*-infected mice secreted lower levels of MOG-specific IL-17A and higher levels of IL-4, whilst MOG-specific IFN-γ was similar between infected and control EAE mice (**Figures 2G–I**). Overall, these results suggest that infection with *H. polygyrus* induces immunological changes that can mitigate EAE disease, however whether the parasite infection is driving these changes or if the proteins secreted by the adult worm are responsible had yet to be determined.

H. polygyrus Excretory/Secretory Product Marginally Delays EAE Onset

Like many helminth parasites, *H. polygyrus* is known to secrete a number of proteins with immunosuppressive properties (30), and hence we next investigated whether proteins secreted by *H. polygyrus* adult worms are able to suppress EAE disease. As mice infected with *H. polygyrus* showed a significant increase in the frequency of Tregs in the inguinal lymph nodes (**Figure 2A**), we first hypothesized that one mechanism by which the parasite is offering protection is through the induction of Tregs. Recent studies in our lab have identified a protein, *Hp*-TGM, secreted by *H. polygyrus* that is able to induce *de novo* Tregs by mimicking the action of TGF-β (22). We therefore next tested if administration of this protein can induce Tregs and suppress EAE symptoms. However, when *Hp*-TGM was administered either during disease induction (**Figure 3A**) or disease onset (**Figure 3B**), no protection was seen compared to untreated EAE mice. However, we also did not see an expansion of Tregs in either the inguinal lymph nodes (**Figures 3C,D**) or spleen (**Figures 3E,F**), suggesting that if Treg induction is the mechanism by which *H. poygyrus* suppresses disease, adminstration of *Hp*-TGM in this way is not effective at inducing Tregs in this model.

Further, we aimed to assess whether the whole *H. polygyrus* excretory/secretory product, HES, can suppress EAE symptoms. To do this, HES was collected from adult *H. polygyrus* as previously described (29) and continuously administered into the peritoneal cavity by osmotic minipumps that were surgically implanted 2 days prior to immunization with pMOG (for initiation of EAE). While HES minipumps only resulted in a slight delay of EAE disease symptoms compared to control sham surgery mice (**Figure 4A**), there was a significant reduction in the number of cells in the inguinal lymph nodes and a trend to reduction in the spleen (**Figures 4B,C**), which indicates that HES reduced the local inflammation but could only delay progression of the disease. No significant changes were seen in Treg frequencies in either the lymph node (**Figure 4D**) or spleen (**Figure 4E**). Treatment with HES significantly increased the proportion of ST2 expressing $CD4^+$ T cells in both the lymph node and spleen, indicating an induction in Th2-type cells (**Figures 4F,G**). There was also evidence for higher expression of PD-1, in the spleen alone (**Figures 4H,I**) suggesting that HES-treated mice had a immunological shift toward a more suppressive Th2-type response which is associated with a reduced EAE disease severity.

An immune deviation effect was further supported by the responses of splenocytes restimulated *in vitro* with pMOG, in which HES-treated mice produced lower levels of the disease-driving cytokine IL-17A (**Figure 4J**), with some suggestion of elevated IL-5 (**Figure 4K**), a surrogate Th2 cytokine in this experiment. Altogether these results indicate that treating mice with HES during the early EAE induction phase leads to no more than a marginal delay in disease symptoms, perhaps through a modest reduction of cell infiltration to the lymph nodes that drain the site of immunization and dampening of inflammatory IL-17A cytokine responses.

Together the results from *Hp*-TGM and HES administration during EAE indicate that proteins secreted by the parasite may only play a small role in EAE disease suppression by *H. polygyrus,* and that the immune response to the parasite itself may be more crucial.

Early but Not Chronic Infection With *H. polygyrus* Is Able to Suppress EAE

Because *H. polygyrus* infection on the day of EAE induction protects against disease, while adult secreted immunomodulatory products do not, we postulated that a key factor may be the early events following parasite entry into the intestinal tract. Here, larvae invade the duodenal submucosa for ∼7 days, where they molt twice and grow to the adult stage that regains the lumen by day 8, having stimulated a potent type 2 response in the tissues. We therefore compared mice infected, as previously, at

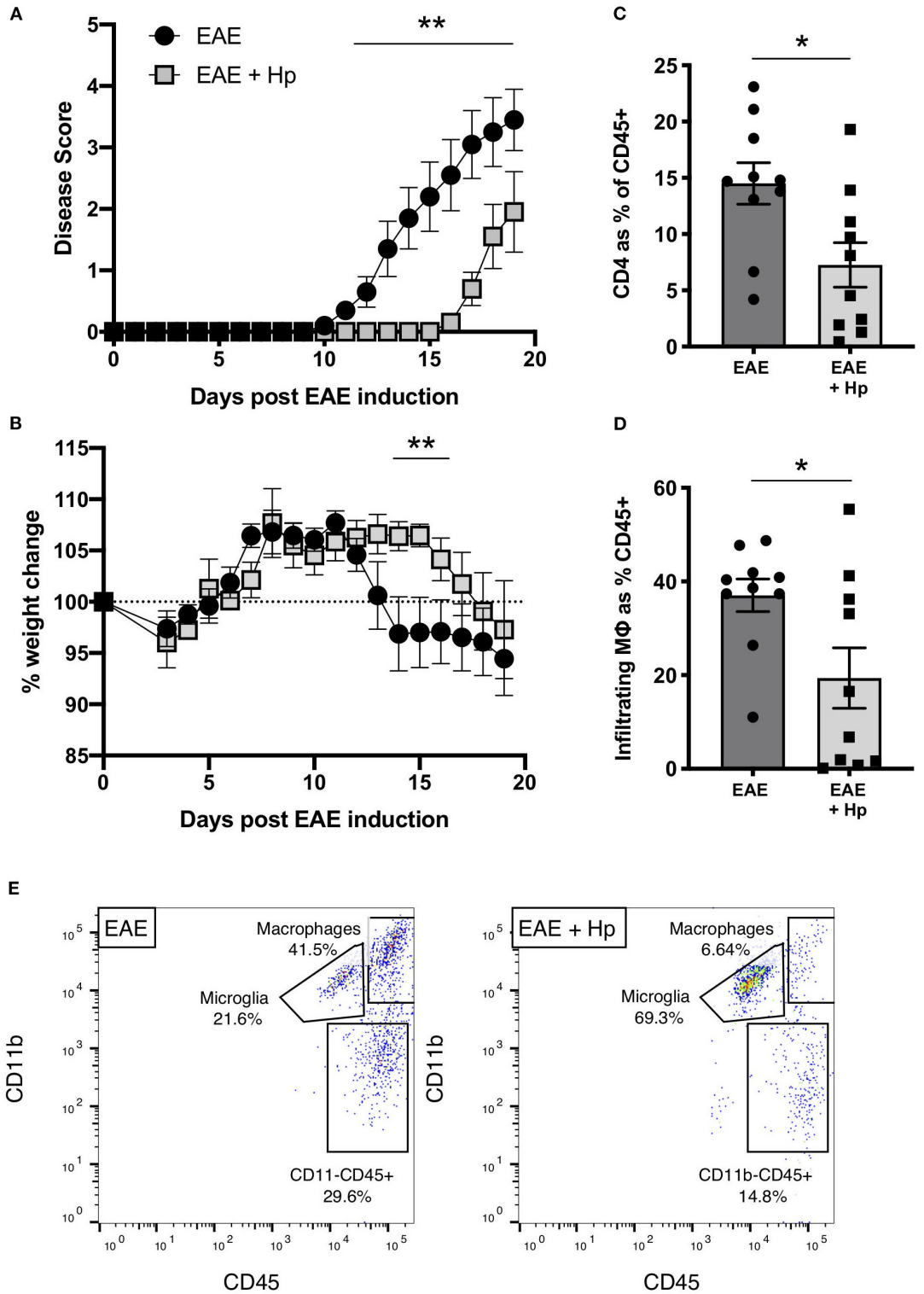

FIGURE 1 | *H. polygyrus* suppresses EAE disease in mice infected on the day of immunization. Female C57BL/6 mice were immunized for EAE on day 0 and were either left untreated (EAE), or received 200 L3 *H. polygyrus* larvae also on day 0 (EAE + Hp). **(A)** Disease score (see Methods). **(B)** Weight change. **(C)** Spinal cord $CD4^+$ T cell infiltration at euthanisation on day 19. **(D)** Spinal cord macrophage infiltration ($CD11b^+CD45^{hi}$ cells as gated in **(E)** and detailed in **Supplementary Figure 1**) at euthanisation on day 19. **(E)** Within the gate of live single leukocytes, $CD45^+$ spinal cord cells were further gated into non-myeloid cells ($CD45^{hi}CD11b^-$), microglia ($CD45^{int}CD11b^+$), and infiltrating macrophages ($CD45^+CD11b^+$) as detailed in **Supplementary Figure 2**. Plots shown are taken from individual mice with median values in **(D)**. Data are pooled from two independent experiments, with a total $n = 10$, and show arithmetic means and standard errors. Data were analyzed by 2-way ANOVA with Sidak's multiple comparisons test **(A,B)** or by Mann–Whitney non-parametric test **(C,D)**, $^*p < 0.05$, $^{**}p < 0.01$.

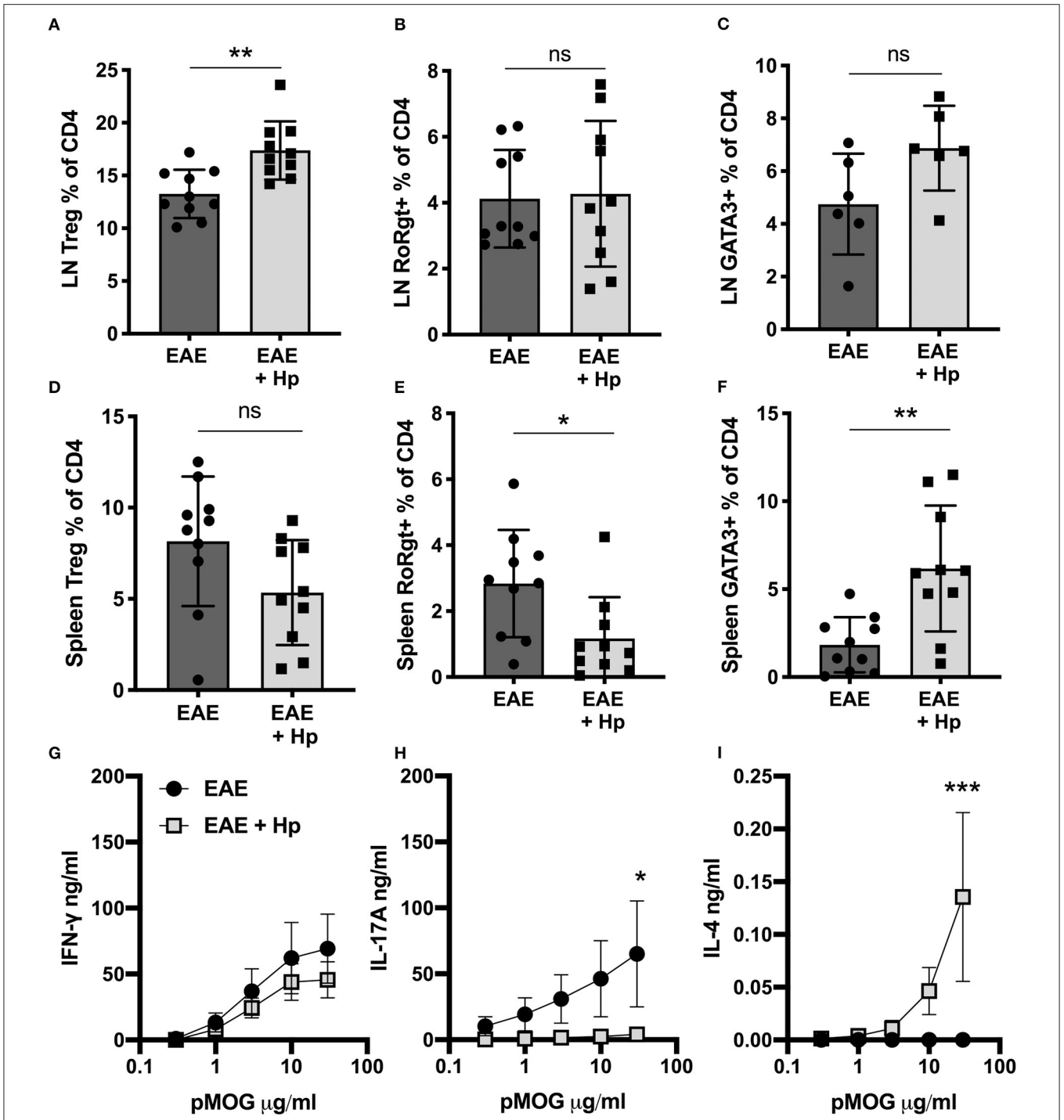

FIGURE 2 | Modes of immune response shift following *H. polygyrus* infection. Female C57BL/6 mice were immunized for EAE on day 0 and were either left untreated (EAE), or received 200 L3 *H. polygyrus* larvae also on day 0 (EAE + Hp). **(A)** Foxp3$^+$ Tregs in draining inguinal lymph nodes (LNs), measured by flow cytometry at euthanisation on day 19. **(B)** Percentage of CD4$^+$ T cells expressing the Th17 cell marker, RORγt, in inguinal lymph nodes. **(C)** Percentage of CD4$^+$ T cells expressing the Th2 marker, GATA3, in inguinal lymph nodes. **(D)** Percentage of Foxp3$^+$ Tregs in the spleen. **(E)** Percentage of RORγt$^+$ Th17 cells in the spleen. **(F)** Percentage of GATA3$^+$ Th2 cells in the spleen. **(G)** MOG-specific responses of spleen cells, IFNγ measured by ELISA in supernatants 72 h post culture. **(H)** MOG-specific responses of spleen cells, IL-17A. **(I)** MOG-specific responses of spleen cells, IFNγ. All data are pooled from two independent experiments, with a total $n = 10$, except for **(C)** in which $n = 6$, and shown are the means ± SEM; **(A–F)** were statistically analyzed by unpaired *t*-tests with Welch's correction is SDs were not equal; **(G–I)** were tested by 2-way ANOVA with Bonferroni's mulitple comparisons tests. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

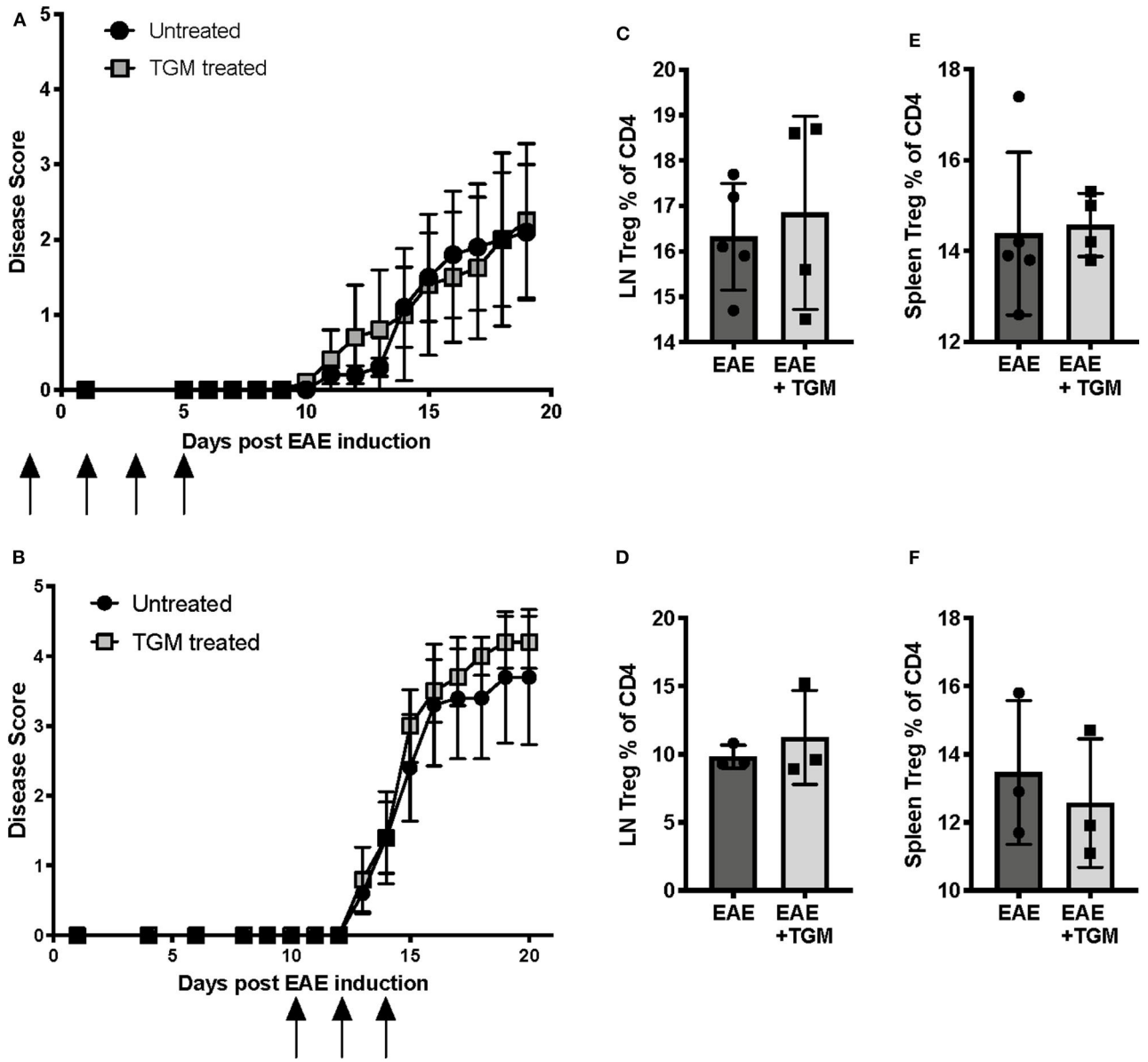

FIGURE 3 | *H. polygyrus* Transforming Growth Factor-β Mimic (*Hp*-TGM) does not protect mice from development of EAE. Female C57BL/6 mice were immunized for EAE on day 0 and were either left untreated (EAE) or received *Hp*-TGM (EAE + Hp), spleens and inguinal lymph nodes were assessed for Treg populations at euthanisation at days 19 **(A,C,E)** or 21 **(B,D,F)**. **(A)** *Hp*-TGM was given on days −1, +1, 3, and 5 relative to EAE induction, as indicated by the black arrows. **(B)** *Hp*-TGM was given on days +10, 12, and 14 relative to EAE induction, as indicated by the black arrows. **(C)** Foxp3$^+$ Tregs as percentage of all inguinal lymph node CD4$^+$ T cells from mice in **(A)**, as measured by flow cytometry at euthanisation. **(D)** Foxp3$^+$ Tregs as percentage of all inguinal lymph node CD4$^+$ T cells from mice in **(B)**. **(E)** Foxp3$^+$ Tregs as percentage of all splenic CD4$^+$ T cells from mice in **(A)**. **(F)** Foxp3$^+$ Tregs as percentage of all splenic CD4$^+$ T cells from mice in **(B)**. Data in **(A**, **B)** each represent individual experiments with no statistical differences between groups, $n = 5$ at the start of both experiments however 2 mice in each group from experiment graphs in **(B)** were culled due to experimental endpoints being reached.

the time of EAE induction, with animals that had been infected 28 days earlier, a timepoint known to be immunoregulatory in other models such as airway allergy (31).

When mice infected at the different time points were compared for the course of disease, it was clear that animals bearing a chronic infection showed little protection against disease, unlike mice that were infected on the day of EAE induction and experiencing the early, tissue-invasive phase of infection (**Figure 5A**). As previously, co-incident infection with EAE induction reduced cell infiltration into the inguinal lymph nodes (**Figure 5B**), and increased Treg frequencies (**Figure 5C**), neither of which were observed in chronically-infected mice. More notably, the pMOG-specific cytokine responses of spleens from mice infected 28 days prior to EAE induction did not differ

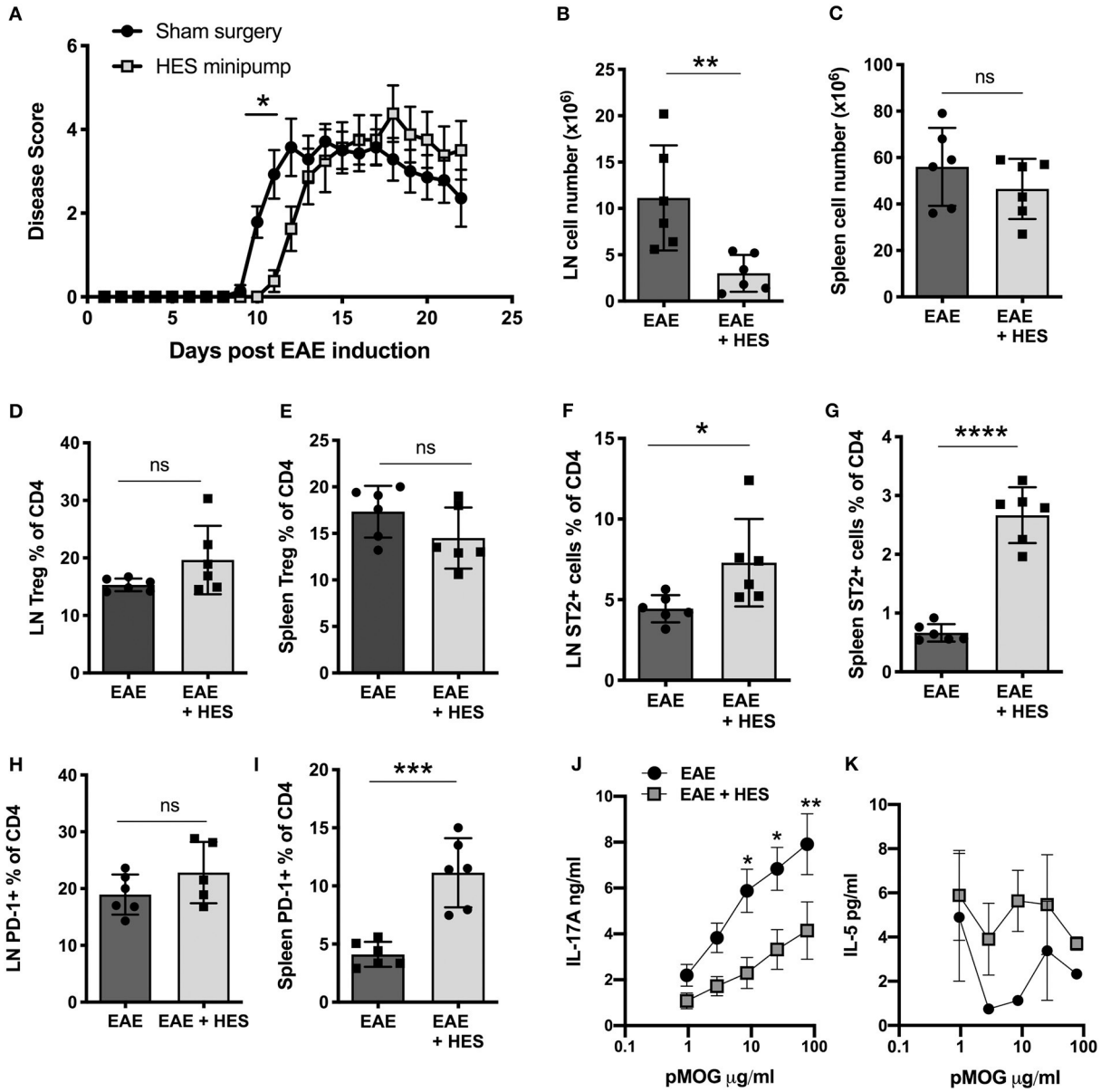

FIGURE 4 | *H. polygyrus* ES administration delays but does not prevent EAE. Female C57BL/6 mice were immunized for EAE on day 0 and received either a sham surgery (EAE) or Alzet mini-osmotic pump surgically implanted i.p, releasing HES at a continuous rate of 0.25 μl per h for 14 days (EAE + HES). **(A)** HES was given by intraperitoneal osmotic minipump 2 days prior to EAE induction. **(B,C)** Total cell numbers in inguinal lymph node **(B)** and spleen **(C)** at day 22. **(D,E)** Foxp3$^+$ cell frequencies in inguinal lymph node **(D)** and spleen **(E)**. **(F, G)**. ST2$^+$ T cell frequencies in inguinal lymph node **(F)** and spleen **(G)**. **(H,I)** PD-1$^+$ T cell frequencies in inguinal lymph node **(F)** and spleen **(G)**. **(J)** MOG-specific responses of spleen cells, IL-17A measured by ELISA at day 22. **(K)** MOG-specific responses of spleen cells, IL-5. Data are from one of two similar independent experiments, with $n = 6$ per group per experiment; **(A,J,K)** were tested by 2-way ANOVA with Bonferroni's mulitple comparisons tests. **(B–I)** were statistically analyzed by unpaired *t*-tests with Welch's correction if SDs were not equal. Shown are the means ± SEM and $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

significantly from the uninfected group, while simultaneously-infected mice showed a significant reduction in pMOG-specific IL-17A and sharply elevated pMOG-specific IL-4 responses (**Figures 5D,E**). These results indicate that *H. polygyrus* is most able to suppress EAE disease during the tissue-invasive stages of infection and during this phase MOG-specific T cells are polarized toward the Th2 mode which has been found to offer protection in previous studies of the EAE model (19, 20).

H. polygyrus Disease Suppression Is IL-4Rα-Dependent

In view of the marked skew toward Th2 during EAE in mice infected with *H. polygyrus,* including induction of pMOG-specific IL-4 responses, we aimed to assess whether signalling through the IL-4Rα plays a critical role in EAE disease suppression by this helminth. We therefore tested mice that were deficient for IL-4Rα (IL-4Rα$^{-/-}$), the shared receptor

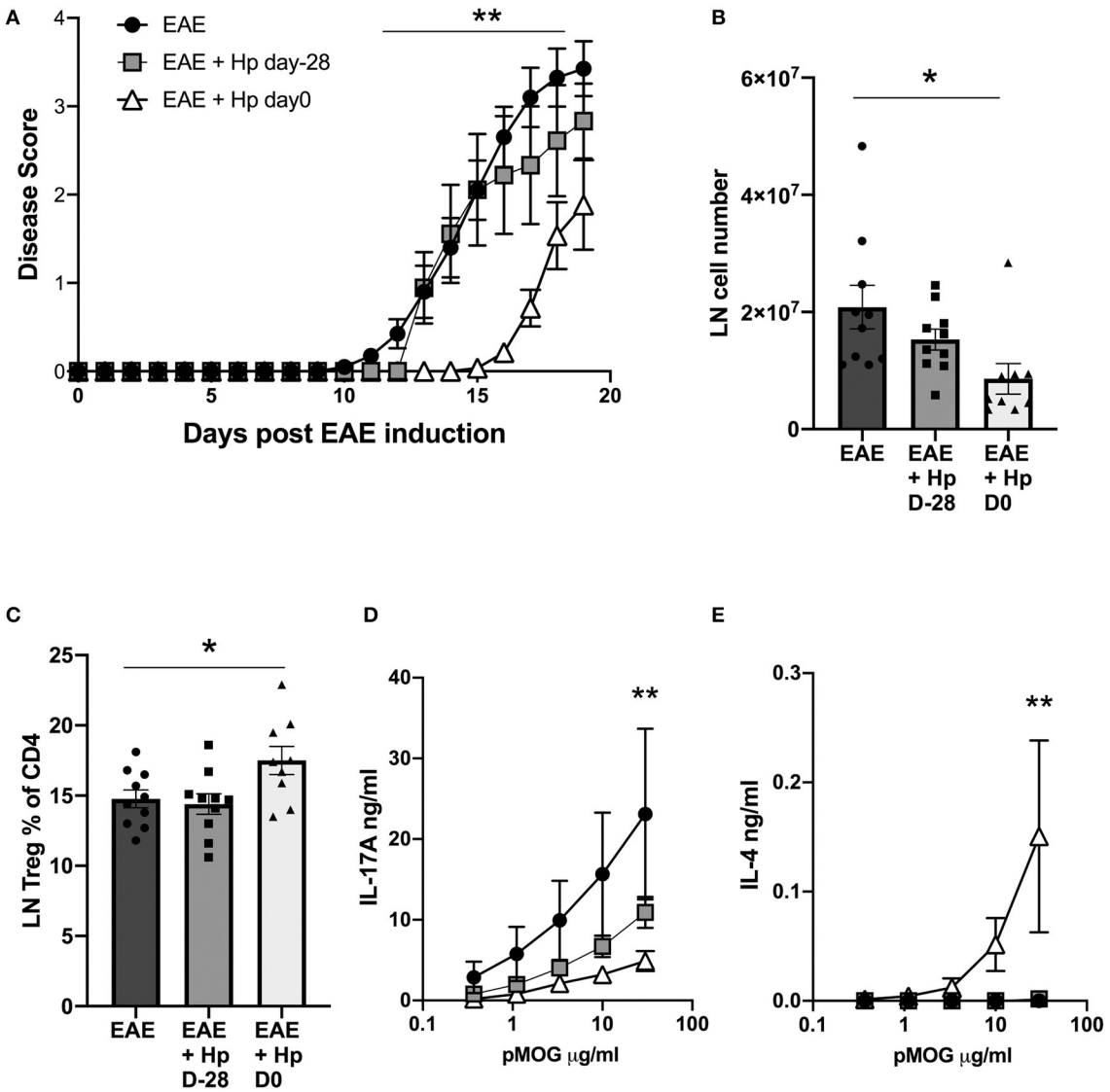

FIGURE 5 | Early, but not chronic, *H. polygyrus* infection is required to inhibit EAE. *H. polygyrus* infection was administered 28 days prior to, or on the day of EAE induction. Female C57BL/6 mice were immunized for EAE on day 0 and were either left untreated (EAE), or received 200 L3 *H. polygyrus* larvae also on day 0 (EAE + Hp D0), or on day−28 (EAE + Hp D-28) prior to EAE immunization. **(A)** Disease course in mice with EAE with or without *H. polygyrus* infection at d0 or day−28. **(B)** Total cell numbers in inguinal lymph nodes at day 19. **(C)** Foxp3$^+$ cell frequencies in inguinal lymph node. **(D)** MOG-specific responses of spleen cells, IL-17A measured by ELISA on day 19. **(E)** MOG-specific responses of spleen cells, IL-4. All data are pooled from two independent experiments, with a total $n = 10$, and shown are the means ± SEM; **(A)** was tested by mixed-effects analysis with a multiple comparisons post-tests. **(B,C)** were tested by 1-way ANOVA with Tukey's multiple comparisons tests. **(D,E)** were tested by 2-way ANOVA with Bonferroni's multiple comparisons tests. Shown are the means ± SEM $^*p < 0.05$, $^{**}p < 0.01$.

component that transduces signals for both IL-4 and IL-13. In these gene-targeted mice, *H. polygyrus* was unable to significantly suppress EAE disease unlike C57BL/6 wild-type control mice (**Figure 6A**) even though their worm load was slightly higher (**Figure 6B**) and this lack of protection was accompanied by minimal Th2 and ST2$^+$ CD4 T cell expansion compared to wild-type mice (**Figures 6C,D**). However, both IL-4Rα$^{-/-}$ and C57BL/6 mice showed an increase in PD-1$^+$ CD4 T cells with *H. polygyrus* infection, suggesting that despite their association with immune down-regulation, this cell subset is not involved in dampening EAE in this model (**Figure 6E**). Neither genotype showed any rise in Treg frequency at this time-point (day 26 post-infection), and while the baseline Treg proportions were actually higher in IL-4Rα$^{-/-}$ mice (**Figure 6F**), there was no correlation with protection, as disease severity was similar between IL-4Rα$^{-/-}$ and wild-type mice. Together, these results indicate that the ability to mount a strong Th2 response to *H. polygyrus* is central to the suppression of EAE disease by the parasite, and the timing of the anthelminthic Th2 response is pivotal for suppression of disease.

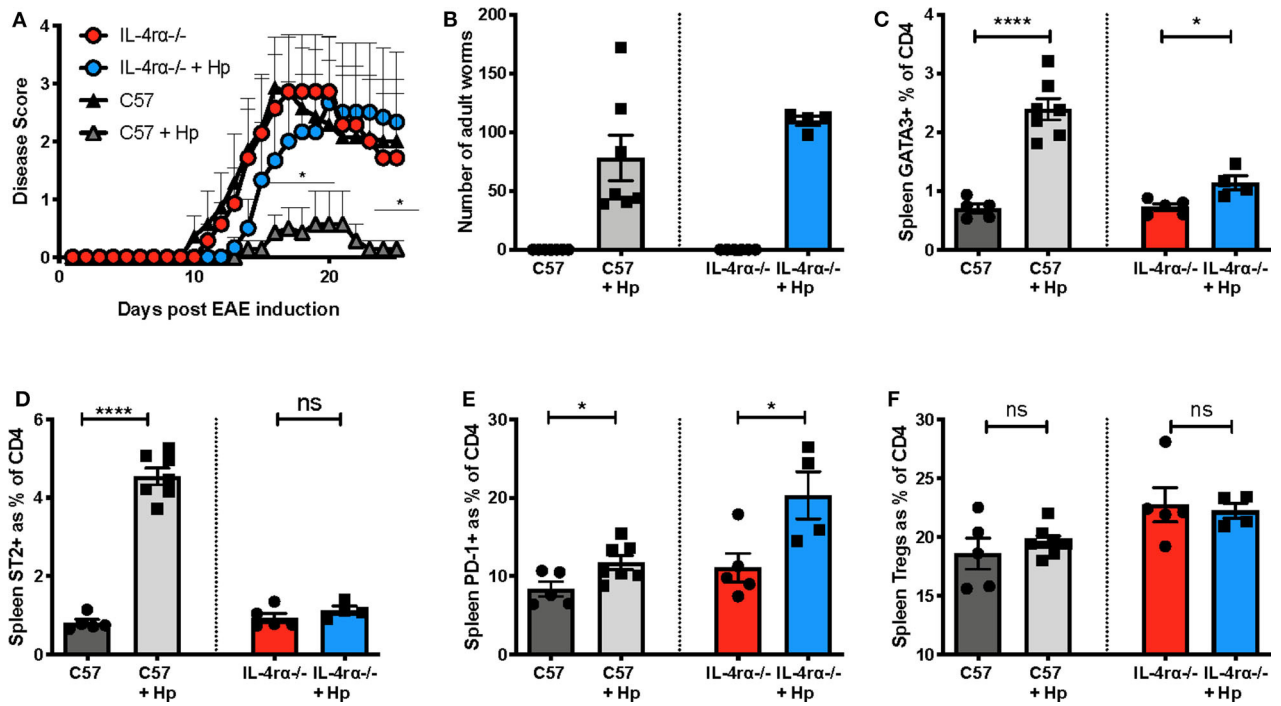

FIGURE 6 | *H. polygyrus* fails to protect against EAE in IL-4Rα-deficient mice. Female C57BL/6 or IL-4Rα-deficient mice were immunized for EAE on day 0 and were either left untreated or received 200 L3 *H. polygyrus* larvae also on day 0 (+Hp). **(A)** Disease course of EAE in uninfected and infected C57BL/6 wild-type and IL-4Rα-deficient mice. **(B)** Adult worm burdens in infected C57BL/6 wild-type and IL-4Rα-deficient mice at the time of euthanisation, day 26. **(C)** Frequency of $CD4^+$ T cells expressing the Th2 marker, GATA3, in spleen at day 25 as measured using flow cytometry. **(D)** Frequency of $CD4^+$ T cells expressing ST2 in spleen. **(E)** Frequency of $CD4^+$ T cells expressing PD1 in spleen. **(F)** Frequency of $Foxp3^+$ Tregs in the spleen. Data are from one of two similar independent experiments with $n = 4$–6 per group; **(A)** was tested by a 2-way ANOVA with Bonferroni's multiple comparisons test. **(C–F)** were statistically analyzed by unpaired *t*-tests with Welch's correction if SDs were not equal. Shown are the means ± SEM and $^*p < 0.05$ and $^{****}p < 0.0001$.

DISCUSSION

Helminth parasites have been hypothesized as important environmental regulators for immune tolerance in both model systems and in human populations (2, 17, 32), resulting in numerous studies assessing the use of helminths or their products as therapeutic agents in the fight against autoimmune disease. In the current study we used a natural mouse parasite *H. polygyrus*, a model for human intestinal helminth infection, to understand the mechanism(s) by which helminths are able to suppress an animal model of multiple sclerosis with the aim to further understand the immune pathways involved in suppressing autoimmune disease.

Infection with *H. polygyrus* is known to induce many immunoregulatory pathways including Tregs, alternatively activated macrophages and regulatory dendritic cells, as well as regulatory B cells acting through IL-10 (33), suggesting various potential mechanisms by which *H. polygyrus* is capable of ameliorating inflammatory diseases (34). Here we show that infection with *H. polygyrus* protects mice from developing severe EAE and that this disease suppression is in fact mediated through IL-4R signaling and Th2 immune deviation rather than a Treg mechanism, as IL-4R$\alpha^{-/-}$ mice infected with *H. polygyrus* developed EAE symptoms similar to uninfected mice. We also show that some of this protection is not limited to infection with the live parasite, as administration of the excretory/secretory product, HES, is able to delay disease onset. Although these mice do succumb to EAE, they also show reduced cell infiltration in the immunization draining lymph nodes and reduced pMOG-specific IL-17A release.

Interestingly, chronic *H. polygyrus* infection (day 28) prior to EAE induction was not protective, although may limit the disease severity which indicates active immune deviation to Th2 may be essential to suppress developing Th17/Th1 responses in the EAE model. Furthermore, day 28 of infection coincides with adult *H. polygyrus* residing in the gut, where they secrete HES proteins, therefore these results are consistent with HES administration only having a limited effect on the EAE disease severity. In contrast, our finding that protection coincides with the early stages of active infection are consonant with recent reports from another laboratory, that infection of mice at the peak of EAE disease results in a rapid amelioration of symptoms, accompanied by evidence of downregulation within the myeloid compartment (24, 25). During the initial 7 days of infection, serum IL-4 reaches a peak and declines thereafter (23), supporting our findings that the early phase of infection is the most protective. Further, type 2 innate lymphoid cell (ILC2) responses are at their most prominent in early infection (35, 36), and IL-4/IL-13 from this

subset is likely to contribute to the Th2 skewing observed in our model.

Our results are also consistent with other helminth infections that have been shown to potently modulate EAE such as *S. mansoni* (18), *T. peusdospiralis* (37), *F. hepatica* (21), *Trichinella spiralis* (38), and *Taenia crassiceps* (39) with protection in these infections mostly being attributed to Th2 immune deviation and suppressed levels of pMOG-specific TNF, IFN-γ, and IL-17A. These studies also highlighted other cell populations as potential suppressors of T cell activation such as alternatively activated macrophages (AAM) and myeloid-derived suppressor cells (MDSC) that regulate CNS infiltration and EAE progression. During *H. polygyrus* infection AAM are induced by Th2 cells after epithelial damage alerts the immune system of parasite infection, resulting in IL-4 release (40) and phenotypic shifts in both peritoneal and CNS macrophage populations (25). It is therefore possible that myeloid cells are also crucial for EAE protection with *H. polygyrus* as it is known that AAM and MDSCs are able to ameliorate EAE (41, 42) and therefore this may be one of the ways in which IL-4 signaling is protective in *H. polygyrus*-infected EAE mice.

In some instances the suppressive effect of helminth infection on EAE can be mimicked by the parasites' excretory/secretory products (ES). However, in the current study we found HES was only able to delay disease onset indicating that the immunological changes produced by HES were not strong enough to recapitulate the effect seen with live parasite infection. In contrast, a study with *Taenia crassiceps* ES (TcES), which showed that in addition to Th2-deviation, ES was able to sequester inflammatory cells into the periphery and therefore inhibit the induction of EAE by preventing trafficking of cells to the CNS (43), a mechanism that could well underpin the protective ability of live *H. polygyrus*. Soluble egg antigens (SEA) from *S. japonicum* and *T. spiralis* ES also ameliorate EAE disease in a way that was similar to whole parasite infection, protection that was related to a Th2 shift in both the periphery and CNS (19, 44).

Although HES treatment in our study did not offer long-term protection from EAE, a significant reduction in pMOG-specific IL-17A indicated that some aspects of autoimmunity are suppressed, although overall disease symptoms eventually appear regardless. Consistent with HES, a moderate reduction in IL-17A was seen in mice with a chronic *H. polygyrus* infection where adult *H. polygyrus* parasites would be present in the intestine secreting the HES products, indicating these ES proteins alter the autoimmunity status of cells in the periphery but ultimately disease symptoms progress. Interestingly, IL-17A is thought to downregulate the tight-junction proteins therefore increasing the permeability of the BBB and allowing CNS infiltration to occur (45). In addition, IL-17A is also thought to recruit activated neutrophils and monocytes into the CNS, facilitating further damage and demyelination which exacerbates symptoms of EAE disease further (46). Given that HES and chronically infected *H. polygyrus* mice had a reduced IL-17A response and a delay or slight reduction in EAE symptoms in our study, it is possible that this reduction suppressed early EAE disease and was then overcome as disease progressed further. It is also possible that the lack of efficacy in our study may be due to the dose of HES used, we chose 2 μg/mouse/day by continuous infusion while other studies administered up to 250 μg of protein by subcutaneous or intraperitoneal injection every other day (43, 47, 48).

While many studies have identified Th2-immune deviation as the dominant mechanism by which helminths suppress EAE disease, there is also mounting evidence that IL-33 signaling also plays a central role. One study looked at the role of the IL-33 receptor subunit, ST2, which when knocked out abrogates resistance to EAE in the partially resistant BALB/c mouse strain. The adoptive transfer of pMOG-specific $CD4^+$ T cells from $ST2^{+/+}$ mice were unable to induce EAE in $ST2^{-/-}$ mice indicating signaling through the IL-33 receptor on $CD4^+$ T cells is suppressive in the EAE model (49). In the case of *F. hepatica* ES (FHES), administration induced Th2 responses, but disease protection was independent of IL-4, IL-10 and Tregs, although it was IL-33-dependent and the transfer of FHES-induced eosinophils conferred protection in the EAE model (50). Together these studies suggest that in some helminth infection models signaling through IL-33 may be essential for EAE disease protection. In the current study we identified that both HES and infection with *H. polygyrus* induced ST2 expression on $CD4^+$ T cells and interestingly in the IL-4Rα$^{-/-}$ mice, which were not protected with *H. polygyrus,* there was very little expression of ST2 suggesting that IL-33 signaling may also play a role in EAE protection in this model.

The programmed cell-death (PD)-1/PD-L1 pathway is extensively studied in cancer research; however this tolerogenic pathway is also critical for maintaining homeostasis and deficits can lead to the development of autoimmunity. Binding of PD-1 on the surface of T cells with its ligands, PD-L1 or PD-L2 which are widely expressed on both haematopoetic and non-haematopoetic cell types, suppresses T cell activation and in the presence of TGF-β may promote *de novo* generation of Tregs (51). Therefore, expression of PD-1 and PD-L1/PD-L2 during helminth infection may result in suppressed T cell responses which is not only beneficial for the host but may also suppress bystander inflammatory diseases such as EAE. In the current study, $CD4^+$ T cells from *H. polygyrus* infected and HES-treated mice expressed higher levels of PD-1 compared to control EAE mice, indicating that expression of this marker may also contribute to the disease suppression seen. It is important to note however that PD-1 expression was also elevated in *H. polygyrus*-infected IL-4Rα$^{-/-}$ mice, but these animals were not significantly protected from EAE, indicating that PD-1 expression may only have a small role in the mechanism of *H. polygyrus.*

The CNS is now recognized to have a close relationship with the gastrointestinal tract, and therefore helminth infections within the gut may disrupt the normal gut-brain axis and interfere with disease progression in a more indirect manner. Studies have shown that alteration of the gut microbiota can have profound effects on EAE disease development, with antibiotic-mediated depletion of intestinal microbes impairing the development of EAE through an IL-10-dependent mechanism (52). These results indicate that changes in the gut can have striking effects on CNS disease development and also be mediated through an alteration in the peripheral immune response. There are also significant changes to the

gut microbiota with *H. polygyrus* infection (53–55), which are unlikely to recur with the systemic administration of ES products, further contributing to the requirement for live infection for amelioration of disease. In our study with *H. polygyrus* this could also be one of the key differences in the level of disease protection seen with HES compared to infection.

In conclusion our study has further supported work by other researchers assessing the effect of intestinal helminths in the amelioration of an animal model of MS. Notably, we identified that infection with *H. polygyrus* was able to suppress EAE in an IL-Rα-dependent manner, with further evidence to suggest that a lack of IL-4 signaling also ablates any disease protection mediated through the IL-33/ST2 receptor. Perhaps surprisingly, it was found that ES products from *H. polygyrus* were unable to mimic disease protection seen with live infection unlike results from other helminths. Altogether our results indicate that both a strong Th2 shift as well as parasite mediated damage in the small intestine is required to ameliorate EAE with *H. polygyrus* infection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Glasgow Animal Welfare and Ethical Review Board.

AUTHOR CONTRIBUTIONS

MW, CJ, JG, JK, and RO'C performed the experiments. SA and RM supervised the laboratory work. MW drafted the manuscript which was edited by CJ and RM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01830/full#supplementary-material

Supplementary Figure 1 | *H. polygyrus* suppresses cellular infiltration in mouse model of EAE disease. Female C57BL/6 mice were immunised for EAE on day 0 and were either left untreated (EAE), or received 200 L3 *H. polygyrus* larvae also on day 0 (EAE + Hp). **(A)** Spinal cord $CD4^+$ T cell infiltration at euthanisation on day 19, relative to the number of glial cells. **(B)** Spinal cord macrophage infiltration ($CD11b^+CD45^{hi}$ cells as gated detailed in **Supplementary Figure 2**) at euthanisation on day 19, relative to the number of glial cells. Data are pooled from two independent experiments, with a total $n = 10$, and show arithmetic means and standard errors. Data were analyzed by Mann-Whitney nonparametric test **(C,D)**, $^*p < 0.05$.

Supplementary Figure 2 | Gating strategy for spinal cord infiltrating macrophages. Female C57BL/6 mice were immunized for EAE on day 0 and were either left untreated (EAE), or received 200 L3 *H. polygyrus* larvae also on day 0 (EAE + Hp). Within the gate of sinle live leukocytes, $CD45^+$ spinal cord cells were gated into non-myeloid cells ($CD45^+CD11b^-$), microglia ($CD45^{int}CD11b^+$) and infiltrating macrophages ($CD45^+CD11b^+$). Further gating from non-myeloid cells into T cells ($CD3^+$) and finally CD4 ($CD4^+$) and CD8 ($CD8^+$) T cells.

Supplementary Figure 3 | Alterations in cell numbers following *H. polygyrus* infection in EAE mice. Female C57BL/6 mice were immunised for EAE on day 0 and were either left untreated (EAE), or received 200 L3 H. polygyrus larvae also on day 0 (EAE + Hp). **(A)** Cell numbers in draining inguinal lymph nodes (LNs), measured by flow cytometry at euthanisation on day 19. **(B)** Cell numbers in spleen, measured by flow cytometry at euthanisation on day 19. All data are in **(A)** are from a single experiment ($n = 5$/group) and in **(B)** pooled from two independent experiments, with a total n = 10/group, and shown are the means ± SEM; **(B)** was statistically analysed by unpaired *t*-test with Welch's correction for unequal variances, $^*p < 0.05$, $^{**}p < 0.01$.

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REVIEW
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Soil-Transmitted Helminth Vaccines: Are We Getting Closer?

Ayat Zawawi[1]* *and Kathryn J. Else*[2]*

[1] Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia, [2] Manchester Academic Health Science Centre, Faculty of Biology, Medicine, and Health, School of Biological Sciences, Lydia Becker Institute of Immunology and Inflammation, University of Manchester, Manchester, United Kingdom

Parasitic helminths infect over one-fourth of the human population resulting in significant morbidity, and in some cases, death in endemic countries. Despite mass drug administration (MDA) to school-aged children and other control measures, helminth infections are spreading into new areas. Thus, there is a strong rationale for developing anthelminthic vaccines as cost-effective, long-term immunological control strategies, which, unlike MDA, are not haunted by the threat of emerging drug-resistant helminths nor limited by reinfection risk. Advances in vaccinology, immunology, and immunomics include the development of new tools that improve the safety, immunogenicity, and efficacy of vaccines; and some of these tools have been used in the development of helminth vaccines. The development of anthelminthic vaccines is fraught with difficulty. Multiple lifecycle stages exist each presenting stage-specific antigens. Further, helminth parasites are notorious for their ability to dampen down and regulate host immunity. One of the first significant challenges in developing any vaccine is identifying suitable candidate protective antigens. This review explores our current knowledge in lead antigen identification and reports on recent pre-clinical and clinical trials in the context of the soil-transmitted helminths *Trichuris*, the hookworms and *Ascaris*. Ultimately, a multivalent anthelminthic vaccine could become an essential tool for achieving the medium-to long-term goal of controlling, or even eliminating helminth infections.

Keywords: helminth, hookworm, *Trichuris*, *Ascaris*, vaccine, vaccine-induced immunity

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***Correspondence:**
Ayat Zawawi
atzawawi@kau.edu.sa
Kathryn J. Else
kathryn.j.else@manchester.ac.uk

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INTRODUCTION

Neglected tropical diseases (NTDs) remain a global public health issue. Their impact is especially felt in areas with poor hygiene and sanitation, and are related to extreme poverty and lack of health education (1). Some of the most "neglected" diseases are caused by soil-transmitted helminths (STHs), which together infect more than a quarter of the world's population (2). The four most prevalent STHs worldwide are the Hookworms *Necator americanus* and *Ancylostoma duodenale*, the roundworm *Ascaris lumbricoides*, *Strongyloides stercoralis*, together with the whipworm *Trichuris trichiura* (3). The highest intensity of infection for *A. lumbricoides* and *T. trichiura* is typically seen in school-aged children and adolescents, whilst in hookworm infections infection intensity tends to increase with age, plateauing in to adulthood. Those who travel to and from regions where STH infections are endemic are also at risk of getting infected (4, 5). Although little is known about *S. stercoralis* in comparison to *Ascaris*, *Trichuris* and hookworm despite prevalence estimates of up to 40% in tropical countries (6–9) there is a growing understanding of the immune response to infection (10, 11). However, a biochemical understanding of the parasite remains

limited and thus, this review will focus on vaccines against the human hookworms (12), whipworm and roundworm (13).

The ability to control STH infections currently depends almost exclusively on mass anti-helminthic drug administrations (MDA) such as albendazole or mebendazole to at-risk populations (4, 5, 14, 15) in conjunction with education and improved sanitation including WASH initiatives (16, 17). However, post-treatment reinfection is common, especially for helminth species such as *Ascaris* and *Trichuris,* where the robust parasite eggs are nearly ubiquitous in the environment. Such extensive contamination of soil with parasite eggs limits the ability of MDA programs, global control and elimination efforts to interrupt the transmission cycle within a community (18). Moreover, the appearance of anthelmintic-resistant parasites threatening human drug treatment programmes (19, 20) has increased interest in developing vaccines, or a pan-anthelminthic vaccine to provide a cost-effective, long-term immunological method to control multiple helminth infections (21–23).

This review explores our current knowledge, prospects, and challenges for anti-human STHs vaccine design. Although of significant importance, the review does not cover veterinary vaccines which have been reviewed recently (24). We include the importance of understanding the underpinning immune responses required to eliminate STHs from their host, opportunities for antigen-presenting cell targeting of candidate antigens, and include recent pre-clinical and clinical trials in the context of *Ascaris*, *Trichuris* and the hookworms.

PROSPECTS AND CHALLENGES FOR ANTI-STHs VACCINE DESIGN

The development of efficient anti-STH vaccines is thought to represent a greater challenge compared to developing anti-bacterial or anti-viral vaccines. This is partly a result of the complex lifecycles of STHs, combined with an incomplete immunological knowledge of the host-parasite interactions and the immune mechanisms conferring protection (25). Moreover, STHs have complex genomes and proteomes (26–30). This complexity makes it difficult to identify antigenic targets for the development of an effective vaccine (31). Despite these obstacles, the development of vaccines against the STHs has progressed over the decades.

Identifying Lead Antigens to Include in Vaccines

Crude Antigen Preparations

The earliest anthelmintic vaccines included attenuated or irradiation-killed parasites (32, 33). Since this time, extensive studies across many helminth spp have explored the immunogenicity of native molecules excreted and/or secreted by parasites (the so-called ES molecules) (34–37) and parasite-derived extracellular vesicles (EVs) in promoting resistance to infection (38–41). Excreted and secreted parasite molecules make excellent vaccine candidates as they sit at the host-parasite interface, playing critical roles in both the modulation of host immunity and in inducing Th2-skewed immune responses (35, 42).

Recombinant Proteins

Instead of targeting the immune response against a mixture of antigens, a particular antigen from the pathogen can be produced and expressed in a heterologous expression system to focus the immune response toward a specific antigen of the pathogen to prevent the infection (43, 44). Such an approach ensures that safer and more reliable vaccines are developed, with an example being the licensed recombinant hepatitis B vaccine (45). The key therefore to an effective recombinant vaccine is to identify that particular conserved antigen or combination of antigens secreted or extracted from the pathogen that can overcome the low protective immunity naturally generated by the infection (44, 46). However, recombinant vaccines often require an adjuvant and multiple immunizations to elicit a protective and long-lasting immune response (47). Moreover, the production of recombinant proteins using bacterial heterologous expressions can prove challenging. Thus, antigens in which proteolytic stability, higher production yield and post-translational modifications (e.g., phosphorylation and glycosylation) are needed, other expression systems such as yeast, insect, plant, or mammalian cells should be considered (44). Of the most successful helminth recombinant vaccines are the two against hookworm disease caused by Necator americanus, which consist of aspartic protease-1 (Na-APR-1) and glutathione-S-transferase-1 (Na-GST-1). These vaccines are currently in phase 1 of clinical trials (48–50).

