1 **Host protection to intestinal worm infections: the importance of activated and armed**
2 **innate effector cells at the host parasite interface.**

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22 responses

24 **ABSTRACT**

25

26 Intestinal roundworms cause chronic debilitating disease in animals, including humans.

27 A lack of effective vaccines and the emergence of widespread drug resistance only

28 increase the need to better understand parasite clearance mechanisms within the host.

29 *Heligmosomoides polygyrus* larvae induce a strong intestinal granuloma response within

30 their murine host, which has been associated with resistance. Immune cells, mostly

31 alternatively activated macrophages and eosinophils, accumulate around the tissue

32 encysted parasites to immobilize and damage/kill developing worms. In a one dose

33 (bolus) experimental infection, infected C57Bl/6 mice are unable to clear parasites

34 which results in chronic infection with high worm burdens. However, using a frequent

35 dose trickle model of infection, we, like others, have found that C57Bl/6 mice can clear

36 infection. We found that the clearance is associated with higher granuloma numbers,

37 but no changes in systemic/intestinal Th2 responses. Within the granulomas, we found

38 that myeloid cells had a different transcriptional profile in each of the infected groups,

39 and that high IgG1, but not IgG2c, IgA or IgE, levels were observed around the larvae of

40 only trickle-infected mice. Our results highlight the importance of the granuloma in the

41 host's ability to clear *H. polygyrus* and emphasise the need to study this key tissue in

42 more depth, rather than using correlates such as general intestinal or systemic

43 responses.

45 **AUTHOR'S SUMMARY**

46

47 Despite decades of research on intestinal parasitic worms, we are still unable to clearly
 48 point to why so many people (approximately 1.8 billion) and most livestock/wild animals
 49 are infected with these parasites. We have made progress in understanding how the
 50 immune system responds to parasitic worms, and how these parasites manipulate our
 51 immune system. However, identifying effective clearance mechanisms is complex and
 52 context dependent. We have used a model of trickle infection (multiple low doses of
 53 parasites) to simulate how people/animals get infected in the real world. Using this
 54 model, we have identified the host/parasite interface (the granuloma) within the
 55 intestinal tissue to be key in determining the host's ability to clear worms. Specific gene
 56 expression signatures in granuloma immune cells and the presence/absence of
 57 antibodies within the granuloma are key factors associated with parasite clearance.
 58 Surprisingly, more common identifiers of parasitic worm infections (increased serum
 59 antibody levels and/or generalized immune markers) did not associate with protection.
 60 These novel findings contribute to a better understanding of the mechanisms underlying
 61 effective parasitic worm clearance.

63 INTRODUCTION

64

65 Gastrointestinal nematodes are parasites adept at causing chronic recurring infections.

66 Hosts mount a strong immune response to these parasites, essential to control worm

67 burden and host tissue damage. However, the efficacy of the response is dependent on

68 genetics, infection dynamics and environment.

69

70 *Heligmosomoides polygyrus* is an enteric nematode parasite of mice [1],[2]. Ingested

71 larvae encyst in the host intestinal wall and mature into adults that escape into the

72 lumen. Adults remain in the intestinal lumen for the duration of infection. *H. polygyrus*

73 tissue dwelling stages cause the release of alarmins from epithelial cells as they damage

74 the intestinal wall [3]–[5]. Alarmin-activated innate lymphoid cells and Th2 polarized

75 CD4⁺ T cells produce Th2 cytokines [6]–[9] which promote innate immune cell influx to

76 the intestine [2],[6],[10]. The accumulation of immune cells is referred to as a

77 granuloma [7],[11],[12]. Increased granuloma size and number are associated with

78 increased resistance to nematodes [13].

79

80 Within the granuloma, the host response focuses on damaging or killing the parasitic

81 nematodes, as well as healing the damage caused by the growing worm (reviewed in

82 [14]). Eliminating tissue stage parasites is thought to rely on antibody dependent cell

83 mediated cytotoxicity (ADCC) by macrophages and eosinophils [7],[15],[16], the main

84 cellular players within the granuloma. Antibodies are key to this process. Resistant

85 strains of mice have been shown to develop faster and more intense parasite specific
 86 antibody responses following *H. polygyrus* infections, as compared to susceptible
 87 strains, where isotypes IgG₁, IgA and IgE have been linked to worm clearance
 88 [2],[17],[18]. Passive transfer of serum, and specifically IgG₁, from infected mice results
 89 in decreased adult worm burden and fecundity [19]–[22]. *H. polygyrus* adult numbers
 90 are increased in infected mice lacking IgA [23]. IgG₁ and IgE have also been negatively
 91 correlated with worm survival across different strains of mice [24]–[26].
 92
 93 Alternatively activated macrophages (AAMs) isolated from *H. polygyrus*-induced
 94 granulomas have increased surface levels of FcγRs as well as complement receptor
 95 CD11b and surface binding of IgG₁ and IgG₃ [27]. However, the exact mechanisms
 96 responsible for parasite adherence and killing remain controversial. *In vitro* observations
 97 have yet to be validated *in vivo*. For example, the CD11b receptor on bone marrow
 98 derived macrophages can directly bind *H. polygyrus* larvae using complement 3 when
 99 cultured with immune serum. *In vitro*, after adhering to the larvae via the CD11b-C3
 100 interaction [27], FcγR1 on macrophages can interact with parasite bound IgG_{2a/c} (but not
 101 IgG₁) antibodies to immobilize the larvae [28]. However, this binding does not reduce
 102 the infectivity of larvae *in vivo* [27]. In contrast, mice vaccinated with *H. polygyrus*
 103 excretory/secretory products (HES) are protected from chronic infection as a result of
 104 the IgG₁ response [5]. IgG₁ antibodies are thought to bind and neutralize parasite
 105 excretory secretory products, that in unvaccinated mice are able to interfere with the

106 functions of protective innate immune cells [5]. However, passive transfer of purified
 107 IgG₁ did not induce sterile immunity suggesting that other mechanisms are at play [5].
 108 As well as being involved in parasite damage and death, AAMs and eosinophils are both
 109 also involved in host tissue repair [14]. AAMs produce immunoregulatory and wound
 110 healing molecules [29],[30] which promote extracellular matrix (ECM) deposition during
 111 helminth infections [31]–[34]. *H. polygyrus* infections induce Ym1 and RELM- α secretion
 112 from AAMs [2], both linked with the wound healing phenotype [35]. Eosinophils also
 113 produce RELM- α [36], TGF- α , TGF- β and fibroblast growth factors [37]–[39]. In addition,
 114 Arginase 1, a marker for AAMs has been found to be essential in both parasite expulsion
 115 and wound healing during *H. polygyrus* infections (9,40). Collagen is a major component
 116 of the ECM and excessive collagen deposition leads to fibrosis and scarring during
 117 chronic helminth infections [41].
 118
 119 Immune responses are not only generated to tissue dwelling parasitic stages but also to
 120 the adults found in the intestinal lumen. The cytokines IL-4 and IL-13 enhance smooth
 121 muscle contractility of the intestine via STAT6 dependent pathways [42] to help
 122 eliminate adult worms [43]–[45]. IL-4, IL-9 and IL-13 also regulate goblet cell hyperplasia
 123 and increase mucus production during gastro-intestinal (GI) nematode infections
 124 [46],[47], which makes it more difficult for adult parasites to coil around intestinal villi.
 125 In addition, RELM- β produced by goblet cells interferes with the ability of adult parasites
 126 to feed, thus limiting their numbers [48]. Finally, *H. polygyrus* infections induce

127 polyclonal and parasite specific serum antibody responses, which function to limit adult
128 female egg production [5],[23],[27],[28].

