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Chronic helminth infection promotes immune regulation *in vivo* through dominance of CD11c^{lo}CD103^{-} dendritic cells

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Abstract

Gastrointestinal helminth infections are extremely prevalent in many human populations, and are associated with down-modulated immune responsiveness. In the experimental model system of *Heligmosomoides polygyrus*, a chronic infection establishes in mice, accompanied by a modulated Th2 response and increased regulatory T cell activity. To determine if DC populations in the lymph nodes draining the intestine are responsible for the regulatory effects of chronic infection, we first identified a population of CD11c^{lo} non-plasmacytoid DCs which expand following chronic *H. polygyrus* infection. The CD11c^{lo} DCs are under-represented in magnetic bead-sorted preparations, and spared from deletion in CD11c-DTR mice. Following infection, CD11c^{lo} DCs did not express CD8, CD103, PDCA, or Siglec H and were poorly responsive to TLR stimuli. In DC:T cell co-cultures, CD11c^{lo} DCs from naïve and *H. polygyrus*-infected mice could process and present protein antigen, but induced lower levels of antigen-specific CD4^{+} T cell proliferation and effector cytokine production, and generated higher percentages of Foxp3^{+} T cells in the presence of TGF-β. Treg generation was also dependent on retinoic acid receptor signaling. In vivo, depletion of CD11c^{hi} DCs further favored the dominance of the CD11c^{lo} DC phenotype. Following CD11c^{hi} DC depletion, effector responses were dramatically inhibited but the expansion in regulatory T cell numbers following *H. polygyrus* infection barely compromised, showing a significantly higher regulatory:effector CD4^{+} T cell ratio compared to CD11c^{hi} DC-intact animals. Thus, the pro-regulatory environment of chronic intestinal helminth infection is associated with the in vivo predominance of a newly defined phenotype of CD11c^{lo} tolerogenic DCs.

Introduction

Helminth parasites infect over two billion humans in the world today, the vast majority of these infections being with gastrointestinal nematodes (1). Each species of gut nematode is able to neutralize host immunity and survive for many months or years. Moreover, there is

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an inverse relationship between helminth infection and the incidence of immunopathological diseases targetting allergens (2-4) or autoantigens (5-7). Hence, it is thought that active diversion and suppression of the immune system occurs, rather than simple ignorance, and that these parasites can generate or sustain an immunoregulatory environment.

To model this important relationship, we have studied dendritic cell (DC) populations in gastrointestinal nematode infections. DCs are a phenotypically diverse set of cells which play a fundamental role in sensing the presence of pathogens and selecting the mode of immune response mounted by antigen-specific T cells (8-10). The crucial role of DCs, rather than other cell types such as basophils, in generating anti-helminth effector responses has very recently been confirmed (11-13). The ability of DCs to sense and discriminate between different pathogen types enables them to direct $CD4^+$ and $CD8^+$ T cell responses to varying pathogens in a highly effective manner.

It is now recognized that DCs also play an essential immunoregulatory role, maintaining immunological tolerance (14-16) or dampening effector responses through the induction of regulatory cytokines and T cells (17-19). Notably, a diverse range of DC subtypes are able to act in this fashion, including *ex vivo* mucosal $CD103^+$ DCs (20), $CD11c^{lo}CD45RB^{hi}$ DCs (21), splenic $CD8^+CD205^+$ cells (22) as well as myeloid DCs treated *in vitro* with pro-regulatory agents such as IL-10, TGF-β and retinoic acid (20, 23, 24). Regulatory DCs may themselves produce IL-10 (25) or indoleamine 2,3-dioxygenase (IDO) (26), or act through surface ICOS-L (27), CTLA-4 and PD-1 (28). It is interesting to note reports of enhanced regulatory DC activity in a number of pathogen contexts such as *Bordetella pertussis* (29), mycobacteria (30), *Candida albicans* (31) and malaria (32).

Several alternative models exist to explain how DCs differ in their ability to positively or negatively influence immunity. First, it has been known for some time that immature DCs which have not been classically activated (for example by TLR ligation) are able to induce regulatory, IL-10-producing T cells (33-35). Secondly, DCs derived from mucosal tissues have a high intrinsic pro-regulatory capacity, including but not restricted to the $CD103^+$ subset mentioned above (36-38). Finally, rather than activation status or location *per se*, it has been suggested that specialized subsets of DCs are dedicated to the maintenance of tolerance; in particular, plasmacytoid DCs (pDCs) are more closely associated with immunoregulatory outcomes than other, conventional, types of DCs (27, 39).

Helminth infection is closely associated with the induction of a Th2 immune response characterized by the production of IL4 and IL13 by $CD4^+$ T cells, exemplified by the model nematode *Nippostrongylus brasiliensis*, which is expelled after a short-lived acute infection (40). In this and other helminth systems, *in vitro*-derived DCs pulsed with parasite antigens are able to induce strongly Th2-polarized immune responses on adoptive transfer to naïve hosts (41, 42). Certain parasites, such as *Heligmosomoides polygyrus*, can also elicit the expansion of regulatory T cells, which suppress effector T cell proliferation, attenuate pathology and may allow survival within the host for many months or years (43-46). Although little information is yet available on the *in vivo* phenotype and function of DCs over the course of gastrointestinal nematode infection, we hypothesised that a pro-regulatory subset of DC would become more prominent during chronic *H. polygyrus* infection. This

hypothesis is consistent with a study showing that pooled splenic and MLN DCs from *H. polygyrus*-infected mice suppressed immunity in *Citrobacter rodentium*-infected recipients (47). We therefore investigated whether DC populations during both acute and chronic infection models displayed altered phenotypic and functional characteristics consistent with the stimulation of an immunoregulatory environment.

Our results, presented below, demonstrate significant changes in DC composition during infection, in particular highlighting a novel $CD11c^{lo}$ subset. We have previously reported that, within the $CD11c^{hi}$ population purified by magnetic bead sorting, $CD8\alpha^{int}$, $CD103^{+}$ DCs diminish in frequency in the MLN, but that overall $CD11c^{hi}$ DCs from infected mice remain immunogenic (48). We now show that infection also expands a population of MLN $CD11c^{lo}$ DCs identified by flow cytometry but which is poorly represented in magnetic bead-sorted preparations, and escapes deletion in CD11c-DTR mice. Critically, these DCs are most efficient at *de novo* induction of $CD4^{+}Foxp3^{+}$ Tregs, and predominate during chronic infection. Because primarily $CD11c^{hi}$ DCs are eliminated in DTx-mediated depletion in CD11c-DTR mice, we are also able to show that the $CD11c^{lo}$ subset which preferentially survives DTx treatment, maintains the frequency of Treg cells *in vivo*.

Materials and Methods

Mice

BALB/c, C57BL/6 and DO11.10 mice were bred and maintained in a specific pathogen-free facility at the University of Edinburgh. CD11c.DOG mice on the C57BL/6 background expressing the human diphtheria toxin receptor and the ovalbumin fragment aa 140-386 under the control of the CD11c promoter were rederived into the same facility from the line previously described (49, 50).

H. polygyrus and *N. brasiliensis* infection and parasite antigen preparation

Mice were infected with 200 *H. polygyrus bakeri* L3 larvae using a gavage tube or injected subcutaneously with 250 *N. brasiliensis* L3 larvae and harvested 7 days post-infection. Excretory-secretory antigens from adult *H. polygyrus* (HES) and *N. brasiliensis* (NES) were prepared as previously described (51, 52).

Dendritic cell phenotyping

Mesenteric lymph nodes (MLNs) were removed into HBSS before digesting in 0.5 mg/ml Liberase CI (Roche) for 30 minutes at 37°C in a shaking incubator with the addition of 0.02 M EDTA (pH 7.3) for the last 5 minutes. MLNs were then washed and homogenized in HBSS, centrifuged at 400 *g* for 5 minutes before resuspending in FACS buffer (0.5 % BSA, 0.05% sodium azide, 1×PBS). DCs were phenotyped by labeling with 1/200 FITC-conjugated antibody to MHC class II (clone M5/114.15.2; BioLegend), F4/80 (MF48020; Caltag), NK1.1 (clone PK136), CD24 (clone M1/69; eBioscience); PE-conjugated anti-CD40 (clone 3/23), CD80 (clone 16-10A1), CD86 (clone GL1), 1/25 PDCA-1 (clone JF05-1C2.4.1; Miltenyi); PerCP-conjugated anti-B220 (clone RA3-6B2), APC-conjugated anti-CD11c (clone N418; Biolegend), Ly6C (clone AL-21); PE-Cy7-conjugated anti-CD8 (clone 53-6.7; eBioscience); APC-ef780-conjugated anti-B220 (clone RA3-6B2;

eBioscience), CD11c (clone N418; ebioscience), a combination of biotin-conjugated anti-CD103 (clone 2E7, eBioscience), CD8α (clone 53-6.7, eBioscience), CD11b (clone M1/70; BioLegend), Siglec-H (clone 551.3D3; Miltenyi) and streptavidin-conjugated PerCP (BioLegend) or PerCP-Cy5.5 (Biolegend) or the relevant isotype controls before analysing by FACS. All fluorochrome-labeled antibodies were obtained from BD Pharmingen unless otherwise stated, and samples were analyzed by flow cytometry using Becton-Dickinson Canto or LSR-II flow cytometers.

