

Primary *Heligmosomoides polygyrus bakeri* infection induces myeloid-derived suppressor cells that suppress CD4⁺ Th2 responses and promote chronic infection

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Primary infection with the gastrointestinal nematode *Heligmosomoides polygyrus bakeri* is chronic in C57BL/6 (B6) mice whereas challenge infection is rapidly eliminated. F4/80⁻ CD11b⁺ Gr⁺ cells, presumed to be neutrophils, were reported to accumulate around encysting larvae in intestinal tissue during primary infection, but their exact identity and role remain unclear. We observed significant increases in F4/80⁻ CD11b^{hi} Gr1^{hi} cells in mesenteric lymph nodes (MLNs) and spleen after primary but not challenge infection; a high proportion of these cells expressed Ly6G and Ly6C. These cells, which phenotypically resemble myeloid-derived suppressor cells (MDSC), increased in lamina propria (LP) early during primary infection. Increased MDSC were associated with low numbers of alternatively activated macrophages (AAMØ) in LP and CD4⁺ GATA3⁺ T cells and AAMØ in MLN and spleen. Purified CD11c⁻ CD11b⁺ Gr1⁺ cells from *H. polygyrus bakeri*-infected mice suppressed OVA-specific CD4⁺ T-cell proliferation via a nitric oxide-dependent mechanism and parasite-specific IL-4 secretion *in vitro*. Adoptive transfer of CD11c⁻ CD11b⁺ Gr1⁺ cells from mice with primary infection resulted in significantly higher adult worm burdens and increased egg production in naïve B6 recipients infected with *H. polygyrus bakeri*. Altogether, these findings indicate that primary *H. polygyrus bakeri* infection induces a novel subset of MDSC that suppress CD4⁺ Th2 responses and promote chronic infection.

INTRODUCTION

The worldwide prevalence of infections with gastrointestinal (GI) nematodes is estimated at over 1.7 billion cases with approximately 25% due to hookworm, 47% to *Ascaris lumbricoides*, and 27% to *Trichuris trichiura* infections.¹ Such infections are not a major cause of mortality, but their impact on morbidity is enormous.^{1,2} For example, hookworm infections cause anemia that may be severe in children and pregnant women.^{3,4} In malaria-endemic regions in sub-Saharan Africa where there is also a high prevalence of GI nematode infections, co-infections with hookworm and malaria are common and may contribute to more severe malaria and increased malaria-related deaths.^{5,6} Moreover, GI nematode infections

in livestock such as cattle and pigs have important economic consequences.⁷

A major characteristic of GI nematodes is their ability to establish chronic infections in their hosts. This is despite their ability to induce a highly polarized Th2 immune response that is considered to be protective.^{8,9} *Heligmosomoides polygyrus bakeri* is a natural pathogen of mice that provides a useful laboratory model to study immune responses to GI nematodes that infect humans and livestock.¹⁰ Primary *H. polygyrus bakeri* infection is chronic and can persist for many weeks in some inbred mouse strains, including C57BL/6 (B6) mice.¹⁰ Following anthelmintic treatment to clear adult worms, challenge infection is rapidly controlled by a mechanism that involves

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Received 4 December 2015; accepted 15 March 2016; published online 13 April 2016. doi:10.1038/mi.2016.36

memory CD4⁺ T cells, IL-4, alternatively activated macrophages (AAM \emptyset), and parasite-specific antibody.^{11,12} Similar to other helminths, *H. polygyrus bakeri* suppresses immune responses to by-stander antigens and unrelated pathogens such as malaria and dampens immune responses to co-infections with unrelated helminths.^{13,14} Chronic infections established by helminths including *H. polygyrus bakeri* may be due to their ability to induce diverse regulatory mechanisms including regulatory T cells (Tregs), tolerogenic dendritic cells (DCs), AAM \emptyset , and regulatory B cells.^{14,15} These responses may contribute to immune evasion by these parasites to maintain a commensal-like relationship with their hosts to prevent their own elimination or severe pathology. However, the exact roles of these cells in suppressing immunity to a parasite developing within its host are unknown.

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature cells that have an important role in regulating immune responses during cancer and chronic inflammation.^{16–18} In mice, MDSC are F4/80[–]CD11b⁺Gr1⁺ cells.¹⁹ MDSC can be separated into two groups based on phenotype and morphology: CD11b⁺Ly6G⁺Ly6C^{lo} granulocytic MDSC and CD11b⁺Ly6G[–]Ly6C^{high} monocytic MDSC. Although the relationship between neutrophils and granulocytic MDSC remains unclear, both granulocytic and monocytic MDSC suppress immune responses.^{17,19} MDSC accumulate during chronic infections in mice due to intracellular pathogens such as protozoan parasites, viruses, bacteria, and helminth infections.^{20–25} In contrast to suppressing antigen-specific responses in infections with intracellular parasites, MDSC have been reported to enhance the clearance of adult worms in mice infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis*.^{24,25}

Here, we observed that primary *H. polygyrus bakeri* infection induced marked expansion of F4/80[–]CD11b^{hi}Gr1^{hi} cells, a phenotype consistent with MDSC, in intestinal and systemic lymphoid tissues in B6 mice. A high proportion of *H. polygyrus bakeri*-induced MDSC expressed both Ly6G and Ly6C and, thus, could not be characterized exclusively as either granulocytic or monocytic MDSC. MDSC purified from mice with primary *H. polygyrus bakeri* infection suppressed parasite-specific CD4⁺ Th2 responses *in vitro* and adoptive transfer of these cells resulted in high adult worm burdens and increased egg production in naïve B6 recipients.

RESULTS

High adult worm burden during primary *H. polygyrus bakeri* infection is associated with decreased Th2 responses

Previous studies showed that fewer CD4⁺ T cells and AAM \emptyset accumulate at the host–parasite interface after primary vs. challenge *H. polygyrus bakeri* infection in BALB/c mice, which are considered to be relatively resistant to primary infection and expel the adult worms after several weeks.^{11,26} To investigate whether differences in cellular responses between primary and challenge *H. polygyrus bakeri* infection contribute to a primary infection that is chronic, we compared the number and type of cells that accumulated in local and systemic lymphoid tissues in

B6 mice.¹⁰ Consistent with previous observations, adult worm burdens were significantly higher after primary compared with challenge *H. polygyrus bakeri* infection (**Figure 1a**). Total mesenteric lymph node (MLN) and spleen cells increased significantly in infected compared with naïve mice, but there were significantly higher numbers of total cells in both tissues after challenge compared with primary infection (**Figure 1b**). CD4⁺GATA3⁺ T cells increased significantly in the MLN after both primary and challenge infection compared with naïve mice, while there was a significant increase in the spleen compared with naïve mice only after challenge infection (**Figure 1c**). Importantly, the numbers of CD4⁺GATA3⁺ T cells were significantly higher in both tissues after challenge compared with primary infection.

Next, we assessed the accumulation of AAM \emptyset in the MLN and spleen of infected B6 mice by determining the frequency and number of F4/80⁺CD11b⁺ cells that expressed CD206, the macrophage mannose receptor upregulated in response to IL-4.²⁷ Although the numbers of F4/80⁺CD11b⁺CD206⁺ cells increased after primary infection compared with naïve mice, the differences were not significant in the MLN or spleen (**Figure 1d,e**). After challenge infection, the frequencies and numbers of F4/80⁺CD11b⁺CD206⁺ cells increased dramatically in both tissues, especially in the spleen, compared with mice after primary infection or naïve mice. Together, these observations suggest that the accumulation and possibly the function of cells involved in adaptive immunity to a GI nematode may be suppressed leading to enhanced and persistent survival of adult worms after primary *H. polygyrus bakeri* infection in B6 mice.

Primary *H. polygyrus bakeri* infection induces MDSC accumulation in local and systemic lymphoid tissues

Previous studies reported a population of F4/80[–] cells that express CD11b and Gr1, a phenotype consistent with neutrophils, accumulate around encysting *H. polygyrus bakeri* larvae in the muscularis externa of the small intestine early after primary infection.^{11,26,28} However, neither the exact identity of these cells nor their role is clear. We observed dramatic increases in the frequencies as well as the numbers of F4/80[–]CD11b^{hi} cells in the MLN, and to an even greater magnitude in the spleen, after primary *H. polygyrus bakeri* infection (**Figure 2a,b**). Interestingly, the F4/80[–]CD11b^{hi} cells expressed high levels of Gr1, a phenotype characteristic of MDSC.^{19,29} In contrast, there were no significant increases in this cell population after challenge infection.

