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

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

4

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23

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3

1 **Abstract**

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

1 **1. Introduction**

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

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

16 Helminth infections evoke Th2 and regulatory immune responses. Both of these
17 responses can counteract Th1 development. Accordingly, worm infection is
18 proposed to impair immune responses that control mycobacteria (4-6). Infection with
19 worms has also been associated with a reduced ability to respond to BCG
20 vaccination (7, 8). Geographically, areas of high TB incidence and poor TB vaccine
21 efficacy typically have a high prevalence of intestinal helminth infections (9).
22 However, the impact helminths have on vaccine efficacy and other, secondary
23 infections remains an open question. Indeed, a number of studies report a lack of
24 correlation between intestinal worms and secondary infections (10-13). In common
25 for many of the studies describing an association between worms and increased

1 susceptibility to secondary infection, or reduced inflammatory response in
2 experimental autoimmune disease, is that the effects have been observed in tissue/s
3 in direct or close contact with the worm (14, 15). On the other hand, the effects of
4 gastrointestinal (GI) worms on infections distal to the worm itself remain poorly
5 characterized.

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

16 Chronic infestation with worms is the norm in animals and humans. Thus, *H.*
17 *polygyrus* provides a relevant model to study the effects GI nematode infection on
18 immune responses to secondary infections. Further, *H. polygyrus* only causes
19 moderate intestinal pathology and the infection is typically asymptomatic in wild-type
20 mice. Thus, secondary infections can be delivered in animals that are seemingly
21 healthy. We employed this model to investigate the effect of *H. polygyrus* infection on
22 the outcome of mycobacteria-triggered Th1 responses at distal sites. Our results
23 show that *H. polygyrus* infection can inhibit priming and recall responses to BCG and
24 promote mycobacterial growth *in vivo*. Our data reinforce TGF-β signaling as a key
25 component of *H. polygyrus*-mediated immune suppression. Based on our findings we

1 also suggest caution in the use of skin-test reactivity based diagnostic when
2 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 37°C 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 1×10^{6} 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.

1 *Leishmania major*, Freidlin, (a kind gift from Dr. D. Sacks, NIAID, NIH), was
2 maintained in M199 supplemented with 20% fetal calf serum (FCS), 2mM L-
3 glutamine, 100U/ml Penicillin, 200mM/ml streptomycin. $1x10^5$ metacyclic
4 promastigotes, enriched form stationary cultures using Ficoll 400 gradient separation
5 (24) were injected into the ear dermis. Control animals were either left untreated or
6 injected with PBS/medium as indicated in figure legends.
7 Detection of promastigote growth was done by microscopy. To determine the burden
8 of *Leishmania* parasites, tissue homogenates were prepared as previously described
9 (25) and cultured in limiting dilutions in 96-well plates using M199 medium
10 supplemented with 20% FCS, 2mM L-glutamine, 100U/ml Penicillin, 200mM/ml
11 streptomycin.
12
13 ***Histopathological analysis***
14 To enumerate and measure granulomas, livers and spleens from infected mice were
15 fixed in 4% paraformaldehyde (PFA) in PBS for 12 hours or more followed by
16 dehydration and paraffin embedding. The tissue was cut into 6µm thick section and
17 stained with hematoxylin and eosin (H&E). Processing and H&E staining of PFA-
18 fixed samples was performed at the CCK pathology laboratory, Karolinska University
19 Hospital, Stockholm, Sweden. Analysis was performed by microscopy on 10 fields
20 per sample at a final magnification of 100X. To allow pooling of granuloma area the
21 individual experiment was normalized by dividing the sample value with the mean
22 value of all samples in the experiment.
23
24 ***Antigens***