Dendritic cell purification

Following liberase digestion as described above, MLN were resuspended in FACS buffer (0.5 % BSA, 0.05% sodium azide, 1×PBS). Following labeling of cells with 1/200 PE-conjugated anti-CD3 (clone 17A2; BD Pharmingen) at 4°C for 15 minutes, cells were incubated with anti-PE microbeads (Miltenyi Biotec) and the flow-through collected from an LS column. After centrifugation at 400 *g* for 5 mins, cells were resuspended at 10^6/ml FACS buffer and labeled with 1/200 APC-conjugated anti-CD11c (clone N418; BioLegend) for 20 minutes, before washing as previously. Cells were sorted on a FACS Aria based on high and low expression of CD11c ($CD11c^{hi}$ and $CD11c^{lo}$). Live cells were >90% pure following sorting. For experiments where B and T cells were depleted, cells were labelled with 1/200 PE-conjugated anti-IgM (clone RMM-1; Biolegend) and anti-IgD (clone 11-26c.2a; BD pharmingen) as well as anti-CD3 before collecting the flow through. Cells were sorted for CD11c expression and the absence of PE and CD49b PeCy7 (clone DX5; eBiosciences).

Dendritic cell antigen presentation, regulatory T cell induction and T cell polarization

Sorted $CD11c^{hi}$ and $CD11c^{lo}$ dendritic cells were plated in triplicate at 10:1, 1:1 and 1:10 ratios with 2×10^4 CFSE-labeled $CD4^+CD25^-$ DO11.10 T cells in the presence of 0.03 μg/ml OVA peptide or 4mg/ml filter sterilised grade V Ovalbumin protein (Sigma). Having determined the optimal proliferative responses in the T cell population for both antigen conditions, regulatory T cell induction experiments were performed with a 1:1 ratio DC:T cell, 0.03ug/ml OVA peptide + 2 ng/ml recombinant TGF-β, 0.1 μg/ml OVA peptide + 2 ng/ml recombinant TGF-β, 0.03 μg/ml OVA peptide + 2 ng/ml recombinant TGF-β + 1 μM of the retinoic acid receptor antagonist LE540 (Tocris Bioscience), or 0.03 μg/ml OVA peptide alone. Cells were fed at days 2 and 4 with 5 ng/ml recombinant IL-2 and harvested at day 5. Antigen presentation and DO11.10 proliferation was assessed by CFSE dilution within the $CD4^+KJ126^+$ T cell population. Regulatory T cell induction was assessed by Foxp3 expression using PE-conjugated anti-Foxp3 (clone FJK-16s; eBiosciences) within the cultured $CD4^+KJ126^+$ T cell population and comparing it to freshly sorted $CD4^+KJ126^+$ T cells (99.8% $Foxp3^-$). Supernatants from the co-cultures were harvested to analyse for T cell polarization by IFN-γ, IL4, IL-6, IL-10 and IL-17 production using commercially available cytokine ELISA (BD pharmingen).

Isolation of naïve transgenic $CD4^+$ T cells for in vitro regulatory T cell induction

The spleen and peripheral lymph nodes were removed from a DO11.10 mouse, homogenized and labeled with CD4 (L3T4) microbeads (Miltenyi Biotec) before positive selection using an LS column. Cells were then labeled with PerCP-conjugated anti-CD4 (clone GK1.5; Biolegend) and PE-conjugated anti-CD25 (clone PD61 5.3; Caltag) for 15

minutes at 4°C before sorting the $CD4^+CD25^-$ population on a FACSAria. Cells were labeled with 5 μM CFSE before co-culture using standard protocols (53).

In vivo depletion of dendritic cells

CD11c.DOG mice were depleted of $CD11c^{hi}MHCII^+$ dendritic cells by intraperitoneal injection of 8 ng/g diphtheria toxin daily from day −1 to 6 after infection (49, 50). The efficacy of this depletion was assessed by liberase digestion of the spleen and MLN followed by flow cytometry using a combination of the aforementioned fluorochrome-conjugated antibodies specific for surface CD11c, MHC class II and B220. Plasmacytoid ($PDCA\text{-}1^+$) DCs were depleted by administration of 100μg of 120G8 ip on days 0, 2, 4, 5, 8 and 11 post-infection with *H. polygyrus*.

Ex vivo MLN analysis following DC depletion

The MLNs of infected and naïve mice were removed for intracellular cytokine staining, regulatory T cell profiling and antigen-specific restimulation. For intracellular staining, 6×10^6 cells/well were plated in a 24-well plate with 0.5 μg/ml PMA and 1 μg/ml ionomycin for 1 hour before the addition of 10 μg/ml Brefeldin A for a further 3 hours. Cells were then washed, and blocked by resuspension in FACS buffer containing FcR block for 15 mins. After washing, cells were incubated with 1/200 anti-CD8-FITC and anti-CD4-PerCP for 20 minutes, washed again then fixed for 20 minutes with 200 μl Fix/Perm buffer (BD Pharmingen). Fixation buffer was removed with two washes with permeabilization buffer (BD Pharmingen) and samples were split and subsequently stained for intracellular cytokines using 1/200 anti-IFN-gamma-APC, anti-IL-4-PE, anti-IL-9 PE, anti-IL-10-APC, anti-IL-13-APC, anti-IL-17-PE or the relevant isotype control for 20 minutes in Perm buffer. After another wash in Perm Buffer, samples were resuspended in FACS buffer and analysed by flow cytometry using a Becton-Dickinson Canto or LSR-II flow cytometer. For regulatory T cell profiling, 10^6 cells were blocked as previously, then surface stained for 20 minutes with 1/200 anti-CD4-PerCP, anti-CD25-PE and anti-CD103-biotin with a combination of Streptavidin-PerCP. After washing, these cells were fixed with eBioscience Fix/Perm buffer for between 45 minutes and 18 hours in accordance with the manufacturer's instructions, before washing twice with Perm buffer and staining with 1/200 anti-Foxp3-APC in Perm Buffer for 30 minutes. Samples were then washed in Perm buffer and resuspended in FACS buffer before analysis by flow cytometry. For antigen-specific restimulation, 10^6 cells were plated in the presence of media or 2 μg/ml HES for 72 hours at 37°C for 5% CO_2 before, centrifuging at 400 g for 5 minutes and freezing the supernatants at −20°C, which were then analysed for IFN-γ, IL-4, IL-5, IL-10, IL-13 and IL-17 by commercially available ELISA (BD Pharmingen).

Statistical analysis

For statistical analysis comparing two groups, data were assessed for normality and equal variances, log transforming if necessary to achieve normality and equal variance. If the data fulfilled these criteria an unpaired t test was used, while if this was not possible the non-parametric Mann-Whitney U test was used. Where more than three groups were being tested, data was assessed and if necessary transformed similarly for variance and normality

and either a parametric 1 way ANOVA (followed by Tukey's multiple comparison test) or a non-parametric Kruskal-Wallis test (followed by a Dunns multiple comparison) used.

Results

Predominance of CD11c^{lo} DC population in the MLN following chronic helminth infection

Gastrointestinal helminth infections stimulate powerful Th2-dominated immune responses both to parasite and bystander antigens (40), as well as in certain cases Foxp3$^+$ Treg expansion (43, 44). Although we and others have previously shown that the Th2 response to helminths such as *N. brasiliensis* (42) and *Schistosoma mansoni* (41) can be recapitulated through antigen-pulsed DCs, it is not yet known whether helminth-stimulated DC populations can favor the expansion of regulatory T cell activity.

We therefore examined CD11c$^+$ DC populations in the draining MLNs of mice infected with the nematode *H. polygyrus,* which establishes a chronic infection and drives Treg expansion, and *N. brasiliensis* which is expelled by a potent Th2 response after an acute infection of 6-8 days. MLNs were found to contain all the common subsets of DCs, conventional CD11c^{hi} (both CD11b$^+$ and CD11b$^-$), CD11c^{hi}CD103$^+$ and plasmacytoid CD11c^{lo}B220$^+$. However, 7 days following *H. polygyrus* infection, at a time point associated with magnified Treg numbers (43) the proportion of CD11c^{lo} to CD11c^{hi} DCs showed a striking increase (Fig 1 A), which remained in place at 28 days (Fig 1 A, B). Within the MLNs of *H. polygyrus* infected mice, conventional CD11c^{hi} represented only 30% of the DC population, with a downshift in peak expression levels. In contrast, no significant alteration occurred in mice mounting effective immunity to expel *N. brasiliensis* (Fig 1 A, B). This change reflected preferential expansion of the CD11c^{lo} subset in *H. polygyrus* infection, which stimulates a doubling or more of total lymph node cell numbers (Fig. 1 C). Moreover, we could confirm a previous report (49), that the CD11c^{lo} population is under-represented in preparations sorted on anti-CD11c beads, and found this was true for both PDCA$^+$ and PDCA$^-$ populations. Thus, flow-sorted DCs were used for all subsequent functional analyses.

Expansion of non-plasmacytoid CD11c^{lo} DCs in infection

To determine which DC subsets are most altered during *H. polygyrus* infection, more extensive phenotypic data were collected. The CD11c^{hi} population included most CD11b^{hi} (Fig. 2 A, B), CD8α^{hi} and CD8α^{int} (Fig. 2 C) cells. In contrast, the CD11c^{lo} subset was found to be largely CD11b-negative and CD8α-negative, consistent with our previous report that CD11c$^+$CD8α^{int} DCs are lost from the MLNC during these nematode infections (48).

Because plasmacytoid dendritic cells (pDCs) express lower levels of CD11c and have been strongly implicated to have tolerogenic or pro-regulatory activity (27, 39, 54), we next analyzed the CD11c^{lo} population for markers of this cell type, namely B220, PDCA-1 (55) and Siglec H (56). In naïve mice, the CD11c^{lo} gate included most of the B220$^+$PDCA-1$^+$Siglec H$^+$ pDCs, but following infection expression of both PDCA-1 (Fig. 2 D) and Siglec H (Fig. 2 E) actually declined. In addition, while a proportion of the CD11c^{lo} cells expressed B220 (Fig 2 F) and CD19 (Fig. 2 G), and the monocyte marker Ly6C (Fig. 2

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H) they did not express F4/80 (Fig. 2 I), NK1.1 (Fig. 2 J) or the eosinophil marker Siglec F (Fig. 2 K).