MDSC are a heterogeneous group of myeloid-derived cells identified initially in tumor-bearing mice and cancer patients that regulate innate and adaptive immune responses.^{17,29,30} MDSC can be characterized further as granulocytic or monocytic, respectively, based on their expression of Ly6G or Ly6C. Remarkably, we observed significant increases in a population of CD206[–]CD11b⁺ cells that expressed both Ly6G and Ly6C in the MLN as well as in the spleen after primary infection, while there were no significant increases in the Ly6G[–]Ly6C⁺ population in either tissue in infected compared

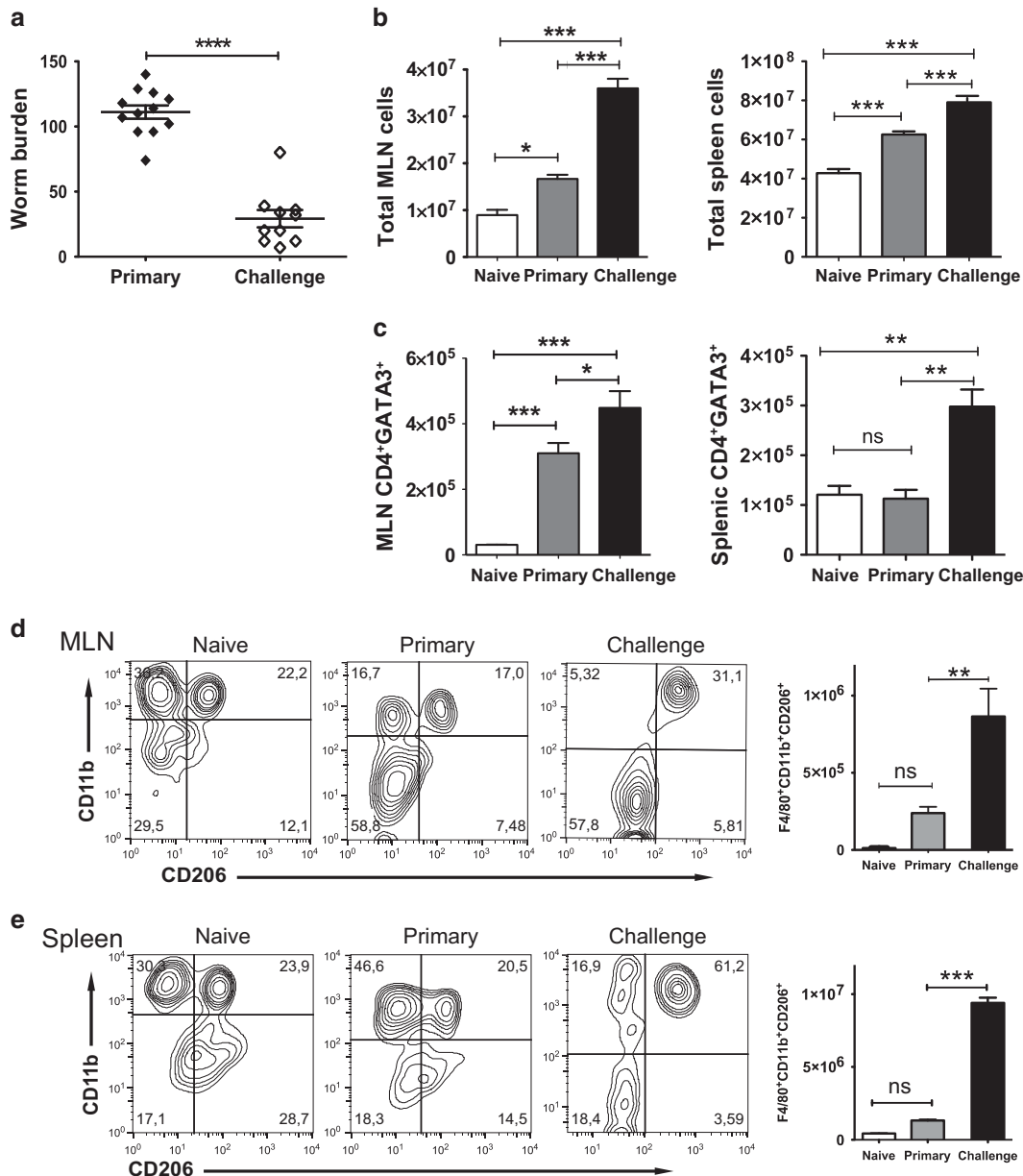


Figure 1 Differences in cellular composition of mesenteric lymph node (MLN) and spleen after primary or challenge infection with *H. polygyrus bakeri*. (a) Adult worm burdens in *H. polygyrus bakeri*-infected B6 mice on day 14 after primary or challenge infection. Data from individual mice pooled from three independent experiments are shown. Data are presented as mean \pm s.e.m. **** P < 0.0001. (b) Total cell numbers in MLN and spleen on day 7 after primary or challenge infection. * P < 0.05; *** P < 0.001. (c) Total CD4⁺GATA3⁺ T cells in MLN and spleen on day 7 after primary or challenge infection. ns, not significant; * P < 0.05; ** P < 0.01; *** P < 0.001 (d and e) CD11b⁺CD206⁺ cells were determined by flow cytometry in gated F4/80⁺ MLN (d) and spleen cells (e) from naive and infected mice on day 7 after primary or challenge infection. The left panel shows representative contour plots from one mouse indicating the frequency of F4/80⁺CD11b⁺CD206⁺, and the right panel shows the absolute cell number. Data are presented as mean \pm s.e.m. (n = 5 mice per group) and are representative of three independent experiments. ns, not significant; ** P < 0.01; *** P < 0.001.

with naïve mice (Figure 2c and Supplementary Figure 1 online). Although there were relatively few F4/80[−]CD11b^{hi}Gr1^{hi} cells that expressed Ly6G in the MLN or spleen of naïve or infected mice, this population increased significantly in both tissues after primary infection.

To determine whether temporal differences in the accumulation of AAM \emptyset vs. MDSC contribute to inefficient clearance of adult worms after primary compared with

challenge *H. polygyrus bakeri* infection, we performed kinetic analysis of increases in F4/80⁺CD11b⁺CD206⁺ and F4/80[−]CD11b^{hi}Gr1^{hi} MDSC in the MLN and spleen during the first 2 weeks post infection (p.i). In this experiment, we observed significant increases in F4/80⁺CD11b⁺CD206⁺ AAM \emptyset on day 7 p.i. in MLN and on day 14 p.i. in spleen after primary infection (Figure 2d, left panels). Significant increases in F4/80[−]CD11b^{hi}Gr1^{hi} MDSC were apparent as early as