1 Adult worms were isolated from the small intestines by allowing the worms to migrate
2 through a fine mesh in a 50 ml tube with RPMI-1640 with 100U/ml penicillin,
3 200mM/ml streptomycin and 0.5 mg/ml gentamycin at 37°C for 3-4 hours. Collected
4 worms were washed extensively, incubated over-night in RPMI containing antibiotics
5 following by further washing in the same medium. The washed worms were used for
6 preparation of whole soluble worm antigen (SWAg) by re-suspending adult worms in
7 10mM TRIS-HCl and adding complete protease inhibitor (Roche) according to
8 manufacturer's instructions. The worms were homogenized by repeated freeze-thaw
9 cycles followed by mechanical disruption and pulsed sonication. After centrifugation,
10 protein concentration in the supernatant was determined by optical density at 280nm.
11 For injection of larval antigens, L3 larvae were collected from cultures and washed
12 extensively in water containing 200U/ml penicillin and 400mM/ml streptomycin. The
13 "PPD" footpad was conditioned with 200 L3 larvae, as previously described by others
14 (26). Control mice were injected with the water used in the last wash of the L3 larvae
15 preparation.
16 *H. polygyrus* excretory-secretory products (HES) were isolated from adult worm
17 cultures as previously described (27). Purified protein derivative (PPD; Statens
18 Serum Institute, Copenhagen, Denmark) was used to measure recall responses *in*
19 *vivo* by injecting 10 μg PPD in the footpad. Whole freeze-thawed *Leishmania* antigen
20 (LAg) (28) was prepared from stationary-phase promasitgote cultures by repeated
21 freeze-thawing cycles. Protein concentration was measured at 280nm and 50μg
22 antigen was injected in the footpad.
23 All antigens injected in the footpad were delivered in a volume of 30μl.
24
25 ***Cell transfer***

1 For *in vivo* assessment of antigen-specific T cells, total lymph nodes (LN) from P25-
2 TCRTg RAG-/- mice were collected and single cell suspensions prepared. Isolated
3 P25 TCRTg (P25) cells were labeled with 1μM CFSE (Invitrogen) in PBS for 10 min
4 at room temperature. The labeling reaction was stopped by adding FCS, and
5 subsequently washed in RPMI supplemented with 10% FCS, 100U/ml penicillin and
6 200 mM/ml streptomycin (cRPMI) to remove any unbound dye. $1x10^5$ CFSE-labeled
7 P25-TCRTg T cells ($CD45.2^+$) were injected *i.v.* into the tail vein of recipient
8 ($CD45.1^+$) congenic mice receiving BCG. Control animals received $1x10^6$ cells.
9
10 ***In vitro re-stimulation***
11 Single cell suspension of the tissue of interest was prepared as previously described
12 (23). Isolated cells were diluted in cRPMI to a concentration of $2x10^6$ cells/ml and
13 stimulated in 96-well tissue cultures plates with 10μg/mL PPD, 2μg/mL purified anti-
14 CD3 antibody (BD) or worm antigens as indicated in figures for three days at 37°C in
15 5% CO_2. Irradiated (30 Gy) splenocytes from naive mice were used as antigen-
16 presenting cells (APCs) at a ratio of 1:5. Supernatants were collected and stored at -
17 80°C until cytokines were measured by ELISA.
18
19 ***ELISA***
20 Cytokine levels in culture supernatants were measured by sandwich ELISA.
21 Immulon 2B plates (Nunc) were coated overnight, 4°C, with capture antibody. For
22 IFN-γ, the capture antibody was diluted in carbonate buffer (0.1M Na_2CO_3, 0.1M
23 $NaHCO_3$, 1mM NaN3, pH 9.6), all other capture antibodies were diluted in PBS. The
24 plates were then blocked for 2 hours at 37°C with 5% milk in diluent solution (1%
25 BSA, 0.05% Tween-20 in PBS) and subsequently incubated overnight, 4°C, with the

