**Chronic gastrointestinal nematode infection mutes immune responses to mycobacterial infection distal to the gut**

Running title: Inhibition of T cell responses to BCG by *H. polygyrus*

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**Abstract**

Helminth infections have been suggested to impair the development and outcome of T-helper (Th) 1 responses to vaccines and intracellular microorganisms. However, there are limited data regarding the ability of intestinal nematodes to modulate Th1 responses at sites distal to the gut. We have here investigated the effect of the intestinal nematode *Heligmosomoides polygyrus bakeri* on Th1 responses to *Mycobacterium bovis* BCG. We found that *H. polygyrus* infection localized to the gut can mute BCG-specific CD4+ T-cell priming in the both the spleen and skin draining lymph nodes. Furthermore, *H. polygyrus* infection reduced the magnitude of delayed type hypersensitivity (DTH) to PPD in the skin. Consequently, *H. polygyrus*-infected mice challenged with BCG had a higher mycobacterial load in the liver compared to worm-free mice. The excretory-secretory product from *H. polygyrus* (HES) was found to dampen IFN-γ production by mycobacteria-specific CD4+ T cells. This inhibition was dependent on the TGF-βR signaling activity of HES, suggesting that TGF-β signaling plays a role in the impaired Th1 responses observed co-infection with worms. Similar to results with mycobacteria, *H. polygyrus*-infected mice displayed an increase in skin parasite load upon secondary infection with *Leishmania* *major*, as well as a reduction in DTH responses to *Leishmania* antigen. We show that a nematode confined to the gut can mute T-cell responses to mycobacteria and impair control of secondary infections distal to the gut. The ability of intestinal helminths to reduce DTH responses may have clinical implications for the use of skin test-based diagnosis of microbial infections.

**1. Introduction**

Control of mycobacteria and other intracellular infections of macrophages are dependent on the generation of Th1 cells. Th1 cells produce IFNγ, which is required to activate macrophages into killing the infecting organism (1). Development of such responses can be measured by a delayed type hypersensitivity (DTH) skin test reactions in both mice and humans. Indeed, the Mantoux test for tuberculosis (TB) and the Montenegro test for leishmaniasis, are still used to screen for infection with *Mycobacterium* and *Leishmania* respectively. Skin test reactivity may suggest the generation of protective immune responses, but depending on the size of the induration, can also be indicative of disease and warrant further examination (<http://www.cdc.gov/tb/>), (2).

The only available vaccine against TB is infection with live attenuated *Mycobacterium bovis* Bacille Calmette-Gúerin (BCG), normally given in the skin. This infection/vaccination regimen has limited and highly variable efficacy in different parts of the world (3).

Helminth infections evoke Th2 and regulatory immune responses. Both of these responses can counteract Th1 development. Accordingly, worm infection is proposed to impair immune responses that control mycobacteria (4-6). Infection with worms has also been associated with a reduced ability to respond to BCG vaccination (7, 8). Geographically, areas of high TB incidence and poor TB vaccine efficacy typically have a high prevalence of intestinal helminth infections (9). However, the impact helminths have on vaccine efficacy and other, secondary infections remains an open question. Indeed, a number of studies report a lack of correlation between intestinal worms and secondary infections (10-13). In common for many of the studies describing an association between worms and increased susceptibility to secondary infection, or reduced inflammatory response in experimental autoimmune disease, is that the effects have been observed in tissue/s in direct or close contact with the worm (14, 15). On the other hand, the effects of gastrointestinal (GI) worms on infections distal to the worm itself remain poorly characterized.

The nematode *Heligmosomoides polygyrus bakeri* (from here on referred to as *H. polygyrus*) causes an infection strictly confined to the gut. In resistant hosts, *H. polygyrus* infection stimulates a strong Th2-type response that drives the expulsion of the worm (16, 17). Despite the generation of a protective Th2 response, the worm can persist and establish long-lasting infection in most laboratory mouse strains, reviewed in (18). This is facilitated by the regulatory responses *H. polygyrus* evoke. In the chronic phase of *H. polygyrus* infection there is an expansion of regulatory Foxp3+ T cells (Treg) in the gut (17). These Treg cells, driven in part by a TGF-β-like activity released from the parasite (19), dampen effector T cell responses aiding persistent worm infection.

Chronic infestation with worms is the norm in animals and humans. Thus, *H. polygyrus* provides a relevant model to study the effects GI nematode infection on immune responses to secondary infections. Further, *H. polygyrus* only causes moderate intestinal pathology and the infection is typically asymptomatic in wild-type mice. Thus, secondary infections can be delivered in animals that are seemingly healthy. We employed this model to investigate the effect of *H. polygyrus* infection on the outcome of mycobacteria-triggered Th1 responses at distal sites. Our results show that *H. polygyrus* infection can inhibit priming and recall responses to BCG and promote mycobacterial growth *in vivo*. Our data reinforce TGF-β signaling as a key component of *H. polygyrus*-mediated immune suppression. Based on our findings we also suggest caution in the use of skin-test reactivity based diagnostic when performed in worm-infected individuals.