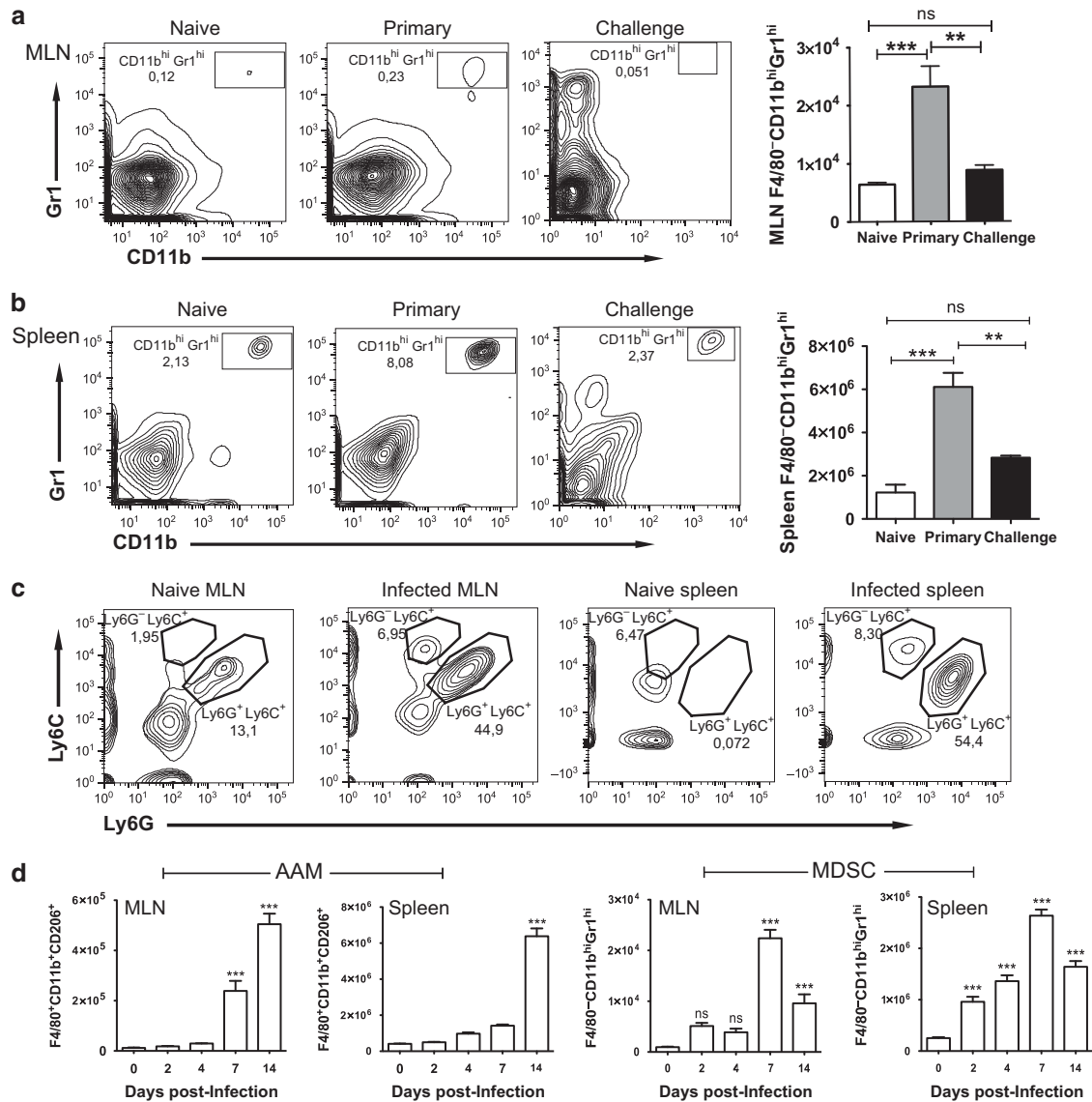


Figure 2 Primary *H. polygyrus bakeri* infection induces accumulation of F4/80⁻CD11b^{hi}Gr1^{hi} myeloid-derived suppressor cells (MDSC) that express both Ly6G and Ly6C. (a and b) CD11b^{hi}Gr1^{hi} cells were determined by flow cytometry in gated F4/80⁻ cells in mesenteric lymph node (MLN) (a) and spleen cells (b) from naive and infected mice on day 7 after primary or challenge infection. The left panel shows representative contour plots from one mouse indicating the frequency of F4/80⁻CD11b^{hi}Gr1^{hi} cells, and the right panel shows the absolute cell number. Data are presented as mean \pm s.e.m. ($n=5$ mice per group) and are representative of three independent experiments. ns, not significant; ** $P<0.01$; *** $P<0.001$. (c) Cells were gated on the CD206⁻CD11b⁺ population and analyzed by flow cytometry for expression of Ly6G and Ly6C. Representative contour plots of MLN and spleen cells from one mouse on day 7 after primary infection are shown ($n=5$ mice). (d) Accumulation of F4/80⁺CD11b⁺CD206⁺ and F4/80⁻CD11b^{hi}Gr1^{hi} cells in MLN and spleen after primary infection. Data are presented as mean \pm s.e.m. ($n=4$ mice per time point). Left panels, F4/80⁺CD11b⁺CD206⁺ cells. Right panels, F4/80⁻CD11b^{hi}Gr1^{hi} cells. ns, not significant; *** $P<0.001$.

day 2 p.i. in both tissues, the numbers peaked on day 7 p.i., and were declining by day 14 p.i. in both MLN and spleen (Figure 2d, right panels).

To establish whether MDSC are present at the site of infection, we harvested small intestines from naive and infected B6 mice during primary infection, isolated leukocytes from the lamina propria (LP), and analyzed F4/80⁻CD11b^{hi}Gr1^{hi} MDSC and F4/80⁺CD11b⁺CD206⁺ AAM \emptyset by flow cytometry. Compared with LP cells from naive mice, the frequency of F4/80⁻CD11b^{hi}Gr1^{hi} MDSC increased on day 2 p.i. while

the frequency of F4/80⁺CD11b⁺CD206⁺ AAM \emptyset was similar to naive mice (data not shown). There was a marked increase in the frequency of F4/80⁻CD11b^{hi}Gr1^{hi} MDSC on day 7 p.i. while the increase in F4/80⁺CD11b⁺CD206⁺ AAM \emptyset was marginal (Figure 3a). The numbers of F4/80⁻CD11b^{hi}Gr1^{hi} MDSC increased significantly in LP of infected compared with naive mice whereas the increase in F4/80⁺CD11b⁺CD206⁺ AAM \emptyset was not significant (Figure 3b). We observed a high frequency of cells with morphology resembling that described for MDSC¹⁹ in infected mice in both LP (Figure 3c,d) and