DNA Based Vaccines

The availability of genome sequences opened up the prospect of the DNA vaccine approach to validate novel vaccine candidates individually or combined to improve vaccine elicitation of cell-mediated and mucosal immunity (51, 52). DNA vaccines are simple rings of DNA containing a gene encoding a specific vaccine antigen under the regulation of a promotor (53). DNA vaccine technology has been intensively used to develop vaccines against various pathogens (54, 55), cancer (56), and autoimmune disorders (57). For example, the human immunodeficiency virus-1 (both as a prophylactic and an immunotherapeutic vaccine) (58, 59), Zika virus (60), and Ebola virus vaccines (61) are currently in clinical trials. Comparatively, little progress has been made toward developing DNA vaccines against parasitic diseases, although several pre-clinical studies have shown promising results against hookworm infections (62, 63), malaria (64, 65), leishmaniasis (66, 67), and schistosomiasis (68, 69). Even though DNA vaccines offer several advantages when compared with recombinant vaccines, such as safety, improper protein folding and production cost, they have not been shown to be sufficient to induce a protective immune response (70). Therefore, DNA encapsulation, plasmid alterations, co-expressing cytokines and heterologous prime-boost approaches have been explored to enhance the immune responses induced by the DNA vaccine (71, 72).

Epitope-Based Vaccines

The advent of the genome era and the tremendous advances in immunological and bioinformatics tools have enabled the reverse vaccinology approach (RV) as a new effective strategy for lead antigen identification in vaccine development (73–75). One of the advantages of the RV approach is that every antigen encoded in the worm genome can be screened *in silico* to determine its ability to induce an immune response (31, 74). Thus, it can overcome some of the limitations of conventional methods of screening vaccine candidates (76–78). Antigen selection can also be carefully prescribed based on clear inclusion and exclusion criteria. For example, Zawawi et al. employed a systematic, multi-stage process to identify *Trichuris* epitope vaccine antigens based on the RV approach (79). The epitopes were identified from secreted, and surface-exposed proteins and any protein with any degree of homology to humans or mice were excluded to eliminate potential autoimmune reactions. Most of the identified vaccine antigens were stage-specific and had essential functions in the parasite biological process, associated with host-parasite interaction, parasite metabolism, development and fecundity (80).

The Search for a Pan-Anthelmintic Vaccine

Coinfections with two or multiple STHs are extremely common in sub-Saharan Africa and elsewhere in the developing countries (1, 81). The three STHs hookworm, *Ascaris* and *Trichuris* also share highly conserved antigens that are likely to have very similar biological functions (46). Thus, researchers have proposed a single pan-anthelmintic vaccine against the three major human STHs to generate strong, lasting immunity with minimal side effects (23). RV methodologies have much to offer in this context. Thus, with the availability of parasite genomes and bioinformatics tools to select out IgE-inducing epitopes *in silico*, the development of a pan-anthelmintic vaccine based on protective epitopes from cross-protective antigens may represent an exciting alternative to the use of whole antigens.

The choice of lead antigen also determines the biomechanical requirements of production, and the design of the laboratory and clinical trials (82–84). For example, vaccines based on native antigens, are known to generate significant immunity against many STHs in pre-clinical models (85–87). However, they have many manufactory limitations, such as high cost, time consumption, difficulty of purifying large quantities of worm antigens, low stability, shelf-life, shortage of *in vitro* methods for the culture of parasites and control over differences in batches to develop a commercially stable vaccine (88–90). Approaches such as recombinant (91), DNA (92), and epitope-based vaccines (93) overcome some of the limitations associated with native antigens. However, most vaccines against STHs remain in early-stage development or are undergoing pre-clinical evaluation.

Adjuvants: Key Components in Modern Vaccinology

The effectiveness of lead antigens is often restrained by an inherent lack of immunostimulation. Therefore, efforts have focused on co-administering antigens with vaccine adjuvants as a key component in modern vaccinology, aiming to intensify the immune response and generate effective immunological memory (94). In addition, adjuvants are important in overcoming immune senescence seen in the elderly (95), and expanding the antibody repertoire generated (96). Adjuvants may also allow dose-sparing (97) and careful choice of adjuvants used provides the ability to guide the type of immune response generated (98, 99). The specific type of T helper response generated plays critical role in the efficacy of the protective immune response, as a Th1-type response is critical in developing vaccines against intracellular pathogens, whereas Th2-type responses are critical in developing vaccines against extracellular parasites (100).

Among the earliest adjuvants used in experimental antibody production were Freund's adjuvants (101, 102). However, as these adjuvants are associated with pain, inflammation, and tissue destruction they are no longer used and have been replaced by other adjuvants that can produce equal or superior antibody responses with less inflammation and tissue destructions (103).

The two adjuvants that are licensed for use in humans, aluminum salt and squalene oil-based emulsion (MF59), mainly promote Th2-biased immune responses (99, 104). Each adjuvant has its own immunological signature. For example, alum increased antibody titers, whereas MF59 induces strong antibody and IL-5 responses in mice (105). However, their mechanisms of action remain only partially understood.

Nanoparticulate vaccine adjuvants and delivery vehicles have also been incorporated into vaccine design to enhance the humoral and cellular immune responses (106, 107). Vaccine delivery systems include synthetic nano- and micro-particles, immunostimulatory complexes, liposomes, virosome, and virus-like particles (VLP) (108). These approaches promote the uptake of antigens by different antigen-presenting cells, promote their migration and maturation from the site of vaccine uptake and protect antigens from degradation (106, 109, 110). VLPs, for example, possess the immunostimulatory and self-adjuvanting properties of natural viruses but do not contain genetic material (111, 112). In addition, they are very stable and can withstand adverse environments, such as those with acidic pH, making VLPs an attractive carrier for mucosally administered vaccines (113). RTS, S (Mosquirix) was the first licensed VLP-based vaccine generated against parasitic disease (114). It is composed of three tandem repeat (R) and cell (T) epitopes from the circumsporozoite protein of the *P. falciparum* malaria parasite, which are displayed on hepatitis B surface particles (HBs-Ag) (S), co-expressed in *Saccharomyces cerevisiae* (S) and reconstituted with an AS01 adjuvant (115). The development of vaccines against *Toxoplasma gondii* (116), *T.* spiralis (117), *Clonorchis sinensis* vaccine (118), and *T. trichiura* (79) have all embraced VLP technology.

Antigens adjuvanted with toll-like receptor (TLR) agonist such as monophosphoryl lipid A (MPLA) and Glucopyransoyl lipid A (GLA) also enhance immune responses by inducing high antibody titres, driving the production of Th1 cytokines and rapid immune responses (119, 120). The TLR4 agonists are approved for use in human vaccines and are being studied in leishmaniasis (121), schistosomiasis (122), tuberculosis (123), and influenza vaccines (124). The combination of vaccine antigen with different TLR agonists has also been studied in various

formulations such as microparticles, nanoparticles, and lipid emulsions to enhance the immune responses induced by the vaccine (125, 126). For example, mice immunized with a yellow fever vaccine containing antigens combined with TLR4 and TLR7 agonists and conjugated to synthetic nanoparticles, induced synergistic antigen-specific neutralizing antibodies compared to immunization with antigens coupled with a single TLR ligand (127). Further, an HIV vaccine conjugated with a TLR5 agonist combined to a nanoparticle synthesized from a synthetic poly lactic-*co*-glycolic acid (PLGA) showed increased immunogenicity in a preclinical mouse model and reduced the immunogenic dose of the vaccine candidate (128). Moreover, the formulation of a TLR4 ligand contained within a PLGA-based nanoparticle plus a malaria proteins (Pfs25) noted for its poor immunogenicity, improved vaccine induced-immunity (129).

Given the range of adjuvants available it is recommended to test several adjuvants or immunostimulants with each potential vaccine antigen at the pre-clinical stage of development in order to optimize the quality of the immune response and to generate effective protective immunity.

Challenges to Vaccine Development

Having identified a lead antigen and selected an appropriate adjuvant, other hurdles remain to be overcome in the development of an anti-helminth vaccine. These include avoiding unwanted side effects, choosing a suitable animal model for testing immunogenicity and protective immunity, the immunization schedule, and the administration route to shape the immune response induced by the vaccine (82, 83).

Beyond the laboratory, other obstacles exist, including geopolitical barriers, unreliable pharmaceutical manufacturer markets, and low industry interest. Further, the rising anti-vaccine movement in the US and elsewhere have had a significant effect on STHs vaccine development (130). Thus, the development of new vaccines is a complex and multidisciplinary task that requires an understanding of host-pathogen interactions, epidemiology, and manufacturing parameters (131). Most importantly, vaccine researchers must have an understanding of the immune mechanisms involved in diseases and protection in order to select appropriate antigenic targets and delivery systems to shape the immune response induced by the vaccine (82, 83).

IMMUNE RESPONSES TO HOOKWORM, *ASCARIS*, AND *TRICHURIS* INFECTIONS

Th2 Immune Responses Are Center Stage in Acquired Immunity to Infection

There is an abundance of literature documenting the need for strong Th2 biased immune responses to generate protective immune responses to STHs in animal models (132, 133). This is also clear in humans, where immunity to the human hookworms, *Ancylostoma duodenale* and *Necator americanus*, the roundworm *A. lumbricoides*, and the whipworm *T. trichiura* require the generation of strong Th2 immune responses and the production of Th2-associated cytokines including interleukin (IL)-4, IL-5, IL-6, IL-9, and IL-13 (134–136).

Infection of man with *N. americanus* and *T. trichiura* induces a mixed Th1/Th2 response characterized by the up-regulation of IFN-γ (Th1) and a strong Th2 response. As worm burdens decrease with age, or in the context of resistance to reinfection post drug treatment, so parasite-specific IgE levels increase accompanied by Th2 cytokines such as IL-5, IL-9, and IL-13 (134–138). The immune response to *A. lumbricoides* infection in 12–17 year old hosts living in endemic communities is more Th2 biased (IL-4 and IL-5) with no detectable production of IFN-γ in response to *Ascaris* antigens (139–141). Turner et al. (142) divided his study population of *Ascaris* infected individuals in to two age cohorts, 4–11 year olds and 12–36 year olds. This study concurred with Cooper et al. (139), reporting significant negative correlations between the Th2 cytokines IL-9 and IL-13 and infection intensity, but only in the older (over 12) age cohort. Thus, children below the age of 12 showed no inverse correlation between Th2 cytokines and intensity of infection (134, 142), suggesting that protection is conferred only after decades of exposure.

Collectively, therefore, there is strong evidence from both animal models and human field studies that any anti-STH vaccine should aspire to promote Th2 immunity.

Identifying the Th2-Controlled Effector Mechanisms

Exactly how Th2 cytokines culminate in worm expulsion has been debated at length for many years, with our evidence base largely accruing from mouse models where the mechanism of action is best unpicked. Peripheral blood eosinophilia is a hallmark feature of the immune response to helminth infections (143–145). However, is it not clear whether eosinophils kill parasitic worms. IL-5 and eosinophils appear not to be essential for *Trichuris* and hookworm expulsion, as no difference in worm expulsion was observed following infection of IL-5 knockout mice (146, 147). In contrast, early studies demonstrated that eosinophils can kill infected larval stages of most helminth species investigated *in vitro* in the presence of specific antibodies or complement (148–150). A recent study also showed that eosinophils were recruited to the site of infection by immune serum activated macrophages, leading to the immobilization of migrating *A. suum* larvae (151).

Several studies have also highlighted a variety of Th2 regulated immune-mediated mechanisms associated with *Trichuris* expulsion including epithelial cell turn over (152, 153), increased muscle hyper-contractility (154), goblet cell hyperplasia and production of mucins (155), with specific Th2 cytokines identified as key. For example, several studies have provided evidence that IL-13, not IL-4 plays a critical role in increasing epithelial cell turnover and in the production of mucins to promote *Trichuris* worm expulsion (153, 156–159). IL-9 has been shown to be important in the stimulation of intestinal smooth muscle contractility, which drives *T. muris* expulsion (154, 160). In keeping with this, impaired IL-9 expression often results in chronic helminth infection (161).

Our understanding of mechanisms of immunity to hookworm and roundworm is less well-defined, largely due to the lack of robust mouse models. However, similar Th2-controlled effector mechanisms have been put forward for hookworm (12). For *Ascaris,* liver-stage immunity during the migratory stages of infection has been associated with oxidative phosphorylation and the production of reactive oxygen species (162), the lung-stage with an eosinophilia (163, 164), and expulsion from the gut with increased muscle contractility (165).

The role of the B cells and antibody in helminth infection remains unclear, with roles embracing both antibody production and antibody-independent cellular regulation. Thus, B-cells may play roles in stimulating the generation and/or polarization of T-cell responses by either cytokine secretion or antigen presentation in addition to their more widely appreciated role in antibody production (166, 167). In mouse models, both antibody-dependent and independent roles for the B cell have been proposed. Using a B-cell depletion strategy in a mouse model, a recent study suggested that the development of a Th2 type immune response to *Trichuris* infection is dependent on the host's genetic background and is independent of antibodies (168). Indeed in the context of *Trichuris* infections, evidence points toward antibody-independent worm expulsion mechanisms (169, 170). However, for other rodent helminths, roles for antibody have been well-documented (171, 172). For example, parasite-specific IgG1 is thought to play a role in immunity to the rodent hookworm *Heligmosomoides polygyrus* (173).

In humans, CD11c+ B cells have been shown to be the main IL-10 producers in Indonesian-STH-infected individuals compared to Europeans and Indonesians not exposed to helminths, inferring a regulatory function (174). The robust production of total and parasite-specific IgG1 and IgG4 have been associated with age, but may simply reflect the intensity of helminth infection (85, 134, 175). For example, children with repeatedly heavy infections with *A. lumbricoides* produced significantly higher levels of *A. lumbricoides*-specific (IgGl, IgG4, and IgE) compared to the repeatedly lightly infected children (140, 176). King et al. also suggested that antibody responses may not predict future levels of infection or confer protection from current infection or re-infection with *A. lumbricoides* but may only reflect infection intensity (177). In contrast, *Trichuris*-specific IgE has been shown to be negatively correlated with infection intensity and positively correlated with age, suggesting that IgE is associated with protection (134, 136). Total levels of IgE have also been correlated with the activation and degranulation of mast cells, basophils, and eosinophils (178). Further, negative associations between hookworm-specific intestinal and serum IgA and hookworm infection were observed in humans and the hamster model of *A. ceylanicum* hookworm infection, suggesting that antibodies may act in concert with other components of the mucosal and systemic immune response to promote protective immunity against hookworm infection (179–181). Overall, data supports the view that B cells are important in immunity to STHs, but the precise mechanisms by which B-cells and antibody support protective immunity remains only partially understood.

Regulation of Host Immunity by STHs—A Challenge for Vaccine Development

Experimental model systems, both rodent and human, have demonstrated that helminth infections regulate host immunity, dampening pathology at the expense of efficient worm expulsion. Thus, helminth-derived products have been shown to suppress of Th1/Th17 responses, with suppression associated with the production of IL-10, IL-22 and transforming growth factor-β (182, 183). Peripheral blood from hookworm-infected individuals also show higher levels of circulating regulatory T cells expressing CTLA-4, GITR, IL-10, TGF-β, and IL-17 than healthy non-infected donors (184). In the context of animal models, mice deficient in IL-10 are susceptible to *T. muris* infection characterized by elevated levels of IFN-γ and TNF-α, and fatal intestinal pathology (185). Furthermore, depletion of regulatory T-cells during *T. muris* infection enables worm expulsion at the expense of increased intestinal inflammation (186). In the context of hookworm, and using a mouse model of colitis, *A. caninum* ES products were shown to suppress colitis. The suppression was associated with potent induction of IL-4 and IL-10 by CD4+ T cells in the draining lymph nodes and the colon together with the recruitment of alternatively activated (M2) macrophages and eosinophils to the site of ES administration (187).

Helminth induced immune regulation has been embraced by "worm therapy" advocates (182, 188, 189), and immune regulation by helminths is important in protecting the infected host from potentially life-threatening immunopathology (190, 191). However, the inherent dampening of the immune response associated with chronic worm infections represents a significant challenge in STH vaccine research as well as potentially compromising immunity to other vaccines and influencing the outcome of infection with co-infecting pathogens (192).

UNDERSTANDING HOW VACCINES ELICIT IMMUNITY: CAN WE OVERCOME THE CHALLENGES?

Vaccination is one of the greatest advances in global health; however, most successful vaccines have been made empirically. Despite a reasonable body of literature from animal models, describing how Th2 immune responses confer protection against STHs in a primary infection, there is very little data regarding the mechanism(s) of vaccine-driven immunity, and this represents a significant gap in our knowledge. Thus, although strong evidence exists to support the need for a vaccine to promote Th2 immune response against STHs in the context of potently regulated environments, how the Th2 immune response culminates in worm expulsion is unknown and may well differ to the Th2 controlled effector mechanisms at play in immunity to a primary infection. Further, we still have little insight into the mechanisms by which vaccines trigger Th1 or Th2 biased immune responses, strong B cell responses or long-lived memory T-cell responses, despite formation of T-cell memory being critical to protection

against infectious diseases. Antigen delivery to the right antigen-presenting cells is critically important for the quality of the T-cell response, yet targeting of specific populations of antigen-presenting cells is often a neglected aspect in the design of vaccines.

There is a substantial evidence base to support the principle of dendritic targeting in vaccine development (193), although it has not been applied to many vaccines against STHs. Incorporating monoclonal antibodies that recognize, for example, dendritic cell surface molecules into delivery platforms, offers the prospect of direct delivery of antigen to specific antigen-presenting cell subsets *in vivo* (194–196), thus taking control of the quality of the subsequent vaccine-driven immune response (197). For example, delivery of antigen to CD8+ dendritic cells via Clec9A has been shown to promote CD4 T-cell responses and efficient development of T-follicular helper cells, important in antibody production (198). Interestingly, B-cells have been shown to be the dominant antigen-presenting cell-activating naïve CD4+ T-cells in response to virus-like particles (199), highlighting the importance of understanding how vaccines are presented *in vivo* as a prerequisite to developing antigen-presenting cell-targeting strategies.

SOIL-TRANSMITTED HELMINTH VACCINES: ARE WE GETTING CLOSER?

Experimental Hookworms Vaccine Candidates

Hookworms (*Necator* americanus, *Ancylostoma duodenale,* and *Ancylostoma ceylanicum*) infect around 500 million people worldwide and are of significant concern due to their voracious blood-feeding (1). *N. americanus* and *A. duodenale* infect humans whereas *A. ceylanicum* and *A. caninum* are zoonotic and rarely infect humans. Hookworms can live for years in the host's small intestine, causing severe iron-deficiency anemia in humans (1).

Pre-clinical hookworm vaccine studies have focused on identifying vaccine antigens from either the dog hookworm *A. caninum*, *A. ceylanicum*-golden hamster, or a laboratory strain of *N. americanus* adapted to golden hamsters (46). For example, Miller et al. developed the first hookworm vaccine using whole irradiated *A. caninum* L3 larvae antigens (200). Dogs immunized with the vaccine candidate showed between 37 and 90% protection depending on the route of administration (200, 201). As a result, this vaccine was commercialized in the United States in 1973 for canines. However, it was withdrawn after 2 years because of the high cost, storage, stability, and the lack of sterilizing immunity (202).

The human hookworm vaccine initiative of the Sabin Product Development Partnership has been directed toward identifying a hookworm vaccine (49, 203). Significant efforts have been made in identifying vaccine antigens from the infective larval L3 stages as they play critical roles in host invasion, modulation of host immunity and parasite establishment (203, 204). Several L3 proteins, especially enzymes, showed promising results as recombinant vaccines in different animals, and expression systems, including the tissue invasion-related Astacin-like metalloprotease (Ac-MTP-1) (205–208), Ac-16 (209), and the two *Ancylostoma* secreted proteins (ASP-1 and ASP-2) (206, 210–212). Of these, ASP-2 was considered a lead hookworm vaccine candidate as it showed the most promising results in animal models and pre-clinical vaccine trials (206, 213, 214). For example, Bethony et al. showed that laboratory dogs immunized with recombinant ASP-2 formulated with the GlaxoSmithKline Adjuvant (AS03) significantly reduced worm burdens and clinical pathology and induced strong antibody titers compared to control animals (204). Furthermore, the sera obtained from the immunized dogs significantly inhibited the migration of L3 through tissue *in vitro* compared to sera from control dogs, suggesting that antibodies might play a critical role in protection by decreasing the number of L3 that reach the gastrointestinal tract (204). Rats immunized with Na-ASP-2 formulated with Alhydrogel also induced strong antibody response (IgG1, IgG2a, and IgM) and induced a Th2 skewed immune response (215, 216). However, after offering so much promise, the rNa-ASP-2/Alhydrogel vaccine was halted in 2008, having reached Phase I clinical trials, due to generalized urticarial reactions characterized by a high prevalence of IgE antibodies to larval antigens in individuals previously infected with or exposed to *N. americanus* in endemic populations (202, 203, 210). Thus, the mechanisms of protection from IgE-mediated disorders, typically associated with helminth infection, seemed unable to protect adults living in hookworm endemic areas from developing allergic reactions after immunization with Na-ASP-2. It was hypothesized that the induced immediate-type hypersensitivity was due to the intrinsic structural or biological properties of the Na-ASP-2 molecule (217, 218).

The Identification of Aspartic Protease-Hemoglobinase (Na-APR-1) and Glutathione S-Transferase-1 (Na-GST-1) as Lead Antigens

The failure of Phase I clinical trial focused attention back on to antigen selection and led scientists toward identifying vaccine antigens that were less likely to be recognized by IgE antibodies induced by the natural infection (49). Strategies included examining IgE responses in the sera from populations in countries with endemic STH infections, mutating the antigenic epitopes recognized by host IgE and using bioinformatics tools that can screen for allergenicity (212, 219). Adult hookworms suck blood from damaged vessels in the gut mucosa and digest hemoglobin using haemoglobinases (220, 221). Since neutralization of these critical enzymes would result in starvation of the parasites, leading to parasite death, these antigens were selected for the development of hookworm vaccines (49, 222). Two promising vaccines derived from the adult stage parasite were identified, aspartic protease-hemoglobinase (Na-APR-1) and glutathione S-transferase-1 (Na-GST-1) (203, 222, 223). APR is an enzyme that helps digest hemoglobin (221, 224), whereas GST is essential for parasite survival and heme detoxification (Blood-feeding pathway) (48, 225).

In pre-clinical testing, recombinant Na-APR-1 induced neutralizing antibodies (IgG1 and IgG2) against the hookworm

haemoglobinase and resulted in significantly reduced blood loss, adult parasite burdens, and fecal egg counts in immunized dogs when challenged with hookworm larval (222, 224). Likewise, hamsters immunized with Ac-APR-1 showed a high level of protection (226). Importantly, IgE from individuals with hookworm infections did not recognize Na-APR-1 (224). Developing APR further as a lead antigen, Skwarczynski et al. developed an epitope-based subunit vaccine based on the $A_{291}Y$ B-cell epitope identified from the Na-APR-1, incorporated into a self-adjuvant system (Lipid Core Peptide). Interestingly, the vaccine candidate induced potent enzyme-neutralizing antibodies in mice (227). However, this study did not assess the protective immune response *in vivo*. Pearson et al. also explored a multi-antigen peptide-based vaccine against schistosomiasis and hookworm containing $A_{291}Y$ peptide from Na-APR-1, a *S. mansoni* Sm-tetraspanin-2 and Na-GST-1 antigens (228). A more recent study also showed that mice immunized with a lipopeptide-based vaccine consisting of a B-cell epitope (p3) derived from the Na-APR-1 and attached to a T-helper epitope (p25) induced a strong humoral immune response and resulted in >98% reduction in worm and egg burden following challenge infection with the rodent model hookworm, *Nippostrongylus brasiliensis* (229). Further, the same vaccine nanoparticle, when incorporated into natural and unnatural hydrophobic amino acids, also significantly reduced both worm and egg burden in orally vaccinated mice following *N. brasiliensis* challenge without the need for adjuvant (93). Pre-clinical studies also suggested that GSTs from *N. americanus*, *A. caninum,* or *A. ceylanicum* to be promising vaccine candidates (230). For example, Na-GSTs from the dog hookworm *A. caninum* (Ac-GST-1) elicited a significant reduction in adult hookworm burdens following challenge infection compared to control animals (225, 231).

On the basis of these and other pre-clinical data, the two lead vaccine candidates (Ac-GST-1 and Ac-APR-1) formulated individually with Th2 adjuvant Alhydrogel and TLR4 agonist (GLA) are in Phase 1 trials in the United States, Brazil, and Africa (48, 232, 233). Co-administration of both vaccines is also undergoing a clinical trial in Gabon (234).

Other Hookworm Vaccine Candidates

Promising results have also been achieved in hamsters immunized with DNA-based vaccines encoding the *A. ceylanicum* metalloprotease 6 (Ace-MEP-6) (63) or Ace-MEP-7 as an alternative strategy to recombinant protein production (62). Both vaccines induced significant reductions in worm burden. Additionally, a 78% egg count reduction was observed in hamsters immunized with Ace-MEP-7 (62).

Recent genomic and transcriptomic analysis of all three species of hookworms (26, 29, 30) have also helped to identify additional vaccine candidates including the two intestinally-enriched, putatively secreted, cathepsin B cysteine proteases (AceyCP1, AceyCPL) and the Kunitz-type protease inhibitor (AceySKPI3) (29). Vaccination of hamsters with AceyCP1/Alhydrogel induced a high level of protection associated with the production of high levels of antigen-specific antibodies (IgG). These antibodies also reduced the motility of the adult worms *in vitro* and induced Th2 responses (IL-4, IL-5, and IL-13) in re-stimulated splenocytes (235). Mechanistically, vaccinated animals were thought to be protected as a result of antibodies that neutralized the catalytic activity of the hookworm antigens in the gut (224, 236), although the full mechanism through which protection is conferred remains unclear. This study and others proved that parasite-secreted cysteine proteases involved in parasite nutrition are valid targets for the development of anti-parasitic vaccines (236). **Table 1** summarizes candidate hookworm vaccine antigens.

In the context of human hookworm vaccine development, the existence of a human model system for the testing of hookworm vaccines is a significant advantage. Thus, controlled human hookworm infections will likely improve the early stages of vaccine efficacy testing (50).

Experimental *Ascaris* Vaccine Candidates

Ascariasis caused by *Ascaris lumbricoides,* or *Ascaris suum* remains the most prevalent NTD worldwide. Indeed, the use of *Ascaris* egg-contaminated sewage sludge in agriculture has meant that, even in Europe, exposure to *Ascaris* is surprisingly common (237–239). *A. lumbricoides* infects around 819 million people worldwide, with children especially susceptible (240). *A. suum*, the pig roundworm, causes serious economic losses in meat-producing livestock species worldwide (241). Both parasites have a cosmopolitan distribution, an identical life cycle and are morphologically, genetically, and antigenically very similar (242–245). Consequently, efforts have been made in the past to identify vaccine antigens derived from the larval stages and ultraviolet irradiated attenuated embryonated eggs of the pig roundworm *A. suum* (246). However, the large size of the pigs, the cost and the complexities of animal husbandry, has driven many researchers to use mice and other rodent hosts as a convenient alternative for purposes of vaccine development (247). However, it is important to note that there is no rodent model that enables the *Ascaris* parasite to complete its life cycle.

Over the last decade, efforts have been made to identify crude extracts (248), recombinant proteins (249, 250), defined native molecules (251) and extracellular vesicles (252) as potential *Ascaris* vaccine candidates. These include the 66 kDa gut antigenic protein, the most immunodominant protein identified from homogenized *A. suum* adult worm fractions (251), but which was never tested *in vivo* for immunoprophylactic purposes (251). Other studies have explored targeting nematode haemoglobins as they can break down nitric oxide and hydrogen peroxide, thus potentially providing protection against innate host defenses (253, 254). However, immunizing pigs with *A. suum* hemoglobin (AsHb) in combination with QuilA adjuvant, failed to induce protective immunity following *A. suum* egg challenge (255).

Further, targeting nematode haemoglobins may offer cross species protection. For example, passive immunization of mice with a monoclonal antibody (48Eg) against hemoglobin of the nematode *Anisakis pegreffii* prior to infection with *Nippostrongylus brasiliensis* (rodent hookworm) enhanced protective Th2 immunity and significantly reduced worm burdens (256). This study also showed that 48Eg cross-reacts with the hemoglobin of several nematodes including *A.*

TABLE 1 | Major hookworm vaccine candidates.

Antigen	Parasite spp.	Adjuvant	Animal model	Vaccine type	Protection %	References
Glutathione-S transferase 1 (GST-1)	*A. caninum* (Ac-GST-1)	Alhydrogel	Mesocricetus auratus golden hamsters	r-protein	50.6% reduction in worm burden	(226)
Aspartic protease 1 (APR-1)	*N. americanus* (Na-APR-1)	Alhydrogel and a CpG	Dogs	r-protein	ND 66.6% reduction in egg count	(224)
	A. caninum (Ac-APR-1)	AS03	Dogs	r-protein	33% reduction in worm burden	(222)
	A. caninum (Ac-APR-1)	Alhydrogel	Mesocricetus auratus golden hamsters	r-protein	44.4% reduction in worm burden	(226)
Metalloprotease 6 and 7 (MEP-6 and MEP-7)	*A. ceylanicum* (AceyMEP-6)	ND	Syrian golden hamsters	DNA-based	80% reduction in worm burden	(63)
	A. ceylanicum (AceyMEP-7)	ND	Syrian golden hamsters	DNA-based	50% reduction in worm burden 78% reduction in egg count	(62)
Cysteine proteases 1 and 2 (CP-1 and CP-2)	*A. ceylanicum* (AceyCP1)	Alhydrogel	Syrian golden hamsters	r-protein	40–54% reduction in worm burden 54–60% reduction in egg count	(235)
	N. americanus (Na-CP-2)	Freund	Mesocricetus auratus golden hamsters	r-protein	29.3% reduction in worm burden	(226)

ND, Not done; r-protein, Recombinant protein.

lumbricoides, *N. brasiliensis*, *Anisakis pegreffii*, *Pseudoterranova decipiens,* and *Contracaecum* spp.

The public availability of the genome, and gene expression data for both *A. lumbricoides* and *A. suum* (28, 243, 245), also helped in identifying *Ascaris* vaccine peptides based on the RV approach. Indeed a recent study identified CD4 Th cell epitopes in *A. suum* ES products based on *in vitro* antigen processing and quantitative proteomic tools (257). However, the selected epitopes have yet to be tested *in vitro* or *in vivo* for their ability to induce immune responses.

To date the five major *A. suum* immunodominant antigens tested as possible vaccine candidates are; *A. suum* 16-kilodalton As16 (249), As14 (258), As24 (259), As37 (260), and As-Enol (enolase) (247). As14 and As16, identified from the sera of infected mice with *A. suum,* are localized in both larval and adult stages, as well as in the ES products of both the human and pig roundworms (23) and are homologous to the *A. ceylanicum* (Ac-16) vaccine candidate (209). Intranasal immunization of mice with recombinant rAs14 (258) and rAs16 (249) expressed in *E. coli* and coupled with cholera toxin B subunit (CTB), produced significant protection (64 and 58% respectively), compared to non-vaccinated mice following *A. suum* infection. Furthermore, recombinant rAs16 induced a 58% reduction in the recovery of the lung-stage in a pig animal model, associated with high levels of IL-4 and IL-10 and high titers of rAs16-specific mucosal IgA and serum IgG antibody (261). Through the use of sera from rAs16-CTB immunized mice, As16 was localized to the worm hypodermis and intestine (249, 261). Sera from rAs16-CTB immunized mice was also shown to inhibit molting of *A. suum* L3 *in vitro* (261). Interestingly, subcutaneous vaccination of mice with yeast-expressed As16 formulated with Montanide ISA720 adjuvant significantly reduced larval recovery and induced a Th2 immune response against challenge infection. In contrast mice immunized with rAs14 formulated with ISA720 failed to induce protection (262). Other studies have used plants as alternative, attractive, vaccine producing factories. For example, mice fed with As16-transgenic rice fused with CTB showed a significant reduction in the number of larvae following challenge infection (263).

Promising results were seen in mice immunized with the nematode-specific protein (As24) expressed in *E. coli* and emulsified with Freund's Complete Adjuvant (FCA), although the potential for translating these studies to man is limited by the choice of adjuvant. The vaccine resulted in a 58% reduction in lung larval burden post-challenge and induced a Th1/Th2-mixed type immune response characterized by elevated levels of IgG, IFN-γ and IL-10 (264). As24 homologous proteins were also identified in *Ascaris lumbricoides* and the dog parasitic nematode *Toxocara canis* (259). Anti-As24 IgG also inhibited molting of *A. suum* lung stage, suggesting As24 plays a critical role in the development of *Ascaris* larvae (259). Further, the protective immune response to *A. suum* larvae correlated with the induction of IgG1 and IgM, and not with IgG2 in pigs immunized with As14, and As24 fractions from adult worms (265, 266).

It has also been proposed that As37, a member of the immunoglobulin superfamily, identified from the adult and larval stages as well as in the hypodermis and muscle of *A. suum* is a potential *Ascaris* vaccine candidate (260, 267). Mice immunized with rAs37 formulated with AddaVax adjuvant induced a significant 49.7% larval worm reduction after challenge infection compared to control animals. Protection was associated with the production of high levels of serological IgG1 and IgG2a and stimulated the production of IL-4, IL5, IL-10, and IL-13 cytokines, suggesting that both Th1 and Th2 immune responses are essential for worm expulsion (250). Interestingly, the AddaVax adjuvant performed better than the Th1 adjuvant MPLA and the Th2 adjuvant Alhydrogel. Remarkably, sequence analysis revealed that As37 is highly conserved in other STHs

including *N. americanus*, *A. ceylanicum*, *A. caninum,* and *T. muris*, but not in humans, suggesting that the nematode-conserved antigen could serve as a pan-helminth vaccine antigen (250).

The inorganic *A. suum* pyrophosphatase is another promising vaccine candidate expressed throughout the life cycle and localized in the surface and adult reproductive tissues (268). Knockdown of *A. suum* pyrophosphatase by RNAi has indicated the importance of this phosphatase in larval development and molting (269). Furthermore, immunization of mice with rAs-PPase expressed in *E. coli*, and formulated with TiterMax Gold adjuvant resulted in >70% protection against the L3 stage, drove a high serum IgG1 response, and significant production of splenic IL-10 (270, 271).

Moreover, enolase (As-Enol-1), found in the *A. suum* larva, adult ES and EVs has been shown to play a critical role in larval development (272, 273), and triggering *in vitro* macrophage nitric oxide production (274). Vaccination of Kunming mice with recombinant As-Enol-1 leads to a 61.13% reduction ($P < 0.05$) in larval recovery and elicits a Th1/Th2 (IFN-γ, IL-2, IL-4, and IL-10) immune response (247).

Gazzinelli-Guimaraes et al. (248) also evaluated the immunological, potential clinical impact and protective immune responses of three different *Ascaris* extract vaccines formulated with the MPLA adjuvant. Mice immunized with crude extract of adult worm (ExAD) exhibited a significant reduction (51%) in the total number of migrating larvae recovered in the lung tissue and bronchoalveolar lavage; crude extract of adult worm cuticle (CUT) 59%, and crude extract of infective larvae (L3) (ExL3) 61% compared to the non-immunized mice. Protection was associated with a marked systemic production of *Ascaris*-specific IgG1 and IgG3 subclasses and a significant increase in systemic IL-5 and IL-10 (pre-challenge) and lung IL-10 (post-challenge). ExL3 and CUT protection was also associated with less tissue damage and pulmonary tissue inflammation as well as reduced pulmonary dysfunction following *Ascaris* challenge. Furthermore, the passive transfer of purified antigen-specific IgG antibodies from mice immunized with ExL3, CUT, and ExAD into naïve mice induced a significant reduction in parasite burdens in lungs of 65, 64, and 64%, respectively (248). These results suggest that vaccine induced antibodies play a crucial role in reducing larval migration and subsequent larval burden in the lungs.

Overall, the protective anti-*Ascaris* immunity (**Table 2**) observed in all the experimental animal model points toward a Th2-biased immune response, associated with the production of high levels of parasite-specific IgG1. Analyses of vaccine-driven immunity has suggested more mixed Th1/Th2 immune responses may be at play (250); however, over robust Th1-type responses are counter protective. Again, as with hookworm, the immunological mechanisms underpinning vaccine induced immunity to *Ascaris* infections are only partially understood.

TABLE 2 | Major Ascaris vaccine candidates.

Antigen	Vaccine type	Protection % (Reduction in lung larval burden)	References
As14	r-protein	64%	(258, 266)
As16	r-protein	58%	(249, 261, 262)
As24	r-protein	58%	(259, 264)
As37	r-protein	69%	(275)
As42	r-protein	67%	(266)
Enol-1	DNA	61%	(247)
As66k	ND	ND	(251)
• Crude extract of adult worm (ExAD) • Crude extract of adult worm cuticle (CUT) • Crude extract of infective larvae (L3) (ExL3)	Crude extract	• ExAD 51% ($p < 0.01$) • CUT 59% ($p < 0.001$) • ExL3 61% ($p < 0.001$)	(248)

ND, Not done; r-protein, Recombinant protein.

TABLE 3 | Major Trichuris vaccine candidates.

Antigen/adjuvant	Vaccine type	Protection % (Reduction in worm burden)	References
ES/FIA	Crude	97%	(85)
EVs and EVs fractions	Crude	Significant reduction (% was not indicated)	(276)
rPP2A/OVS	r-protein	97.90%	(277)
• rTm-WAP49/Montanide ISA 720 • rTm-WAP-F8+Na-GST-1/Montanide ISA 720	r-protein	• 48% • 38%	(91)
VLPs expressing *Trichuris* T-cell epitopes	Epitope-based	50%	(79)

OVS, Oleic-vinyl sulfone; FIA, Freund's incomplete adjuvant.

Experimental *Trichuris* Vaccine Candidates

Historically, the global health community have focused their vaccine research on hookworm and ascariasis and somewhat neglected trichuriasis, despite the fact that trichuriasis is the second most common STH infection after ascariasis (13). Around 477 million people are estimated to be infected with *Trichuris* infection, with the highest intensity of infection seen in school-aged children (1, 2).

One of the first attempts at identifying a non-living vaccine for *T. trichiura* was conducted in the mouse model *T. muris*, using adult and larval worm somatic antigens to stimulate protective immunity in infected animals (278). Vaccination of mice with *T. muris* somatic antigens that were isolated from NIH mice and emulsified in Freund's incomplete adjuvant stimulated protective immunity and a 92% reduction in worm burden after infection (278). Wakelin and Selby (278) also demonstrated that soluble antigens from the anterior region of adult worms were more effective in stimulating immunity than antigens prepared from the posterior region. *T. muris* adult worm homogenate and ES products from both adult and larval stages have been used in early-stage vaccine development (279, 280). Route of

antigen delivery has also been explored. For example, a level of protection can be achieved against *T. muris* using homogenized adult antigens combined with cholera toxin as an oral vaccine (281). However, a 100% reduction in worm burden can be achieved through subcutaneous (s.c.) vaccination with adult antigens emulsified in Freund's complete adjuvant (281). Moving to slightly less crude antigen preparations, but also exploring the route of administration, Jenkins and Wakelin (279) showed that vaccinating mice subcutaneously with 100 μg of the excreted and secreted ES products of adult *T. muris* parasites without adjuvant, was more effective than intraperitoneal vaccination, also in the absence of adjuvant. More recently, Dixon et al. (85) showed that subcutaneous vaccination of naturally susceptible AKR mice with *T. muris* ES emulsified with Freund's incomplete adjuvant (IFA) induced expulsion of a high-dose infection. This study also described priming of the immune response to subcutaneous vaccination as occurring in peripheral lymph nodes draining the site of vaccination, that the ensuing protective immune response was of a mixed Th1/Th2 type and eluded to the effector mechanisms involving some form of intestinal antibody-mediated cellular immune response (85).

Interest in identifying host protective material in the extracellular vesicle (EV) components within helminth secretions has increased recently (282–284). For example, Shears et al. (276, 285) showed that vaccinating C57BL/6 mice with either ES fractions or EVs isolated from *T. muris* without an adjuvant and prior to infection with a low-dose of *T. muris* eggs significantly reduced the worm burden compared to the PBS-injected group, inducing a mixed Th1/Th2 response. Vaccination of mice with ES fractions stimulated long-lasting protection against chronic infection characterized by the production of high levels of IL-9 and IL-13. However, the sera from ES vaccinated mice did not protect naïve mice from *T. muris* chronic infection, suggesting that anti-parasite antibodies did not play a critical role in protection (285). Shears et al. also demonstrated that vaccination with EVs boosted IgG1 antibody production against *T. muris* ES proteins, suggesting that there is extensive overlap in protein content between the EVs and ES (276).

Gomez-Samblas et al. (277) have also identified a vaccine candidate that potentially could work against a variety of helminth parasites, including *T. muris*. This candidate is based on the recombinant serine/threonine phosphatase 2A from the nematode *Angiostrongylus costaricensis* (rPP2A), formulated as a lipopeptide and conjugated with a self-adjuvant oleic-vinyl sulfone (OVS). Interestingly, intranasal immunization of AKR mice with the vaccine candidate prior to *T. muris* challenge led to a marked reduction in the number of adult parasites. The immunized mice also developed a combined Th17/Th9 response orchestrated by the cytokines IL-25, IL-17, and IL-9 (277). The same vaccine candidate has also been tested in lambs and was found to provide significant protection against the ovine helminth *Haemonchus contortus* and *Teladorsagia circumcincta* infection (286).

Briggs et al. (91) had developed two vaccines against trichuriasis based on *T. muris* whey acidic protein (rTm-WAP49) and *T. muris* WAP fragment fusion protein (rTm-WAP-F8+Na-GST-1) formulated with Montanide ISA 720 adjuvant. Vaccinating AKR mice with the vaccine candidates three times at 2-week intervals prior to *T. muris* challenge induced a partial reduction in worm burden (48 and 38%, respectively) (91). The authors also showed that both humoral and cellular immune responses were induced and characterized by elevated antigen-specific IgG1 and IgG2c antibodies and Th2 (IL-4, IL-9, and IL-13) cytokines in the draining inguinal LNs, draining mesenteric LNs and spleens of vaccinated mice (91).

Despite these promising results (**Table 3**), subunit vaccines often require substantial adjuvant and often do not provide sufficient protective cellular immunity compared with other vaccine approaches (76). Thus, a recent study identified a promising vaccine candidate based on major histocompatibility complex class II (MHC-II) T-cell epitopes identified from the whole genome of the *Trichuris*, incorporated into Hepatitis B core antigen VLP (79). The four epitopes were identified from chitin-binding domain-containing proteins and chymotrypsin-like serine proteases. *In vitro* studies showed that the VLPs were internalized and co-localized in the antigen-presenting cells (dendritic cells and macrophages) lysosomes and stimulated the production of pro-inflammatory and anti-inflammatory cytokines. Remarkably, immunization of mice with four VLPs expressing *Trichuris* T-cell epitopes induced a significant reduction in worm burden following challenge infection compared to control animals without the need for an adjuvant. Protection was associated with the induction of a mixed Th1/Th2 immune response characterized by the production of *Trichuris*-specific IgM and IgG2c and the production of mesenteric lymph node-derived Th2 cytokines and goblet cell hyperplasia.

CONCLUSION AND LESSONS LEARNED

STH infections are common in the world's poorest people living in low- and middle-income countries and are linked to detrimental effects on maternal and child health. Several studies have been conducted over the years to develop vaccines as cost-effective methods to control STHs infection (49). However, the process of vaccines development against STH parasites is complicated and faces several challenges. Thus, multiple broad-ranging factors have to be taken in to account when developing a protective vaccine to meet the immunogenicity, safety, and efficacy criteria of regulatory institutes such as the US Food and Drug Administration. These include identifying protective antigens that do not trigger unwanted allergic-type immune responses, the best route of administration, the vaccine type and adjuvant to be used and how to elicit protective immunity in the face of regulated immune environments typical of chronic STH infections (287). Underpinning all these factors is a need to understand the immune response induced by STHs and the nature of vaccine-driven protective immune responses, such that one can strategically design vaccines to drive the right sort of quality of immune response in susceptible hosts. There is a significant problem in vaccinology in that subunit vaccines, which are composed of antigenic proteins, are often poorly immunogenic and fail to stimulate memory immune responses. Furthermore, there is a knowledge gap in understanding the

mechanisms of action of vaccines and how they stimulate the required T- and B-cell responses. Targeting of antigen to specific antigen-presenting cell subsets is a promising strategy going forward. Coupling of a monoclonal antibody to antigen poses technical challenges, requiring fusion protein engineering or cross-linking, both of which can fail or produce low yields. However, overcoming these technical barriers is likely to be far-reaching in the context of STHs, where vaccine delivery is likely to occur in the context of pre-existing chronic infections. Despite an increase in STH vaccine research, it remains a disappointing truth that no human anti-STH vaccine currently exists. Among the lessons learned over the last decade is the importance of gaining international pharma company attention and support in order to bring antihelminth vaccines to trials. Further, raising public health awareness of the enormous threat presented by STH infections to the economy and health of at-risk populations, including their impact on susceptibility to other pathogens (49, 192) is critical. This has never been more relevant than now, given today's climate of emerging infectious diseases including SARS-CoV-2.

AUTHOR CONTRIBUTIONS

AZ researched the literature, drafted the manuscript, and compiled the tables. KE edited the manuscript and revised the content. All authors contributed to the article and approved the submitted version.

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OPINION
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COVID-19 Lethality in Sub-Saharan Africa and Helminth Immune Modulation

Luis Fonte[1], Armando Acosta[2]*, Maria E. Sarmiento[2], María Ginori[3], Gissel García[4] and Mohd Nor Norazmi[2]**

[1] Department of Parasitology, Institute of Tropical Medicine "Pedro Kourí", Havana, Cuba, [2] School of Health Sciences, Universiti Sains Malaysia, Kelantan, Malaysia, [3] Department of Teaching, Polyclinic "Plaza de la Revolución", Havana, Cuba, [4] Department of Medical Genetic, Hospital "Hermanos Ameijeiras", Havana, Cuba

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Jürgen Schwarze, The University of Edinburgh, United Kingdom

***Correspondence:**
Luis Fonte
luisfonte@infomed.sld.cu
Armando Acosta
armando@usm.my
Mohd Nor Norazmi
norazmimn@usm.my

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The acronym COVID-19 (Coronavirus Disease 2019) identifies the human disease caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (1). Since the outbreak of COVID-19 in Wuhan city, China, in December 2019, it has rapidly spread through 185 countries in all continents (2). SARS CoV-2 infection, and its related disease, is now a major health problem, with 23,057,288 infected individuals and 800,906 deaths confirmed worldwide as of August 24, 2020 (3).

The natural progression of SARS CoV-2 infection is extremely variable. It ranges between an asymptomatic course or mild clinical expression, which generally occurs in children and healthy adults, and the development of pneumonia and severe multi-organ failure, more frequent in the elderly and in patients of chronic diseases. This broad spectrum of clinical expression is the consequence of another one at immunological level: SARS CoV-2 infection activates innate and adaptive immune responses that, in the most frequent and benign of evolutions, lead to the containment of viral replication and recovery and, in the most unfavorable of sequences, can stimulate an intense pulmonary inflammatory reaction that, leading to more severe complications, can end in death (4).

SARS-CoV-2, unlike its close genetic relative SARS CoV, has the ability to infect and reproduce in the upper respiratory tract (5). There, type I/III interferons, tumor necrosis factor alpha (TNF-α-) interleukin-1 (IL-1), IL-6, and IL-18, among other components of the innate immunity, control the infection in the majority of the individuals (6). However, if SARS-CoV-2 passes through that first control and spreads, along the conducting airways, to the alveoli, it can replicate there more rapidly, causing pneumonia and other severe clinical complications (7).

Severe COVID-19 evolution is associated with an increase in the proportion of Th1 and Th17 cells, with their corresponding cytokines IFN-γ, IL17, IL-23, and TNF-α (8). At the same time, there is an activation of the inflammatory CD14+CD16+ monocytes, with an amplified production of cytokines, such as IL-6, and chemokines, such as CC- chemokine ligand 2 (CCL2), CCL3 and CXC- chemokine ligand 10 (CXCL10) (8, 9). The triggering of these cellular types, and the release of their mediators, leads to an increase of inflammation, vascular permeability and leakage with severe lung damage (8).

COVID-19 has shown significant differences in its lethality rate between continents, regions and countries (3). Of them, the more notable is the higher rates registered in economically developed regions with robust health systems, such as Europe and the United States, compared to countries having poor economies and insufficient health services, in particular, almost all the nations that constitute the Sub-Saharan Africa (SSA) (**Table 1**) (3). Some factors, or combinations of them, have been mentioned to explain the unexpected evolution: diagnostic test unavailability, age and genetic background of the population, mutational variations of SARS-CoV-2 in relation with

TABLE 1 | COVID-19 lethality rates in Europe, United States and Sub-Saharan Africa as of August 23, 2020.

Region	Confirmed cases	Deaths	Lethality (%)
Europe	3,970,890	216,478	5.45
United States	5,567,217	174,246	3.12
Sub-Saharan Africa	959,311	18,897	1.96

***Source**: (3).*
***Confirmed Case**: person positive by Polymerase Chain Reaction (PCR) test for SARS CoV-2.*
***COVID-19 death**: a COVID-19 death is defined as a death resulting from a clinically compatible illness in a probable or confirmed COVID-19 case, unless there is a clear alternative cause of death that cannot be related to COVID-19 disease (e.g., trauma). There should be no period of complete recovery between the illness and death.*

geographic settings, environmental temperature and humidity non-favorable for viral replication, BCG vaccination policies and endemicity of other infections (10–12). Here, we hypothesize the possible role of helminth immune modulation in the low COVID-19 lethality in SSA.

In 2009, Hotez and Kamath, in a landmark paper analyzed the striking connection between living conditions and prevalence of Neglected Tropical Diseases (NTDs), linking the world's greatest concentration of poverty with helminth infection prevalence in SSA region (13). In this region, "73% of the population lives on <US$2 per day, the most common NTDs, such as the soil-transmitted helminth infections, schistosomiasis, lymphatic filariasis and onchocerciasis, affected more than 500 million people" (13). For example, "of the world's 207 million estimated cases of schistosomiasis, 93% occur in SSA (192 million)" (13). Since then, little has changed in that part of the planet.

The "equilibrium" occurring in individuals chronically infected with helminths is the result of hundreds of millions of years of host-parasite coevolution. That prolonged interaction has led to the development of defensive responses by the human hosts and to the achievement of complex immune modulatory means by the helminths. The host protective responses against helminths, which are multicellular and large organisms, include wound repair mechanisms, which reduce the tissue damage that these parasites may cause as they move through body organs.

The cellular damage resulting from helminths migration through tissues is the major stimulus of the innate immunity against those parasites, as danger associated molecular patterns (DAMPs) are released and induce the production of cytokine alarmins (IL-25, IL-33, and thymic stromal lymphopoietin -TSLP-) by epithelial cells (14). IL-25 and IL-33 trigger the production of IL-4, IL-5 and IL-13, the principal mediators of type 2 responses, by type 2 innate auxiliary cells (14). On the other hand, TSLP limits IL-12 production by dendritic cells, the main promoter of type 1 responses (15).

For controlling the helminth infections, the adaptive immunity of the host usually develops type 2 immune responses, including the development of Th2 cells and the release of cytokines such as IL-4, IL-5, and IL-13 (16). This host-helminth interaction has, at least, two additional outcomes: (i) the classical and best-known down-regulation of type Th1 and type Th17 responses (and its related cytokines IL-12, IFN-γ, IL17, IL-23, TNF-α) by the Th2 cytokines (16, 17) and (ii) the helminths limitation of both host type1 and type 2 responses by enhancing FOXP3+ T regulatory cells, B regulatory cells and alternatively activated macrophages (AAMs) activities, which together cause the release of regulatory cytokines such as IL-10 and transforming growth factor (TGF-β) (18).

The modulation by helminths of the immune responses of their hosts has relevant clinical and epidemiological consequences: increased susceptibility to some infections, decreased frequency and intensity of allergic, autoimmune and inflammatory diseases, inadequate responses to vaccines and, as is possible in the case of SARS-CoV-2 infection, may inhibit the inflammatory processes that characterize infection by other microorganisms (17).

Helminths modulation has the ability to suppress inflammatory responses present during infection by protozoon, bacteria and virus: (i) when *Plasmodium falciparum* infection occurs in an individual infected with helminths, the effects of pro-inflammatory cytokines (IFN-γ and TNF-α) that characterize severe forms of malaria are attenuated by the action of anti-inflammatory mediators (IL-10 and TGF-β) and, consequently, decrease the chances of developing severe inflammatory conditions, including cerebral malaria (19); (ii) mice infected by *Nippostrongylus brasiliensis* showed increased susceptibility to *Mycobacterium tuberculosis*. Apparently, AAMs with impaired killing capacity in a less inflammatory Type 2 pulmonary milieu function as a mycobacteria reservoir (20); (iii) *Trichinella spiralis* infection limits inflammatory pulmonary damage induced by influenza virus in mice (21).

Nevertheless, and analyzing the helminth-virus relationship from a more holistic perspective, it is necessary to mention that helminths can enhance anti-viral mechanisms leading to a better control of viral load. Two examples: (i) during helminth infection IL-4 can expand and condition virtual memory CD8+ T cells (T_{VM} cells) for more rapid CD8 responses against subsequent cognate antigen encounter. Apparently, immunity against helminths has evolved a safety mechanism through induction of highly responding T_{VM} cells to counterbalance anti-inflammatory effects related to type 2 immunity on the development of effective antiviral responses (22); (ii) mice infection by the rodent roundworm, *Heligmosomoides polygyrus*, significantly reduce pulmonary lung damage and viral load following intranasal infection with respiratory syncytial virus. Interestingly, those effects were independent of adaptive immune responses because protection was lost in germ free mice, denoting a possible role of intestinal microbiota (23).

Taking into account the arguments described above, it is plausible to consider other factors, such as the inhibition of inflammatory processes by regulatory mechanisms induced by helminths, to provide an explanation to the low lethality of COVID-19 in SSA. Interestingly, and probably in connection with it, the historical data relating to SARS-CoV and Middle East respiratory syndrome-CoV epidemics reveal that these viruses caused very limited health problems, if any, in Sub-Saharan countries (24).

In a very recent paper, Bradbury et al., suggested that immune modulation by helminths could reduce the human resistance to SARS CoV-2 infection. Nevertheless, they called upon the research community to investigate whether helminth co-infection with COVID-19 could influence the pandemic spread through the helminth endemic regions of the world (25). Here, contrary to the opinion by Bradbury et al., we argue that helminth coinfection, in conjunction with at least part of the factors mentioned above, may be related to the low lethality of COVID-19 in SSA.