**2. Material and Methods**

***Mice***

C57BL/6, congenic CD45.1 (Ly5.1) and P25-TCRTg RAG-1-/- (20) x RAG-1-/- ECFP (kindly provided by Dr. R. Germain, NIAID, USA) were bred and maintained under specific-pathogen-free conditions at MTC, Karolinska Institutet, Sweden. Female mice were used if not otherwise mentioned. All experiments were conducted in accordance to ethical regulations following approval by Stockholm’s Norra Djurförsöketiskanämnd.

***Infections***

All infections were performed in wild type (C57BL/6 or congenic Ly5.1/CD45.1) mice.

At 4-5 weeks of age mice were infected by oral gavage with 200 *H. polygyrus* L3 larvae, obtained as described previously (21, 22). The worm-infections were considered chronic after 28 days. At the end of each experiment the worm burden was estimated by counting viable worms that had migrated out of the opened intestine through a fine net into a tube containing RPMI-1640 at 37oC within 3-4 hours.

*Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) strain SSI 1331 was obtained from Statens Serum Institut (Copenhagen, Denmark) expanded in 7H9 medium as previously described (23) and inoculated at 1×106 colony forming units (CFU) in the footpad, ear pinnae or *i.v.* into the tail vein . For quantification of mycobacterial load in tissue, single cell suspensions were plated onto 7H11 agar supplemented with OADC (BD) and cultured at 37°C for 21 days.

*Leishmania major*, Freidlin, (a kind gift from Dr. D. Sacks, NIAID, NIH), was maintained in M199 supplemented with 20% fetal calf serum (FCS), 2mM L-glutamine, 100U/ml Penicillin, 200mM/ml streptomycin. 1x105 metacyclic promastigotes, enriched form stationary cultures using Ficoll 400 gradient separation (24) were injected into the ear dermis. Control animals were either left untreated or injected with PBS/medium as indicated in figure legends.

Detection of promastigote growth was done by microscopy. To determine the burden of *Leishmania* parasites, tissue homogenates were prepared as previously described (25) and cultured in limiting dilutions in 96-well plates using M199 medium supplemented with 20% FCS, 2mM L-glutamine, 100U/ml Penicillin, 200mM/ml streptomycin.

***Histopathological analysis***

To enumerate and measure granulomas, livers and spleens from infected mice were fixed in 4% paraformaldehyde (PFA) in PBS for 12 hours or more followed by dehydration and paraffin embedding. The tissue was cut into 6μm thick section and stained with hematoxylin and eosin (H&E). Processing and H&E staining of PFA-fixed samples was performed at the CCK pathology laboratory, Karolinska University Hospital, Stockholm, Sweden. Analysis was performed by microscopy on 10 fields per sample at a final magnification of 100X. To allow pooling of granuloma area the individual experiment was normalized by dividing the sample value with the mean value of all samples in the experiment.

***Antigens***

Adult worms were isolated from the small intestines by allowing the worms to migrate through a fine mesh in a 50 ml tube with RPMI-1640 with 100U/ml penicillin, 200mM/ml streptomycin and 0.5 mg/ml gentamycin at 37oC for 3-4 hours. Collected worms were washed extensively, incubated over-night in RPMI containing antibiotics following by further washing in the same medium. The washed worms were used for preparation of whole soluble worm antigen (SWAg) by re-suspending adult worms in 10mM TRIS-HCl and adding complete protease inhibitor (Roche) according to manufacturer’s instructions. The worms were homogenized by repeated freeze-thaw cycles followed by mechanical disruption and pulsed sonication. After centrifugation, protein concentration in the supernatant was determined by optical density at 280nm. For injection of larval antigens, L3 larvae were collected from cultures and washed extensively in water containing 200U/ml penicillin and 400mM/ml streptomycin. The “PPD” footpad was conditioned with 200 L3 larvae, as previously described by others (26). Control mice were injected with the water used in the last wash of the L3 larvae preparation.

*H. polygyrus* excretory-secretory products (HES) were isolated from adult worm cultures as previously described (27). Purified protein derivative (PPD; Statens Serum Institute, Copenhagen, Denmark) was used to measure recall responses *in vivo* by injecting 10 μg PPD in the footpad. Whole freeze-thawed *Leishmania* antigen (LAg) (28) was prepared from stationary-phase promasitgote cultures by repeated freeze-thawing cycles. Protein concentration was measured at 280nm and 50μg antigen was injected in the footpad.

All antigens injected in the footpad were delivered in a volume of 30μl.

***Cell transfer***

For *in vivo* assessment of antigen-specific T cells, total lymph nodes (LN) from P25-TCRTg RAG-/- mice were collected and single cell suspensions prepared. Isolated P25 TCRTg (P25) cells were labeled with 1μM CFSE (Invitrogen) in PBS for 10 min at room temperature. The labeling reaction was stopped by adding FCS, and subsequently washed in RPMI supplemented with 10% FCS, 100U/ml penicillin and 200 mM/ml streptomycin (cRPMI) to remove any unbound dye. 1x105 CFSE-labeled P25-TCRTg T cells (CD45.2+) were injected *i.v.* into the tail vein of recipient (CD45.1+) congenic mice receiving BCG. Control animals received 1x106 cells.

***In vitro re-stimulation***

Single cell suspension of the tissue of interest was prepared as previously described (23). Isolated cells were diluted in cRPMI to a concentration of 2x106 cells/ml and stimulated in 96-well tissue cultures plates with 10μg/mL PPD, 2μg/mL purified anti-CD3 antibody (BD) or worm antigens as indicated in figures for three days at 37°C in 5% CO2. Irradiated (30 Gy) splenocytes from naive mice were used as antigen-presenting cells (APCs) at a ratio of 1:5. Supernatants were collected and stored at -80°C until cytokines were measured by ELISA.