129

130 Most of the murine studies on helminth infection use a bolus model of infection (one
131 large dose), with some groups adopting a drug clearance model (bolus infection, drug
132 clearance, bolus infection) to simulate mass drug administration programs [7],[49].

133 However, under natural conditions, GI nematodes are ubiquitous in the environment
134 [50] and hosts are constantly coming into contact with them. While many hosts are

135 infected, few have life threatening levels of worms implying immune regulatory

136 mechanisms are at play [5]. Most hosts are unable to clear infection but can limit

137 excessively damaging worm burdens [13]. Hence, we (and others) have set up

138 experimental infection models using trickle infections to study parasite clearance of GI

139 nematodes in a more natural setting [18],[51]–[53]. We use multiple low doses of

140 larvae, given over a specific time period to achieve this.

141

142 *H. polygyrus* trickle infections in genetically resistant and susceptible strains of mice

143 reveal that the frequency of infection is an important determinant of parasite expulsion,

144 where frequently infected mice eliminate worms more rapidly than mice infected with

145 the same total number of larvae but in less frequent doses [53]. The aim of our study

146 was to identify the host protective immune mechanisms underlying these results.

147 Previous studies speculated that improved antibody and innate immune cell responses

148 to tissue dwelling parasites were key elements [18],[53]. We were able to reproduce the

149 data demonstrating that in susceptible mice, trickle infection results in reduced worm
150 burdens. However, we were also able to show that this reduction was associated with
151 increased levels of antibodies bound to tissue larvae and a specific gene expression
152 signature in the granulomas. All other correlates of Th2 immunity measured between
153 the bolus- and trickle-infected mice were similar including systemic Th2 cytokine
154 responses and antibody levels, as well as local physiological, mucosal and immunological
155 responses in the small intestine. Our results highlight the importance of the granuloma
156 in the host's ability to clear *H. polygyrus* and emphasise the need to study this key tissue
157 in more depth, rather than using correlates such as general intestinal or systemic
158 responses.

159

160 MATERIALS AND METHODS

161

162 *Mice, parasites and antigen*

163

164 Female and male C57Bl/6 mice aged 6-8 weeks (bred and maintained at the animal care
165 facility, Department of Biological Sciences, University of Calgary or University of
166 California, Riverside, USA) were used for all experiments. All animal experiments were
167 approved by the University of Calgary's Life and Environmental Sciences Animal Care
168 Committee (protocols AC17-0083 and AC17-0240) and the University of California,
169 Riverside's Institutional Animal Care and Use Committee
170 (<https://or.ucr.edu/ori/committees/iacuc.aspx>; protocol A-20180023). All protocols for

171 animal use and euthanasia were in accordance with either the Canadian Council for
172 Animal Care (Canada) or National Institutes of Health (USA) guidelines. Animal studies
173 are in accordance with the provisions established by the Animal Welfare Act and the
174 Public Health Services (PHS) Policy on the Humane Care and Use of Laboratory Animals.
175
176 Female BALB/c mice and Swiss Webster mice aged 6-8 weeks were purchased from
177 Charles River Laboratories (Senneville, Quebec). Infected mice were orally gavaged with
178 200 third stage *Heligmosomoides polygyrus* larvae (maintained in house, original stock
179 was a gift from Dr. Allen Shostak, University of Alberta, Canada) and euthanized at
180 either 7, 14, 21 or 28 days post initial infection. Mice were infected according to the
181 bolus or trickle infection regimes (Fig 1A, 1B & 8A). To avoid differences in counts during
182 the trickle infections, on day 0, two identical bolus solutions were made up (200
183 worms/100ul). One was used to infect the bolus infected mice and one was used for the
184 trickle infected mice and diluted as necessary according to the number of trickle doses.
185
186 *H. polygyrus* antigen was prepared by collecting live adult worms from 14-day infected
187 mice using modified Baerman's apparatus. Worms were washed multiple times and
188 homogenized in PBS using a glass homogenizer. The resulting solution was centrifuged
189 (13, 000 g, 10 minutes, 4°C) and the supernatant filtered (0.2µm filter, Nalgene). The
190 protein concentration was calculated using the Bradford assay. The antigen was stored
191 at 15 mg/ml at -80°C.
192

193 *Adult worm burden and granuloma number*

194

195 Small intestines of infected mice were harvested and opened longitudinally. The number
196 of adult worms present in the intestinal lumen and of granulomas present along the
197 length of the small intestine were counted using a dissection microscope.

198

199 *Transit time*

200

201 Gastrointestinal transit time was measured one day prior to euthanasia. Mice were fasted
202 for 6 hours and 200 µl of 5% Evans blue (Sigma) in 5% gum arabic (ACROS organics) was
203 orally gavaged using a ball tip 20 gauge 1.5", 2.25mm curved animal feeding needle. Each
204 mouse was labelled, with the time of dye administration recorded. Mice were transferred
205 to clean empty cages and the time to pass the first blue fecal pellet was recorded.
206 Gastrointestinal transit time was calculated for each mouse.

207

208 *Cell isolation and in vitro re-stimulation assay*

209

210 MLN and SPL were mechanically dissociated into single cell suspensions. Cells were
211 counted using a Beckman-Coulter ViCell XR. MLN and SPL were cultured at 1×10^6
212 cells/ml for 48 hours in RPMI medium, 10% FCS, 1% L-glutamine, 1% penicillin/
213 streptomycin (supplemented RPMI 1640) in the presence of 10 µg/ ml *H. polygyrus*

214 antigen or 2 µg/ml concanavalin A (Sigma) at 37 °C with 5% CO₂. Supernatants were
215 collected for cytokine measurements. Measurements for antigen specific production
216 were not included in the analysis unless cytokine production was observed in the wells
217 with concanavalin A stimulation.

218

219 *Serum*

220

221 Blood samples were collected using a terminal cardiac bleed. Blood was left to clot for
222 30 minutes and then centrifuged twice at 11, 000 g at 4°C for 10 minutes. Serum was
223 collected and used either fresh or stored at -80°C.

224

225 *Intestinal Tissue homogenates*

226

227 Small intestines were opened longitudinally and washed with PBS to remove luminal
228 content. The mucosal surface was identified under a dissecting microscope. The mucosal
229 surface (with its mucus) was gently scraped using a glass slide. Scrapings were weighed,
230 added to 500 µl lysis buffer (10 µM tris HCl, 0.025% sodium azide, 1% tween 80, 0.02%
231 phenylmethylsulfonyl fluoride) with one complete protease inhibitor tablet (Roche
232 diagnostics GmbH, Germany) and homogenized using a bead beater (40 seconds at
233 speed 6 using the Fast-prep-24 bead beater, MP biomedical). The homogenate was
234 centrifuged at 11, 000 g at 4°C for one hour. Supernatants were collected and used fresh
235 or stored at -80°C.

236

237 *ELISAs*

238

239 Cytokines in serum and intestinal tissue homogenates were measured by ELISA
240 according to manufacturer's guidelines (R & D systems, DY404 (IL-4), DY413 (IL-13),
241 DY594 (IL-21). Total IgE (BD, 555248) and IgA (capture antibody, BD, 556969, detection
242 antibody, BD, 556978) levels were measured by ELISA according to manufacturer's
243 instructions.

244

245 Antigen specific antibody responses were also measured by ELISA. ELISA microplates
246 were coated with 10 µg/ml *H. polygyrus* antigen in carbonate buffer (0.1mM NaHCO₃,
247 pH 9.6), overnight at 4°C. Plates were blocked with 2% BSA in TBS/0.05% tween 20 for 2
248 hours at 37°C. Sera were diluted in TBS Tween and added to wells overnight at 4°C.
249 Antigen- specific IgG₁ was detected with HRP-conjugated corresponding detection
250 antibodies (anti-IgG₁ (BD, 553441) with TMB peroxidase substrate (T3405, Sigma). The
251 reaction was stopped using 1M H₂SO₄ solution and the colour change was read at
252 450nm.