1 sample supernatants or the respective standards. Bound protein was detected with
2 biotin-labeled detection antibodies for 2 hours at 37°C followed by incubation with
3 peroxidase-labeled streptavidin (2 hours, 37°C) and development with ABTS
4 peroxidase substrate (both KPL). Plates were read at 405nm. The following antibody
5 clone pairs were used: IFN-γ (R4-6A2/ XMG1.2), IL-5 (TRFK5/ TRFK4), IL-10 (JES5-
6 2A5/ JES5-16E3), all from BD Biosciences and Jackson Immuno Research. For
7 detection of IL-17 and TGF-β mouse Quantikine kits (RnD Systems) were used
8 according to manufacturer's instructions.
9
10 ***In vitro stimulation of P25-TCRTg cells***
11 P25-TCRTg cells were isolated and in some experiments CFSE labeled, as
12 described above. Splenic DCs were isolated from collagenase IV and DNAse-1
13 treated spleens as previously described (23), followed by magnetic enrichment of
14 $CD11c^+$ cells using CD11c microbeads (Miltenyi Biotec) according to manufacturer's
15 instructions. Splenic DCs were incubated with HES and SWAg at various
16 concentrations for 2 hours, and subsequently co-cultured with P25-TCRTg at a ratio
17 of 1:5 and stimulated with MOI 1 of BCG 1331. In some experiments HES and SWAg
18 were given to DCs in the presence of 5µM ALK5 inhibitor SB-431542 (Torics),
19 treatment with 5ng rhTGF-β (RnD Systems) +/- SB-431542 was used as control.
20 Positive control stimulations were with 2µg/mL $Ag85B_{240-254}$ peptide and 100ng/mL
21 lipopolysaccharide (LPS) (Sigma Aldrich).
22
23 ***Flow cytometry staining***
24 Single-cell suspensions from tissues were incubated with various combinations of
25 flourochrome-conjugated rat anti-mouse monoclonal antibodies specific for CD4

1 (RM4-5), CD11b (M1/70), CD11c (HL3), MHC-II I A/I E (M5/114.15.2), CD44 (IM7),
2 CD45.2 (104), CD69 (H1.2F3), (BD Biosciences), CD326/EpCAM (G8.8), CD103
3 (2E7), (Biolegend), CD4 (RM4-5), B220 (RA3-6B2), LAP (TW7-16B4) (eBioscience),
4 for 45 min in FACS buffer (2% FCS in 5mM EDTA, 0.1% azide) containing 0.5 mg/ml
5 anti-mouse FcγIII/II receptor (2.4G2) (BD Biosciences). Live-dead staining was done
6 using live-dead dye (Life Technologies). For analysis of intracellular cytokine
7 production, cells were stimulated ex vivo for 6 hours with 10 μM $Ag85B_{240-254}$ peptide
8 in the presence of 10 μg/ml Brefeldin A (Sigma) prior to surface staining, followed by
9 fixation in 2% paraformaldehyde (Electron Microscopy Sciences) and
10 permeabilization with 1% saponin (Sigma) and staining with anti-IFN-γ (XMG1.2) (BD
11 Biosciences). For staining of Foxp3 we first surface stained cells and then prepared
12 cells for intra-nuclear/-cellular staining using eBioscience Foxp3 staining set (FJK-
13 16S) according to manufacturer's instructions. Irrelevant isotype-matched antibodies
14 were used to determine levels of non-specific binding. Cell proliferation was
15 measured by CFSE dilution.
16 To track cell migration from the skin to the draining lymph node (LN) 20 μL of 0.5mM
17 CFSE in PBS were injected into the same footpad in which BCG vaccination had
18 been delivered 48 hours earlier, as previously described (29). The popliteal lymph
19 node (pLN) was collected 24 hours after CFSE injection. Single cells suspensions of
20 pLN were stained for expression of CD11c, CD11b, MHC class II, CD103 and
21 CD326. Detection and phenotypic characterization of $CFSE^{hi}$ cells were done by
22 FACS. FACS acquisition was performed using CyAn (Beckman Coulter), LSRII or
23 FACSCanto (BD Biosciences). Analysis was done on single cells gated as
24 lymphocytes by forward-side scatter using FlowJo software (TreeStar).
25

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

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

10

11 **Statistical analysis**

12 Unpaired Student´s t-test was used for comparison between two groups using Prism
13 (version 5.0a; GraphPad Software). Outliers were excluded from analysis of following
14 Grubb's test for one outlier, alpha=0.05 (GraphPad, QuickCalcs). P-values below
15 0.05 were considered to indicate significant differences between the groups.