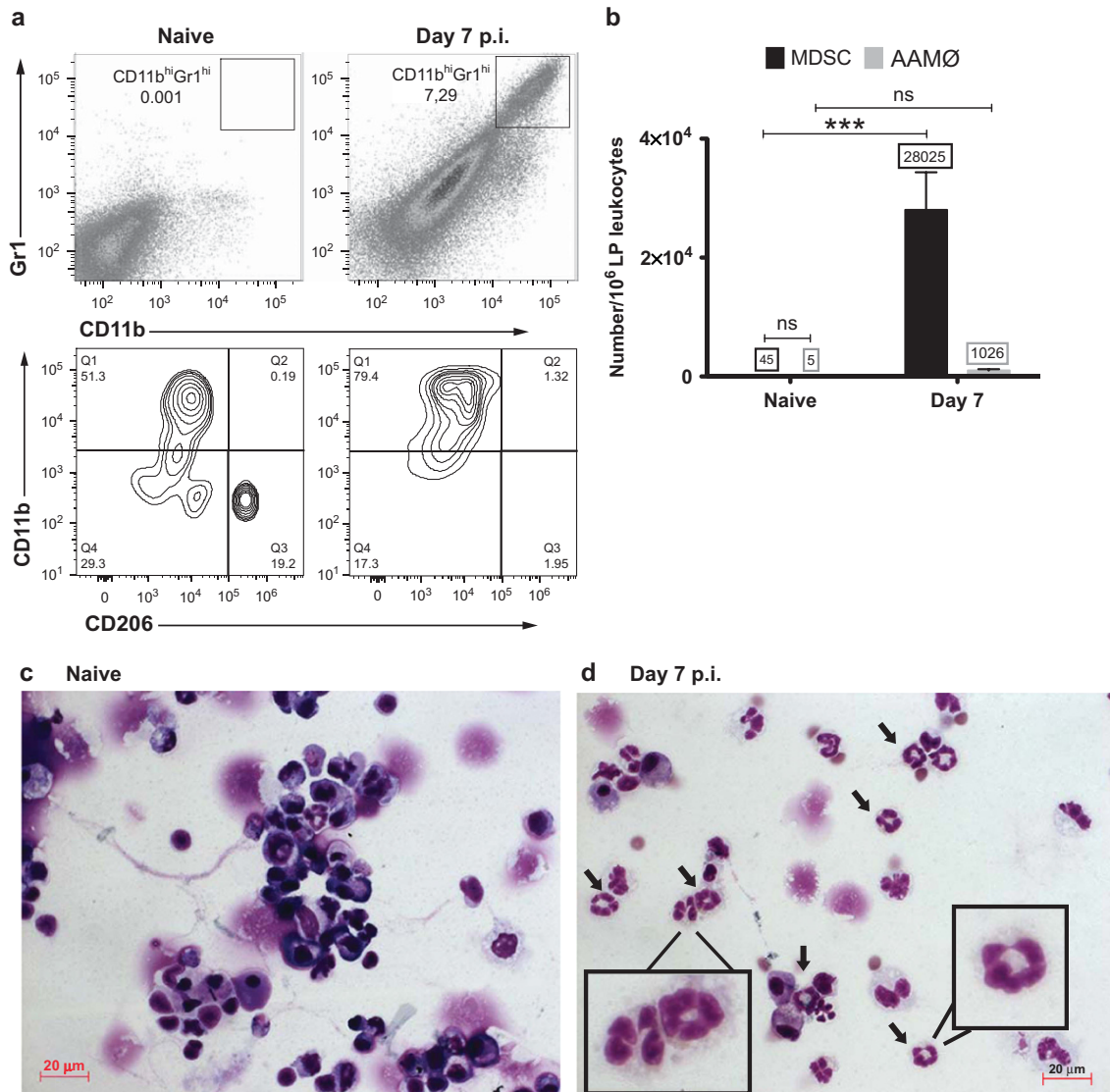


Figure 3 F4/80⁻ CD11b^{hi}Gr1^{hi} cells increase in the lamina propria (LP) after primary *H. polygyrus bakeri* infection. Leukocytes were isolated from LP obtained from the small intestines of naive or infected mice on day 7 post infection (p.i.) ($n = 4$ per group). **(a)** The frequency of CD11b^{hi}Gr1^{hi} cells in gated F4/80⁻ cells (top two panels) and CD11b⁺CD206⁺ cells in gated F4/80⁺ cells (bottom two panels) was determined by flow cytometry. **(b)** Numbers of F4/80⁻ CD11b^{hi}Gr1^{hi} myeloid-derived suppressor cells (MDSC) and F4/80⁺ CD11b⁺CD206⁺ alternatively activated macrophages (AAMØ)/10⁶ LP leukocytes in naive and day 7 infected mice. ns, not significant; *** $P < 0.001$ **(c and d)** Diff-Quik stained cytopsin preparations of LP leukocytes obtained from naive **(c)** and infected mice on day 7 p.i. **(d)** Arrows indicate cells with the characteristic donut-shaped nucleus typical of MDSC. Inset boxes show details of nuclear morphology. Magnification = $\times 630$.

spleen (**Supplementary Figures 2C and D**) on day 7 p.i. These data indicate that MDSC accumulate in intestinal and systemic lymphoid tissue during primary *H. polygyrus bakeri* infection and may negatively regulate macrophage polarization to AAMØ.

***H. polygyrus bakeri*-induced MDSC suppress effector CD4⁺ T-cell responses**

MDSC have been observed to accumulate during chronic infections with intracellular pathogens such as protozoan parasites where they have diverse roles including suppressing effector CD4⁺ T cells.^{20–23,31} To determine whether MDSC

induced by primary *H. polygyrus bakeri* infection suppressed effector CD4⁺ T-cell responses, we co-cultured purified CD11c⁻CD11b⁺Gr1⁺ cells and carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-II spleen cells at various ratios and stimulated the co-cultures with OVA_{323–339} peptide. CD11c⁻CD11b⁺Gr1⁺ cells from the MLN of infected mice potently suppressed CD4⁺ T-cell proliferation compared with the same cell population from naive mice (**Figure 4a**). The suppressive capacity of CD11c⁻CD11b⁺Gr1⁺ cells from the spleens of naive and infected mice was similar at higher ratios, but CD11c⁻CD11b⁺Gr1⁺ cells from infected mice were more suppressive at a ratio of 1:4.

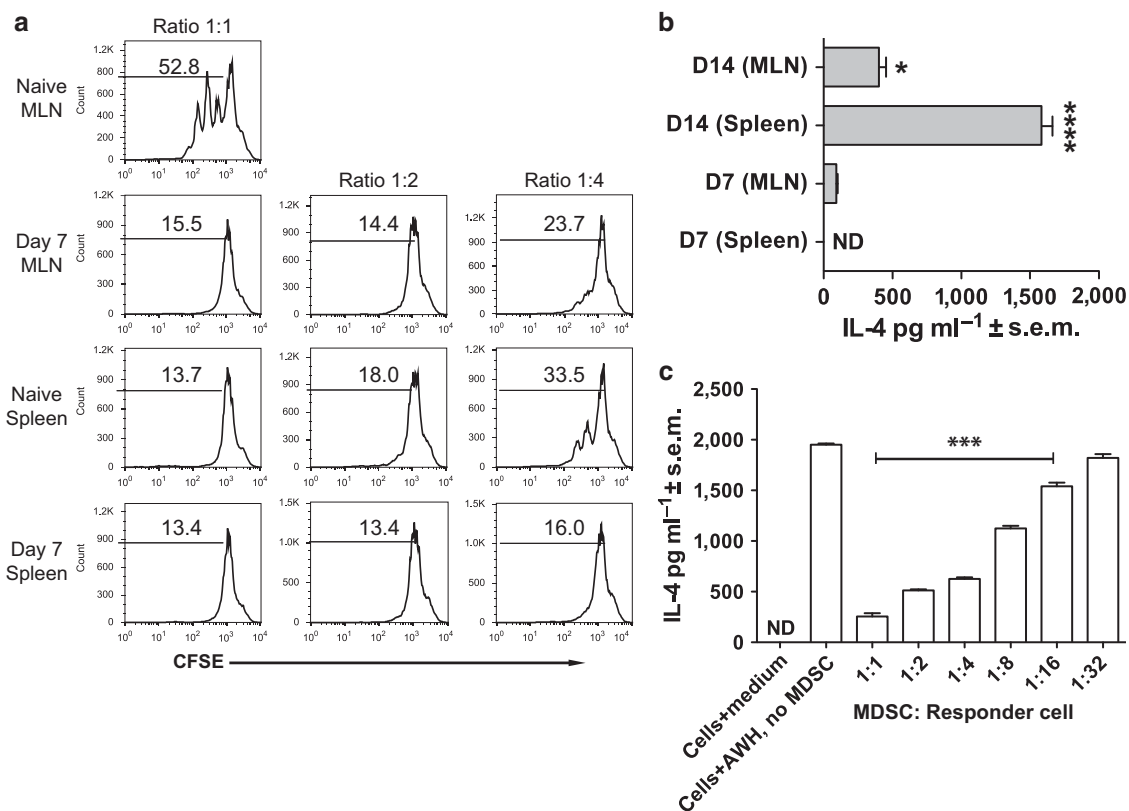


Figure 4 *H. polygyrus bakeri*-induced CD11c⁻CD11b⁺Gr1⁺ cells suppress CD4⁺ Th2 cell function *in vitro*. (a) CD11c⁻CD11b⁺Gr1⁺ cells were purified from mesenteric lymph node (MLN) (top two panels) and spleen cells (lower two panels) obtained from naive and infected mice on day 7 after primary infection, co-cultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-II spleen cells at the indicated ratios, and stimulated with 1 nM OVA₃₂₃₋₃₃₉ peptide. Seventy-two hours later, the cells from triplicate wells were harvested and pooled, stained with APC-conjugated anti-CD4 mAb and the CFSE dilution was analyzed by flow cytometry in gated CD4⁺ cells. Data are representative of three independent experiments. (b) IL-4 levels secreted by adult worm homogenate (AWH)-stimulated MLN and spleen cells on days 7 and 14 after primary infection. **P* < 0.05 for MLN cells on day 14 vs. day 7 p.i.; *****P* < 0.0001 for spleen cells on day 14 vs. day 7 p.i.; ND, not detected. (c) CD11c⁻CD11b⁺Gr1⁺ cells were purified from MLN and spleen cells of infected mice on day 7 after primary infection, pooled, and co-cultured with unfractionated spleen cells obtained from infected mice on day 14 p.i. at the indicated ratios. Co-cultures were stimulated with 50 μg ml⁻¹ adult worm homogenate (AWH), and IL-4 levels were determined in supernatants collected at 48 h by ELISA. ****P* < 0.001 compared with spleen cells without CD11c⁻CD11b⁺Gr1⁺ cells.