***ELISA***

Cytokine levels in culture supernatants were measured by sandwich ELISA. Immulon 2B plates (Nunc) were coated overnight, 4°C, with capture antibody. For IFN-γ, the capture antibody was diluted in carbonate buffer (0.1M Na2CO3, 0.1M NaHCO3, 1mM NaN3, pH 9.6), all other capture antibodies were diluted in PBS. The plates were then blocked for 2 hours at 37°C with 5% milk in diluent solution (1% BSA, 0.05% Tween-20 in PBS) and subsequently incubated overnight, 4°C, with the sample supernatants or the respective standards. Bound protein was detected with biotin-labeled detection antibodies for 2 hours at 37°C followed by incubation with peroxidase-labeled streptavidin (2 hours, 37°C) and development with ABTS peroxidase substrate (both KPL). Plates were read at 405nm. The following antibody clone pairs were used: IFN-γ (R4-6A2/ XMG1.2), IL-5 (TRFK5/ TRFK4), IL-10 (JES5-2A5/ JES5-16E3), all from BD Biosciences and Jackson Immuno Research. For detection of IL-17 and TGF-β mouse Quantikine kits (RnD Systems) were used according to manufacturer’s instructions.

***In vitro stimulation of P25-TCRTg cells***

P25-TCRTg cells were isolated and in some experiments CFSE labeled, as described above. Splenic DCs were isolated from collagenase IV and DNAse-1 treated spleens as previously described (23), followed by magnetic enrichment of CD11c+ cells using CD11c microbeads (Miltenyi Biotec) according to manufacturer’s instructions. Splenic DCs were incubated with HES and SWAg at various concentrations for 2 hours, and subsequently co-cultured with P25-TCRTg at a ratio of 1:5 and stimulated with MOI 1 of BCG 1331. In some experiments HES and SWAg were given to DCs in the presence of 5μM ALK5 inhibitor SB-431542 (Torics), treatment with 5ng rhTGF-β (RnD Systems) +/- SB-431542 was used as control. Positive control stimulations were with 2μg/mL Ag85B240-254 peptide and 100ng/mL lipopolysaccharide (LPS) (Sigma Aldrich).

***Flow cytometry staining***

Single-cell suspensions from tissues were incubated with various combinations of flourochrome-conjugated rat anti-mouse monoclonal antibodies specific for CD4 (RM4-5), CD11b (M1/70), CD11c (HL3), MHC-II I A/I E (M5/114.15.2), CD44 (IM7), CD45.2 (104), CD69 (H1.2F3), (BD Biosciences), CD326/EpCAM (G8.8), CD103 (2E7), (Biolegend), CD4 (RM4-5), B220 (RA3-6B2), LAP (TW7-16B4) (eBioscience), for 45 min in FACS buffer (2% FCS in 5mM EDTA, 0.1% azide) containing 0.5 mg/ml anti-mouse FcγIII/II receptor (2.4G2) (BD Biosciences). Live-dead staining was done using live-dead dye (Life Technologies). For analysis of intracellular cytokine production, cells were stimulated ex vivo for 6 hours with 10 μM Ag85B240-254 peptide in the presence of 10 μg/ml Brefeldin A (Sigma) prior to surface staining, followed by fixation in 2% paraformaldehyde (Electron Microscopy Sciences) and permeabilization with 1% saponin (Sigma) and staining with anti-IFN-γ (XMG1.2) (BD Biosciences). For staining of Foxp3 we first surface stained cells and then prepared cells for intra-nuclear/-cellular staining using eBioscience Foxp3 staining set (FJK-16S) according to manufacturer’s instructions. Irrelevant isotype-matched antibodies were used to determine levels of non-specific binding. Cell proliferation was measured by CFSE dilution.

To track cell migration from the skin to the draining lymph node (LN) 20 μL of 0.5mM CFSE in PBS were injected into the same footpad in which BCG vaccination had been delivered 48 hours earlier, as previously described (29). The popliteal lymph node (pLN) was collected 24 hours after CFSE injection. Single cells suspensions of pLN were stained for expression of CD11c, CD11b, MHC class II, CD103 and CD326. Detection and phenotypic characterization of CFSEhi cells were done by FACS. FACS acquisition was performed using CyAn (Beckman Coulter), LSRII or FACSCanto (BD Biosciences). Analysis was done on single cells gated as lymphocytes by forward-side scatter using FlowJo software (TreeStar).

***Gene expression and real-time PCR***

Total mRNA was isolated from tissue using Trizol (Sigma–Aldrich) according to manufacturer’s instructions. RNA concentration was determined by spectrometry and first strand cDNA generation and real-time PCR was performed as previously described (29, 30), using the T100 and CFX 384 Real-time System (BioRad), respectively. Expression of HRPT was used as baseline and the relative expression of gene expression was determined as follows: ΔCT between gene of interest and HRPT in the sample / ΔCT between gene of interest and HRPT in the in assigned unstimulated (control) sample.