16

17

1 **3. Results**

2

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

4 To test the effect of an underlying *H. polygyrus*-infection on induction of
5 mycobacteria-specific CD4 T cells we infected mice *i.v*. with BCG and assessed
6 activation of P25-TCRTg cells in animals with or without worms introduced 28 days
7 previously. P25-TCRTg cells recognize the major CD4 T cell epitope of antigen
8 (Ag)85B, present in both BCG and *M. tuberculosis* (20).
9 Antigen-specific T-cell priming was clearly impaired in BCG-infected mice with
10 chronic worm infection compared to animals free of worms. The expansion and
11 proliferative capacity of mycobacteria-specific P25-TCRTg cells was substantially
12 reduced in *H. polygyrus* co-infected animals, as measured by total number (figure
13 1A) and percentages (figure 1B) of P25-TCRTg cells in the spleen. This was
14 accompanied by a reduced ability of P25-TCRTg cells to produce IFN-γ upon re-
15 stimulation with Ag85B (figure 1C, D). While separated, the spleen and the gut are
16 still in relative proximity and Th2 responses to *H. polygyrus* can be detected in the
17 spleen (28). To increase the distance between the site of worm infestation and the
18 secondary infection we delivered BCG in the footpad. Similar to the observations
19 described above, fewer P25-TCRTg T cells and fewer IFN-γ^+ P25-TCRTg T cells
20 were found in the skin-draining popliteal LN (pLN) six days after BCG footpad
21 infection in mice with chronic *H. polygyrus* infection compared to worm-free mice
22 (figure 1E, F). Furthermore, the cellularity of the BCG-draining pLN was notably lower
23 in *H. polygyrus* (HP)-infected mice compared to worm-free mice (lymphocytes per
24 pLN 6 days after BCG infection; HP/BCG: $4.56x10^6 \pm 8.68x10^5$; BCG: $13.15x10^6 \pm$
25 $2.48x10^6$, N=5/group; p=0.0115, one out of three or more experiment with similar

1 results). The viability of CD4 T cells in the BCG draining LN was high (95%) and
2 similar between *H. polygyrus* infected and worm-free mice. This indicates that the
3 impaired T cell response observed in worm infected mice is not due to increased T
4 cell death. These observations demonstrate that an intestinal worm infection can
5 affect T-cell priming to infection/vaccination at sites distal to the gut.
6

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

8 To investigate the effect of *H. polygyrus* on recall responses we measured DTH to
9 PPD in BCG-infected/vaccinated animals with or without worm infection. In these
10 experiments BCG infection was delivered in the right footpad and the magnitude of
11 the DTH response measured two weeks later by recording swelling upon PPD
12 delivery in the other footpad. This measurement can be seen as an equivalent of the
13 PPD skin tests done in humans to assess BCG vaccination or TB infection. In
14 addition, we also studied the effect of depositing worm antigens proximal to the site
15 of PPD challenge. To that end, footpads were pre-conditioned by injecting *H.*
16 *polygyrus* larvae four weeks prior to PPD re-stimulation.
17 Interestingly, footpad swelling was significantly reduced when PPD was given in
18 footpads pre-conditioned with L3 larvae (figure 2A). However, no significant effect on
19 footpad swelling was observed in mice tested for PPD reactivity four weeks after
20 worm infection and two weeks after BCG infection, compared to BCG-infected,
21 worm-free mice (figure 2A). Since most helminth infections are chronic in nature, we
22 prolonged the time to BCG infection, allowing the worm to become chronic before
23 infection with BCG was given. Interestingly, we now found that the DTH responses
24 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

1 susceptibility to mycobacterial infection. The impaired control of mycobacteria was
2 coupled to reduced infiltration of both DCs and macrophages and lower expression of
3 iNOS and IFN-γ in livers of co-infected animals (figure 3D, E, G). Assessment of
4 CFU in spleen also indicated an increase in bacterial load in worm-infested animals,
5 although this did not reach statistical significance (data not shown).
6 The impaired immune response and control of BCG in worm-infected mice was not
7 associated with an increase in Foxp3 expression or more Foxp3+ CD4 T cells (S2 A,
8 B, C).
9 The numbers of viable worms were similar in mice infected with *H. polygyrus* alone
10 and *H. polygyrus*-infected mice given BCG *i.v.* (figure 3H). Thus, the established
11 worm infection was not affected by the superimposed BCG infection in our model.
12 To investigate if another Th1-controlled infection was affected by an underlying,
13 chronic *H. polygyru*s infection, animals were co-infected with *L. major* in the ear. We
14 found that *H. polygyrus*-infected mice had double the amount of parasites in the ear
15 (figure 4A) and a reduced cellularity in the ear-draining LN (figure 4 B) compared to
16 worm-free mice. *In vitro* recall response to LAg by an equal number of LN cells were
17 similar in worm-free and worm-infected mice (not shown), indicating that a more
18 sensitive model, e.g. one involving TCRTg cells, is needed to detect such
19 differences. In line with the observations shown in figure 2, *H. polygyrus* and *L. major*
20 co-infected mice had diminished DTH responses to LAg in the footpad compared to
21 worm-free mice (figure 4C). This shows that a pre-existing intestinal helminth
22 infection can influence host control of secondary infections occurring distal to the
23 worm.
24

