Helminth Antigen Exposure Enhances Early Immune Control of *Mycobacterium tuberculosis* in Monocytes and Macrophages

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Abstract

Helminth and *Mycobacterium tuberculosis* (Mtb) coinfection is common and suggested to influence the risk of developing active tuberculosis (TB). It is known that helminths in contrast to TB induce a strong Th2 response in the host. However, the direct impact of helminth antigen exposure on host immunity against TB is largely unknown. Our aim was to explore the effects of helminth antigen exposure on the early immune control of Mtb in monocytes and macrophages. *Ascaris lumbricoides* (ASC) and *Schistosoma mansoni* (SM) protein antigens were used to study the immediate effect of helminth antigen exposure in monocytes, on monocyte-to-macrophage differentiation, or mature macrophages, in the control of virulent Mtb H37Rv. Pre-exposure of peripheral blood mononuclear cells reduced Mtb growth in monocytes, especially with SM, but no Th1/Th2 cytokines or activation markers indicated involvement of T cells. Monocytes exposed before maturing into macrophages reduced

Mtb growth in macrophages (ASC), and pre-exposure of mature macrophages reduced (ASC) or kept Mtb growth at control levels (SM). This in vitro model shows how helminth infection directly affects the monocyte-macrophage axis at an early stage before cell-mediated immunity develops. During acute helminth coinfection or when helminth antigen concentration is elevated at the site of Mtb infection, these helminths provide an enhanced control and killing of Mtb owing to the direct stimulatory effect of helminth antigens on phagocytic cells.

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Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is a major persisting global health challenge and one of the deadliest infectious diseases, resulting in 10 million people developing active disease and 1.2 million deaths from Mtb in 2018 (WHO report, 2019). In addition to the high morbidity and mortality assigned to TB, recent evaluation reported that one-quarter of the world's population is latently infected with Mtb [1]. On a similar scale, it has been estimated that up to one-third of

the world's population is infected with helminths and that TB and helminths overlap geographically, with 20–35% of people with TB being infected with helminths [2, 3] (WHO, 2017). Despite multiple studies showing an impact of helminths on cell-mediated immunity, there is yet limited evidence directly linking helminthic infections to the reactivation of latency or the impact on TB disease progression [4]. In fact, recent studies [5, 6] show that helminth exposure may also enhance immunity to mycobacteria and that there is a need to focus on the effects of specific helminth species and at different stages of Mtb infection. Thus, there is a lack of understanding in what effect helminths and their antigens have on innate immune responses to Mtb and more specifically their effect on the main effector cells for Mtb – the macrophage.

It is well established that an efficient Th1 immune response is required to control intracellular pathogens such as Mtb. Activation and polarization of macrophages toward an M1-type is believed to play a central role. Part of the proinflammatory M1 macrophage signature is the release of TNF, IL-1 β , IL-12, and IL-6, whereas M2 macrophages release IL-10 and TGF- β . Helminth infection skews the immune response towards a Th2 response with the common signature of IL-4, IL-10, IL-13, and TGF- β , which have been linked to induction of M2 macrophage polarization [7]. Studies showing a negative effect of helminth infection on the host defense against Mtb include reduced BCG vaccine efficacy [8], attenuation of Mtb-specific Th1 responses [8–10], downregulation of costimulatory molecules [11], triggering of T-cell anergy [12, 13], and reduced therapeutic response in patients with pulmonary TB [14]. However, helminth infection may not affect tuberculin test results in humans [15], mycobacterial infection [16], or even enhance the control of mycobacteria [5, 6]. Thus, the immune responses described during Mtb-helminth coinfection are variable and depend on the helminth species or experimental design [17]. Studies with *Nippostrongylus brasiliensis*-mycobacterial coinfection show contrasting results with respect to Mtb growth control [5, 18, 19], showing that there is large heterogeneity in the effect of helminth-Mtb interaction on host immunity.

In the current study, we have focused on the effect of 2 prevalent helminths, the nematode *Ascaris lumbricoides* and the trematode *Schistosoma mansoni*, which infect over 1 billion people worldwide [20–23]. *Schistosoma mansoni* induces a strong Th2-type response, and treatment with *Schistosoma mansoni* soluble egg antigens has been shown to reverse experimental colitis [24]. *Ascaris lumbricoides* infection shows a similar pattern with a Th2

response in concert with dendritic cells, associated with the production of IL-4 and IL-5 [25, 26]. The role of *Ascaris lumbricoides* infection is not well characterized in Mtb infection, but the effect of *Ascaris lumbricoides* on the tuberculin skin test in children [27, 28] suggests that *Ascaris lumbricoides* may reduce the immune response following Mtb exposure.

The aim of this study was therefore to analyze the early effects of *Ascaris lumbricoides* whole worm antigen (ASC) and *Schistosoma mansoni* soluble egg antigen (SM) during virulent Mtb H37Rv infection along the monocyte-macrophage axis, that is, the effect on undifferentiated monocytes, the effect on monocyte-to-macrophage differentiation, and the effect on mature monocyte-derived macrophages. Our in vitro model shows that helminth infection directly affects the monocyte-macrophage axis at an early stage before cell-mediated immunity develops.