**Statistical analysis**

Unpaired Student´s t-test was used for comparison between two groups using Prism (version 5.0a; GraphPad Software). Outliers were excluded from analysis of following Grubb’s test for one outlier, alpha=0.05 (GraphPad, QuickCalcs). P-values below 0.05 were considered to indicate significant differences between the groups.

**3. Results**

***H. polygyrus infection impairs priming of mycobacteria-specific T cells***

To test the effect of an underlying *H. polygyrus*-infection on induction of mycobacteria-specific CD4 T cells we infected mice *i.v*. with BCG and assessed activation of P25-TCRTg cells in animals with or without worms introduced 28 days previously. P25-TCRTg cells recognize the major CD4 T cell epitope of antigen (Ag)85B, present in both BCG and *M. tuberculosis* (20).

Antigen-specific T-cell priming was clearly impaired in BCG-infected mice with chronic worm infection compared to animals free of worms. The expansion and proliferative capacity of mycobacteria-specific P25-TCRTg cells was substantially reduced in *H. polygyrus* co-infected animals, as measured by total number (figure 1A) and percentages (figure 1B) of P25-TCRTg cells in the spleen. This was accompanied by a reduced ability of P25-TCRTg cells to produce IFN-γ upon re-stimulation with Ag85B (figure 1C, D). While separated, the spleen and the gut are still in relative proximity and Th2 responses to *H. polygyrus* can be detected in the spleen (28). To increase the distance between the site of worm infestation and the secondary infection we delivered BCG in the footpad. Similar to the observations described above, fewer P25-TCRTg T cells and fewer IFN-γ+ P25-TCRTg T cells were found in the skin-draining popliteal LN (pLN) six days after BCG footpad infection in mice with chronic *H. polygyrus* infection compared to worm-free mice (figure 1E, F). Furthermore, the cellularity of the BCG-draining pLN was notably lower in *H. polygyrus* (HP)-infected mice compared to worm-free mice (lymphocytes per pLN 6 days after BCG infection; HP/BCG: 4.56x106 ± 8.68x105; BCG: 13.15x106 ± 2.48x106, N=5/group; p=0.0115, one out of three or more experiment with similar results). The viability of CD4 T cells in the BCG draining LN was high (95%) and similar between *H. polygyrus* infected and worm-free mice. This indicates that the impaired T cell response observed in worm infected mice is not due to increased T cell death. These observations demonstrate that an intestinal worm infection can affect T-cell priming to infection/vaccination at sites distal to the gut.

***Mice with chronic* H. polygyrus *infection have reduced DTH responses to PPD***

To investigate the effect of *H. polygyrus* on recall responses we measured DTH to PPD in BCG-infected/vaccinated animals with or without worm infection. In these experiments BCG infection was delivered in the right footpad and the magnitude of the DTH response measured two weeks later by recording swelling upon PPD delivery in the other footpad. This measurement can be seen as an equivalent of the PPD skin tests done in humans to assess BCG vaccination or TB infection. In addition, we also studied the effect of depositing worm antigens proximal to the site of PPD challenge. To that end, footpads were pre-conditioned by injecting *H. polygyrus* larvae four weeks prior to PPD re-stimulation.

Interestingly, footpad swelling was significantly reduced when PPD was given in footpads pre-conditioned with L3 larvae (figure 2A). However, no significant effect on footpad swelling was observed in mice tested for PPD reactivity four weeks after worm infection and two weeks after BCG infection, compared to BCG-infected, worm-free mice (figure 2A). Since most helminth infections are chronic in nature, we prolonged the time to BCG infection, allowing the worm to become chronic before infection with BCG was given. Interestingly, we now found that the DTH responses were significantly diminished in worm-infected animals (figure 2B), implying that it takes time before the impact of worm-mediated inhibition can be observed on immune responses at distal sites.

When the PPD-injection site was pre-conditioned with L3 larvae, *in vitro* IFN-γ recall-responses to PPD in the draining LN were reduced by almost 65% compared to control LN (not shown). However, this was not seen in LNs from worm-infected animals. There were also no significant effects of *H. polygyrus* infection on *in vitro* production of IL-5, IL-12 or IL-17 in LN cultures (not shown).

To determine if fewer total T cells and antigen-specific CD4+ T cells accumulated at the site of recall response in worm-infected mice compared to worm-free mice, we measured the infiltration of total and P25-TCRTg CD4+ T cells in the skin where PPD was delivered. To facilitate the isolation of cells from skin, PPD was in these experiments injected in the ear pinnae. We found that worm-infected mice had fewer T cells and fewer antigen-specific P25-TCRTg cells in the skin (figure 2C, D), suggesting that the impaired DTH response is linked to a reduction of antigen-specific T cells in the PPD-challenged tissue. There was however, no difference in the number or frequency of P25-TCRTg cells in PPD-draining auricular LNs (figure S1A, B).

**H. polygyrus *infection increases susceptibility to Th1-controlled infections***

In line with the observations that both T-cell priming and recall responses were reduced in *H. polygyrus*-infected mice, we found that these animals had a 2.3-fold higher bacterial load in the liver when given a systemic BCG infection (figure 3A). Interestingly, BCG-induced granulomas were both fewer and smaller in *H. polygyrus-*infected mice compared to worm-free mice (figure 3B, C). This indicates that worm-infection can interfere with granuloma formation, leading to an increased susceptibility to mycobacterial infection. The impaired control of mycobacteria was coupled to reduced infiltration of both DCs and macrophages and lower expression of iNOS and IFN-γ in livers of co-infected animals (figure 3D, E, G). Assessment of CFU in spleen also indicated an increase in bacterial load in worm-infested animals, although this did not reach statistical significance (data not shown).