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

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

19 The activity of HES was sensitive to heat treatment (figure 5D). HES has been shown
20 to have a heat-sensitive TGFβ-like activity (19), suggesting that the TGF-βR
21 activating molecule of HES-mediated inhibition of P25-TCRTg-cell IFN-γ production.
22 In support of this, the effect of HES was reverted by inhibition of TGF-βR signaling,
23 using the TGF-βR1/ALK5 inhibitor SB431542 (figure 5E). The SWAg-mediated effect
24 also appeared to involve TGF-βR signaling, as the inhibition of BCG-induced
25 responses was reversed by blockade of TGF-βR signaling (figure 5E). These results

1 thus propose a role for TGF-βR signaling in worm-mediated inhibition of T-cell
2 priming to BCG. The inhibitory effect of worm antigens in these *in vitro* cultures were
3 likely through a direct inhibition on the T cells, since both HES and SWAg inhibited
4 αCD3/αCD28 induced T cell activation in absence of DCs (not shown). Further, it
5 was not enough to simply pre-incubate (4 hours) DCs with HES prior to co-culture
6 with T cells in order to observe the inhibitory effect on IFNγ production. That said,
7 direct effects of worm antigen on DCs have been shown by others (31) and cannot
8 be excluded.
9
10 ***H. polygyrus* infection reduces BCG triggered migration of dendritic cells from**
11 **the skin to the draining lymph node.**
12 Given the above, we decided to investigate there were effects of *H. polygyrus* on
13 antigen-presenting cells (APC) *in vivo*. In this regard, migration of APC from the site
14 of infection to the draining LN is fundamental for the initiation of a primary T-cell
15 response. The number of APC reaching the LN will accordingly influence the
16 magnitude of that response (32). Given that BCG-specific T-cell expansion was
17 reduced in worm-infected animals, we tested the effect of chronic *H. polygyrus*
18 infection on migration of APC from the site of BCG infection to the LN draining. To
19 track migratory cells, we injected CFSE into the skin of the footpad and quantified the
20 number of $CFSE^{+}$ cells detected in the draining pLN. Following BCG footpad infection
21 we found that the majority of migrating ($CFSE^{hi}$) cells were MHC class-II^{hi} $CD11c^{+/int}$,
22 consistent with the phenotype of migratory DC (figure 6A). The majority of these cells
23 were also $CD11b^{+}$ (figure 6A). To address if *H. polygyrus* affected such cell
24 migration, we measured the number of $CFSE^{hi}$ MHC classII^{hi} $CD11c^{+/int}$ cells in the
25 pLN after infection with BCG. Mice with chronic *H. polygyrus* infection had fewer

1 CFSE^{hi}MHC-II$^{+/hi}$CD11c$^{+/int}$ cells in the pLN after BCG injection compared to worm-
2 free mice (figure 6B, C). This may in part explain the reduction in antigen-specific T-
3 cell response found in the worm-infected mice (as shown in figure 1). TGF-β is a key
4 cytokine in *H. polygyrus*-induced immune regulation (33), with the capacity to block
5 antigen-specific CD4-T-cell activation, as shown above, and with the potential to
6 down-modulate the migratory capacity of DCs (34). To test if worm-derived
7 molecules and TGF-β could influence BCG-induced DC migration, we conditioned
8 the footpad with TGF-β or HES 10 days prior to BCG inoculation. As a control we
9 heat-inactivated HES (HiHES), thereby eliminating its TGF-β-like activity (19). In line
10 with studies of skin cancer (34), our data suggest that TGF-β lowers the migratory
11 capacity of DCs. Fewer CFSE^{hi}MHC-II^{hi}CD11c$^{int/+}$ cells were found in the draining
12 pLN after BCG infection when the site of BCG injection was pre-conditioned with
13 TGF-β (figure 5D). HES-conditioning of the footpad also reduced the migratory
14 response of MHC-II^{hi} CD11c$^{int/+}$ cells to BCG, while Hi-HES was found to be a less
15 potent inhibitor of the same (figure 6D). This finding supports worm-driven TGF-β
16 signaling as a potential mechanism underlying impaired responses to BCG.
17
18