Materials and Methods

Ethics Statement

Normal human serum, heparinized whole blood and buffy coat preparations from whole blood that was the source of peripheral blood mononuclear cells (PBMCs), and monocytes were from the Linköping University Hospital blood bank and Jönköping hospital blood bank, and all donors were anonymized. All the work was carried out in accordance with the Declaration of Helsinki, not requiring a specific ethical approval according to paragraph 4 of the Swedish law.

Helminth Antigens

To evaluate the overall effect of helminth exposure and not restrict interpretations to one specific protein from each respective helminth, whole worm protein extracts of *Ascaris lumbricoides* (ASC) from Allergen AB Thermo Fisher Scientific and *Schistosoma mansoni* soluble egg antigen (SM) donated by Professor Mike Doenhoff, Nottingham University, Nottingham, UK, were used. The protein concentration of each antigen was determined by Bradford assay and stored at -80°C till used. Based on initial titration experiments, the helminth antigen concentration chosen was validated to have no effect on human monocyte-derived macrophages (hMDMs) viability and separately to have no effect on Mtb growth in absence of cells (data not shown). The pre-exposure time with helminth antigens before Mtb infection for PBMCs was set to 20 h to limit the time for monocytes to differentiate before infection (model 1), and for mature macrophages (model 3), we used 48 h in accordance with our previous work [17] and that of the work with macrophage exposure by others [29].

Infection of PBMCs

For infection of PBMCs, SepMate™ PBMC isolation from freshly drawn blood (Na-heparin tubes) was used only in order to accelerate the PBMC isolation process. For standardization, the number of monocytes was kept constant as this is the dominant

Fig. 1. Early models for timing of helminth antigen exposure and Mtb infection. To cover several helminth exposure scenarios along the monocyte-macrophage axis, 3 models were used. PBMC, from Swedish healthy blood donors, was exposed with ASC or SM helminth antigens, and 20 h later, Mtb was added for 5 days (without antigen removal) (model 1). PBMC was the source of monocytes for generating monocyte-derived macrophages (models 2–3), with the difference that PBMCs were exposed before monocyte selection and differentiation to macrophages (model 2), or fully mature macrophages were exposed for 2 days (model 3) before the 1.5-h

Mtb infection and 5 days incubation. The total time from PBMC isolation to Mtb infection was 9 days for both models 2 and 3. Post-Mtb infection treatment in model 3 was with or without conditioned medium from the 48-h pre-exposure with helminth antigens. Mtb MOI = 5 for model 1 and MOI = 4 for models 2–3. PBMC, peripheral blood mononuclear cell; MOI, multiplicity of infection; Mo, monocytes; Mo sel., selection of monocytes by adherence; MQ, monocyte-derived macrophages; ASC, *Ascaris lumbricoides* total protein antigen; SM, *Schistosoma mansoni* soluble egg antigen; Mtb, luciferase-expressing Mtb H37Rv.

phagocytic cells in the PBMC mix. SepMate™ tube (Stem Cell Technologies, Grenoble, France) with 15 mL of LymphoPrep was layered with a 1:1 mix of heparinized diluted blood and PBS with 2% heat-inactivated FBS. The SepMate™ tubes were centrifuged at 1,200 g for 10 min, and the isolated PBMCs were washed twice with Krebs-Ringer Glucose buffer (KRG) without calcium and then resuspended in antibiotic-free complete DMEM medium (specified below). The cells were counted with the Swelab Alfa cell counter (Boule Medical AB, Stockholm, Sweden) and plated at a concentration of 10,000 monocytes per well (about 10⁵ PBMCs) in triplicates in a 96-well plate and rested for 20 h with or without 5 µg/mL of helminth antigen and then infected with luciferase-expressing Mtb without removal of helminth antigen or change of medium. For infection of PBMCs, an add-on approach was used (model 1), and for infection of hMDMs (models 2–3 explained below), the bacteria were removed after 1.5 h and extracellular bacteria removed by washes in plain DMEM and cells incubated in antibiotic-free complete DMEM medium for the indicated time. The 3

Mtb infection models for analyzing early effect of helminth antigen exposure were (1) exposure of PBMCs and Mtb infection before any maturation of cells (PBMC model), (2) exposure of PBMCs before monocyte-to-macrophage maturation and Mtb infection (long-term exposure), and (3) a tissue macrophage model using helminth antigen exposure of mature macrophages prior to Mtb infection (short-term exposure) and are shown in Figure 1 and further explained below.

Monocyte-to-Macrophage Maturation in Presence of the Helminth Antigen and Conditioned Medium

Model 2. PBMCs were isolated by density gradient and hMDMs generated essentially as previously described [17], but with an extended adhesion time allowing for a helminth antigen exposure of monocytes in presence of surrounding PBMCs. In brief, the buffy coat was diluted with 0.9% NaCl and layered on LymphoPrep (Alere Technology AS, Oslo, Norway) and centrifuged for 40 min at 480 g at room temperature. PBMCs were collected and

washed 3 times with cold PBS, pH 7.3 containing heparin (0.5 IE/mL), followed by washes in cold KRG without calcium. PBMCs were split into 3 groups (control, ASC treated, and SM treated). Cells were allowed to adhere in 6-well plates overnight in DMEM medium containing 10% normal human serum, HEPES, L-glutamine, penicillin and streptomycin (complete DMEM medium), and with or without 5 µg/mL of helminth antigen for 20 h, and thereafter the medium was collected and the nonadherent cells washed away. The collected medium from the overnight treatment was centrifuged at 300 g for 5 min to obtain a cell-free conditioned medium. After the 20-h exposure, monocytes were cultured in 50% of the conditioned medium and 50% of the fresh complete DMEM medium, and at day 3, the same procedure was repeated, and 50% of the conditioned medium was added back and cells cultured until day 8. On day 8, the hMDMs were detached and plated in triplicates at 100,000 cells per 96 well in antibiotic-free complete DMEM and rested overnight, before hMDM phenotype analysis by flow cytometry or infection with Mtb was performed on day 9.