To further test whether the small number of pDCs within the CD11c^{lo} population play a tolerogenic role in *H. polygyrus* infection, we tested the effects of pDC depletion with the anti-PDCA-1 antibody 120G8 (55), prior to and during *H. polygyrus* infection. We continued the depletion protocol until day 11, when the adult *H. polygyrus* have emerged from the gut wall and mice were analysed at d28 to determine the effect of depletion on chronic worm burden and T cell responses. Depletion did not alter adult worm survival (Fig. 3 A) or the increase in regulatory T cell numbers seen with *H. polygyrus* infection at this time point (Fig. 3 B). Th2 responses, as measured by intracellular IL-4 (Fig. 3 C) and IL-10 (Fig 3 D), as well as IL-5 and IL-13 recall responses to HES antigen (data not shown) were undiminished in anti-120G8 treated mice.

CD11c^{lo} DCs do not express high levels of CD103

Expression of CD103, the α_E chain of integrin $\alpha_E\beta_7$, has been reported to be an important attribute of mucosal DCs involved in immune regulation, as demonstrated with magnetic bead-purified (ie CD11c^{hi}) cells (20, 38, 57). We therefore measured levels of this marker in MLN DCs from naive and infected mice. Surprisingly, despite the overall pro-regulatory environment associated with *H. polygyrus* infection, we found that the proportion of CD103-expressing CD11c^{+} cells declined sharply. Few CD11c^{lo} DCs expressed CD103 either in the naïve or infected MLN, and by d28, the predominant DC phenotype (>50%) was CD11c^{lo}CD103^{-} (Fig. 4 A). Notably, CD103 expression was restricted to the CD11c^{hi} population (Fig. 4 A), but even within the latter, CD103 intensity waned following infection (Fig. 4 B).

Co-stimulatory phenotype of DC populations in the MLN following infection

We then compared the expression of CD40, CD80, CD86 and MHCII among CD11c subsets following infection with *H. polygyrus*. In both naïve and infected mice, the CD11c^{lo} DC population expressed lower levels of CD40, CD80, CD86 and MHC class II compared to the CD11c^{hi} population, but the two subsets showed converse trends as a result of infection. Thus, each surface marker was downregulated in the CD11c^{hi} population of DCs from infected mice (Fig. 5 A-D), while CD40 and MHCII expression were significantly increased in the population of CD11c^{lo} DCs following infection (Fig. 5 A, D). Hence, the CD11c^{hi} population in infection presents with a lower level of maturation and activation markers than is found in naïve animals, while the CD11c^{lo} subset displays significant upregulation of MHCII and CD40.

Characterization of CD11c^{lo} DC function following *H. polygyrus* infection

Having demonstrated significant increases in the ratio of CD11c^{lo}:CD11c^{hi} DC in the MLN of *H. polygyrus* infected mice, we compared the functional profile of these cells in terms of responsiveness to innate ligands and potential for antigen presentation to T cells. To isolate required numbers of each cell type, MLNs from naïve and *H. polygyrus*-infected mice were pooled, treated with liberase/EDTA, depleted of CD3^{+} cells, and flow sorted on CD11c

expression. Purities of >90% were achievable, with <2% of CD11c^{hi} DCs within the CD11c^{lo} preparation, and vice versa (Fig. 6 A).

The two subsets from naive and infected mice were first tested for responsiveness to TLR ligands LPS and poly-IC. Naive CD11c^{hi} DCs were highly responsive to stimulation, producing IL-6 (Fig. 6 B), IL-10 (Fig 6 C) and IL-12p40 (Fig. 6 D); however, production of the latter two cytokines was diminished in equivalent cells from *H. polygyrus*-infected mice. In contrast, CD11c^{lo} DCs failed to respond to TLR ligation with IL-10 and IL-12p40. Production of IL-6 by CD11c^{lo} DCs was in response to LPS alone and was unaffected by infection status. The restricted TLR responsiveness corresponded to the relatively low levels of TLR-2, -3, -4 and -9 expressed by the CD11c^{lo} population as determined by RT-PCR (data not shown).

T cell polarization

To test the capacity of these cells to stimulate an antigen-specific CD4^{+} effector T cell response, CD11c^{hi} and CD11c^{lo} cells from naïve and *H. polygyrus*-infected mice were pulsed with OVA peptide in co-cultures with CFSE-labelled CD4^{+}CD25^{-} DO11.10 T cells. While CD11c^{hi} DCs were able to drive a high level of T cell proliferation, this was not the case for the CD11c^{lo} subset from both naïve and *H. polygyrus*-exposed mice (Fig. 7 A). In similar *in vitro* cultures, cytokine production in response to presentation by each subtype was measured. Naive CD11c^{hi} DCs strongly stimulated IFN-γ, IL-4, IL-6, IL-10 and IL-17 (Fig. 7 B-F); following infection, responses elicited by this subset were similar, but with higher IL-4 showed a skew towards Th2. CD11c^{lo} DCs were consistently poorer at generating T cell cytokine responses; following infection, this subset showed enhanced stimulation of IL-10 responses (Fig. 7 E), but it was notable that negligible IL-6 or IL-17 production was elicited by CD11c^{lo} DCs (Fig. 7 D, F).

Because the CD11c^{lo} DC subset was less effective at presenting peptide to T cells, we tested whether they were functional at the level of antigen processing. Flow-sorted DCs of each phenotype were incubated with ovalbumin protein antigen, and the response of co-cultured CFSE-labelled CD4^{+}CD25^{-} DO11.10 T cells measured by CFSE dilution (Fig. 7 G) and cytokine release (Fig. 7 H). The results showed that CD11c^{lo} DCs are capable of processing and presenting intact protein antigen, although stimulating a significantly weaker IFN-γ response from naive transgenic T cells.

CD11c^{lo} DCs preferentially induce regulatory T cells

Because CD11c^{lo} DCs were found to be ineffective stimulators of the effector T cell response, we aimed to determine whether this reflected the absence of an activated phenotype (Fig. 5) or if they were able to induce regulatory T cell activity *in vitro*, potentially suppressing antigen-specific T cell proliferative and cytokine responses. We found that CD11c^{lo} cells, from either naïve or infected mice, showed dramatically enhanced ability to induce antigen-specific CD4^{+}Foxp3^{+} T cells in culture compared to CD11c^{hi} cells when tested in the presence of exogenous TGF-β. Conversely, induction of CD4^{+}Foxp3^{+} cells was significantly reduced by including the retinoic acid receptor antagonist LE540, or by increasing the concentration of OVA antigen (Fig. 8 A, B). The requirement for

exogenous TGF-β reflected the low levels of TGF-β1 mRNA detected by RT-PCR in the $CD11c^{lo}$ population (data not shown).

To exclude any influence of either B or T lymphocytes that may be present in the CD11clo population, further experiments were undertaken in which these cell types were depleted by magnetic bead negative selection using anti-CD3, anti-IgM and anti-IgD prior to flow sorting by CD11c expression levels. B- and T-cell-depleted CD11c DCs from *H. polygyrus*-infected mice were then tested for their ability to induce a regulatory T cell phenotype and were found to strongly drive Foxp3 expression in naive $CD4^+CD25^-$ DO11.10 T cells, with a significantly greater effect induced by the $CD11c^{lo}$ subset.

CD11c DC depletion *in vivo*

In order to test the *in vivo* role of $CD11c^{lo}$ DCs, we altered the ratio of $CD11c^{lo}$:$CD11c^{hi}$ DCs *in vivo* through the use of CD11c.DOG mice, depleting $CD11c^{hi}$ MHC class II^+ DCs using daily administration of 8 ng/g DTx (50). This regime resulted in effective depletion of $CD11c^{hi}$ DCs from the MLN and spleen of *H. polygyrus*-infected mice (Fig. 9 A), and an increase in the proportion of $CD11c^{lo}$ to $CD11c^{hi}$ DCs in both the MLN and spleen (Fig. 9 B). Notably, DTx treatment achieved a systemic depletion, whereas in *H. polygyrus* infection the loss of $CD11c^{hi}$ DCs occurred only in the MLN. Following 7 days of *H. polygyrus* infection, intracellular cytokine staining demonstrated that $CD11c^{hi}$ MHC class II^+ depletion resulted in a significant reduction in the percentage of IL-4 and IL-10 cytokine-synthesizing $CD4^+$ T cells in the MLN (Fig. 9 C-E). Antigen-specific re-stimulation of total MLN cells also demonstrated dramatic reduction in IL-5 and IL-13 secretion following $CD11c^{hi}$ MHC class II^+ depletion (Fig. 9 F, G), confirming that the effector Th2 response had been greatly diminished in the absence of $CD11c^{hi}$ DCs. In contrast, depletion with DTx did not abolish the expansion in $CD4^+Foxp3^+$ Treg MLN cell numbers resulting from *H. polygyrus* infection (Fig. 9 H). The combination of these effects resulted in a markedly higher regulatory:effector ratio as measured by Foxp3 and IL-4 expression respectively (Fig. 9 I).

Discussion

Many parasitic infections are accompanied by, and may owe their longevity to, heightened immunoregulatory responses in their host (58-60). Regulatory cell populations may be directly stimulated by pathogens for their survival (61), by commensal organisms in the steady state (62) or activated by host mechanisms to forestall pathology, depending on the particular setting in question (63). Here, we studied a gastrointestinal nematode, *H. polygyrus*, in which a marked immunoregulatory response develops, including the expansion of $Foxp3^+$ regulatory T cells (43, 44, 46) during chronic infection.