Furthermore, and looking ahead, we believe that helminth modulation on both type 1 and 2 immunity should be an important factor to consider during the design and evaluation of vaccines against SARS CoV-2 in those countries. The requirements of triggering type 1 responses for controlling viral replication and the development of type 2 immunopathology events observed during challenge experiments in animal models immunized with some coronavirus vaccine candidates support that reflection (26).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Host Immunity and Inflammation to Pulmonary Helminth Infections

Jill E. Weatherhead[1,2,3], Pedro Gazzinelli-Guimaraes[4], John M. Knight[5], Ricardo Fujiwara[6], Peter J. Hotez[2,3,7,8,9,10], Maria Elena Bottazzi[2,3,7,8] and David B. Corry[5,11,12]*

[1] Department of Medicine, Infectious Diseases, Baylor College of Medicine, Houston, TX, United States, [2] Department of Pediatrics, Pediatric Tropical Medicine, Baylor College of Medicine, Houston, TX, United States, [3] National School of Tropical Medicine, Baylor College of Medicine, Houston, TX, United States, [4] Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, MD, United States, [5] Department of Medicine, Pathology and Immunology, and the Biology of Inflammation Center, Baylor College of Medicine, Houston, TX, United States, [6] Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [7] Texas Children's Center for Vaccine Development, Houston, TX, United States, [8] Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, United States, [9] Department of Biology, Baylor University, Waco, TX, United States, [10] Hagler Institute for Advanced Study at Texas A&M University, College State, TX, United States, [11] Department of Medicine, Immunology, Allergy, Rheumatology, Baylor College of Medicine, Houston, TX, United States, [12] Michael E. DeBakey VA Center for Translational Research in Inflammatory Diseases, Houston, TX, United States

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Paul Giacomin, James Cook University, Australia

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James Hewitson, University of York, United Kingdom
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Roland Ruscher, James Cook University, Australia

****Correspondence:***
Jill E. Weatherhead
weatherh@bcm.edu

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Helminths, including nematodes, cestodes and trematodes, are complex parasitic organisms that infect at least one billion people globally living in extreme poverty. Helminthic infections are associated with severe morbidity particularly in young children who often harbor the highest burden of disease. While each helminth species completes a distinct life cycle within the host, several helminths incite significant lung disease. This impact on the lungs occurs either directly from larval migration and host immune activation or indirectly from a systemic inflammatory immune response. The impact of helminths on the pulmonary immune response involves a sophisticated orchestration and activation of the host innate and adaptive immune cells. The consequences of activating pulmonary host immune responses are variable with several helminthic infections leading to severe, pulmonary compromise while others providing immune tolerance and protection against the development of pulmonary diseases. Further delineation of the convoluted interface between helminth infection and the pulmonary host immune responses is critical to the development of novel therapeutics that are critically needed to prevent the significant global morbidity caused by these parasites.

Keywords: hookworm, *Ascaris*, *Schistosoma*, lung pathologies, inflammation, host immune response

INTRODUCTION

Helminths are multicellular parasitic organisms belonging to a diverse taxonomic group of metazoans that compromise the phylum Platyhelminths, known as flatworms, including cestodes and trematodes, and Nematoda, known as roundworms, including *Ascaris*, hookworm, whipworms, filarial parasites, and others. Helminths overwhelmingly infect people living in extreme poverty in tropical and sub-tropical regions. Helminths cause significant disease burden globally, particularly in young children, infecting 1.5 billion people worldwide, nearly 20% of the world's population (1). Together, the impact of

helminth infections contributes to more than 12 million disability adjusted life years (DALYS), a measure of significant global mortality and morbidity (2). Helminth induced morbidity is largely due to the direct impact of the helminth on host tissues and indirectly from the host inflammatory response reflecting the complex helminth-host interface.

Host organs such as the lungs are a frequent target of helminth infection. For some human helminth infections, such as paragonomiasis, the adult stage of the parasite takes up its final residence in the lung (3). More commonly, the lung hosts the migratory pathways of the helminth larval stages either through the lung parenchyma or vasculature. As a general principle, helminth tissue invasion causes profound mechanical and chemical damage to the human lung and is linked to vigorous host inflammatory responses. The consequences are huge but the global burden of pulmonary disease as a result of helminths remains unknown.

A coordinated type-2 innate and adaptive immune response aimed at pathogen containment and management of tissue restoration occurs during helminth larval migration. This highly regulated type-2 immune response is communicated through the production of the cytokines interleukin (IL)-4, IL-5, IL-9, IL-10, and IL-13 and chemokines targeting the recruitment and activation of immune cells (4). In addition to the direct tissue damage incurred from the worm itself, helminths also release inflammatory mediators, typically through shedding of the outer chitin layer during molting and through release of soluble excretory-secretory (ES) product or extracellular vesicles (EVs) that contain immunomodulatory proteins including proteases and protease inhibitors, glycolytic enzymes, allergens, and lectins (5, 6). ES product and EVs are released by helminths at all stages of development, and can have an impact on the local environment in a paracrine-like mechanism, but also can influence the immunologic milieu in distant tissues (7, 8). The antigen profile of ES product is diverse amongst helminthic species but remains essential for the maturation and migration of these organisms (9–11). Because parasites are macropathogens, unable to be phagocytized by classic antigen presenting cells (APC), antigens within ES product or EVs are a major mode of communication with the host immune system leading in some cases to immune activation and in others to immunmodulation (12). The variation in antigenic components and concentration within ES product and EVs in different helminths and at different developmental stages contributes to the challenges in understanding the direct relationship between helminth derived factors and host immune responses. Given the link between helminth products, helminth survival, and modification of the host immune response, understanding the communication mechanism between the helminths and host may provide targets for future therapeutic interventions and highlights the need for further investigation.

Helminth Life Cycles

The impact of helminths on the host lungs is varied and largely depends on the burden of disease and the life cycle of the helminth species. Large burden of helminth disease contributes to a greater degree of tissue damage and a more profound immunologic response aimed at larval control and tissue repair (13). Additionally, helminths have distinct, complex life cycles, which support maturation of larvae into adult worms, as they migrate through varying host tissue compartments including the lungs (14). Several helminths including *Ascaris*, *Strongyloides*, and hookworms (*Necator americanus*, *Ancylostoma duodenale*) have mandatory but transient life cycles that involve larval migration through human lung tissue as an essential larval developmental step. In contrast, *Toxocara*, *Dirofilaria*, and *Echinococcus* larvae, all of which are zoonotic infections, travel to the human lungs and are unable to complete their life cycle; while filaria (*Wuchereria bancrofiti* and *Brugia malayi*) and *Schistosoma* larvae travel through the pulmonary vasculature (15, 16). *Anisakis* and *Trichinella* larvae do not typically migrate to the lungs but do induce a profound systemic inflammatory response during their migration cycle that impacts lung function. The purpose of the species-specific life cycle, particularly one that involves the lungs, remains unclear. Despite the extreme energy expenditure endured during migration, larval migration likely provides a survival advantage allowing for more rapid maturation and larger body size for some species and may also serve as an immune-evasion mechanism during larval development (13, 17). However, the transient migration process of helminths can have a significant impact on the host lungs through activation of the pulmonary and systemic immune responses.

Helminth Associated-Lung Diseases

Lung pathology induced by helminths occurs in three general categories: 1) diffuse lung disease, 2) focal lung lesions 3) and systemic inflammatory responses and hypersensitivity (**Table 1**). Helminths that cause lung disease typically have either a larval migratory phase directly through the lung parenchyma or through the lung vasculature, form cyst, and nodules in the lung tissue or have indirect systemic effects on the lungs (**Figure 1**).

Diffuse Lung Disease

Organisms that have a larval migration stage through the lung parenchyma include the major soil-transmitted helminths, *Ascaris lumbricoides* and *Ascaris suum*, *Strongyloides stercoralis*, hookworm (*Necator americanus*, *Ancylostoma duodenale*), and the zoonotic infection *Toxocara canis* and *cati* (14). These interactions can lead to transient, diffuse lung infiltrates (14), and eosinophilic pneumonia termed Löeffler's Syndrome (18, 19). Similar to allergic diseases such as asthma, this syndrome is characterized by a severe type-2 immune response with eosinophilia, goblet cell hyperplasia, increased mucus production and manifests clinically as coughing and wheezing and, in severe cases, respiratory failure (13). Given the similar clinical features, helminths such as ascarids may be a major cause of allergic airway disease globally (20). In addition, there are clinical consequences due to mechanical and chemical tissue destruction from these pulmonary helminth invasions, which can produce manifestations that resemble chronic obstructive pulmonary disease (COPD) (21).

Moreover, pulmonary vascular helminths, including filaria (*Wuchereria bancrofti* or *Brugia malayi*) and schistosomes also induce diffuse lung disease. The filarial parasites, for instance,

TABLE 1 | Lung pathology induced by helminth infection.

Lung Pathology	Organisms	Lung Appearance	Symptoms
Diffuse Lung lesions	*Schistosoma* spp. *Ascaris lumbricoides Ascaris suum* *Wuchereria bancrofti* *Brugia malayi* *Strongyloides stercoralis* *Necator americanus*, *Ancylostoma duodenale* *Toxocara canis* and *cati*	Transient Diffuse lung infiltrates eosinophilic pneumonia	Coughing and wheezing Respiratory failure
Focal Lung lesions	*Dirofilaria* *Paragonimus* *Echinococcus spp* *Schistosoma* spp.	Cysts Single lesions	Asymptomatic Hemoptysis Cough
Systemic Inflammatory Response	*Anisakis simplex* *Trichinella spiralis*	none	Anaphylaxis Dry cough Dyspnea

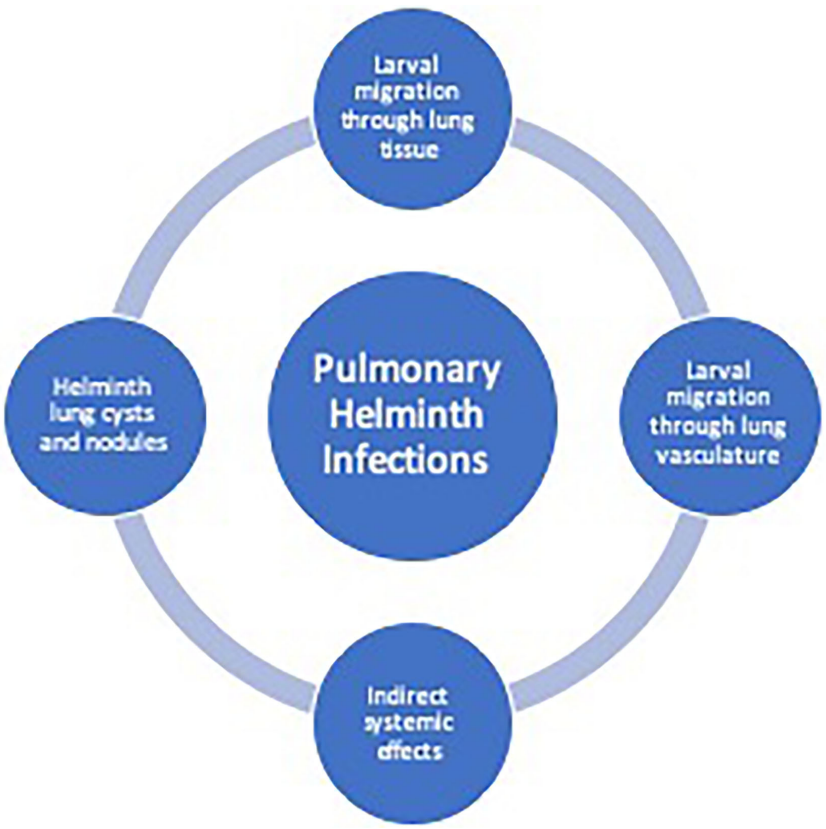

FIGURE 1 | Helminth-induced pathogenesis of human pulmonary disease. Helminth infections can cause pulmonary pathology due to larval migration through the lung vasculature or through the lung tissue causing a diffuse lung disease. Additionally, trapped larvae or eggs can cause focal lesions within the lungs. Indirect systemic effects from helminths may cause pulmonary disease from release of parasite-derived or host-derived factors leading to system inflammation.

cause a severe pulmonary syndrome called Tropical Pulmonary Eosinophilia (TPE) as a result of marked immunologic hyperresponsiveness to microfilariae trapped in the pulmonary vasculature. Within the pulmonary vasculature, microfilariae degenerate and release antigenic components eliciting a robust local inflammatory processes (22). Clinical manifestations include nocturnal cough, dyspnea, and wheezing in addition to interstitial infiltrates on chest radiograph, predominantly restrictive but also obstructive lung function abnormalities, peripheral blood eosinophilia, and markedly elevated levels of anti-filarial antibodies (23, 24). Other examples of vascular helminths that may trigger diffuse lung diseases are *Schistosoma*

spp., primarily associated with *Schistosoma mansoni*, which can lead to different pathways of disease from either the larvae or the eggs. The larval migratory stage known as lung-stage schistosomula sometimes leads to an acute febrile illness known as Katayama fever which can include fever, rash, cough and associated diffuse, interstitial pulmonary infiltrates secondary to release of larvae derived products (25, 26). Additionally, chronic pulmonary schistosomiasis can cause nodular lesions composed of granulomatous inflammation from egg deposition in lung tissue appearing as "ground-glass" on pulmonary imaging (27). Late stage, chronic schistosomiasis can lead to pulmonary arterial hypertension (Sch-PAH). The etiology of Sch-PAH is unknown but may be due to egg deposition in pulmonary vessels causing vascular inflammation or due to portal hypertension. PAH most commonly presents clinically as dyspnea and reduced exercise tolerance. Given the widespread distribution, schistosomiasis may be a leading cause of PAH globally (27–29).

Focal Lung Lesions and Cysts

Other organisms such as *Dirofilaria* and *Echinococcus*, in which humans are accidental hosts and do not permit completion of the life cycle, worms are sequestered in the lungs and induce an inflammatory response manifesting as focal lung lesions (13). *Dirofilaria immitis*, the dog heartworm, cause formation of granulomas in the pulmonary vasculature seen on pulmonary imaging as "coin lesions". Likewise, Echinococcus causes fluid-filled hydatid cysts in the lungs (13). *Paragonimus* also forms focal lung lesions. Larvae migrate to the lungs and develop into adult worms in pulmonary cysts manifesting clinically as cough, fever, chest pain and hemoptysis (16). Because paragonimiasis geographically overlaps with tuberculosis in East Asia, it is not uncommon to confuse these two causes of hemoptysis (30).

Indirect Effects of Systemic Inflammation

Several helminths, which do not directly infect host lungs, cause significant systemic inflammation through parasite-derived immunomodulatory molecules (13). Heavy infection with *Anisakis simplex*, thorough ingestion of raw or uncooked fish or handling fresh fish, can cause allergic airway disease or life-threatening anaphylaxis despite no direct contact with the host lungs. Fish processing workers specifically are at increased risk of developing *Anisakis*-induced bronchial hyperresponsiveness from consuming, touching or even inhaling *Anisakis* proteins (31). *Trichinella spiralis* larvae obtained from consuming undercooked pork or wild game can migrate through the lung vasculature on the way to striated muscle causing a dry cough (32). However, more commonly lung pathology occurs from either the systemic release of larval products during acute trichinellosis causing cough and dyspnea or from larvae developing into cysts in the chest wall accessory muscles and the diaphragm impacting lung dynamics (33).

These distinct clinical manifestations occur regularly in helminth endemic areas or can occur seasonally based on region specific transmission patterns (34). However, understanding the role of the host immune response to helminths and the resulting clinical impact on human lungs can be challenging and has lead to contradictory outcomes in clinical studies (20).

HELMINTHS AND THE HOST IMMUNE RESPONSE

The challenge in gaining an in-depth understanding of the host innate and adaptive pulmonary immune response to helminths is multifactorial. Human studies have led to varying outcomes based on inconsistent diagnostic capabilities, variation in study populations, seasonality of infection, genetic predisposition, population age, and disease burden. As a result, animal models have been used to gain a greater understanding of the host pulmonary immune response to helminths. *Nippostrongylus brasiliensiss, Strongyloides venezuelensis*, *Schistosoma mansoni*, *Brugia malayi*, and *Ascaris suum* have all been used to evaluate the impact of helminths on host lung function in animal models (35, 36). These animal models are critical to elucidate the complex balance between helminth induced innate and adaptive lung immunity. Mouse models of Nippostrongylus, *Schistosoma*, and *Ascaris* have been especially helpful in elucidating immunological mechanisms. It is critical to dissect the complex lung innate and adaptive immune response as a result of helminth infection in order to inform on-going therapeutic and vaccine development that is needed to reduce global morbidity.

Innate Immune Response in the Lungs to Helminthic Infections

Early recognition of helminths by the host pulmonary innate immune response is critical for disease control. Helminth molecules, glycans and lipids, serve as pathogen-associated molecular patterns (PAMPs). Recognition and processing of helminth PAMPS by APCs through pattern recognition receptors (PRR) such as toll-like receptors (TLR), C-type lectins and intracellular nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors are initial steps in the host innate immune response engagement (17, 37, 38). Intracellular signaling as a result of PRR activation, leads to release of cytokines, particularly IL-4, IL-5, IL-9, IL-13, IL-25, IL-33, and IL-17 as well as chemokines that propagate the host immune response. Cytokines and chemokines activate and recruit additional innate inflammatory cells including neutrophils, macrophages, basophils, eosinophils, dendritic cells, mast cells, natural killer (NK) cells, and type 2 innate lymphoid cells (ILC2) to the lungs, further propagating the innate immune response (13, 39). Helminths also expel cytokine- and chemokine-like natural products and molecules displaying immunologic mimicry, further aiding in cellular recruitment and activation. The recruitment and activation of innate cells in coordination with epithelial cell release of alarmins (e.g., IL-33; HMGB-1) and cellular hyperplasia, goblet cell production and release of intraluminal mucous and smooth muscle contraction are all necessary for the eradication of parasites from host lungs. A variety of specialized innate cells direct type-2 immune polarization and subsequent synchronization with adaptive immune pulmonary responses against helminth infections. The major innate immunity effector mechanisms to helminth infections include the following major elements.

Complement System

The classical, lectin and alternative complement pathways are prominently activated in early helminth pulmonary infection (40). Mice deficient in C3 infected with *Strongyloides* have increased larval survival (41). Despite activation of complement during helminth infection, helminths are able to evade complement mediated immunity potentially through binding complement inhibitory protein factor H, during molting, a process allowing for shedding of the larval outer layer (40), and by expressing surface proteinases that continuously degrade complement proteins binding to the helminth cuticle. While not necessarily playing a large role in opsonization and pathogenic killing, complement remains an important regulator of leukocyte-mediated immunity during helminth infection. Specifically, C3a and C5a act as chemotactic factors aiding eosinophil recruitment (40). Despite all three pathways being engaged, the independent role of the three individual pathways may differ depending on the helminth species, helminth developmental stage and the location of infection (40). In *N. brasilliensis* infection, the alternative pathway, mediated by hydrolysis of C3, plays a major role in eosinophil adherence in the infective larval stage (L3) occurring in the host lungs. In contrast, during the adult stage of *N. brasilliensis*, which resides within the gastrointestinal tract, lectin pathway plays a larger role in helminthic control (40).

Mucosal Barrier (Epithelium, Smooth Muscle, Mucus Production)

Direct helminth-induced damage to and death of lung epithelial cells can be one of the initial triggering events in helminth infection, leading to release of epithelial derived alarmins IL-25, IL-33, thymic stromal lymphopoietin (TSLP), and chemokines CXCL1, CXCL2, CXCL8, and eotaxins (42, 43). These alarmins signal to innate cell populations such as Innate lymphoid cells type 2 (ILC2), basophils, eosinophils, neutrophils, macrophages, and dendritic cells during the early phases of infection (43). The influx of type-2 cytokines into the lung compartment as a result of innate immune cell activation, particularly through the action of IL-4, IL-9, and IL-13, aids in goblet cell hyperplasia andmucin production needed for helminth expulsion and epithelial turn-over and repair (37, 43). In *Schistosoma mansoni* infection in the lung, rapid influx of IL-9 leads to robust generation of goblet cell hyperplasia (44). IL-4 receptor *alpha* (IL-4Rα) signaling *via* IL-4 and IL-13 cytokines also plays a role in mucin production and smooth muscle responsiveness during *Nippostrongylus* infection (45). Smooth muscle cells responding to IL-4Rα activation on smooth muscle leads to airway hyperresponsiveness, the exaggerated tendency of the airway to constrict, during *Nippostrongylus* infection in addition to T cell recruitment to the lungs (45). Mice deficient in smooth muscle IL-4Rα lack coordination of acetylcholine responsiveness with reduced M3 muscarinic receptor expression, delayed goblet cell hyperplasia, reduced type-2 cytokine production, and have a delayed ability to expel *Nippostrongylus* (46). Independent of IL-4Rα signaling, secreted proteins Ym1 and Restin-link molecule *alpha* (RELMα/Fizz1) from epithelial cells in the lungs contribute to lung repair through inducing IL-17A and neutrophilic infiltration promoting type-2 immunity and remodeling (47). Pulmonary tuft cells, termed brush cells, along the airway epithlium contain microvilli and potentially function as chemosensory cells playing an additional role in innate epithelial immunity (48). Tuft cells in the intestines have been shown to be involved in the generation of type-2 immunity to helminths (49). However, more research is needed to evaluate the interaction between helminths and pulmonary tuft cells.

Neurons and Neurotransmitter Signaling

The role of the neuroimmune network in helminth infection has more recently been highlighted as a critical modulator in helminth control and tissue repair particularly at heavily innervated mucosal sites (50). Pulmonary neuroendocrine cells, located at airway branching points release neuropeptides and neurotransmitters that influence smooth muscle tone (51). Bovine lungworm infection, *Dictyocaulus vivparus*, leads to upregulation of nictotinic acetylcholine receptors and increased cholinergic signaling on immune cells including epithelium, leukocytes, and macrophages (52). Additionally, helminth-derived acetylcholinesterase (AChE) has been identified in ES product of lung stage helminth larvae in different species. AChE secreted by *Nippostrongylus* regulates hydrolysis of endogenous Ach in mucosal tissue and co-localizes with cholinergic mucosal neurons that express the neuropeptide neuromedin U (NMU) suggesting that helminth derived products are involved in neurotransmitter modulation in mucosal tissue (50, 52, 53). NMU is particularly involved in coordination of the host innate response in the lungs during helminth infections. Neuron stimulation by either host alarmin IL-33 or IL-25 or *Nippostrongylus* ES product leads to direct neuronal release of NMU showing that neurons are sensing host damage and larval products (54). High concentrations of IL-25 in combination with NMU are associated with enhanced expression of type-2 cytokines IL-5 and IL-33 (55). Furthermore, intranasal administration of NMU has been linked to initiation of type-2 immune responses such as increased eosinophils, decreased tissue hemorrhage, enhanced mucus production and reduced infectious burden (50, 54). This may in part be an indirect effect of NMU given its role in enhancing maturation, proliferation and cytokine expression of lung ILC2s. ILC2 and NMU seem to have an inter-dependent relationship during helminth infection in the lungs. ILC2 selectively express neuromedin U receptor 1 (*Nmur1*), deletion of which impairs type-2 immune response and parasite control (54). NMU neuroimmune effects during helminth infection is counter regulated by the neuropeptide CGRP. The cognate receptor of CGRP is highly expressed on IL-5 secreting ILC2 populations, and when activated, decreases ILC2 populations (56).

ILC2s

ILC2s are innate tissue-resident cells particularly abundant at mucosal barriers including the lungs. They are found in high concentrations around the adventitia of lung bronchi and large vessels and co-localize with subsets of dendritic cells, T regulatory (Treg) cells, adventitial stromal cells, and mesenchymal fibroblasts (57). ILC2 activation and proliferation in the lungs is dependent on the release of alarmins IL-25, IL-33, and TSLP, in addition to the neuropeptide NMU. ILC2s also express IL-4Rα enabling the cells

to respond to the local type-2 cytokine milieu during helminth infection. Responding to IL-4 and IL-13 signals leads to further cellular expansion of ILC2s and a robust release of type-2 cytokines particularly IL-5 and IL-13 in a STAT6-independent mechanism (58, 59). The significant influx of IL-5 and IL-13 supports eosinophil recruitment, epithelial cell hyperplasia, and goblet cell hyperplasia (60). STAT6 is also not required for IL-13 production in ILC2s response to *Nippostrongylus* in the lungs (59). Once thought to be a homogenous population, single cell analysis during *Nippostrongylus* infection demonstrates significant heterogeneity within ILC2 populations in the lungs (56). Transient IL-25 responsive ILC2 (iILC2) are circulating ILC2s that mobilize to lung tissue in response to chemokines and cytokines (58, 61). Basic Leucine Zipper ATF-Like Transcription Factor (BATF), an AP-1 superfamily transcription factor, is critical for activation of iILC2 during *Nippostrongylus* infection (58). BATF-deficient *Nippostrongylus* infected mice lack iILC2 in lungs, have reduced early influx of type-2 cytokines and impaired mucosal barrier integrity. Conversely, tissue resident ILC2 (nILC2) are IL-33 responsive and not BATF- dependent (58, 61). Migration of ILC2 to the lungs may be secondary to increased expression of prostaglandin D2 (PGD2) receptor CRTH2 (chemoattractant receptor-homologous molecule) on the cell service (62). IL-33 coordinates the PGD2–CRTH2 pathway further regulating ILC2 migration patterns to the lungs (63).

Granulocytes (Basophils, Mast Cells, Eosinophils)

In addition to ILC2, basophils, mast cells, and eosinophilia constitute IL-4– and IL-13–producing cells of the innate immune system during lung helminth infection. The role of basophil and eosinophil production of IL-4 and IL-13 is sufficient enough to induce allergic airway disease in defense against migrating worms (64).

Basophils are granulocytes that produce type-2 cytokines including IL-4, IL-5, and IL-13 in addition to histamine, leukotrienes, and prostaglandins during degranulation as a result of helminth infection (65). Production of IL-4 by basophils is STAT-6 independent. *Nippostrongylus* infection as well as *Schistosoma* egg antigen (SEA) increase the number of basophils in the lungs as a result of FcR cross-linkage by IgE or IgG, complement activation of C5a or cytokine stimulation through T cell derived IL-3 or IL-18 (66–69). As a result, basophils can potentially serve as an early source of IL-4 during helminth lung infection prior to T cell activation. *Schistosoma* ES product IPSE/alpha-1 can trigger basophil production of IL-4 and IL-13 and influence cellular commitment toward type-2 immunity such as alternatively activated macrophage (AAM) formation, smooth muscle activity, goblet cell hyperplasia, and eosinophilic infiltration into the lungs (65, 70). Basophils are also capable of processing antigen to naïve CD4 T cells and promoting Th2 differentiation *via* MHC Class II expression (65, 71). While basophils are not essential for Th2 differentiation they are likely a critical initial trigger in the type-2 immune cascade (65). Recently, up-regulation of Notch2 receptor in basophils was shown to cause increase cytokine production, including IL-4 and IL-6, in intestinal helminth infections. Moreover, basophil-intrinsic Notch signaling promoted worm clearance and type 2 inflammation in the cecum (72). Whether basophil Notch signaling occurs in the lungs remains unclear.

Mast cells located along the epithelial lining of the host lungs additionally play a role in helminth infection. At baseline, mast cells are rare resident cells in host lung tissue but can rise significantly during helminth infection. However, the inciting chemoattractants that induce mastocystosis in the lungs during helminth infection remains largely unknown (73). IL-9 signaling through IL-9R on mast cells is an important factor for maturation and activation of mast cells. Depletion of IL-9 during *Schistosoma mansoni* infection demonstrates 8-fold fewer mast cells in the lungs (44). Additionally IL-3, derived from varying cell sources including T cells, may contribute to further influx of mast cells. In IL-3 deficient, *Strongyloides* infected mice, mast-cell development is stalled and parasite killing is impaired (67). Degranulation and rapid release of inflammatory mediators (histamine, leukotrienes, and prostaglandins), cytokines (IL-4, IL-6, and TNFα), and proteases (mcpt1 and mcpt2) occurs secondary to high affinity mast cell FcεR1 bound to IgE, which plays an important role in helminth killing (11, 43). However, release of these mediators including IL-4 from mast cells is not required to generate a robust type-2 immune response. Mast-cell deficient mice infected with helminths can still generate normal type-2 immune response in the lungs (65).

Eosinophils are critical effector cells during helminth infection through direct killing of parasites and, potentially acting as APCs directing T cell differentiation to type-2 immune cells, although this function remains controversial (39, 74, 75). The early presence of helminth larvae in the lungs triggers rapid eosinophilic activation and recruitment through the release of chemoattractants eotaxins and MIP-1a as well as cytokine IL-5 production (76). Eosinophilic degranulation occurs with engagement of the FcεR1 on eosinophils surface to Fc of IgE bound parasites (11). Eosinophilic cellular cytotoxicity of helminthic larvae in the lungs occurs through release of major basic protein (MBP) and eosinophil peroxidase (EPO)-dependent mechanisms particularly during secondary infection and through induction of histamine release from mast cells (75, 77). The release of eosinophil DNA-based extracellular traps (EETs) may be an additional mechanism of helminth larvae-killing or immune response against extracellular pathogens (78). The eosinophilic immune response restricts larval development and reduces parasite burden in the lungs particularly in secondary exposure to helminths or in hosts with allergen presensitization (79). Presensitized lung tissue and airways in HDM-allergic lungs or after multiple helminth exposures, contain IL-4 and IL-13-rich AAMs and eosinophil-dominated type 2 cellular infiltration, which dramatically reduces the burden of disease, including lower hemorrhage and mechanical damage in the tissue as a result of parasite migration through tissue (76, 79). Likewise, deletion of eosinophils in animal models has been shown to increase parasite survival (38, 39). Reduction of eosinophils through anti-IL-5 or anti-CCR3 monoclonal antibody blocks innate protective immunity to *Strongyloides* and leads to reduced control of larvae (39). However, eosinophils may not be essential for larval killing as neutrophils provide redundency (77). In the complete absence of

eosinophils during *Strongyloides* infection, neutrophils were capable of partial control of infection (39). While eosinophils release IL-4 and aid in type 2 T cell polarization, eosinophil deficient mice still mount a normal T helper type 2 (Th2) response to helminth infection (65).

Neutrophils

Neutrophils also perform as effector cells during helminthic infection in the lungs. Facilitated by the release of myeloperoxidase, neutrophils can independently kill *Strongyloides* larvae in the lungs (39). Interestingly, *Strongyloides* larvae alone can directly induce the activation and recruitment of neutrophils to infected tissues (80). *Strongyloides*-specific molecules released during the larval stage promote neutrophil release of chemokines MIP-2 and KC, further enhancing recruitment of neutrophils (39). Additionally, chitinase-like proteins Ym1 and Ym2 lead to expansion of IL-17A–producing γδT cells with increased IL-17A production and subsequent neutrophil recruitment. The enhanced neutrophil recruitment prevents parasite survival but at the expense of enhanced lung injury (81). Likewise *Anisaki* release of ES products elicits neutrophil recruitment to the lungs *via* production of IL-6, IL-8, and CXCL1 which has been shown to further enhance lung inflammation and contribute to lung disease during anisakiasis despite no lung larval migratory phase (82). Such tissue damage is not seen to the same degree with type 2 immunity-dependent, eosinophil predominant inflammation, suggesting that type 2 immunity may have evolved to provide control over chronic, inescapable parasitic infections while minimizing bystander tissue injury.

Natural Killer Cells

NK cells contribute to helminthic control in the lungs through direct cytotoxicity and cytokine production. NK cells expand early in helminth lung infection potentially secondary to recognition of helminth ES product (83, 84). Filarial infective-stage larvae and microfilariae modulate NK cell activation and release of cytokines including gamma interferon (IFN-γ) and tumor necrosis factor-α (TNF-α) (85). In filarial disease animal models, *in vivo* depletion of NK cells increased worm burden in the pleural cavity and influenced IL-4 and IL-5 plasma levels (84). Likewise, pulmonary schistosomiasis increases NK cells within the lungs and NK cells localize near *Schistosoma* pulmonary granulomas (86). NK cells additionally promote dendritic cell maturation through IL-12 production and directly interact with T cells providing a link between innate and adaptive immunity (86).

Professional APCs (Macrophages and Dendritic Cells)

Dendritic cells (DCs) serve as mediators in the innate and adaptive orchestration of type-2 immunity during lung helminth infection (87). DCs recognize helminth ES-derived antigens or parasite surface molecules. Proteins and lipids that are highly glycosylated are recognized by different innate PRR including TLR, c-type lectin receptors, and NOD-like receptors (11, 87, 88). DCs capture helminthic antigen and present to T cells propagating the host type-2 immune response (11). *Schistosoma* and filaria both activate TLR4 on dendritic cells to engage type-2 immunity in the lungs potentially through reduction in dendritic cells ability to produce IL-12. However, evaluation of th immunologic pathways involving TLR4's role in the development of type-2 immunity is on-going (11).

Activated macrophages in the lungs serve as APCs that aid in transitioning to a type-2 adaptive immune response during helminthic infection and selectively polarize toward type-2 mediated AAMs. In the context of a predominant type-2 immune response environment, IL-4 and IL-13 signaling *via* the STAT6 pathway induce expression of mediators that promote AAM. The IL-4/IL-13 and STAT6 signaling pathway allow for upregulation of genes associated with AAM (YM1, YM2, RELMα, ARG1) and upregulation of class II MHC on macrophages to further promote type-2 immune responses (89, 90). Amplified concentrations of AAM during helminth infection in the lungs impedes parasite migration and are sufficient to kill *Nippostrongylus in vitro (*80). Additionally, AAM associated RELMα specifically supports tissue repair, extracellular matrix turnover and homeostasis and is essential to prevent fatal lung damage as a result of helminth infection in the lungs (89, 90).

Additional cells that play a role in innate immunity including γδT cells (81) and coagulation factors (platelets, thrombin, fibrinogen) (91, 92) may also contribute to helminth disease control and tissue damage in the lungs. However, additional studies are required. Overall, the coordinated work of innate cells play a critical role in early recognition and termination of helminth infections as well as initiation of tissue repair in the lungs. However, perhaps the most important role of the innate cells during pulmonary helminthic infections is engagement and activation of the adaptive immune response (**Figure 2**).

Adaptive Immune Response in the Lungs to Helminthic Infections

Innate immunity is not just important as the first line of defense but also influences the nature of the adaptive response (93), by creating environments with signature cytokines that have the ability to drive the proliferation and differentiation of the effector T helper (Th) cells. In response to helminth infection, naive $CD4^+$ T-cells differentiate into several possible effector subsets, including Th2, Th17, T regulatory cells (Treg), and T follicular helper cells (Tfh) as well as influence the maturation of B cells and isotype switching of immunoglobulins favoring a type-2 immune response (94). Conversely, Th1 cells that secrete principally IFN-γ are activated most commonly in intracellular parasitic infections such as protozoa (e.g., *Leishmania* and *Plasmodium*) and are not predominately linked to helminth infections with some exceptions (95). Several cytokines, mostly originated from innate cells, and transcription factors tune the differentiation and expansion of these cell populations. Overall, helminth-induced immunoregulation occurs through the induction T cell subsets and B cell maturation influencing the immunologic landscape during helminth infection, providing effector function, tissue repair, and memory in the lungs. Of note, very few studies have analyzed $CD8^+$ T cell responses to helminth infection in the lung tissue. While, $CD4^+$ T cells classically play a major effector role in pulmonary helminthic

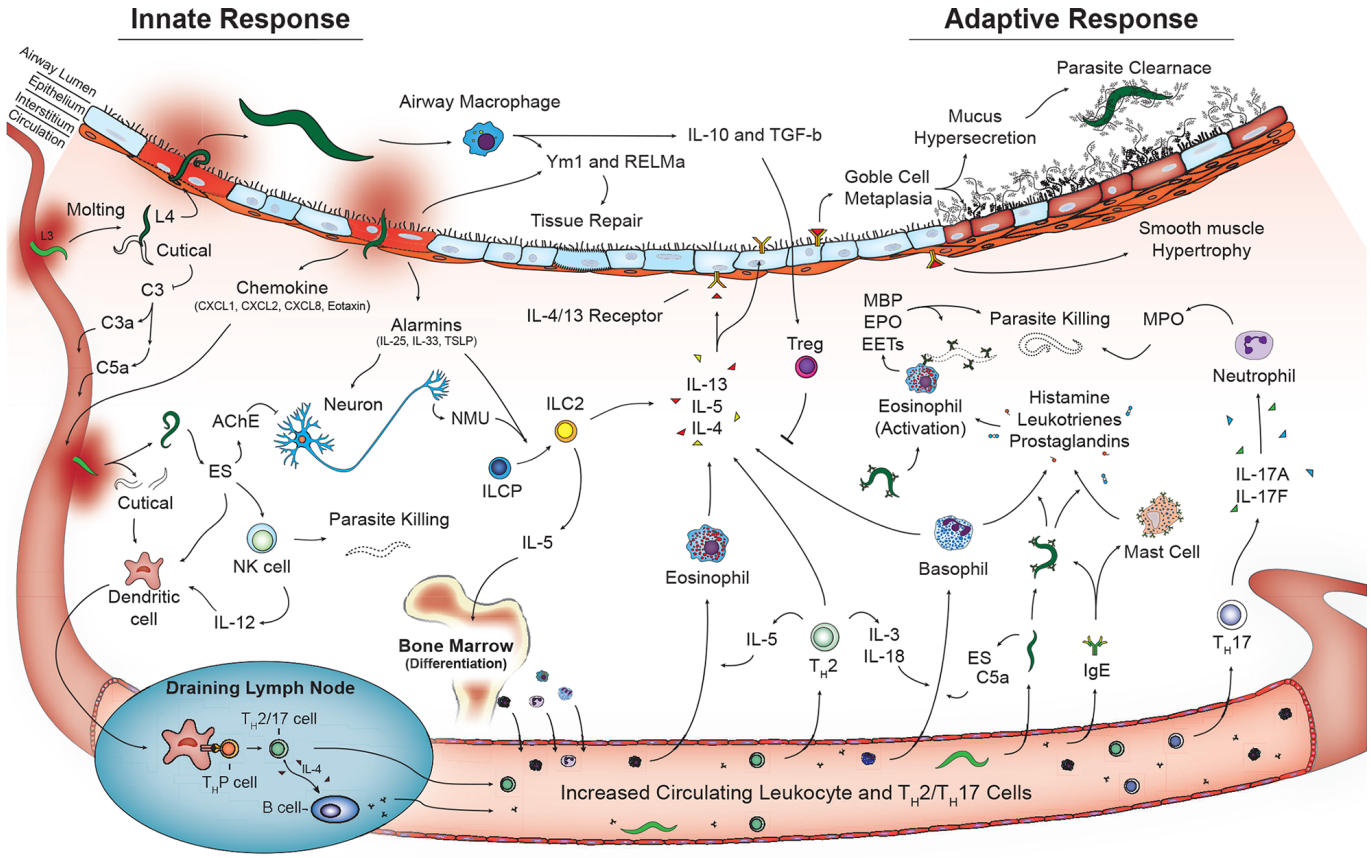

FIGURE 2 | Activated innate and adaptive immune pathways in the lungs during helminth infection. Innate phase of immune activation to helminths in the lungs is mediated by antigen presenting cells including dendritic cells and macrophages in addition to release of alarmins from damaged epithelium that drive a type-2 immune response. Release of type-2 cytokines IL-4, IL-5, and IL-13 contributed from granulocytes and type 2 innate lymphocytes promote a lung phenotype with goblet cell metaplasia and mucus hypersecretion and smooth muscle hypertrophy. T lymphocytes including Th2, Th17 in combination with B lymphocyte aiding immunoglobulin class switching further contribute to type-2 immune response promoting parasite killing and clearance as well as tissue repair and recovery.

disease, $CD8^+$ cells can enhance granulomatous inflammation in the lungs to schistosomiasis (96). More detailed analysis of the role of $CD8^+$ cells during pulmonary helminth infection are needed. While helminths classically create a type-2 immune response, the specific balance of adaptive immune cells is intrinsically associated with the helminth species, the stage of infection (acuteness or chronicity) and what organ/tissue has been affected. In the lung tissue, as most of the pulmonary helminthic infections are transient, the role of the adaptive response can be divided in 2 phases: helminth control and tissue repair.

Helminth Control in the Lungs

During parasitic invasion of the lungs, Th2 effector cells are essential to rapidly polarize and amplify a multifactorial type-2 inflammatory response to control parasite burdens. Although the mechanisms are not fully elucidated, the circumstances that promote the acute Th2 cell protective immunity in the lungs occurs by cross-talk with innate immunity through cytokines, chemokines and growth factors that result in the establishment of a robust type-2 inflammatory environment rich in AAM and dominated by eosinophils. Initial Th2-cell differentiation largely involves the cytokines IL-2, IL-4, and collectively IL-25, TSLP, and IL-33 as well as the transcription factor GATA3 directing effector function in the lungs (95, 97, 98). The Th2 effector response to helminth infections in the lungs is thus typified by the differentiation of antigen-specific $CD4^+$ T cells to Th2, high levels of IL-4, IL-5, and IL-13, accompanied by eosinophilia, mucus production, and abundant IgE production, phenotypically similar to allergic airway disease (99).

IL-4–, IL-13–, and IL-5–producing $CD4^+$ Th2 cells have been implicated as one of the most important effector mechanism in adaptive pulmonary immunity against helminths. IL-5–producing Th2 cells provide support for IL-5–mediated eosinophil-dependent larval killing. IL-5 enhances the differentiation, maturation, and survival of eosinophils derived from bone marrow precursors (100, 101). In animal models of *Strongyloides stercoralis*, Th2 production of IL-5 is not only required to elicit additional effector function but also to induce protective antibodies (102). IL-4– and IL-13–producing Th2 cells induce STAT6-dependent changes in epithelial-cell function resulting in goblet cell hyperplasia and increased mucus production *via* IL-4Rα and IL-13Rα1 signaling. Additionally, IL-4Rα expression on lung-resident $CD4^+$ T cells is required to generate a protective recall immunity to *N. brasiliensis* re-infection (103). In subsequent infection, the presences of primed memory Th2 cells aid in the accumulation of eosinophils expressing high levels of MBP. The recruitment of eosinophils by memory Th2 cells facilitates more rapid reduction of helminth burden in the lungs compared to primary infection (104).

Th2 effector cells and Tfh cells in germinal centers coordinate the humoral response in type-2 inflammation by promoting a helminth-specific B cell response and stimulating B cell class switching to high-affinity IgE, IgG1, and IgG4 (38). Tfh is reliant on Notch signaling in an IL-4 dependent pathway and the transcription factor BCL6. However, deficiency of Tfh does no impact worm clearance, overall IL-4 production or Th2 mediated immunity (105, 106). Antibodies are the quintessential adaptive immune effector molecules and are a central feature of Th2 immunity (97). Moreover, B cell activation has been investigated over the years as the target for effective vaccine development against helminth parasites and antigen-specific IgG antibodies generate a protective adaptive immune response against *Ascaris* parasites specifically (107).

Lung Tissue Repair and Restoration

After parasites have migrated through the lungs, Th2 effector cells are critical to activating efficient tissue repair responses. IL-4– and IL-13–producing Th2 cells are important for differentiation and maintenance of AAM in the tissue, encoding genes such as YM1, RELMα, and Arg1, key players in tissue repair and remodeling (108, 109). The crosstalk between Th2 effectors cells and macrophages has been demonstrated in experimental models of the filarial nematode *Brugia malayi*. Filarial infection in recombinase activating gene (RAG) or MHC class II-deficient mice (mice lacking T cells) demonstrate a striking lack of AAM, suggesting that Th2 cells are required for maintenance and full activation of AAM directed repair immunity to helminth parasites (110).

Treg populations, "natural" Tregs and "induced" Tregs, are activated in an effort to dampen host immune response through production of IL-10 and TGF-β during helminthic infection (111). The role of Treg varies depending on the developmental stage and the parasite species. Lung damage, blood clots, damage-associated molecular patterns (DAMPS) as a result of helminth infection in the lungs, promotes differentiation of macrophages and release of macrophage-derived growth factors and anti-inflammatory IL-10 and TGF-β that activate Treg (38). Depletion of Treg heightens Th2 cell proliferation and clearance of infection; however, this is associated with increased lung damage due to prolonged effector activation (112). Conversely, Treg production early in infection can promote worm survival by dampening the acute Th2 effector cell response but promoting tissue integrity (95). In helminth re-infection models, Tregs suppress memory Th2 cells in order to reduce the recruitment of eosinophils into the lungs and prevent excessive lung damage and further pathology (104, 111). Additionally, Treg activation during helminth infection can generate non-specific host immune suppression across tissue compartments and thus can be important in parasitic co-infection models particularly regarding progression of malaria and tuberculosis (112).

Additional T helper subtypes can play important roles during helminthic infection. Driven largely by pro-inflammatory cytokines, IL-6 and IL-17A and the transcription factor RORγt and maintained by IL-23, Th17 cells at mucosal barrier sites aid in mucosal defense and tissue restoration (95). Lung tissue expression of receptors for the Th17 cytokines IL-17A, IL-21, GM-CSF, and IL-22 allows for both protective and pathogenic responses in the lungs to helminth challenge (113). During *Nippostrongylus* infection in the lungs, Th17 activation is associated with early release of IL-17A which aids in IFNγ suppression and type-2 immunity, recruitment of neutrophils and subsequent pulmonary damage and hemorrhage (38, 114).

Prolonged Th17 activation and excessive early IL-17A can be associated with immune-mediated disease in the lungs including asthma (114). It is also linked with the extravasation of eosinophils from the bone marrow and eosinophilic infiltrations in host lung tissues. However, once established, IL-17A can also act as a negative regulator of the type-2 response in the lungs (114). On that basis Th17 responses, mediated through IL-17A, may be as important as Th2 responses in the pathogenesis of helminth-inducted pulmonary disease (115). However, Th17-driven IL-22 aids in epithelial cell repair and regeneration of mucosal barrier after helminth infection in the lungs (116). The IL-17/IL-22 axis is critical to the generation of epithelial homeostasis post-infection. IL-6 promotes differentiation of Th22 cells and release of IL-22, which also likely aids in tissue repair although the role of IL-22 in pulmonary helminth infections remains unknown. Additionally, Th9 in gastrointestinal helminth infection has been shown to cause rapid worm expulsion and basophil activation however further investigation is needed in the lungs (117).

Systemic Impact of the Adaptive Immune Response

The complexity of the adaptive immune response to helminths is varied and can be influential in co-infection and co-morbid models of disease. Helminth co-infection models with different infected host compartments impacts not only the local adaptive immune response but also the host immune response to helminth infection in distant tissues. *H. polygyrus* intestinal infection induces a IL-33–mediated activation of IL-5 secreting Th2 cells and causes upregulation of IL-5–mediated eosinophils, leading to a significant increase in immune-mediated killing of *N. brasiliensis* larvae in the lungs of the co-infected mice (118). Additionally, co-morbidities may also influence the adaptive immune response to helminth infections. In an animal model of house dust mite (HDM) allergic sensitization, subsequent *Ascaris* infection is mitigated by a primed memory Th2 response in the lungs. Allergen-driven inflammation, increases IL-5– and IL-13–producing Th2 cells in the lungs leading to an IL-4– and IL-13–rich environment that drives the differentiation of lung macrophages toward an AAM phenotype expressing arginase-1, as well as, an eosinophil infiltration. This strict type-2 immune milieu leads to a marked reduction in the number of lung-stage *Ascaris* larvae, reducing the intensity of infection, inducing lower pulmonary hemorrhage and mechanical damage in the lung tissue during larval migration (79). These results suggest a sophisticated and efficient feedback loop among Th2 cells, eosinophils and AAM in coordinating innate and adaptive immunity against lung tissue helminths (95).

Implications for Vaccines

Several human helminth vaccines are in phase 1 or phase 2 stages of clinical development, including hookworm infection and schistosomiasis (119). For human hookworm infection, vaccine immunity appears to operate *via* directing anti-enzyme antibody responses to parasite gut digestive enzymes, including a glutathione S-transferase and a hemoglobinase, while schistosome vaccines focus on larval and adult surface antigens (120). Extensive studies in mice show how vaccine immunity against schistosomes and possibly other helminths operate through adaptive immune responses in the lungs. In some cases, these responses actually trap larval helminths in the lung and block further migration (121). Furthermore, *Ascaris* vaccines targeting larval-stage immunodominant proteins that are highly conserved between helminths suggest the possibility of developing a pan-helminth vaccine that may prevent larval migration (122). Therefore, helminth vaccine development targeting effector immunity in the lung may prevent larvae from completing their life cycle while preventing host lung damage.

HELMINTHS AND IMMUNE MODULATION

Helminths can directly cause lung pathology as a result of direct larval migration or indirectly through systemic activation of a type-2 immune response. Many of the molecules released from helminths have been associated with development of lung disease, including allergic airway disease. *Ascaris* ABA-1 fatty acid binding protein, a glutathione transferase (GST) involved in distribution of lipids, is released at different stages of *Ascaris* development including the lung stage. ABA-1 has significant homology to mite and cockroach GST and has been linked to the development of asthma (123). Conversely, *Anisakis*, which is the most allergenic helminth and does not typically have a lung larval stage, contains the allergens molecules Ani s 1, Ani s 4, and Ani s 9, in ES product, all of which are proteins central to the development of systemic anaphylaxis and lung pathology (123). Similar proteins are found in the ES product of hookworm and *Schistosoma* spp. that have been linked to lung disease (123). This acute lung pathology can lead to chronic lung abnormalities. *Nippostrongylus* larval migration causes destruction of alveoli, long-term airway hyperresponsiveness and chronic low level hemorrhaging in the lungs through chronically activated AAM and release of matrix metalloproteinase 12 (MMP-12; macrophage metalloelastase) (21). The release of high levels of IL-13 causes persistent type-2 immune polarization of macrophages leading to on-going, chronic lung damage over months (14). However, these acute and chronic changes in the lungs are likely developmental stage- and helminth-specific.

While some larval stages induce end organ disease within the lungs, others, particularly those without larval migration through the lungs or once the larvae develop into adult worms, induce immunomodulation and may prevent underlying inflammatory disease states. Components of ES product as well as secreted EVs can be associated with immune evasion and immunomodulatory mechanisms (5). Helminths can produce mediators within the ES product that influence regulatory immunity using immune mimicry through production of TGFβ cyotkine-like molecules and altering the gut microbiome to promote Treg differentiation (38, 124). Additionally, helminth derived chemokine-like molecules such as IPSE/alpha-1 have also been linked to immune modulation (70). *Ascaris* ES product at larval and adult stages contains protein-1 of *Ascaris suum* (PAS-1) which contributes to increased concentrations of IL-10, a cytokine that is both made by and influences the generation of Treg and thus an immunomodulatory environment (125). Likewise, anti-inflammatory protein-2 (AIP-2) secreted from hookworms, has also been noted to enhance Treg and suppress airway inflammation.

This influence over the host immune response balance can lead to long-term protection of lung structure and function (14). Animal models of *Heligmosomoides polygyrus*, a non-pathogenic helminthic infection in the gastrointestinal tract, has been shown to attenuate airway inflammation (38, 126, 127). Helminth-derived immunomodulatory molecules can influence all facets of the host immune response including cytokine and chemokine signaling and gene expression (126). This immunomodulation and immune-mimicry allows parasites to live within the host for extended time periods. Further characterization of helminthic molecules and their cognate host receptors may contribute knowledge in the development of future therapeutic approaches in the treatment of diverse inflammatory conditions.

CONCLUSIONS

Helminth infections remain a significant global issue impacting the lives of billions of people. Several human helminth infections lead to profound morbidity including pulmonary disease. Conversely, some helminths may prevent the development of pulmonary disease. This dichotomy in outcomes in the lungs and the global impact on pulmonary disease is likely helminth-species and helminth-stage specific. Dissecting the intricacies of the parasite-host pulmonary immune response is critical knowledge in order to develop therapeutic strategies to reduce helminth burden worldwide.

AUTHOR CONTRIBUTIONS

JW developed, researched, wrote, edited, and reviewed the manuscript. PG-G wrote, edited, and reviewed the manuscript. JK wrote, edited, and reviewed the manuscript. RF reviewed the manuscript. PH edited and reviewed the manuscript. MB edited and reviewed the manuscript. DC edited and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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A Vaccine Based on Kunitz-Type Molecule Confers Protection Against *Fasciola hepatica* Challenge by Inducing IFN-γ and Antibody Immune Responses Through IL-17A Production

Leonardo Silvane[1,2], *Daiana Pamela Celias*[1,2], *Pablo Alberto Romagnoli*[3,4], *Belkys Angélica Maletto*[1,2], *María Fernanda Sanchez Vallecillo*[1,2], *Laura Silvina Chiapello*[1,2], *Santiago Daniel Palma*[5,6], *Daniel Alberto Allemandi*[5,6], *Rodrigo Eduardo Fabrizio Sanabria*[7,8], *César Iván Pruzzo*[7], *Claudia Cristina Motrán*[1,2] and *Laura Cervi*[1,2]*

[1] Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [2] Centro de investigaciones en Bioquímica Clínica e Inmunología (CIBICI), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Córdoba, Argentina, [3] Centro de Investigación en Medicina Traslacional Severo Amuchastegui (CIMETSA), Córdoba, Argentina, [4] Instituto Universitario de Ciencias Biomédicas de Córdoba (IUCBC), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Córdoba, Argentina, [5] Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina, [6] Unidad de Investigación y desarrollo en Tecnología Farmacéutica (UNITEFA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Córdoba, Argentina, [7] Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Argentina, [8] Instituto Tecnológico Chascomús (INTECH), Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de San Martín (CONICET/UNSAM), Chascomús, Argentina

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***Correspondence:**
Laura Cervi
laura.cervi@unc.edu.ar

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Fasciola hepatica is helminth parasite found around the world that causes fasciolosis, a chronic disease affecting mainly cattle, sheep, and occasionally humans. Triclabendazole is the drug of choice to treat this parasite. However, the continuous use of this drug has led to the development of parasite resistance and, consequently, the limitation of its effectiveness. Hence, vaccination appears as an attractive option to develop. In this work, we evaluated the potential of *F. hepatica* Kunitz-type molecule (FhKTM) as an antigen formulated with a liquid crystal nanostructure formed by self-assembly of 6-O-ascorbyl palmitate ester (Coa-ASC16) and the synthetic oligodeoxynucleotide containing unmethylated cytosine-guanine motifs (CpG-ODN) during an experimental model of fasciolosis in mice, and we further dissected the immune response associated with host protection. Our results showed that immunization of mice with FhKTM/CpG-ODN/Coa-ASC16 induces protection against *F. hepatica* challenge by preventing liver damage and improving survival after *F. hepatica* infection. FhKTM/CpG-ODN/Coa-ASC16-immunized mice elicited potent IFN-γ and IL-17A with high levels of antigen-specific IgG1, IgG2a, and IgA serum antibodies. Strikingly, IL-17A blockade during infection decreased IgG2a and IgA antibody levels as well as IFN-γ production, leading to an increase in mortality of vaccinated mice. The present study

highlights the potential of a new vaccine formulation to improve control and help the eradication of *F. hepatica* infection, with potential applications for natural hosts such as cattle and sheep.