1 **4. Discussion**

2 The efficacy of BCG vaccination is highly variable in different parts of the world.
3 Worms, through the immune responses they evoke, have been suggested as one
4 factor that can impair BCG vaccine efficacy and increase susceptibility to
5 mycobacterial infection (6, 35). In support of this there is ample evidence that worms
6 and worm products can counteract Th1 immunity and down-modulate inflammatory
7 responses to secondary antigens (36). However, most of these observations have
8 been made in systems where the worm or the worm antigen is either in direct contact
9 with or in close proximity to the site of inflammation/infection. Yet, most parasitic
10 worms live in the gut and are therefore not proximal to the site where injection based
11 vaccines are delivered. While some findings indicate that intestinal worms have more
12 systemic effects on immunity (7, 8, 37, 38), evidence that intestinal nematodes
13 modulate immune response in tissue distal to the worms and thereby impair immune
14 responses to vaccination and secondary infections is scarce and remains questioned
15 (12, 26, 39, 40).

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

24 Similar to observations made in humans (8), we found that DTH responses to PPD
25 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

1 shown). However, FACS analysis did reveal a modest decrease in the percentage of
2 T cells in skin-draining LN of mice with chronic *H. polygyrus* infection (percentage of
3 lymphocytes gated as T cells in pLN; naïve mice: 60.9±0.9%, mice with chronic *H.*
4 *polygyrus* infection: 54.8±1.5%, N=10, p=0.0034). This indicates that an intestinal
5 worm may affect lymphocyte composition in skin-draining LN, which in turn could
6 influence the subsequent ability to respond to infection/vaccination.
7 Reduced immunogenicity of BCG in people chronically infected with worms has been
8 associated with increased production of TGF-β by PBMC (7). Indeed, TGF-β is also
9 induced by *H. polygyrus* infection. Although primarily found at the site of infection,
10 increased levels of serum TGF-β have also been reported in *H. polygyrus*-infected
11 mice (41). We found that splenic CD4 cells from mice with chronic *H. polygyrus*
12 infection express more TGFβ latency associated peptide (LAP) compared to naïve
13 mice (S2 D-F). TGF-β is a pluripotent, mainly anti-inflammatory, cytokine, which can
14 limit both Th1 and Th2 responses. *H. polygyrus* and other intestinal worms exploit
15 this cytokine in order to facilitate the chronic establishment in the host (18, 42).
16 Experimentally, TGF-β has been found to be important for the control of worm-
17 mediated inhibition of several inflammatory diseases (43). Many nematode species
18 express TGF-β homologues (44) and some, including *H. polygyrus,* secrete products
19 that can signal through the TGF-βR (19, 45). HES from *H. polygyrus* has previously
20 been shown to drive regulatory (Foxp3) T cell responses and to increase production
21 of both TGF-β and IL-10, another immune-modulatory cytokine (19). We found no
22 evidence for an increase in Foxp3 expression in *H. polygyrus* co-infected animals.
23 While we cannot exclude involvement of IL-10, using a IL-10 GFP reporter mice (46)
24 we did not find more IL-10 expressing cells in the BCG draining pLN in worm infected
25 compared to worm free mice (not shown).