Dendritic cell populations play a decisive role T cell subset differentiation in response to pathogens (8), and are of integral importance for intestinal immunity (64) and homeostasis (65). DCs pulsed with helminth products selectively drive the development of Th2 cells (41, 42, 66-68), and the depletion of DCs dramatically impairs Th2 induction by *Schistosoma mansoni* (13) and, as we now confirm, *H. polygyrus*. While a critical role for basophils, rather than DCs, has been suggested for Th2 reactivity to the colon-dwelling nematode

Trichuris muris (69), this does not hold for other infections: the magnitude and balance of Th2 responsiveness to *S. mansoni* (13) and *N. brasiliensis* (11) are unaffected by basophil depletion, as we have also found for *H. polygyrus* infection (Smith, K.A. et al, unpublished data). Hence, it is appropriate to place due emphasis on how host DC subsets, and their ability to induce regulatory T cells, change with infection. For this reason, we analyzed the DC population within the immune tissue most affected by gastrointestinal infection, the MLN.

A significant transformation takes place within the MLN DC population, with important regulatory consequences. First, preferential expansion of a non-plasmacytoid DC subset, with a $CD11c^{lo}CD103^{-}$ phenotype, occurs within 7 days of infection and eclipses conventional $CD11c^{hi}$ subsets. Not only is the $CD11c^{lo}$:$CD11c^{hi}$ ratio reversed, but the remaining $CD11c^{hi}$ cells show diminished expression of functional markers, including CD103. Secondly, the $CD11c^{lo}$ cells are poor stimulators of T cell proliferation or cytokine production, while being potent inducers of *de novo* $Foxp3^{+}$expression. Reflecting this, *in vivo* depletion of the $CD11c^{hi}MHCII^{+}$ population accentuates $CD11c^{lo}$ predominance, resulting in a greatly reduced effector response and a higher percentage of regulatory T cells. Thus, chronic infection is associated with the emergence of a $CD11c^{lo}$ DC population which is directly implicated in the induction of regulatory T cell responses *in vitro* and *in vivo*.

$CD11c^{lo}$ DCs are mostly, in naïve mice, classified as plasmacytoid by their $B220^{+}PDCA\text{-}1^{+}Siglec\text{-}H^{+}$ phenotype. However, in *H. polygyrus* infection, the frequency of pDCs declines and $CD11c^{lo}$ cells do not mount an IFN-α response to TLR ligation (data not shown). Further, pDCs do not appear to play a critical role in *H. polygyrus* infection, as their depletion with anti-120G8-mediated impacts on neither Th2 responsiveness nor $CD4^{+}Foxp3^{+}$ T reg expansion. The induced $CD11c^{lo}$ subset expresses low levels of F4/80, CD11b and CD8 and is therefore distinct from tolerogenic lymphoid-derived $CD8^{+}$ DCs (70). They do not express NK1.1 or high levels of Ly6C, and so are unlikely to represent a population of interferon-producing killer DCs (71), pulmonary inflammatory DCs (72), or the $Ly6C^{+}CD11c^{lo}$ DCs found in *Leishmania* infection (73). Interestingly, $CD11c^{lo}$ populations are quite apparent in published studies of Peyer's patches (74), lamina propria (36) and MLNs (75).

A diverse range of DC types have been demonstrated to be capable of driving Treg induction and/or expansion (18). In steady-state conditions, where many DCs are immature and activation signals are lacking, most DC subsets are more tolerogenic and able to induce regulatory T cell gene expression (28, 33). Certain phenotypes are endowed with intrinsic pro-regulatory properties, such as the $CD11c^{hi}CD103^{+}$ cells from MLN and lamina propria which produce endogenous TGF-β and retinoic acid to induce Foxp3 expression in T cells in vitro (20, 76). In non-mucosal systems, both plasmacytoid (39) and non-plasmacytoid (32, 77) $CD11c^{lo}$ cells are tolerogenic and elicit regulatory cytokines, while in the skin Tregs are induced by CD103-negative $CD11c^{hi}$ DCs (78). Hence, our finding of regulatory T cell induction by mucosal $CD11c^{lo}$ DCs highlights a novel function of a subset which shares some, but not all, phenotypic properties of previously described pro-regulatory cells.

Consistent with their attenuated co-stimulatory profile, CD11c^{lo} DCs can weakly induce T cell proliferation, but may also influence T cell polarization by eliciting low levels of IFN-γ, IL-4 and IL-10 while failing to stimulate any IL6 or IL-17 release by CD4^{+} T cells. More strikingly, these cells efficiently induce CD4^{+}Foxp3^{+} cells *in vitro*. This ability requires exogenous TGF-β and is inhibited by excess antigen and the retinoic acid receptor inhibitor LE540 in a similar manner to mucosal CD103^{+}CD11c^{hi} dendritic cells (20). However, CD103 expression is downregulated on CD11c^{hi} cells following *H. polygyrus* infection, in tune with their reduced propensity to induce CD4^{+}Foxp3^{+} T cells *in vitro*. Thus, although CD11c^{hi}CD103^{+} cells contribute to CD4^{+}Foxp3^{+} T cell induction, this capacity declines following infection and is surpassed by the ability of CD11c^{lo} cells to perform this function.

Due to the lack of a unique marker for the CD11c^{lo} population, we were restricted in determining their function *in vivo*. However, we were able to model the predominance of the CD11c^{lo} population described following *H. polygyrus* infection by administrating diphtheria toxin (DTx) into CD11c.DOG mice. Here, we find effective depletion of CD11c^{hi} DCs with a compensatory increase in CD11c^{lo} frequency in the MLN and spleen of DTx-treated mice, which is more extensive than the MLN-restricted effect observed with active *H. polygyrus* infection. CD11c^{hi} depletion of infected mice decreased effector T cell activation while raising the percentage of CD4^{+}Foxp3^{+} T cells. Interestingly, in mice with transgenic genetic ablation of CD11c^{hi} DCs, generation of Foxp3^{+} Tregs was found to be intact (79), again implying a pro-regulatory function for CD11c^{lo} DCs of one or another type.

Several key biological questions now need to be addressed. The primary pro-regulatory pathway invoked by CD11c^{lo} DCs we have identified is that of TGF-β/retinoic acid dependent induction of Foxp3, but as these are required exogenously, the intrinsic pro-regulatory characteristics of the CD11c^{lo} DC population remain to be identified. A second issue is whether the numerical expansion of DCs during infection represents increased migration of gut-derived antigen-laden cells, the accumulation of CD103^{-} blood-borne precursors, or expansion in situ of resident cells (80, 81). Within the steady-state MLN, CD103^{+} DCs, and not CD103^{-} cells, are those arriving from the mucosal sites (38, 82) suggesting that CD11c^{lo} cells differentiate from the precursor population, consistent with the fact that pre-DCs retain B220 expression prior to completing their maturation (83). Further, we have previously reported that the CD8α^{int} population, which is restricted to cells migrating from the mucosa to the MLN (74), is diminished in *H. polygyrus* infection. If antigen-loaded DC migration to the MLN is impaired, while recruitment of tolerogenic DC is enhanced, the balance of response to antigen in the lymph node may primarily be a regulatory one.

Finally, it would be interesting to determine if the dominance of CD11c^{lo} DCs is a result of a parasite immune modulation strategy, or a host mechanism to control potentially damaging responses in the gut. *H. polygyrus* is known to secrete products that interfere with the pro-inflammatory functions of DCs (84, 85), and blocking mucosal DC migration to the MLN may be one result of this. On the other hand, the expansion of CD11c^{lo} DCs can occur in mice exposed to LPS (32) as well as other pathogens, and may represent a physiological response to infection stress. In combination, these two factors may result in the outcome we describe here. Further work is now in progress to resolve these issues.