Keywords: Th17-dependent protection, nanostructure, ascorbyl palmitate, kunitz type molecule, vaccine, *Fasciola hepatica*

INTRODUCTION

Fasciolosis is a zoonotic and chronic disease caused by a helminth parasite, *F. hepatica,* that causes huge economic losses in animal production worldwide. These losses have been estimated to be US$ 3 billion due to a reduction in milk, wool, and meat production in cows and sheep (1, 2). On the other hand, the World Health Organization (WHO) has reported that approximately 2.4 million people are infected by this parasite worldwide. Fasciolosis has recently been declared as an emerging disease in humans with an increased number of cases in some regions of the planet (3). The frontline drug against fasciolosis is triclabendazole. However, the emergence of resistance to this drug in diverse *F. hepatica* populations (4, 5), and its high cost suggest the need for other control strategies. In this sense, developing a vaccine against this parasite would be a better preventive control strategy. Moreover, vaccines are considered safe and environmentally friendly because their use ensures the absence of chemical residues in food, as well as in pasture (1).

Both cattle and humans are infected by the ingestion of metacercariae, the infective stage, encysted in aquatic plants. After that, the newly excysted juveniles (NEJ) fluke emerges in the intestine and penetrates the intestinal wall in its migration through the peritoneum and liver parenchyma to finally allocate in the bile ducts.

During its migration, the parasite releases an array of molecules from its intestinal content called excretory-secretory products (FhES) or its tegumental coat (FhTeg), which are the main source of immune-modulatory molecules (6–8).

These complex parasite-derived molecules can interact with the immune system and inhibit the Th1-driven protective pro-inflammatory responses through the induction of M2 macrophages (9), mast cells (10), and Th2-type responses (11, 12), and promote regulatory T (Treg) cell development (6, 13). Thus, the induction of an ineffective immune response against this parasite allows the development of a chronic infection.

Over the last 25 years, there have been numerous attempts to formulate a successful vaccine against *F. hepatica* by using parasite extracts or individual antigens (14, 15). These formulations achieved different levels of protection in experimental models of mice, rats, sheep, and cattle (1, 16, 17) by the induction of an antibody response and/or Th1/Th17-mediated cellular immunity (18–22).

However, a commercially viable vaccine against *F. hepatica* with an appropriate level of efficacy is not available yet.

Among the molecules released by the parasite, the most abundant are proteases and protease inhibitors (14, 23, 24). The proteases secreted by *F. hepatica* allow its migration through the tissues and modulate the immune system, which enables its establishment and permanence in the host. However, a tight control of this enzymatic activity should be regulated by protease inhibitors. Kunitz type molecule (FhKTM) is a member of the inhibitory protease family expressed in the FhES and FhTeg during the juvenile stage, suggesting an essential role in controlling proteolytic activity (25). Thus, the physiological function of FhKTM may be to protect the parasite from the host and parasite proteases by inhibiting its activity. In our study we tested a FhKTM peptide as a vaccine antigen.

On the other hand, new approaches have focused on the design of innovative methods to improve immune response involving mainly suitable adjuvant strategies (26). Over the last decade, an area of extreme development has been the application of nanomaterials to vaccine development. In this line, the adjuvant capacity of the synthetic oligodeoxynucleotide containing unmethylated cytosine-guanine motifs (CpG-ODN) (agonist of TLR9) formulated with liquid crystal-type nanostructures formed by self-assembly from ascorbyl 6-O-palmitate ester (Coa-ASC16) has been demonstrated. The immunization of mice with the ovalbumin (OVA) protein, together with the adjuvant CpG-ODN/Coa-ASC16, induced a potent antigen-specific antibodies and Th1/Th17/CD8 + T-cell cellular responses without toxic systemic effects (27, 28).

In this work, we evaluated the potential of an FhKTM peptide formulated in a nanostructure based on CpG-ODN/Coa-ASC16 as a vaccine during an experimental model of fasciolosis in mice and we further dissected the immune response associated with host protection.

MATERIALS AND METHODS

Animals

Wild-type 8- to 10-week-old female BALB/c mice were obtained from the Faculty of Veterinary Sciences, National University of Litoral (UNL, Argentina) and housed in the Animal Facility of the Faculty of Chemical Sciences, National University of Córdoba.

Ethics Statement

All animal experiments were approved by and conducted in accordance with the guidelines of the committee for Animal Care and Use of the Faculty of Chemical Sciences, National University of Córdoba (Approval Number HCD 881) in strict accordance with the recommendation of the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (OLAW Assurance number A5802-01).

Antigens and Adjuvant

A FhKTM peptide according to the sequence described by Bozas et al. (29) was synthesized by ONTORES

TABLE 1 | Processing mice in each group.

Groups	Treatments
Untreated	Non-immunized and uninfected
Infected	Non-immunized and infected
CpG-ODN/Coa-ASC16	Immunized with adjuvant and infected
FhKTM/CpG-ODN/Coa-ASC16	Immunized with FhKTM/CpG-ODN/Coa-ASC16 and infected

Biotechnologies (Zhejiang, China). The identity and purity of the peptide was analyzed by analytical reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry MALDI-TOF (purity >95%). Class-B CpG-ODN 1826 (5'-TCCATGACGTTCCTGACGTT-3') with total phosphorothioate-modification was provided by Operon Technologies, Alameda, CA, United States. To prepare Coa-ASC16-based formulations, FhKTM peptide and/or CpG-ODN were added to a dispersion of 2% (w/v) ASC16 in 5% dextrose solution, heated up to 72°C for 15 min, and then allowed to reach room temperature as described previously (27).

Vaccination With FhKTM/CpG-ODN/Coa-ASC16

BALB/c mice were randomly divided into four groups (n = 4–5) as described in **Table 1**. Immunizations were performed three times at one-week intervals over 2 weeks. Each mouse was subcutaneously immunized with an entire dose (250 μl) equally distributed at five sites: tail, back, neck region, and both hind limbs (50 μl/site). CpG-ODN was administered at 75 μg/mouse/dose. The FhKTM dose was 10 μg/mouse/dose. One week after the last immunization, all groups were orally infected with 6 metacercariae of *F. hepatica* (Sanabria Laboratory, Universidad Nacional de la Plata, La Plata, Argentina). Mice were sacrificed at three different days, 0, 4, and 24, after infection.

Cytokine Detection Assay

Peyer patches (PPs) were harvested from the small intestine of mice and then incubated in RPMI 1640 medium (Gibco BRL, Life Technologies, Grand Island, NY) containing 0.5 mg/ml collagenase, 2% (V/V) fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/ml penicillin, and 100 μg/ml streptomycin for 30 min. The PPs cells were filtered through a cell strainer (100 μm; BD) and washed with the medium without collagenase. The cells were suspended in RPMI 1640 medium containing 10% (V/V) FBS, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin and then cultured at 1.0×10^5 cells/well in a U-bottom 96-well plate stimulated with FhKTM (2 μg/well) for 3 days at 37°C under 5% of CO_2 and 95% air. Spleen, mesenteric lymph nodes (MLNs), and inguinal lymph node (ILNs) cells were obtained, homogenized, and suspended in RPMI 1640 medium (Gibco BRL, Life Technologies, Grand Island, NY) supplemented with 10% FCS (Gibco), 1 mM sodiumpyruvate, 2 mM l-glutamine, 100 U of penicillin/ml, and 100 μg/ml of streptomycin (complete medium). Cultures were incubated at 37°C in a humidified atmosphere (5% CO_2) and stimulated with FhKTM (2 μg/ml) for 72 h. At the end of the incubations, cell culture supernatants were collected, aliquoted, and frozen at −80°C until being analyzed for IFN-γ, IL-17A, IL-4, IL-5, and IL-10 by sandwich ELISA according to the manufacturer's guidelines (BD Pharmingen, San Jose, CA, United States).

Treatment With αIL-17A Antibody

Monoclonal antibody was applied to the vaccinated group to induce the functional inhibition of IL-17A. Two days before and after infection, FhKTM/CpG-ODN/Coa-ASC16 vaccinated and infected mice were injected with 250 μg (100 μl i.p./mouse/dose) of αIL-17A antibody (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States). The FhKTM/CpG-ODN/Coa-ASC16 and infected groups were alternatively injected with the non-specific isotype control IgG (Invitrogen,Thermo Fisher Scientific, Waltham, MA, United States) (100 μl i.p./mouse/dose) (30).

Survival Curves

Mortality and survival of mice in different groups were observed until the completion of the experiment and survival curves were plotted until day 75 post-infection by using GraphPad Prism 6.01 software (GraphPad Software, San Diego, CA).

Liver Analysis

The analysis of livers consisted of two parts. Gross lesions were scored (range 0 to 5) according to the method described by Changklungmoa N et al. (31) taking into account the extension of damage on the surface of livers. The histopathological examination was done after livers were fixed in 10 % neutral-buffered formalin for 48 h, followed by paraffin embedding. Sections of 5 μm were stained with hematoxylin and eosin (HE). Histological samples were scored according to Chien-Chang Chen et al. (32) with modifications. The lesions were scored between 0 and 9 based on the following findings: infiltration of inflammatory cells (score range, 0 to 3), together with the evaluation of liver tissue damage (necrosis, hemorrhagic foci, fibrosis, score range 0 to 3), and presence of tunnels and flukes (score range, 0 to 3), with 0 as normal and 9 as the most diseased.

Antigen Specific Antibody Titers

FhKTM-specific titers of IgA in fecal extracts and IgG isotypes (IgG1 and IgG2a) in serum were determined by ELISA. Fecal extracts were prepared by suspending five fecal pellets in 0.5 ml of extraction buffer (100 μg/ml soybean trypsin inhibitor, Sigma Aldrich St. Louis, MO, United States), 10 mg/ml bovine serum albumin (Sigma Aldrich, St.Louis, MO, United States), and 30 mM disodium EDTA in PBS (pH=7.6). After homogenization and centrifugation at 4°C, the supernatants of the fecal extracts were used for IgA determination in feces (33). Blood was allowed to clot, and serum was removed and stored at −20°C until use. The small intestinal contents were flushed out with 3 mL of PBS. The intestinal lavage fluids were centrifuged at 9,200 g for 5 min at 4°C and the supernatants were stored at −80°C until analysis. For ELISA, FhKTM was diluted at 10 μg/ml in

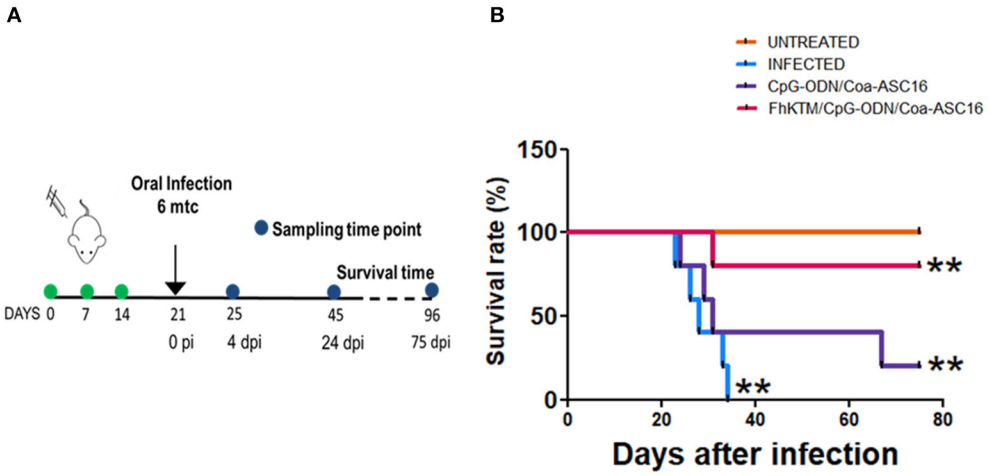

FIGURE 1 | Vaccination with FhKTM/CpG-ODN/Coa-ASC16 increases the survival rate in *F. hepatica* infected mice. **(A)** Scheme of the vaccination and challenge experiments. BALB/c mice were injected s.c. in the upper and dorsal region on days 0, 7, and 14 with FhKTM/CpG-ODN/Coa-ASC16, CpG-ODN/Coa-ASC16, or PBS (infected). One week later, the mice were oral-challenged with 6 metacercariae of *F. hepatica*. Non-vaccinated non-infected (untreated) mice were used as a negative control group. The samples were obtained at 4 and 24 dpi. **(B)** Survival was monitored for >75 days. Each group comprised of five mice. Survival was significantly higher in the FhKTM/CpG-ODN/Coa-ASC16-immunized mice than in the control groups. Kaplan–Meier curves were generated and survival was compared across groups using the log-rank test $^{**}p < 0.05$. This figure is representative of two experiments with similar results.

NaHCO3 1M (pH=9.6), and ELISA plates were coated in 100 µl/well overnight at 4°C. Plates were blocked with 5% bovine serum albumin (BSA) in PBS at 37°C for 1 h and washed with PBS-Tween 0.05%. Samples were incubated for 2 h at room temperature and after washing, rat anti-mouse IgA-HRP (BD Pharmingen, San Jose, CA, United States) or anti-mouse IgG-HRP (Invitrogen, Thermo Fisher Scientific, Waltham, MA, United States) diluted in 1% PBS–BSA were added for 1 h at room temperature. Finally, detection was performed with BD Opt EIA™ TMB Substrate Reagent Set (BD, San Diego, CA, United States). Titers were calculated as the reciprocal of the last serum dilution that yielded an absorbance at 490 nm above that of twice the mean value of blank. The sera from the non-immunized group (untreated) were represented by a full line.

ALT and AST Measurement

The serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a kinetic-UV method by BIOCON laboratory, Cordoba, Argentina, under the established manufacturer's protocols.

Statistical Analysis

Data were analyzed using GraphPad Prism 6.01 software (GraphPad Software, SanDiego, CA). Data analysis included one-way ANOVA followed by a Tukey's post-test for multiple comparisons and the unpaired Student's *t*-test. In survival experiments, Kaplan–Meier curves were analyzed with log-rank test. All data were considered statistically significant for *p*-values of $* < 0.05$, $** < 0.01$ or $*** < 0.001$ depending on the experiment.

RESULTS

FhKTM/CpG-ODN/Coa-ASC16 Vaccination Protects Against *F. hepatica* Infection

To study whether FhKTM/CpG-ODN/Coa-ASC16 protects against *F. hepatica* infection, we followed an experimental procedure of immunization and infection described in **Figure 1A**. Samples from mice were obtained on days 25 and 45 after the first immunization (**Figure 1A**). In addition, the survival rates of infected mice were evaluated until 75 days post infection (dpi). **Figure 1B** shows that all infected mice died by day 32 pi, while immunization with FhKTM/CpG-ODN/Coa-ASC16 effectively increased mice survival, showing no significant differences with untreated animals. Moreover, mice injected only with CpG-ODN/Coa-ASC16 showed a survival impairment, with their survival significantly lower than it was observed for FhKTM/CpG-ODN/Coa-ASC16-vaccinated mice (**Figure 1B**). Taking into account that vaccination with FhKTM/CpG-ODN/Coa-ASC16 prolonged infected mice survival, we investigated the level of damage in the liver, the target organ of infection, establishing a macroscopical score (range 0 to 5) according to the extension of surface liver lesions. The infected and CpG-ODN/Coa-ASC16-injected mice showed significantly higher scores of liver lesions than the FhKTM/CpG-ODN/Coa-ASC16 vaccinated group, which did not present damage in the liver (**Figure 2A**). All vaccinated mice exhibited a microscopically preserved liver architecture comparable to the untreated group (**Figure 2B**). In contrast, livers from both infected and CpG-ODN/Coa-ASC16-injected mice presented migratory tunnels (T) containing young flukes (thin arrows), large areas of fibrosis that replace hepatic parenchyma (thick arrows), and large leukocyte infiltrates

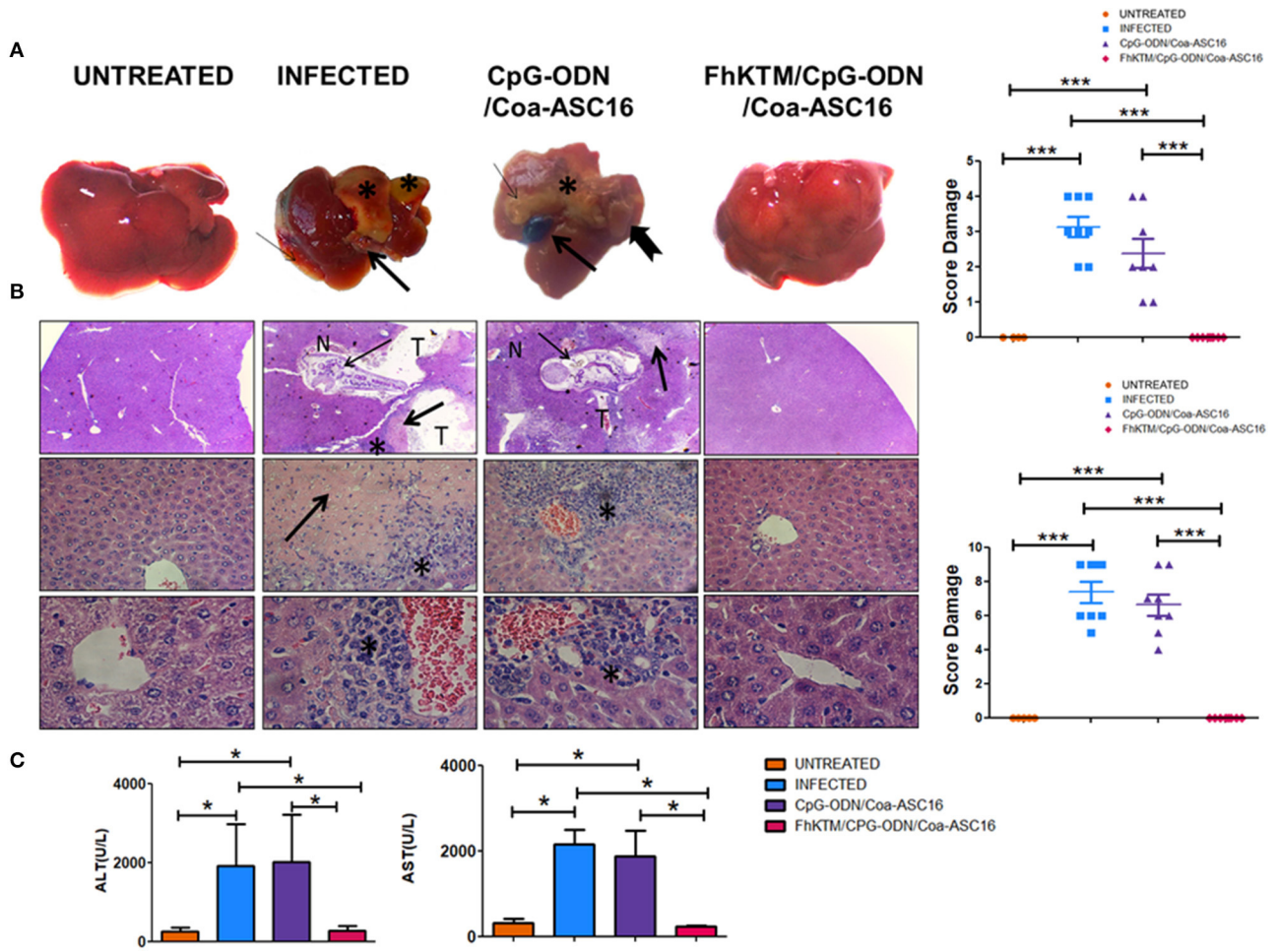

FIGURE 2 | FhKTM/CpG-ODN/Coa-ASC16 immunization in mice prevented liver damage caused by *F. hepatica* infection. Gross lesions of livers from mice after 24 days of the infection with *F. hepatica* or without infection. **(A)** Untreated: tissue from mice without treatment showed a standard architecture with no observed lesions. Infected: there are marked irregularities of capsule (thin arrow), changes in the tissue color (asterix) belonging to the growing fibrosis generated, and enlargement of bile ducts (thick arrow). CpG-ODN/Coa-ASC16: multiple foci of fibrosis (asterix), also enlargement, thickening, and color changes due to hemorrhages in the gallbladder (thick arrow) and the presence of worms on the liver surface (notched arrow). FhKTM/CpG-ODN/Coa-ASC16: the livers maintained a structure similar to the untreated control mice without visible lesions. Right, the score was assessed by the extensión of liver damaged (score range 0 to 5). The data shown are pooled from two independent experiments with total (n = 5–8 per group). **(B)** Histopatological evaluation of livers from the different groups was performed 24 days after infection. Left, untreated control mice showed the expected mice hepatic architecture (left panel) [H/E, 100 × (top) 400× (middle panel) 900× (below)]. The infected group exhibited migrating newly excysted juvenile fluke (NEJ) in the hepatic parenchyma (thin arrow), associated tract (T), extensive Inflammatory infiltrate (*), moderate hepatocyte necrosis (N), and fibrotic connective tissue proliferation (thick arrow) at the chronic stage of the infection (center left panel). Likewise, CpG-ODN/Coa-ASC16 control evidenced the presence of NEJ and the consequences thereof (center right). FhKTM/CpG-ODN/Coa-ASC16 mice maintained the liver parenchyma structure despite infection (right panel). Right, histological samples were scored between 0 and 9 based on the following findings: infiltration of inflammatory cells (score range, 0 to 3), together with the evaluation of liver tissue damage (necrosis, haemorragic foci, fibrosis, score range 0 to 3), and the presence of tunnels and flukes (score range, 0 to 3), with 0 as normal and 9 as the most diseased. The data shown are pooled from two independent experiments with total (n = 5–8 per group). **(C)** Serum was collected at the time of euthanasia, 24 days after challenge. The levels of AST (right), aspartate aminotransferase; ALT (left), and alanine aminotransferase were determined by using a kinetic-UV method, under the established manufacturer's protocols. Data were analyzed by one-way ANOVA and Tukey's post-test *$P < 0.05$; ***$P < 0.001$. Data are shown as mean ± SD. All data are representative of two individual experiments.

(asterisk) (**Figure 2B**). Results showing the histopathological analysis of the livers are summarized in **Figure 2B**. Accordingly, FhKTM/CpG-ODN/Coa-ASC16-immunized mice showed serum ALT and AST levels similar to those observed in untreated animals (**Figure 2C**). As expected, ALT and AST levels were significantly increased in sera from infected and CpG-ODN/Coa-ASC16-injected mice (**Figure 2C**). In summary, high survival rates and no significant changes in the liver structure, together with normal concentrations of hepatic enzymes (ALT and AST), demonstrate the effectiveness of the FhKTM/CpG-ODN/Coa-ASC16 vaccine to protect mice against *F. hepatica* infection.

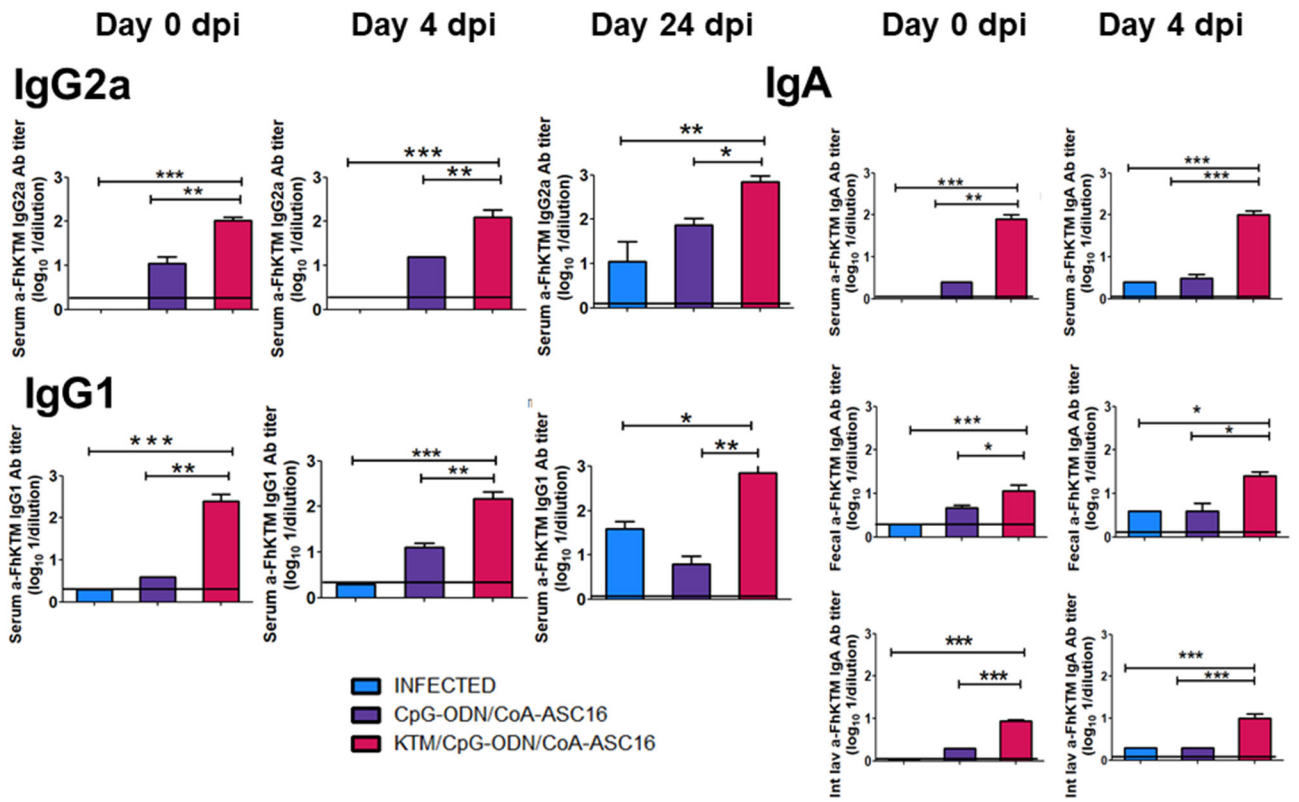

FIGURE 3 | FhKTM-specific IgG antibodies detection. **(A)** IgG1 and IgG2a subclass antibodies in the sera of the immunized mice by enzyme-linked immunosorbent assay (ELISA). Serum samples were collected from the mice by retro-orbital bleeding at 0, 4, and 24 dpi. Humoral immune responses were analyzed. **(B)** IgA titles were detected in the acute infection on days 0 and 4 in sera, feces, and intestinal lavage. Results have been expressed as the mean of OD 450 ± SD values ($n = 15$) and are representative of at least three independent experiments. The data were analyzed by one-way ANOVA and Tukey's multiple comparison tests*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

FhKTM/CpG-ODN/Coa-ASC16 Immunization Elicits Strong Antigen-Specific Humoral Immune Responses

It has been reported that one important protective mechanism against *F. hepatica* is the humoral response (14, 18, 34, 35). Therefore, to evaluate whether FhKTM/CpG-ODN/Coa-ASC16 promotes an antigen-specific antibody response and primes the infection-induced humoral response, the levels of IgG1 and IgG2a antibodies against FhKTM were determined by ELISA in sera from FhKTM/CpG-ODN/Coa-ASC16-immunized, infected, and CpG-ODN/Coa-ASC16-injected mice on 0, 4, and 24 dpi. The anti-FhKTM IgG2a and IgG1 titers are depicted in **Figure 3A**, with the levels of anti-FhKTM obtained in untreated mice indicated as a line. Seven days after the third immunization (0 dpi, **Figure 1A**), FhKTM/CpG-ODN/Coa-ASC16-immunized mice showed significantly higher titers of FhKTM-specific IgG1 and IgG2a antibodies than CpG-ODN/Coa-ASC16-injected mice (**Figure 3A**). In addition, immunization worked as an effective stimulus for boosting the infection-induced antibody response, because at 4 and 24 dpi, FhKTM/CpG-ODN/Coa-ASC16-immunized mice showed significantly higher titers of IgG1 and IgG2a anti-FhKTM than those observed in serum from the other two infected experimental groups (infected and CpG-ODN/Coa-ASC16) (**Figure 3A**). Next, to evaluate the IgA immune responses induced by the vaccine formulation systemically and at a mucosal level, titers of FhKTM-specific IgA in serum, fecal pellets, and intestinal lavage were determined by ELISA (**Figure 3B**). According to what was observed in the systemic response for IgG1 and IgG2a, strong IgA responses were observed by vaccination with FhKTM/CpG-ODN/Coa-ASC16. Together, these data indicate that this vaccine is effective at inducing a specific antibody response at a systemic level and also in the intestine, with the latter being really important considering the migration period of the parasite through the host intestine wall at an early time after infection.

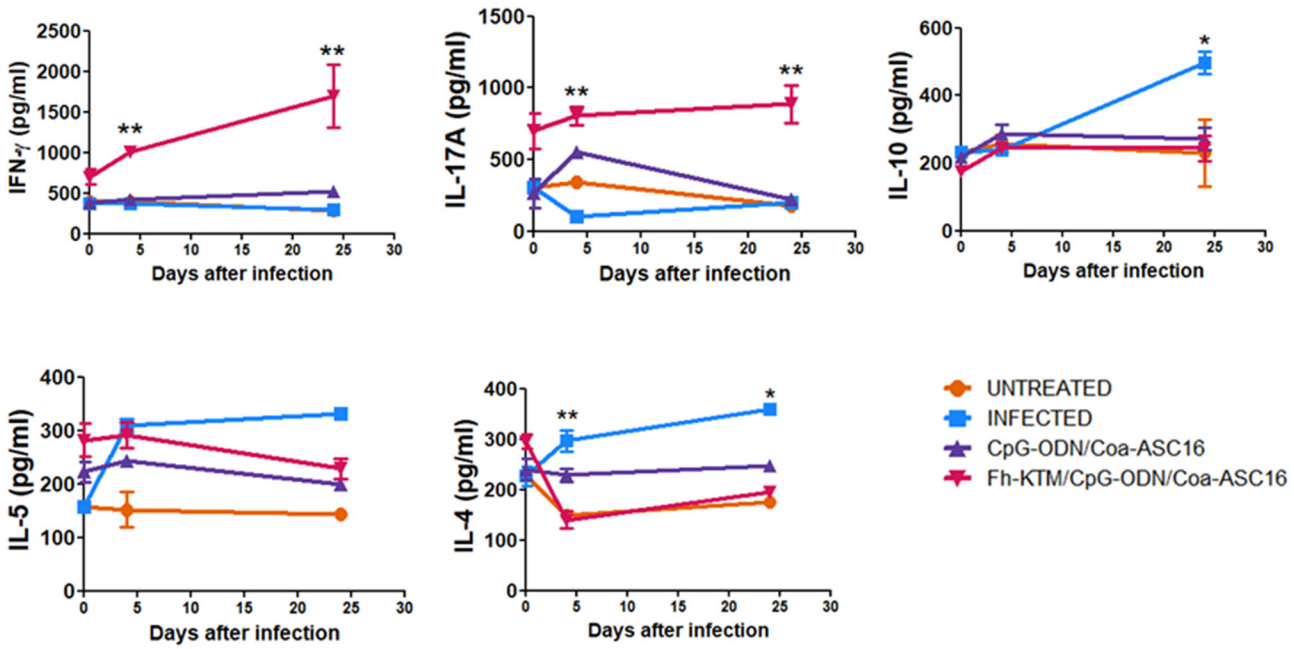

FIGURE 4 | FhKTM-specific cytokine immune responses in BALB/c mice after vaccination. At 0, 4, and 24 dpi, spleens were collected and splenocytes were stimulated *in vitro* with FhKTM for 72 h. The culture supernatants from spleen cells were assessed for the production of IL-17A, IFN-γ, IL-10, IL-5, and IL-4 by ELISA. Results are shown as mean ± SD and levels of significance as indicated by *p*-values and are representative of two or three independent experiments. They were assessed by one-way ANOVA and Tukey's multiple comparison tests $^{*}p < 0.05$; $^{**}p < 0.01$.

Immunization With FhKTM/CpG-ODN/Coa-ASC16 Enhances Antigen-Specific IL-17A and IFN-γ Production

Next, we evaluated whether vaccination with FhKTM/CpG-ODN/Coa-ASC16 is also able to promote an antigen-specific cellular response. To this end, BALB/c mice that were vaccinated and infected according to the scheme of **Figure 1A** were euthanized on 0, 4, and 24 dpi and the capacity of splenocytes to produce IFN-γ, IL-17A, IL-10, IL-5, and IL-4 after restimulation with FhKTM was assessed in the culture supernatants by ELISA. As shown in **Figure 4**, vaccination with FhKTM/CpG-ODN/Coa-ASC16 was able to induce a strong cellular response characterized by enhanced secretion of IL-17A and IFN-γ, whereas only a weak production of IL-4 and IL-5 was observed, thereby suggesting the stimulation of dominant Th17 and Th1 responses. Moreover, the IL-17A and IFN-γ production was markedly increased after the infection (**Figure 4**). As previously described (36), *F. hepatica* infection induces an increase in IL-4-, IL-5-, and IL-10-producing splenocytes, while FhKTM/CpG-ODN/Coa-ASC16-immunized mice not only did not increase IL-10- production but also decreased IL-4-producing splenocytes after the infection (**Figure 4**). Taking into account that an early IL-17A production has been previously demonstrated to promote IgA class switching in lymph organs (37), and considering our data showing increased IgA in feces as well as in intestinal lavage, we examined cytokine production by lymphatic organs for mucosal immunity, such as MLNs and PPs. **Figure 5** shows that after three immunizations and prior to infection, MLNs or PP cells from FhKTM/CpG-ODN/Coa-ASC16-immunized mice produced high levels of IL-17A and IFN-γ after antigen-specific stimulation. In addition, MLNs or PP cells from vaccinated mice secreted higher levels of these cytokines compared to those secreted by cells of MLNs or PP from CpG-ODN/Coa-ASC16 and infected mice (**Figure 5**).

These data suggest that IL-17A and IFN-γ production could generate an inflammatory environment during the parasite migration which might contribute to its elimination.

In vivo Neutralization of IL-17A Abolishes the Protective Capacity of FhKTM/CpG-ODN/Coa-ASC16 Vaccination

IL-17 production has been associated with plasma cells switching to IgG2a antibodies and the promotion to IgA isotype (38, 39). In addition, IFN-γ has been involved in the protection against *F. hepatica* (19, 20, 40). Moreover IL-17A can act synergistically with IFN-γ to activate antiparasitic mechanisms by macrophages (41). Taking into account these reports and our results showing high levels of IL-17A after vaccination, we decided to investigate the role of IL-17A on vaccine-induced protection. Groups of FhKTM/CpG-ODN/Coa-ASC16-immunized or PBS-treated (infected) mice received neutralizing IL-17AmAb or isotype-matched control mAb 2 days before and after the oral challenge with the metacercariae (**Figure 6A**). Injection of neutralizing α-IL-17A mAb, but not control mAb, significantly decreased the serum levels of vaccine-induced IgG2a

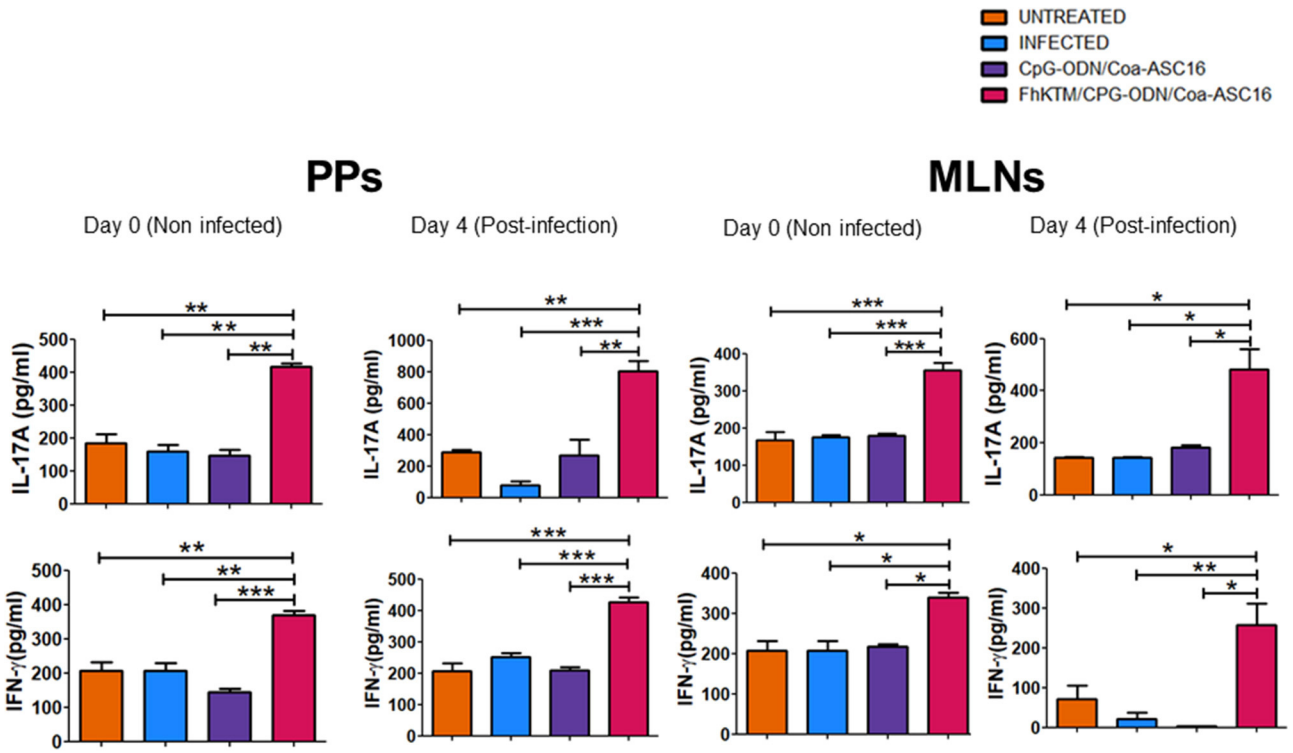

FIGURE 5 | KTM/CpG-ODN/Coa-ASC immunized mice showed increased production of IFN-γ and IL-17A in supernatants from PPs and MLNs. Cell suspensions from PPs and MLNs from treated mice at day 0 (uninfected) and 4 dpi were cultured for 3 days with FhKTM. The IFN-γ and IL-17A were measured in the culture supernatants by ELISA test. Results are shown as mean ± SD and levels of significance as indicated by *p*-values, were assessed by one-way ANOVA and Tukey's multiple comparison tests $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

and IgA as well as fecal IgA titles, but did not significantly affect IgG1 production (**Figure 6B**). Likewise, splenocytes from vaccinated mice that were treated with α-IL-17A mAb, but not control mAb, showed diminished IFN-γ production after antigen stimulation in culture (**Figure 6C**). In addition, the vaccine-induced protection appeared to be mediated by an IL-17A-driven immune response because treatment of mice with neutralizing α-IL-17A mAb abolished the protective effect evaluated as survival rate (**Figures 7A,B**). Thus, FhKTM/CpG-ODN/Coa-ASC16-immunized mice and those treated with anti-IL17A neutralizing antibody decreased their survival compared with vaccinated animals without treatment (lines violet vs. fucsia) (**Figure 7B**) and increased the gross liver damage (**Figure 7C**). Interestingly, the IL-17A neutralization decreased the survival of *F. hepatica* infected mice with or without treatment (lines pink and light blue), suggesting an important role of IL-17A in the protection against *F. hepatica*. These data highlight the remarkable role played by IL-17A as a regulator of IFN-γ production and specific antibody response which correlates with the survival levels found in mice after the infectious challenge.

DISCUSSION

The production of an efficient vaccine against *F. hepatica* remains a major challenge for the scientific community for different reasons. The anti-helminthic resistance, high rates of reinfection in endemic areas, and the acute infection cases provoking animal death have raised the need for developing a vaccine against fasciolosis (1, 14). However, the most important challenge in vaccine design against this helminth is the possibility of beating the Th2-type or immunosuppressive responses with a view to an efficient response to eliminate the parasite. So far, numerous vaccination attempts have included different purified parasite molecules or their recombinant forms in cattle, sheep, and goats with varying levels of protection (14, 42–44).

To date, the vaccination trials developed against *F. hepatica* are not reproducible among animal models, in which variable levels of protection are achieved regardless if the antigen is native or recombinant. A number of antigens have been tested as promising vaccine candidates in mice (22, 45, 46). However, partial protection, insufficient improvement in animal survival, or hepatic damage is not be enough to merit progress in the development of a commercially viable vaccine for livestock production. For these reasons, it is still important to define new vaccine candidates and efficient adjuvant formulations in murine models prior to the examination of the protective capacity in natural hosts such as cows or sheep.

The rationale for the vaccine design in this work was based on the properties of FhKTM. Apart from being an abundant protein within the parasite gut, the parenchymal tissue, and the tegument of juvenile (NEJ) (25) and adult (29), its role as protease inhibitor enables the parasite to avoid both its own and the

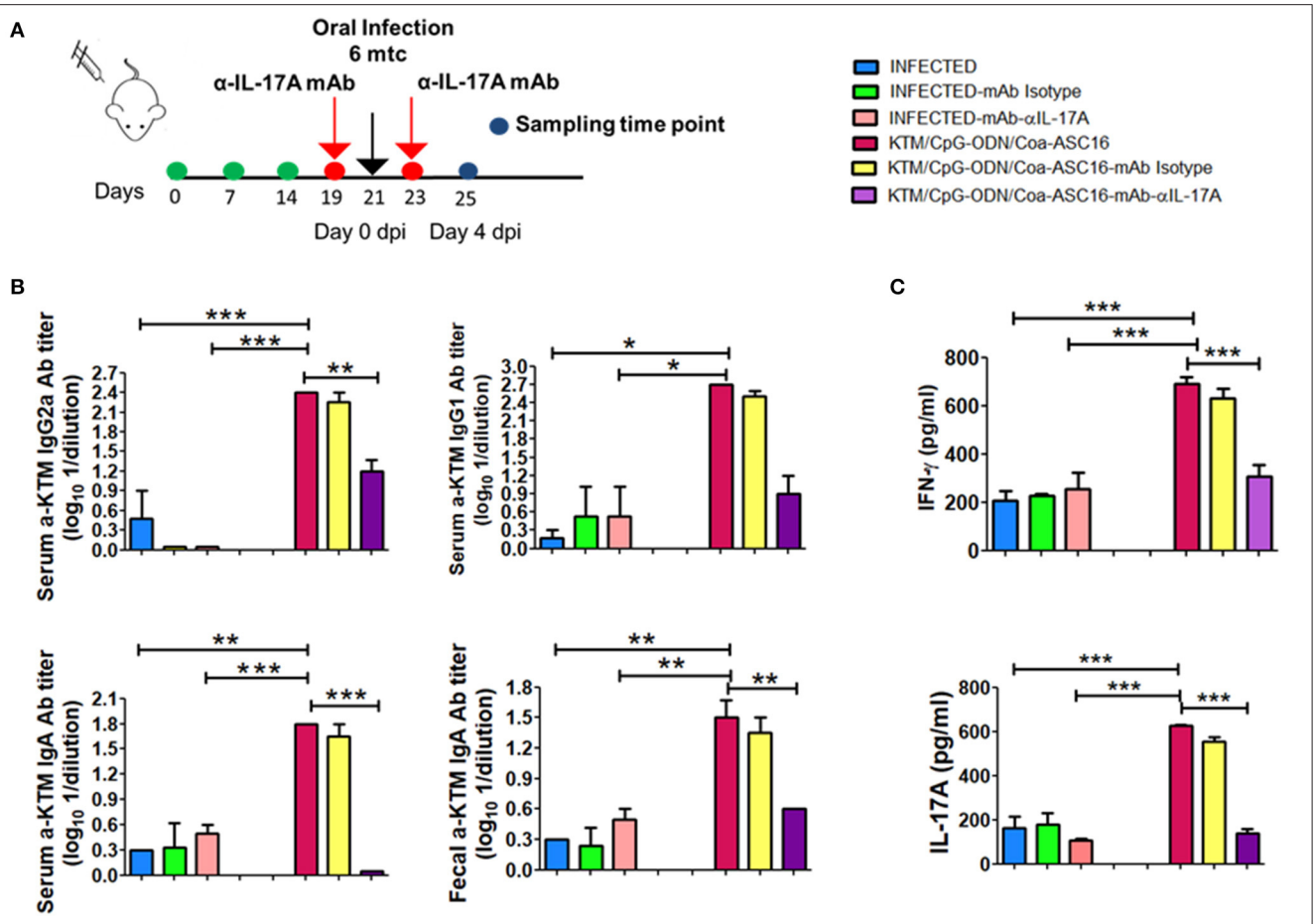

FIGURE 6 | IL-17A neutralizing reduced the immune response against FhKTM inFhKTM/CpG-ODN/Coa-ASC16 immunized mice. **(A)** Mice were administered with anti-IL-17A antibody and the corresponding isotype control (250 μg i.p) 2 days before and after infections. **(B)** Twenty-five days after the first immunization, serum and feces samples were obtained and FhKTM antibody levels were measured by ELISA. Their titers were detected by serial serum dilution and the limit was determined by twice the value of the blanks mean. **(C)** Both IL-17A and IFN-γ secretion were evaluated in the splenocytes supernatant culture by ELISA. dpi: days post infection. Data are representative of two independent experiments. One-way ANOVA + Tukey'multiple comparison tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

host's deleterious protease action. Along with this, the antigen was formulated with a novel adjuvant strategy, CpG-ODN/Coa-ASC16, that constitutes a nanoplatform. This adjuvant strategy is able to induce potent Th1 and Th17 responses, and elicit long-term antibody responses (27, 28). In this study we demonstrated that the immunization of mice with FhKTM/CpG-ODN/Coa-ASC16 increases the survival against *F. hepatica* challenge. Accordingly, the immunized animals presented a highly preserved liver structure, suggesting that mice vaccination somehow prevented worms from reaching the liver. This idea is also supported by results showing that immunization induces early production of specific antibodies and cytokines associated with INF-γ and IL-I7 protection, both systemically and locally in MLNs and PPs after infection. On the one hand, high levels of FhKTM-specific IgA in the intestinal content as well as in feces from vaccinated mice could favor the hypothetical expulsion of parasites. Although *F. hepatica* is a trematode that remains for a short period of time in the intestine, the mechanisms of parasite expulsion in the gut as a result of vaccination could also be operating against the larval stage of this parasite. As described by others, the transference of IgA or IgG1 antibodies from resistant mice to helminth infections confers partial resistance to different nematodes (47, 48), probably through their neutralizing effect on secreted parasite antigens, or by trapping larvae (49–51). In addition, we cannot not rule out the possibility that antibodies generated during immunization with the vaccine might participate in mechanisms of antibody-dependent cell-mediated cytotoxicity (ADCC) and reactive oxygen and nitrogen species (ROS and NOS), according to results reported in *in vitro* studies by Piedrafita et al. (52). On the other hand, a critical role for IL-17A in the protective immunity against *F. hepatica* shown in this study is an interesting finding, whereas the Th1 profile has been the response mostly associated with protective mechanisms (19, 20, 53). The fact that IL-17A was crucial to induce the IgA isotype in the fecal content of vaccinated mice correlates with the ability of Th17 cells shown by other authors to become precursors for the follicular helper T cells in PPs and to induce IgA class switching (54). This fact could be explained

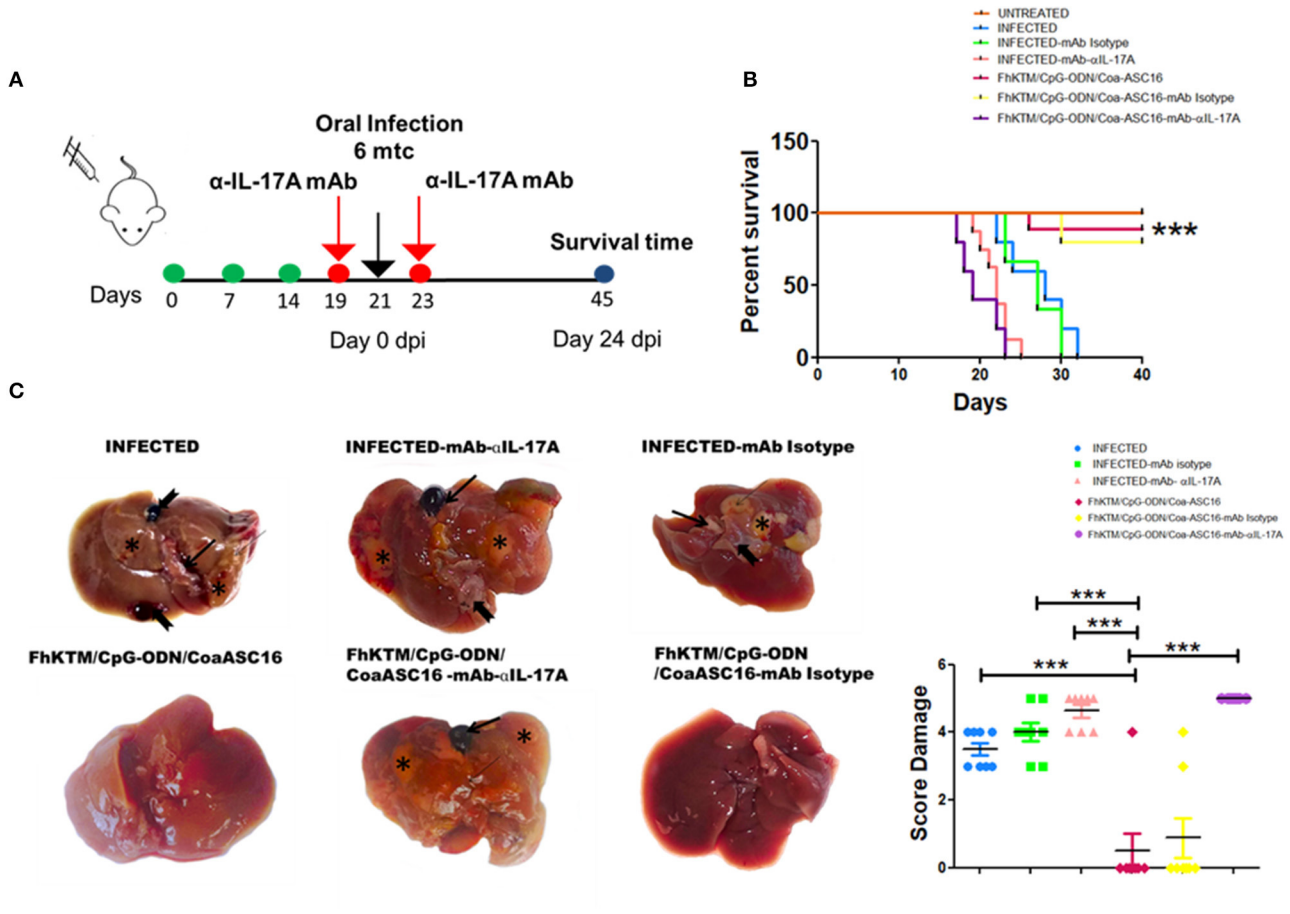

FIGURE 7 | IL-17A neutralization decreased the efficacy of FhKTM/CpG-ODN/Coa-ASC16 vaccination. **(A)** Mice were administered with anti-IL-17A antibody and the corresponding isotype control (250 μg i.p) 2 days before and after infections. **(B)** Kaplan–Meier graphs show survival curves for those mice immunized three times with FhKTM/CpG-ODN/Coa-ASC16 or PBS. IL-17A-mAb and corresponding isotype control (mAb Isotype) were administered 2 days both before and after infection. dpi: days post infection. **(C)** Left: infected liver showing marked enlargement with distended gallbladder (thick arrow), areas of fibrosis extending (asterix), and larvae on the parenchyma surface (notched arrow). FhKTM/CpG-ODN/Coa-ASC16: liver showing normal size with no observed lesion except in 1 out of 8 mice. Infected-mAb-IL-17A: the liver showing marked severe macroscopic damage (thin arrow) and presented irregular surface denoting hepatic fibrosis (asterix). The presence of larvae on the surface can be seen (notched arrow). FhKTM/CpG-ODN/Coa-ASC16 -mAb-IL-17A: The hepatic parenchyma is severely reduced, firm, and presents an irregular surface (thin arrow) denoting hepatic fibrosis (asterix). Abnormal gross appearance of the distended gallbladder with presence of blood inside (thick arrow). infected-mAb Isotype: the liver is smaller than normal and presents irregular surface tissue denoting hypertrophied (thin arrow) and replaced with fibrosis (asterix) and a fluke released after the rupture of the gallbladder (notched arrow). FhKTM/CpG-ODN/Coa-ASC16-mAb Isotype: liver showing no apparent gross pathology except for in 2 out of 8 mice. Right, the score damage was assessed by the extensión of liver damaged (score range 0 to 5). The data shown are pooled from two independent experiments with total ($n = 5$–8 per group). The statistical differences among survival curves were calculated by using Mantel–Cox test. ***$p <$ 0.001.

by the capacity of IL-17A to increase the transport and secretion of IgA into the intestinal lumen (38). Moreover, Th17 cell-deficient mice had an impaired antigen-specific intestinal IgA after immunization with cholera toxin, pointing out that Th17 cells were responsible for inducing the switch of GC B cells toward the production of high-affinity T cell–dependent IgA (54). Given the important role of IL-17 in the protective immunity induced by the vaccine, we cannot rule out the presence of innate as well as adaptive cells as a possible source of Th17, since both $CD4^+$ and $CD4^-$IL17A-producing cells were found in the spleen of vaccinated animals (data not shown). Among the effector mechanisms of IL-17A there appears the ability to recruit neutrophils, which destroy the pathogen through the production of cytokines, chemokines, and anti-microbial peptides or myeloid cells which in turn restrict pathogen survival through activation and recruitment of Th1 cells (55). In the present study, a low neutrophil recruitment at the peritoneal cavity was observed in all experimental mice without significant differences among the groups (data not shown). On the other hand, the passage of worms through the intestinal wall that could induce neutrophil recruitment is random and transitory, so its finding might be difficult. The uncoupled IL-17A-dependent effector mechanisms from the neutrophil response have already been demonstrated in barrier tissues, in mouse models oropharyngeal, or skin fungal infections where IL-17A provided immunity through anti-microbial peptide generation (56), independently of neutrophils

(57). These data suggest that the mechanism by which IL-17A plays a crucial role in the protective immunity against *F. hepatica* might be independent from neutrophil recruitment, and is still to be determined.