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

1 Taken together our data support worm-induced TGF-βR signaling as a mechanism
2 behind helminth-mediated immune modulation of effector-T cell responses.
3 In summary, we show that a chronic worm infection confined to the gut impacts both
4 primary and recall immune responses to secondary microbial challenge delivered in
5 tissue distal to the gut. Compared to worm-free animals, mice with a chronic
6 intestinal nematode infection had impaired T-cell priming in responses to BCG,
7 reduced DTH responses in the skin and a higher bacterial / parasite load when
8 infected with BCG and *L. major* respectively. This implies that worms negatively can
9 affect the diagnosis as well as the control of intracellular infections with
10 *Mycobacterium* and *Leishmania*. We propose worm-evoked TGF-βR signaling as a
11 part of the explanation as to why helminth-infected individuals are more susceptible
12 to Th1-controlled infections and respond less well to immunizations dependent on
13 such responses.
14

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19
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25

1 FIGURE LEGENDS

2

3 **Figure 1. *H. polygyrus* infection interferes with T cell priming in response to**
4 **mycobacteria infection**
5 P25-TCRTg cells (1x10^5 to those receiving BCG and 1x10^6 to control animals/PBS
6 group) were seeded in Ly 5.1 mice the day before BCG infection. The P25-TCRTg
7 were assessed in the spleen 6 days after *i.v.* injection of 1x10^6 CFU BCG in mice
8 with chronic (28-day) *H. polygyrus* (HP) infection or free of worms. A) Total number
9 and B) frequency of P25-TCRTg cells, C) number and D) frequency of IFN-γ-positive
10 P25-TCRTg cells following 6 hours *in vitro* re-stimulation with Ag85B peptide in mice
11 infected as described above. E) Total number of T cells, F) number and G) frequency
12 of IFN-γ+ P25-TCRTg cells in BCG-draining pLN 6 days after BCG footpad infection
13 in mice with chronic *H. polygyrus* infection or free of worms. Data show is
14 representative of two or more experiments with 3-5 mice per group. Significant
15 differences are indicated as follows * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

16

17 **Figure 2. DTH responses are reduced in sites pre-conditioned with worms and**
18 **in animals with chronic *H. polygyrus* infection.**
19 DTH response to PPD in C57Bl/6 mice either infected orally with *H. polygyrus* (HP)
20 or pre-conditioned with *H. polygyrus* L3 larvae in the “PPD” draining left footpad
21 (L.Fp). A) Footpad swelling in animals infected with 1x10^6 CFU BCG in the right
22 footpad (R.Fp) 14-days after worm infection/ larval pre-conditioning and 14 days after
23 BCG (=day 28) given PPD in the contralateral footpad B) Footpad swelling in animals
24 infected with BCG in the footpad 28 days after *H. polygyrus* infection (chronic
25 infection) and PPD challenged in the contralateral footpad 14 days after BCG

1 vaccination (=day 42) C) Total T (CD3+) cells and D) seeded P25-TCRTg cells in ear
2 dermis 48 hours after PPD injection in Ly 5.1 mice infected with *H. polygyrus* and
3 BCG infected as above and challenged with PPD in the ear 14 days later. P25-
4 TCRTg cells ($1x10^5$ in BCG infected and $1x10^6$ in control animals) were transferred
5 one day before BCG infection. Data shown is representative of two or more
6 experiments with 3-5 mice per group. Significant differences are indicated as follows
7 * P<0.05; ** P<0.01; *** P<0.001.
8
9 **Figure 3. Control of mycobacteria is impaired in mice with chronic *H. polygyrus***
10 **infection**
11 A) Bacterial load, B) granulomas and C) relative granuloma area in livers 21 days
12 post *i.v.* infection with $1x10^6$ CFU BCG in C57Bl/6 mice chronically infected with *H.*
13 *polygyrus* (HP) or free of worms. Data shown is pooled from 2-3 experiments (n=10-
14 15 per group). D) Frequency of DC ($CD11c^+$ $MHCII^{hi}$) and E) monocytes
15 ($CD11b^+Ly6C^{hi}MHCII^+$) in livers 14 days after *i.v.* infection with $1x10^6$ CFU in mice
16 with chronic *H. polygyrus* or free of worms at the time of BCG infection. Results are
17 pooled from 2 experiments (n=10/group) F) iNOS, G) IFN-γ and H) TNF-α mRNA
18 expression in livers from mice infected as in E and D. The control group received
19 PBS injection. Data is representing two independent experiments (n=5/group). I)
20 Viable adult worms in intestines of C57Bl/6 mice 42 days post *H. polygyrus* infection.
21 Mice given BCG were infected *i.v.* 28 days after *H. polygyrus* infection (as in D).
22 Results are pooled from 2 experiments (n=10/group). Significant differences are
23 indicated as follows * P<0.05; ** P<0.01; *** P<0.001. Groups given BCG were
24 significantly p<0.05 different from the uninfected PBS control group.
25