Generation of hMDMs before Helminth Antigen Stimulation

Model 3. For helminth antigen stimulation of mature hMDMs, PBMCs isolated from buffy coats as above were plated and allowed to adhere for 1.5–2 h. The nonadherent cells were removed by washes with KRG, and adherent monocytes were cultured in the complete DMEM medium for up to 6 days with medium change at day 3. At day 6, the mature hMDMs were detached and plated in triplicates at 100,000 cells per 96 well in antibiotic-free complete DMEM medium and rested overnight. From day 7 to 9 (48 h), the hMDMs were treated with or without 5 µg/mL of helminth antigen in antibiotic-free complete DMEM medium, and thereafter the hMDM phenotype was analyzed by flow cytometry, or these short-term-treated hMDMs were infected with Mtb on day 9.

Bacterial Preparation and Infection

Mtb H37Rv (ATCC) carrying a luciferase construct (Mtb luciferase) [30] was cultured in Middlebrook 7H9 broth supplemented with 0.05% Tween-80 and 10% albumin-dextrose-catalase enrichment (ADC; Becton Dickinson, Franklin Lakes, NJ, USA) and 100 µg/mL hygromycin (Sigma) at 37°C. Log phase bacterial cultures were centrifuged twice for 5 min at 5,000 g, and pellets were separated by passage through a sterile 27-gauge needle with syringe 10 times in PBS with 0.05% Tween-80 after each centrifugation step to generate a clump-free uniform bacterial suspension. The OD₆₀₀ value of the bacterial suspension in plain DMEM medium was measured to determine the concentration, and the bacteria were diluted in antibiotic-free complete DMEM medium to the appropriate multiplicity of infection (MOI, as specified in figure legends).

Fig. 2. Helminth antigen pretreatment of PBMCs induces controlled growth of Mtb and regulates proinflammatory cytokines. PBMCs were treated with ±5 µg/mL *Ascaris lumbricoides* (ASC) or *Schistosoma mansoni* (SM) protein antigen for 20 h and then infected with luciferase-expressing Mtb (Mtb-lux; MOI 5) (model 1). **a** Total Mtb growth at 2 and 5 days after infection, as RLU. **b** Concentration of cytokines in the cell culture supernatants at day 2

Cell Viability of hMDMs and Bacterial Luciferase Measurement

The hMDM viability and Mtb luciferase were measured as described earlier [17]. In brief, cell supernatants were collected, and cells were washed with PBS. For determining the viability, the cells were incubated with 0.4% calcein-AM for 30 min, and the fluorescence was recorded. For quantification of total Mtb replication, the luciferase signal in both supernatant and cell lysate was measured using decanal (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. Uninfected hMDMs were used as reference for the viability and for background subtraction of the Mtb luciferase signal. In order to calculate the total Mtb growth (intracellular and extracellular bacteria) in each well, the relative luminescence values from lysate and supernatant were summed up. The median value of each triplicate was then presented using the absolute of the relative luminescence unit or normalized to the day 0 medians (day of infection) of the same donor and treatment to create an Mtb fold change to day 0. The current work uses the same strain of virulent Mtb and the same protocol for detection, for which we previously determined that 1 relative luminescence unit correspond to approximately 2 bacteria by CFU plating [31].

T-Cell Activation Marker Analysis by Flow Cytometry

The cells from triplicate wells were pooled and centrifuged for 5 min at 300 g at 4°C. For flow cytometry staining, CCR7 PE, CTLA4 PerCP-Cy5.5, CD45RA AF647, PD1 AF700, CD4 Pacific Blue, OX40 PerCP-Cy5.5, CD28 FITC, CCR9 PerCP-Cy5.5, ICOS AF700, HLA-DR AF488, TIM3 FITC, and CD38 PE, all from BioLegend (San Diego, CA, USA), CD40L FITC (BD Pharma), CD3 Pacific Orange (EXBIO Praha), and CCR4 AF700 (R&D Biosystems) were used. The cell-free supernatant was stored at –80°C for cytokine analysis, and the cell pellets were resuspended and stained with 30-µL FACS antibody cocktail for 30 min and then washed with FACS buffer. Further, the cells were fixed with 4% paraformaldehyde (PFA) for 30 min before acquired using a Gallios flow cytometer (Beckman Coulter), and total marker expression (MFI, mean fluorescence intensity) was analyzed using FlowJo version 10.5.3.

Analysis of Polarization of hMDMs

Flow cytometric analysis of macrophage phenotype was carried out according to our previous work [17]. hMDMs analysis was performed just prior to Mtb and 24 h after Mtb infection. The cells were gently detached on the day of analysis using Accutase (Stem Cell Technologies, Grenoble, France) and centrifuged at 500 g for 5 min before they were stained with an antibody cocktail containing CD206 FITC, CD163 PE, CCR7 AF647, CD86 AF750, and CD14 Pacific Blue to enable analysis of the M1/M2 spectrum. After staining, infected and uninfected hMDMs were fixed with 4% PFA

after infection (**b**) or at day 5 after infection (**c**). Data presented as mean ± SD of 8 independent donors. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, using repeated measures ANOVA with Tukey's multiple comparison test. Mtb, *Mycobacterium tuberculosis*; PBMCs, peripheral blood mononuclear cells; RLU, relative luminescence unit; MOI, multiplicity of infection.