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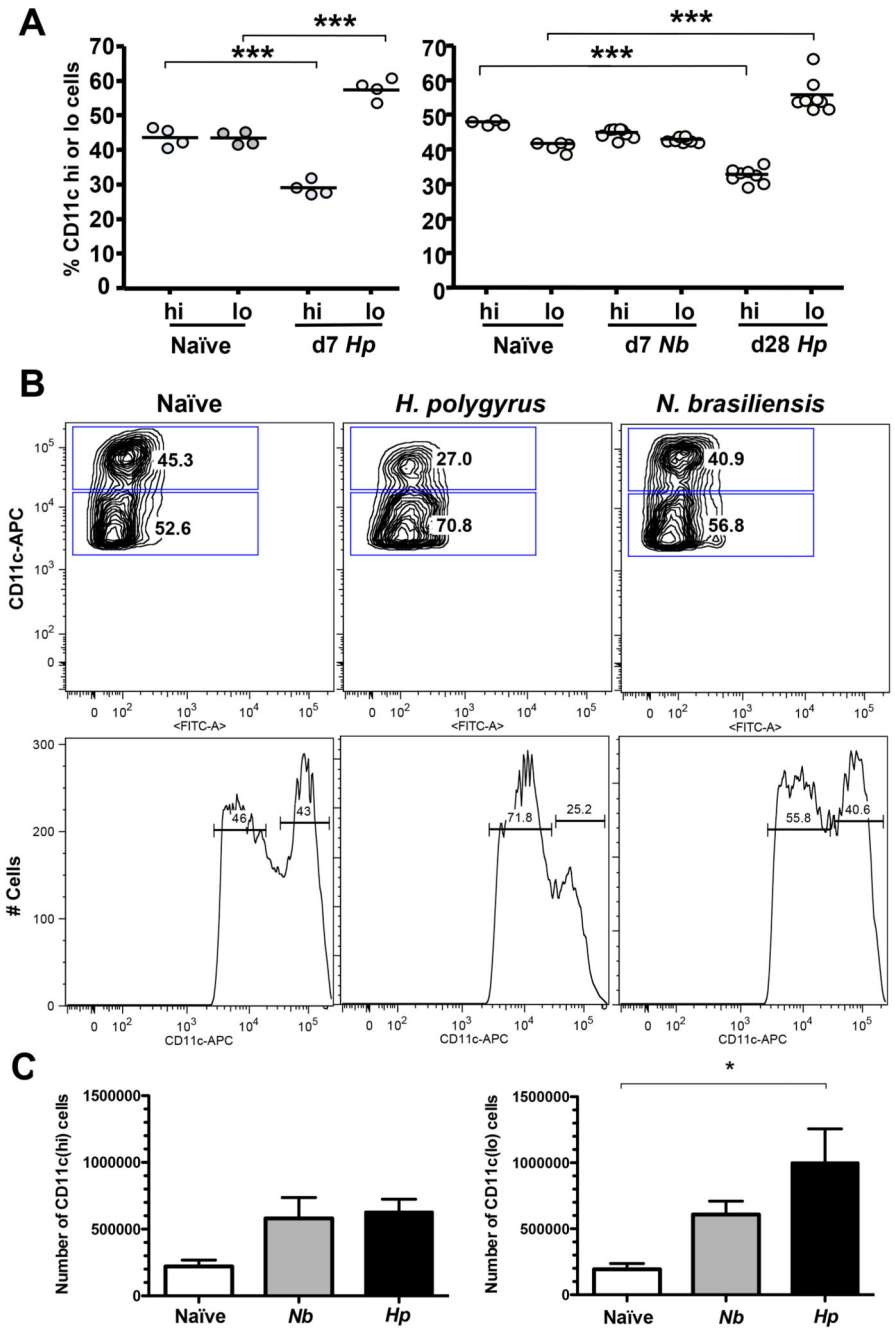

Figure 1. CD11c^{hi} and CD11c^{lo} DC Subsets in Naïve and Infected MLNC

A. Proportions of CD11c^{hi} and CD11c^{lo} DC subsets in MLNs of groups of naïve and infected mice. Left-hand panel shows naïve and d7 *H. polygyrus* infections; right hand panel d28 *H. polygyrus* and d7 *N. brasiliensis* infections. Significant differences in the percentage of CD11c^{hi} and CD11c^{lo} cells are denoted by ***($p \leq 0.001$).

B. Representative CD11c expression analyzed by flow cytometry in MLNs from individual naïve, d28 *H. polygyrus*-infected and d7 *N. brasiliensis*-infected mice, showing gatings used and the proportions of CD11c^{hi} and CD11c^{lo} populations.

C. Total numbers of $CD11c^{hi}$ and $CD11c^{lo}$ DC subsets in MLN for naïve, *H. polygyrus* and *N. brasiliensis*-infected infected mice. Significant difference is denoted by * ($p \leq 0.05$).

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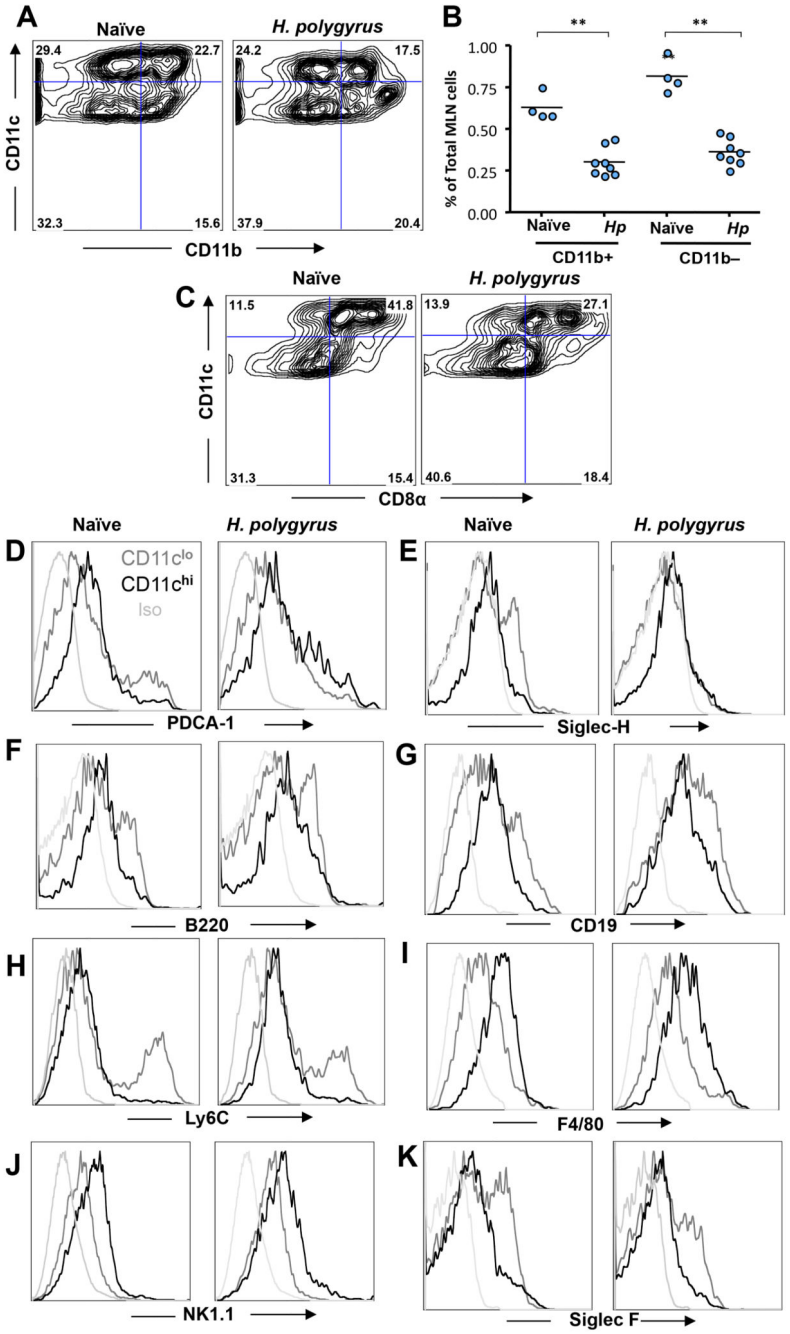

Figure 2. Phenotypes of CD11c^{hi} and CD11c^{lo} DC subsets in MLNC

A. Representative flow cytometry plots of CD11b expression by CD11c^{hi} and CD11c^{lo} DC populations within naïve and d28 *H. polygyrus*-infected MLNs.

B. Percentage of total MLN cells represented by CD11c^{hi} and CD11c^{lo} DCs of either CD11b^{+} or CD11b^{-} phenotype within naïve and d28 *H. polygyrus*-infected mice. Significant differences are denoted by ** ($p \leq 0.01$).

C. Representative plots of CD8α expression by DC populations of naïve and *H. polygyrus*-infected mice.

D-K. Overlay of histograms showing expression levels among $CD11c^{hi}$ (Black) and $CD11c^{lo}$ (dark gray) DCs of the markers of PDCA-1 (**D**), Siglec H (**E**), B220 (**F**), CD19 (**G**), Ly6C (**H**), F4/80 (**I**), NK1.1 (**J**) and Siglec F (**K**). Isotype controls for each antibody are shown as light gray lines.

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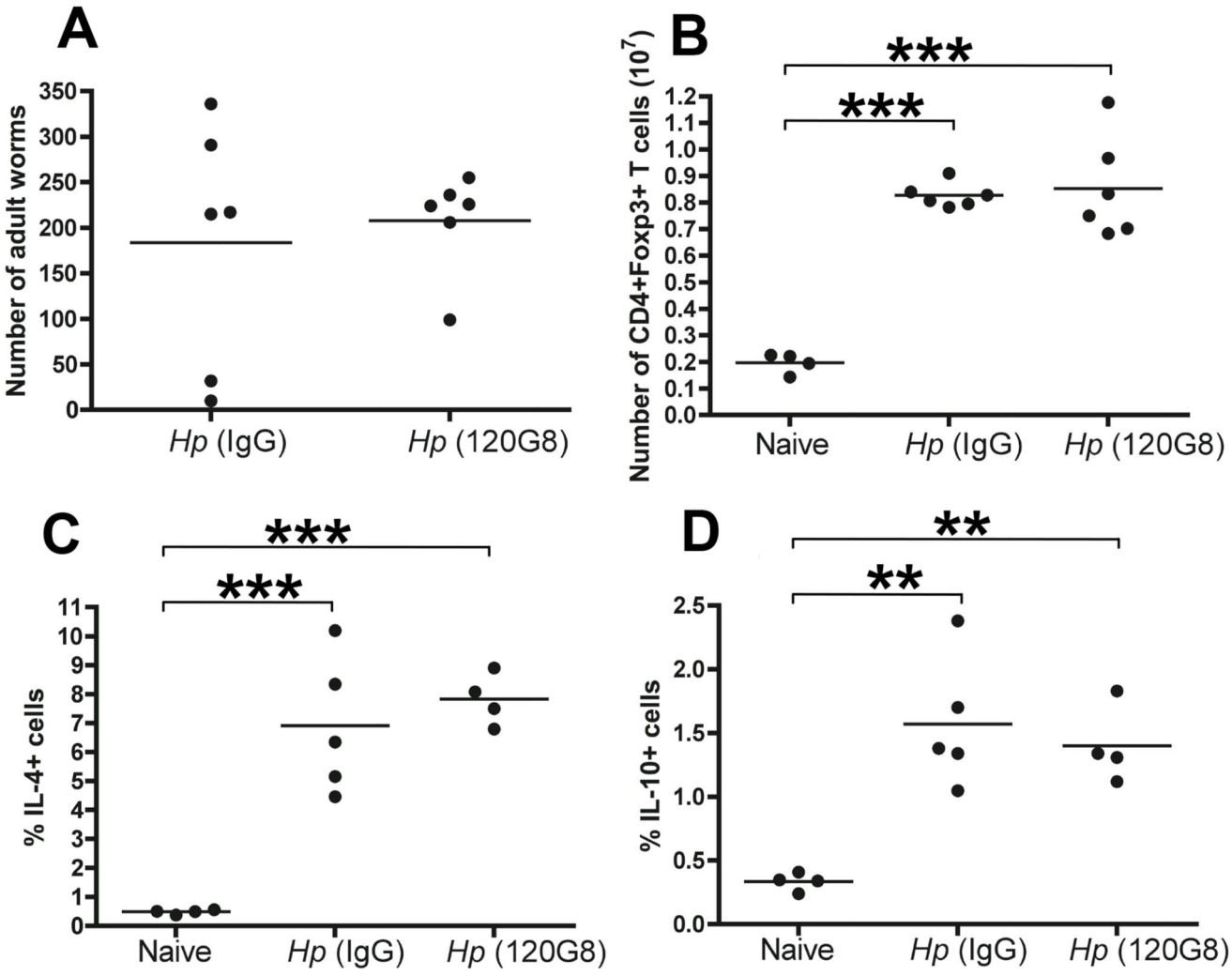