Urban *et al.*³² showed earlier that treatment with a long-acting formulation of IL-4 consisting of IL-4 and an anti-IL-4 monoclonal antibody (mAb) complex delivered in three doses starting on day 10 p.i. during primary *H. polygyrus bakeri* infection results in decreased egg production and almost total elimination of adult worms. These findings suggest that IL-4 secretion by CD4⁺GATA3⁺ T cells may be suboptimal during primary *H. polygyrus bakeri* infection. Previously, we observed that spleen cell secretion of IL-4 in response to adult worm homogenate (AWH) peaks at day 14 after primary *H. polygyrus bakeri* infection in B6 mice.¹³ Here, we observed that MLN and spleen cells stimulated with AWH *in vitro* secreted low or undetectable levels, respectively, of IL-4 on day 7 after primary infection while IL-4 secretion by both cell types was higher on day 14 p.i. (Figure 4b). Importantly, purified CD11c⁻CD11b⁺Gr1⁺ cells from mice on day 7 after primary *H. polygyrus bakeri* infection, even at low ratios, significantly suppressed IL-4 secretion in co-cultures of spleen cells from infected mice stimulated with AWH (Figure 4c).

Increased L-arginine metabolism by MDSC via the *Arg1* and *Nos2* pathways has been implicated in the ability of these cells to inhibit T-cell responses.³³⁻³⁵ To determine whether either pathway contributed to MDSC suppression of CD4⁺ T responses during *H. polygyrus bakeri* infection, we co-cultured purified CD11c⁻CD11b⁺Gr1⁺ cells from infected mice with purified CFSE-labeled CD4⁺ T cells from OT-II mice and mitomycin C-treated CD11c⁺ DC from naïve B6 mice. The co-cultures were stimulated with OVA₃₂₃₋₃₃₉ peptide in the presence of *N*(omega)-hydroxy-nor-L-arginine (nor-NOHA) or 1400W to inhibit arginase or nitric oxide (NO) production, respectively, or both inhibitors were added to the cultures. Proliferation was robust in control cultures containing CD4⁺ T cells and DC in medium alone, while the response was almost totally suppressed in the co-cultures containing CD4⁺ T cells, DC, and CD11c⁻CD11b⁺Gr1⁺ cells (Figure 5a). Nor-NOHA had no effect on the ability of CD11c⁻CD11b⁺Gr1⁺ cells to suppress CD4⁺ T-cell proliferation while 1400W either alone or in combination with nor-NOHA fully restored antigen-specific CD4⁺ T-cell

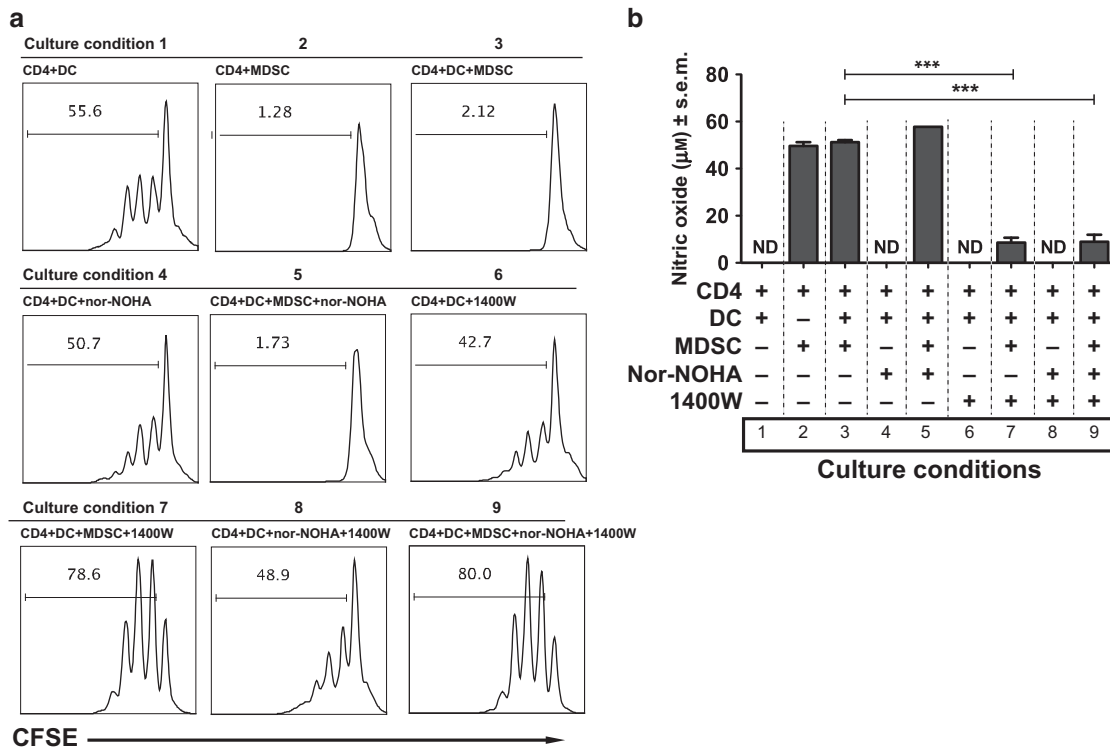


Figure 5 Suppression of CD4⁺ T cells by *H. polygyrus bakeri*-induced CD11c⁻CD11b⁺Gr1⁺ cells is nitric oxide (NO) dependent. (a) CD11c⁻CD11b⁺Gr1⁺ cells purified from mesenteric lymph node (MLN) and spleens of infected mice on day 7 after primary infection were co-cultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled splenic CD4⁺ T cells purified from OT-II mice in the presence of mitomycin-C-treated splenic CD11c⁺ dendritic cell (DC) purified from naïve mice. The co-cultures were stimulated with 1 nM OVA₃₂₃₋₃₃₉ peptide, and the cells from triplicate wells were harvested 72 h later and pooled. The cells were stained with APC-conjugated anti-CD4 monoclonal antibody (mAb), and the CFSE dilution was analyzed by flow cytometry in gated CD4⁺ cells. (b) Supernatants were collected from the triplicate wells for each culture condition in **a**, and NO levels were determined using Griess reagent. Data are presented as mean ± s.e.m. ****P* < 0.001. Culture conditions indicated as numbers are as follows: (1) CD4⁺ T cells + DC with medium; (2) CD4⁺ T cells + myeloid-derived suppressor cells (MDSC) with medium; (3) CD4⁺ T cells + DC + MDSC with medium; (4) CD4⁺ T cells + DC with *N*(omega)-hydroxy-nor-L-arginine (nor-NOHA) (300 µM); (5) CD4⁺ T cells + DC + MDSC with nor-NOHA (300 µM); (6) CD4⁺ T cells + DC with 1400W (100 µM); (7) CD4⁺ T cells + DC + MDSC with 1400W (100 µM); (8) CD4⁺ T cells + DC with both inhibitors; and (9) CD4⁺ T cells + DC + MDSC with both inhibitors. Data from one of two independent experiments are presented.

proliferation in the co-cultures. Coincidentally, NO levels were significantly reduced to low levels in the co-cultures containing 1400W (**Figure 5b**). Together, these findings indicate that NO but not arginase contributes to suppression of antigen-specific CD4⁺ T-cell proliferation by MDSC from *H. polygyrus bakeri*-infected mice.

Adoptive transfer of MDSC suppresses worm expulsion in recipient mice

To investigate whether MDSC suppress adaptive immunity to primary *H. polygyrus bakeri*, we treated infected mice with anti-Gr-1 mAb (clone RB6-8C5) during primary infection to deplete MDSC *in vivo*. Using this approach, we observed F4/80⁻CD11b⁺Gr1⁺ MDSC were depleted at day 7 p.i., but the depletion was transient. Flow cytometry analysis revealed that the numbers of F4/80⁻CD11b⁺Gr1⁺ MDSC were significantly higher in the tissues of the antibody-treated group compared with control mice later on day 14 p.i. (unpublished observation). This finding is consistent with published observations of a dramatic increase in F4/80⁻CD11b⁺Gr1⁺ MDSC in the periphery after anti-Gr-1 mAb administration to

mice.³⁶ Our observations suggest that MDSC depletion using anti-Gr-1 mAb may result in increased proliferation of bone marrow myeloid precursors leading to subsequent expansion and repopulation of these cells in peripheral tissues during *H. polygyrus bakeri* infection.