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for 30 min at room temperature. All the samples along with unstained or fluorescence minus one control stained were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed using Kaluza software version 1.2. MFI values with subtracted control and percent marker positive cells using controls for setting positive gates were determined.

Cytokine Analysis of Cell Culture Supernatants by Cytokine Bead Array

Human PBMCs isolated from buffy coats were stimulated with or without 5 µg/mL of ASC or SM antigen for 20 h at 37°C in 6-well plates. Aliquots of these supernatants were saved and stored at -80°C till analyzed, and the remaining were used as conditioned medium for the monocyte-to-macrophage maturation. In conjunction with the previous work, for their effect on monocyte differentiation, we selected certain cytokines for analysis such as IL-4, IL-5, IL-6, IL-1β, IL-10, IL-13, TNF, IFN-γ, TGF-β, GM-CSF, IL-33, and IL-27. IL-33, IL-27, and GM-CSF were determined by using the multiplex bead-based assay panel according to the manufacturer's protocol (LEGENDplex™ from BioLegend), and remaining cytokines were analyzed by the cytometric bead array from BD Biosciences, San Diego, CA, USA, according to the manufacturer's protocol. Bead fluorescence was detected by a Gallios flow cytometer (Beckman Coulter), and cytokine concentration was analyzed using Kaluza software version 1.2 (Beckman Coulter, Brea, CA, USA).

For the analysis of the day 2 and day 5 infected PBMCs, supernatants were assayed by the Human Th1/Th2 Cytokine Kit II (BD Biosciences) according to the manufacturer protocol. In addition, the supernatants from the infected PBMC were assayed for IL-1β as specified below. The day 5 postinfection hMDM supernatant analysis was customized according to our need using the human soluble protein master buffer kit from BD Biosciences (San Diego, CA, USA) and including the flexi cytokines IL-1β, IL-6, TNF, IL-12/IL-23/p40, and IL-10, and the protocol was followed as per the manufacturer's instructions. After staining, the samples were fixed with 4% PFA for 30 min and acquired using a Gallios flow cytometer (Beckman Coulter). The concentration of each cytokine was determined from a standard curve analyzed by Kaluza 1.2 software (Beckman Coulter).

Statistical Analysis

All statistical analyses were performed with GraphPad prism software. The data passed normality testing and were considered normally distributed. The data were presented as means ± SD and analyzed using repeated measures ANOVA with the post hoc Tukey's multiple comparison test, unless otherwise specified in legend. *p* values <0.05 were considered significant.

Fig. 3. ASC antigen-pretreated PBMCs have delayed T-cell activation upon Mtb infection, and SM did not elicit a T-cell response. T-cell activation markers in PBMCs treated with ±5 µg/mL ASC or SM antigen for 20 h followed by infection with luciferase-expressing Mtb MOI 5 for 2 or 5 days (as in Fig. 2; model 1). PBMCs were washed and stained with T-cell markers against CD3, CD4, CD45RA, HLADR, CD38, CTLA4, and PD1, analyzed by flow cytometry, and total marker expression expressed as MFI. T-cell activation marker analysis in Mtb-infected control (Cont), ASC, and SM-pretreated cells in the CD4 naïve (CD3⁺CD4⁺CD45RA⁺) and

Results

ASC and SM Antigens Show Distinct Modulatory Effects in PBMCs during Coinfection with Mtb

To evaluate early effect of helminth antigen exposure along the monocyte-macrophage axis, 3 models were used. As a first step to understand the immunomodulatory effect of helminths on the innate control of Mtb, PBMCs from blood donors were exposed to helminth antigens for 20 h prior to infection with live virulent Mtb H37Rv. Since monocytes constitute the dominating phagocytic cells in the myeloid fraction of the PBMCs, this offered a way to study the impact of lymphocytes during the monocyte Mtb infection and their influence during early helminth/Mtb coinfection. Both at day 2 and day 5 after Mtb infection, the helminth antigen SM significantly improved intracellular control of Mtb (Fig. 2a). The ASC antigen pretreatment showed the same tendency of controlling Mtb (Fig. 2a). To explore potential mechanisms for how SM improved control of Mtb in monocytes, both the cytokine pattern and activation state of the T cells in the PBMC-culture were analyzed. The levels of IL-2, IL-4, and IL-10 remained unchanged at day 2 and day 5 after Mtb infection (Fig. 2b, c) and did not increase compared to supernatants from PBMCs before Mtb infection (i.e., after the initial 20-h pretreatment; online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000512279). IFN-γ, known to be involved in the protective immune response toward Mtb [32], increased with infection and was at similar levels in control and the SM pretreated group at both 2 and 5 days after infection. ASC pretreatment, on the other hand, resulted in a >50% reduction of IFN-γ at day 2 after infection, explaining the partial lack of Mtb control by ASC. The proinflammatory cytokines IL-6, TNF, IL-1β, and IL-12p40 all increased during infection with Mtb, and from these data, it was also apparent that the SM-mediated control of Mtb in monocytes was mirrored by a significant reduction of TNF,

memory (CD3⁺CD4⁺CD45RA^{neg}) T cells within the lymphocyte gate at day 2 after infection (a) and day 5 after infection (b). c T-cell activation marker analysis in CD3⁺CD4^{neg} T cells within the lymphocyte gate, as proxy for CD8 T cells at day 2 and day 5 after infection. Data presented as mean ± SD from 12 independent donors. *, *p* < 0.05, using repeated measures ANOVA with Tukey's multiple comparison test. Mtb, *Mycobacterium tuberculosis*; PBMCs, peripheral blood mononuclear cells; MFI, mean fluorescence intensity; MOI, multiplicity of infection.