In addition, high systemic production of IFN-γ and IgG2A levels in vaccinated animals is in agreement with previous reports showing the association of these responses with increased levels of protection against the parasite (34). A close relationship between IL-17A and IFN-γ production was demonstrated in this work since the blockade of IL-17A significantly decreased IFN-γ levels in splenocyte supernatants and, consequently, animal survival. The precise mechanism by which the production of IFN-γ is dependent on IL-17 is unknown, however, it could be speculated that after this cytokine is produced, responder cells such as epithelial or myeloid cells through IL-17R signaling might induce the recruitment of Th1 cells. These cells could secrete pro-inflammatory cytokines, chemokines, and anti-microbial peptides to restrict the pathogenesis of the disease (55). Although these mechanisms have been proposed in different bacterial (58, 59) or protozoal infections (60), we cannot rule out that IL-17A might play a similar role during *F. hepatica* infection.

Data from other authors support the idea of a synergistic effect between IFN-γ and IL-17A in protective mechanisms against different pathogens through the potentiation of NO production in macrophages (41, 61, 62). Although a mucosal response in the intestine after subcutaneous immunization with FhKTM/CpG-ODN/Coa-ASC16 might seem surprising, recent findings have shown that parenteral immunization can generate a potent IgA response in mucosal tissues (63, 64). This fact could be explained in two hypothetical ways: in one of them, the antigen is captured by APC in the injection site and then transported to mucosal-associated lymphoid tissues (MALT) for antigen presentation. In the other, the antigen can be presented peripherally to naive T cells and B cells which are in turn home to mucosal tissues. The high levels of IFN-γ and IL-17A observed in inguinal lymph nodes after immunization with FhKTM/CpG-ODN/Coa-ASC16 (data not shown) suggest that the APC carrying the antigen might spread to the draining peripheral lymph nodes, either prior to or simultaneously with the antigen presentation to lymphocytes in the MALT. One limitation in this type of approach to studying protective immunity is the difficulty to decide whether protection comes from mucosal or systemic immunity, suggesting that induction of mucosal immunity by parenteral injection is an important issue for vaccine design. Finally, we believe that the CpG-ODN/Coa-ASC16 platform might allow FhKTM-long term release. Coa-ASC16 nanostructure have a certain rigidity, which can either modulate the release of molecule/s into the biological medium or provide stability to loaded molecules (65). Previously, it has been reported by *in vitro* approaches that Coa-ASC16 generates a sustained release of both OVA and CpG-ODN (27). In addition, Coa-ASC16 could exert a protective effect, avoiding FhKTM antigen protease degradation. This strategy may work *in vivo* as a depot effect, which often makes it possible to reduce the dose and/or the number of immunizations required for an optimal response.

The precise protective immunity mechanism as induced by FhKTM remains to be investigated. However, our data highlight the importance of designing vaccines that induce a potent response in mucosa and systemic levels capable of preventing the parasite from reaching the liver. Given the high levels of protection shown in mice susceptible to the infection, our next step is the validation of this vaccine system to the natural hosts of the infection, such as sheep, upon which our regional livestock economy is based.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LC and LS conceived of and designed the experiments and wrote the paper. LS, DC, PR, and MS performed the experiments. LC, LS, PR, LSC, CM, and BM analyzed the data. BM, SP, and DA conceived, developed, and tested the adjuvant capacity of CpG-ODN/Coa-ASC16. RS and CP produced the metacercariae. LC, CM, LSC, and PR contributed reagents, materials, and analysis tools. PR, LSC, BM, and CM revised the manuscript. All authors contributed to the article and approved the submitted version.

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Defined Intestinal Regions Are Drained by Specific Lymph Nodes That Mount Distinct Th1 and Th2 Responses Against *Schistosoma mansoni* Eggs

Johannes U. Mayer[1,2*], *Sheila L. Brown*[3], *Andrew S. MacDonald*[3] *and Simon W. Milling*[1]

[1] Centre for Immunobiology, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow, United Kingdom, [2] Malaghan Institute of Medical Research, Wellington, New Zealand, [3] Lydia Becker Institute of Immunology and Inflammation, Manchester Collaborative Centre for Inflammation Research, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, United Kingdom

The balance of type 1 and type 2 immune responses plays a crucial role in anti-helminth immunity and can either support chronic infection or drive type 2 mediated expulsion of the parasite. Helminth antigens and secreted molecules directly influence this balance and induce a favorable immunological environment for the parasite's survival. However, less is known if the site of infection also influences the balance of type 1 and type 2 immunity. Here, we report that tissue-specific immune responses are mounted against helminth antigens, which elicited strong IL-4 responses when injected into the skin, while the same antigen, delivered into the intestinal subserosa, induced increased IFN-γ and reduced Th2 responses. Immune responses in individual mesenteric lymph nodes that drain defined regions of the intestine furthermore displayed a site-specific pattern of type 1 and type 2 immunity after *Schistosoma mansoni* or *Heligmosomoides polygyrus* infection. *S. mansoni* egg-specific Th2 responses were detectable in all mesenteric lymph nodes but Th1 responses were only present in those draining the colon, while *H. polygyrus* infection elicited mixed Th1 and Th2 responses in the lymph nodes associated with the site of infection. Similar site-specific type 1 and type 2 immune responses were observed in the draining lymph nodes after the controlled delivery of *S. mansoni* eggs into different segments of the small and large intestine using microsurgical techniques. Different subsets of intestinal dendritic cells were hereby responsible for the uptake and priming of Th1 and Th2 responses against helminth antigens. Migratory $CD11b^{+}CD103^{-}$ and especially $CD11b^{+}CD103^{+}$ DC2s transported *S. mansoni* egg antigens to the draining lymph nodes to induce Th1 and Th2 responses, while $CD103^{+}$ DC1s induced only IFN-γ responses. In contrast, *H. polygyrus* antigens were predominantly transported by $CD11b^{+}CD103^{-}$ DC2s and $CD103^{+}$ DC1s and all DC subsets induced similar Th1 but weaker Th2 responses, compared to *S. mansoni* egg antigens. The development of adaptive anti-helminth immune responses is therefore influenced by the antigen itself, the

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***Correspondence:**
Johannes U. Mayer
jmayer@malaghan.org.nz

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uptake and priming characteristics of antigen-positive dendritic cell subsets and the site of infection, which shape the level of Th1 and Th2 responses in order to create a favorable immunological environment for the parasite.

Keywords: mucosal immunology, th1/th2 balance, helminth antigen, mesenteric lymph node, microsurgery, dendritic cells, *Schistosoma mansoni*, *Heligmosomoides polygyrus bakeri*

INTRODUCTION

The balance of different types of immune responses plays an important role in orchestrating the optimal immunological environment to appropriately counter infections. Numerous studies [reviewed in (1–4)] show that this is particularly relevant in the context of helminth infections, where type 2-biased immune responses promote parasite killing and worm expulsion, whereas type 1 immunity usually results in ineffective responses and chronic infection.

This effect is perhaps best demonstrated during the infection of the nematode *Trichuris muris* where Interleukin-4 (IL-4) and IL-13 mediated Th2 responses lead to rapid expulsion in resistant mouse strains, whereas susceptible mouse strains produce high levels of Interferon-gamma (IFN-γ), IL-12 and IL-18, which are characteristic of a predominant Th1 response (5, 6). IFN-γ depletion in normally susceptible animals furthermore promotes the development of protective Th2 responses, which renders these animals resistant to infection (7). Similarly, *T. muris*-resistant mice treated with IL-12 develop chronic infection (8), with similar observations made during *Nippostrongylus brasiliensis* infection, where parasitic worm expulsion is delayed after the injection of recombinant IL-12 (9). A direct requirement for IL-4/IL-13 signaling in worm expulsion is demonstrated by IL-4 receptor antibody blocking experiments, and in IL-4R$^{-/-}$ and STAT6$^{-/-}$ mice, dramatically limiting worm expulsion during *T. muris* (7), *Heligmosomoides polygyrus* (10) or *N. brasiliensis* (11) infection.

Th1 and Th2 responses also play an important role in *Schistosoma mansoni* infection, which afflicts over 200 million people worldwide (12). In humans, acute schistosomiasis manifests as an incapacitating febrile illness through the production of pro-inflammatory cytokines (13). With the onset of egg production by the female parasites a Th2-driven immune response is initiated, resulting in a mixed Th1/Th2 response which leads to chronic infection and granuloma formation in the liver and intestines (13, 14). In murine infection models, low levels of Th1/2 responses against developing worms become dominated by the potent type 2 immune response that develops against *S. mansoni* eggs, limiting damage to the host (15). In the absence of these Th2 responses, IL-4$^{-/-}$ mice infected with *S. mansoni* experience severe TNF-α-mediated acute cachexia, hepatotoxicity, and high mortality (16), as well as intestinal pathology and detectable serum levels of LPS (17). Through experimental immunization models, *S. mansoni* eggs have been shown to induce both Th1 and Th2 responses in an antigen-specific manner (18, 19), and several antigens that can initiate and influence Th2 responses have been identified to date (20–23). An assessment of the ratios of type 1 and type 2 immune responses induced by the injection of *S. mansoni* eggs, however, suggests that other factors such as the different routes of immunization can influence anti-parasite immunity (18, 24, 25).

The idea that the tissue environment can influence T cell immunity has been well studied for tissue resident memory cells, which display tissue-specific signatures and functions (26–29). While tissue resident memory T cells acquire most of these characteristics after priming, a location-dependent preferential development of effector T cells has recently been identified in the intestinal draining lymph nodes (LNs) (30). The analysis of RORγt$^+$ Th17 and Foxp3$^+$ T regulatory cell development after controlled antigen delivery into different segments of the intestine revealed that tolerogenic responses preferentially develop in LNs draining the proximal intestine while Th17 responses are more pronounced in the LNs draining the distal intestine (30). This observation demonstrated that effector T cell differentiation can directly be influenced by location-specific factors, suggesting that the distinct phenotypes and proportions of pro-inflammatory and T regulatory cells, reported in numerous tissues and LNs (31–33), might not only be maintained but also initiated by location-specific cues.

Here we report that the site of immunization can indeed influence the balance of Th1 and Th2 responses against helminth antigens in a tissue- and site-specific manner. After immunization with *S. mansoni* eggs or *H. polygyrus* antigens in the footpad or the intestinal subserosa, antigen-specific CD4$^+$ T cell responses in the lymph nodes showed a contrasting Th1/Th2 profile with higher IFN-γ responses observed after intestinal immunization, compared to increased Th2 responses after immunization of the skin. Distinct type 1 and type 2 immune responses were also observed in the individual mesenteric lymph nodes during live parasite infection, and stronger Th2 responses were observed in the LNs draining the proximal intestine compared to distal intestine. Similar observations were made after the controlled delivery of *S. mansoni* eggs into different segments of the small and large intestine, which induced increased Th1 and reduced Th2 responses in LNs draining the lower colon compared to the small intestine. Different helminth antigens were furthermore taken up by distinct subsets of intestinal migratory dendritic cells, which induced distinct levels of Th1 and Th2 responses, indicating that lymph node-specific type 1 and type 2 immune responses against helminth antigens are modulated by the antigen itself, antigen-positive dendritic cells and the site of infection.

MATERIALS AND METHODS

Mice

C57BL/6 were obtained from Envigo, UK or bred at the University of Manchester, UK or the Malaghan Institute of

Medical Research, Wellington, New Zealand. Mice were housed under specific pathogen free conditions and age- and gender-matched adult animals were used in each individual experiment.

Surgical Procedures

All surgical procedures were carried out under aseptic conditions using inhalation anesthesia with Isoflurane (Abbot Animal Health). Agents, diluted in a maximum volume of 20 µl, were injected into the footpad using Micro-Fine Plus Hypodermic Syringes (29 G × 12.7 mm; BD Bioscience). To display the murine intestine and the mesenteric lymph nodes laparotomy surgery was performed. For lymph cannulations and MLN subcapsular injection, mice were gavaged with 0.2 ml Oliver oil 30 min prior to surgery to visualize the lymphatics. Buprenorphine (0.1 mg/kg; Vetergesic, Reckitt Benckiser Healthcare) and Carprofen (5 mg/kg; Rimadyl, Pfizer) were given subcutaneously into the flank as prophylactic analgesics. The abdominal area was shaved and sterilized, and the mouse placed on its back. A small incision into the skin of the animal's midline was made using a scalpel. This incision was extended to up to 3 cm using scissors. Similarly, the muscle layer was incised at the midline, and the incision extended with scissors. The intestine was carefully displayed onto a moistened surgical cloth using cotton buds as shown in **Figure 2A**. Organs were moistened every 2–5 min during surgery to prevent them from drying out. Using a Leica M651 surgical microscope, agents, diluted in a maximum volume of 10 µl, were injected into the subserosal layer of the different segments of the intestine or under the MLN capsule using Micro-Fine Plus Hypodermic Syringes (29G × 12.7 mm; BD Bioscience). The intestinal segment of interest was gently held in place with forceps, and the needle was horizontally inserted with the bevel facing upwards. A small injection pocket would form at the injection site confirming that the intestinal lumen was not penetrated. For non-recovery ink injection experiments 10 µl of ink was injected into the intestinal subserosa and lymphatic drainage visualized using a Leica M651 surgical microscope (6×) with an attached Nikon camera. Different dyes were injected, which resulted in the following observations.

Injected material	Observations
2% Chicago Blue dye (Sigma)	Wide-spread deposition and drainage *via* the lymphatics within minutes
2% Evans Blue dye (Sigma)	Wide-spread deposition and drainage *via* the lymphatics within minutes
Graphite particles (<20 µm, Sigma) resuspended in PBS	Very localized deposition but no lymphatic drainage
Black Chinese calligraphy ink (AMI)	Local deposition and drainage *via* the lymphatics within seconds
Black India ink (Pelikan)	Local deposition but no lymphatic drainage

After injection, the intestines were replaced into the body cavity, the muscle layer was sutured with 6.0 Vicryl absorbable sutures (Johnson and Johnson) using discontinuous stitching, and the skin was closed using surgical clips (Autoclip Wound Clip System, Harvard Apparatus). Mice were closely monitored post-surgery to ensure full recovery from the anesthesia and monitored on a daily basis thereafter. To collect lymph migrating DCs, MLNs were removed from 6-week-old male mice by laparotomy and blunt dissection, as previously described (25, 34). After 6 weeks, the thoracic lymph duct was cannulated by the insertion of a polyurethane medical grade intravascular tube (2Fr; Linton Instrumentation) and fluorescently labeled soluble parasite antigens were injected in the intestinal serosa. Lymph was collected for 18 h on ice in PBS (Gibco) supplemented with 20 U/ml of heparin sodium (Wockhardt UK).

Schistosoma mansoni Infection

Mice were infected percutaneously with ~180 *S. mansoni* cercariae. Seven weeks after infection MLNs were collected. Infective *S. mansoni* cercariae were obtained from infected *B. glabrata* snails provided by the NIAID Schistosomiasis Resource Centre of the Biomedical Research Institute (Rockville, MD) through NIH-NIAID Contract HHSN272201700014I and distributed through BEI Resources. Eggs were isolated under sterile conditions from the livers of infected C57BL/6 mice prior to cryopreservation. SEA was prepared by homogenization and ultracentrifugation of *S. mansoni* eggs and concentrated by vacuum dialysis to 1 mg/ml in DPBS (Life Technologies). SEA was fluorescently labeled using an AF660 Microscale Antibody Labelling Kit (Life Technologies) following the manufacturer's instructions.

Heligmosomoides polygyrus Infection

The *H. polygyrus* life cycle was maintained as previously described (35). For experimental infections, C57BL/6 mice were infected with 200 L3 larvae by oral gavage at six weeks of age. 17 days after infection MLNs and intestines were collected. Adult worm burden was quantified by mounting opened intestines inside a 50 ml falcon filled with PBS. After a 3 h incubation at 37°C, worms were collected from the bottom of the tube and counted under a microscope. HES was collected from adult parasites that were maintained for 21 days in serum-free tissue culture medium and concentrated by vacuum dialysis to 1 mg/ml in DPBS (Life Technologies). HES was fluorescently labeled using an AF660 Microscale Antibody Labelling Kit (Life Technologies) following the manufacturer's instructions.

Lymph Node Harvest and Cell Isolation

Individual LNs were identified and collected as described throughout the manuscript and depending on the experiment either all, pooled, or individual lymph nodes were collected. For restimulation cultures 1×10^6 MLN cells were cultured in X-vivo 15 media (Lonza) supplemented with 1% L-glutamine (Invitrogen), 0.1% 2-mercaptoethanol (Sigma-Aldrich), and 7.5 µg/ml SEA or HES in round bottom 96-well plates (Corning) at 37° C and 5% CO2. Supernatants were collected after three days and cytokines detected using the IL-4, IL-5, IL-13, and IFN-γ "ready-set-go" ELISA kits (eBioscience) or paired antibodies (Biolegend).

For dendritic cell isolation, lymph was washed in FACS buffer or MLNs were digested with RPMI 1640 (Life Technologies) supplemented with 8 U/ml Liberase and 10 mg/ml DNase (both Sigma-Aldrich) for 45 min at 37°C in a shaking incubator and single-cell suspensions were prepared using a 40 µm cell strainer

(Greiner Bio One). For intracellular staining of cytokines, lymph nodes were passed through a 40 μm cell strainer (Greiner Bio One) to create a single cell suspension. 2×10^6 cells were incubated in RPMI 1640 (Life Technologies) supplemented with 2.5 ng/ml PMA (Sigma- Aldrich), 1 mg/ml ionomycin (Invitrogen), 0.5% GolgiStop (BD Bioscience) and 10% FCS for 4 h at 37°C, after which cell surface markers were stained. Cells were fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (eBioscience), and intracellular staining was performed following the manufacturer's instructions.

Antibodies for Flow Cytometric Analysis

The following combination of fluorescently labeled primary antibodies against cell surface markers and intracellular cytokines were used: anti-CD3 (17A2), anti-CD4 (clones GK1.5 and RM4-5), anti-CD8a (53–6.7), anti-CD44 (IM7), anti-CD45R/B220 (RA3-6B2), anti-CD11c (N418), anti-CD103 (M290), anti-Ly6C (HK1.4), anti-I-A/I-E (M5/114.15.2), anti-IL17 (TC11-18H10.1), and anti-IL4 (11B11) from Biolegend, and anti-IFN-γ (XMG1.2) and anti-IL-13 (eBio13A) from eBioscience. Cells were analyzed using a LSRII flow cytometer running FACSDiva Software (BD Bioscience) and analyzed using FlowJo Software (Tree Star). Fixable Viability Dye eFluor780 (eBioscience), DAPI or 7-AAD Viability Staining Solution (Biolegend) were used according to the manufacturer's instructions to exclude dead cells from analysis.

Statistical Analysis

Experimental group sizes ranging from three to five animals were chosen to ensure that a twofold difference between means could be detected with a power of at least 80%. Prism 8 Software (GraphPad) was used to calculate the SEM and the statistical significance using an ordinary one-way ANOVA with Holm–Sidak's multiple comparisons test, two-way ANOVA with Dunnett's multiple comparisons test or unpaired t-tests as indicated in the figure legends. Statistical significance was defined as $p \leq 0.05$.

RESULTS

Increased IFN-γ and Decreased Th2 Responses Are Induced When *S. mansoni* Eggs Are Injected Into the Intestinal Subserosa Compared to the Footpad

In previous studies (18, 24, 25), different levels of Th1 and Th2 responses had been observed when *S. mansoni* eggs were injected into different locations, such as the tail vein, the footpad or the intestine. To formally compare whether the injection of *S. mansoni* eggs into different tissues induced different levels of type 1 and type 2 immune responses, we injected 2,500 *S. mansoni* eggs into the footpad or the intestinal subserosa of C57BL/6 mice and collected the draining lymph nodes 5 days after immunization. In line with previous reports, these immunizations resulted in robust Th1 and Th2 responses but revealed differences in the quality of the response when compared to each other. In the popliteal lymph nodes (pLNs) approximately 2% of PMA/ionomycin stimulated $CD4^+$ T cells produced IFN-γ after egg injection into the footpad, whereas up to 4% of $CD4^+$ T cells in the mesenteric lymph nodes (MLNs) were IFN-γ^+ after subserosal egg injection (**Figures 1A, B**; **Supplementary Figure 1A**). Conversely, in the same experiment IL-4^+ T cells were four times more abundant in the pLNs than in the MLNs, resulting in a much higher ratio of IFN-γ^+ T cells in the MLNs (**Figure 1 C**). As the subserosal injection required laparotomy surgery, we also assessed IFN-γ and IL-4 responses under mock surgery conditions and confirmed that the injection itself did not induce IFN-γ or IL-4 T cell responses, with no significant levels of IFN-γ^+ or IL-4^+ T cells detected in naïve or PBS injected mice (**Figure 1B**).

To assess antigen-specific cytokine responses in these experiments, we restimulated pLN or MLN cells with soluble egg antigen (SEA) for three days and measured cytokine production by ELISA. Similar to our observations for PMA/ionomycin stimulated $CD4^+$ T cells, high levels of IFN-γ were produced by MLN cells after subserosal egg injection, whereas pLN cells secreted higher levels of IL-5 and IL-13 after egg injection into the footpad (**Figure 1D**). Despite similar levels of IL-4 production in the pLN and MLN, the ratio of secreted IFN-γ compared to each of the Th2 cytokines was always greater in the MLN (**Figure 1E**).

Thus, increased antigen-specific IFN-γ and reduced Th2 responses were observed in the draining LNs when *S. mansoni* eggs were injected into the intestine compared to the footpad, indicating that tissue-specific signals can influence the balance of type 1 and type 2 immunity after experimental immunization.

Th1 and Th2 Responses Develop in Individual Intestinal Lymph Nodes After Live *S. mansoni* Infection or Experimental Egg Immunization

To assess if the site of *S. mansoni* egg deposition during live infection also affected type 1 and type 2 immune responses in the intestinal draining LNs, we infected C57BL/6 mice with 180 *S. mansoni* cercariae and analyzed the MLNs after 7 weeks, which coincides with the onset of egg production (15).

Although egg granuloma formation has been observed both in the small and large intestines (36, 37), it remains unclear which regions of the intestine are most affected and if different levels of immune responses are initiated. To monitor antigen-specific T cell immunity in the segment-specific draining LNs we identified the individual mesenteric LNs, according to previous reports (25, 30, 38–40). We termed the separate larger LN closest to the caecum 'cMLN1' (colon draining MLN 1), the adjacent string of four similar sized LNs draining the small intestine 'small intestine draining MLNs 1–4' (sMLN1–4), and the smaller lymph nodes draining the lower colon and the caudal LN 'cMLN2' and 'cLN', respectively (**Figure 2A**).

To assess location-dependent antigen-specific immune responses during live infection, LNs were individually collected and cultured for three days with media or SEA. As expected, MLN cellularity increased after infection, due to activation of the immune system by eggs passing through or becoming lodged in

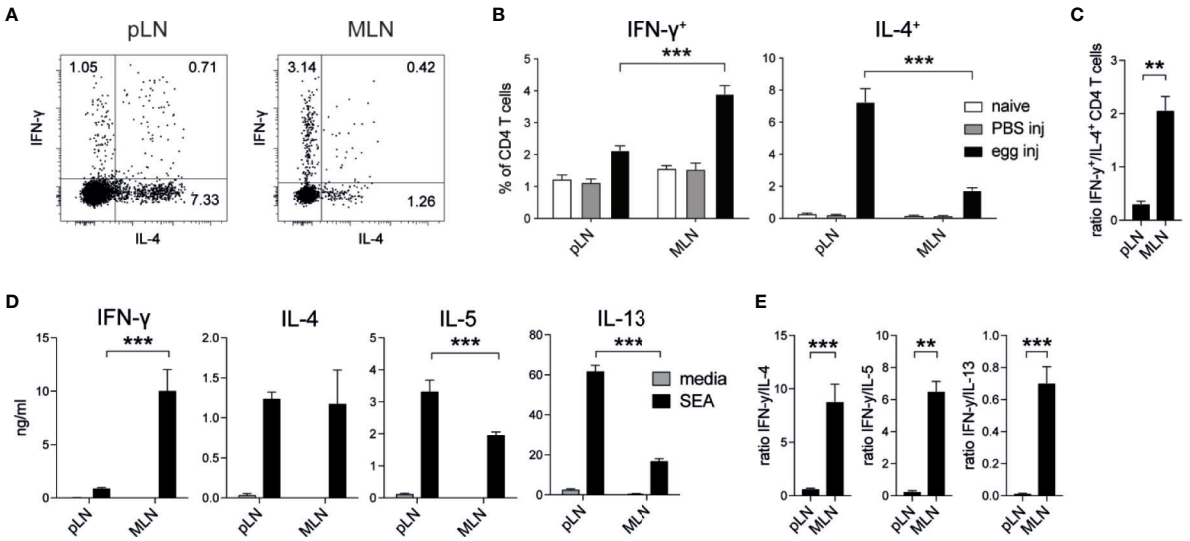

FIGURE 1 | *Schistosoma mansoni* eggs induce increased IFN-γ and decreased Th2 responses when injected into the intestine compared to the footpad. **(A, B)** Lymph node IFN-γ^+ and IL-4$^+$ CD4$^+$ T cell responses in naïve mice or 5 days after the injection of 2,500 *S. mansoni* eggs or PBS in the footpad or intestinal subserosa (n = 5 mice per group, combined data from three independent experiments; mean ± SEM; unpaired t-tests compare LN responses within each experimental group; ***p ≤ 0.001). **(C)** Ratio of IFN-γ^+ and IL-4$^+$ CD4$^+$ T cell responses after *S. mansoni* egg injection from data shown in **(B)** (mean ± SEM; unpaired t-test compares LN responses; **p ≤ 0.01). **(D)** From the same experiments, lymph node cells were restimulated with soluble egg antigen (SEA) or media for 3 days *in vitro* and Th1 and Th2 cytokines were measured by ELISA (mean ± SEM; unpaired t-tests compare LN cytokine responses within each treatment group; ***p ≤ 0.001). **(E)** Ratio of IFN-γ and Th2 cytokine levels after SEA restimulation from data shown in **(D)** (mean ± SEM; unpaired t-test; **p ≤ 0.01, ***p ≤ 0.001).

the intestinal tissue (**Figure 2B**), and the cytokine profile of cultured LN cells revealed strong immune activation. Significant levels of IFN-γ, IL-4 and IL-13 were observed in the absence of antigen and increased after SEA restimulation (**Figure 2C**). In contrast to previous studies (24, 41), levels of IFN-γ were low in our study, and IFN-γ was not detectable in pooled MLNs. However, IL-4 and IL-13 were detected at similar levels to those previously described (24, 41). Peak IL-4 responses were observed in sMLN4, cMLN1, and cMLN2, whereas peak IL-13 responses were observed in sMLN1, sMLN2, sMLN3, and cMLN2. IFN-γ could only be detected in the colon draining LNs cMLN1 and cMLN2, suggesting a bias towards Th1 responses in the large intestine.

It is difficult to determine whether the T cell responses observed in the individual LNs during live infection resulted from T cells primed within that same individual lymph node or had recirculated from other sites. To develop a more controlled model, we delivered *S. mansoni* eggs directly into the ileal subserosa and assessed T cell responses 5 days later. A single injection of 1,000 eggs into the ileum was sufficient to drive a robust antigen-specific T cell response in the draining LNs (**Figure 2D**), which were only detected in one individual LN (sMLN4), indicating that T cell priming occurs in individual LNs that drain the injection site.

In summary, both live *S. mansoni* infection and *S. mansoni* egg injection elicited robust antigen-specific immune responses in the MLNs but showed clear differences depending on which individual LNs were affected. All individual LNs draining the small and large intestines mounted similar levels of Th2 responses, while Th1 responses were restricted to the LNs draining the large intestine after live infection, whereas *de novo* primed T cells only responded in an individual LN after subserosal egg immunization.

H. polygyrus Antigens Induce Increased IFN-γ and Decreased Th2 Responses When Injected Into the Intestinal Subserosa Compared to the Footpad

To test whether tissue-specific differences in the induction of type 1 and type 2 immune responses would also affect other helminth antigens, we assessed immune responses against *Heligmosomoides polygyrus*, a gut dwelling nematode.

Soluble excretory/secretory antigens from *H. polygyrus* (HES) were injected into the footpad or the intestinal subserosa of C57BL/6 mice and IFN-γ^+ and IL-4$^+$ CD4$^+$ T cells or cytokine production after HES restimulation were assessed in the respective draining LNs after 5 days. Similar to our observations after *S. mansoni* egg injection, more IFN-γ^+ CD4$^+$ T cells were observed in the MLNs after HES injection into the ileal subserosa compared to injections into the footpad, whereas IL-4$^+$ CD4$^+$ T cells were more abundant in the pLNs after footpad injection (**Figure 3A**, **Supplementary Figure 2A**). This increased ratio of IFN-γ^+ CD4$^+$ T cells after subserosal injection (**Figure 3B**) was also observed after *in vitro* restimulation of LN cells with HES and showed increased levels of antigen-specific IFN-γ and decreased levels of IL-4 after subserosal injection, compared to injections into the footpad (**Figures 3C, D**). The observation that greater antigen-

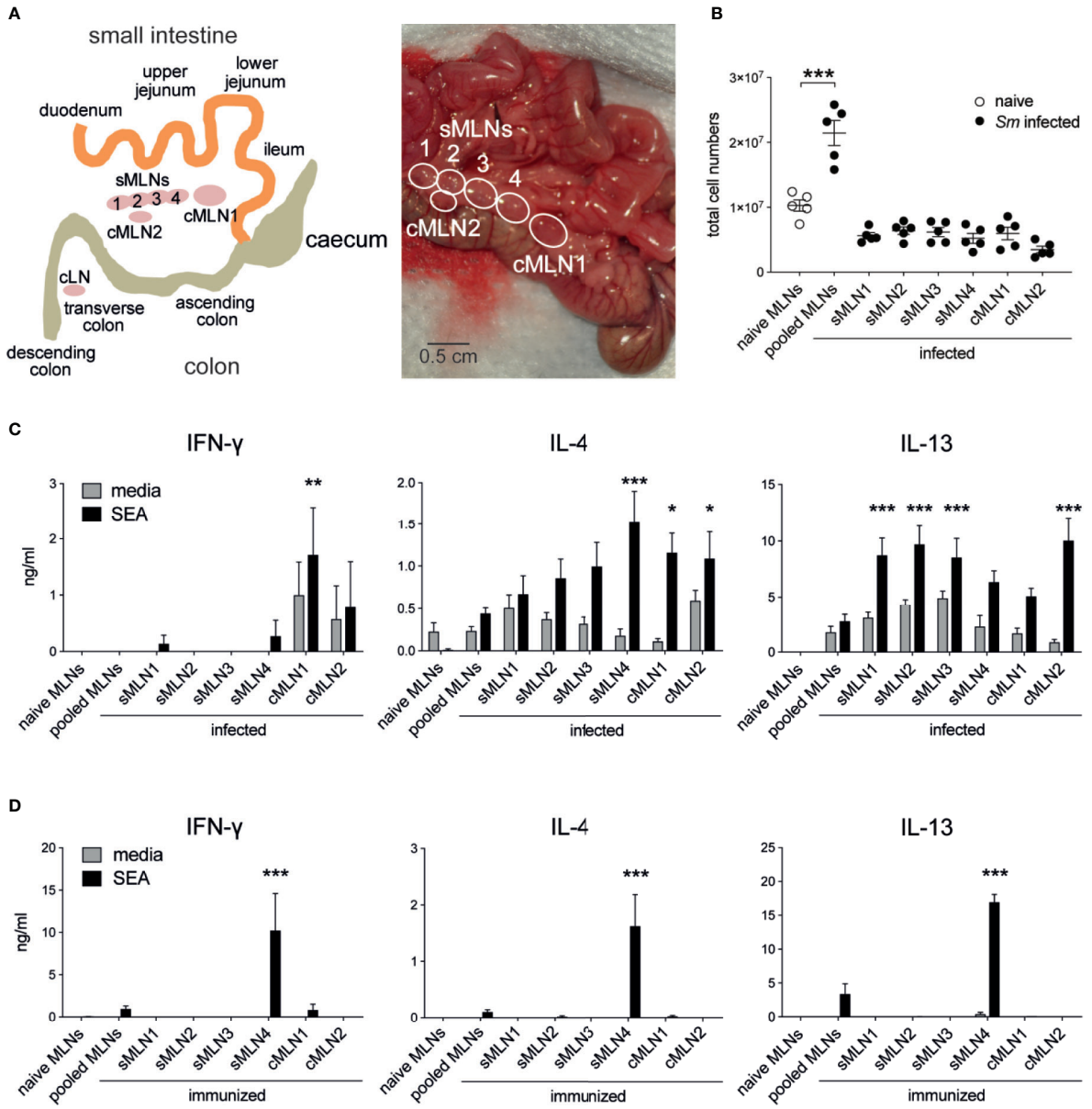

FIGURE 2 | Th1 and Th2 responses are detected in individual mesenteric lymph nodes after *Schistosoma mansoni* infection and egg immunization. **(A)** Schematic and photograph of the murine intestine illustrate the different segments and indicate the position and nomenclature of the individual draining lymph nodes. **(B)** Total cell counts of pooled or individual mesenteric lymph nodes (MLNs) seven weeks after *S. mansoni* (*Sm*) infection (n = 5 mice per group, representative of two independent experiments; mean ± SEM; unpaired t-tests compare cell numbers between naïve and infected groups; $^{**}p \leq 0.01$). **(C)** From the same experiments, pooled or individual lymph node cells were restimulated with SEA or media for 3 days *in vitro* and Th1 and Th2 cytokines were measured by ELISA (mean ± SEM; two-way ANOVA followed by Dunnett's multiple comparisons test compare samples to pooled infected MLNs within each restimulation group; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). **(D)** 1,000 *S. mansoni* eggs were injected into the ileal subserosa and pooled or individual lymph nodes were collected after 5 days. Cells were restimulated with SEA or media for 3 days *in vitro* and Th1 and Th2 cytokines were measured by ELISA (n = 3 mice per group, combined data from three independent experiments; mean ± SEM; two-way ANOVA followed by Dunnett's multiple comparisons test compare samples to pooled infected MLNs within each restimulation group; $^{***}p \leq 0.001$).

specific IFN-γ and reduced Th2 responses were observed in the MLNs against helminth products from both *S. mansoni* and *H. polygyrus* suggests that tissue-specific mechanisms can influence the balance of type 1 and type 2 immune responses against multiple helminth antigens.

To assess $CD4^+$ T cell responses in individual intestinal draining MLNs after live *H. polygyrus* infection, C57BL/6 mice were infected with 200 L3 *H. polygyrus* larvae by oral gavage and the individual MLNs were collected after 17 days. Similar to previous reports (42–44), the majority of adult worms were detected in the upper small

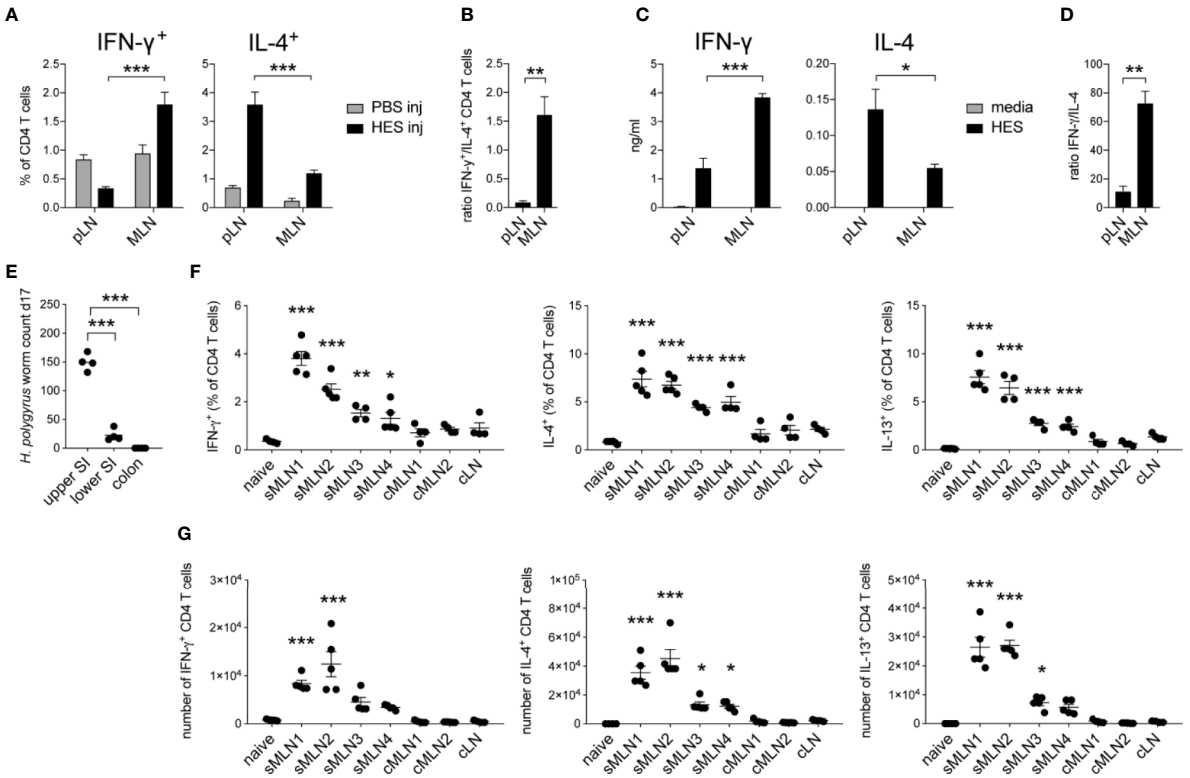

FIGURE 3 | *Heligmosomoides polygyrus* antigens induce stronger Th1 and reduced Th2 responses in the intestinal draining lymph nodes compared to the footpad. **(A)** 20 µg of *H. polygyrus* ES antigen (HES) was injected in the footpad or ileal subserosa. IFN-γ^+ and IL-4$^+$ CD4$^+$ T cell responses were analyzed 5 days after injection in the popliteal (pLN) or mesenteric (MLN) lymph nodes (n = 5 mice per group, combined data from two independent experiments; mean ± SEM; unpaired t-tests compare LN responses within each experimental group; ***p ≤ 0.001). **(B)** Ratio of IFN-γ^+ and IL-4$^+$ CD4$^+$ T cell responses after HES injection as shown in **(A)** (mean ± SEM; unpaired t-test compares LN responses; **p ≤ 0.01). **(C)** From the same experiments, LN cells were restimulated with HES or media for 3 days *in vitro* and Th1 and Th2 cytokines were measured by ELISA (mean ± SEM; unpaired t-tests compare LN responses within each experimental group; *p ≤ 0.05, ***p ≤ 0.001). **(D)** Ratio of IFN-γ and IL-4 cytokine levels after HES restimulation as shown in **(C)** (mean ± SEM; unpaired t-test compares LN responses; **p ≤ 0.01, ***p ≤ 0.001). **(E)** Worm counts from the upper half of the small intestine (upper SI), lower half of the small intestine (lower SI) and the colon 17 days after infection with 200 L3 *H. polygyrus* larvae (n = 4 mice, representative of two independent experiments; mean ± SEM; unpaired t-tests compare worm counts to upper SI numbers; ***p ≤ 0.001). **(F, G)** Mice were infected with 200 L3 *H. polygyrus* larvae by oral gavage and individual MLNs were collected after 17 days. Frequency and number of IFN-γ, IL-4, and IL-13 producing CD4$^+$ T cells in the individual MLNs are shown (n = 5 mice per group, representative of two independent experiments; mean ± SEM; ordinary one-way ANOVA followed by Holm–Sidak's multiple comparisons test compare LN responses to naïve controls; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

intestine (SI), with few worms present in the lower SI and no worms present in the colon (**Figure 3E**). Correspondingly, we observed a significant increase in total cell numbers, CD4$^+$ T cells and IFN-γ^+, IL-4$^+$ and IL-13$^+$ T cells in the individual MLNs draining the proximal intestine, with responses declining in the MLNs that drained the lower areas of the intestine (**Figures 3F, G**, **Supplementary Figures 2B, C**).

Individual Mesenteric Lymph Nodes Drain Specific Intestinal Segments After Subserosal Dye Injection

To identify what exact segments of the intestine were drained by these individual MLNs we set out to map the lymphatic drainage within the intestine *in vivo*. The luminal injection of dyes (30, 38, 39) or gavaging of olive oil (45) (**Supplementary Figure 1B**) has previously allowed researchers to map intestinal regions to individual LNs. Yet, due to the diffusion and uptake characteristics of the different dyes that were used in these studies, a consistent assessment of all regions of the intestine is still missing.

To identify a dye that would result in fast uptake and labeling of the lymphatics, while displaying limited diffusion characteristics, we trialled the injection of different dyes into the intestinal subserosa of anesthetized animals and monitored the lymphatic vessels and LNs for uptake of the dye. Similar to previous studies (38, 39), 2% Chicago Blue or 2% Evans Blue rapidly diffused along the intestine, the lymphatics and the MLNs, making it difficult to detect segment-specific draining LNs, while a suspension of graphite particles or black India ink resulted in a very localized deposition, but no uptake by the lymphatics. In contrast, the injection of black calligraphy ink resulted in localized coloration of the injected segment and lymphatic drainage to individual MLNs within seconds (**Figure 4**, **Supplementary Video 1**). In line with previous findings (30, 38–40), subserosal dye injection into the duodenum labeled

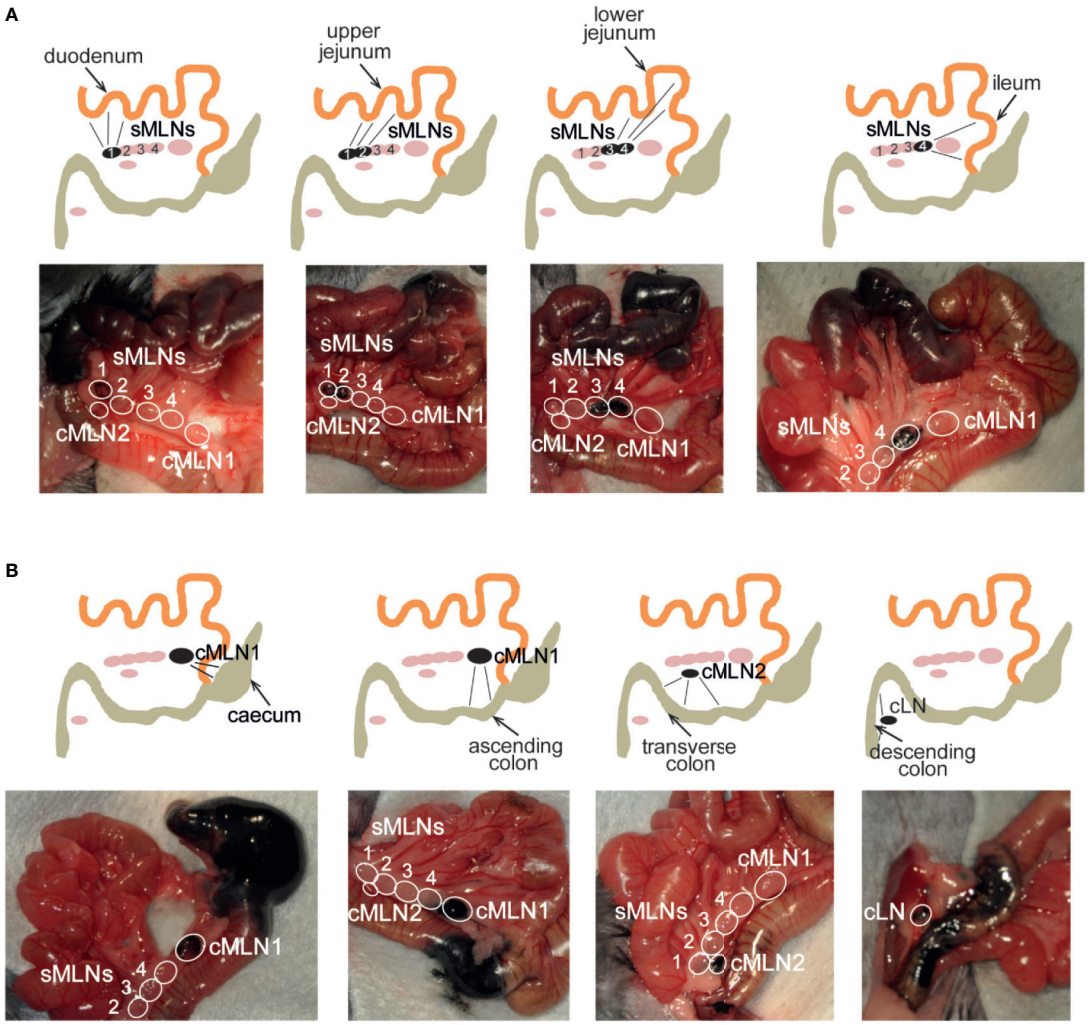

FIGURE 4 | Subserosal dye injection along the murine intestine identifies individual segment draining lymph nodes. Anesthetized animals were injected with 10 µl of black calligraphy ink into different segments of the small **(A)** or large intestine **(B)**. Photographs were taken 1 min after dye injection. Schematics indicate injection site, labeled lymphatics and individual draining lymph nodes as seen in each photograph.

sMLN1, while injections into the upper jejunum, lower jejunum or ileum labeled sMLN1 and sMLN2, sMLN3 and sMLN4 or sMLN4 respectively (**Figure 4A**). Subserosal dye injections into different segments of the large intestine demonstrated that injections into the caecum or ascending colon labeled cMLN1, while the transverse or descending colon was drained by cMLN2 or the cLN, respectively (**Figure 4B**). By subserosal injection of a dye that had fast lymphatic uptake and limited diffusion characteristics, we were thus able to map the lymphatic drainage of all segments of the intestine and define the individual segment-specific draining lymph nodes.

Individual Mesenteric Lymph Nodes Mount Distinct Th1 and Th2 Responses Against Subserosally Injected *S. mansoni* Eggs

As we had observed distinct LN-specific immune responses after live *S. mansoni* infection, we investigated whether *S. mansoni* eggs would induce similar LN-specific responses after controlled egg delivery into different sites of the intestine. To precisely control the dose, location, and timing of helminth antigen delivery, 1,000 *S. mansoni* eggs were injected subserosally into each segment of the small and large intestines. After 5 days individual MLNs were collected, LN cells were restimulated with SEA for 3 days *in vitro* and antigen-specific cytokine responses were measured by ELISA.

We detected IFN-γ, IL-4, IL-5 and IL-13 responses in the draining LNs of all immunized groups, which were localized to the same segment-specific LNs as identified by our ink injection experiments. We observed that the cytokine levels generated from the small intestine-draining MLNs ranged from 2 to 15 ng/ml for IFN-γ, 1 to 3 ng/ml for IL-4, 0.5 to 2 ng/ml for IL-5, and 5 to 35 ng/ml for IL-13 after egg injection into the different segments of the small intestine. Of note, injection of the duodenum resulted in a low IFN-γ response (2 ng/ml)

compared to the other small intestinal sites, whereas IL-4 and IL-5 responses were comparable, and IL-13 responses decreased along the small intestine (**Figure 5A**, **Supplementary Figure 3A**).

Injection of eggs into specific regions of the large intestine resulted in lower IL-4, IL-5, and IL-13 responses in the LNs compared to the small intestine, which ranged from 0.2 to 0.8 ng/ml for IL-4, 0.1 to 0.5 ng/ml for IL-5 and 10 to 20 ng/ml for IL-13, whereas IFN-γ responses were in a similar range (2–12 ng/ml). After caecal injection, cMLN1 exhibited low responses of both IFN-γ and IL-4, compared to the other large intestinal injections, whereas the cLN displayed high levels of IFN-γ accompanied by lower levels of IL-4 and IL-5 after egg injection into the descending colon (**Figure 5B**, **Supplementary Figure 3B**). Both, small and large intestinal immune responses were localized to the same segment-specific LNs that we had identified in our ink injection experiments (summarized in **Figure 5C**), clearly demonstrating that site-specific immune responses were induced, which varied between the responding LNs.

When we analyzed the ratio of IFN-γ to the different Th2 cytokines in the individual MLNs after segment-specific subserosal *S. mansoni* egg injection, we observed patterns that indicated gradual changes in the ratio of type 1 and type 2 immune profiles along the intestine (**Figure 5D**). A low ratio of IFN-γ to Th2 responses was observed in the LNs draining the proximal intestine, with the lowest ratio of IFN-γ to all Th2 cytokines observed in sMLN1 after duodenal injection. A pronounced increase in the ratio of IFN-γ to IL-4 and IL-5 was observed in cMLN2 and cLN after subserosal egg injections in the transverse and descending colon due to low levels of Th2 cytokines being detected in these LNs (**Figure 5B**, **Supplementary Figure 3B**), demonstrating a distinct and increasing proportion of IFN-γ responses in the distinct LNs draining the length of the intestine.

The ratio of IFN-γ to IL-4 and IL-5 responses in the individual draining LNs correlated well with each other, while IL-13 responses followed a different pattern, similar to our observations during live *S. mansoni* infection (**Figure 2C**). IL-13 levels gradually decreased throughout the LNs draining the small intestine from 30 ng/ml in sMLN1 to 15 ng/ml in sMLN4 and stayed within a range of 15–20 ng/ml in the large intestinal draining LNs. The resulting IFN-γ to IL-13 ratio thus increased gradually within the LNs draining the length of the small intestine, decreased in cMLN1 after caecal injection (due to low IFN-γ levels) and returned to a higher ratio for the remaining large intestinal LNs.

While our model does only approximate the individual LN immune responses, it suggests that different levels of antigen-specific type 1 and type 2 cytokines are produced in individual intestinal LNs after controlled immunization of different intestinal segments with *S. mansoni* eggs.

Different Subsets of Intestinal Dendritic Cells Transport and Present Soluble Helminth Antigens in the MLNs

Dendritic cells (DCs) play a crucial role in priming $CD4^+$ T cell responses against parasite antigens (25, 46–50). To confirm that DCs were also responsible for the uptake of subserosally injected helminth antigens and their delivery to the individual draining MLNs, we injected fluorescently labeled AF660-SEA into the ileum, collected the individual MLNs 24 h after injection, and assessed antigen-positive cells by flow cytometry. As expected, $MHCII^{hi}$ $CD11c^+$ migratory DCs were the only antigen-presenting cell population labeled with AF660-SEA in the MLNs (**Figure 6A**, **Supplementary Figure 4A**). Around 1% of DCs were SEA-positive in pooled MLNs, whereas up to 4% SEA^+ DCs were detected in sMLN4 but no other individual LN (**Figure 6B**). Thus, locally delivered antigens were transported to the individual MLN that we had previously identified, and a dilution of signal occurred when LNs were pooled.

To investigate which specific subsets of migratory DCs were involved in the uptake and active transport of helminth antigens from the intestine to the draining MLNs, we injected fluorescently labeled SEA or HES into the ileum of mesenteric lymphadenectomized (MNLx) mice, performed thoracic duct cannulations and collected migrating intestinal dendritic cells over the course of 18 h. We observed that SEA was mainly associated with CD11b-positive DCs, as previously reported (25) and that $CD11b^+CD103^+$ DCs were the most frequent SEA^+ migratory DC population (**Figure 6C**). To investigate if these DC subsets were also sufficient to prime antigen-specific immune responses, we transferred antigen-loaded DCs into naïve MLNs using microsurgical techniques we had previously developed (25, 34). We collected lymph DC subsets from naïve MLNx mice and loaded them with SEA *in vitro* for 18 h to ensure a controlled uptake of the antigen. After washing off excess antigen, 30,000 cells of each DC subset were transferred under the MLN capsule of naïve mice to assess if these subsets were sufficient to drive antigen-specific immune responses. After 5 days the injected MLNs were collected, cells were cultured with SEA for 3 days *in vitro*, and antigen-specific cytokine responses were assessed by ELISA. Similar to our previous findings (25) and in accordance with the observation that CD11b-positive DCs were responsible for transporting SEA *in vivo*, we found that both $CD11b^+CD103^-$ and $CD11b^+CD103^+$ DC2 subsets were able to prime IFN-γ and Th2 cytokine producing T cell responses, when antigen-loaded DCs were transferred under the MLN capsule (**Figure 6D**). CD103 single-positive DC1s were also able to induce antigen-specific immune responses in this system but did only induce IFN-γ responses with no IL-4 or IL-13 being detected.