As an alternative approach, we adoptively transferred CD11c⁻CD11b⁺Gr1⁺ cells purified from infected mice on day 7 after primary *H. polygyrus bakeri* infection to naïve recipients (**Figure 6a**). A separate group of mice received CD4⁺CD25⁻CD103⁻ T cells pooled from MLN and spleen cells obtained on day 28 p.i. Previous studies by Rausch *et al.*³⁷ demonstrated that adoptive transfer of this cell population results in significantly decreased worm burdens in recipient mice. As a control, a group of mice were treated with phosphate-buffered saline (PBS).

We observed that transfer of effector CD4⁺ T cells resulted in a significantly higher number of CD4⁺GATA3⁺ cells and significantly low number of F4/80⁻CD11b^{hi}Gr1^{hi}MDSC compared with control mice and MDSC recipients (**Figure 6b,c**). The numbers of F4/80⁺CD11b⁺CD206⁺AAMØ were not significantly different in CD4⁺ T-cell

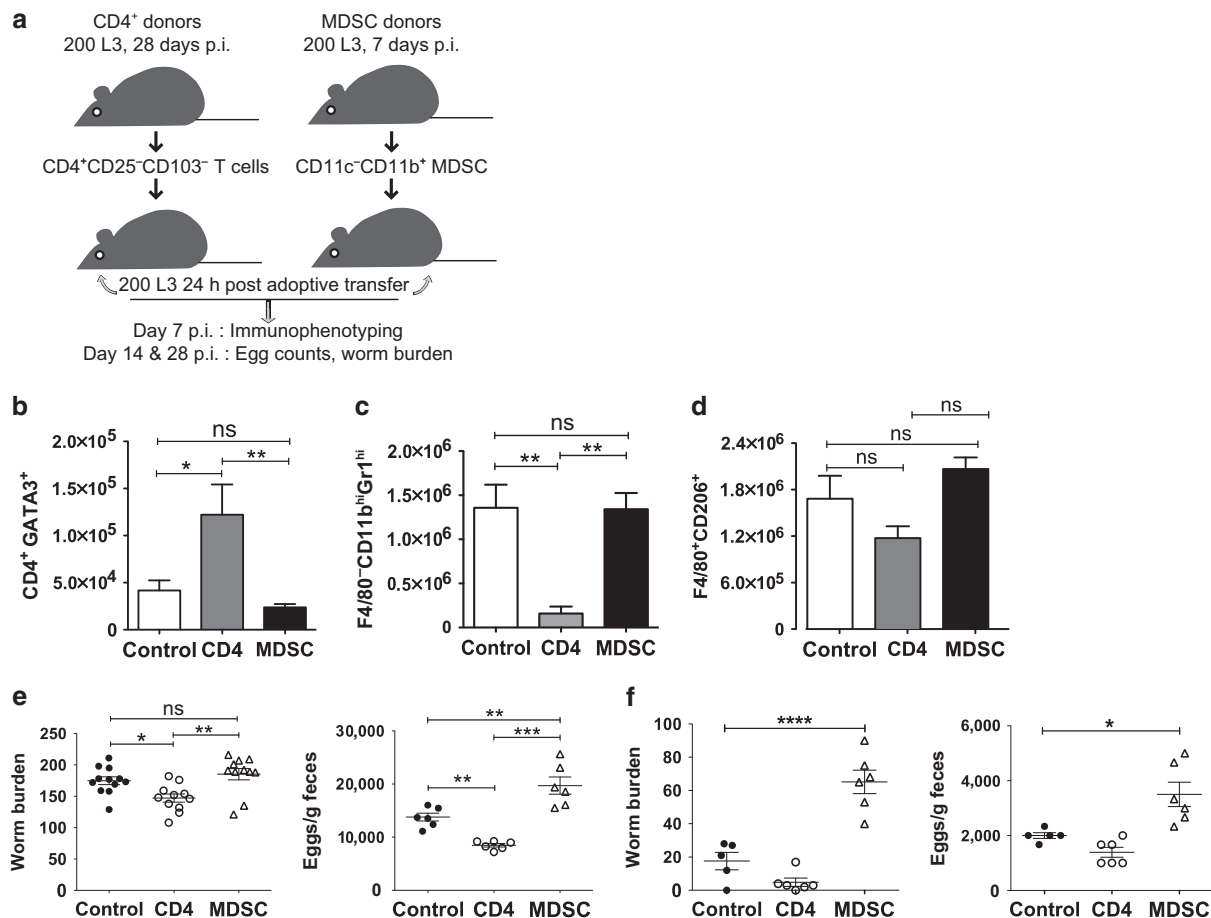


Figure 6 Adoptive transfer of CD11c⁻CD11b⁺Gr1⁺ cells from *H. polygyrus bakeri*-infected mice results in increased egg production and high adult worm burdens in recipient mice. **(a)** Scheme of the adoptive transfer experiment. Recipient mice adoptively transferred by i.v. injection of 2×10^6 CD4⁺CD25⁻CD103⁻ or CD11c⁻CD11b⁺Gr1⁺ cells and control mice injected with 200 μ l phosphate-buffered saline (PBS) were infected per os (p.o.) with 200 third-stage larvae (L3) 24 h later. Mice were killed on day 7 post infection (p.i.) for immunophenotyping. Adult worm burdens and egg production were determined in individual mice on days 14 and 28 p.i. **(b–d)** Bar graphs showing the absolute numbers of CD4⁺GATA3⁺ T cells **(b)**, F4/80⁻CD11b⁺Gr1^{hi} cells **(c)**, and F4/80⁺CD206⁺ cells **(d)** in the spleens of PBS control mice and recipients of effector CD4⁺ T cells or CD11c⁻CD11b⁺Gr1⁺ cells. Data are presented as mean \pm s.e.m. ($n=5$ mice per group). ns, not significant; * $P<0.05$; ** $P<0.01$ **(e and f)** Adult worm burdens and egg counts were determined on days 14 **(e)** and 28 **(f)** p.i. Data for individual mice are shown and the mean \pm s.e.m. of each group is indicated ($n=5$ mice per group). ns, not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$. Data shown in **(b–d)** are representative of three independent experiments. Data shown in **(e)** are from individual mice pooled from two independent experiments. Data shown in **(f)** are from individual mice from one experiment.

recipients compared with MDSC recipients or control mice (**Figure 6d**). There were no significant differences in the numbers of CD4⁺GATA3⁺ T cells or MDSC between the recipients of CD11c⁻CD11b⁺Gr1⁺ cells and PBS controls and no difference in the number of AAM \emptyset compared with PBS controls or CD4⁺ T-cell recipients.

On day 14 p.i., there were significantly fewer adult worms in the recipients of CD4⁺ T cells as previously shown while worm burdens were similar in recipients of CD11c⁻CD11b⁺Gr1⁺ cells and PBS control mice (**Figure 6e**). Egg production, however, was significantly increased in CD11c⁻CD11b⁺Gr1⁺ cell recipients compared with CD4⁺ T-cell recipients or PBS control mice, indicating increased fecundity of the worms in the former mice. On day 28 p.i., the adult worm burden was markedly and significantly higher together with significantly higher egg production in the CD11c⁻CD11b⁺Gr1⁺ cell

recipients compared with effector T-cell recipients or PBS control mice (**Figure 6f**). Collectively, these data indicate that *H. polygyrus bakeri*-induced MDSC suppress adaptive immune responses that involve CD4⁺GATA3⁺ T cells during primary infection.

DISCUSSION

The time required for expulsion of adult worms after primary *H. polygyrus bakeri* infection varies depending on the genetic background of the host.^{10,38,39} Inbred mouse strains such as SWR and SJL mice expel primary infection within 4–6 weeks and are considered to be rapid responders while others such as CBA and C3H mice are slow responders. After drug-cure and challenge infection with *H. polygyrus bakeri*, adult worm expulsion occurs rapidly in most inbred mouse strains including B6 mice and is critically dependent on memory

CD4⁺GATA3⁺ cells, IL-4, AAMØ, and parasite-specific antibody.^{10–12,15} Previously, it was observed that fewer CD4⁺ T cells and AAMØ accumulate around encysting larvae in intestinal tissue after primary vs. challenge *H. polygyrus bakeri* infection in BALB/c mice, which are fast responders and require up to 8 weeks for expulsion of adult worms after primary infection.^{11,26} In the present study, we questioned whether similar differences in the cellular composition of local and systemic lymphoid tissues are evident in B6 mice. Primary *H. polygyrus bakeri* infection is chronic in B6 mice and adult worms persist for several months indicating an intermediate level of responsiveness.