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IL-1 β , and IL-12p40 at day 2 after infection in the SM pretreated group. TNF and IL-12p40 levels remained reduced in the SM group also at day 5 after infection. From these data, we can conclude that SM antigen exposure induced enhanced control of Mtb replication with a concomitant downregulation of TNF and IL12/23p40 secretion and also transiently affected IL-1 β . ASC antigen did not significantly alter Mtb replication in the PBMC model.

Fig. 4. Monocytes exposed with ASC during their maturation into hMDMs acquire enhanced capacity to control Mtb. PBMCs were treated with $\pm 5 \mu\text{g/mL}$ ASC or SM antigen for 20 h, before the adherent monocytes were cultured for 8 days into hMDMs using 50% conditioned cell-free medium from the initial 20-h exposure (long-term treatment; model 2). Thereafter, the hMDMs were replated without conditioned medium and infected with luciferase-expressing Mtb at MOI 4 for 1.5 h, before extracellular bacteria were removed and hMDMs incubated for 5 days. The total luminescence signal was measured at day 0 (after 1.5 h of infection) and at day 5 after infection (p.i.). The fold increase from day 0 to day 5 is presented. Total luminescence values expressed as day 5 p.i. over day 0 (left), and hMDM viability at day 5 p.i. determined by calcein-AM uptake (right). Data presented as mean $\pm$ SD from $n = 8$ independent donors. *, $p < 0.05$, using repeated measures ANOVA with Tukey's multiple comparison test. PBMCs, peripheral blood mononuclear cells; hMDMs, human monocyte-derived macrophages; Mtb, *Mycobacterium tuberculosis*; MOI, multiplicity of infection.

Fig. 5. ASC long-term treatment induces CD206 expression in macrophages, whereas SM-induced polarization is more evident after Mtb infection. **a** PBMCs were treated with $\pm 5 \mu\text{g/mL}$ ASC or SM antigen for 20 h, before the adherent monocytes were cultured for 8 days into hMDMs using 50% conditioned cell-free medium from the initial 20-h exposure. Thereafter, the hMDMs were replated without conditioned medium and rested overnight (as in Fig. 4), and macrophage polarization was analyzed by flow cytometry (uninfected) or the rested macrophages infected with luciferase-expressing Mtb at MOI 5 for 24 h before being analyzed (infected). Results show the MFI values of the M1/M2 surface mark-

ASC Antigen Treatment Leads to Modest T-Cell Activation and SM Does Not Elicit Early T-Cell Responses

To investigate if T-cell activation was part of the enhanced Mtb control in monocytes during helminth antigen exposure of PBMCs, CD4 T cells were first analyzed for changes in expression levels (MFI) of costimulatory, coinhibitory, and activation markers. There were no changes in the CD3⁺CD4⁺ T cell (CD4 T cells) levels of CD40L, CTLA4, PD1, TIM3, OX40, ICOS, CD28, CCR9, and CCR4 after ASC or SM antigen pretreatment and infection with Mtb for 3 days (online suppl. Fig. 2). HLADR, CD38, CTLA4, and PD1 expression was analyzed in both CD4 T cells and CD3⁺CD4^{neg} T cells at day 2 and day 5 after infection to further delineate the effect of helminth antigens on T-cell activation. The activation marker CD38 was marginally but significantly upregulated in response to ASC treatment at day 5 (Fig. 3b), but not at day 2 after infection (Fig. 3a), with enhanced expression in both the naive and memory compartments of CD4 T cells. CD3⁺CD4^{neg} T cells showed a similar delayed expression of CD38 at day 5 after infection with ASC treatment (Fig. 3c). For SM, there was no modulation in CD3⁺CD4^{pos/neg} T cell activity in any of the markers studied that would indicate that the enhanced control of Mtb in monocytes during SM antigen exposure of PBMCs was involving activation of T cells.

Monocytes Differentiated to Macrophages in Presence of ASC Helminth Antigen Show Enhanced Ability to Control Mtb Replication

As T cells showed negligible involvement in this in vitro model, we next examined the effect of helminth antigens on monocyte differentiation and maturation into macrophages prior to infection with Mtb (model 2). PBMCs were exposed to helminth antigens for 20 h, and then adhered monocytes were left to differentiate to

ers in uninfected and infected macrophages. **b** Cytokine profile in the cell-free culture supernatants of the 20-h antigen-exposed PBMCs and conditioned medium used for the macrophage culture in Figures 4 and 5a; model 2. Data presented as mean $\pm$ SD from 18 independent donors for uninfected and 6 independent donors for infected in **a** and from $n = 8$ independent donors in **b**. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, using repeated measures ANOVA with Tukey's multiple comparison test. PBMCs, peripheral blood mononuclear cells; hMDMs, human monocyte-derived macrophages; Mtb, *Mycobacterium tuberculosis*; MFI, mean fluorescence intensity; MOI, multiplicity of infection.