253

254 *Antibody detection in the granuloma*

255

256 Consecutive formalin fixed paraffin embedded mouse small intestinal sections were
257 deparaffinized using two, five-minute xylene washes, rehydrated by washing in 95%

258 ethanol and 70% ethanol for 5 minutes. Slides were incubated in 2% sodium borohydride
 259 (VWR, BDH4604) in PBS for 40 minutes at RT to remove auto fluorescence. Antigens were
 260 retrieved using 2.5% trypsin (Thermo scientific, 15090046) in 0.1% HEPES buffer,
 261 incubated at 37 °C for 25 minutes. Blocking steps were performed following PBS washes.
 262 All samples were blocked with starting block (Thermo Scientific, 37578) for 1 hour at RT
 263 and rat/rabbit/goat serum for 30 minutes at RT. Following blocking steps, slides were
 264 incubated with rat anti-mouse IgG₁ (BD, 562026), IgE conjugated to FITC (BD, 553415) or
 265 rat anti-mouse IgA (BD, 559354) overnight at 4°C. For IgG_{2c} staining, slides were incubated
 266 overnight at 4°C with unconjugated rabbit anti-mouse IgG_{2c} antibody (Invitrogen, SA5-
 267 10221) followed by incubation with goat anti-rabbit IgG conjugated to Alexafluor488
 268 (ImmunoResearch Laboratories, 111-545-003) for 2 hours at RT. Slides were washed in
 269 PBS for 15 minutes and mounted with Fluoroshield with DAPI (Sigma, F56057). Images
 270 were acquired using Thorlabs Tide whole-slide scanning microscope, x20 objective and
 271 analysed using Fiji 5.59.05 software.

272

273 *Nanostring nCounter gene expression assay*

274

275 Intestinal tissue from naïve mice or dissected pooled granulomas from infected mice were
 276 snap frozen in liquid nitrogen and RNA was isolated using phenol-chloroform extraction
 277 (TRIZOL, Sigma). RNA was quantified using a nanodrop and 50 ng was used for the Myeloid
 278 Innate Immunity V2 panel (NanoString) according to the manufacturer's guidelines. Gene
 279 expression analysis was conducted in R (1). Gene counts obtained via the nanostring

280 hybridization assay were normalized with NanostringNorm (2) using the negative control
 281 probes, positive control probes and housekeeping genes Eif2b4, Polr1b, and Edc3. Of the
 282 20 housekeeping genes included in the assay Eif2b4, Polr1b, and Edc3 were the only ones
 283 found to have consistent expression among all samples in preliminary comparisons that
 284 used all 20 housekeeping genes for normalization. Therefore Eif2b4, Polr1b, and Edc3
 285 were the only housekeeping genes used for normalization in subsequent analyses. The
 286 normalized counts were then compared using DESeq2 (3) to find differentially expressed
 287 genes in pairwise comparisons between treatment groups. A false discovery rate adjusted
 288 p-value cut off of 0.05 and a fold-change cutoff of 2 were used to identify genes that were
 289 differentially expressed in each pairwise comparison. The data discussed in this
 290 publication have been deposited in NCBI's Gene Expression Omnibus (4) and are
 291 accessible through GEO Series accession number GSExxx (number will be available upon
 292 acceptance).

293

294 *Statistical analysis (except for nanostring results)*

295

296 Mann-Whitney and Kruskal Wallis tests with Dunn's multiple comparisons were used to
 297 assess differences between either two or more experimental groups using GraphPad
 298 Prism.

299

300

301

302

303 RESULTS

304

305 *C57Bl/6 mice develop a resistant phenotype when infected using the trickle regimen*

306

307 Two inbred strains of mice, C57Bl/6 (genetically susceptible) and BALB/c (genetically
308 resistant) [13] were infected with *H. polygyrus* according to the bolus or trickle adult
309 infection regimes (Fig. 1A & 1B). When given a bolus infection of 200 worms, BALB/c mice,
310 being partially resistant to *H. polygyrus*, eliminated the majority of their worms by 14 days
311 post-infection (mean worm burden of 53, SD +/- 30, Fig. 1C). Worm burdens declined over
312 time until day 28 post infection, where the average number of worms per mouse was 8.5.
313 SD +/- 13 (Fig. 1C). In contrast, C57Bl/6 mice being susceptible to *H. polygyrus* harboured
314 high numbers of adult worms (an approximate mean value of 100 worms per mouse, Fig.
315 1D) in the intestinal lumen at all time points tested (Fig. 1D left). When infected according
316 to the trickle protocol, infection dynamics in BALB/c mice were similar to those infected
317 with the bolus regimen, whereby mice had low burdens at all time points post-infection,
318 with near complete clearance by day 28 post-infection (Fig. 1C). However, C57Bl/6 mice
319 infected according to the trickle protocol also eliminated most of the adult worms by 28
320 days post infection (mean worm burden 11, SD +/- 22, Fig. 1D). This mirrored the BALB/c
321 mice results (Fig. 1C) but was in stark contrast to the results obtained for C57Bl/6 mice
322 infected according to the bolus protocol despite similar worm burdens at day 14 post-
323 infection (bolus: 129, SD +/- 38, trickle: 106, SD +/- 24, Fig. 1D). Administering *H. polygyrus*

larvae in low frequent doses to C57Bl/6 mice changed their susceptibility to infection (Fig. 1D).

Systemic IL-4 and IL-13 responses do not differ between bolus and trickle infected mice.

H. polygyrus clearance has been associated with a strong Th2 response, specifically increases in IL-4 and IL-13 cytokines [54]–[56]. As the Th2 immune response develops in response to *H. polygyrus*, MLN (mesenteric lymph nodes) and SPL (spleen) cell numbers increase [57]. Despite BALB/c mice having higher numbers of cells than C57Bl/6 mice at all time points in both organs, we found no differences between the trickle and bolus groups in either strain at any of the time points in either of the organs (Fig. 2A-D, left panel).

We measured the levels of the antigen-specific Th2 cytokine production (IL-4 and IL-13) in both the MLN and SPL as well as in the serum of mice by ELISA. Levels of cytokines measured in BALB/c mice were in general higher than in C57Bl/6 mice, as has previously been reported [13],[58],[59]. In BALB/c animals, levels were increased early and remained elevated over the course of infection for IL-4 in the MLN (increased from day 7, Fig. 2A middle panel) and the SPL (increased from day 14, Fig. 2C middle panel) and IL-13 in the MLN (increased from day 14 but decreased by day 28, Fig. 2A right panel) and SPL (increased from day 7, Fig. 2C right panel). In C57Bl/6 animals, levels were increased early but decreased by the later time points for IL-4 in the MLN (decreased from day 21, Fig. 2B

middle panel) and the SPL (decreased from day 21, Fig. 2D middle panel) and IL-13 in the MLN (decreased by day 28, Fig. 2B right panel) and SPL (increased over the whole infection, Fig. 2D right panel). Levels of IL-4 and IL-13 were undetectable in the serum, as seen by others [60].

350

Taken together, we show that despite differences in cytokine levels according to mouse strain, organ and post-infectious time-point, no differences were detected between bolus and trickle infected mice in any of the conditions tested (Fig. 2).

354

Serum antibody levels do not differ between trickle and bolus infected mice.

356

IgE and parasite specific IgG1 have both been shown to increase during primary and secondary *H. polygyrus* infection [49]. IgG1 has been associated with parasite clearance [5] while IgE is thought to reduce parasite fecundity [23]. We investigated changes in serum antibody responses following bolus and trickle infections in both BALB/c and C57Bl/6 mice over the course of infection.