The impaired immune response and control of BCG in worm-infected mice was not associated with an increase in Foxp3 expression or more Foxp3+ CD4 T cells (S2 A, B, C).

The numbers of viable worms were similar in mice infected with *H. polygyrus* alone and *H. polygyrus*-infected mice given BCG *i.v.* (figure 3H). Thus, the established worm infection was not affected by the superimposed BCG infection in our model.

To investigate if another Th1-controlled infection was affected by an underlying, chronic *H. polygyru*s infection, animals were co-infected with *L. major* in the ear. We found that *H. polygyrus*-infected mice had double the amount of parasites in the ear (figure 4A) and a reduced cellularity in the ear-draining LN (figure 4 B) compared to worm-free mice. *In vitro* recall response to LAg by an equal number of LN cells were similar in worm-free and worm-infected mice (not shown), indicating that a more sensitive model, e.g. one involving TCRTg cells, is needed to detect such differences. In line with the observations shown in figure 2, *H. polygyrus* and *L. major* co-infected mice had diminished DTH responses to LAg in the footpad compared to worm-free mice (figure 4C). This shows that a pre-existing intestinal helminth infection can influence host control of secondary infections occurring distal to the worm.

**H. polygyrus*-secreted products inhibit T-cell priming* in vitro *through their TGF-β receptor signaling capacity***

To avoid host immune responses and to establish chronic infection without causing detrimental pathology, helminths produce and secrete molecules with immuno-modulatory capacity (27). Such molecules could, directly or indirectly, be the cause of the diminished T-cell priming in response to BCG observed in worm-infected mice. To test if worm-derived products interfere with T-cell priming, splenic DCs were treated with soluble worm antigen (SWAg) or the excretory-secretory product of *H. polygyrus* (HES) prior to stimulation with BCG and co-culture with P25-TCRTg cells. SWAg was a relatively poor inhibitor of BCG-induced P25-TCRTg-cell IFN-γ production, and significant inhibition was only seen when a high concentrations of SWAg concentration was used (figure 5A). SWAg also inhibited BCG-induced IL-5 in a dose-dependent manner (figure 5B), reinforcing the general down-modulation of effector-T cell responses by worms. HES strongly inhibited IFN-γ production by P25-TCRTg cells, while pre-exposure to BSA or LAg did not significantly affect IFN-γ production by P25-TCRTg CD4+ T cells (figure 5C), indicating that the effect observed was specific to HES. IL-10 was not detected in BCG-stimulated cultures and was not induced by HES or SWAg (data not shown).

The activity of HES was sensitive to heat treatment (figure 5D). HES has been shown to have a heat-sensitive TGFβ-like activity (19), suggesting that the TGF-βR activating molecule of HES-mediated inhibition of P25-TCRTg-cell IFN-γ production. In support of this, the effect of HES was reverted by inhibition of TGF-βR signaling, using the TGF-βR1/ALK5 inhibitor SB431542 (figure 5E). The SWAg-mediated effect also appeared to involve TGF-βR signaling, as the inhibition of BCG-induced responses was reversed by blockade of TGF-βR signaling (figure 5E). These results thus propose a role for TGF-βR signaling in worm-mediated inhibition of T-cell priming to BCG. The inhibitory effect of worm antigens in these *in vitro* cultures were likely through a direct inhibition on the T cells, since both HES and SWAg inhibited αCD3/αCD28 induced T cell activation in absence of DCs (not shown). Further, it was not enough to simply pre-incubate (4 hours) DCs with HES prior to co-culture with T cells in order to observe the inhibitory effect on IFNγ production. That said, direct effects of worm antigen on DCs have been shown by others (31) and cannot be excluded.

***H. polygyrus* infection reduces BCG triggered migration of dendritic cells from the skin to the draining lymph node.**

Given the above, we decided to investigate there were effects of *H. polygyrus* on antigen-presenting cells (APC) *in vivo*. In this regard, migration of APC from the site of infection to the draining LN is fundamental for the initiation of a primary T-cell response. The number of APC reaching the LN will accordingly influence the magnitude of that response (32). Given that BCG-specific T-cell expansion was reduced in worm-infected animals, we tested the effect of chronic *H. polygyrus* infection on migration of APC from the site of BCG infection to the LN draining. To track migratory cells, we injected CFSE into the skin of the footpad and quantified the number of CFSE+ cells detected in the draining pLN. Following BCG footpad infection we found that the majority of migrating (CFSEhi) cells were MHC class-IIhi CD11c+/int, consistent with the phenotype of migratory DC (figure 6A). The majority of these cells were also CD11b+ (figure 6A). To address if *H. polygyrus* affected such cell migration, we measured the number of CFSEhi MHC classIIhi CD11c+/int cells in the pLN after infection with BCG. Mice with chronic *H. polygyrus* infection had fewer CFSEhiMHC-II+/hiCD11c+/int cells in the pLN after BCG injection compared to worm-free mice (figure 6B, C). This may in part explain the reduction in antigen-specific T-cell response found in the worm-infected mice (as shown in figure 1). TGF-β is a key cytokine in *H. polygyrus*-induced immune regulation (33), with the capacity to block antigen-specific CD4-T-cell activation, as shown above, and with the potential to down-modulate the migratory capacity of DCs (34). To test if worm-derived molecules and TGF-β could influence BCG-induced DC migration, we conditioned the footpad with TGF-β or HES 10 days prior to BCG inoculation. As a control we heat-inactivated HES (HiHES), thereby eliminating its TGF-β-like activity (19). In line with studies of skin cancer (34), our data suggest that TGF-β lowers the migratory capacity of DCs. Fewer CFSEhiMHC-IIhiCD11cint/+ cells were found in the draining pLN after BCG infection when the site of BCG injection was pre-conditioned with TGF-β (figure 5D). HES-conditioning of the footpad also reduced the migratory response of MHC-IIhi CD11cint/+ cells to BCG, while Hi-HES was found to be a less potent inhibitor of the same (figure 6D). This finding supports worm-driven TGF-β signaling as a potential mechanism underlying impaired responses to BCG.