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macrophages for a total of 9 days in presence of conditioned media from the initial 20-h exposure before being infected with Mtb. After infection, there was similar levels of relative luminescence of Mtb regardless of the helminth antigen pre-exposure (day 0 after infection relative luminescence values, mean $\pm$ SD: 19,298 $\pm$ 5,148, 19,566 $\pm$ 10,463, and 15,141 $\pm$ 7,910, for untreated, ASC, and SM-treated hMDMs, respectively), indicating that there was no difference in uptake of Mtb. At day 5 after infection, ASC had enhanced the capacity of macrophages to control Mtb infection, whereas SM had no effect on Mtb growth (Fig. 4). This was in contrast to the PBMC model, where SM induced Mtb protection in monocytes, and indicates that different helminth antigens affect the monocyte-macrophage axis at different stages.

Long-Term Exposure with Helminth Antigens Induces Macrophage Polarization towards an M2 Profile

The effect of this long-term exposure of helminth antigen (model 2) on macrophage M1/M2 polarization was evaluated before and during Mtb infection. Polarization was evaluated by surface staining against surface markers CD206 (mannose receptor; M2a, indicative of IL-4 macrophages), CD163 (M2c, also known as IL-10 macrophages), CCR7 (M1 macrophage marker), and CD86 and CD14 as markers for activation (or as surrogate M1 markers). Before infection, macrophages exposed to ASC for 9 days showed a significant increase in CD206 (Fig. 5a). This modulated response by ASC did not sustain after infection with Mtb. For SM, CD163 levels were significantly decreased, but after infection, CD206, CD163, CD86, and CD14 all showed a significant decrease in the SM-treated group. One obvious trend in unexposed control macrophages was the increased expression of CD14 and CD86 after Mtb infection, something that SM-exposed macrophages either did not show (CD14 stayed at the same level as before infection) or

behaved opposite to (CD86 decreased upon infection). This indicates that long-term helminth antigen exposure alone can polarize macrophages to a more M2 phenotype (ASC increased CD206) or drastically change their polarization in response to Mtb infection (SM affected several markers).

In search of possible mediators that could have skewed the monocyte differentiation (as evident in Fig. 5a), we screened the conditioned medium that was used for the macrophage culture (model 2) for possible cytokines and factors that could have affected the polarization of macrophages in this long-term exposure model. There were negligible levels for Th2 cytokines (IL-4, IL-5, and IL-13) and IFN- γ (Fig. 5b). There was no induction of IL-10 or TNF, whereas there was a significant increase in IL-6 and IL-1 β in the ASC-treated group although still at low levels. SM exposure increased IL-6 to similar levels as that for ASC. These data indicate that there was an early induction of IL-6 with both antigens and that extended exposure with the ASC antigen for 9 days enables macrophages to control Mtb replication.

Effects of Mtb Infection on Mature Macrophages following Short-Term ASC and SM Exposure

For investigating the ability of the ASC and SM antigens to influence mature macrophages (model 3), the helminth antigens were added 48 h prior to infection of macrophages or after infection. This experimental setup was used to mimicking the scenario of helminth-TB coinfection of tissue macrophages. To avoid the omission of any possible secretory products or the helminth antigen itself, the 48-h antigen conditioned macrophage medium was added back after infection, as one of the conditions tested. Similar to model 2, there was no difference in uptake of Mtb by helminth antigen pre-exposure at day 0 after infection (data not shown) although at day 5 after infection, ASC and SM antigen short-term

Fig. 6. ASC and SM antigen exposure of mature macrophages: condition and timing influence the outcome of Mtb infection. Fully matured macrophages (hMDM) were treated with ± 5 μ g/mL of ASC or SM antigen for 48 h (48-h pretreat; model 3) and then infected with luciferase-expressing Mtb (MOI 4) for 1.5 h, before being washed, and received 100% fresh medium or 50% fresh medium with 50% of its own cell-free conditioned medium from the 48-h pretreatment (CM). Alternatively, 5 μ g/mL of ASC or SM antigen was added in the fresh medium after the infection (postinfection). Total Mtb-luminescence values at day 5 after infection (p.i.) over day 0 in the ASC treatment group (**a**) and the SM treat-

ment group (**b**). Corresponding hMDM viability in the different groups was determined by calcein-AM uptake at day 5 p.i. Cytokine profile in the cell-free culture supernatants at day 5 p.i. in the ASC treatment group (**c**) and in the SM treatment group (**d**). Data presented as mean $\pm$ SD from 12 independent donors for **a**, **b** and from 6 independent donors for **c**, **d**. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$, using repeated measures ANOVA with Tukey's multiple comparison test. hMDM, human monocyte-derived macrophage; Mtb, *Mycobacterium tuberculosis*; MOI, multiplicity of infection.