In contrast, HES was mainly transported by $CD11b^+CD103^-$ DC2s and $CD103^+$ single-positive DC1s in lymph, while $CD11b^+CD103^+$ double-positive DCs were the least frequent HES^+ DCs (**Figure 6E**). Similar, to our observation of SEA-loaded DCs, HES-specific IFN-γ responses were induced by all three migratory DC subsets, when HES-loaded DC subsets were transferred under the MLN capsule (**Figure 6F**). In contrast to SEA-loaded DCs, all HES-loaded DC subsets induced similar levels of IL-4 and low levels of IL-13 responses, indicating that HES is transported and presented by all subsets of intestinal migratory DCs to drive

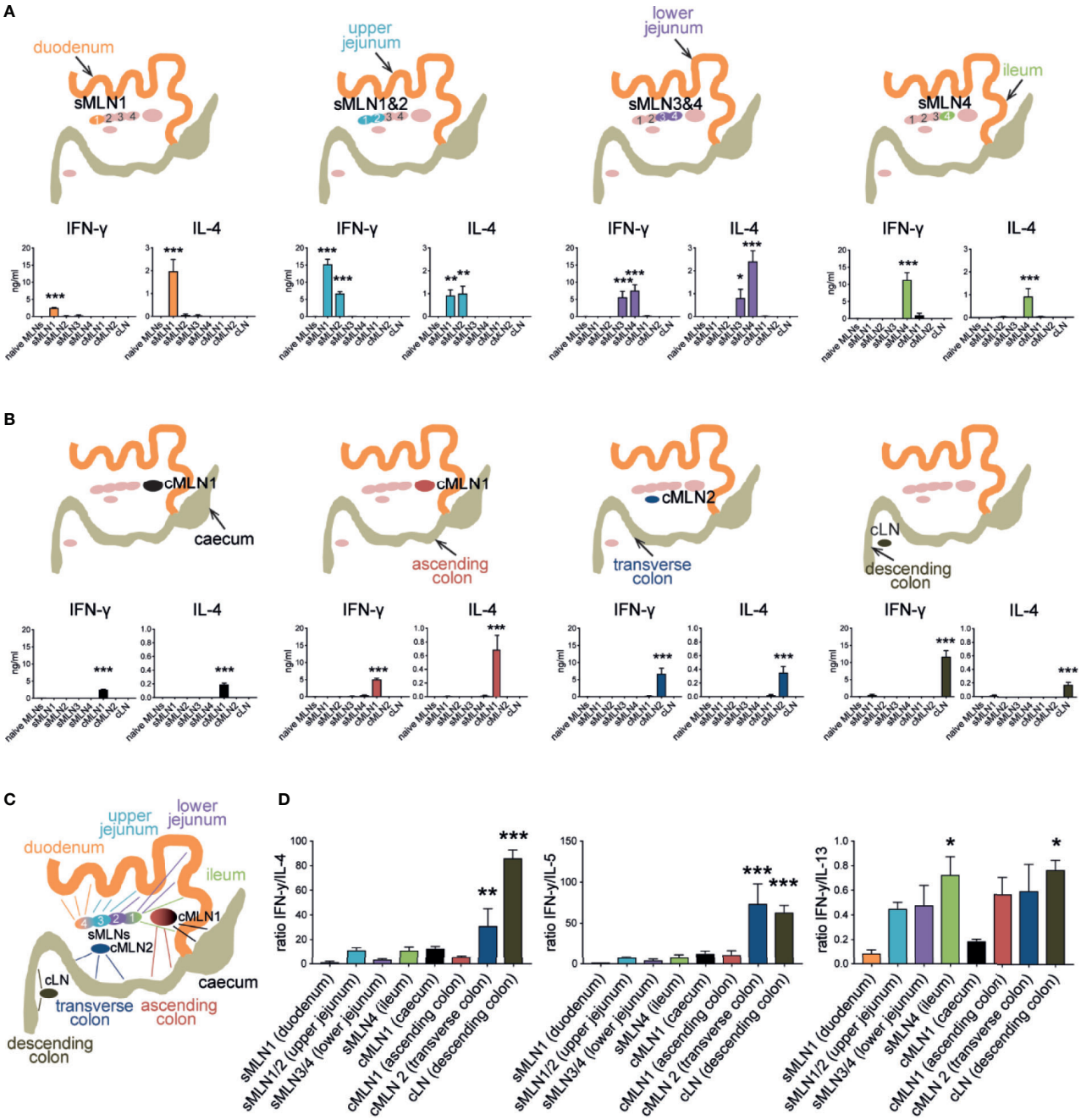

FIGURE 5 | Subserosal injection of *Schistosoma mansoni* eggs induces segment-specific Th1 and Th2 responses in the individual MLNs. **(A)** 1,000 *S. mansoni* eggs were injected into different segments of the small intestine. Individual MLNs were collected five days after injection; LN cells were restimulated with SEA *in vitro* and IFN-γ, and IL-4 were measured by ELISA. Schematics indicate injection site and responding LNs (n = 3 mice per group, combined data from three independent experiments; mean ± SEM; ordinary one-way ANOVA followed by Holm–Sidak's multiple comparisons test compare LN responses to naïve controls; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). **(B)** Similar to **(A)**, 1,000 *Schistosoma mansoni* eggs were injected into the different segments of the large intestine. After 5 days LNs were collected, restimulated *in vitro*, and assessed for antigen-specific cytokines by ELISA (n = 3 mice per group, combined data from three independent experiments; mean ± SEM; ordinary one-way ANOVA followed by Holm–Sidak's multiple comparisons test compare LN responses to naïve controls; ***p ≤ 0.001). **(C)** Schematic of the murine intestine indicating the draining pattern of the different intestinal segments to their individual MLNs. **(D)** Segment-specific ratio of IFN-γ and Th2 cytokine levels after SEA restimulation from experiments described in **(A, B)** and **Supplementary Figure 3** (mean ± SEM; ordinary one-way ANOVA followed by Holm–Sidak's multiple comparisons test compare samples to sMLN1 ratios; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

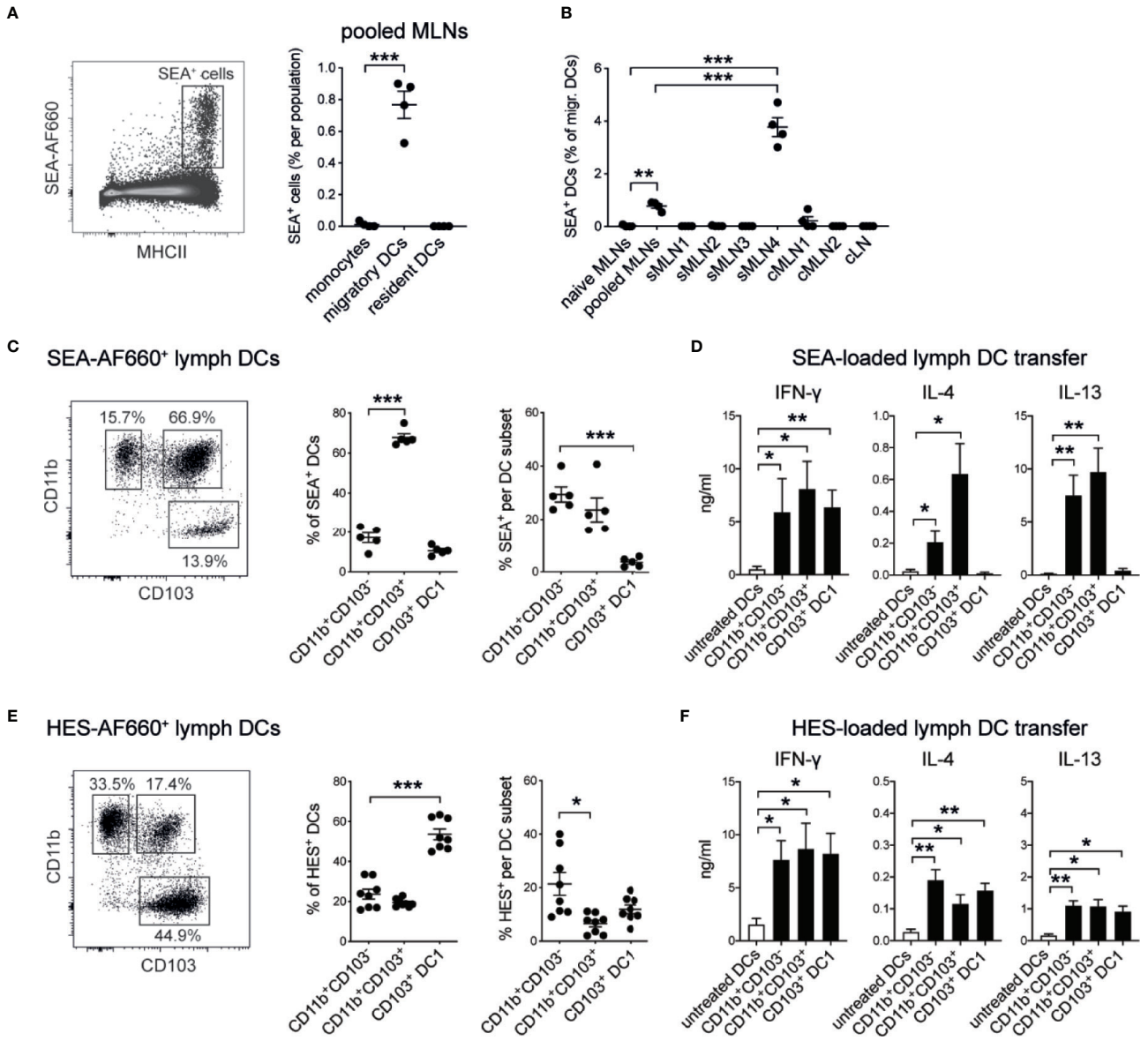

FIGURE 6 | Soluble helminth antigens are transported to the MLN by distinct subsets of intestinal dendritic cells that prime Th1 and Th2 responses. 15 µg of AF660-labeled antigens were injected into the intestinal serosa and antigen-positive cells were assessed 24 h after injection. **(A)** Representative dot plot of viable single MLN cells and frequency of SEA-AF660$^+$ cells in pooled MLNs 24 h after ileal injection (n = 4 mice per group, representative of two independent experiments; mean ± SEM; unpaired t-tests compare cell frequencies to monocytes; ***p ≤ 0.001). **(B)** Frequency of SEA-AF660$^+$ dendritic cells in pooled or individual MLNs 24 h after ileal injection (n = 4 mice per group, representative of two independent experiments; mean ± SEM; ordinary one-way ANOVA followed by Holm–Sidak's multiple comparisons test compares DC frequencies to naïve controls; unpaired t-test compares pooled MLN to sMLN4 responses; **p ≤ 0.01, ***p ≤ 0.001). **(C)** DC subset distribution (left and middle) and frequency (right) of SEA-AF660$^+$ lymph DCs collected over 18 h after ileal injection of SEA-AF660 (n = 2–3 mice per group, combined data from two independent experiments; mean ± SEM; unpaired t-tests compare cell distribution and frequencies to CD11b$^+$CD103$^-$ DCs; ***p ≤ 0.001). **(D)** Lymph DC subsets were collected from naïve mice over 18 h, sorted into subsets and incubated with or without 1 mg/ml SEA for 18 h. 30,000 cells were then transferred under the MLN capsule of naïve mice. After 5 days LNs were collected, restimulated with SEA *in vitro*, and assessed for antigen-specific cytokines by ELISA (n = 2–3 mice per group, combined data from two independent experiments; mean ± SEM; unpaired t-tests compare cytokine responses from transferred SEA-treated DC subsets to untreated DCs; *p ≤ 0.05, **p ≤ 0.01). **(E)** DC subset distribution (left and middle) and frequency (right) of HES-AF660$^+$ lymph DCs collected over 18 h after ileal injection of HES-AF660 (n = 2–3 mice per group, combined data from two independent experiments; mean ± SEM; unpaired t-tests compare cell distribution and frequencies to CD11b$^+$CD103$^-$ DCs; *p ≤ 0.05, ***p ≤ 0.001). **(F)** Lymph DC subsets were collected from naïve mice over 18 h, sorted into subsets and incubated with or without 1 mg/ml HES for 18 h. 30,000 cells were then transferred into the MLN subcapsule of naïve mice. After 5 days LNs were collected, restimulated with HES *in vitro*, and assessed for antigen-specific cytokines by ELISA (n = 2–3 mice per group, combined data from two independent experiments; mean ± SEM; unpaired t-tests compare cytokine responses from transferred HES-treated DC subsets to untreated DCs; *p ≤ 0.05, **p ≤ 0.01).

Th2 responses that show a different cytokine profile and ratio of immune responses than those observed with SEA (**Supplementary Figure 4B**).

Thus, location-specific variation of the immune response, as well as antigen-dependent uptake and presentation by different subsets of intestinal dendritic cells, likely shape the resulting immune responses to helminth antigens, resulting in a complex regulation of local type 1 and type 2 immunity.

DISCUSSION

In this study we report that the delivery of helminth antigens from *S. mansoni* and *H. polygyrus* into the intestine promotes higher Th1 and lower Th2 responses in the draining LNs compared to delivery into the footpad. In a more detailed analysis of intestinal immune responses initiated during live infection or by experimentally delivering *S. mansoni* eggs into different sites within the intestine itself, we furthermore show that distinct levels of type 1 and type 2 immune responses develop in the individual draining MLNs, with higher ratios of IFN-**γ** responses associated with infection/immunization of the large compared to the small intestine. Soluble helminth antigens are hereby transported by lymph migrating DCs, and different DC subsets transport and prime antigen-specific immune responses in an antigen-dependent manner in the draining MLNs.

Our observation that the controlled delivery of the same antigen into different sites of the intestine induces distinct Th1 and Th2 responses shares similarities with a previous study by Esterházy et al. (30), which demonstrates that the preferential development of Th17 or T regulatory cell responses is determined by location-specific signals within the intestine that include distinct stromal and dendritic cell signatures.

While LN stromal cells have been shown to influence T cells homing (51), support T regulatory cell induction (51), and limit or support inflammatory T cells during immunization or infection (52, 53), little is known if LN stromal cells also play a role in type 2 immunity against helminths. While helminth infection has been shown to promote stromal cell remodeling and *de novo* B cell follicle formation to promote total and helminth-specific antibody production (54), with IL-4 being critical to promote stromal cell expansion (55), it remains unknown if LN stromal cells can also directly affect Th2 development.

The involvement of DCs in the induction of type 2 immune responses against helminths is in turn better understood (47). $CD11c^+$ DCs are necessary for the induction of type 2 immune responses against *S. mansoni* and SEA *in vivo* (46) and IRF4-dependent migratory DC2s are required for effective type 2 immunity in the intestine (25, 48), lung (56), and skin (48, 57). Further DC subsets that express CD301b or are dependent on KLF4 are required to drive type 2 immune responses in the skin (49, 58, 59) but have not been described in other tissues (47). Our comparison of type 2 immune responses after intestinal or footpad immunization of SEA (**Figure 1**) or HES (**Figure 3**) demonstrates that $IL\text{-}4^+$ cells are more frequent and Th2-associated cytokine levels are higher after restimulation in the pLN compared to the MLN, indicating that the skin represents a Th2-promoting environment, which could be influenced by skin-specific subsets of DCs.

While the proportion of DC2 subsets within the intestine changes from a dominant $CD11b^+CD103^+$ phenotype in the small intestine to a $CD11b^+CD103^-$ phenotype in the colon (60, 61), both populations are sufficient to drive type 2 immune responses against *S. mansoni* eggs (25) and can drive SEA-specific immune responses when transferred *in vivo* (**Figure 6**). Our observation that SEA and HES display distinct uptake characteristics by migratory intestinal DC subsets and promote different levels of IL-4 and IL-13 immune responses when transferred *in vivo* (**Figure 6**) suggests that type 2 immune responses are mounted in a highly antigen-dependent manner, which could elicit distinct location dependent immune responses within the intestine.

We show that in contrast to SEA and most reported Th2-inducing antigens (47), which are presented by $IRF4^+$ DC2s, intestinal BATF3-dependent $CD103^+$ single-positive DCs (62) can also take up fluorescently labeled HES and are sufficient to induce HES-specific immune responses when transferred *in vivo* (**Figure 6**). While the direct involvement of CD103 single-positive DC1s in priming Th2 responses in this system requires further investigation, BATF3-dependent migratory DCs are known to suppress helminth-driven type 2 immunity in the intestine through the expression of IL-12 (63). As $CD103^+$ single-positive DCs are most prominent in the distal intestine (60, 61), it is possible that this subset could directly or indirectly contribute to the increased IFN-γ responses (**Figure 2C**) and IFN-γ to Th2 ratio (**Figure 5**) that we observed in the LNs draining the large intestine.

Apart from cellular differences that exist within the LNs, several studies have also shown that antigen-specific immune responses in the intestine and MLNs can be affected by the intestinal microbiota. A direct link between anti-helminth immunity and the microbiota was observed in antibiotic-treated *S. mansoni* infected mice that develop smaller granulomas and produce lower levels of IFN-γ in the intestine (41). Similar observations have been made in $Myd88^{-/-}$ mice, which cannot respond to TLR signals, and displayed reduced IFN-γ but intact Th2 responses after *S. mansoni* infection (64). As *S. mansoni* eggs are themselves weak inducers of TLR responses compared to bacterial compounds such as LPS (64–66), it is likely that these changes are the result of defective microbiota signaling. Bacterial compounds have also been shown to directly act on DCs and limit their potential to drive type 2 immune responses (67, 68) and could also act through innate immune cells that in turn alter their production of Th2-stimulting alarmins. As increasing microbial burden and diversity have been reported along the length of the intestine (60, 69), it is conceivable that they could also influence the ratio of Th1 and Th2 responses after *S. mansoni* egg immunization along the intestine. Experiments in germ-free or antibiotic-treated mice would clarify to what extend the

microbiota is involved in regulating site-specific anti-helminth immune responses.

Given the co-evolutionary development between helminths and their hosts, several factors likely influence local immune responses and directly or indirectly modulate anti-helminth Th2 immunity to create a favorable immunological environment for the parasite. Our study demonstrates that such site-specific differences exist between the skin and the intestine, and that within the intestine *S. mansoni* infection promotes distinct levels of type 1 and type 2 responses in individual MLNs, which we also observe when a controlled dose of *S. mansoni* eggs is experimentally delivered into distinct segments of the intestine. While we could not determine if these location-dependent differences are influenced by distinct immune cell populations or external stimuli, our observation suggests that Th1 and Th2 responses against helminth antigens are distinctly regulated in different regions of the intestine.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

Animal experiments carried out at the University of Glasgow were performed under licenses issued by the UK Home Office (Project License 60/4500) and were approved by the Ethical Review Panel of the University of Glasgow. Animal experiments carried out at the University of Manchester adhered to UK Home Office regulations (project license 70/7815) and were approved by the Ethical Review Panel of the University of Manchester. Animal experiments carried out at the Malaghan Institute of Medical Research adhered to the regulations of the Ministry of Primary Industries New Zealand (licenses 2014R22M, 24432) and were approved by the Animal Welfare Ethical Review Board of the Victoria University of Wellington.

AUTHOR CONTRIBUTIONS

JM and SLB performed the experiments. ASM provided reagents and, with SM, helped direct the study. JM conceived the project and together with SM and ASM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.592325/full#supplementary-material

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REVIEW
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Impact of Helminth Infections on Female Reproductive Health and Associated Diseases

Alisha Chetty[1*], *Millicent A. Omondi*[1], *Claire Butters*[1], *Katherine Ann Smith*[1,2], *Gnatoulma Katawa*[3], *Manuel Ritter*[4], *Laura Layland*[4*] and *William Horsnell*[1,5*]

[1] Institute of Infectious Disease and Molecular Medicine and Division of Immunology, University of Cape Town, Cape Town, South Africa, [2] School of Biosciences, Cardiff University, Cardiff, United Kingdom, [3] Ecole Supérieure des Techniques Biologiques et Alimentaires, Université de Lomé, Lomé, Togo, [4] Institute for Medical Microbiology, Immunology and Parasitology (IMMIP), University Hospital Bonn (UKB), Bonn, Germany, [5] Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom

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Edited by:
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***Correspondence:**
Alisha Chetty
alisha.chetty@uct.ac.za
Laura Layland
laura.layland@sbcomputing.de
William Horsnell
wghorsnell@gmail.com

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A growing body of knowledge exists on the influence of helminth infections on allergies and unrelated infections in the lung and gastrointestinal (GI) mucosa. However, the bystander effects of helminth infections on the female genital mucosa and reproductive health is understudied but important considering the high prevalence of helminth exposure and sexually transmitted infections in low- and middle-income countries (LMICs). In this review, we explore current knowledge about the direct and systemic effects of helminth infections on unrelated diseases. We summarize host disease-controlling immunity of important sexually transmitted infections and introduce the limited knowledge of how helminths infections directly cause pathology to female reproductive tract (FRT), alter susceptibility to sexually transmitted infections and reproduction. We also review work by others on type 2 immunity in the FRT and hypothesize how these insights may guide future work to help understand how helminths alter FRT health.

Keywords: Helminths, female reproductive tract, sexually transmitted infections, fertility, Systemic immunity

BURDEN OF DISEASE

Helminth infections are widespread and are characterized by sophisticated host immune modulation and evasion. Helminth infections are a global health concern, with more than 1.7 billion affected worldwide, particularly in tropical and subtropical regions (1). A feature of helminth infections are the parasites' ability to alter immunity and susceptibility to unrelated diseases (2–7). Of particular interest is the potential impact of helminth immune-regulation on susceptibility to sexually transmitted infections (STIs), given their high incidence in developing regions and detrimental impact on public health (8). For example, Ivan et al. (9) studied a cohort of 328 Rwandan pregnant women on anti-retroviral therapy, 38% of whom were stool positive for helminth infections (9). Mkhize-Kwitshana et al. (10) reported 66% of HIV+ study participants from an helminth endemic region of South Africa, were helminth egg positive and/or helminth-specific IgE seropositive (10). Likewise, Abossie and Petros (11) reported 68% of study participants in Ethiopia were co-infected with helminths and HIV, 35% were women (11). In this review we

address how the geographical overlap between helminth exposure and STIs can result in parasite-induced changes to female reproductive health (12–14).

HELMINTH IMMUNITY

Host immunity to helminths has been studied in depth using mouse models reflective of human infection and immunity (15–18). Typically, helminths induce a type 2-skewed immune response, associated with the production of the canonical cytokines interleukin (IL)-4, IL-5, and IL-13 (19–26). These cytokines amplify alternatively activated macrophages (AAMs; M2) (27–29), eosinophilia (30–32), smooth muscle contraction and goblet cell hyperplasia; cellular and physiological responses that underlie the 'weep and sweep' worm expulsion from the intestine (21, 23, 24, 26, 33, 34). Consistent with *in vivo* studies, epidemiological studies also report type 2-biased immune responses in humans infected with roundworm *Ascaris lumbricoides* (35–37), whipworm *Trichuris trichiura* (36–38), and hookworm *Necator americanus* (39). Furthermore, experimental infections of participants with hookworm has been shown to result in strong mucosal and systemic type 2 cytokine responses (40). Helminth infections also elicit regulatory immune responses, characterized by transforming growth factor-β (TGF-β), IL-10 and expansion of FoxP3-expressing regulatory T cells, involved in immune polarization and controlling inflammation (2, 41–48).

Antagonism between type 1 and type 2 immunity is central to our understanding of the T helper (Th) 1 cells (Th1)- T helper 2 cells (Th2) immune paradigm: Mosmann et al., first described Th1 and Th2 $CD4^+$ T cell differentiation and cytokine responses (49, 50), and Fernandez-Botran et al. (51) first demonstrated Th subtype regulation of each other (51). Furthermore, Reese et al. (52) demonstrated that IL-4 and STAT6 signaling can competitively inhibit interferon (IFN)-γ production (52). This paradigm has been expanded beyond T cell responses, as what is known as type 1 and type 2 immunity and regulation. For example, AAMs are a key feature of helminth infection induced by IL-4, -13 and -10. AAMs synthesize high levels of the enzyme arginase-1, which inhibits nitric oxide (NO) production (53). In addition, AAMs downregulate inflammatory Th1 immune responses mediated by TGF-β (54), which induce the development of regulatory T cells (41). Considering the opposing responses of type 1 and type 2 immunity, it is hypothesized that canonical type 2 immunity induced by helminths, can influence Th1- and Th17-mediated immune protection against STIs in the female reproductive tract (FRT).

HELMINTH-INDUCED IMMUNE MODULATION

Co-evolution of parasitic worms with the host is thought to have resulted in their ability to evade host's immunity through highly sophisticated responses. Helminths actively promote the expansion of regulatory T cell populations, promoting helminth persistence as well as host survival following infection (41, 44, 45, 55, 56). This can be achieved by the helminths release of excretory/secretory products, which effectively target and inhibit specific components of anti-parasite immune mechanisms or induce favorable immune regulation (43). For example, *Heligmosomoides polygyrus* excretory/secretory products (HES) contain a TGF-β mimic, the importance of this is supported by blockade of HES TGF-β mimic *in vivo* resulting in parasite expulsion in susceptible C57BL/6 mice (41). Bancroft et al. (57) recently identified the immunomodulatory molecule p43, a major secreted protein by murine whipworm *T. muris*, which binds to and inhibits IL-13 activity (57). Helminth-induced immune modulation benefits parasite survival by supporting asymptomatic or chronic infections. This has been demonstrated by individuals with asymptomatic lymphatic filariasis who display regulatory T and B cell responses (58), as well as skewed Th2 and regulatory T cell cytokine profiles i.e. favorable IL-4 and TGF-β, over IFN-γ and IL-17 production (46, 59–61). Alternatively, symptomatic patients had dominant pro-inflammatory responses, i.e. Th1, Th17 inflammatory responses and uncontrolled Th2 responses, resulting in immune-mediated damage of colonized tissue leading to severe symptoms like dermatitis in hyperreactive onchocerciasis or elephantiasis in lymphatic filariasis (62, 63).

Importantly, helminth-induced immune modulation has bystander effects on unrelated conditions such as allergies, autoimmune and inflammatory disorders, and unrelated infections. McSorley et al. (2) reported the suppression of type 2 allergic lung inflammation from treatment with HES (2), associated with TGF-β-like activity (41). Furthermore, Johnston et al. (42) demonstrated the suppression of skin allograft rejection by treatment with a TGF-β mimic isolated from HES (42). In support, Li et al. (64) demonstrated suppression of allograft rejection with *H. polygyrus*-induced Th2 and regulatory T cell bystander immunity (64). Recombinant hookworm anti-inflammatory proteins have been shown to reduce inflammation during experimental colitis (65) and asthma (66), associated with the induction of regulatory T cells. Layland et al. (67) demonstrated the suppression of allergic airway inflammation mediated by *S. mansoni*-induced regulatory T cells *in vivo* (67). Furthermore, Straubinger et al. (68) showed reduced susceptibility to ovalbumin (OVA)-induced allergic airway inflammation in mice born to mothers infected with *S. mansoni* during pregnancy. Osbourn et al. (69) described the ability of *H. polygyrus* Alarmin Release Inhibitor (HpARI) secreted protein to bind to and suppress IL-33 activity, reducing ILC2 and eosinophil responses, and promoting parasite survival (69). Interestingly, Zaiss et al. (70) demonstrated that infection with GI *H. polygyrus* resulted in changes to host intestinal microbiota and increased microbial-derived short chain fatty acids, which contributed to helminth-induced suppression of allergic lung inflammation (70). Conversely, Pinelli et al. (71) reported exacerbated ova-induced

allergic airway inflammation in mice infected with *Toxocara canis* (71). In humans, Jõgi et al. (5) reported increased risk of allergy manifestations in Norwegian children with anti-*T. canis* IgG4 seropositivity (5).

In addition to modulation of allergies and autoimmunity, Darby et al. (72) recently demonstrated how pre-conception maternal helminth exposure influences offspring immunity to helminth infection. Prior murine hookworm, *Nippostrongylus brasiliensis* infection imprinted Th2 immunity in female mice, which was transferred *via* breast milk and conferred protection against the parasite in their offspring. Protection was associated with maternally-derived Th2 primed $CD4^+$ T cells (72). Helminth-induced bystander immunity has also been implicated in altered vaccine responses (73–77) and immunity to unrelated infections. This highlights the potential significance of a transgenerational axis of influence on immunity by helminth infections.

Helminth-induced bystander immunity has also been implicated in altered vaccine responses and immunity to unrelated infections (73–77). For example, mouse infection with *T. spiralis* and *H. polygyrus* can impair immunity to murine norovirus (MNV) in the co-colonized intestine, mediated through impaired type 1 responses by type 2 activation of macrophages (78). Changes to lymphoid lineage function are demonstrated by Rolot et al. (7), who show helminth-mediated expansion of virtual memory $CD8^+$ T cells which enhance control of subsequent murine γ-HV respiratory infection (7). McFarlane et al. (79) showed that infection with murine nematode *H. polygyrus*, altered gut microbiota, which systemically increased proinflammatory type I IFN, and protected against subsequent respiratory viral infection (79). Additionally, *in vivo* infection with *T. spiralis* reduced pathological inflammation of the airways following influenza A virus infection (80). Helminth infection also impacts on control of bacterial infections. *N. brasiliensis* infections have been shown to impair natural and vaccine elicited T cell and B cell responses against *Salmonella typhimurium* infection *in vivo* (4). Protection against bacterial infections has also been reported; with reduced pulmonary mycobacterial burdens during concurrent nematode infection in mice, that required helminth-modified alveolar macrophage responses (3). Human studies have also identified helminth-associated changes to myeloid responses that relate to protection against MTb. For example, a negative association between hookworm infection and latent Mtb infection in Nepalese immigrants to the UK, was associated with elevated eosinophil numbers (6). Coincidence of filarial infection has also been associated with moderate protective immunity during latent Mtb infection (62, 81) and in a recent study, *S. stercoralis* infection in latent tuberculosis patients, was associated with down-regulated chemokine responses (82). Associations between soil-transmitted helminth (STH) infection and higher risk of concurrent bacterial and protozoal infections, and lower risk of concurrent viral infections in children and adults have also been reported (83). Recent studies have also demonstrated that prior nematode infection can confer resistance to subsequent infection by a different nematode species (84, 85). Together this existing body of work shows that helminths infections can have diverse influences on unrelated disease at sites distal to the anatomical location of the helminth in the host.

HELMINTHS, FEMALE REPRODUCTIVE TRACT, AND SUSCEPTIBILITY TO STIS

Immune imprinting on helminth infected hosts is therefore a feature of tissues not colonized by the parasite (86), including the FRT (87). The impact of helminth infection on immunity in the FRT and subsequent immune responses to sexually transmitted infections is not well studied, but it is apparent that significant effects on disease control in the FRT are likely.

Immune Control of STIs

The vaginal mucosa, the entry point for most STIs, is a unique and dynamic mucosal site under the cyclic influence of female sex hormones, and is made up of stratified squamous epithelial, lined by mucous, commensal bacteria and other anti-microbial defenses (88–91). In addition, the vaginal submucosa is surveyed by resident immune cells such as dendritic cells (DCs), which mount the response against invading pathogens (92–95). Host immune control of STIs is strongly correlated with the pattern of cytokine production in the host. Differential activation of Th1 cells, producing IL-2 and IFN-γ, mediate cellular immune responses, whereas Th2-like cells producing IL-4, IL-5, and IL-13, facilitate humoral immunity (96). Persistence of STIs can also be influenced by the production of IL-10 (97) and activation of regulatory T cells (98). While many STIs are initially asymptomatic, lack of treatment can result in an increased risk of acquiring another STI, infertility, organ damage, cancer, or death.

The most common sexually transmitted viral infections (STVIs) of the FRT are Herpes Simplex Virus type II (HSV-2), Human Papillomavirus (HPV) and Human Immunodeficiency Virus (HIV). Control of STVIs is typically associated with type 1 immune responses (99, 100). With the exception of HIV, killing of virally infected cells requires Th1 polarization of $CD4^+$ T cells (101), production of type 1 cytokines such as IFN-gamma (IFN-γ) (102, 103) and cytotoxic T cell responses (104–106) (**Figure 1**). Th1 immunity is also critical for early control of HIV, however, this response is insufficient to resolve infection (110), due to the virus' ability to rapidly mutate and evade $CD8^+$ T cell responses (107). Pre-existing inflammation and increased presence of $CD4^+$ target T cells in the FRT are major risk factors for increased susceptibility to HIV infection (111). Elimination of $CD4^+$ T cells by HIV is a hallmark of acquired immune deficiency syndrome (AIDS), resulting in increased susceptibility to opportunistic infections (112) and viral-associated cancers (113).

Similarly to STVIs, bacterial infections of the FRT require a Th1 and/or Th17 response to clear the infection (114, 115). *Chlamydia trachomatis* is a common bacterial STI worldwide, with women carrying the burden of this disease (116). IFN-γ

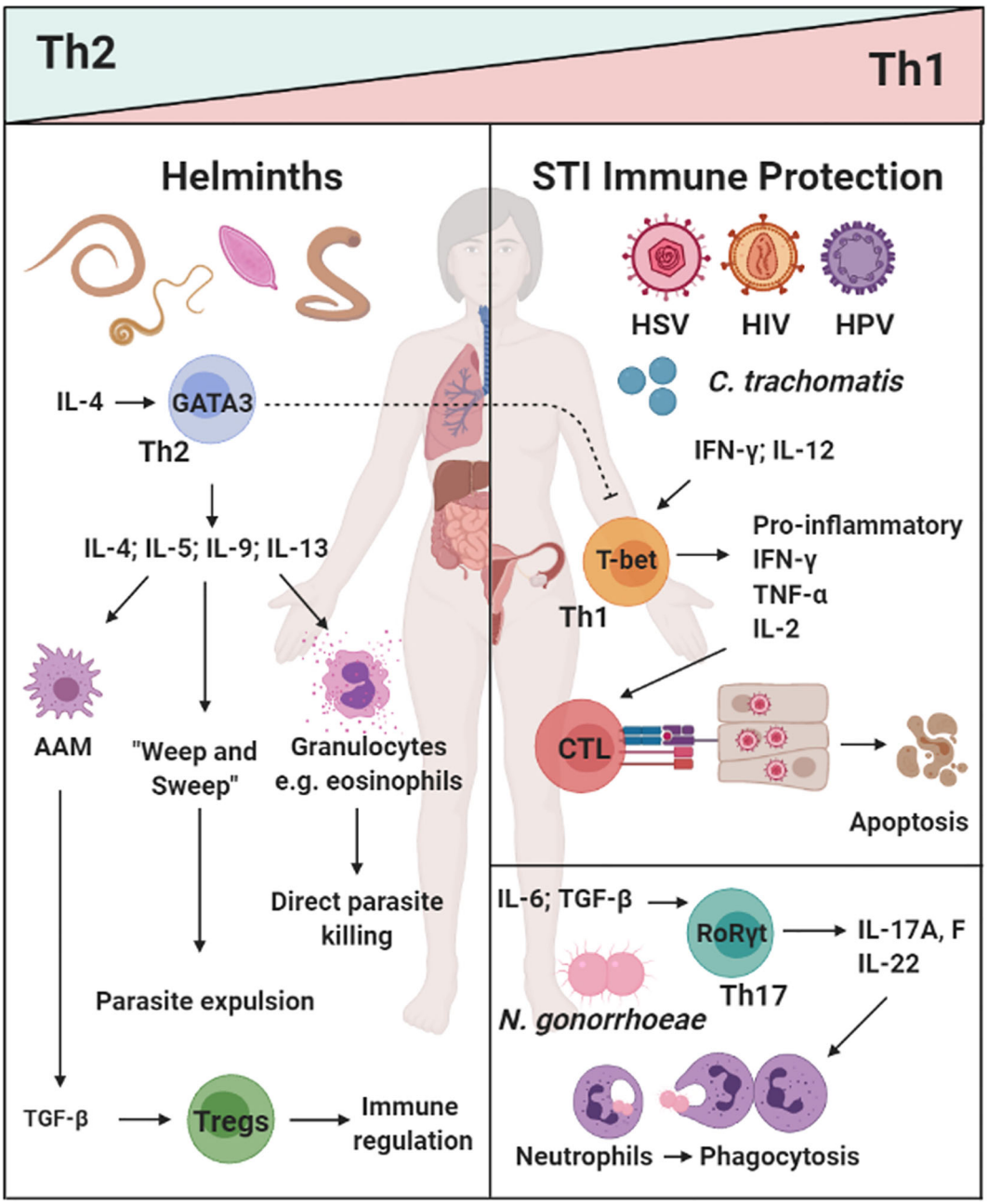

FIGURE 1 | The dichotomy of helminth-induced Th2/type 2 and regulatory immunity, and protective responses against sexually transmitted infections (STIs) in the female reproductive tract (FRT): Helminth infections (e.g. *A lumbricodes, T. trichiura, Schistome eggs*) commonly induce a potent Th2/type 2 immune response characterized by type 2 cytokines IL-4, IL-9, and IL-13, which induce a potent type 2 effector cells and functions (e.g. eosinophils, alternatively activated macrophages(AAMs), "weep and sweep" responses) (20, 21, 35, 36, 38–40). Prevalent viral [Herpes Simplex Virus type II (HSV-2), Human Immunodeficiency Virus (HIV), and Human Papillomavirus (HPV)] and bacterial (*C. tranchomatis and N. gonorrhoeae*) vaginal infection are a serious health concern for women in low- and middle-income countries (LMICs). Protective immunity against these pathogens can be classified a Th1/type 1 and Th17 responses i.e. cytotoxic killing of infected cells or phagocytosis of extracellular pathogens (101–107–109). How helminth exposure and immune modulation may influence susceptibility and control of STIs, is not fully understood. Created with BioRender.com.

production by Th1 $CD4^+$ T cells have been shown to be important for the resolution of *C. trachomatis* infections (117, 118). Cytotoxic T lymphocyte (CTL) responses are not required for clearance of this infection and instead have been shown to promote tissue pathology in the upper genital tract (108, 109). Another common bacterial STI is *Neisseria gonorroeae*, the causative agent of gonorrhea. In a murine model of infection, Th17 immune responses were shown to be favorable for

N. gonorrhoeae clearance (114). Considering the established counterbalance between Th2/Treg immunity and Th1/Th17 responses (50, 52, 119, 120), it is important to understand the consequence of helminth-induced immunity on susceptibility to co-endemic STIs (**Figure 1**).

Genital Schistosomiasis

Schistosoma haematobium infections have profound effects on female genital health. *S. haematobium* larvae (cercariae) emerge from aquatic snails and infect the human host through skin penetration. The larvae develop into schistosomula and migrate through the vasculature. Eventually, these mature into adult parasites, pair up and reside for years in the pelvic venous plexus. *S. haematobium* eggs produced here lodge in the urinary bladder wall and FRT, causing urogenital schistosomiasis (121). In chronically infected individuals, vaginal pathology here is acute with reported itching, pain, hematuria and ulceration in *S. haematobium*-infected individuals (122–125). Pathology is driven by eggs traversing host tissue and the formation of calcified granulomas in the female urinary and reproductive tract. The World Health Organization (WHO) International Agency for Research on Cancer (IARC) declared *S. haematobium* a group 1 carcinogen, as the correlation between urogenital schistosomiasis and the occurrence of bladder cancer has been extensively proven (126).

In the mouse model of urinary schistosomiasis, injection of eggs into the urinary bladder results in formation of a granuloma around the eggs made up of neutrophils, eosinophils and macrophages, as well as the onset of fibrosis in the surrounding bladder tissue (127). Furthermore, in this model *S. haematobium* eggs induced a strong type-2 response characterized by eosinophilia and elevated IL-4, IL-13 and IL-5 in the tissue surrounding the eggs. A compromised FRT epithelium is associated with increased HIV risk (128). The bystander tissue damage resulting from *S. haematobium* egg-induced inflammation (129, 130), increased immune activation (131) and lesions in the FRT is reasonably hypothesized to increase host risk of HIV infection, by providing routes for viral entry and increased number of target cells at the site of infection (132) (**Figure 2**). Furthermore, the type 2 response induced during *S. haematobium* infection (127) may dampen type 1 responses required for protection against viral pathogens such as HIV. These hypotheses are supported by clinical findings, where women infected with *S. haematobium* may have up to a 3-fold increased risk of acquiring HIV (133–135).

Following treatment with the anti-helminthic drug, praziquantel, the immune response in treated individuals shifts from a type 2 and regulatory T cell immune response (131, 136, 137) to a pro-inflammatory state, with elevated levels of egg antigen-specific TNF-α, IL-6, IFN-γ, IL-12p70, IL-8 and Th17 cytokines (IL-17, IL-21, and IL-23) post-treatment (138). If this inflammatory state results in reduced susceptibility to HIV infection is yet to be explored.

Filariasis

Filarial-driven immune modulation (i.e. induction of Th2, regulatory immune responses and suppression of inflammatory/Th1 responses) may increase susceptibility to viral and bacterial infections in the FRT, as Th1/inflammatory responses are important for the defense against these pathogens (139, 140). This is supported by identification of an association between infection with the filarial nematode *Wuchereria bancrofti* and increased risk of HIV infection (141). This increased HIV susceptibility may be associated with systemic increase in proportions of $CD4^+$ T cells expressing HLA-DR and HLA-DR/CD38, as well as effector memory $CD4^+$ T cells in lymphatic filariasis patients, i.e. an increase in HIV target cells in these patients (142). This supports *in vitro* findings demonstrating increased HIV infection of PBMC from lymphatic filariasis patients in comparison to uninfected individuals (12). Increased inflammation has also been reported in lymphatic filariasis patients (62, 143), with systemic IL-17 and IFN-γ elevated in response to PBMC stimulation with filarial antigen in these individuals. With chronic filarial infections, a type 2 immune signature, i.e. elevated IL-4 and IL-5, is detected in antigen-stimulated host PBMCs (143, 144). In contrast to schistosomiasis, regulatory T cells were reduced in lymphatic filariasis cases (62, 144) however type 1 responses (IFN-γ production) were suppressed in these patients (144). These studies suggest that chronic filarial infections could alter susceptibility to common FRT pathogens requiring type 1-mediated immune control. Surprisingly, genital manifestations of *W. bancrofti* infection have not been associated with any changes to fertility or pathology in the FRT (145).

Soil-Transmitted Helminths

Unlike schistosomiasis that causes direct pathology to the FRT, evidence has emerged of the potential systemic effect of helminths at sites that are not colonized by these pathogens. In a STH endemic region of Peru, Gravitt et al. (87) reported an increased prevalence of HPV among older women (30–45 years old) infected with STHs, which included *T. trichiura, A. lumbricoides, Ancylostoma duodenale* and *Strongyloides stercoralis*. Importantly, the life cycle of these helminths does not involve any larval transit through, or egg deposition in the FRT. The type 2 cytokine IL-4 was detected in cervicovaginal lavages of these women and IL-4 levels correlated positively with other cytokines involved in anti-helminth immunity; IL-25, IL-21, IL-5, IL-10, IL-8, and IL-31 (87). The authors hypothesized that the increased HPV prevalence among older women in STH-endemic regions, is mediated by helminth-induced immune regulation which may impair viral control, supported by a *in vivo* studies which demonstrate IL-4-mediated impairment of anti-viral immunity (52, 78, 146) (**Figure 3**). This study therefore suggests a systemic skewing of the immune response towards a type 2 phenotype detectable in the FRT impairing host ability to control HPV *via* type 1-mediated mechanisms. In contrast, murine hookworm *N. brasiliensis* antigen has been shown to inhibit HPV-16 pseudovirion uptake by human cervical cell lines. Furthermore, murine hookworm antigen exposure and *in vivo* infection decreased expression of cell surface vimentin or total vimentin expression in the cell line or the FRT, respectively (152). Cell surface vimentin has previously been described as a restriction factor that mediates internalization of HPV pseudovirion particles (153). This suggests that helminth

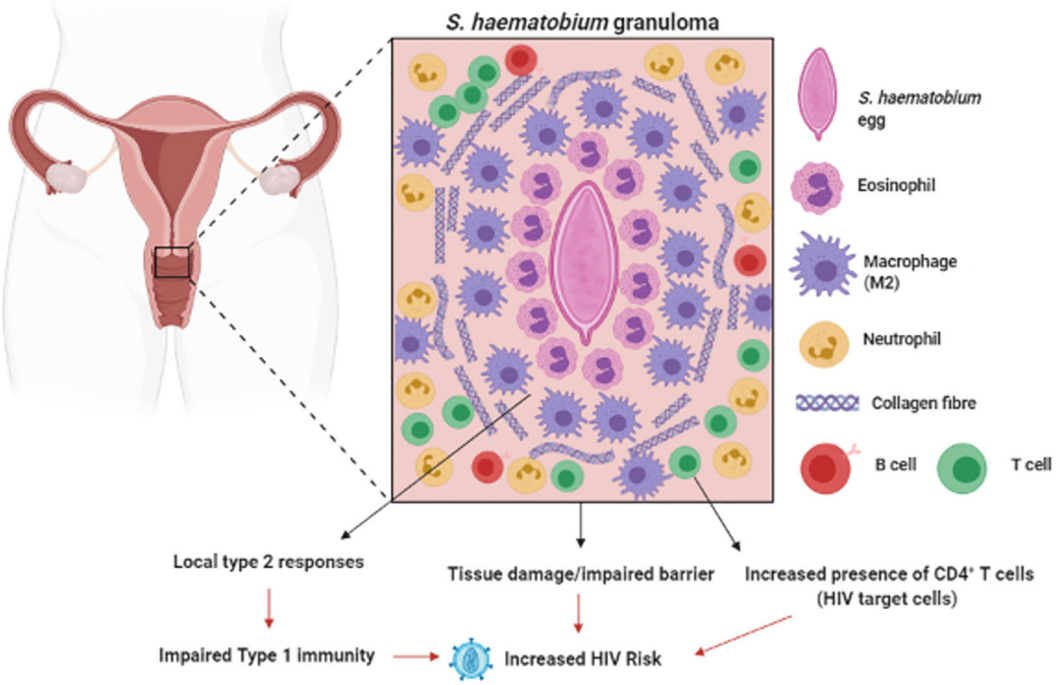

FIGURE 2 | Genital Schistosomiasis: In S. *haematobium infected* women, eggs can become lodged in the cervix, resulting in inflammation around the schistosome eggs (granula) and bystanders tissue damage. Genital schistosomiasis is common and can impair vaginal immunity and increase Human Immunodeficiency Virus (HIV) risk (129–133). Created with BioRender.com.

exposure may alter cervical epithelial susceptibility to HPV infection. Further, *N. brasiliensis* L3 somatic antigen decreased migration of cervical cancer cells in motility assays, suggesting a possible downmodulation of cancer cell metastasis by this helminth. Further studies are required to fully understand the complex consequences of helminth infection on HPV infection and pathogenesis.

HPV, Cancer, and Type 2 Immunity

Persistent HPV strains evade protective host immune responses, which are the first steps to the development of high-grade cervical lesions and cancer (154–156). Interestingly, type 1/type 2 antagonism can be manipulated by oncogenic HPV, to suppress anti-viral responses, promote persistence and tumor development (157). For example, Lepique et al. (147) described an association between M2-like macrophages and the suppression of anti-tumor responses and tumor progression during HPV-related cancer (147). Here, they identified tumor-associated macrophages (TAMs) as a dominate population in tumors, with high baseline Arginase I and IL-10 expression, and low iNOS activity, when stimulated with LPS/IFN-γ. Additionally, Petrillo et al. (148) reported a correlation between increased ratio of M2:M1 macrophages and poor responses to treatment and survival (148). Regulatory cytokines IL-10, TGF-β and prostaglandin E2 (PGE_2) produced by M2-like TAMs, promote the accumulation of regulatory T cells, which are associated with viral persistence and tumor development (158–160). Production of type 2 cytokines (e.g. IL-4, IL-13) by M2-like TAMs promotes Th2 polarization, reducing Th1 and CTL responses (149, 161–164). Moreover, Xie et al. (150) reported high levels of eosinophils in cervical cancer lesions and demonstrated that thymic stromal lymphopoietin (TSLP)-mediated eosinophil infiltration and activation promoted proliferation of cancer cells *in vitro* (150). Considering the significance of type1/type 2 imbalances during HPV persistence and related cancer progression, we hypothesize that helminth-induced type 2 immunity may impair anti-viral and anti-tumor immune responses, resulting in the promotion of tumor progression in the FRT (**Figure 3**).

Type 2 Immunity in the FRT

The role of type 2 immunity in modulating immune responses in the FRT has been demonstrated by Oh et al. (165), where induction of the type 2-associated 'alarmin' IL-33 in the genital mucosa, increased susceptibility to the HSV-2 pathology *in vivo* (165). The mediator of this effect was vaginal dysbiosis, which promoted IL-33 and impaired recruitment of memory T cells and reduced IFN-γ production in the FRT. These mice also demonstrated marked eosinophil accumulation and elevated IL-5 in the FRT (165). Furthermore, administration of recombinant IL-33 or protease-mediated induction of IL-33 in the vagina resulted in heightened susceptibility to HSV-2 (165). Oh et al. (166) elaborated on this model of IL-33-mediated type 2 immune

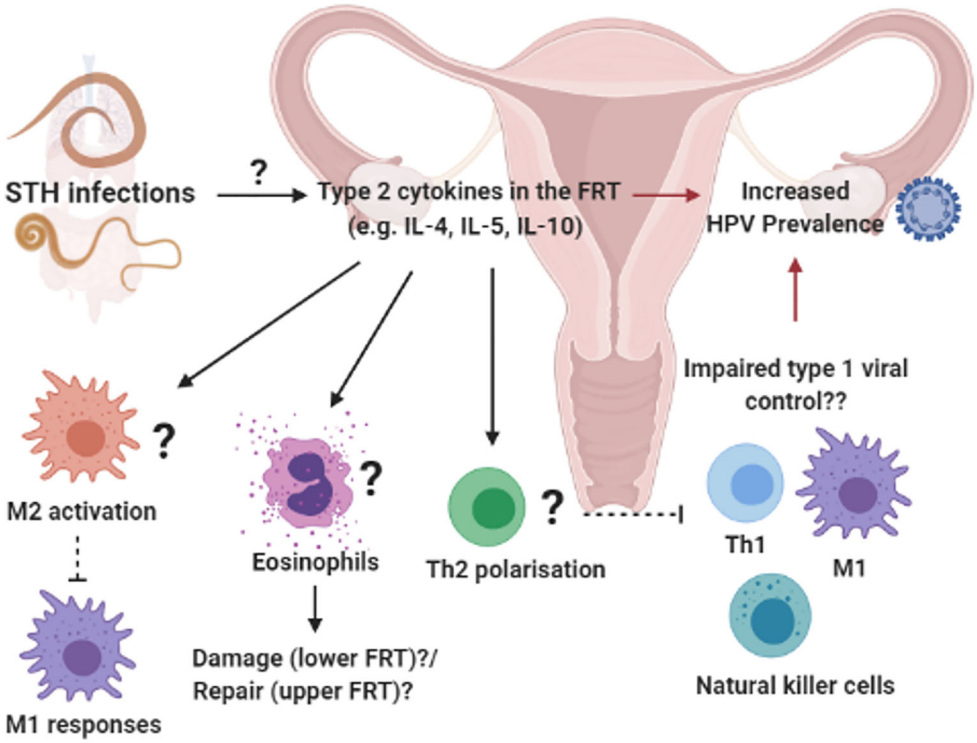

FIGURE 3 | Systemic influences of soil-transmitted helminths on uncolonized female reproductive tract (FRT): Prevalent soil-transmitted helminth (STH) infections, which transit the lung and GIT, can systemically alter host immunity in biological compartments not directly colonized by the parasite. For example, STH exposure was associated with increased HPV risk and a helminth-associated type 2 cytokine profile in vagina fluid of women in a STH endemic region (87). We hypothesize that the induction of type 2 immunity in the FRT e.g. type 2 cytokines activating M2 macrophages, eosinophils an Th2 differentiation of $CD4^+$ T cells, could impair protective type 1 immune responses and increased susceptibility to viral STIs (52, 147–151). Created with BioRender.com.

induction in the FRT, through administration of the serine protease papain. Here, papain-induced IL-33 in the vagina lead to the accumulation of vaginal eosinophils and production of canonical type 2 cytokines IL-4, IL-5, and IL-13 in genital lymph node T cells (166). Furthermore, elevated levels of type 2-associated IgE and IgG1 were detected in vaginal washes of papain-treated mice. Although elevated levels of IL-5 and eosinophils were detected in the FRT, papain induction of type 2 immunity in the FRT was not dependent on eosinophil recruitment, but rather on myeloid differentiation primary response gene 88 (MyD88) signaling and $PDL2^+CD301b^+$ dendritic cells under the control of interferon regulatory factor 4 (IRF4) (166).

Conversely, Vicetti Miguel et al. (151) demonstrated the protective role of type 2 immunity during *in vivo C. trachomatis* infection. *Chlamydia*-induced damage of the upper genital tract was prevented by IL-4 producing eosinophils, which promotes proliferation of endometrial stromal cells and tissue repair (151). Together, these studies demonstrate the significance of type 2 immunity in the FRT during STI infections and highlight potential differences in the role of type 2 responses at different sites in the FRT.

Helminths and Fecundity

The dichotomy of type 1: type 2 immune responses has been studied during the stages of pregnancy and labor, with a type 2 bias contributing to immune tolerance and a successful pregnancy (167). This would suggest that type 2-inducing helminth infections may systemically influence pregnancy in infected mothers in a positive manner. Interestingly, *in vivo* studies have demonstrated that helminth infection can result in pregnancy loss and failure of implantation of fertilized eggs (168), as well as reduced fecundity in parasitized hosts (169). Using a *Schistosoma mansoni* mouse model, Straubinger et al. (68) demonstrated that infected female mice gave birth to pups with lower birth weights during the Th2 phase of the immune response, as opposed to uninfected mice (68). In humans, Kurtis et al. (170) reported an association between maternal schistosomiasis and increased levels of inflammatory cytokines in mothers', placental and cord blood (170). As mother-to-child transmission of the schistosomes has not been reported in humans, the authors hypothesized the inflammatory response is likely due to helminth antigen movement across the placenta (170, 171). Furthermore, McDonald et al. (171) measured increased levels of pro-fibrotic proteins in the cord blood of

neonates born to *S. japonicum*-infected mothers (171). Clinical trials by Ndibazza et al. (172) and Olveda et al. (173) reported that treatment of pregnant women in endemic regions with anti-Schistosome drug praziquantel, did not significantly alter birth outcomes (172–174).

For maternal STH infections, Blackwell et al. (175) reported an association between hookworm infection and delayed age of first pregnancy and lower odds of successive pregnancies after the initial pregnancy. The converse was observed with *Ascaris* infection, which positively associated with conception at a younger age and shortened intervals of subsequent pregnancies after the first, among women younger than 32 years of age living in helminth endemic regions (175). The authors hypothesized that the opposing observations in fecundity between hookworm and *Ascaris* infections, is associated with the differing immune responses to the parasites; *A. lumbricoides* is associated with a polarized Th2 response (37) whereas hookworm infections may induce a mixed Th1/Th2 response (176). Together these studies suggest that helminth infections can have profound effects on female reproductive health, experimental investigation is required to better understanding of these effects.

CONCLUDING REMARKS

In this review, we have outlined the local and potential systemic effects of helminth infections on female reproductive health and susceptibility to STIs. Considering the great geographical overlap between STI and helminth prevalence, as well as the reduced access to health care and poor female health in helminth endemic regions, the study of helminth influences on the FRT should be a priority going forward, with focus on systemic effects of these parasites on uncolonized mucosal sites. Importantly, further comprehension on the systemic effects of GI helminths is needed, to direct health care strategies to mitigate the burden of helminth infections on the female reproductive health in those most at risk.

AUTHOR CONTRIBUTION

All authors contributed to the article and approved the submitted version.