**4. Discussion**

The efficacy of BCG vaccination is highly variable in different parts of the world. Worms, through the immune responses they evoke, have been suggested as one factor that can impair BCG vaccine efficacy and increase susceptibility to mycobacterial infection (6, 35). In support of this there is ample evidence that worms and worm products can counteract Th1 immunity and down-modulate inflammatory responses to secondary antigens (36). However, most of these observations have been made in systems where the worm or the worm antigen is either in direct contact with or in close proximity to the site of inflammation/infection. Yet, most parasitic worms live in the gut and are therefore not proximal to the site where injection based vaccines are delivered. While some findings indicate that intestinal worms have more systemic effects on immunity (7, 8, 37, 38), evidence that intestinal nematodes modulate immune response in tissue distal to the worms and thereby impair immune responses to vaccination and secondary infections is scarce and remains questioned (12, 26, 39, 40).

In this study, we have experimentally addressed how a nematode infection confined to the gut influences Th1 responses to secondary infections at sites separated from the worm infection. Our results support the view that intestinal worms diminish immune responses to secondary vaccinations and infections. We found that CD4+ T-cell priming in response to BCG was reduced in mice chronically infected with *H. polygyrus*, compared to worm-free animals. Likewise, pre-exposure to *H. polygyrus* antigens *in vitro* dramatically decreased IFN-γ production by mycobacteria-specific CD4+ T cells.

Similar to observations made in humans (8), we found that DTH responses to PPD following footpad BCG infection/vaccination and to *L. major* antigen following experimental leishmanization were smaller in mice with chronic *H. polygyrus* infection compared to worm-free mice. This may reflect vaccine efficacy, but more importantly it suggests that intestinal worms can influence skin test-based diagnostics and possibly reduce DTH-based detection of tuberculosis and leishmaniasis. The notion that worms decrease the sensitivity of recall response-based diagnosis is something that would need more careful investigation in clinical studies.

The proximity between worm and co-infections appear to influence the suppressive effects of *H. polygyrus* on responses to BCG. The inhibitory effect was most evident when the distance to the BCG effector site was small, e.g. when BCG was delivered systemically (*i.v*.) or when the effector site itself had been pre-conditioned with worm antigens (figure 2A). Time is another factor that may influence the inhibitory effects of *H. polygyrus.* The DTH responses to PPD were attenuated in mice with chronic (> 4 week) but not acute (2-week) worm infection, at the time when infected with BCG.

Systemic dissemination of worm-induced Th2 or regulatory T cells could explain how an infection confined to the gut can modulate immunity at peripheral sites. During the first weeks of *H. polygyrus* infection, worm-induced Th2 cells can be found in the spleen (28, 37). An increase in Th2 cells could underlie the reduced priming of BCG-specific CD4+ T cells observed in the spleen. Moreover, using IL-4 reporter mice, Mohrs et al. found that *H. polygyrus-*induced Th2 cells that spread systemically have a preference for non-lymphoid organs, such as the liver (37). This could in turn explain why the impact of *H. polygyrus* on BCG load was more evident in the liver compared to the spleen. We could, however, not find any evidence for dissemination of Th2 cells to pLN in mice with chronic *H. polygyrus* infection. There were no differences in mRNA expression of IL-4 or T cell transcription factors GATA-3, T-bet and Foxp3 in pLN when comparing *H. polygyrus*-infected and worm-free mice (not shown). However, FACS analysis did reveal a modest decrease in the percentage of T cells in skin-draining LN of mice with chronic *H. polygyrus* infection (percentage of lymphocytes gated as T cells in pLN; naïve mice: 60.9±0.9%, mice with chronic *H. polygyrus* infection: 54.8±1.5%, N=10, p=0.0034). This indicates that an intestinal worm may affect lymphocyte composition in skin-draining LN, which in turn could influence the subsequent ability to respond to infection/vaccination.