362

Despite an increase in total serum IgE over the course of infection in both mouse strains (BALB/c maximum levels on day 14 post infection with 559 ng/ml, SD +/- 94 compared to naïve levels at 68 ng/ml, SD +/- 58 and C57Bl/6 maximum levels on day 21 post-infection with 624 ng/ml, SD +/- 445 compared to naïve levels of 40 ng/ml, SD +/- 21), there was

367 no difference in levels between bolus- and trickle-infected groups at any post-infection
368 time point (Fig. 3A & B).

369

370 We also measured an increase in parasite specific IgG1 over time, as has previously been
371 reported [23]. In BALB/c mice, at both days 21 and 28 post-infection, titers were above
372 10^4 (Fig. 3C), while in C57Bl/6 mice, despite an increase in titers, these remained below
373 10^4 at all time points (Fig. 3D). However, again, no differences were observed between
374 trickle and bolus infected animals in either strain, at any time point. Also, no detectable
375 levels of *H. polygyrus* larval or adult parasite antigen specific IgE, IgG2c or IgA were
376 observed in the serum of bolus- or trickle-infected mice at any post-infection time point.

377

378 ***Local physiological, mucosal and immunological responses in the small intestine are***
379 ***not responsible for the improved protection observed in trickle-infected C57Bl/6 mice***

380

381 Worm infections result in physiological changes in the small intestine that have been
382 linked to promoting worm expulsion. These include increased intestinal smooth muscle
383 contractility [61], and therefore, decreased intestinal transit time, as well as increased
384 mucus production. We found that transit time (as measured by the time to pass dyed
385 gavigated material) was reduced in infected C57Bl/6 animals by day 7 post infection, in
386 both trickle and bolus infected groups by approximately 20% (Fig. 4A, left panel).
387 However, this decrease was only apparent in trickle infected animals by day 21 post-
388 infection (approximate 12% difference between the trickle- and bolus-infected groups).

389 At day 28 post-infection, the difference between bolus- and trickle-infected groups
390 disappeared (approximate 3% difference between the trickle and bolus infected
391 groups). We also measured mucus production indirectly through intestinal tissue
392 weights. Infected mice had significantly higher intestinal tissue weights at 7 days
393 (approximately 1.5 times naïve weights in both trickle- and bolus-infected mice) and 21
394 days (approximately 2.5 times naïve weights in both trickle- and bolus-infected mice)
395 compared to naïve animals. Levels had returned to naïve levels by 28 days post-infection
396 (Fig. 4A, right panel). There was no difference between the bolus- and trickle- infected
397 groups.

398
399 Next, we measured the levels of the Th2 cytokines IL-4 and IL-13 by ELISA in intestinal
400 tissue, since both these cytokines have been associated with stimulating increased
401 mucus production in the small intestine [62]. While levels of IL-13 were increased at day
402 21 and 28 post-infection, they were not different between the trickle- and bolus-
403 infected groups (naïve: mean level 125 pg/ml, SD +/- 141, day 21: bolus mean level 558
404 pg/ml, SD +/- 268, trickle mean level 309 pg/ml, SD +/- 231, day 28: bolus mean level
405 363 pg/ml, SD +/- 254, trickle mean level 366 pg/ml, SD +/- 216, Fig. 4B, right panel).
406 Intestinal IL-4 levels were not increased at any point (approximately 40pg/ml) apart
407 from at day 21, where they were significantly increased in the bolus-infected group (132
408 pg/ml, SD +/- 85) and different to the trickle-infected group. No levels of IL-5, IL-9 or IL-
409 10 were detectable in intestinal tissue from control or infected mice.

410

411 Mucosal IgA levels, regulated by the cytokine IL-21 [63], are also increased in the
 412 presence of intestinal dwelling parasites [64]. We therefore measured IgA and IL-21
 413 levels in the small intestine (Fig. 4C). Intestinal IgA levels did not differ between bolus-
 414 or trickle-infected mice over the course of infection (Fig. 4C, right panel). Interestingly,
 415 levels in both infection groups were similar to naïve levels (1292 pg/ml, SD +/- 924) at
 416 day 7 and 21 post-infection, but decreased at day 28 post-infection (bolus: 832 pg/ml,
 417 SD +/- 754; trickle: 555 pg/ml, SD +/- 93). Contrastingly, IL-21 levels were increased at
 418 day 21 and 28 post-infection (from 98 pg/ml, SD +/- 84 in naïve animals to
 419 approximately 950 pg/ml in infected animals, Fig. 4C, left panel). Like IgA levels, they
 420 were not different between trickle- and bolus-infected animals.

421
 422 In summary, at the level of the small intestine, very few differences were observed
 423 between bolus- and trickle-infected animals, and these differences were not associated
 424 with reduced worm burden.

425
 426 ***Unlike bolus infection, trickle infection results in stable granuloma numbers over the***
 427 ***course of infection in C56BL/6 mice.***

428
 429 Since few differences were recorded between trickle- and bolus-infected mice in the
 430 small intestine, we focused on the host/parasite interface, the intestinal granuloma.

431

Granulomas are a characteristic response to intestinal roundworms [14]. In response to *H. polygyrus*., they are round, opaque hard structures protruding from the small intestinal wall, easily identifiable and quantifiable using a dissection microscope (Fig. 5A). We measured these structures in both C56Bl/6 and BALB/c mice over the course of infection (days 7, 14, 21 and 28 days post-infection). We found that, BALB/c mice, both trickle- and bolus-infected, had consistently high granuloma numbers over the first three time points (granuloma numbers >45, Fig. 5B). At day 28 post-infection, numbers dropped below this. In C57Bl/6 mice, a similar pattern was observed for the bolus-infected mice: at days 7 and 14, granuloma numbers were high, and >45, at days 21 and 28, granuloma numbers were reduced to ~17 (Fig. 5C). However, in trickle-infected C57Bl/6 mice, granulomas remained > 45 for the entire time course (Fig. 5C).

In C56Bl/6 mice, granulomas from bolus and trickle- infected mice have different patterns of gene expression.

Since protective immune responses to *H. polygyrus* infection are thought to be localized in the granuloma (92), we isolated all granulomas along the small intestine of bolus- and trickle-infected C57Bl/6 mice at days 7 and 21 post-infection. To identify the granuloma transcriptional profiles, we extracted the mRNA and quantified transcript levels using the nanostring myeloid innate immunity V2 panel. For naïve mice that do not have any granulomas along their small intestine, we harvested intestinal tissue from similar areas to those harvested in infected mice.

454 Using principal component (PC) analysis, we found that infection is responsible for the
455 largest differences observed between the three groups (naïve, bolus-infected and
456 trickle-infected) at both post-infection time points. Naïve and infected groups clustered
457 distantly from each other, with infection explaining 62.1% of the variation (PC1) at day 7
458 post-infection and 90.5% of the variation (PC1) at day 21 (Fig. 6). Along the second
459 principal component, granulomas from day 21 (but not day 7) infected mice clustered
460 separately according to their infection mode (trickle or bolus, Fig. 6).