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REVIEW
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The Two Faces of Nematode Infection: Virulence and Immunomodulatory Molecules From Nematode Parasites of Mammals, Insects and Plants

*Sarah D. Bobardt[1], Adler R. Dillman[2] and Meera G. Nair[1]**

[1] *Division of Biomedical Sciences, School of Medicine, University of California, Riverside, Riverside, CA, United States,*
[2] *Department of Nematology, University of California, Riverside, Riverside, CA, United States*

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***Correspondence:**
Meera G. Nair
meera.nair@medsch.ucr.edu

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Helminths stage a powerful infection that allows the parasite to damage host tissue through migration and feeding while simultaneously evading the host immune system. This feat is accomplished in part through the release of a diverse set of molecules that contribute to pathogenicity and immune suppression. Many of these molecules have been characterized in terms of their ability to influence the infectious capabilities of helminths across the tree of life. These include nematodes that infect insects, known as entomopathogenic nematodes (EPN) and plants with applications in agriculture and medicine. In this review we will first discuss the nematode virulence factors, which aid parasite colonization or tissue invasion, and cause many of the negative symptoms associated with infection. These include enzymes involved in detoxification, factors essential for parasite development and growth, and highly immunogenic ES proteins. We also explore how these parasites use several classes of molecules (proteins, carbohydrates, and nucleic acids) to evade the host's immune defenses. For example, helminths release immunomodulatory molecules in extracellular vesicles that may be protective in allergy and inflammatory disease. Collectively, these nematode-derived molecules allow parasites to persist for months or even years in a host, avoiding being killed or expelled by the immune system. Here, we evaluate these molecules, for their individual and combined potential as vaccine candidates, targets for anthelminthic drugs, and therapeutics for allergy and inflammatory disease. Last, we evaluate shared virulence and immunomodulatory mechanisms between mammalian and non-mammalian plant parasitic nematodes and EPNs, and discuss the utility of EPNs as a cost-effective model for studying nematode-derived molecules. Better knowledge of the virulence and immunomodulatory molecules from both entomopathogenic nematodes and soil-based helminths will allow for their use as beneficial agents in fighting disease and pests, divorced from their pathogenic consequences.

Keywords: entomopathogenic nematode, inflammatory disorders, vaccination, excretory and secretory products, soil-transmitted helminth

INTRODUCTION

Parasitic nematodes infect hosts from almost all branches of the tree of life, often using conserved strategies to successfully invade host tissue while evading the rapid immune response against them (Blaxter and Koutsovoulos, 2015). Their ability to manipulate host immunity is incredible. Consider a 21-year-old woman who presents with no pathology, inflammation, or any symptoms other than the sensation of something moving in her eye (Shah and Saldana, 2010). On examination, a live two cm nematode was found in the superior subconjunctival space of her eye and removed. Subsequent blood testing revealed microfilaremia (i.e., the presence of thousands of juvenile parasitic nematodes in her blood and likely other tissues). Her medical history revealed that she had picked up this infection on a trip to Nigeria 6 years earlier. This carefully coordinated infection is facilitated in part through immunomodulatory excretory/secretory (ES) products that allow the parasite to establish long-lived infection. Given their potent immunomodulatory properties, understanding these nematode-derived products may lead to the development of new anthelminthics and vaccines to overcome host immune suppression, or instead exploited for new therapies in allergy and inflammatory diseases.

Nematode-derived products comprise a diverse range of molecules, including proteins, lipids, carbohydrates, and nucleic acids (Maizels et al., 2018). They have a multitude of effects on the host, from toxic virulence factors that cause tissue damage to powerful immunomodulators. Some of these molecules are homologous to host molecules, allowing the parasite to manipulate immune cell function by mimicking host proteins or producing miRNAs that target host gene expression (Buck et al., 2014; Johnston et al., 2017). Others are unique to parasites and are not found in the host (Periago and Bethony, 2012; Flynn et al., 2019). These virulence factors are important for the parasite's infectivity and growth within the host, and can cause host tissue damage.

Entomopathogenic nematodes (EPN), which infect and kill their insect hosts within days, are currently used for controlling agricultural pests (Cooper and Eleftherianos, 2016). These parasites also provide a valuable model of parasitic nematode infections with significantly lower costs than rodent models. EPNs produce venom proteins with significant similarity to many proteins found in mammalian pathogenic nematodes (Lu et al., 2017; Chang et al., 2019). EPNs, like nematodes that infect mammals, need to suppress and/or evade the initial immune response (Bai et al., 2013; Brivio and Mastore, 2018). For this reason, identification of EPN-derived molecules and their effects on the host may be translational to nematode parasites of mammals.

Several recent reviews have provided significant insight into the immunomodulatory role of nematode-derived molecules, in particular excreted and secreted proteins (McSorley et al., 2013; Gazzinelli-Guimaraes and Nutman, 2018; Maizels et al., 2018; Zakeri et al., 2018; Maizels, 2020; Ryan et al., 2020). In this review, we highlight recently characterized nematode-derived molecules involved in host-parasite interactions, expand our discussion to non-protein molecules (e.g., lipids, nucleic acids) as potential for therapeutic targets, and investigate the utility of non-mammalian model systems (e.g., insect, plant) to understand host-nematode interactions. We first describe nematode virulence factors, how they mediate host tissue damage, and their utility as anthelminthic or vaccine targets. Next, we discuss nematode-derived products that suppress the host immune response and can be harnessed therapeutically for their immunomodulatory properties. Finally, we evaluate what can be learned from EPNs and plant-parasitic nematodes (PPNs) as models for mammalian-pathogenic nematodes to identify new parasite virulence and immunomodulatory molecules for immunotherapies and drug targets. Studying nematode-derived products' role in host-parasite interactions provides valuable insight for novel treatments to fight off infection or alleviate allergic and inflammatory diseases.

VIRULENCE FACTORS

Many parasitic nematodes transition to parasitism from a developmentally arrested lifecycle stage in order to obtain resources, complete their lifecycle, and find a long-lasting niche (Hotez et al., 2008). To this end, they can produce a wide variety of molecules to assist in their ability to attack, invade, and digest host tissue (Periago and Bethony, 2012). Their arsenal of host virulence molecules can be targeted to develop vaccines or design anthelminthics to reduce worm burden and mitigate host pathology in infected individuals. Significant research on anthelminthics and vaccines is performed in preclinical rodent models to find potential targets for vaccine development prior to the initiation of human research and clinical trials (**Table 1** and **Figure 1**). Here, we highlight promising nematode-derived molecules for anthelminthic and vaccine targets. These include enzymes involved in host tissue invasion and parasite feeding, nematode-derived molecules necessary for parasite development, and immunogenic ES proteins as vaccine candidates.

Nematode-Derived Enzymes

Nematodes produce a variety of enzymes for host tissue digestion and feeding that provide useful targets for vaccine development or anthelminthics. For example, enolases are plasminogen-binding surface proteins that are involved in assisting parasites in invading host tissue by promoting the degradation of fibrin (Ayón-Núñez et al., 2018; Jiang et al., 2019). Vaccination with a *Trichinella spiralis* enolase resulted in the production of specific antibodies in mice, however, this did not lead to a striking reduction in worm burden (Zhang et al., 2018). Additionally, parasite-derived enolases were found in the serum of *Brugia malayi*-infected individuals, indicating its potential as a diagnostic molecule (Reamtong et al., 2019).

For hookworms, which feed on the blood of their host causing anemia, enzymes involved in processing blood hemoglobin have demonstrated promising vaccine potential (Periago and Bethony, 2012; Hotez et al., 2013). Specifically, the *N. americanus* hemoglobinase aspartic protease (Na-APR-1) and the heme detoxifying Na-Glutathione S-transferase (Na-GST-1), are

TABLE 1 | Nematode-derived molecules and their potential for therapeutic use as vaccine, anthelminthic or anti-inflammatory treatments.

	Therapeutic potential	Name	Function
Preclinical	Anthelminthic/Vaccine	UDP-glucuronosyltransferase (UGT)	Detoxifies molecules Saeed et al., 2018
		Enolase	Binds to plasminogen and assists in invading host tissue Jiang et al., 2019
		Endocannabinoid (eCB)	Regulates parasite metabolism and development Batugedara et al., 2018; Ma et al., 2019b
		Dafachronic acid (DA)	
		Serpin	Inhibits host blood coagulation Yi et al., 2010
		α-Gal	Induces host production of protective antibodies Hodžić et al., 2020
Clinical		Hemoglobinase aspartic protease (APR-1)	Involved in the hemoglobin detoxification pathway Hotez et al., 2013
		Glutathione S-transferase (GST-1)	
		Ancylostoma secreted protein-2 (ASP-2)	Although effective in animal models, caused generalized urticaria in clinical trial Diemert et al., 2012
Preclinical	Anti-Inflammatory	Anti-inflammatory protein-2 (AIP)	Promotes Tregs to suppress airway inflammation Navarro et al., 2016
		Alarmin release inhibitor (ARI)	Binds to IL-33 and inhibits its release Osbourn et al., 2017
		Binds ARI (BARI)	Binds to receptor for IL-33 Vacca et al., 2020
		Glutamate dehydrogenase (GDH)	Induces anti-inflammatory eicosanoid switch de Los Reyes et al., 2020
		Cystatin	Reduces inflammatory cytokines and promotes Tregs Hartmann et al., 2002
		Macrophage inhibitory factor (MIF)	Suppresses the immune system through structures homologous to host cytokines Johnston et al., 2017; Cho et al., 2011
		TGF-β mimic (TGM)	
		p43	Binds and inhibits IL-13 Bancroft et al., 2019
		ES-62	Interacts with a broad range of immune cells to downregulate an inflammatory response Suckling et al., 2018
		DNase	Impairs worm killing by degrading neutrophil extracellular traps Bouchery et al., 2020
		Extracellular vesicles (EVs)	Disrupt immune cell function and contain miRNAs Eichenberger et al., 2018

currently in clinical trials for a hookworm vaccine, with early results showing the vaccine is safe and immunogenic (Zhan et al., 2010; Bottazzi, 2013, 2017; Diemert et al., 2017). Several phase 1 clinical trials have been completed with recombinant Na-APR-1 vaccine (Bottazzi, 2013). Administration of the recombinant Na-GST-1 vaccine to hookworm-naïve individuals as well as those from hookworm endemic regions in Brazil was found to be safe and immunogenic, and phase 2 clinical trials are underway (Bottazzi, 2017). The success of these trials suggest that safe and effective vaccines could be developed by targeting enzymes integral to nematode feeding and survival. For example, in filarial nematodes, enzymes have provided promising targets for anthelminthic and vaccine development. These include GST, which protects the nematode parasite by neutralizing host cytotoxic products (e.g., reactive oxygen species) and mediating drug resistance, and UDP-glucuronosyltransferase (Bm-UGT), a detoxifying enzyme expressed in the *B. malayi* intestinal lumen that was essential for its survival (Saeed et al., 2018; Flynn et al., 2019).

Nematode-Derived Molecules Involved in Growth and Metabolism

Lipid-derived molecules are involved in a variety of biological functions in parasitic nematodes including metabolism and development, making them of particular interest as potential novel anthelminthics that target parasite fitness and development (Ma et al., 2020). Endocannabinoids are lipid-derived molecules important for metabolic homeostasis and immunity, among other functions, and are produced by both mammals and nematodes (Batugedara et al., 2018). The specific function of parasitic nematode-derived endocannabinoids in the host is unclear. However, functional studies for endocannabinoids have been possible in the free-living nematode *C. elegans*, where endocannabinoids, 2-arachidonoyl glycerol and anandamide played a significant role in metabolism and aversion to pain (Oakes et al., 2017; Galles et al., 2018). Understanding the interplay between host and parasitic nematode-derived endocannabinoids might reveal new immune and metabolic targets to reduce parasite fitness or improve the host response.

The steroidal hormone dafachronic acid (DA) modulates nematode lipid metabolism and development, and the ligand-binding domain for the steroid hormone nuclear receptor for DA (DAF-12) is highly conserved among nematode species, such as *C. elegans, N. brasiliensis, Haemonchus contortus,* and *Strongyloides stercoralis*, therefore targeting these receptors may have therapeutic potential to impair parasite fitness (Ogawa et al., 2009; Patton et al., 2018; Ma et al., 2019a,b; Ayoade et al., 2020). In *C. elegans,* the DAF-12 system acts to inhibit dauer formation, and in *H. contortus*, DA promoted transition from free-living to parasitism by modulating dauer-like signaling (Ogawa et al., 2009;

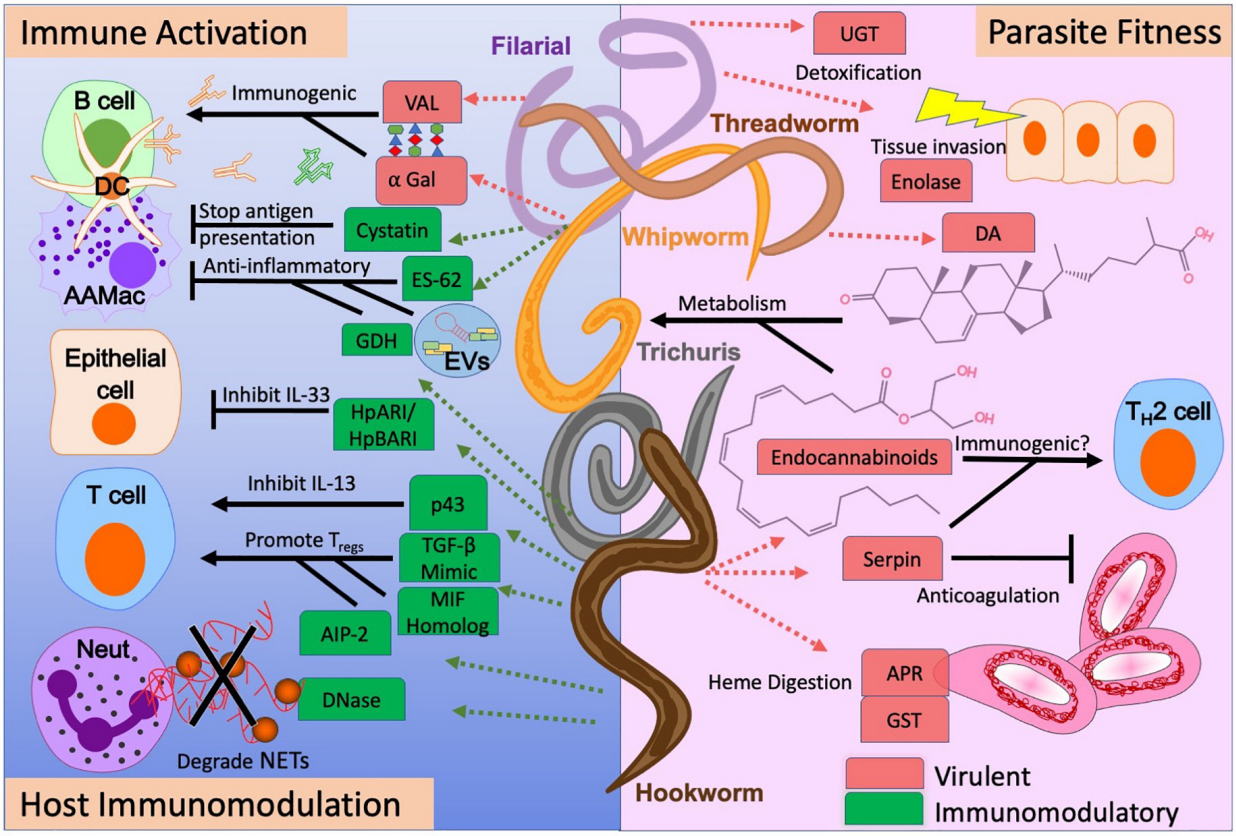

FIGURE 1 | The pleiotropic functions of nematode-derived molecules. Functions range from promoting or inhibiting the host immune response (left), to providing essential physiologic functions for the nematode parasite (right). Understanding the virulence (red) and immunomodulatory (green) potential for specific nematode-derived molecules allows us to determine their utility as vaccines, anthelminthics, or new therapeutics for allergic or inflammatory diseases.

Ma et al., 2019a,b). Recent investigation of the DAF-12 system in *S. stercoralis* hyperinfection supports the therapeutic potential of inducing this steroid hormone pathway. In a mouse model of *S. stercoralis* hyperinfection, which is an often fatal condition in immunocompromised individuals, DA treatment was protective and reduced *S. stercoralis* parasite burdens by suppressing the development of autoinfective L3a larvae (Patton et al., 2018).

Immunogenic Nematode ES Proteins

Molecules integral to the parasite's growth and ability to colonize and feed on the host offer promising vaccine and anthelminthic targets. However other weapons of warfare employed by the worms, such as excreted molecules that are immunogenic and promote a protective anti-helminthic immune response can also be considered as vaccines or adjuvants.

The family of venom allergen-like proteins (VAL) family is one such example. This family has been extensively studied especially given their high expression in many parasitic nematodes (Wilbers et al., 2018). While these proteins are conserved among a wide variety of nematodes, their functions are diverse—including examples of both pro-inflammatory and immunosuppressive molecules. Here we explore some of the most pertinent examples from this family, focusing on the VAL proteins that have demonstrated immunogenic properties for vaccine potential. VAL proteins are homologs of vespid (wasp) venom proteins, the latter of which are locally toxic and can induce allergic and inflammatory responses in humans (King and Valentine, 1987; Tawe et al., 2000; Murray et al., 2001). This makes nematode-derived homologs of these proteins of particular interest in understanding host-nematode interactions that lead to the excessive pathology for the host (King and Valentine, 1987; Wilbers et al., 2018; Tawe et al., 2000; Murray et al., 2001). VAL proteins are conserved in several parasitic nematodes, including *Heligmosomoides polygyrus*, *B. malayi*, *Trichinella pseudospiralis*, and *Teladorsagia circumcincta* (Ellis et al., 2014; Wang et al., 2017a; Asojo et al., 2018; Darwiche et al., 2018; Wilbers et al., 2018). Notably, VALs are highly expressed: a study of the secreted products from the gastrointestinal murine parasite *H. polygyrus* revealed that members of the VAL protein family were the most abundant product (Hewitson et al., 2011). Due to their abundance and conserved structure, VALs have been considered as vaccine candidates. For instance, the *B. malayi* protein Bm-VAL-1 is highly immunogenic, promoting antibody and T cell responses in humans, and conferring protection in vaccination models in mice and jirds (Maizels et al., 1995; Kalyanasundaram and Balumuri, 2011; Darwiche et al., 2018). To target larval

stages, Bm-VAL-1 and *B. malayi* abundant larval transcript-2 (Bm-ALT-2) were combined in a multivalent vaccine, which successfully increased antibody titers and provided enhanced worm killing in a challenge infection in mice (Kalyanasundaram and Balumuri, 2011; Anugraha et al., 2013). The importance of combining antigens as a vaccine strategy for a more effective immune response is increasingly being recognized. In *H. polygyrus* infection, immunization with a cocktail of three *H. polygyrus* VALs induced antibody production that protected mice from challenge *H. polygyrus* infection (Hewitson et al., 2015). The biological function of VALs in infection is unclear, however, given that they are secreted sterol-binding proteins, they may bind immunomodulatory molecules such as prostaglandins and leukotrienes, which have both immune stimulatory and regulatory roles (Honda and Kabashima, 2019).

The immunogenic properties of VALs have made them potential targets for vaccine development. Na-ASP-2 (Ancylostoma-secreted protein), another member of the VAL family, initially showed promise as a hookworm vaccine (Bethony et al., 2005; Diemert et al., 2012). This allergen-like protein was associated with the production of IgE and IgG4 antibody responses that correlate with reduced risk of high *Necator americanus* burdens in endemically affected areas. Further, validation studies in dogs confirmed that Na-ASP-2 specific antibodies were protective in challenge infections (Bethony et al., 2005). However, early clinical trials resulted in generalized urticarial reactions in many individuals, associated with pre-existing Na-ASP-2-specific IgE (Diemert et al., 2012). This failed clinical trial is a cautionary tale for vaccine development against parasitic nematodes. First, the potential for non-protective allergic-immune responses in previously exposed individuals in endemic areas needs to be considered. Additionally, anti-inflammatory nematode-derived molecules that are necessary to mitigate host tissue damage and inflammation may need to be carefully considered before being used as vaccine or therapeutic targets, since inhibiting these may be more pathogenic than beneficial for the host. Although the effectiveness of VALs as vaccine targets for helminths is challenged by these recent studies, their immunogenic potential may be harnessed for use as adjuvants against other infectious pathogens. For example, recombinant ASP-1 derived from the filarial nematode *Onchocerca volvulus,* Ov-ASP-1, has shown promise as an adjuvant for vaccines against viral infections, such as HIV, SARS-CoV, and influenza, augmenting viral antigen-specific antibody titers in immunization studies in mice (MacDonald et al., 2005).

Serine protease inhibitors (serpins) constitute another highly conserved family of nematode ES proteins, identified in many nematodes, including *B. malayi*, *Anisakis simplex*, and *H. contortus* (Zang et al., 2000; Bennuru et al., 2009; Yi et al., 2010; Valdivieso et al., 2015). *In vitro* studies of a serpin derived from *H. contortus* showed that it reduced blood coagulation (Yi et al., 2010). The anti-coagulation function of serpins is likely an important feeding mechanism for blood-feeding nematodes. *B. malayi* microfilariae secrete serpins, perhaps to mitigate a coagulation response to excess circulating microfilariae in the bloodstream during chronic infection (Zang et al., 2000). *B. malayi* serpins are also immunogenic and stimulate mouse and human B and T cell responses, however, this immune response is short-lived, suggesting that serpins alone are not effective vaccine candidates for long-term immunity (Zang et al., 2000). It is interesting to note that despite many preclinical studies on nematode-secreted proteins, only nematode enzymes are currently the subject of ongoing vaccine clinical trials (**Table 1**). This suggests that targeting virulence factors, which are integral components of the worm's physiology may offer the most promising vaccination targets.

IMMUNOMODULATORY MOLECULES

Parasitic nematodes have evolved multiple mechanisms to evade the host immune system, allowing persistence in their host, in some cases for decades, without being killed (Maizels et al., 2018). Nematode-derived products are key to the immunomodulatory capabilities of the parasites, and investigating their mechanism of action may identify novel therapies for allergic and inflammatory diseases. Reviewing current research into which molecules show the most promise for the development of immunotherapies is an ongoing conversation that has already received a significant amount of attention (Smallwood et al., 2017; Maizels et al., 2018; Bohnacker et al., 2020; Coakley and Harris, 2020; Ryan et al., 2020; White et al., 2020). Here we contribute to that conversation by contextualizing the most current advances in our understanding of immunomodulatory capabilities of nematodes and identifying the molecules that appear to show the most promise for further research.

Identifying the specific molecules within parasitic nematode ES or extract that have the strongest immunomodulatory potential is a main focus in the field of "helminth therapeutics" (Ryan et al., 2020). Nematode-derived immunomodulatory molecules include mimics of host immune mediators as well as novel molecules that are unique to parasites themselves. Many of the products with extensive characterization are proteins. However non-proteins products, such as carbohydrates and small RNAs, are currently being studied for their potential role in immunomodulation (Prasanphanich et al., 2013; Hokke and van Diepen, 2017; Maizels et al., 2018). Mechanisms of immunomodulation for the main nematode-derived molecules discussed here are summarized in **Figure 1**.

Glycans

The differential glycosylation of lipids and proteins during the lifecycle of parasitic nematodes provides a unique opportunity for the development of vaccines and novel anthelminthics (Prasanphanich et al., 2013; Hokke and van Diepen, 2017).

Glycosylation patterns unique to the parasite are potential vaccine targets, because they are distinct from host glycosylation patterns and potentially more immunogenic, acting as pathogen-associated molecular patterns. On the other hand, glycosylation patterns that mimic the host can be explored for their immunomodulatory potential, providing novel immunotherapies. Gala1–3GalNAc-R (α-Gal), a parasite-specific glycan epitope produced by the sheep pathogen

H. contortus induced an IgG response in lambs and is implicated in protection against *H. contortus* challenge infections (van Stijn et al., 2010). Similarly, glycosylation patterns are essential for the host to recognize the glycans on the surfaces of mucin-like proteins expressed by *T. canis*, and led to pro-inflammatory cytokine expression by human THP-1 macrophages (Długosz et al., 2019).

In the anaphylactic reaction known as α-Gal Syndrome (AGS), humans produce IgE in response to α-Gal present in red meat. However, humans infected with *T. canis* had reduced IgE antibodies to α-Gal caps on N-glycans, indicating that the parasites may be able to downregulate the allergic response, even though this is not an epitope that the worms themselves make (Hodžić et al., 2020). The ability of the parasite to suppress the immune response to oligosaccharides provides evidence for the "hygiene hypothesis" which argues that an increased sensitivity to a wide variety of allergens may result from a reduction in helminth infections in countries with stronger sanitation infrastructure (Yazdanbakhsh et al., 2002). In another example of immunomodulation, N-glycans produced by the canine heartworm *Dirofilaria immitis* allowed the worm to hide from the host immune system, by imitating host glycosylation patterns and also using unique glycosylation patterns that interfered with host binding to other nematode-derived molecules, a technique known as glycol-gimmickry (Martini et al., 2019). Further, changing the glycosylation patterns on proteins in *H. polygyrus* resulted in an increase in proinflammatory cytokines and a decrease in nematode-specific IgG1 in Balb/c mice (Doligalska et al., 2013). Together these studies show the importance of glycosylation patterns in both immunogenicity and evasion of the host immune system by parasitic nematodes.

Proteins

ES proteins have systemic effects on the immune system, which could be harnessed as therapies for allergy and inflammatory diseases. For example, *in vitro* treatment of macrophages with *T. spiralis* ES generated a regulatory phenotype that prevented airway allergic inflammation in mice (Kang et al., 2019). Similarly, in the dog hookworm *A. caninum,* the secreted anti-inflammatory protein-2 (AIP-2) suppressed airway inflammation in an asthma model in mice in a dendritic cell and Treg-dependent pathway (Navarro et al., 2016). The *H. polygyrus* ES proteins, *H. polygyrus* Alarmin Release Inhibitor (HpARI) and *H. polygyrus* Binds Alarmin Receptor and Inhibits (HpBARI), were identified due to their ability to downregulate the initiation of both type 2 allergic and parasitic responses through the IL-33-ST2 pathway. HpARI bound to the alarmin IL-33 in necrotic cells and prevented its release, while HpBARI binds IL-33 receptor ST2, preventing IL-33 engagement (Osbourn et al., 2017; Vacca et al., 2020). Intranasal administration of HpARI followed by infection with the skin-penetrating *Nippostrongylus brasiliensis* lead to greater intestinal worm burdens in comparison to untreated infected mice, indicating that a *H. polygryus*-specific product could impair immune responses to a different but related parasitic worm. HpBARI administration suppressed Th2 inflammatory responses to the extract from the allergenic fungus *Alternaria*. While *H. polygyrus* is a nematode parasite of mice, and HpBARI targets murine ST2, a homolog of HpBARI (HpBARI_Hom2), was identified that could effectively suppress the human ST2, supporting the clinical relevance of these findings. A similar strategy to inhibit Th2 cytokine responses is employed by *T. muris* with p43, the most abundant protein in its excretome/secretome (Bancroft et al., 2019). *T. muris* p43 contains structural domains homologous to thrombospondin and the IL-13 receptor, which allowed it to tether to matrix proteoglycans and bind and inhibit IL-13. Functionally, p43 inhibits its function in promoting worm expulsion. Another recently identified candidate for immune modulation is the enzyme *H. polygyrus*-derived *Hpb* glutamate dehydrogenase (GDH), which reduced allergic airway inflammation in mice by inducing a switch from pro-inflammatory to anti-inflammatory eicosanoids (e.g., prostaglandins). *Hpb*-GDH was effective at suppressing inflammatory pathways in both mouse and human macrophages and granulocytes by inhibiting the 5-lipooxygenase and instead promoting the cyclooxygenase pathway leading to the synthesis of prostanoids and the downregulation of 5-LOX metabolites (de Los Reyes et al., 2020). Identifying nematode-derived enzymes that target host immune-metabolic pathways with the resulting effect of suppressing inflammatory responses is an exciting new research avenue that may offer novel immunotherapeutics of allergic diseases.

Nematode-derived cysteine protease inhibitors (cystatins) also have demonstrated anti-inflammatory functions. Cystatin from the filarial nematode *Acanthocheilonema viteae* downregulated Th2 cytokine responses in an airway allergy model in Balb/c mice, including decreased IL-5 and IL-13 in the broncho-alveolar lavage (Daniłowicz-Luebert et al., 2013). In *in vitro* microglial cultures from cells harvested from rat brains and stimulated with LPS, *A. viteae* cystatin downregulated nitric oxide and TNFα expression as well as mRNA expression of the pro-inflammatory cytokines iNOS and COX-2, providing promise for therapies for neurodegenerative diseases, such as Parkinson's disease (Behrendt et al., 2016). Cystatins from filarial nematode *B. malayi* were also immunosuppressive: treatment with recombinant Bm cystatin was able to reduce dextran sulfate sodium (DSS)-induced colitis in mice (Bisht et al., 2019). Specifically, Bm cystatin led to increased Tregs in the colon and alternative activation of peritoneal macrophages. Recently, cystatin from the ES products of the zoonotic nematode *T. spiralis,* rTsCstN, was discovered as structurally homologous to human cystatin (Kobpornchai et al., 2020). Functionally, rTsCstN suppressed inflammatory cytokine production by LPS-treated mouse bone marrow derived macrophages. Cystatins are found in a wide variety of nematode species, including *O. volvulus, H. contortus, and B. malayi* (Hartmann et al., 2002; Hartmann and Lucius, 2003; Gregory and Maizels, 2008; Arumugam et al., 2014; Wang et al., 2017c) and their potential as immunomodulators is being explored.

Cytokines are commonly mimicked by parasitic nematodes in their efforts to modulate the host immune system. One such ortholog is Macrophage Inhibitory Factor (MIF), which is produced by many nematodes including *B. malayi*, *Anisakis*

simplex, *Wuchereria bancrofti,* and *H. contortus* (Bennuru et al., 2009; Chauhan et al., 2015; Wang et al., 2017b; Harischandra et al., 2018). Murine MIF is important for alternative activation of macrophages, promotes the Th2 response during nematode infection, and is required for optimal worm clearance (Filbey et al., 2019). On the other hand, nematode MIF homologs appear to have an immunosuppressive effect (Cho et al., 2011). MIF isolated from *A. simplex* increased Treg responses and reduced colitis severity. Here, mice treated with rAs-MIF regained previously lost weight and had lower disease activity indices in DSS-induced colitis. Another modulator of Tregs are TGF-β orthologs which are found in several parasitic nematodes, including *H. polygyrus*, *N. brasiliensis*, and *T. circumcincta* (McSorley et al., 2010). Hp-TGM was recently identified as a TGF-β mimic produced by *H. polygyrus* (Johnston et al., 2017). Interestingly, this mimic is structurally distinct from mammalian TGF-β, however, it is able to bind to mouse and human TGF-β receptors and induce Foxp3 expression in Treg cells. Furthermore, Hp-TGM was immunosuppressive in an allogenic skin graft model where it delayed graft rejection.

Across the proteomes of parasitic nematodes, there is consistency in the presence of amino acid motifs recognizable by T-cell receptors, known as T-cell-exposed motifs or TCEMs (Homan and Bremel, 2018). Using bioinformatics to analyze the proteomes of a wide variety of nematodes, many proteins have been identified with extremely high indices of predicted immunosuppression, indicating that the protein is likely to promote Treg responses. For instance, the hookworm, *A. ceylanicum* alone had over 500 peptides with a highly suppressive index (Homan and Bremel, 2018). Given its rapidity and cost-effectiveness, the ability to screen *in silico* for immunotherapeutic nematode-derived proteins may constitute an important frontier for research in nematode immunomodulation.

Some products have an effect on a wide range of immune cells. These include ES-62, a secreted protein from the nematode *A. viteae*, which interacts with B cells, dendritic cells, macrophages, and mast cells to downregulate inflammatory responses (Pineda et al., 2014). This protein's anti-inflammatory potential is reliant on post-translational modification including the attachment of phosphorylcholines. Current research on characterizing small molecule analogs for ES-62 is an example of the potential for nematode-derived products to be the impetus for the development of immunotherapies (Suckling et al., 2018).

A recent study highlighted nematode-secreted DNases as a novel mechanism for impairing neutrophil-mediated killing (Bouchery et al., 2020). Within hours post-infection with rat hookworm *N. brasiliensis*, host neutrophils swarmed invading nematodes and released neutrophil extracellular traps (NETs) comprised of nucleic acids, histones, and granular proteins. This provides evidence that NETs, originally identified in bacterial killing, are also used against helminths. However, at the same time, hookworms have developed an excretory/secretory deoxyribonuclease protein, known as Nb-DNase II, that can degrade the NETs, in both *in vitro* and *in vivo* models. This new finding provides an exciting avenue of targeting parasitic nematode DNAses as vaccine or therapeutic targets to promote NET-mediated nematode killing.

Extracellular Vesicles

Extracellular vesicles (EVs) released by parasitic nematodes during infection may provide a powerful strategy for the parasitic nematode to generate widespread effects on host cells (Coakley et al., 2015). Of therapeutic promise, treatment with EVs from *T. spiralis* and *N. brasiliensis* suppressed colitis of mice and protection was associated with reduced proinflammatory cytokines and increased Th2 and Treg responses (Eichenberger et al., 2018; Yang et al., 2020). In addition to containing lipids and proteins that may be immunomodulatory, EVs may also serve as cargos to deliver small RNAs to host cells, such as macrophages and intestinal cells, where they target and suppress host RNA. sRNAs have been identified in EVs generated by several parasitic nematodes, including *T. spiralis*, *N. brasiliensis*, *Trichuris muris*, and *H. polygyrus*, where they are predicted to target host immune gene networks (Tritten et al., 2017; Eichenberger et al., 2018; Chow et al., 2019; Yang et al., 2020). For example, *H. polygyrus* EVs were able to suppress macrophage responses and IL-33 signaling, and contained miRNAs that specifically targeted host DUSP1 RNA, a regulator of MAPK signaling (Buck et al., 2014; Coakley et al., 2017). miRNA generation has been reported in several nematode species, including *Ascaris suum*, which infects pigs, where miRNA sequence analysis predicted that they targeted the host Th2 immune response (IL-13, IL-25, IL-33) (Hansen et al., 2019). Circulating filarial nematode-derived miRNAs were detected in the blood of *Litomosomoides sigmodontis*-infected mice, *O. volvulus*-infected humans, *Loa loa*-infected baboons and *Onchocerca ochengi*-infected cattle (Buck et al., 2014; Tritten et al., 2014; Quintana et al., 2015), however, whether they were present in EVs, or targeted host gene expression, is unclear.

The products included in EVs can differ by lifecycle stage and sex of the nematode (Gu et al., 2017; Harischandra et al., 2018). Examining the molecules found in EVs for all lifecycle stages will allow for the discovery of a wide variety of potential drug targets. Molecules found in all developmental stages associated with the host could be strong candidates for the development of vaccines, allowing the immune system to recognize parasites throughout an infection, such as Galectin-2 (Hertz et al., 2020). On the other hand, molecules unique to adults may assist the worm in evading the host immune system in order to maintain a chronic infection, making them of particular interest for the development of anthelminthics and as immunotherapies. Interestingly, adult female *B. malayi* EVs had far more complex proteomes than males, with nearly four times as many proteins, including a MIF homolog, which may be involved in regulating the immune system (Harischandra et al., 2018). EVs may offer containment and protection from degradation of a multitude of immunogenic nematode antigens that could allow for more effective host immune responses to helminths. For example, intact EVs, but not lysed EVs, from *T. muris* were able to reduce egg burden in a subsequent infection of this worm, making them potential vaccine candidates for improved immunogenicity (Coakley et al., 2017; Shears et al., 2018). EVs present a unique

opportunity to study the parasitic lifecycle, allowing for a greater understanding of molecules that are required to initiate and maintain an infection.

NON-MAMMALIAN MODEL SYSTEMS: ENTOMOPATHOGENIC NEMATODES AND PLANT PARASITIC NEMATODES

Model systems have proven to be valuable to the study of human disease (Rubin et al., 2000). For example, initial analysis of the *Drosophila melanogaster* genome identified that over 60% of human disease-associated genes had orthologs in the fly. Studies of fly immunity led to the discovery and description of the Toll pathway and subsequently Toll-like receptors in mammals (Lemaitre, 2004). Similarly, determining the mechanistic function of conserved parasitic nematode effectors may benefit from the use of the EPN-insect model system, which is cheaper, faster, and allows for more individual hosts to be used per experiment than could possibly be done in a mammalian system. Effectors could be mechanistically described in the model system, providing an elevated starting point for experimentation in parasitic infections of mammals.

EPNs form a mutualistic relationship with bacteria, carrying them inside their intestine when they infect their hosts, releasing the bacteria into the hemolymph of their insect host. The bacteria assist in killing the host, and, along with liquified host tissue, serve as a food source (Bai et al., 2013; Brivio and Mastore, 2018). Similar to skin-penetrating nematode parasites of mammals, the initial infection process is entirely dependent on the ability of the nematode to enter the host and suppress its immune system. EPNs suppress the host immune system early in infection, causing it to tolerate not only nematode parasites but their symbiotic bacteria, until the host succumbs to infection (Toubarro et al., 2013). The specific molecules excreted/secreted from EPNs could be used for pest control in agricultural settings and also for immunoregulatory studies. Here we discuss the current research and known functions of EPN-derived virulence and immunomodulatory molecules, and how they relate to molecules employed by mammalian pathogenic nematodes. We also discuss main virulence factors that are present in plant parasitic nematodes, highlighting the striking conservation of these parasitic virulence mechanisms across the tree of life.

The ES products from the EPN *Heterorhabditis bacteriophora* are lethal to their insect hosts at high concentrations (Kenney et al., 2019). Treatment of *Drosophila* with proteins extracted from the supernatant of activated *H. bacteriophora* suppressed expression of antimicrobial protein diptericin, a product of the immune deficiency (Imd) pathway in insects (Kenney et al., 2019). This immunomodulatory mechanism is swift enough to allow for the infection of not just the nematode, but its mutualistic bacterial co-infector *Photorhabdus luminescens*, which would otherwise be killed by its insect host. In a similar manner, *S. carpocapsae* suppresses the immune response of its *Drosophila* host, allowing for the propagation of the endosymbiotic bacteria *Xenorhabdus nematophila* (Garriga et al., 2020). Shortly after infection, and before the bacteria is released from the gut of the infected nematode, there is a significant reduction in total insect hemocytes, suggesting that the nematode itself is capable of suppressing the host immune system, to the benefit of its endosymbiotic bacteria. The mechanism for immunomodulatory products from EPNs remains to be determined, however it appears to be time sensitive. After 3 h of exposure to live *S. carpocapsae,* insect hemocytes had reduced phagocytic activity, which was not apparent after only 1 h (Brivio et al., 2018). Identification of the specific EPN-derived molecules that target this innate immune Imd pathway in *Drosophila*, and whether they are conserved in mammalian parasitic nematodes could allow discovery of new anthelminthics and immunotherapies.

There are striking differences between the EPN lifecycle and that of mammalian pathogenic nematodes, most importantly the fate of the host, which is swiftly killed by EPNs in contrast to mammalian parasitic nematodes which establish chronic infections (Cooper and Eleftherianos, 2016). In order to evade the host immune system, contribute to host killing, and then feed on the dead body, the parasite must be able to successfully suppress the host immune response, release toxins, and then digest host tissue, making EPNs a strong model for identifying anti-inflammatory molecules as well as strong virulence factors. Recently, studies characterizing the specific proteins present in EPN ES revealed the remarkable resemblance to mammalian parasitic nematode-derived proteins with regards to structure and function. These include VAL proteins, enolases, serpins, and cystatins (**Figure 2**). For instance, *Steinernema glaseri* was shown to express enolases only at the activated infectious juveniles (IJs) stage, suggesting that the protein has a role in staging an infection, likely to digest the insect tissue (Liu et al., 2012). In infected insects, this secreted enolase was present in the insect hemolymph, and alone was sufficient to allow for quicker propagation of the bacteria, *Xenorhabdus poinarii*. Many venom proteins, with similarity to mammalian parasitic nematode VAL proteins, were identified in the ES of activated *Steinernema carpocapsae* and *S. feltiae* infective juveniles (IJs), and may contribute to the high toxicity of these parasites to their insect hosts (Lu et al., 2017; Chang et al., 2019). *S. carpocapsae* also expressed the serpin-like inhibitor Sc-SRP-6. Sc-SRP-6 impaired clot formation in its insect host by preventing the incorporation of melanin, known as melanization, which is an important defense mechanism in insects (Toubarro et al., 2013). Likewise, Sc-SRP-6 inhibited the hydrolysis of insect gut juices, a function that is thought to be conserved in *A. ceylanicum*. This serpin-like protein not only modulated the immune system, but inhibited digestion as the nematode passes through the gut of its host. Similar serpin-like genes were also found in mammalian-pathogenic nematodes, such as *B. malayi* (Zang et al., 1999). ES products from EPNs therefore have similar functions to those from mammalian-pathogenic nematodes, and may serve as powerful models for rapid discovery of useful targets for anthelminthics, given the comparative affordability and shorter lifecycles of EPNs.

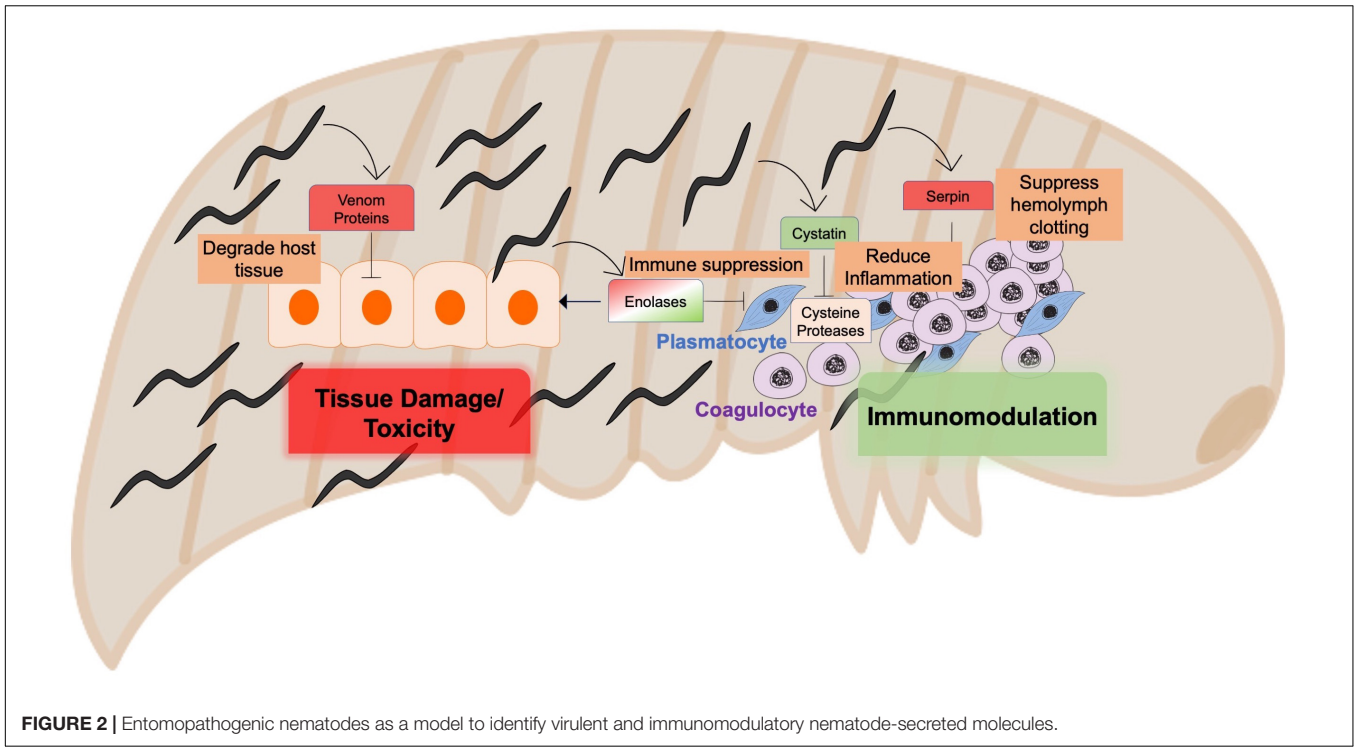

FIGURE 2 | Entomopathogenic nematodes as a model to identify virulent and immunomodulatory nematode-secreted molecules.

EPNs also produce cystatin, particularly when they detect insect hemolymph, as a location cue for their presence in the insect (Hao et al., 2008). Further research comparing the similarities between cystatins from EPNs and mammalian pathogenic nematodes would be valuable in validating the connections between these models.

Like nematode parasites of animals, plant-parasitic nematodes (PPNs) are masters of immune modulation, most of which is mediated by their secreted proteins and molecules. Because of their devastating effects in agriculture, PPNs are well-studied, and hundreds of secreted effectors have been identified, though, similar to other nematodes, few have been studied in mechanistic detail (Rehman et al., 2016; Vieira and Gleason, 2019). A detailed discussion of PPN effectors is beyond the scope of this review, however several recent reviews focus on PPN virulence factors and host-pathogen interaction (Goverse and Smant, 2014; Rehman et al., 2016; Vieira and Gleason, 2019). Here we discuss PPN virulence factors that are shared with EPN and mammalian parasitic nematodes, including VALs and cystatins. VAL genes have been identified in many PPNs, and their expression is associated with host invasion and migration through host tissues (Lozano-Torres et al., 2014; Wilbers et al., 2018). PPN VALs have been shown to be important for modulating host immunity, especially in the early stages of infection (Kang et al., 2012; Wang et al., 2007). Several VAL-family proteins have been characterized, and they appear to modulate similar processes in both plants and animals, suggesting that mechanistic studies in one model system will be valuable to our understanding of how these effectors work in general. Many cystatin genes have been predicted in plant-parasitic nematode genomes, but little is known about their role in parasitism. A recent description of a cystatin from the pine wood nematodes (PWN) *Bursaphelenchus xylophilus*, found that *Bx-cpi-1* is involved in the development and pathogenic process of the nematode (Xue and Wu, 2019).

The shared ancestry and parasitic behavior of these nematodes could be an explanation for their similar strategies of immune modulation. The conservation of molecular mechanisms of parasitism could allow for the identification of more proteins as well as small molecules that could be used to optimize an immune response during nematode infections, balancing an inflammatory response with worm burden. Further research will allow for ES products to be harnessed for the modulation of the immune system in mammals beyond the context of nematode infections, with applications in allergy and inflammatory diseases, without the risks of infection with live worms. The early stages of EPN infections are of particular interest, as the parasite focuses on suppressing the immune system without killing its host, prior to the release of mutualistic bacteria. This highly immunosuppressive stage may have applications with the mammal-infecting parasites that persist in their hosts for months or even years, such as hookworms and filarial nematodes (Hotez et al., 2008).

CONCLUSION

Here we discussed some of the main nematode-derived effectors in virulence and host immunomodulation. With improved high throughput technologies, further research is being conducted to identify more specific molecules that are involved in host-parasite

interaction, that could provide therapeutic insight for controlling helminth infections, and at the same time curing debilitating inflammatory diseases. We also discussed EPNs and PPNs as cheaper and faster models for understanding how nematode-derived molecules influence host-pathogen interactions. The combined strategies from screening for novel nematode-derived molecules, to determining their therapeutic abilities by mechanistic studies in vertebrate, invertebrate, and plant models, and finally testing them in clinical studies will allow for faster development of anthelminthic drugs, vaccines, and therapies for allergy and inflammatory disease.

AUTHOR CONTRIBUTIONS

MN and SB contributed writing as well as the organization and idea to the work. AD contributed significant writing. All authors have approved it for publication.

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An Evaluation of the *Fasciola hepatica* miRnome Predicts a Targeted Regulation of Mammalian Innate Immune Responses

Alison Ricafrente[1], *Hieu Nguyen*[1], *Nham Tran*[2] *and Sheila Donnelly*[1]*

[1] Faculty of Science, School of Life Sciences, The University of Technology Sydney, Ultimo, NSW, Australia, [2] Faculty of Engineering and Information Technology, School of Biomedical Engineering, The University of Technology Sydney, Ultimo, NSW, Australia

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***Correspondence:**
Sheila Donnelly
Sheila.Donnelly@uts.edu.au

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Understanding mechanisms by which parasitic worms (helminths) control their hosts' immune responses is critical to the development of effective new disease interventions. *Fasciola hepatica*, a global scourge of humans and their livestock, suppresses host innate immune responses within hours of infection, ensuring that host protective responses are quickly incapacitated. This allows the parasite to freely migrate from the intestine, through the liver to ultimately reside in the bile duct, where the parasite establishes a chronic infection that is largely tolerated by the host. The recent identification of micro(mi)RNA, small RNAs that regulate gene expression, within the extracellular vesicles secreted by helminths suggest that these non-coding RNAs may have a role in the parasite-host interplay. To date, 77 miRNAs have been identified in *F. hepatica* comprising primarily of ancient conserved species of miRNAs. We hypothesized that many of these miRNAs are utilized by the parasite to regulate host immune signaling pathways. To test this theory, we first compiled all of the known published *F. hepatica* miRNAs and critically curated their sequences and annotations. Then with a focus on the miRNAs expressed by the juvenile worms, we predicted gene targets within human innate immune cells. This approach revealed the existence of targets within every immune cell, providing evidence for the universal management of host immunology by this parasite. Notably, there was a high degree of redundancy in the potential for the parasite to regulate the activation of dendritic cells, eosinophils and neutrophils, with multiple miRNAs predicted to act on singular gene targets within these cells. This original exploration of the *Fasciola* miRnome offers the first molecular insight into mechanisms by which *F. hepatica* can regulate the host protective immune response.

Keywords: fasciola, helminth, miRNA, immune modulation, dendritic cell, eosinophil, neutrophil

INTRODUCTION

Fasciolosis is a major production limiting disease of ruminant livestock globally (1). Infection with *F. hepatica*, the liver fluke parasite, results in substantial delays in animals reaching slaughter weight with increased levels of worm burden in the liver directly correlating with reduced growth rates of animals (2). The impact of infection on the production of meat, wool, and milk is estimated to result in economic losses over US$3.2 billion annually (3). Due to the close proximity of people with their livestock, humans are incidental hosts and fasciolosis is now recognized as an emerging human disease. The World Health Organization has estimated that at least 2.4 million people are infected in more than 70 countries worldwide, with several millions at risk, and have thus classified liver fluke infection as one of the food-borne trematode priority diseases (4). Despite this status, the only option to treat the infection is Triclabendazole and although effective at reducing worm burden, it does not prevent re-infection. Furthermore, over reliance on this single drug and its frequent widespread use has resulted in the emergence of resistant flukes (5). The global scale of *F. hepatica* infection, combined with limited treatment options, raises an urgent need to develop novel control strategies. To achieve this, a deeper understanding of the parasite's mechanisms of invasion and colonisation are necessary.

FASCIOLA HEPATICA MANIPULATES THE HOST IMMUNE RESPONSE TO SUPPORT SUCCESSFUL INVASION

All mammalian hosts of *F. hepatica* become infected by ingestion of vegetation that is contaminated with the encysted dormant larvae (metacercariae). In the duodenum, the newly excysted juveniles (NEJ) emerge and penetrate the intestinal epithelium to migrate through the peritoneal cavity to reach the liver. Within the liver these parasites spend many weeks feeding on tissue and blood to mature, after which, they migrate to the bile duct, where they take up residence, often for decades, producing thousands of eggs, which are excreted from their mammalian host to continue their life cycle (6).

In naturally infected animals, there is no evidence of the typical host protective, pro-inflammatory Th1 type, immune response that would be expected in response to infection with a pathogen (7). Instead, the Fasciola-specific immune response is predominantly Th2, which becomes more potent as the parasite migrates through the liver. Once the worm is established in the bile duct and the infection becomes chronic, the parasite-specific immune response switches to a combination of regulatory T cells and anergic effector T cells (8–12). Notably, vaccine trials have shown that when a parasite-specific Th1 response is activated, significant levels of protection against infection are achieved (13, 14). Collectively, these observations suggest that by inhibiting the immediate host protective immune response, the parasite ensures survival of the NEJs, permitting their safe passage from the intestine, across the peritoneal cavity and on to the liver, at which point the host response switches to a Th2 phenotype to mediate tissue repair mechanisms. Indeed, in mice deficient in Th2 immunity, worm burden and size were unaffected, suggesting there was no impact to the maturation of the parasite. However, the infected mice displayed significantly more damage to liver tissue and succumbed to premature deaths (15).

While the host innate immune response is activated by the presence of the parasite, resulting in an influx of dendritic cells (DC), eosinophils, neutrophils, and macrophages to the peritoneal cavity immediately after infection (16, 17), evidence suggests that the anti-pathogenic, pro-inflammatory activities of these immune cells are inhibited by the NEJs. The DCs display low expression of CD80, CD40, MHC class II, and CD86 and high expression of CCR5 (17, 18), a phenotype that is indicative of an immature DC. Functionally, these DCs, are unable to promote the differentiation of Th1 cells, and instead induce the expansion of anergic T cells (17). Similarly, macrophages have a low expression of MHC-II and are impaired in their ability to produce pro-inflammatory mediators such as iNOS and TNF, instead adopting a regulatory profile by secreting IL-10 and TGFβ (17, 19, 20). Although not demonstrated *in vivo*, the exposure of bovine neutrophils to the intra-mammalian life stages of *F. hepatica in vitro*, failed to induce significant production of reactive oxygen species or NETosis, suggesting that the parasite was impairing the antimicrobial activities of neutrophils (21). Likewise, there is no evidence of eosinophil degranulation in the peritoneal cavity, which indicates that these cells are not activated or have an alternative phenotype that is not contributing to parasite killing (16).

Understanding the mechanisms that *F. hepatica* employs to disarm the host's response during the early stages of infection offers the opportunity to counteract these strategies to target the NEJs, which would prevent penetration of the liver capsule and thus the disease pathology. While research to date has largely focused on the characterisation of immune modulating proteins/glycans secreted by helminths during infection [reviewed in (22, 23)], it has recently become apparent that parasitic worms also actively release micro(mi)RNAs which may have a role in the regulation of host immune cells.