Consistent with the findings in BALB/c mice, we observed significant increases in total cells and CD4⁺GATA3⁺ T cells in both tissues after primary or challenge infection but, in each case, the cell numbers were significantly higher after challenge infection. Although F4/80⁺CD11b⁺ cells increased after primary and challenge infections (data not shown), CD206⁺ AAMØ increased significantly only during challenge infection such that CD11b⁺CD206⁺ cells accounted for >50% of the total F4/80⁺ macrophages. This resulted in a 2- to 2.5-fold increase in AAMØ in the MLN and spleen after primary infection whereas increases in AAMØ in the tissues after challenge infection were >10-fold. It is important to point out that there were 8- to 10-fold more AAMØ in the spleen than in the MLN after challenge infection. This finding is consistent with observations of splenomegaly in *H. polygyrus bakeri*-infected mice in association with increased type 2 cytokine gene expression.⁴⁰ The remarkable ability of *H. polygyrus bakeri* to induce potent systemic responses, including immunoregulatory networks, provides compelling evidence supporting the usefulness of this GI nematode as well as other helminths as therapy to modulate disease promoting immune responses in distal sites such as type 1 diabetes and allergic responses.

Importantly, we observed significant increases in F4/80⁺ cells that expressed high levels of CD11b and Gr1 in the MLN and spleen after primary but not challenge *H. polygyrus bakeri* infection. A population of F4/80⁺CD11b^{hi}Gr1^{hi} cells also increased in the LP of infected mice early after primary infection. Cells with a similar phenotype have been reported to accumulate around larvae, presumably L4-stage larvae, encysting in the muscularis of the small intestine within a few days after primary infection.^{11,26,28} Although these cells were initially presumed to be neutrophils, neither their identity nor their role has been investigated fully. To further characterize the phenotype of these cells, we analyzed the expression of Ly6G and Ly6C, cell-surface markers used to distinguish MDSC, myeloid-derived cells phenotypically similar to the cells induced by *H. polygyrus bakeri* infection, as either granulocytic or monocytic, respectively.^{17,29,30} There were significant increases in F4/80⁺CD11b^{hi}Gr1^{hi}Ly6G⁺Ly6C⁺ MDSC in the MLN and spleen of infected mice after primary *H. polygyrus bakeri* infection. However, this population, akin to granulocytic MDSC, accounted for relatively few of the total CD11b^{hi}Gr1^{hi} cells. In contrast, dramatic and significant increases in F4/80⁺CD11b^{hi}Gr1^{hi} cells that expressed both Ly6G and

Ly6C were evident in the MLN and spleen after primary infection. This suggests that a unique population of MDSC is induced during primary *H. polygyrus bakeri* infection.

MDSC are known to regulate innate and adaptive immune responses, including effector CD4⁺ T-cell responses during cancer and chronic inflammation as well as during parasite infections.^{20–23,31} To determine whether the F4/80⁺CD11b^{hi}Gr1^{hi} MDSC induced during primary *H. polygyrus bakeri* infection suppressed Th2 responses, we performed *in vitro* and *in vivo* experiments. First, we purified CD11c⁺CD11b⁺Gr1⁺ cells from naïve and infected mice and co-cultured these cells with CFSE-labeled OT-II spleen cells in the presence of OVA_{323–339} peptide. At high ratios, CD11c⁺CD11b⁺Gr1⁺ cells from infected mice suppressed OVA-specific CD4⁺ T-cell proliferation compared with cells from naïve mice. To investigate whether *H. polygyrus bakeri*-induced F4/80⁺CD11b^{hi}Gr1^{hi} MDSC contribute to low levels of IL-4 secretion by CD4⁺ Th2 cells during primary infection, CD11c⁺CD11b⁺Gr1⁺ cells from infected mice were co-cultured with spleen cells harvested on day 14 after primary infection when IL-4 secretion is maximal in B6 mice, and the cultures were stimulated with AWH. Even at a ratio of 1:16, CD11c⁺CD11b⁺Gr1⁺ cells purified from infected mice on day 7 after primary infection significantly suppressed IL-4 secretion.

Studies in tumor-bearing mice and cancer patients have identified various mechanisms whereby MDSC mediate their suppressive effects on effector T-cell responses.⁴¹ Increased L-arginine metabolism via either the *Arg1* or *Nos2* pathway has emerged as a major pathway. Although both granulocytic (Ly6G⁺) and monocytic (Ly6C⁺) MDSC utilize the *Arg1* pathway to mediate their suppressive activity, only Ly6C⁺ MDSC produce high levels of NO.⁴¹ Co-culture of CD11c⁺CD11b⁺Gr1⁺ cells from *H. polygyrus bakeri*-infected mice with OT-II CD4⁺ T cells and DC revealed that inhibiting NO secretion reversed suppression of OVA-specific proliferation whereas the arginase inhibitor nor-NOHA had no effect. It is well established that AAMØ secrete arginase that is responsible together with specific antibody for elimination of adult worms after challenge infection with *H. polygyrus bakeri*.^{11,12} Indeed, *in vivo* treatment with the arginase inhibitor S-(2-boronoethyl)-L-cysteine during challenge infection results in higher worm burdens, reduced egg production, and increased larval recovery.^{11,12} Together, these studies support a critical role for arginase in eliminating challenge infection with *H. polygyrus bakeri*. NO secretion by macrophages or other myeloid-derived cells in response to the Th1 cytokine IFN- γ has not been described during either primary or challenge infection with this parasite. Nevertheless, in our hands, treatment with the NO inhibitor 1400W almost completely restored OVA-specific proliferation and significantly reduced NO levels in the co-cultures. Finally, we demonstrated that adoptive transfer of CD11c⁺CD11b⁺Gr1⁺ cells from mice with primary *H. polygyrus bakeri* infection to naïve mice resulted in significantly higher adult worm burdens and increased egg counts on days 14 and 28 p.i.

Our findings that MDSC suppressed Th2 immune responses to *H. polygyrus bakeri* are divergent from reports on the role of these cells in *N. brasiliensis* and *T. spiralis* infected mice. In these models, MDSC were observed to enhance adult worm clearance by increasing IgE-mediated cytokine production by mast cells.^{24,25} While mucosal mastocytosis correlates with adult worm expulsion during *H. polygyrus bakeri* infection, the exact role of mast cells in immunity against *H. polygyrus bakeri* is unclear.¹⁰ Although *N. brasiliensis* and *H. polygyrus bakeri* are closely related rodent GI nematodes, primary *N. brasiliensis* infection is rapidly eliminated in mice within 2 weeks. In contrast, primary *H. polygyrus bakeri* infection is chronic in B6 mice used in our studies. Thus, differences in how these parasites establish infection and how the host immune system responds may explain the different role of MDSC in these infections.

Primary *H. polygyrus bakeri* infection induces Foxp3⁺ Tregs in intestinal tissue as well as in the MLN and spleen, but the reported effect of depletion of these cells on worm expulsion and intestinal pathology is variable.^{37,42–45} However, these studies consistently showed that depletion of Tregs results in increased Th2 responses. Furthermore, it appears that there is no correlation between susceptibility to primary *H. polygyrus bakeri* infection and the number of tissue Foxp3⁺ Tregs among inbred mouse strains although the suppressive potential of cells from mice with varying levels of susceptibility was not examined.⁴⁶

Taken together, our findings demonstrate that primary *H. polygyrus bakeri* infection induced the accumulation of F4/80[−]CD11b^{hi}Gr1^{hi} MDSC in local and systemic lymphoid tissues. A high proportion of these cells express Ly6G and Ly6C, and thus may represent a novel class of MDSC. *H. polygyrus bakeri*-induced MDSC suppressed antigen-specific CD4⁺ T-cell proliferation *in vitro* via an NO-dependent mechanism. Importantly, infection-induced MDSC suppressed parasite-specific IL-4 secretion *in vitro* and possibly CD4⁺GATA3⁺ T-cell function *in vivo*. A role for MDSC in promoting the establishment of chronic primary *H. polygyrus bakeri* infection in B6 was demonstrated by adoptive transfer studies. Understanding how MDSC are induced during GI nematode infection and their interplay with other regulatory cells such as Foxp3⁺ Tregs to dampen protective Th2 responses will be important in devising vaccines and new therapeutic approaches to alleviate the burden of infections with these parasites in humans and livestock, especially in regions where co-infections are prevalent.