Figure 3. Depletion of PDCA-1$^+$ plasmacytoid DCs does not alter response to infection

Plasmacytoid DCs were depleted in vivo by administration of 120.G8 antibody from d0-d11 of infection with *H. polygyrus*, and parasitological and immunological parameters measured at d28. Data are representative of two similar experiments and significant differences are denoted by ** ($p \leq 0.01$) and ***($p \leq 0.001$). No significant differences were found between recipients of 120G.8 and control IgG antibodies.

A. Intestinal worm burden

B. Foxp3$^+$ T cell numbers in MLN

C, D Intracellular staining for IL-4 and IL-10, gated on CD4$^+$ T cells

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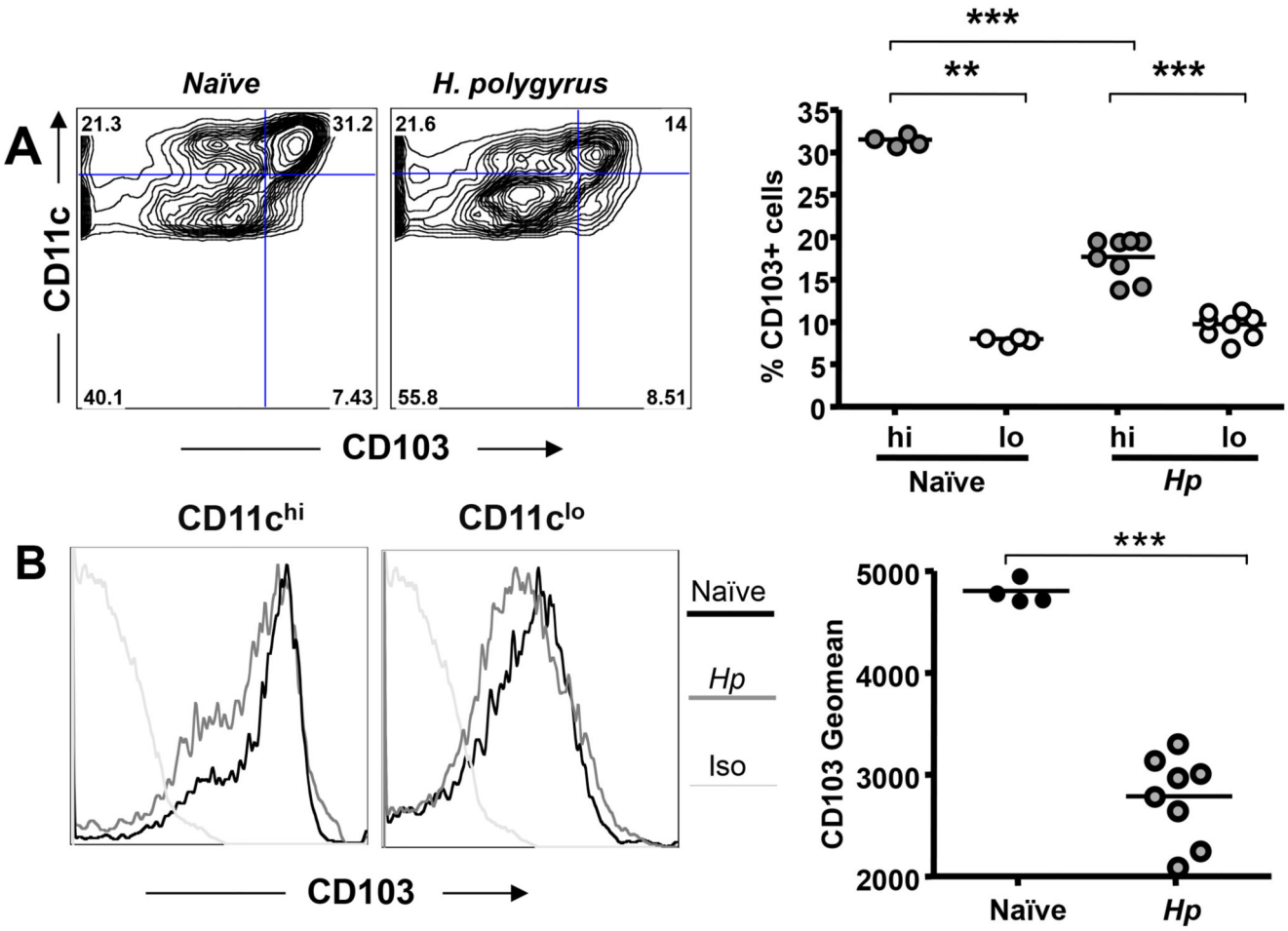

Figure 4. CD103 Expression on DCs during Gastrointestinal Nematode Infection

A. Expression of CD103 within the $CD11c^+$ DC population in naïve and d28 *H. polygyrus*-infected mice; left hand panel shows representative bivariant plots, right hand panel presents percentages of the indicated $CD11c^+$ subset expressing CD103 in MLNs from experimental groups of 4-8 mice. Significant differences in the expression of CD103 are denoted by **($p \leq 0.01$) and ***($p \leq 0.001$).

B. Intensity of CD103 expression on $CD11c^{hi}$ and $CD11c^{lo}$ MLN DCs from naïve and day 28 *H. polygyrus* infected mice; isotype controls are shown in light gray. Representative histograms (left panels) and mean MFI (right panel) from experimental groups of 4-8 mice are presented.

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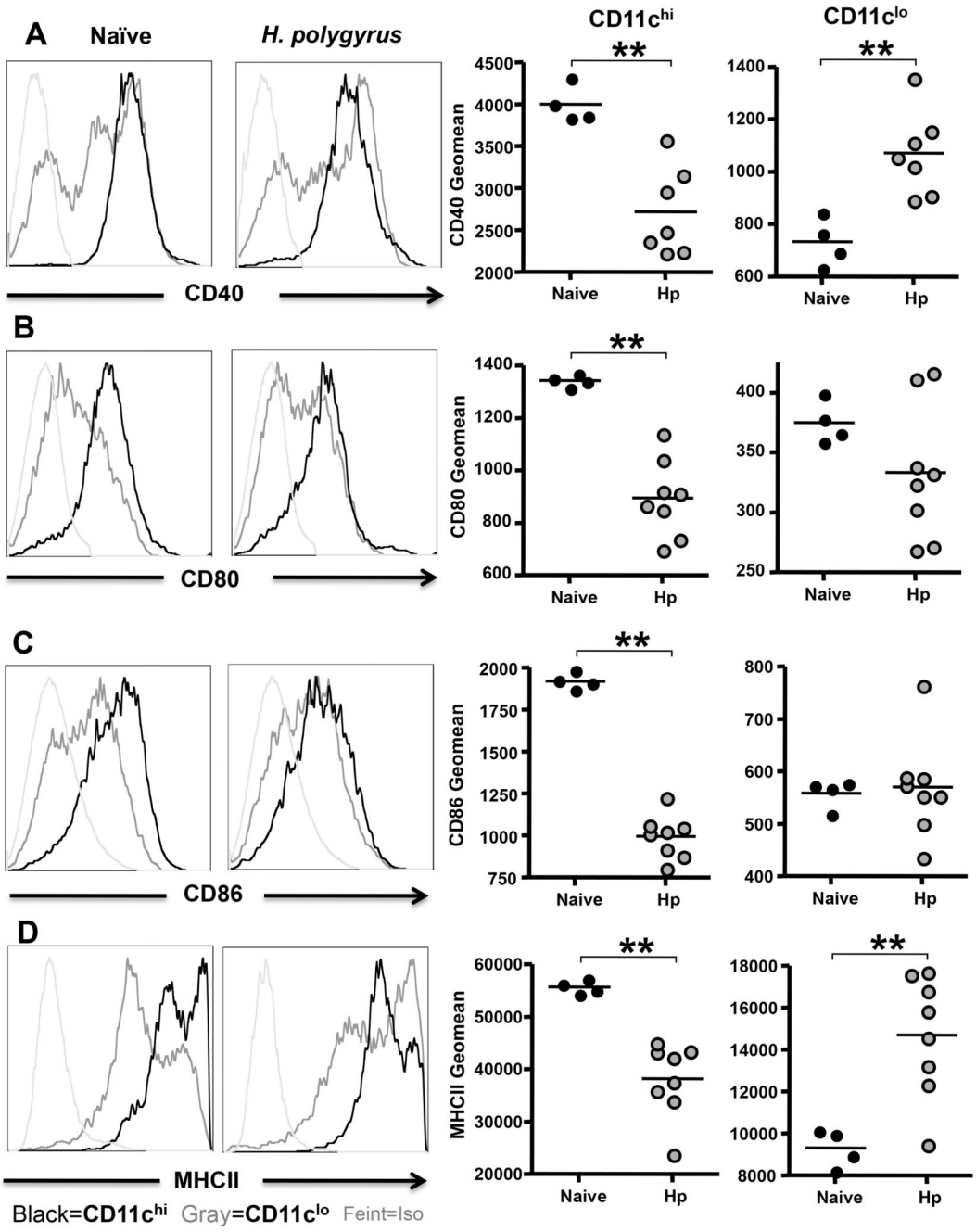