Reduced immunogenicity of BCG in people chronically infected with worms has been associated with increased production of TGF-β by PBMC (7). Indeed, TGF-β is also induced by *H. polygyrus* infection*.* Although primarily found at the site of infection, increased levels of serum TGF-β have also been reported in *H. polygyrus*-infected mice (41). We found that splenic CD4 cells from mice with chronic *H. polygyrus* infection express more TGFβ latency associated peptide (LAP) compared to naïve mice (S2 D-F). TGF-β is a pluripotent, mainly anti-inflammatory, cytokine, which can limit both Th1 and Th2 responses. *H. polygyrus* and other intestinal worms exploit this cytokine in order to facilitate the chronic establishment in the host (18, 42). Experimentally, TGF-β has been found to be important for the control of worm-mediated inhibition of several inflammatory diseases (43). Many nematode species express TGF-β homologues (44) and some, including *H. polygyrus,* secrete products that can signal through the TGF-βR (19, 45). HES from *H. polygyrus* has previously been shown to drive regulatory (Foxp3) T cell responses and to increase production of both TGF-β and IL-10, another immune-modulatory cytokine (19). We found no evidence for an increase in Foxp3 expression in *H. polygyrus* co-infected animals. While we cannot exclude involvement of IL-10, using a IL-10 GFP reporter mice (46) we did not find more IL-10 expressing cells in the BCG draining pLN in worm infected compared to worm free mice (not shown).

However, we found that HES can act directly on T cells and that the TGF-βR signaling capacity of HES was needed to inhibit mycobacteria-specific T-cell priming *in vitro*. DCs typically acquire regulatory properties in the presence of TGF-β (47). While the effect of HES in our *in vitro* co-cultures was mainly on the T cells, *H. polygyrus* and the excretory-secretory (ES) products are also know to facilitate regulatory and Th2 promoting DC (31, 48-50). Interestingly, we found that pre-conditioning the site of BCG infection with TGF-β or HES significantly reduced BCG-triggered migration of DC (MHC class IIhi CD11c+/int cells) to the draining LN. TGF-β can down modulate expression of CCR7 on DCs and inhibit their migratory capacity (Ogata 1999). In mesenteric LNs Léon et al found that *H. polygyrus* infection alters the expression of CCR7 and CXCR5 on DCs, with implications on development of down-stream Th responses (51). We did not find that HES affected the CCR7 or CXCR5 expression in the BCG-draining LN (not shown). The inhibitory effect of HES on DC migration may involve more than TGF-β since HES is a complex mixture (27). Recently we found that the levels of CCR7 does not correlate with the *in vivo* ability of BMDC to migrate in response to BCG (29). Interestingly, IL-12p40 was found to be important for the BCG migration from footpad to the draining LN (29). Others have shown that exposure of DC to *H. polygyrus* ES products down modulates their expression of IL-12p40 (31). Similar observations have been made following exposure of DCs to TGF-β (52). In line with a role for TGF-βR signaling in HES-mediated inhibition of DC migration, heat-inactivation of HES, which destroys the TGF-β-like activity of HES, significantly diminished the inhibitory effect of HES on DC migration. If this effect on migration involves down-regulation of IL-12p40 by ES products remains to be investigated.

Taken together our data support worm-induced TGF-βR signaling as a mechanism behind helminth-mediated immune modulation of effector-T cell responses.

In summary, we show that a chronic worm infection confined to the gut impacts both primary and recall immune responses to secondary microbial challenge delivered in tissue distal to the gut. Compared to worm-free animals, mice with a chronic intestinal nematode infection had impaired T-cell priming in responses to BCG, reduced DTH responses in the skin and a higher bacterial / parasite load when infected with BCG and *L. major* respectively. This implies that worms negatively can affect the diagnosis as well as the control of intracellular infections with *Mycobacterium* and *Leishmania*. We propose worm-evoked TGF-βR signaling as a part of the explanation as to why helminth-infected individuals are more susceptible to Th1-controlled infections and respond less well to immunizations dependent on such responses.

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FIGURE LEGENDS

**Figure 1. *H. polygyrus* infection interferes with T cell priming in response to mycobacteria infection**

P25-TCRTg cells (1x105 to those receiving BCG and 1x106 to control animals/PBS group) were seeded in Ly 5.1 mice the day before BCG infection. The P25-TCRTg were assessed in the spleen 6 days after *i.v.* injection of 1x106 CFU BCG in mice with chronic (28-day) *H. polygyrus* (HP) infection or free of worms. A) Total number and B) frequency of P25-TCRTg cells, C) number and D) frequency of IFN-γ-positive P25-TCRTg cells following 6 hours *in vitro* re-stimulation with Ag85B peptide in mice infected as described above. E) Total number of T cells, F) number and G) frequency of IFN-γ+ P25-TCRTg cells in BCG-draining pLN 6 days after BCG footpad infection in mice with chronic *H. polygyrus* infection or free of worms. Data show is representative of two or more experiments with 3-5 mice per group. Significant differences are indicated as follows \* P<0.05; \*\* P<0.01; \*\*\* P<0.001.

**Figure 2. DTH responses are reduced in sites pre-conditioned with worms and in animals with chronic *H. polygyrus* infection.**

DTH response to PPD in C57Bl/6 mice either infected orally with *H. polygyrus* (HP)or pre-conditioned with *H. polygyrus* L3 larvae in the “PPD” draining left footpad (L.Fp). A) Footpad swelling in animals infected with 1x106 CFU BCG in the right footpad (R.Fp) 14-days after worm infection/ larval pre-conditioning and 14 days after BCG (=day 28) given PPD in the contralateral footpad B) Footpad swelling in animals infected with BCG in the footpad 28 days after *H. polygyrus* infection (chronic infection) and PPD challenged in the contralateral footpad 14 days after BCG vaccination (=day 42) C) Total T (CD3+) cells and D) seeded P25-TCRTg cells in ear dermis 48 hours after PPD injection in Ly 5.1 mice infected with *H. polygyrus* and BCG infected as above and challenged with PPD in the ear 14 days later. P25-TCRTg cells (1x105 in BCG infected and 1x106 in control animals) were transferred one day before BCG infection. Data shown is representative of two or more experiments with 3-5 mice per group. Significant differences are indicated as follows \* P<0.05; \*\* P<0.01; \*\*\* P<0.001.