1 **Figure 4. Intestinal worms can facilitate skin infection with *L. major***
2 Effect of chronic *H. polygyrus* (HP) infection on *L. major* infection ($1x10^5$
3 promastigotes) in the ear dermis: A) parasite load in ear and B) cellularity in ear dLN
4 5 weeks after *L. major* infection. C) DTH response to LAg (50µg) delivered in the
5 footpad 8 weeks after *H. polygyrus* infection and 4 weeks after ear infection with *L.*
6 *major* ($1x10^5$ promastigotes), one out of two experiments with 4-5 mice per group is
7 shown. Significant differences are indicated as follows, * $P<0.05$; ** $P<0.01$; ***
8 $P<0.001$.
9
10 **Figure 5. Worm-derived molecules inhibit IFN-γ production by mycobacterial**
11 **specific T cells in response to BCG in through TGFβ-R signalling.**
12 Cytokine production to BCG in splenic $CD11c^+$:P25-TCRTg cell co-cultures treated
13 with worm antigens. Splenic $CD11c^+$ cells, from C57Bl/6 mice, were preconditioned
14 with worm or control antigens as indicated and treated with BCG (MOI 1). Single-cell
15 suspensions of LN from naïve P25-TCRTg mice were then added to the $CD11c^+$ cells
16 and the cells were co-cultured for 5 days before supernatants were collected.
17 Negative control cultures were treated with medium or diluent A) IFN-γ and B) IL-5 in
18 supernatant of cultures pre-treated with different concentrations (5-50µg/ml) of
19 SWAg. C) IFN-γ in supernatant of cultures treated with 5 or 10 µg/ml of *H. polygyrus*
20 secreted antigens (HES) or control antigens as indicated. D) Effect of heat
21 inactivation on HES (used at 5µg/ml) and SWAg (used at 50µg/ml) on inhibition of
22 BCG induced IFNγ D) Effect of 5µM SB431542 on HES (5µg/ml), SWAg (50µg/ml)
23 and rhTGFβ (5ng/ml) mediated inhibition of BCG induced IFN-γ in P25-TCRTg
24 cultures. Data show mean ± SEM and is representative of two or more experiments
25 generated from triplicate cultures. Stimulations/inhibitions were compared with

1 cultures stimulated with BCG alone. Significant differences, using student's t-test,
2 are indicated as follows, *P<0.05; ** P<0.01; *** P<0.001.
3
4 **Figure 6. *H. polygyrus* affect DC migration in response to BCG vaccination**
5 Tracking of cells migrating to the pLN 48-72 hours after BCG infection was done by
6 labelling the footpad with CFSE by injection 48 hours after BCG vaccination ($1x10^6$
7 CFU) and measuring the number of labelled ($CFSE^{hi}$) cells in the draining pLN 24
8 hours later by FACS. A) Gating strategy for detection of migratory (CFSE positive)
9 cells in response to BCG infection. B) Numbers and C) frequencies of $CFSE^{hi}$MHC-
10 II^{hi} $CD11c^{+/int}$ cells in pLN following BCG vaccination in mice with chronic *H.*
11 *polygyrus* (HP) infection or free of worms. D) $CFSE^{hi}$MHC-$II^{hi}$$CD11c^{+/int}$ cell numbers
12 in pLN, where the BCG infection site (footpad) had been pre-conditioned of with 5 ng
13 TGFβ, 5μg HES or 5μg heat inactivated (Hi)-HES as indicated 10 days prior to BCG
14 injection. Results shown are representative of two or more experiments with 3-5 mice
15 per group. Control mice were preconditioned with 5μg OVA or left untreated and only
16 infected with BCG. Background migration was monitored by injection of PBS in naïve
17 mice. Significant differences are indicated as follows, *P<0.05; ** P<0.01; ***
18 P<0.001.