Figure 5. Activation Phenotypes of DCs in Naïve and Infected MLNC

A. Expression of the costimulatory molecule CD40 by CD11c^{hi} and CD11c^{lo} dendritic cells from the MLN of naïve and d28 *H. polygyrus*-infected mice. Left hand panels: histogram of CD40 expression in naïve mice and *H. polygyrus* infections; black line denotes CD11c^{hi}; gray line, CD11c^{lo}; isotype controls are shown in light gray. Right hand panels: geometric mean fluorescent intensity for CD40 among CD11c^{hi} and CD11c^{lo} dendritic cells; note different scales reflecting substantially lower expression levels on CD11c^{lo} DCs.

B-D. As above, expression of CD80 (**B**), CD86 (**C**) and MHCII (**D**).

Significant differences in costimulatory molecule expression are denoted by **($p \leq 0.01$)

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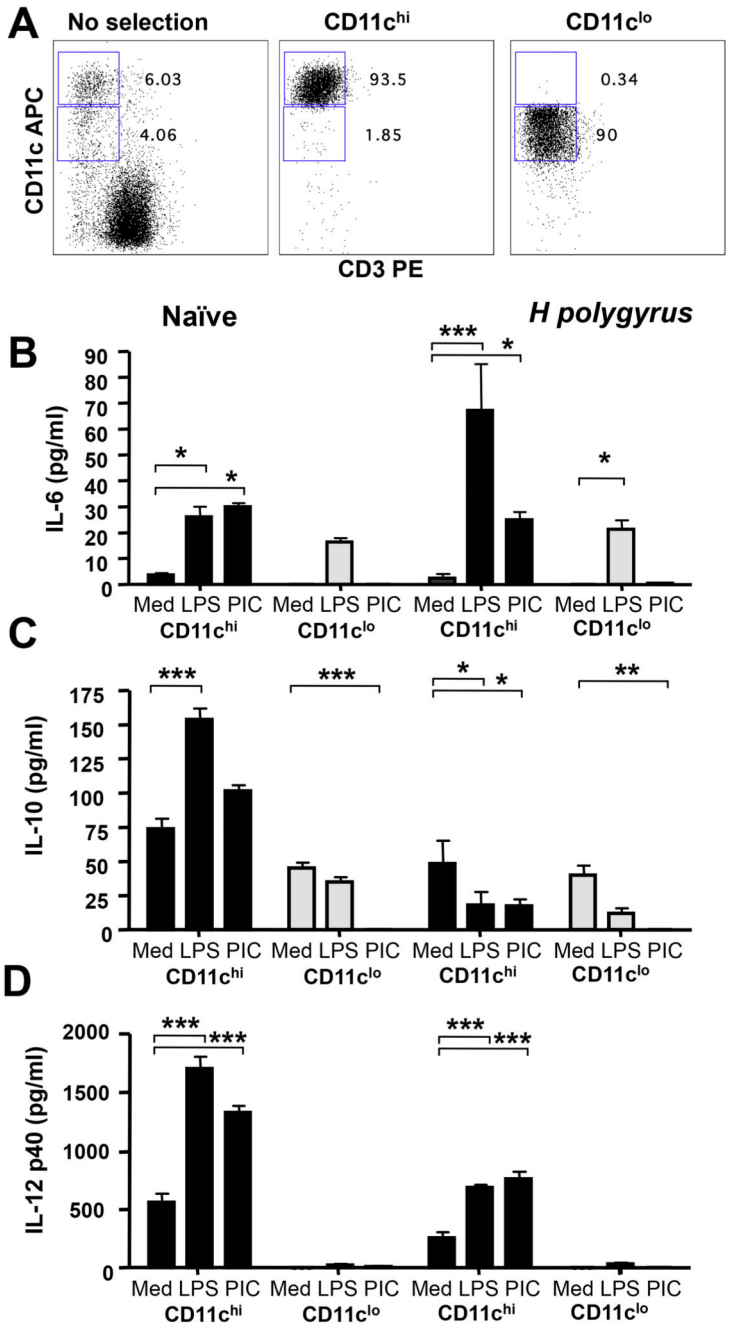

Figure 6. Responses of CD11c^{hi} and CD11c^{lo} DCs to TLR ligation

A. Representative flow cytometry of unseparated MLNs (left-hand panel) and FACS-sorted CD11c^{hi} (center panel) and CD11c^{lo} DCs (right-hand panel).

B-D FACS-sorted CD11c^{hi} and CD11c^{lo} MLN cells from naïve and d7 *H. polygyrus*-infected mice were cultured with medium alone or the TLR ligands LPS or PolyIC. Concentrations of IL-6 (**B**), IL-10 (**C**) and IL-12p40 (**D**) in culture supernatants harvested after 3 days were measured by ELISA. No IL-12p70 or IFN-α was detected. Significant differences are denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$) and ***($p \leq 0.001$).

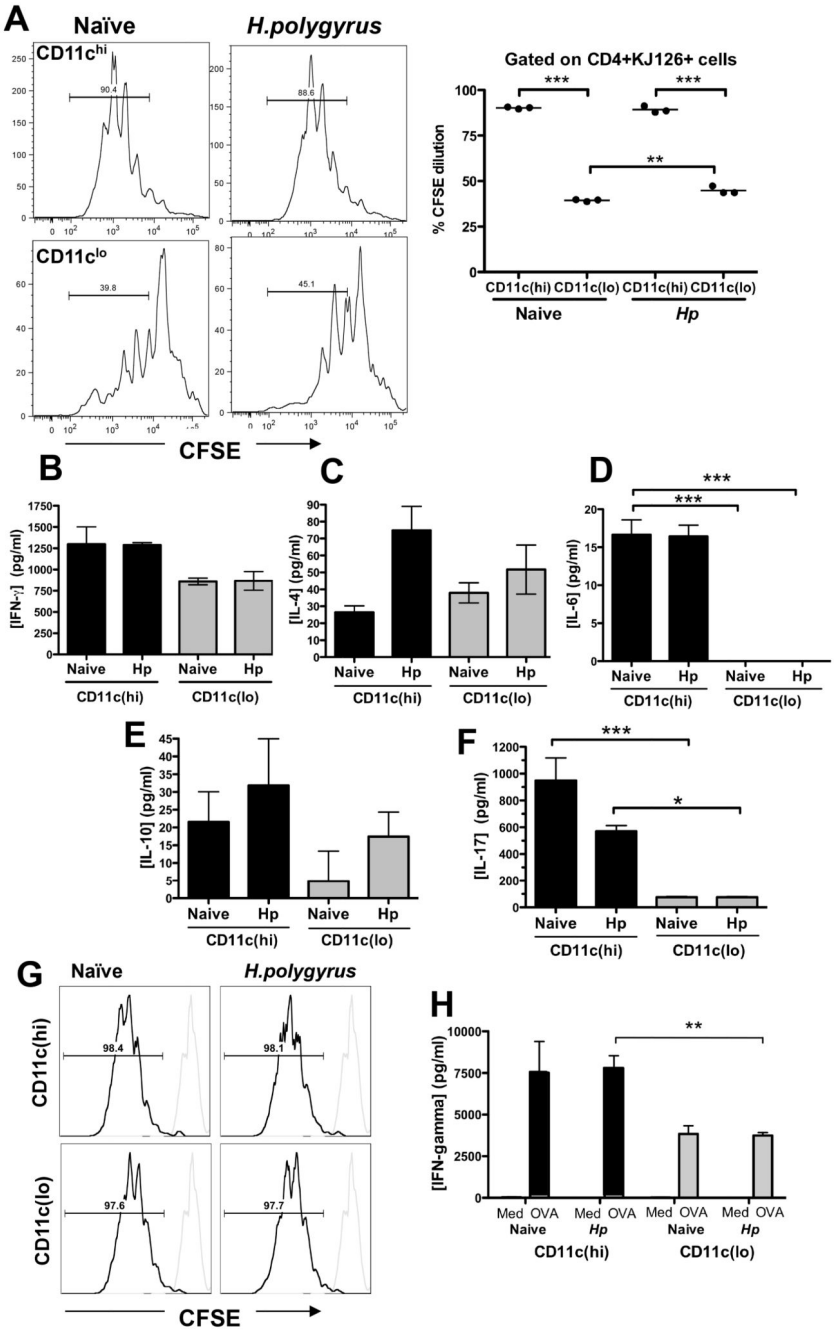

Figure 7. Ability of CD11c^{hi} and CD11c^{lo} DCs to stimulate T cell responsiveness

A. Proliferation of CFSE-labeled DO11.10 CD4^{+}CD25^{-} T cells following incubation with pooled CD11c^{hi} or CD11c^{lo} cells from naïve and *H. polygyrus*-infected mice and 0.03 μg/ml OVA peptide; a representative analysis from each DC type is shown in the left hand set of panels, and the right hand panel presents data from 3 individual wells. Gating was set on unstimulated labeled cells. Results are representative of 2 separate experiments. Significant differences in this and other panels are denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$) and ***($p \leq 0.001$).