461
462 We found a number of genes that were differentially expressed between naïve
463 intestinal tissue (naïve group) and granuloma tissue (infected groups trickle and/or
464 bolus) at day 7 and day 21 post-infection. At day 7, a total of 40 genes were upregulated
465 in infected (NvT and NvB) vs. naïve animals: 9 are involved in cell migration, 11 in
466 chemokine signalling, 6 in ECM and 5 in lymphocyte activation (Fig. 7A, Fig. S1). Over
467 half of the upregulated genes (57%, 23/40) were also found to be upregulated at day 21
468 post-infection. Many of the identified differentially expressed (DE) genes have already
469 been implicated in the immune response to helminths, such as *Retnla*, *Arg1*, *Chil3*, *Chil4*
470 and *Retlnb* [48],[65]. Fifteen of the 40 genes were highly upregulated (more than 16-
471 fold); they are mostly involved in chemokine signaling, likely attracting macrophages
472 and eosinophils to the granuloma. A total of 46 genes were downregulated in infected
473 vs. naïve animals (NvT and NvB) at day 7 post-infection: 9 involved in cytokine signaling,
474 11 in growth factors, 8 in ECM, 6 in metabolism and 9 in lymphocyte activation. Seven of
475 these genes (~15%) were also downregulated at day 21 post-infection. Twenty four of

the 46 genes were highly downregulated, and were associated with all functional categories. At day 21 post-infection, 75 genes were upregulated in infected vs. naïve (NvT and NvB) (Fig. 8A, Fig. S2): 12 involved in cell migration, 12 in chemokine signalling, 17 in ECM, 14 as growth factors, 10 in metabolism and 15 in lymphocyte activation. Forty-one of these 75 genes were highly upregulated; these are involved in attracting macrophages and eosinophils (e.g. *chil4*, *chil3*, *Serpine 1*, *ccl7*, *cxcl3*, *cxcr4*), as well as ECM remodelling (e.g. *mmp12*, *col1a2*, *cma1*, *Arg1*). Twelve genes were downregulated in infected vs. naïve (NvT and NvB): 2 involved in cytokine signalling, 2 in growth factors, 2 in pathogen response and 3 in metabolism. None were highly downregulated: the most downregulated gene was *nos2* at ~7 times.

When looking at differentially expressed genes between trickle- and bolus-infected animals, we also found differences at both time points (day 7 and day 21). At day 7 post-infection, 10 genes were upregulated in trickle (vs. bolus) infected animals (Fig. 7A & 7B): 3 are TLR related, 3 are growth factors and 3 are involved in cytokine signalling. Of the 10 DE genes, 6 are highly upregulated: *Selp*, *Tlr6*, *Bcl2*, *Tlr12*, *Flrt2*, *Il3ra* and one is also upregulated at day 21: *cc/3*. At day 21 post-infection, 24 genes were upregulated in trickle (vs. bolus, Fig. 8A & 8B): 4 are TLR related, 7 are growth factors and 8 are involved in cytokine signaling. Of the 24 DE genes, 3 were highly upregulated: *Adamts4* involved in ECM, *Osm* involved in cytokine production and *Ndc80* involved in cell division. Twelve genes were upregulated in bolus (vs. trickle) at day 21 post-infection compared to none at day 7. Three of these genes were growth factors and three

involved in cytokine signaling. No genes were highly upregulated, all were expressed below 7-fold and were associated with many different functions. The three most highly upregulated in the bolus infected animals (*cxcr3*, *cxcr4*, *ptgdr*) are involved in eosinophil recruitment and Th1 immunity.

Despite having many genes commonly expressed between the granulomas of trickle- and bolus-infected mice, different gene expression signatures were identified between these two modes of infection. Of the genes previously associated with immune responses to helminths, granulomas from day 21 trickle-infected animals had higher levels of *Il13* (associated with worm expulsion [47],[54]), *S100A8* and *S100A9* (associated with neutrophil recruitment [66]), and *Retlnb* (associated with worm death [48]), as well as *ccl3* and *cxc15* (inflammatory chemokines) expression. Surprisingly, we found no differences between the trickle and bolus infections in any genes linked to Fc receptor signalling, despite the importance of antibody-mediated worm killing by macrophages and/or eosinophils within granulomas [14].

IgG1 is the only antibody subtype to accumulate around encysted larvae in trickle infected C57Bl/6 mice.

The ability to immobilize and/or kill parasitic worm larvae has been linked to antibody-mediated binding by myeloid cells in the granuloma [14]. IgG1, IgG2c, IgE and IgA antibody subtypes have all been linked to larval binding and/or damage to varying

520 degrees. Since we found no differences in the expression of Fc receptor signalling genes
521 within the granuloma between bolus and trickle-infected groups, we measured the
522 accumulation of IgG1, IgG2c, IgE and IgA antibodies using immunofluorescence, both
523 within the intestine and focusing on the host parasite interface within the granulomas
524 (Fig. 9-10). First, we found that high levels of IgG1 in the serum at day 21 post-infection
525 (Fig. 3D) correlated with the presence of IgG1 in granulomas in both bolus- and trickle-
526 infected mice (Fig. S3A). At day 7, where antigen specific serum IgG1 could not be
527 detected (Fig. 3D), IgG1 levels were minimal and/or absent (Fig. S3B).

528

529 To study the host/parasite interface, we set up a trickle (larvae) model (Fig. 9A), in
530 which granulomas containing larvae (acute granulomas) and granulomas where larvae
531 had either escaped or been killed (chronic granulomas) could be observed. Using this
532 model, we found a high concentration of IgG1 at the host parasite interface at both day
533 14 and day 21 (Fig. 9B) post-infection in acute granulomas. However, in both trickle and
534 bolus infected animals, levels of IgG1 were similar in chronic granulomas (Fig. 9C). We
535 could not detect any IgG2c, IgE or IgA within acute granulomas of trickle (larvae)
536 infected mice (Fig. 10A). This was despite observing IgG2c in intestinal tissue infected by
537 *C. rodentium* (Fig. S4), IgE in the lamina propria of infected mice (Fig. 10B) and IgA in the
538 lamina propria and Peyer's patches of infected mice (Fig. 10C).

539

540 Overall, our data show that bolus and trickle infection result in similar systemic and
541 tissue-wide immune responses. However, granuloma formation is distinct between the
542 two types of infection, and correlate with different resistant phenotypes.

543

544 **DISCUSSION**

545 The formation of granulomas around tissue encysted *H. polygyrus* worms has been
546 associated with resistance to infection [13]. The innate cells of the granuloma are
547 thought to damage/kill worms in conjunction with antibodies and complement
548 components [18],[27],[28]. We and others [13],[67] have shown that resistant BALB/c
549 mice have more granulomas (Fig. 5) as well as higher levels of Th2 cytokines (Fig. 2) and
550 parasite specific antibodies (Fig. 3) compared to susceptible C57Bl/6 mice. These
551 differences are thought to contribute to the BALB/c resistance phenotype.

552 Using our trickle model, we observed a resistance phenotype in the C57Bl/6 mice as
553 opposed to the susceptible phenotype of bolus infected animals (Fig. 1). The improved
554 immune response we observed is likely due to the continuous stimulation of the host
555 immune system by multiple low doses of larvae resulting in a greater number of more
556 effective granulomas (better responsive myeloid cells and the presence of IgG1
557 antibodies, Fig. 8 & 9) and ultimately fewer adult worms (Fig. 1). Unlike differences
558 observed between resistant BALB/c and susceptible C57Bl/6 mice, we found no
559 significant differences between the systemic (serum antibody and spleen/MLN cytokine
560 response, Fig. 2 & 3) or tissue-wide (intestinal tissue physiological, cytokine and
561 antibody responses, Fig. 4) immune responses of bolus and trickle infected C57Bl/6

562 mice. Only when studying the granulomas themselves, at the host parasite interface,
563 were important differences observed (Fig. 8 & 9).
564
565 Granulomas are made up of myeloid cells (mainly alternatively activated macrophages
566 and eosinophils) and CD4⁺ T cells [7],[12] that accumulate around tissue encysted
567 worms and immobilize them. As expected [14], we identified increased gene expression
568 linked to myeloid cell recruitment, Th2 immunity and ECM deposition in granulomas
569 from both trickle and bolus infected groups, at both time points (Fig. 7 & 8).
570 Overall, the increased levels of myeloid cell chemotactic gene expression within
571 granulomas at day 7 in both bolus and trickle infections highlights the strong response
572 to the tissue dwelling phase of the parasite. Ten of the highly upregulated genes at day
573 7 post-infection (NvT and NvB) have been previously associated with immune responses
574 to helminth infections. And at day 21 post-infection, many of these same genes
575 remained upregulated, with an increase in the expression of genes related to ECM
576 remodelling (Fig. S1 & S2). Granulomas are novel structures created around tissue
577 dwelling worms. They are not observed in naïve animals (Fig. 5A). As such, we observed
578 the increased expression of genes associated with collagen production. At day 7 post-
579 infection, *col1a2* was upregulated with infection (NvT and NvB) as seen in other
580 helminth infections [68],[69]. At day 21 post-infection, *Col3a1*, *Col4a1* and *Col15a1* were
581 also upregulated. This is the first time a direct involvement of type III, type IV and type
582 XV alpha 1 collagens have been linked to wound healing in an intestinal parasitic
583 infection.