Fig. 7. Short-term helminth antigen exposure of mature macrophages and effects on polarization. Fully matured macrophages (hMDM) were treated with $\pm 5 \mu\text{g/mL}$ of ASC or SM antigen for 48 h (as in Fig. 6; model 3), before being stained with surface markers for M1/M2 polarization. Additionally, the 48-h-treated hMDMs were infected with luciferase-expressing Mtb at MOI 5 for 24 h, before analyzed. Results show the MFI values of surface marker

expression in uninfected and infected macrophages. Data presented as mean $\pm$ SD from 6 independent donors for uninfected and 9 independent donors for infected hMDMs. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ using repeated measures ANOVA with Tukey's multiple comparison test. hMDM, human monocyte-derived macrophage; Mtb, *Mycobacterium tuberculosis*; MFI, mean fluorescence intensity; MOI, multiplicity of infection.

exposure resulted in distinct outcomes (Fig. 6a, b). ASC antigen pretreatment alone did not affect the replication of Mtb in macrophages, whereas adding back the pretreatment conditioned medium directly after infection resulted in a 50% reduction of the total bacterial burden compared to untreated at day 5 after infection ($p < 0.05$; $n = 12$). On the other hand, the SM antigen led to increased bacterial replication, but adding back the conditioned medium impaired bacterial replication (Fig. 6b). The viability of macrophages receiving either ASC or SM antigen followed by their respective postinfection add-back of conditioned medium was decreased, yet both had enhanced ability to control Mtb infection. As we measured total bacteria, this indicates that decreased macrophage viability not necessarily translates to reduced capacity to control Mtb; on the contrary, macrophage

apoptosis has been shown important for killing of intracellular Mtb [32].

In the conditioned medium used as postinfection treatment, none of the cytokines analyzed were induced by the 48-h short-term ASC or SM antigen pre-exposure (online suppl. Fig. 3). Coherent for both helminth antigens was that compared to having the antigen only before infection, the add-back of conditioned culture supernatants increased IL-10 and induced a decrease in a wide range of proinflammatory cytokines at 5 days after infection (Fig. 6c, d). The short-term (48 h) helminth antigen exposure of mature macrophages alone did not induce any significant changes in any of the polarization (M1/M2) markers studied (Fig. 7). After infection, SM antigen short-term exposed macrophages showed a downregulated expression of both M1 (CCR7,

CD14, and CD86) and M2 (CD206 and CD163) markers, and although less pronounced, this was a similar trend to that with long-term exposure with the same antigen (Fig. 5a).

Discussion

Previous studies by our group and others [4, 33, 34] have confirmed a link between chronic helminth infection during TB infection and an increased Th2-type immune response with a concomitant impaired immune control of Mtb. In contrast, the impact of early helminth antigen exposure on host immunity against TB is largely unknown. To this end, we used 3 helminth antigen exposure models using human-naïve PBMCs as the source of monocytes for investigating early host control of virulent Mtb growth in monocytes/macrophages. Our main result is that human phagocytes had increased ability to control virulent Mtb during early stages of helminth exposure. Additionally, *Ascaris lumbricoides* or *Schistosoma mansoni* antigens showed differential effects on M1/M2 polarization markers of monocyte macrophages and cytokine expression, which indicated a gradual transition into a Th2-type immune response.

We found that exposure with *Ascaris lumbricoides* proteins (ASC) induced an enhanced capacity to restrict Mtb growth in hMDMs, whereas *Schistosoma mansoni* soluble egg antigen exposure (SM) showed enhanced control of Mtb in monocytes/PBMCs without involving T cells (as no T-cell activation markers or Th1/Th2 cytokines were affected). In PBMCs, SM reduced the Mtb-driven release of IL-1 β , TNF, and IL-12p40, whereas ASC, which had minimal capacity to improve Mtb control in PBMCs, showed a delayed IFN- γ response and a delayed T-cell activation as shown by a small but significant CD38 increase in both CD3⁺CD4⁺ and CD3⁺CD4^{neg} T cells at day 5 after Mtb infection. Apart from this slight difference, T-cell activation by *Ascaris* antigen exposure had limited effects in the short-term PBMC model.

Our exposure models were designed to investigate early events and effects by helminth antigens on monocytes and macrophages, innate immune responses that may be important for the later cell-mediated immune response, and final outcome for the host. However, it is important to note that >2 weeks of SM exposure of naïve PBMCs is required for differentiation of CD4 T cells from their Th0 state [35], making our models difficult to interpret in view of the Th1/Th2 paradigm.

Ascaris exposure leads to an M2-type polarization of the macrophages, whilst the withheld or decreased expression of both M1 and M2 markers in the SM-treated group after infection indicated skewing toward a regulatory or M0 phenotype. It has been shown that IL-1 β can drive IL-6 production [36] and that IL-6 being a pleiotropic cytokine itself or together with IL-4/IL-13 can drive polarization of M2 or alternatively activated macrophages [37, 38]. Elevated IL-6 levels in the conditioned medium of hMDM culture could explain hMDM polarization by ASC and SM to a more M2 phenotype.