B-F. Induction of IFN-γ(**B**), IL-4 (**C**), IL-6 (**D**), IL-10 (**E**) and IL-17 (**F**) from $CD4^+CD25^-$ DO11.10 T cells incubated with pooled $CD11c^{hi}$ and $CD11c^{lo}$ cells from naïve and *H. polygyrus* infected mice and 0.03μg/ml OVA peptide. Supernatants were harvested after 5 days of co-culture and analyzed for cytokines by ELISA. Results are representative of 2 separate experiments.

G-H. Presentation of ovalbumin protein to $CD4^+CD25^-$ DO11.10 T cells incubated with pooled $CD11c^{hi}$ and $CD11c^{lo}$ cells from naïve and *H. polygyrus* infected mice, measured as dilution of CFSE label following proliferation (**G**) and antigen-dependent release of IFN-γ in the presence of OVA protein compared to medium alone (MED) control (**H**). Data are representative of 2 separate experiments.

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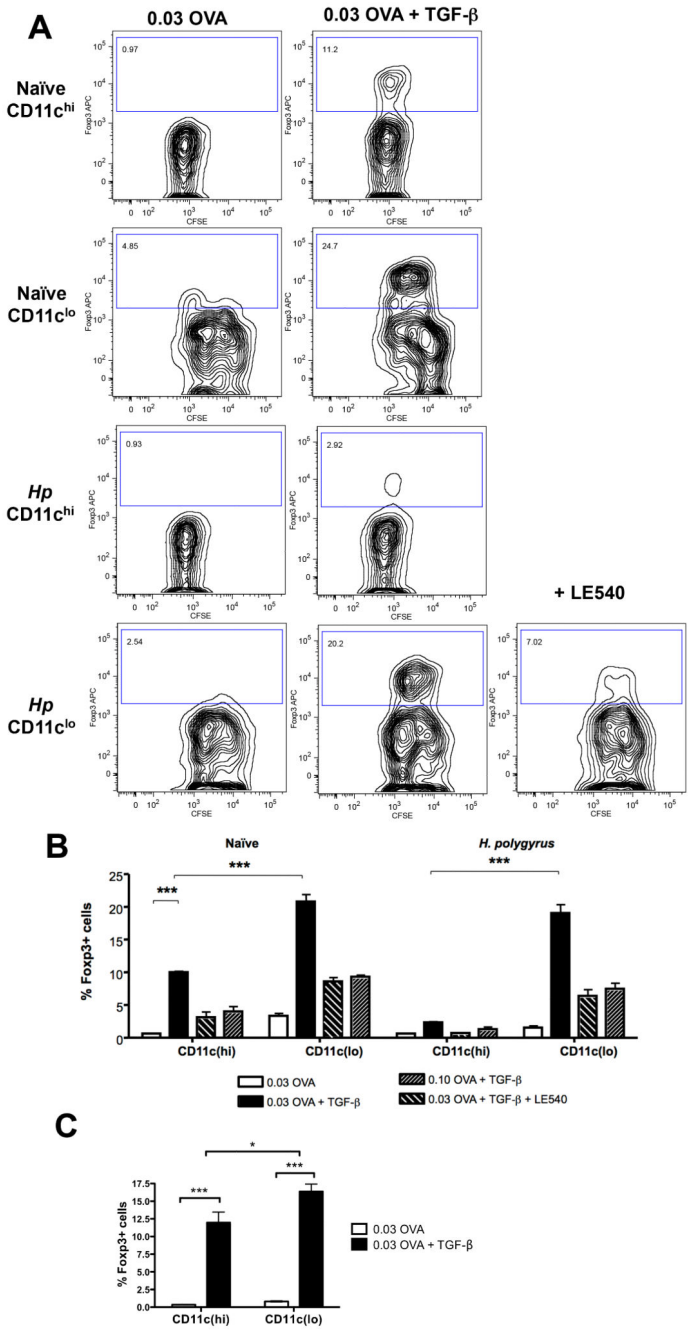

Figure 8. Induction of Foxp3$^+$ T cells by CD11c^{lo} DCs

A. Foxp3 expression by DO11.10 CD4$^+$CD25$^-$ T cells following incubation with FACS-sorted CD11c^{hi} and CD11c^{lo} cells from naïve and *H. polygyrus*-infected mice, with 0.03 μg/ml OVA peptide and 2 ng/ml TGF-β in the presence or absence of 1 μM LE540.

B. Percentage of DO11.10 CD4$^+$ Foxp3$^+$ T cells under conditions of 0.03 μg/ml OVA peptide + 2 ng/ml TGF-β, 0.1 μg/ml OVA peptide + 2 ng/ml TGF-β, 0.03μg/ml OVA peptide + 2 ng/ml TGF-β + 1 μM LE540 or 0.03 μg/ml OVA peptide alone. Data shown are

representative of three separate experiments. Significant differences in this and panel C are denoted by * ($p \leq 0.05$) and ***($p \leq 0.001$).

C. Percentage of DO11.10 $CD4^+$ $Foxp3^+$ T cells following incubation of B- and T-cell depleted, FACS-sorted $CD11c^{hi}$ and $CD11c^{lo}$ cells from naïve and *H. polygyrus*- infected mice, with or without 0.03 μg/ml OVA peptide and 2 ng/ml TGF-β. Data shown are representative of two separate experiments.

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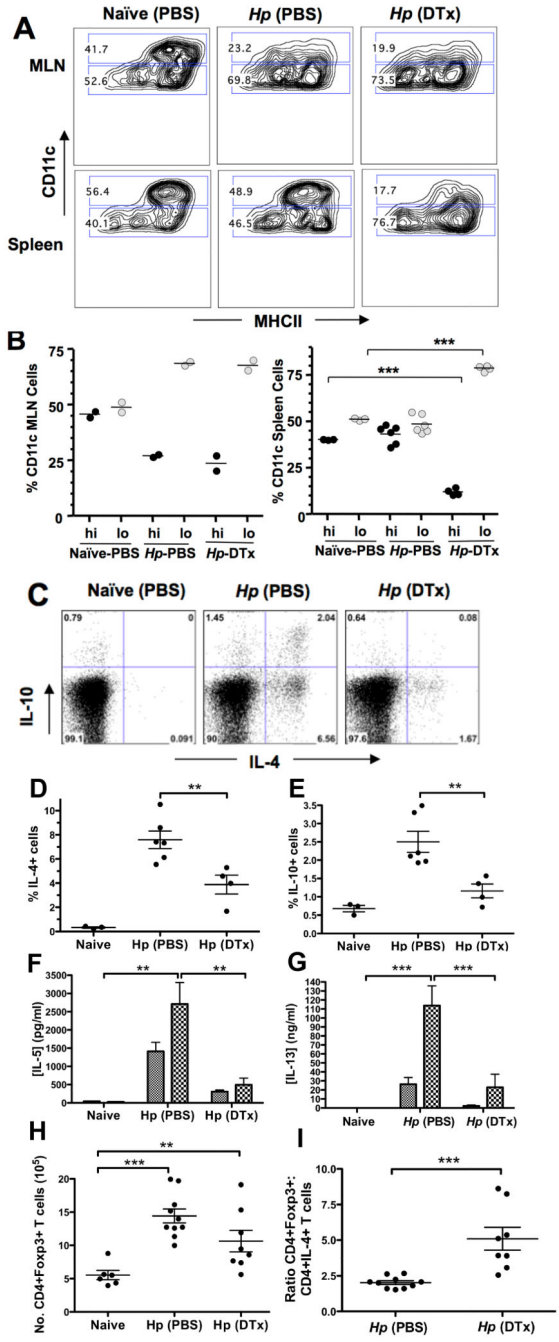

Figure 9. *In vivo* depletion of CD11c^{hi} and predominance of CD11c^{lo} DCs in CD11c.DOG mice

A. CD11c^{hi} depletion in the MLN and spleen of day 7 *H. polygyrus*-infected, DTx-treated or PBS control treated mice, confirmed by flow cytometry based on surface expression of CD11c and MHC class II.

B. Percentage of CD11c^{hi} and CD11c^{lo} DCs in the MLNs and spleens of infected, DTx-treated or PBS control mice.

C-E Cytoplasmic IL-4 and IL-10 expression by CD4^{+} T cells shown by intracellular cytokine staining of MLN cells from infected, DTx-treated or PBS control mice. Panel **C**

shows representative bivariate plot for intracellular IL-4 and IL-10, with data from individual animals shown if **D** for IL-4 and **E** for IL-10. Significant differences in this and other panels are denoted by **($p \leq 0.01$) and ***($p \leq 0.0005$)

F, G Antigen-specific Th2 cytokine responsivness in cultures of MLN cells from infected, DTx-treated or PBS control mice, restimulated with *H. polygyrus* HES antigen and assayed for IL-5 (**G**) and IL-13 (**H**) production.

H, I Numbers of $CD4^+Foxp3^+$ MLN T cells, 7 days following infection in the presence and absence of $CD11c^{hi}MHCII^+$ DC depletion (H)., and ratio of $Foxp3^+$:intracellular $IL\text{-}4^+$ $CD4^+$ T cells in the MLN of *H. polygyrus*-infected CD11c.DOG mice receiving control or DTx administration (I).

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