584 In the bolus-infected mice at day 7 post-infection, a number of genes were
585 downregulated compared to trickle-infected animals (Fig. 7B). These genes are
586 associated with growth factors, as well as TLR and cytokine signalling, in dendritic cells.
587 This could be attributed to the initial parasite dose. Bolus-infected animals were given a
588 dose of 200 worms, known to strongly downregulate the immune response as early as
589 day 7 in the small intestine [70]. The trickle-infected animals received the same total
590 dose, but split over 3 different time points which may have reduced the impact of the
591 parasite. Interestingly, one of the downregulated genes on day 7 post-infection, *Bcl2*
592 (decreased by 33 fold compared to trickle-infected mice), has previously been
593 associated with *H. polygyrus* infection. It was found to be increased in CD4+ T cells in the
594 MLN, 2 weeks post-infection [71]. Similarly, we also found it was increased ~4 fold at
595 day 21 post-infection in the granulomas of both bolus- and trickle-infected animals (Fig.
596 S2).

597 At day 21, the two most highly expressed genes in the trickle-infected mice are involved
598 in tissue remodelling (*Adamts4*: 124 fold, *Osm*: 49 fold, [72]). *Adamts12* (7 fold increase,
599 (5)), *Adamts3* (6 fold increase, (6)), *Hdac5* (3 fold increase, [73]), *Smad2* (2 fold increase
600 [74]), and *Socs 3* (2 fold increase, [75]), were also upregulated and are involved in tissue
601 remodelling. The expression for all these genes is significantly higher compared to both
602 day 21 and day 7 bolus-infected mice. As such, the difference observed is not due to the
603 difference in 'age' of the granulomas, with the bolus day 21 granulomas being 'older'
604 than the trickle day 21 granulomas. Interestingly, to our knowledge, the 2 most highly
605 expressed genes (*Adamts4* and *Osm*) have not been associated with helminth infection

606 before. However, *Osm* is thought to stimulate *adamts4* mediated degradation of the
607 ECM [72], which may be an important process in the regulation of the granuloma
608 structure.

609

610 We also found genes involved in the Th2 response upregulated in the day 21 post-
611 infection trickle-infected animals. *Il-13* was increased 10-fold in the trickle- vs. bolus-
612 infected animals. While *Igf1* was increased 4-fold. *Igf1* is secreted by alternatively
613 activated macrophages, and has been shown to promote worm expulsion. In
614 *Nippostrongylus brasiliensis* infected mice, animals lacking *Igf1* had higher worm counts
615 than their wildtype counterparts [76].

616

617 Granulomas from trickle-infected animals have a different transcriptomic signature to
618 bolus-infected animals, with a stronger Th2 and wound healing response. However,
619 worm killing depends not only on activated myeloid cells, but also antibodies.
620 Antibodies are thought to be the bridge that allows granuloma granulocytes to
621 kill/damage trapped parasites [14]. Antibodies are known to be required for protective
622 immunity against *H. polygyrus* as B cell deficient mice [80] and mice lacking antibody
623 production (J_H or AID deficient, [23]), fail to eliminate adult worms. Of all the antibody
624 isotypes, only IgG₁ from immune mice has been shown to reduce adult worm burdens
625 when administered alone [20], and protect mice from re-infection [22]. Passive transfer
626 of IgG₁ resulted in stunted worms, and *in vitro* assays demonstrated that IgG₁ promotes
627 peritoneal exudate cell attachment to the larval surface [20]. We found that IgG₁ was

the only detectable antibody isotype observed within the granulomas of infected mice (Fig. 9 & 10). No IgE or IgA accumulation was detected (Fig. 10A), despite their presence in the intestinal tissue (Fig. 10B & 10C), serum (Fig. 3B) and intestinal scrapings (Fig. 4C). Interestingly, others have shown that while IgE was found to play no role in parasite clearance in *H. polygyrus* infected animals, IgA contributes to limiting worm development [23]. This was done using IgA deficient animals, and, unlike in our study, levels of antibodies within granulomas were not assessed.

Trickle and bolus infected mice had similar levels of serum IgG₁ (Fig. 3) and of IgG₁ accumulation in their chronic granulomas (containing no tissue dwelling phases) at day 21 post-infection (SupFig. 3A). At this time point, tissue encysted worms had either escaped or been killed and digested within the granulomas in both infected groups. To detect whether IgG₁ was playing a role in damaging/killing incoming worms in the trickle model (as has been suggested by [53]), we examined granulomas earlier (3/4 days as opposed to 10 days) after the last trickle dose in order to observe tissue dwelling worms within granulomas (Fig. 9A). In the granulomas containing worms, IgG₁ was concentrated around the parasites (Fig. 8C). This observation helps explain how antibodies play a role in worm clearance. Two weeks following infection, both bolus- and trickle-infected mice produce a strong parasite specific IgG₁ response (high serum titers, Fig. 3D). However, IgG₁ antibodies can only play a role in ADCC and direct parasite killing/damage in trickle-infected mice when tissue encysted worms and IgG₁ are present simultaneously in the granuloma. In bolus-infected animals, when high parasite

650 specific IgG₁ titers develop after two weeks of infection, larvae have already developed
651 into adults and escaped from the granulomas into the intestinal lumen.

652

653 While the nanostring data obtained offer exciting new avenues of research into the
654 regulation of granulomas, our study has some limitations. It is difficult to compare data
655 from day 7 and day 21 post-infection with accuracy since they were obtained from mice
656 of different sexes. Also, variation within groups was observed. Mouse 2 from the trickle
657 group had much reduced gene expression than the other trickle-infected animals, which
658 could in part be due to the low number of granulomas obtained from this animal.
659 Finally, it would be interesting to study gene expression profiles of acute/chronic
660 granulomas with/without tissue encysted worms from trickle/bolus infected mice using
661 single cell RNA sequencing to provide new and valuable details at a cellular level. This
662 would allow the differentiation of genes that are important during acute and chronic
663 stages of infection, and help identify other mediators involved in antibody accumulation
664 and ADCC as well as wound healing/fibrosis within the granulomas during infection.