**Figure 3. Control of mycobacteria is impaired in mice with chronic *H. polygyrus* infection**

A) Bacterial load, B) granulomas and C) relative granuloma area in livers 21 days post *i.v.* infection with 1x106 CFU BCG in C57Bl/6 mice chronically infected with *H. polygyrus* (HP) or free of worms. Data shown is pooled from 2-3 experiments (n=10-15 per group). D) Frequency of DC (CD11c+ MHCIIhi) and E) monocytes (CD11b+Ly6ChiMHCII+) in livers 14 days after *i.v.* infection with 1x106 CFU in mice with chronic *H. polygyrus* or free of worms at the time of BCG infection. Results are pooled from 2 experiments (n=10/group) F) iNOS, G) IFN-γ and H) TNF-α mRNA expression in livers from mice infected as in E and D. The control group received PBS injection. Data is representing two independent experiments (n=5/group). I) Viable adult worms in intestines of C57Bl/6 mice 42 days post *H. polygyrus* infection. Mice given BCG were infected *i.v.* 28 days after *H. polygyrus* infection (as in D). Results are pooled from 2 experiments (n=10/group). Significant differences are indicated as follows \* P<0.05; \*\* P<0.01; \*\*\* P<0.001. Groups given BCG were significantly p<0.05 different from the uninfected PBS control group.

**Figure 4. Intestinal worms can facilitate skin infection with *L. major***

Effect of chronic *H. polygyrus* (HP) infection on *L. major* infection (1x105 promastigotes) in the ear dermis: A) parasite load in ear and B) cellularity in ear dLN 5 weeks after *L. major* infection. C) DTH response to LAg (50μg) delivered in the footpad 8 weeks after *H. polygyrus* infection and 4 weeks after ear infection with *L. major* (1x105 promastigotes), one out of two experiments with 4-5 mice per group is shown. Significant differences are indicated as follows, \* P<0.05; \*\* P<0.01; \*\*\* P<0.001.

**Figure 5. Worm-derived molecules inhibit IFN-γ production by mycobacterial specific T cells in response to BCG in through TGFβ-R signalling.**

Cytokine production to BCG in splenic CD11c+:P25-TCRTg cell co-cultures treated with worm antigens. Splenic CD11c+ cells, from C57Bl/6 mice, were preconditioned with worm or control antigens as indicated and treated with BCG (MOI 1). Single-cell suspensions of LN from naïve P25-TCRTg mice were then added to the CD11c+ cells and the cells were co-cultured for 5 days before supernatants were collected. Negative control cultures were treated with medium or diluent A) IFN-γ and B) IL-5 in supernatant of culturespre*-*treated with different concentrations (5-50μg/ml) of SWAg. C) IFN-γ in supernatant of culturestreated with 5 or 10 μg/ml of *H. polygyrus* secreted antigens (HES) or control antigens as indicated. D) Effect of heat inactivation on HES (used at 5μg/ml) and SWAg (used at 50μg/ml) on inhibition of BCG induced IFNγ D) Effect of 5μM SB431542 on HES (5μg/ml), SWAg (50μg/ml) and rhTGFβ (5ng/ml) mediated inhibition of BCG induced IFN-γ in P25-TCRTg cultures. Data show mean ± SEM and is representative of two or more experiments generated from triplicate cultures. Stimulations/inhibitions were compared with cultures stimulated with BCG alone. Significant differences, using student’s t-test, are indicated as follows, \*P<0.05; \*\* P<0.01; \*\*\* P<0.001.

**Figure 6. *H. polygyrus* affect DC migration in response to BCG vaccination**

Tracking of cells migrating to the pLN 48-72 hours after BCG infection was done by labelling the footpad with CFSE by injection 48 hours after BCG vaccination (1x106 CFU) and measuring the number of labelled (CFSEhi) cells in the draining pLN 24 hours later by FACS. A) Gating strategy for detection of migratory (CFSE positive) cells in response to BCG infection. B) Numbers and C) frequencies of CFSEhiMHC-IIhi CD11c+/int cells in pLN following BCG vaccination in mice with chronic *H. polygyrus* (HP) infection or free of worms. D) CFSEhiMHC-IIhiCD11c+/int cell numbers in pLN, where the BCG infection site (footpad) had been pre-conditioned of with 5 ng TGFβ, 5μg HES or 5μg heat inactivated (Hi)-HES as indicated 10 days prior to BCG injection. Results shown are representative of two or more experiments with 3-5 mice per group. Control mice were preconditioned with 5μg OVA or left untreated and only infected with BCG. Background migration was monitored by injection of PBS in naïve mice. Significant differences are indicated as follows, \*P<0.05; \*\* P<0.01; \*\*\* P<0.001.