METHODS

Mice, parasite, and infection. C57BL/6 (B6) and BALB/c mice, 8–10 weeks old, were purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada). Breeding pairs of B6.Cg-Tg(TcratTcrb)425Cbn/J OT-II mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained and handled according to the guidelines of the Canadian Council on Animal Care and the Animal Ethics Committee of McGill University. *H. polygyrus bakeri* was maintained and propagated in male BALB/c mice as described previously.^{13,47} Female B6 mice were used for all

experimental infections. For primary *H. polygyrus bakeri* infection, mice were infected by oral gavage (per os, p.o.) with 200 infective third-stage larvae (L3), and the adult worm burden was determined on day 14 p.i. as described previously.⁴⁷ For challenge infection, mice were infected with 200 L3 and treated with pyrantel pamoate (100 mg kg^{−1}) on day 14 p.i. to clear adult worms, allowed to rest for 5 weeks, and challenged with 200 L3. In some experiments, fecundity was assessed by collecting feces to determine the number of eggs as described previously.⁴⁷ AWH was prepared as described previously, the protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), and aliquots were stored at −20 °C until use.⁴⁷

Cell preparation and adoptive transfer. MLN and spleens were harvested from naïve and infected B6 mice at the indicated times p.i., and single-cell suspensions were prepared as described previously.³⁷ Dead cells were removed using a kit from Miltenyi Biotec, Auburn, CA. Immune cell populations were purified using MACS beads (Miltenyi Biotec) according to the manufacturer's instructions. To isolate MDSC, MLN and spleen cells were enriched by negative selection of CD11c⁺ cells using anti-CD11c magnetic beads followed by positive selection of CD11b⁺ cells using anti-CD11b magnetic beads. The resultant cell populations had a purity of >96% CD11c[−]CD11b⁺Gr1⁺ cells. For *in vitro* suppression assays, purified CD11c[−]CD11b⁺Gr1⁺ cells were co-cultured with unfractionated spleen cells or purified CD4⁺ T cells isolated from the spleens of OT-II mice using a mouse CD4⁺ T cell isolation kit (StemCell Technologies, Vancouver, BC). Spleen CD11c⁺ DCs were purified from naïve mice by positive selection using anti-CD11c magnetic beads (Miltenyi Biotec). To obtain CD4⁺CD25[−]CD103[−] cells for adoptive transfer, MLN and spleens were harvested from infected B6 mice on day 28 p.i. Single-cell suspensions were prepared, pooled, and surface stained with PE-conjugated anti-CD25 (clone 7D4; Miltenyi Biotec) and anti-CD103 (clone 2E7; Miltenyi Biotec) mAbs. CD25[−]CD103[−] cells were enriched by negative selection using anti-PE conjugated magnetic beads (Miltenyi Biotec). CD4⁺ cells were positively selected in the resultant population using anti-CD4 magnetic beads (Miltenyi Biotec). The purity of the CD4⁺CD25[−]CD103[−] cells obtained was always >96%. Recipient B6 mice were injected i.v. with 2 × 10⁶ CD4⁺CD25[−]CD103[−] or CD11c[−]CD11b⁺Gr1⁺ cells or 200 μl PBS as a control. Twenty-four hours later, recipient mice were infected with 200 L3 and adult worm burden and egg production were assessed on days 14 and 28 p.i. as described previously.⁴⁷

Leukocytes from the LP of the small intestines were isolated according to the method described by Davies and Parrott.⁴⁸ The viability of the cells obtained was >90%. For photomicrographs, cytopins of the cells were prepared and stained with Diff-Quik. Images were taken at ×630 magnification using the AxioVision (Zeiss, Jena, Germany) software.

***In vitro* suppression assay.** For antigen-specific proliferation, purified CD11c[−]CD11b⁺Gr1⁺ cells from MLN and spleen of naïve or infected mice were co-cultured at various ratios with 1 × 10⁶ CFSE-labeled spleen cells from OT-II mice prepared as previously described⁴⁹ and stimulated with 1 nM OVA_{323–339} peptide (AnaSpec, Fremont, CA). After incubation at 37 °C with 5% CO₂ for 72 h, the cells were harvested and stained with APC-conjugated anti-CD4 mAb (clone GK1.5; eBioscience, San Diego, CA). The CFSE dilution was determined in gated CD4⁺ T cells by flow cytometry and analyzed using the FlowJo software (Tree Star, Ashland, OR). To assess *H. polygyrus bakeri*-specific suppression of cytokine secretion, CD11c[−]CD11b⁺Gr1⁺ cells purified from MLN and spleens of infected mice on day 7 p.i. were pooled and co-cultured at various ratios with spleen cells obtained from infected mice on day 14 p.i. The co-cultures were stimulated with 50 μg ml^{−1} AWH, and IL-4 levels were determined in supernatants collected 48 h later by ELISA as described previously.¹³ In some experiments, 1 × 10⁶ CD11c[−]CD11b⁺Gr1⁺ cells from infected mice were co-cultured with CFSE-labeled CD4⁺ cells purified from the spleens of OT-II mice in the

presence of 2×10^5 CD11c⁺ DCs purified from the spleens of naïve B6 mice and pre-treated with 0.5 mg ml^{-1} mitomycin C (Sigma, St. Louis, MO). *N*(omega)-hydroxy-nor-L-arginine (nor-NOHA; 300 μM ; Calbiochem, San Diego, CA) or 1400W (100 μM ; Sigma) was added to the co-cultures to block arginase or NO production, respectively. After incubation at 37 °C with 5% CO₂ for 72 h, the cells were harvested and the CFSE dilution in gated CD4⁺ cells was determined. Supernatants were harvested and the NO levels were determined using Griess reagent as described previously.⁵⁰

Immunophenotyping. Before staining for immunophenotyping, the viability of all MLN, spleen, and LP cell preparations was determined by flow cytometry using eFluor 780 (eBioscience). Fc receptors were blocked using anti-mouse CD16/CD32 mAb (clone 2.4G2; BD Pharmingen, San Jose, CA). To identify MDSC and AAMØ, the cells were surface stained with PE-conjugated anti-F4/80 (clone BM8, eBioscience), APC-conjugated anti-CD11b (clone M1/70, eBioscience), Per-CP-Cy5.5-conjugated anti-Gr-1 (clone RB6-8C5, eBioscience), Horizon V450-conjugated anti-Ly6G (clone1A8, BD Biosciences, San Jose, CA), and Brilliant Violet 510-conjugated anti-Ly6C (clone HK1.4, BioLegend, San Diego, CA) mAbs. Surface stained cells were then fixed using a fixation and permeabilization kit (eBioscience) before intracellular staining with FITC-conjugated anti-CD206 (clone C068C2, BioLegend, San Diego, CA) mAb. To identify Th2 cells, cells were surface stained with FITC-conjugated, anti-CD4 (clone GK1.5; BioLegend) mAb, fixed, and stained intracellularly with PE-conjugated anti-GATA-3 (clone TWAJ; eBioscience) mAb. Compensation controls included UltraComp eBeads (eBioscience) singly stained with each of the fluorochromes used in the experiment. Gating strategies for AAMØ and MDSC are shown in **Supplementary Figure 2**. Flow cytometry was performed using a FACSCanto or FACSCalibur (BD Biosciences), and the data were analyzed using the FlowJo software.

Statistical analysis. Data are presented as mean \pm s.e.m. Groups were compared and differences were analyzed for significance using an unpaired *t*-test with Welsh's correction. For multiple groups, differences were analyzed for significance using one-way ANOVA followed by Bonferroni *post hoc* test using the GraphPad Prism software (La Jolla, CA). A *P*-value of <0.05 was considered to be significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

ACKNOWLEDGMENTS

This work was supported by grants from NSERC (326946 to T.G.G. and 238294 to A.J.) and CIHR (MOP-81169 and MOP-130369 to M.M.S.). R.M.V. was supported by a Studentship from the Research Institute of the McGill University Health Centre. T.G.G. was supported by a Canada Research Chair (Tier 1). The Centre for Host-Parasite Interactions was supported by Fonds de Québec de recherche sur la nature et les technologies.

DISCLOSURE

The authors declared no conflict of interest.

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