665

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675

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956

957 **FIGURE LEGENDS**

958

959 ***Figure 1: The trickle infection model results in C57Bl/6 mice developing a resistant***

960 ***phenotype.***

961 6-8 week old female C57Bl/6 and BALB/c mice were infected with 200 *H. polygyrus*

962 according to the bolus and trickle infection regimes. Adult worms were counted in the

963 small intestine at each time point using a dissection microscope. (A) Bolus infection
964 regimen: mice are infected with 200 larvae on Day 0 (in grey). Worms develop in the tissue
965 from day 0 to day 7 post-infection (in black), when adults emerge into the lumen and
966 reside there for the duration of infection (in black). Worms are counted in the intestinal
967 lumen on day 14, 21, and 28 post-infection. (B) Trickle infection regime: mice are infected
968 with 200 larvae in total, but in multiple doses over the course of infection (in grey). For
969 mice euthanised on day 7 post-infection (in black), doses of 50 larvae are trickled on days
970 0, 1, 2 and 3 post-infection. This allows enough time for the last dose of larvae to enter
971 the tissue and start developing. For mice euthanised on day 14 (in black), doses of 67
972 larvae are trickled on days 0, 2 and 4 post-infection. For mice euthanised on day 21 (in
973 black), doses of 33 larvae are trickled on days 0, 2, 4, 6, 8 and 10 post-infection. For mice
974 euthanised on day 28 (in black), doses of 20 larvae are trickled on days 0, 2, 4, 6, 8, 10,
975 12, 14, 16 and 18 post-infection. In each case (14, 21 and 28), there is a 10-day window
976 after the final dose to allow parasites to fully develop into adults and migrate from the
977 intestinal tissue to the intestinal lumen. (C) Worm burdens in BALB/c mice (white mouse)
978 infected according to the bolus (black circles) and trickle (white circles) regimes. (D) Worm
979 burdens in C57Bl/6 mice (black mouse) infected according to the bolus (black circles) and
980 trickle (white circles) regimens. For (C) and (D), graphs represent pooled data from 2
981 experiments, bars represent the median, with a minimum of 3 mice per group per
982 experiment. A Kruskal Wallis test with Dunn's multiple comparisons test was performed
983 to test for statistical significance between trickle and bolus groups at each time point, n.s.
984 = not significant, * = $p < 0.05$.

Figure 2: Systemic IL-4 and IL-13 responses do not differ between bolus and trickle infected mice. 6-8 week old female C57Bl/6 and BALB/c mice were infected with 200 *H. polygyrus* larvae according to the bolus and trickle infection regimes. Single cell suspensions were isolated from the mesenteric lymph nodes and spleens; these were cultured for 48 hours in the presence of *H. polygyrus* antigen. IL-4 and IL-13 cytokine levels were measured in the supernatant by ELISA. (A) Viable cell numbers (left) with their IL-4 (middle) and IL-13 (right) production over the course of infection (d0, 7, 14, 21 and 28 post-infection) in the MLN of BALB/c (white) (A) and C57Bl/6 (black) mice (B) as well as the SPL of BALB/c (white) (C) and C57Bl/6 (black) mice (D). Graphs represent pooled data from 2 experiments, bars represent the median, with a minimum of 3 mice per group per experiment. A Kruskal Wallis test with Dunn's multiple comparisons test was performed to test for statistical significance between trickle (white circles) and bolus groups (black circles) at each time point. No differences were found at any time point, in either organ, in either strain.

Figure 3: Serum IgG1 and IgE antibody levels do not differ between bolus and trickle infected mice. 6-8 week old female C57Bl/6 (black) and BALB/c (white) mice were infected with 200 *H. polygyrus* larvae according to the bolus and trickle infection regimes. Serum antibody levels were measured by ELISA for total IgE in Balb/C (A) and C57Bl/6 (B) mice and parasite specific IgG1 for BALB/c (C) and C57Bl/6 (D) mice on days 0, 7, 14, 21 and 28 post infection. Levels in naïve controls and at day 7 post-infection were undetectable for parasite specific IgG1. Graphs represent pooled data from 2 experiments, bars represent

the median, with a minimum of 3 mice per group per experiment. A Kruskal Wallis test with Dunn's multiple comparisons test was performed to test for statistical significance between trickle (white circles) and bolus groups (black circles) at each time point. No differences were found at any time point, for either antibody, in either strain.

Figure 4: Aspects of the intestinal response to *H. polygyrus* differ between bolus and trickle infected C57BL/6 mice at day 21 post-infection. 6-8 week old female C57BL/6 mice were infected with 200 *H. polygyrus* larvae according to the bolus and trickle infection regimes and euthanised on days 7, 21 and 28 post-infection. (A) Mice were fasted for 6 hours followed by Evans Blue administration. Time from dye administration to the passing of dyed fecal pellets was measured and normalised to control animals (left). Small intestines were dissected out and scraped using a glass slide leaving only the serosa. The intestinal scrapings were weighed for each mouse and normalised to control animals (right). (B) IL-4 (left) and IL-13 (right), as well as (C) IL-21 (left) and total IgA (right) were measured in the intestinal scrapings by ELISA. Graphs represent pooled data from a minimum of 2 experiments, bars represent the median, with a minimum of 2 mice per group per experiment. A Kruskal Wallis test with Dunn's multiple comparisons test was performed to test for statistical significance between trickle (white circles) and bolus (black circles) groups at each time point, n.s. = not significant, * = $p < 0.05$.

Figure 5: Granuloma numbers remain stable over time in trickle-infected C56BL/6 mice. 6-8 week old female C57BL/6 (black) and BALB/c (white) mice were infected with 200 *H.*

1029 *polygyrus* according to the bolus and trickle infection regimes. Granuloma were counted
 1030 in the small intestine at each time point using a dissection microscope. (A) Representative
 1031 images of a portion of a naïve (left), a bolus (middle) and a trickle (left) infected small
 1032 intestine at day 14 post-infection in C57Bl/6 mice. White arrows point to granulomas.
 1033 Granuloma number in BALB/c (B) and C57Bl/6 (C) mice infected according to the bolus
 1034 (black circles) and trickle (white circles) regimes. For (C) and (D), graphs represent pooled
 1035 data from 2 experiments, bars represent the median, with a minimum of 3 mice per group
 1036 per experiment. A Kruskal Wallis test with Dunn's multiple comparisons test was
 1037 performed to test for statistical significance between trickle and bolus groups at each time
 1038 point, n.s. = not significant, * = $p < 0.05$, **** = $p < 0.0001$.

1039

1040 **Figure 6: Transcriptional profiles differ between granulomas from bolus and trickle**
 1041 **infected mice.** 6-8 week old female (7 days post-infection) and male (21 days post-
 1042 infection) C57Bl/6 mice were infected with 200 *H. polygyrus* according to the bolus and
 1043 trickle infection regimes. We used the nanostring nCounter mouse myeloid innate
 1044 immunity V2 panel to measure the expression profiles of 754 gene encoding mRNAs
 1045 within the granulomas. PCA plots highlighting differential gene expression at days 7 (left)
 1046 and 21 (right) post infection.

1047

1048 **Figure 7: Ten genes are differentially expressed between granulomas from bolus and**
 1049 **trickle infected mice 7 days post-infection.** . 6-8 week old female (7 days post-infection)
 1050 C57Bl/6 mice were infected with 200 *H. polygyrus* according to the bolus and trickle

1051 infection regimes. We used the nanostring nCounter mouse myeloid innate immunity V2
1052 panel to measure the expression profiles of 754 gene encoding mRNAs within the
1053 granulomas. (A) Summary of differentially expressed genes (*adjusted p* < 0.05; FC >2) in
1054 each pairwise comparison showing differentially expressed genes in red and blue in a
1055 volcano plot, the total number of differentially expressed genes in the middle of the
1056 bidirectional arrows, and arrowheads showing the direction of differential expression for
1057 all and highly (FC = 4–16) differentially expressed genes. (B) Heatmap showing the relative
1058 expression of differentially expressed genes associated with the regulation of myeloid
1059 immune responses between bolus- and trickle-infected mice.