One striking feature with the conditioned media add-back experiments in the tissue macrophage model, which essentially allows for a prolonged antigen exposure, was that for both antigens it resulted in significantly elevated levels of the regulatory cytokine IL-10 after infection. The IL-10 response was not triggered due to concomitant increase in proinflammatory cytokines. IL-10 production is more coherent with regulatory macrophages that usually requires 2 signals for producing IL-10 [39, 40], and in our case, helminth antigens being the first signal and Mtb providing the second signal. Filarial cystatin has been shown to induce regulatory macrophages with an anti-inflammatory activity [41]. It has also been shown that *S. mansoni* E/S products can stimulate IL-10 production in a similar short-term helminth antigen exposure model using BMM [42], and it is known that IL-10 inhibits IL-1 β , IL-6, and TNF on a transcriptional level [43] which may partly explain our observations for reductions of those cytokines by the prolonged *Schistosoma* antigen exposure. By comparison, adding the helminth antigens after the Mtb infection had limited effect on the cytokine output and bactericidal capacity of macrophages, suggesting that it is the pre-exposure with antigens that alters the macrophage response. This may also more adequately mimic the clinical scenario where most patients in high endemic areas are colonized with helminths early in life and some of them later get infected by Mtb and in some cases develop active TB.

We have previously shown that *T. muris* and *H. diminuta* E/S products themselves can trigger a rapid release of proinflammatory cytokines from hMDMs, and when infected, they lose control over Mtb [17]. Herein, SM exposure of hMDM alone did not induce any cytokine response in our broad panels although it substantially enhanced the ensuing Mtb-driven proinflammatory IL-1 β and IL-6 cytokine secretion and reduced Mtb control in contrast to the unchanged or reduced levels of proinflammatory cytokines when adding back the conditioned media. Our observations, and those by others [44–

46], indicate that exaggerated inflammation is detrimental for Mtb control and suggests that regulating or limiting the macrophage infection-driven cytokine response can be beneficial.

In our in vitro assay, helminth antigen-stimulated macrophages showed a slight shift towards an M2 or M0 phenotype with enhanced control of Mtb, also indicating that M2 polarization is not detrimental for the control of Mtb during early exposure of macrophages. In fact, M2 macrophages have previously been shown to attenuate ongoing Mtb infection and decrease lung inflammation [47]. We have previously showed that *T. muris* and *H. diminuta* E/S products stimulate a robust M2-type polarization of macrophages but trigger an enhanced proinflammatory cytokine secretion leading to impaired control of Mtb [17]. Here, we show an M2 (with ASC) and M0 polarization (with SM), with reduced proinflammation, ultimately increasing the control of Mtb. These findings indicate that it is not the surface expression of M1/M2 markers that dictate the outcome after Mtb infection but rather the cytokine environment or effects on the infection-driven cytokine response. Moreover, the increase in IL-10 after Mtb infection shows that early effects of helminth exposure have an ability to trigger a regulatory function in macrophages.

Exaggerated inflammasome activation leads to excessive IL-1 β production in monocytes/macrophages, with uncontrolled inflammation and pathology, as seen in HIV/TB-coinfected patients that develop TB-associated immune reconstitution inflammatory syndrome after antiretroviral therapy [48]. We recently observed a link between gain-of-function polymorphisms in the inflammasome genes *CARD8* and *NLRP3* and the development of extrapulmonary TB and a poor clinical outcome among patients with active TB [49]. This indicates that optimal control of Mtb requires a tight regulation of innate immunity-related proinflammatory genes and production of IL-1 β . Indeed, SM exposure of monocytes (PBMCs) and ASC exposure of hMDMs resulted in an impaired production of Mtb-induced IL-1 β along with an enhanced control of Mtb.

Using cells from naïve donors limited the influence of cell-mediated immunity and allowed us to investigate the direct effect of early helminth antigen exposure along the monocyte-macrophage axis in an unbiased way. It was previously shown that innate immune responses to lung stage helminth infection induce alternatively activated alveolar macrophages before the onset of adaptive immunity [50]. This effect could be seen during the early and acute phases of helminth infection, for example, during

the lung phase of *Ascaris* increasing helminth antigens close to the Mtb infection site. However, as chronic gastrointestinal-restricted helminths have shown to affect the immune response to mycobacteria distal to the gut [51], this would indicate that a helminthic infestation does not necessarily need to be in close proximity to the lung for exerting effects. This study also highlights the differential outcome of helminth antigen exposure directly in monocytes and on macrophages either before or after their maturation from monocytes. Since there is a constant recruitment of phagocytes to the site of Mtb infection, all maturity levels of immune cells exist and should be studied in order to fully understand the effect of a helminth coinfection.

In conclusion, we found that exposure with *Ascaris lumbricoides* protein along several stages of the monocyte-macrophage axis induced an enhanced mycobactericidal capacity in hMDMs, whereas exposure with *Schistosoma mansoni* soluble egg antigens showed enhanced control of Mtb in monocytes/PBMCs. *This to our knowledge is the first study highlighting a beneficial role of early Ascaris lumbricoides antigen exposure in Mtb coinfection.* Increased phagocyte control of Mtb by the helminth antigens was associated with a reduction in the infection-driven proinflammatory cytokine pattern. Although both antigens to some degree polarized the hMDMs into an M2-type or regulatory macrophage, with enhanced secretion of IL-10 upon infection, this phenotype was not hampering with the intra-hMDM control of Mtb and may constitute the first events of the deviation into a Th2-type immune response.

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Statement of Ethics

The paper is exempt from ethics committee approval as all work was carried out in accordance with the Declaration of Helsinki, not requiring a specific approval according to paragraph 4 of the Swedish law.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.B. conceived and designed the experiments; S.K.T. and G.P. performed the experiments; S.K.T., G.P., and R.B. analyzed the data; S.K.T., G.P., and R.B. wrote the paper, with inputs from all authors.

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