HELMINTH-DERIVED MIRNAS REGULATE MAMMALIAN GENE EXPRESSION TO MODULATE HOST IMMUNE RESPONSES

MicroRNAs are small (~22 nucleotides long) non-coding RNA (24), that function to regulate gene expression at the posttranscriptional level through specific binding and subsequent silencing of target messenger RNA (mRNA; **Figure 1**). Target recognition is a highly specific process with complementary binding between the seed region (2–8 nt) at the 5' end of the miRNA and the 3' untranslated region (UTR) of the target mRNA (25). This binding activity eventually leads to either inhibition of the initiation step of translation or promotes mRNA decay through the deadenylation of the poly(A)-tail of the mRNA target (25). With many hundreds of human miRNAs

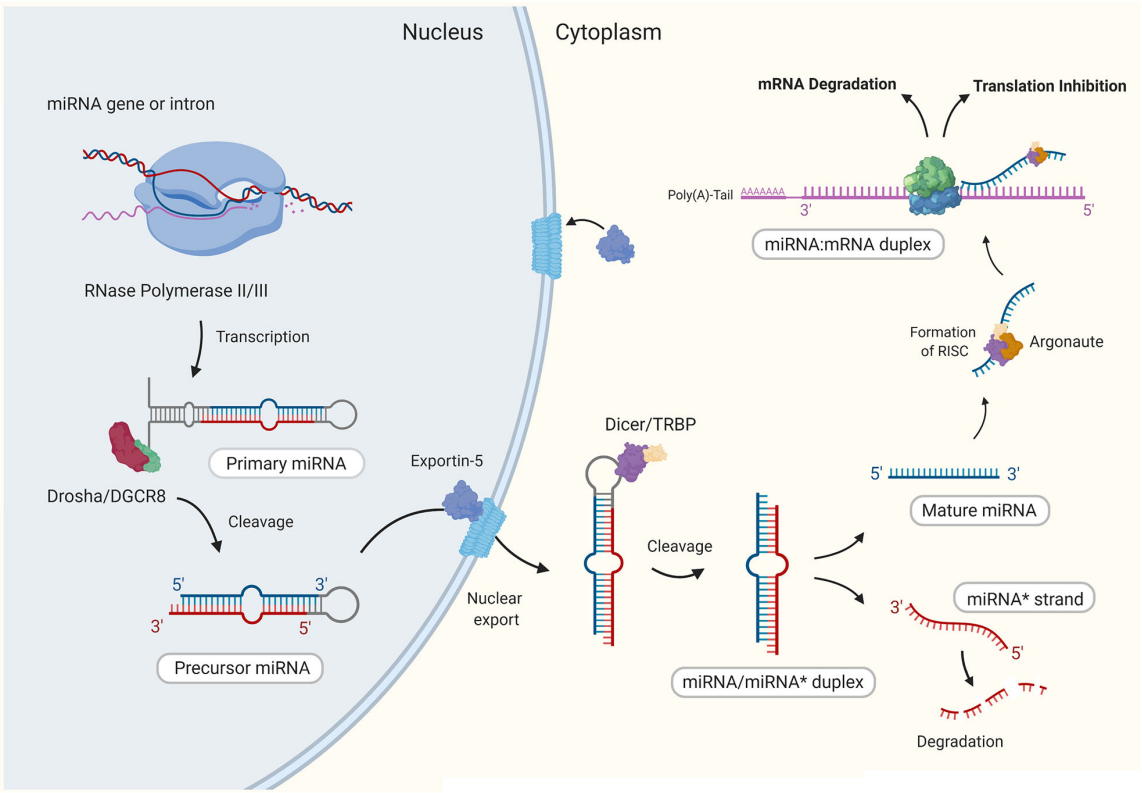

FIGURE 1 | The canonical biogenesis of mammalian mature microRNAs. Generally, through canonical processes miRNAs are first transcribed in the nucleus by RNase polymerase II (Pol-II) to form a stem loop structure that is the primary miRNA (pri-miRNA). Similar to mRNAs, pri-miRNAs present a 5' 7-methylguanosine (m7G)-cap and polyadenylated 3' end. The pri-miRNA is then cropped by RNase III Drosha and the double stranded RNA-binding protein DGCR8 (DiGeorge syndrome critical region 8 gene) to form the precursor miRNA (pre-miRNA), which is then exported to the cytoplasm by Exportin-5 (XPO5). In the cytoplasm, the stem loop of the pre-miRNA is cleaved by RNase III Dicer to form a short miRNA duplex (miRNA/miRNA*) comprising of the guide strand and the passenger strand (miRNA*). The miRNA* is degraded while the guide strand is stably loaded onto an Argonaute protein (AGO) which forms the core of the miRNA induced silencing complex (miRISC). Once bound to the miRISC, the mature miRNA finally has the capacity to bind to a target mRNA and form a miRNA/mRNA duplex. Image created by Biorender.

identified to date, it is not surprising that miRNAs are recognized to take part in virtually every biological process (26). The first indication that miRNAs were involved in the regulation of immune responses, emerged from a study in 2004, which demonstrated the selective expression of a small number of miRNAs in immune cells (27). Since then, numerous miRNAs have been characterized as having roles in the regulation of both innate and adaptive immune responses, in which they control the development of immune cell progenitors, maintenance and differentiation and mature immune cell function [reviewed in (28)].

Importantly, miRNAs are highly conserved through metazoan evolution and are thus considered to be a vital ancient component of gene regulation (29, 30). Comparative analyses of parasite genomes revealed that a number of helminth miRNAs are widely conserved across diverse organisms and share sequence identity with mammalian species known to have an immune regulatory role (31, 32). These observations led to the suggestion that parasite-derived miRNAs could target mammalian genes within the immune cells of their hosts to modulate immune responses (33). Further support for this hypothesis was provided with the discovery that miRNAs, encapsulated in extracellular vesicles (EVs), secreted by parasitic worms can be delivered to host immune cells (34, 35). While definitive proof for worm-derived miRNAs acting on host cells *in vivo* remains to be obtained, enticing evidence has been provided by *in vitro* studies (34, 36). Of particular relevance to *F. hepatica*, it has been recently reported that EVs, derived from the closely related trematode worm *Schistosoma japonicum*, were internalized by murine macrophages resulting in the release of parasite-derived miRNAs intracellularly. One of these schistosome miRNAs, *sja-miR-125b*, incorporated into the host AGO protein resulting in the regulation of the host Toll Like Receptor (TLR) signaling pathway, which consequently modulated the production of cytokines by the macrophages (36). Despite this growing evidence for a functional role for helminth miRNAs in the host-parasite relationship, proper characterisation of the complete miRNomes of these pathogens

is lacking and very little exploration of their putative role in host immune modulation has been performed.

AN EVALUATION OF THE *FASCIOLA HEPATICA* MIRNOME IDENTIFIED TO DATE

Currently, the identification of helminth microRNAs is performed from sequencing reads by applying various algorithms based on sequence structure, evolutionary conservation, thermodynamic stability, and machine learning. Consequently, the output from every discovery pipeline is highly dependent on specific input requirements critical to producing reliable miRNA candidates.

The characterization of *F. hepatica* miRNAs has been reported across three primary discovery projects and was achieved using three distinct pipelines mandated by the availability of the *F. hepatica* genome, the sequencing input, and the use of different analytical tools. Initial explorations by Xu et al. (37), employed strategies that compensated for the absence of an assembled *F. hepatica* genome. The availability of a reference genome is required for predicting the candidate pre-miRNAs (characterized by complementary sequences separated by a hairpin loop). These pre-miRNAs give rise to the mature miRNAs that partake in gene regulation. Therefore, without a reference genome, the ability to predict miRNAs from sequencing data is diminished. To fill this gap, Xu et al., utilized the genome of *S. japonicum* in combination with the Short Oligonucleotide Alignment Program (SOAP) to map putative miRNAs within the RNA sequencing data obtained from adult *F. hepatica*. This approach produced the initial repertoire of 16 F. *hepatica* miRNAs. Matching these miRNA candidates with the known miRNAs of *S. japonicum* from miRBase (version 16.0) revealed that eight were conserved miRNAs between the trematode species (**Tables 1** and **2**), which suggested that the remaining eight miRNAs were unique to *F. hepatica*.

The subsequent study by Fromm et al. (38), also worked with the lack of an available *F. hepatica* genome, and instead utilized the miRCandRef tool to develop assembled contigs from *F. hepatica* genomic data (as part of the 50 Helminth Genomes Initiative) to use as the reference genome. This study used a modified version of the miRDeep2 algorithm to permit a higher sensitivity for predicting miRNA loci within the assembled contigs. Using this approach, the sequencing data from the initial study by Xu et al. was re-analyzed in addition to the sequenced miRNA content of extracellular vesicles (EVs) isolated from adult liver fluke. This analysis produced an expanded list of 55 miRNAs, all of which were found in both the adult fluke and the EVs. This list included the eight conserved miRNAs identified by the earlier study (**Table 1** and **2**). However, the eight novel miRNAs proposed in that study were not found by Fromm et al., suggesting that in fact, they may not be *bone fide* miRNAs and thus we propose should be removed from the listed miRNAs for *F. hepatica*.

The most recent discovery project by Fontenla et al. (40), focused on the miRNA content of newly excysted juveniles (NEJ) 6 h post-excystment as opposed to the adult life stage utilized in the previous two studies. Initially, the conservation of *F. hepatica* miRNAs across mammalian and platyhelminth species was determined *via* a series of filtering processes which compared the NEJ sequencing data against published sequences and databases of miRNA and non-coding RNA such as miRBase (version 20.0), Rfam (41) and the functional RNA database fRNAdb (www.ncrna.org/frnadb).

This identified a total of 46 miRNAs. However, although six of these were classified as miRNAs due to high similarity (>85%) with other helminth miRNAs reported in literature, as they were not found within any of the databases the authors removed these from their final list of proposed miRNAs, thus producing a total of 40 (**Tables 1** and **2**). Of these, 34 shared sequence identity to the miRNAs discovered in the previous analyses of the adult miRNA (37, 38), which suggests that the other 6 miRNAs may be specific to the NEJs. Only three of the eight putative novel miRNAs identified by Xu et al. were identified within this sequencing data but were found to correspond to repetitive sequences within the Fasciola genome, confirming the earlier proposition that they are not genuine miRNAs. To differentiate the Fasciola specific miRNAs from conserved sequences within the panel of 40, a genome assembly was generated using genomic sequence reads downloaded from the Welcome Trust Sanger Centre and used as the reference genome for analysis by miRDeep2. Novel mature miRNA candidates were then aligned to *F. hepatica* genomes PRJEB6687 (42) and PRJNA179522 (43) to confirm the presence and location of mature miRNAs. This analysis resulted in the identification of five *F. hepatica* specific miRNAs, of which four are now officially annotated as fhe-miR-11584 to 11587 on miRBase.

Combined, these studies resulted in the identification of 72 miRNAs (excluding the eight proposed novel miRNAs by Xu et al). Of these, 38 are currently featured in the miRBase database (F_hepaticav1; **Table 1**). Of the remaining 34 miRNAs (**Table 2**), 13 were characterized as novel to *F. hepatica* (i.e. unidentified within the miRNA profile of any other species on miRBase). Although the other 21 sequences have been annotated, within their respective publications, based on their similarity with known miRNAs, they are not listed on the miRBase database, suggesting they were not deemed as authentic miRNAs. This is most likely due to expression and biogenesis criteria for miRNA identification. It is possible that miRNAs identified in the adult flukes were not accepted due to the genome assembly that was used in those discovery pipelines. Although several miRNAs in the NEJ study were localized within the Fasciola genome according to the 2015 genome, these miRNAs may not have satisfied the specific criteria for precursor hairpin structures employed by miRBase (44).

However, these should not be discounted as likely miRNAs, as it has more recently been proposed that the hairpin structures of miRNAs may be more variable than originally proposed (45).

TABLE 1 | Comparison of miRBase *Fasciola hepatica* mature miRNAs to published sequences.

miR BasemiRNA	Mature miRNASequence on miRBase	Other Proposed Annotation	Other Proposed Mature miRNA Sequence	Study					Sample
				Xu	Fontenla	Fromm 2015	Fromm 2017	Ovchinnikov	
fhe-bantam	TGAGATCGCGATTAAAGCTGGT	Fhe-Bantam		+	+	+	+	+	NEJ, Ad, EV
fhe-miR-2b	GTATCACAGCCCTGCTTGGGACA	miR-2b-3p	_TATCACAGCCCTGCTTGGGACAC	+	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-2-P2b	_TATCACAGCCCTGCTTGGGACACA						
		Fhe-Mir-2-P2c							
fhe-miR-2e	GTATCACAGTCCAAGCTTTGG	miR-2e-3p	_TATCACAGTCCAAGCTTTGG	+	+	+	+	+	NEJ, Ad, EV
		fhe-mir-2c-A	_TATCACAGTCCAAGCTTTGGT						
		Fhe-Mir-2-P3b	_TATCACAGTCCAAGCTTTGGTAAA						
fhe-miR-10	AACCCTGTAGACCCGAGTTTGCA	miR-10-5p	AACCCTGTAGACCCGAGTTTG:	+	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-10-P1	AACCCTGTAGACCCGAGTTTGC_						
fhe-miR-71a	TGAAAGACGATGGTAGTGAGATG	Fhe-Mir-71-P1b	TGAAAGACGATGGTAGTGAGAT_	+	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-71-P1b							
fhe-miR-71b	TGAAAGACTTGAGTAGTGAG	Fhe-Mir-71-P1a	TGAAAGACTTGAGTAGTGAGACG	+	+	+	+	+	NEJ, Ad, EV
fhe-miR-124	TTAAGGCACGCGGTGAATGTCA	Fhe-Mir-124	_TAAGGCACGCGGTGAATGTCA	+	+	+	+	+	NEJ, Ad, EV
fhe-miR-2a	GTCACAGCCAGAATTGATGAACG	Fhe-Mir-2-P1b	_TCACAGCCAGAATTGATGAACG	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-2c	ATATCACAGCCGTGCTTAAGGGCT	Fhe-Mir-2-P3a	_TATCACAGCCGTGCTTAAGGGCTT	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-2d	GTATCACAGTCCTGCTTAGGTG	Fhe-Mir-2-P2a	_TATCACAGTCCTGCTTAGGTGACGA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-2f	GTCACAGCCAATATTGATGCC	Fhe-Mir-2-P1a	_TCACAGCCAATATTGATGCCTG	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-7	TGGAAGACTGGTGATATGTTGTT	Fhe-Mir-7-P1		–	+	+	+	+	NEJ, Ad, EV
fhe-miR-8	CTAATACTGTTTGGTAAAGATGCC	Fhe-Mir-8	_TAATACTGTTTGGTAAAGATGCC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-9	TCTTTGGTTATCAAGCAGTATG	Fhe-Mir-9	TCTTTGGTTATCAAGCAGTATGA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-31	TGGCAAGATTATGGCGAAGCTGA	Fhe-Mir-31		–	+	+	+	+	NEJ, Ad, EV
fhe-miR-36a	GTCACCGGGTAGACATTCATTCAC	Fhe-Mir-36-P1	_TCACCGGGTAGACATTCATTCAC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-61	ATGACTAGAAAGTGCACTCACTT	Fhe-Mir-279	_TGACTAGAAAGTGCACTCACTTC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-87	GGTGAGCAAAGTTTCAGGTGTGA	Fhe-Mir-87	_GTGAGCAAAGTTTCAGGTGTGA	–	+	+	+	+	NEJ, Ad, EV
fhe-mir-96	CTTGGCACTTTGGAATTGTCA	Fhe-Mir-96	CTTGGCACTTTGGAATTGTCAC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-125a	TCCCTGAGACCCTAGAGTTTC	Fhe-Mir-10-P2b	TCCCTGAGACCCTAGAGTTTCC	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-125b	CCCCTGAGACTGATAATTGCTC	Fhe-Mir-10-P2a	CCCCTGAGACTGATAATTGCT_	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-10-P2a	CCCCTGAGACTGATAATTGCTCC						
fhe-miR-190	AGATATGTTTGGGTTACTTGGTG	Fhe-Mir-190-P1		–	+	+	+	+	NEJ, Ad, EV
fhe-miR-219	TGATTGTCCATTCGCATTTCTTG	Fhe-Mir-219	TGATTGTCCATTCGCATTTCTT_	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-219							
fhe-miR-277	GTAAATGCATTTTCTGGCCCG	Fhe-Mir-277-P1	_TAAATGCATTTTCTGGCCCGTAA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-745b	GAAAGCTGCCAAGCGAAGGGC	Fhe-Mir-22-P2	:AAGCTGCCAAGCGAAGGGCCAA	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-22-P2	:AAGCTGCCAAGCGAAGGGCCAAG						
fhe-miR-2162	GTATTATGCAACATTTCACTCT	Fhe-Mir-1993	_TATTATGCAACATTTCACTCT	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-3479	GTATTGCACTTTCCTTCGCCTTA	Fhe-Mir-92-P1	_TATTGCACTTTCCTTCGCCTTA	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-11584	CCATTATATAAGATTGAGGCTCT	Fhe-Mir-NOV-1	_CATTATATAAGATTGAGGCTCT	–	+	+	+	+	NEJ, Ad, EV
fhe-miR-46	ATGTCATGGAGTTGCTCTCTACA	Fhe-Mir-281	_TGTCATGGAGTTGCTCTCTACA	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-281	AGGAGGGCAATTTTATGACTTT						
fhe-miR-307	ATCACAACCTACTTGATTGAGGGG	Fhe-Mir-67	_TCACAACCTACTTGATTGAG:_	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-67	CCTCAACAAGAAGGCTGTTGGATG						
fhe-miR-745a	ATGCTGCCTTATAAGAGCTGTG	Fhe-Mir-22-P1	_TGCTGCCTTATAAGAGCTGTGA	–	+	+	+	+	NEJ, Ad, EV
		Fhe-Mir-22-P1	TCAGTTCTCATTAGGCATGACATG						
fhe-miR-1	ATGGAATGTGGCGAAGTATGGT	Fhe-Mir-1-P2	_TGGAATGTGGCGAAGTATGG_	–	+	–	+	+	NEJ, Ad, EV
		Fhe-Mir-1-P2	_TGGAATGTGGCGAAGTATGGTCT						

(Continued)

TABLE 1 | Continued

miR BasemiRNA	Mature miRNASequence on miRBase	Other Proposed Annotation	Other Proposed Mature miRNA Sequence	Study				Sample
				Xu	Fontenla	Fromm 2015	Fromm 2017	Ovchinnikov	
fhe-miR-36b	ACCACCGGGTAGACATTCATC	Fhe-Mir-36-P3	_CCACCGGGTAGACATTCATCCGC	–	+	–	+	+	NEJ, Ad, EV
fhe-miR-750	ACCAGATCTGACTCTTCCAGCTCT	Fhe-Mir-750	_CCAGATCTGACTCTTCCAGCTCT	–	+	–	+	+	NEJ, Ad, EV
		Fhe-Mir-750	_CCAGATCTGACTCTTCCAGCTCTT						
fhe-miR-11585	GACCGGTTTCGTCGTTCAACAC	Fhe-Mir-NOV-6	_ACCGGTTTCGTCGTTCAACACC	–	+	–	+	+	NEJ, Ad, EV
		Fhe-Mir-NOV-6	CGTTGCACCGTTCGGAATTCGGGCA						
fhe-let-7	GAGAGGTAGTGACTCATATGACT	fhe-let-7c	_AGAGGTAGTGACTCATATGACT	–	+	–	–	+	NEJ, Ad, EV
		Fhe-Let-7-P2							
fhe-miR-11586	TGTAAGACGATCGTAGTTGACG			–	+	–	–	–	NEJ
fhe-miR-11587	ATTCCGGCAGCTTAGTACAGCT			–	+	–	–	–	NEJ

Ad, Adult F.hepatica; EVs, Adult F. hepatica extracellular vesicles; NEJ Newly excysted juveniles; featured in study (+); not featured in study (-). Sequences that are featured in a study with identical annotation but a non-identical sequence are characterised with a yellow box. Gray highlighted nucleotides represent nucleotide variability and/or missing nucleotides when compared to respective F. hepatica miRBase miRNA. F. hepatica miRBase miRNAs as featured on miRBase database (version 22.1) and hyperlinked to respective miRNA profile on mirbase.org. Other proposed annotations represent the most up to date annotation of the respective other proposed mature miRNA sequence-colour coded based on the study, Xu et al., 2012 (37) (pink), Fromm et al., 2015 (38) (blue), Fromm et al., 2017 (33) (orange) and Ovchinnikov et al. 2020 (39) (green). Sequences sorted based on presence throughout the featured studies.

This suggestion has influenced the development of a new set of criteria for the annotation of metazoan miRNAs, which is employed by the curated miRNA gene database MirGeneDB. Utilising these criteria to re-evaluate the *F. hepatica* small RNA sequencing data from all three of the discovery projects, Fromm et al. (33) determined that the annotated miRNAs, not present in miRBase were genuine miRNAs (**Table 2**) (33). However, seven of the putative novel miRNAs from the Fromm et al., 2015 study (**Table 2**) and 3 novel miRNAs proposed within the NEJ study were no longer considered as *bona fide* miRNAs (**Table 1** and **2**). This outcome highlights the impact of using different criteria for the assessment of miRNAs, as two of these NEJ miRNAs are currently annotated as genuine miRNAs in miRBase as fhe-miR-11586 and fhe-miR11587 (**Table 1**). In addition to the assessment of previously identified miRNAs, this study also discovered eight new conserved miRNAs within the adult parasites (Fhe-mir-1-P2, Fhe-mir-36-P2, Fhe-mir-36-P3, Fhe-miR-190-P2, Fhe-mir-210-P1, Fhe-mir-210-P2, Fhe-mir-277-P2, Fhe-mir-750). Of these, three were also present in the NEJs (Fhe-mir-1-P2, Fhe-mir-36-P3, Fhe-mir-750) and are listed in miRBase as fhe-miR-36b and fhe-miR-750, with the sequences for both Fhe-mir-1-P2, Fhe-mir-36-P3 aligning to fhe-miR-36b (**Table 1**).

As well as using different criteria for assessment, MirGeneDB employs an internal annotation, which differs from the nomenclature utilized by MirBase. Whereas, the miRBase system assigns the next number in succession (i.e. miR-10 was reported after miR-9 etc.) to new sequences, with paralogs indicated by a letter (if there is a difference of a single nucleotide) or a number (if the mature sequences are identical), the MirGeneDB nomenclature was developed to capture the phylogenetic relationship between miRNAs, where genes of common descent are assigned the same miRNA family name (46). Using this system resulted in the re-classification of Mir-novel-5 (33) as a member of the MIR-2160 family (**Table 2**). Similarly, fhe-miR125 has been assigned to the eumetazoan MIR-10 gene family resulting in the nomenclature Fhe-MiR-10, with the paralogs identified as Fhe-Mir-10-P2a and Fhe-Mir-10-P2b (**Table 1**). Likewise, fhe-miR-745a has been named Fhe-Mir-22-P1.

Most recently, the small RNA sequencing data sets from the adult parasites (37) and their EVs (38) were re-evaluated again using an improved version of MirMiner (39). This study reported the discovery of four conserved miRNAs (Fhe-Let-7-P3, Fhe-Mir-133, Fhe-Mir-278 and Fhe-Mir-2160-P2) and four Fasciola-specific miRNAs. Of the parasite-specific miRNAs, the sequences for Fhe-Mir-NOV-1 and Fhe-Mir-NOV-6 were near identical to miRNAs within the NEJ miRBase dataset, listed as fhe-miR-11584 and fhe-miR-11585 respectively (**Table 1**). Although the other two (Fhe-Mir-NOV-2 and Fhe-Mir-NOV-3) may be classified as new sequences, they each differ in only one nucleotide from the previously identified adult parasite miRNAs fhe-mir-novel-6 and fhe-mir-novel-7 respectively (**Table 2**).

Compiling the findings from all of these studies suggests that in addition to the 38 miRNAs listed on miRBase, *F. hepatica* expresses an additional 39 miRNAs (excluding the seven sequences deemed not to be genuine according to the

TABLE 2 | Published *Fasciola hepatica* mature miRNAs not featured in miRBase database.

Most Recent Published Annotation	Other Published Annotation	Mature miRNA Sequence(s)	Study					Sample
			Xu	Fontenla	Fromm 2015	Fromm 2017	Ovchinnikov	
Fhe-Let-7-P1	let-7 Fhe-Let-7	GGAGGTAGTTCGTTGTGTGG_ GGAGGTAGTTCGTTGTGTGGT	+	–	+	+	+	Ad, EV
Fhe-Mir-1-P1	fhe-mir-1	TGGAATGTTGTGAAGTATGTAC	–	–	+	+	+	Ad, EV
Fhe-miR-2-P4	fhe-mir-2a-B	TATCACAGCCCTGCTTGGAACA: TATCACAGCCCTGCTTGGAACACA	–	–	+	+	+	Ad, EV
Fhe-Mir-7-P2	fhe-mir-7b	TGGAAGACTTGTGATTAAGTTGT_ TGGAAGACTTGTGATTAAGTTGTT	–	–	+	+	+	Ad, EV
Fhe-Mir-10-P3	fhe-mir-10*	CAAGCTCGGGTATACAGGAGCAG	–	–	+	+	+	Ad, EV
Fhe-Mir-12	fhe-mir-12	TGAGTATTTCATCAAGTAGTG_ TGAGTATTTCATCAAGTAGTGA	–	–	+	+	+	Ad, EV
Fhe-Mir-71-P2	fhe-mir-71b	TGAAAGACATGGGTAATGAGGT	–	–	+	+	+	Ad, EV
Fhe-Mir-184-P1 Fhe-Mir-184-P2	fhe-mir-184	TGGACGGAGATTTGTTAAGAGC	–	–	+	+	+	Ad, EV
Fhe-Mir-1175	fhe-mir-1175	TGAGATTCAACTACTTCAGCTG	–	–	+	+	+	Ad, EV
Fhe-Mir-1992	fhe-mir-1992	TCAGCAGTTGCACCATTGACG	–	–	+	+	+	Ad, EV
Fhe-Mir-1989	fhe-mir-1989	TCAGCTGTGTTCATGTCTTCGA	–	–	+	+	+	Ad, EV
Fhe-Mir-2160-P1	fhe-mir-novel-5 Fhe-Mir-2160	TGGCGCTTAGTTATATGTCATCG	–	–	+	+	+	Ad, EV
Fhe-Mir-NOV-2	fhe-mir-novel-6	AGTGGTGATGGTCGAGTGGTTTAG AGTGGTGATGGTCGAGTGGTTTAG_	–	–	+	+	+	Ad, EV
Fhe-Mir-NOV-3	fhe-mir-novel-7	TCAGCACCGGCCGAAACGACAC TCAGCACCGGCCGAAACGACA	–	–	+	+	+	Ad, EV
Fhe-Mir-92-P2	fhe-mir-92b Fhe-Mir-92-P2	GATTGCACTACTCATAGCCTTC AGGCTGTGTGTAGAGCAAGTTG	–	–	+	+	+	Ad, EV
Fhe-Mir-210-P2	fhe-novel-3 Fhe-Mir-219-P2	TAGTCACTGGGCTACGAACACG TGTGCGTAGTTTCAGTGATTAGC	–	–	+	+	+	Ad, EV
Fhe-Mir-NOV-4	fhe-mir-novel-8	ACCCTCATTTAGATCGAAGGT	–	–	+	+	–	Ad, EV
Fhe-Mir-NOV-5	fhe-mir-novel-10	AGACACTCAGAGGACGATCAGT	–	–	+	+	–	Ad, EV
Fhe-Mir-36-P2		TCACCGGGTGTTTTTCACCCTC GGGTGGATACAGTCGGTTATG	–	–	–	+	+	Ad, EV
Fhe-Mir-190-P2		TGATATGTATGGTTTTCGGTTG	–	–	–	+	+	Ad, EV
Fhe-Mir-277-P2		AAAATGCATCATCTACCCGAGA	–	–	–	+	+	Ad, EV
Fhe-Mir-210-P1		TTGTGCGTCGTTTCAGTGACCGAA	–	–	–	+	+	Ad, EV
fhe-let-7-5p		TGAGGTAGTAGGTTGTATAGT	–	+	–	–	–	NEJ
miR-2e-5p		TACCAACTTAGACTGCGTTAT	–	+	–	–	–	NEJ
miR-61-5p		TGTGAGTCTCTTTCTTGTCCATG	–	+	–	–	–	NEJ
miR-190-3p		CCAGTGACCAAACATATTCTC	–	+	–	–	–	NEJ
miR-10-3p		AAATTCGAGTCTACAAGGAAC	–	+	–	–	–	NEJ
miR-205		CGAGGACGTTCAATGGGTTCT	–	+	–	–	–	NEJ
miR-562		TCTAGTCCGACTTTGTGAGGA	–	+	–	–	–	NEJ
miR-598		CGCTGTACGATGATGATGATTT	–	+	–	–	–	NEJ
miR-920		AGGTGTAGAAGTGGTAACACT	–	+	–	–	–	NEJ
miR-1985		TAAAGTGACTGTTAGAATGGT	–	+	–	–	–	NEJ
miR-2478		TCGTATCCCACCTCTGACACCA	–	+	–	–	–	NEJ
miR-3487		TCCCCGTAATCGAACTGTTGT	–	+	–	–	–	NEJ
fhe-miR-3064		CTGGCTGTTGCGGTTAAACC	–	+	–	–	–	NEJ
fhe-novel-5		TAGAGTACCTGTAGATTTAG	–	+	–	–	–	NEJ
fhe-mir-novel-1		GTGGCCTCGTAGCTCAGCTGGTAG	–	–	+	–	–	Ad, EV
fhe-mir-novel-2		TTTGCATATCTAAGTCGGACA	–	–	+	–	–	Ad, EV
fhe-mir-novel-9		GTCAGCGAAGACGTCGGGAA	–	–	+	–	–	Ad, EV
fhe-mir-novel-11		TCCGAAAACGCGATGGAACCT	–	–	+	–	–	Ad, EV
fhe-mir-novel-12		ATGAGACGGTGAGTGATGAATT	–	–	+	–	–	Ad, EV
fhe-mir-novel-13		GACCGGTGGTGGTCGAGTGGGTT	–	–	+	–	–	Ad, EV
Fhe-Mir-36-P2		TCACCGGGTGTTTTTCACCCTC	–	–	–	+	–	Ad, EV
Fhe-Let-7-P3		GAGGTAGTGAGTTGTATGTCTG	–	–	–	–	+	Ad, EV
Fhe-Mir-36-P2		GGGTGGATACAGTCGGTTATG	–	–	–	–	+	Ad, EV
Fhe-Mir-133		TTGGTCCCTATCAACCAGCTAT	–	–	–	–	+	Ad, EV
Fhe-Mir-278		TCGGTGGGAGTATCATTCGTGC	–	–	–	–	+	Ad, EV
Fhe-Mir-2160-P2		AGGCGCTTTGATTGTCCACACTGA	–	–	–	–	+	Ad, EV

Ad; adult fluke, EV; Adult fluke extracellular vesicles NE; Newly excysted juveniles; featured in study (+); not featured in study (-). Annotation of published miRNA as most up to date of that sequence across studies. Other published annotation represents studies by Xu et al. 2012 (37) (pink), Fromm et al. 2015 (39) (blue), Fromm et al. 2017 (33) (orange), and Ovchinnikov et al. 2020 (39) (green). Sequences that are featured in a study with identical annotation but a non-identical sequence are characterised with a yellow box. Sequences sorted based on presence throughout the featured studies.

MirGeneDB criteria for miRNA annotation (33), as described above. Of the 77 miRNAs, 36 were identified in both NEJ and adult parasite, 15 were specific to NEJ and 26 were specific to adult parasites (**Tables 1** and **2**).

Despite the similarities in the profiles of miRNAs identified across all studies, it is important to note the number of variations in the recorded sequences for many of the mature miRNAs (**Table 1**). Differences in nucleotides are particularly evident towards the 5' or 3' end for 26 of the common mature miRNAs. Studies of mammalian miRNAs have indicated that variations in the 3'- and/or 5'-end(s) of canonical miRNA-sequence represent IsomiRs (sequence variants) created either due to imprecise cleavage of miRNA sequence by drosha or dicer enzymes or through the addition of nucleotides at 3' end during miRNA-biogenesis (47). Whether the variations within the Fasciola miRNA sequences represent IsomiRs that correlate with different life stages is an issue that will only be resolved with continued analysis of the fluke's miRNA. Nonetheless, as these changes do not alter the 2–8 nt seed region of these miRNAs, they are not likely to have a significant effect on the specificity of gene that they target.

In contrast, the sequences reported for let-7, miR-1, and miR-71b are quite different between the studies, with nucleotide variations evident throughout the entire sequence of the mature miRNA. Further examination of these sequences suggests that the annotations are correct, but the different sequences may in fact reflect distinct members of the miRNA families (**Table 3**). The sequence of miR-1 identified in the NEJs more closely aligns to the *S. mansoni* miR-1a and other species of fluke, whereas the miR-1 in adult fluke is more closely conserved to *S. mansoni* miR-1b and species of tapeworm. Similarly, miR-71b in NEJs is near identical to only other species of trematode, whereas the adult fluke miR-71b closely resembles other parasitic and non-parasitic helminths outside of the trematode class. Likewise, the sequence of the let-7 within the NEJs is conserved with Planaria while the adult fluke let-7 is closer to other parasitic trematodes and mammalian sequences. This is of particular interest as let-7 is the miRNA that regulates the expression of *Lin41* in *C. elegans*, a gene that controls the transition to adulthood (48). This suggests that perhaps the differences in the listed sequences for Fasciola adult and NEJ miRNAs may in fact reflect the evolution of variants of the same miRNAs specific to the different life stages of Fasciola to ensure the regulation of different gene targets as necessary for maturation of the worm and modulation of host responses in different tissue environments.

Of interest, miR-281, miR-279, miR-67, and miR-1993 identified in adult fluke by Fromm et al. (39), are alternatively annotated as miR-46, miR-61, miR-307, and miR-2162 respectively, by Fontenla et al. (40). An analysis of miR-281 and miR-46 showed that the sequences are in fact very similar, but have been classified as two distinct miRNAs in miRBase. In both fluke miRNA studies; this particular sequence was published as miR-46/miR-281. However, due to increased availability of miRNA sequences within databases, it is now evident that miR-46 is generally found within helminths, and while miR-281 can be found in some species of parasitic helminth it is more prominent in other invertebrates (**Table 4**). This was likely a miRbase consideration to finalize the annotation of this sequence to miR-46 in Fasciola. The same

TABLE 3 | Mature miRNA sequence, study and conservation between fhe-let-7, fhe-miR-1 and fhe-miR-71b.

Source	Species	miRNA	Mature sequence
Fontenla et al	***Fasciola hepatica***	**fhe-let-7**	**GAGAGGUAGUGACUCAUAUGACU**
miRBase	*Melibe leonina*	mle-let-7-5p	_UGAGGUAGUGACUCAUUUUGUU
miRBase	*Schmidtea mediterranea*	sme-let-7c-5p	_UGAGGUAGUGACUCAAAAGGUU
miRBase	*Schmidtea mediterranea*	sme-let-7d	_AGAGGUAGUGAUUCAAAAAGUU
Fromm et al	***Fasciola hepatica***	**fhe-let-7**	**GGAGGUAGUUCGUUGUGUGGU**
miRBase	*Schistosoma japonicum*	sja-let-7	GGAGGUAGUUCGUUGUGUGGU
miRBase	*Schistosoma mansoni*	sma-let-7-5p	GGAGGUAGUUCGUUGUGUGGU
miRBase	*Ovis aries*	oar-let-7b	UGAGGUAGUAGGUUGUGUGGU
miRBase	*Homo sapiens*	hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
Fontenla et al	***Fasciola hepatica***	**fhe-miR-1**	**AUGGAAUGUGGCGAAGUAUGGU**
miRBase	*Schistosoma japonicum*	sja-miR-1	_UGGAAUGUGGCGAAGUAUGGUC
miRBase	*Gyrodactylus salaris*	gsa-miR-1-3p	_UGGAAUGUGGCGAAGUAUGGUC
miRBase	*Schistosoma mansoni*	sma-miR-1a-5p	_UGGAAUGUGGCGAAGUAUGG_
Fromm et al	***Fasciola hepatica***	**fhe-miR-1**	**UGGAAUGUUGUGAAGUAUGUAC**
miRBase	*Schistosoma mansoni*	sma-miR-1b-3p	UGGAAUGUUGUGAAGUAUGUGC
miRBase	*Echinococcus granulosus*	egr-miR-1-5p	UGGAAUGUUGUGAAGUAUGU_
miRBase	*Echinococcus multilocularis*	emu-miR-1-3p	UGGAAUGUUGUGAAGUAUGU_
Fontenla et al	***Fasciola hepatica***	**fhe-miR-71b**	**UGAAAGACUUGAGUAGUGAG**
miRBase	*Schistosoma japonicum*	sja-miR-71b-5p	UGAAAGACUUGAGUAGUGAGACG
miRBase	*Schistosoma mansoni*	sma-miR-71b-5p	UGAAAGACUUGAGUAGUGAGACG
Fromm et al	***Fasciola hepatica***	**fhe-miR-71b**	**UGAAAGACAUGGGUAAUGAGGU**
miRBase	*Gyrodactylus salaris*	gsa-mir-71a	UGAAAGACAUGGGUAAUGAGU_
miRBase	*Schmidtea mediterranea*	sme-mir-71c	UGAAAGACAUGGGUAGUGAGAU
miRBase	*Haemonchus contortus*	hco-mir-71	UGAAAGACAUGGGUAGUGAGAC
miRBase	*Heligmosomoides polygyrus*	hpo-mir-71	UGAAAGACAUGGGUAGUGAGAC

Nucleotides highlighted in grey represent mismatches when compared to the F. hepatica mature miRNA sequence (shown in bold text).

TABLE 4 | Species conservation of miR-46/281.

Species	miRNA	Mature miRNA Sequence	Phylum			
			Platyhelminth	Nematoda	Anthropoda	Mollusc
Fasciola hepatica	**fhe-miR-46**	**ATGTCATGGAGTTGCTCTCTACA**	+			
Schistosoma mansoni	sma-miR-281-3p	_TGTCATGGAGTTGCTCTCTATA	+			
Echinococcus granulosus	egr-miR-281-3p	_TGTCATGGAGTTGCTCTCTATA	+			
Echinococcus multilocularis	emu-miR-281-3p	_TGTCATGGAGTTGCTCTCT__	+			
Ascaris suum	asu-miR-46-3p	_TGTCATGGAGTTGCTCTCTTCA		+		
Panagrellus redivivus	prd-miR-46-3p	_TGTCATGGAGT_GCTCTCTTA_		+		
Haemonchus contortus	hco-miR-46	_TGTCATGGAGTCGCTCTCTTCA		+		
Heligmosomoides polygyrus	hpo-miR-46-3p	_TGTCATGGAGTCGCTCTCTTCA		+		
Caenorhabditis elegans	cel-miR-46-3p	_TGTCATGGAGGCGCTCTCTTCA		+		
Caenorhabditis briggsae	cbr-miR-46	_TGTCATGGAGGCGCTCTCTTCA		+		
Caenorhabditis brenneri	cbn-miR-46	_TGTCATGGAGGCGCTCTCTTCA		+		
Caenorhabditis remanei	crm-miR-46-3p	_TGTCATGGAGTCGCTCTCTTC_		+		
Pristionchus pacificus	ppc-miR-46	_TGTCATGGAGTCGCTCTCTTC_		+		
Tribolium castaneum	tca-miR-281-3p	_TGTCATGGAGTTGCTCTCTTT_			+	
Acyrthosiphon pisum	api-miR-281	_TGTCATGGAGTTGCTCTCTTT_			+	
Bombyx mori	bmo-miR-281-3p	ACTGTCATGGAGTTGCTCTCTT__			+	
Branchiostoma floridae	bfl-miR-281	_TGTCATGGAGTTGCTCTCTTTT			+	
Tetranychus urticae	tur-miR-281-3p	_TGTCATGGAGTTGCTCTCTTTC			+	
Manduca sexta	mse-miR-281	CTGTCATGGAGTTGCTCTCTTT_			+	
Culex quinquefasciatus	cqu-miR-281-3p	_TGTCATGGAATTGCTCTCTTT_			+	
Lottia gigantea	lgi-miR-281-3p	_TGTCATGGAGTTGCTCTCTTTA				+

Nucleotides highlighted in gray represent mismatches when compared to the Fontenla et al/miRbase F. hepatica mature miRNA sequence (shown in bold text).

scenario can be applied to the miRNAs characterized as miR-279, miR-67, and miR-1993, which are now listed as miR-61, miR-307, and miR-2162 respectively on miRBase.

This compilation and comparative analysis of the Fasciola miRNome has highlighted the impact and need for appropriate annotation of all flatworm miRNAs. The complexity of miRNA biogenesis giving rise to isomiRs will add to these challenges in annotation and subsequent curation. It must be noted that confirmation and authenticity of miRNAs is largely dependent on available sequencing data, and therefore will improve as more sequencing data is generated and made available. There are currently 38 Fasciola miRNAs listed in miRBase, which is presently the only repository of flatworm miRNAs. As more sequences become available, it is likely that Fasciola will feature within other curated databases such as MirGeneDB and miROrtho. However, it is clear from this compilation of studies, that a universal system of naming needs to be accepted by the wider Fasciola research community for the sake of clarity. The exciting outcome of adopting a uniform system of annotation is the release of a fully curated and annotated Fasciola miRnome for all life stages.

PREDICTED GENE TARGETS FOR *FASCIOLA HEPATICA* MIRNAS REVEAL A HIGH DEGREE OF REDUNDANCY IN THE REGULATION OF IMMUNE CELL ACTIVATION

Despite the characterization of *F. hepatica* miRNAs, beyond an acknowledgement that some share homology to mammalian miRNAs involved in the regulation of immune responses (33–37, 38, 40), there has been no detailed exploration of the possible mammalian genes that might be targeted by parasite miRNAs as a mechanism for controlling the host immune response. Given that a single miRNA can have multiple targets and that the 3'UTR of the mRNA can include various binding sites for multiple miRNAs, predictive tools provide an in silico method for screening potential targets followed by experimental validation.

As the miRBase *F. hepatica* miRNAs were identified within the NEJ stage, we specifically explored potential mammalian gene targets that were specific to innate immune cells. It is important to acknowledge that highly characterized species such as human, mouse and rat dominate the curated literature that support these predictive tools and databases. Although *F. hepatica* is typically regarded as a parasite of sheep and cattle, due to the lack of information on the biological contribution of genes in these species it is not possible to accurately apply the predictive tools against these hosts. Accordingly, predictions of mammalian gene targets for Fasciola derived miRNAs were performed against a *Homo sapiens* background. Given that the bovine genome is regarded as 80% similar to the human genome (49), and that the profile of immune response to *F. hepatica* is common across all host species (50), it is probable that the predicted gene targets are common to the regulation of immune response during infection for multiple mammalian hosts of Fasciola.

We analyzed the mature sequences of the 38 miRBase Fasciola miRNAs for custom target prediction using miRDB (mirDB.org.) This database was selected as it utilizes the bioinformatic tool MirTarget, which is a compendium of experimentally validated miRNA targets (51). Genes with a target score of >60 were selected for further analysis using

InnateDB (innatedb.com), an integrated analysis platform that has been specifically designed to facilitate systems-level analyses of pathways and genes specific to mammalian innate immune cells (52). Thus, gene targets were further filtered according to their association with immunological responses of innate cells including dendritic cells (DCs), eosinophils, innate lymphoid cells (ILCs), macrophages, monocytes, and neutrophils. Within the miRBase Fasciola miRNome, 26 of the 38 miRNAs were predicted to target genes within all of the innate immune cells examined (**Figure 2**). Of these cells, DCs, eosinophils and neutrophils were the most targeted with 17, 14, and 8 miRNAs, respectively, identified as having gene targets within

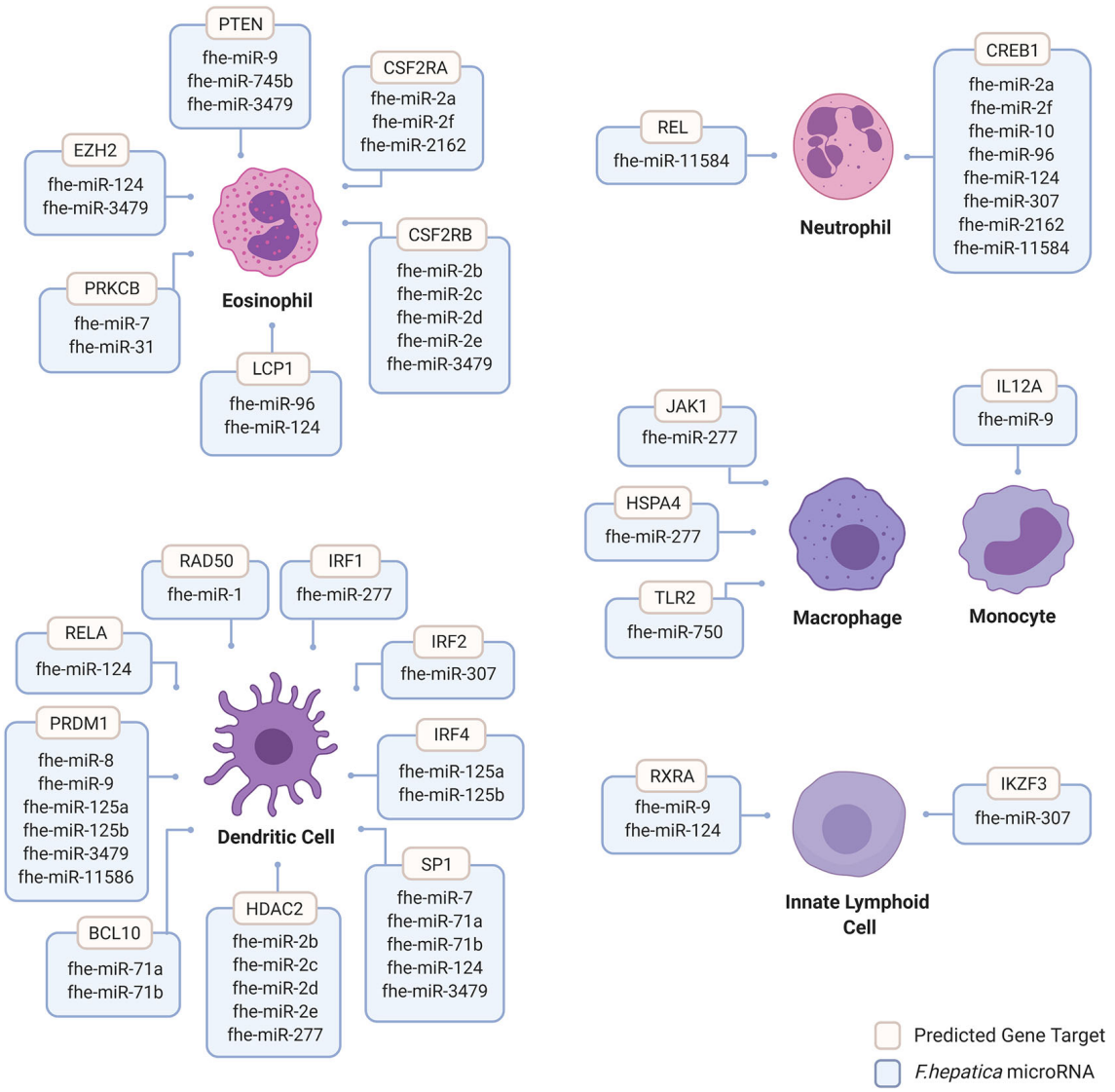

FIGURE 2 | Human innate immune cell genes predicted to be targeted by *Fasciola hepatica* microRNAs. Human genes targeted by *F. hepatica* miRNAs were predicted using the miRNA target prediction tool and database miRDB (mirdb.org). Gene targets are considered with >60 target score produced by miRTarget in miRDB with *Homo sapiens* background using *F. hepatica* miRNA sequences featured in miRbase database version 22 (mirbase.org). Gene targets filtered for innate immune cell background including dendritic cells (DCs), eosinophils, innate lymphoid cells (ILCs), macrophages, monocytes and neutrophils were considered. Dendritic cell targeted genes include B-Cell Lymphoma/Leukemia 10 Signaling Adaptor (BCL10), Histone Deacetylase 2 (HDAC2); Interferon Regulatory Factor (IRF) 1, 2, and 4; Positive Regulatory Domain I-Binding Factor 1 (PRDM1); RAD50 Double Strand Break Repair Protein (RAD50); REL Proto-Oncogene, NF-KB Subunit (RELA) and Sp1 Transcription Factor (SP1). Eosinophil targeted genes include Colony Stimulating Factor 2 Receptor Subunit Alpha (CSFR2A), Colony Stimulating Factor 2 Receptor Subunit Beta (CSFR2B), Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2), Lymphocyte Cytosolic Protein 1 (LCP1), Protein Kinase C Beta (PRKCB) and Phosphatase and Tensin Homolog (PTEN). Innate lymphoid cell gene targets include Ikaros Family Zinc Finger 3 (IKZF3) and Retinoid X Receptor Alpha (RXRA). Macrophage gene targets include Heat Shock Protein Family A Member 4 (HSPA4), Janus Kinase 1 (JAK1) and Toll Like Receptor 2 (TLR2). Monocyte targeted genes include Interleukin 12A (IL12A). Neutrophil targeted genes include CAMP Responsive Element Binding Protein 1 (CREB1) and REL Proto-Oncogene and NF-KB Subunit (REL). Image created by Biorender.

these cells (**Figure 2**). Furthermore, this mapping revealed a high degree of redundancy as many of the miRNAs were predicted to act on the same targets, suggesting a certain selectivity to the genes that are targeted and thus the modulation to immune response that consequently occurs.

Of the 14 miRNAs acting on eosinophils, 8 were predicted to target colony stimulating factor receptor (CSF2R). This receptor is a heterodimer comprised of an alpha and beta chain. The alpha subunit contains a specific binding site for granulocyte macrophage colony-stimulating factor [GM-CSF; (53)]. The beta chain triggers signal transduction and is also present in the receptor complexes for IL-5 and IL-3 (54–56). Many aspects of eosinophil biology are controlled by GM-CSF and IL-5, acting through the CSF2R complex (57–59). Of relevance to infection with *F. hepatica*, and in particular to the excystment and migration of the NEJs, in the context of intestinal inflammation GM-CSF and IL-5 foster the survival of peripheral eosinophils, but only GM-CSF promotes the activation of effector functions (57). By targeting the expression of both subunits, the Fasciola miRNAs are capable of regulating the recruitment and functionality of eosinophils to reduce parasite killing.

Within DCs, the parasite miRNAs primarily targeted three host genes, Histone deacetylase-2 (HDAC2), PRDM1, and SP1. By regulating their expression, the parasite miRNAs would significantly impact the maturation of DCs, as both HDAC and PRDM1 promote the expression of costimulatory molecules [such as CD40, CD86 and MHC-II (60–62)] and the acquisition of CD1a (63), the hallmark of an IL-12 producing, pro-inflammatory DC. Considering the requirement for DC-derived cytokines and co-stimulatory molecules for efficient T-cell activation, regulating the expression of these genes would also inhibit the development of an effector adaptive immune response, as seen during infection with *F. hepatica*. Indeed knockdown of PRDM1 in DCs resulted in a reduced ability for these cells to induce efficient allogeneic T cell proliferation (62), and inhibition of HDACs in DCs, led to the differentiation of T cells with an anergic phenotype (64).

All eight of the Fasciola miRNAs that were determined to target neutrophils, were predicted to target only a single gene; CREB1. This gene encodes a transcription factor that has a central role in the regulation of the functional response of neutrophils (65–68). Specific mutation of this gene, results in a decreased ability of neutrophils to generate inflammatory chemokines and cytokines (65), which reflects the reduction in neutrophil activity when exposed the parasite.

This analysis strongly supports the hypothesis that parasite-derived miRNAs can regulate host genes, and by doing so can manipulate the functional activity of all immune cells. While, the use of a single miRNA target prediction tool (miRDB) has its limitations, particularly on non-model organisms such as the liver fluke, using it in combination with other databases such as (InnateDB and Reactome) captured preliminary insights to the immunoregulatory functions of Fasciola miRNA. More so, the revelation that multiple parasite miRNAs targeted the same genes within eosinophils, DCs and neutrophils suggest that Fasciola miRNAs have been conserved to regulate specific anti-parasitic immune pathways during an infection. With knowledge of the miRNA sequences, this hypothesis can be experimentally tested.

As this review was being finalized, a similar consideration of the biological role of the 46 of the most abundant miRNAs in adult parasites and EVs was published. Using a combination of TargetScan and PITA to predict gene targets within the genome of cattle and humans, and applying Reactome and KEGG pathway analysis to these targets, identified 44 and 23 genes respectively, that were characterized as having a role in the immune system (39).

CONCLUSION

We specifically focused on the innate immune cells as the miRBase list of miRNAs has been validated for the NEJs, a stage of the parasite that is most closely associated with the immediate host immune response. However, based on this analysis the identification of gene targets within every cell suggests that the parasite's ability to modulate host cell behavior is widespread. Therefore, by characterising the expression of specific miRNAs at each stage of the parasite's life cycle, this analytical workflow could be extended to other biologically relevant host cells, such as intestinal epithelium, liver cells, B and T lymphocytes, and the bile duct. The outcomes from this analysis would provide a holistic view of the host-parasite crosstalk.

Before undertaking this type of analysis, a definitive characterization of the Fasciola miRNome is required. Compiling the sequencing data and annotations from all five independent studies reporting the identification of the parasite's miRNAs revealed a surprising level of variation in the sequences and annotation. This outcome illustrates the importance of the research community working together to compare data and to submit these analyses for verification by an independent body. In this manner, the parasite's miRNome will be correctly catalogued (and reviewed), to be used in future studies as a reference point for comparison and continued expansion. At this stage, the Fasciola research field requires the accurate curation of a completed Fasciola genome and robust guidelines for processing of miRNA sequencing data. If we can generate these tools, variations in sequencing will be reduced and more importantly, it will permit the precise identification of parasite miRNAs within immune cells *in vivo* during an infection. This is an essential piece of defining evidence to fully support the hypothesis that Fasciola miRNAs are manipulating host immune cell function. Furthermore, accurate knowledge of the parasite miRNA sequences would also support the targeted knockdown of specific miRNAs within the NEJs, to determine the relative importance of each in supporting the safe passage of these juvenile parasites to the liver.

One additional consideration is the verification that different isomiRs within the same family of miRNAs may be differentially expressed according to the life stage of the parasite. This

possibility was only uncovered as we compared all of the sequences for each annotated miRNA across all of the published studies. It has been shown that the transcriptome of the worm varies greatly between the NEJ and adult stage (69). It is thus, not surprising that the parasite may require different miRNAs to regulate the expression of different parasite genes as it matures. If these isomiRs regulate different targets, this would also represent an adaptation to different immunological environments as the parasite migrates from intestine, through the liver to the bile duct. Further, the fine tuning of single nucleotide in parasitic miRNAs for immune regulation would be a novel area of gene regulation.

In conclusion, the continued characterization and functional analysis of miRNAs in *F. hepatica* will create a new mechanistic framework for the regulation of host immune responses by parasite-secreted miRNAs. This information will also reveal the molecular biological pathways that are unique to parasitism and will be of enormous benefit to the development of novel strategies for infection control.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. Details of the original publications and data are provided in the article.

AUTHOR CONTRIBUTIONS

SD conceived the idea. SD, AR, and HN searched the literature and drafted the manuscript. NT commented on the structure of manuscript, provided critical intellectual input, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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