1060

1061 **Figure 8: Thirty six genes are differentially expressed between granulomas from bolus**
1062 **and trickle infected mice 21 days post-infection.** 6-8 week old male (21 days post-
1063 infection) C57Bl/6 mice were infected with 200 *H. polygyrus* according to the bolus and
1064 trickle infection regimes. We used the nanostring nCounter mouse myeloid innate
1065 immunity V2 panel to measure the expression profiles of 754 gene encoding mRNAs
1066 within the granulomas. (A) Summary of differentially expressed genes (*adjusted p* < 0.05;
1067 FC >2) in each pairwise comparison showing differentially expressed genes in red and blue
1068 in a volcano plot, the total number of differentially expressed genes in the middle of the
1069 bidirectional arrows, and arrowheads showing the direction of differential expression for
1070 all and highly (FC = 4–16) differentially expressed genes. (B) Heatmap showing the relative
1071 expression of differentially expressed genes associated with the regulation of myeloid
1072 immune responses between bolus- and trickle- infected mice.

Figure 9: IgG1 antibodies accumulate around encysted larvae in the acute granulomas of trickle-infected C57Bl/6 mice. 6-8 week old female C57Bl/6 mice were infected with 200 *H. polygyrus* according to bolus and/or trickle models of infection. (A) Trickle infection regimen (acute granulomas): mice are infected with 200 larvae in total but spread out over infection (in grey). For mice euthanised on day 14 (in black), doses of 33 larvae are trickled on days 0, 2, 4, 6, 8 and 10 post-infection. For mice euthanised on day 21 (in black), doses of 20 larvae are trickled on days 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 post-infection. In each case (14 and 21), there is a 3-4 days window after the final dose to allow parasites to start developing in the mucosa and observe the formation of granulomas around them. (B & C) Formalin fixed, paraffin-embedded 6 µm sections were obtained from small intestine swiss rolls. These sections were co-stained with anti-mouse IgG1 and DAPI. Whole sections were studied using the Thorlabs Tide whole-slide scanning microscope, (20x objective). Antibody stain (left), isotype control stain (middle), and DAPI stain (right). (B) Representative acute granulomas (with worms, white dashed lines) from trickle infected mice at 14 (top) and 21 (bottom) days post-infection. (C) Representative chronic granulomas (without worms, white dashed lines) from bolus (top) and trickle (bottom) infected mice at 21 days post-infection. Experiments were performed at least twice (minimum of 5 mice per group) and are representative of a total of 21 (bolus, day 14 post-infection), 19 (of which 3 were acute granulomas, trickle, day 14 post-infection), 7 (bolus, day 21 post-infection), 30 (of which 4 were acute granulomas, trickle, day 21 post-infection) granulomas. Scale bar=100µm.

Figure 10: IgG2c, IgE and IgA are not detected around encysted larvae in the granulomas of trickle infected mice. 6-8 week old female C57Bl/6 mice were infected with 200 *H. polygyrus* according to the trickle (larvae) model of infection. (A-C) Formalin fixed, paraffin-embedded 6 µm sections were obtained from small intestine swiss rolls. These sections were co-stained with antibodies to the IgG2c, IgE and/or IgA antibody subtype, as well as DAPI. Whole sections were studied using the Thorlabs Tide whole-slide scanning microscope, (20x objective). Experiments were performed once (minimum of 5 mice per group). Scale bar=100µm. (A) Representative images of granulomas from trickle infected mice at 21 days post-infection stained with either anti-mouse IgG2c (top), IgE (middle) or IgA (bottom). Antibody stain (left), DAPI stain (middle) and isotype control stain (right). Photographs are representative of a total of 15 granulomas (of which 3 were acute granulomas). The photographs here are of consecutive slides of the same granuloma as depicted in Fig. 8C, bottom. (B) Representative images of intestinal tissue from trickle-infected mice at 21 days post-infection stained with IgE. Antibody stain (left), DAPI stain (middle) and isotype control stain (right). White arrows point to IgE accumulation in the intestinal tissue. (D) Representative Peyer's Patch (top) and intestinal tissue (bottom) from trickle- infected mice at 21 days post-infection stained with IgA. Antibody stain (left), DAPI stain (middle) and isotype control stain (right). White arrows point to IgA accumulation in the intestinal villi (top) and Peyer's patches (bottom).

1117 **Figure S1: Eighty six genes are differentially expressed between granulomas from naïve**
 1118 **and infected mice (NvT and NvB) 7 days post-infection.** 6-8 week old female (7 days post-
 1119 infection) C57Bl/6 mice were infected with 200 *H. polygyrus* according to the bolus and
 1120 trickle infection regimes. We used the nanostring nCounter mouse myeloid innate
 1121 immunity V2 panel to measure the expression profiles of 754 gene encoding mRNAs
 1122 within the granulomas. Heatmap showing the relative expression of differentially
 1123 expressed genes associated with the regulation of myeloid immune responses between
 1124 naïve and infected mice (bolus and trickle).

1125
 1126 **Figure S2: Eighty eight genes are differentially expressed between granulomas from**
 1127 **naïve and infected mice (NvT and NvB) 21 days post-infection.** 6-8 week old male (21
 1128 days post-infection) C57Bl/6 mice were infected with 200 *H. polygyrus* according to the
 1129 bolus and trickle infection regimes. We used the nanostring nCounter mouse myeloid
 1130 innate immunity V2 panel to measure the expression profiles of 754 gene encoding
 1131 mRNAs within the granulomas. Heatmap showing the relative expression of differentially
 1132 expressed genes associated with the regulation of myeloid immune responses between
 1133 naïve and infected mice (bolus and trickle).

1134
 1135 **Figure S3: IgG1 antibodies are present in the chronic granulomas of both bolus- and**
 1136 **trickle-infected mice at day 21 post-infection.** 6-8 week old female C57Bl/6 mice were
 1137 infected with 200 *H. polygyrus* according to bolus and/or trickle regimes. Formalin fixed,
 1138 paraffin-embedded 6 µm sections were obtained from small intestine swiss rolls. These

1139 sections were co-stained with anti-mouse IgG1 and DAPI. Whole sections were studied
1140 using the Thorlabs Tide whole-slide scanning microscope, (20x objective). Representative
1141 granulomas (white dashed line) from bolus- (top) and trickle- (bottom) infected mice at
1142 21 days (A) and 7 days (B) post-infection. Antibody stain (left), isotype control stain
1143 (middle), and DAPI stain (right). For both groups, at day 7 post-infection, granulomas
1144 contained worms (acute granulomas) while at 21 days post-infection they did not (chronic
1145 granulomas). Experiments were performed at least twice (minimum of 5 mice per group)
1146 and are representative of a total of 5 (bolus, day 21 post-infection), 8 (trickle, day 21 post-
1147 infection), 8 (bolus, day 7 post-infection), and 17 (trickle, day 7 post-infection),
1148 granulomas. Scale bar=100µm.

1149

1150 **Figure S4: IgG2c is detectable in the intestinal tissue of *C. rodentium* infected mice.** 6-8
1151 week old female C57Bl/6 mice were infected with 5×10^8 cfu/ mouse *C. rodentium*
1152 DBS100 strain by gavage as a positive control for IgG2c staining. Formalin fixed, paraffin-
1153 embedded 6 µm sections were obtained from small intestinal swiss rolls. These sections
1154 were co-stained with antibodies to the IgG2c (left) as well as DAPI (right). Whole sections
1155 were visualized using the Thorlabs Tide whole-slide scanning microscope, (20x objective).
1156 Experiment was performed once (minimum of 5 mice per group). White arrows point to
1157 IgG2c staining within the lamina propria. Scale bar=100µm.

Figure 1

Figure 2

Figure 3

A.

B.

C.

Figure 4

A.

Naive

Bolus

Trickle

B.

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C.

Figure 5

Day 7

Day 21

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Figure 6

A.

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B.

Figure 7

Figure 8

Figure 10