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Infection and Immunity

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Type 2 Innate Immunity in Helminth Infection Is Induced Redundantly and Acts Autonomously following CD11c$^+$ Cell Depletion

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Infection with gastrointestinal helminths generates a dominant type 2 response among both adaptive (Th2) and innate (macrophage, eosinophil, and innate lymphoid) immune cell types. Two additional innate cell types, CD11c^{high} dendritic cells (DCs) and basophils, have been implicated in the genesis of type 2 immunity. Investigating the type 2 response to intestinal nematode parasites, including *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis*, we first confirmed the requirement for DCs in stimulating Th2 adaptive immunity against these helminths through depletion of CD11c^{high} cells by administration of diphtheria toxin to CD11c.DOG mice. In contrast, responsiveness was intact in mice depleted of basophils by antibody treatment. Th2 responses can be induced by adoptive transfer of DCs, but not basophils, exposed to soluble excretory-secretory products from these helminths. However, innate type 2 responses arose equally strongly in the presence or absence of CD11c^{high} cells or basophils; thus, in CD11c.DOG mice, the alternative activation of macrophages, as measured by expression of arginase-1, RELM-α, and Ym-1 (Chi3L3) in the intestine following *H. polygyrus* infection or in the lung following *N. brasiliensis* infection, was unaltered by depletion of CD11c-expressing DCs and alveolar macrophages or by antibody-mediated basophil depletion. Similarly, goblet cell-associated RELM-β in lung and intestinal tissues, lung eosinophilia, and expansion of innate lymphoid ("nuocyte") populations all proceeded irrespective of depletion of CD11c^{high} cells or basophils. Thus, while CD11c^{high} DCs initiate helminth-specific adaptive immunity, innate type 2 cells are able to mount an autonomous response to the challenge of parasite infection.

Type 2 immunity encompasses a suite of cellular and cytokine-dependent immune responses associated with the Th2 phenotype of interleukin-4 (IL-4), IL-5, and IL-13 production (42, 68). The downstream effector mechanisms mobilized by these cytokines include B cell class switching to IgG1, IgG4, and IgE, eosinophilia, goblet cell hyperplasia, and alternative activation of macrophages, as well as activity on many other target cells (2). The recent recognition of a non-B, non-T (NBNT^2) "natural helper" lymphoid population that can parallel the Th2 phenotype in production of the same canonical cytokines (25, 35, 37, 46, 51) emphasizes that a broader type 2 compartment embraces both innate and adaptive immunity.

Helminth parasites provide highly polarizing stimuli for the mammalian immune response, generating a Th2-dominated profile in nearly every infection setting (14, 43), in part by releasing potent Th2-driving molecular products (11, 21, 59). Helminths and their antigens have thus provided powerful tools to dissect how the immune system discriminates between pathogenic challenges and selects type 2 immunity. A key focus has been to identify innate immune cells, which stimulate naïve Th0 precursors, with the dendritic cell (DC) being the principal player in this process. Thus, helminth antigen-pulsed DCs induce strong Th2 responses (5, 29), and mice depleted of CD11c^{high} DCs through a diphtheria toxin (DTx) receptor (DTR)-linked construct fail to mount Th2 responses to *Schistosoma mansoni* or *Heligmosomoides polygyrus* helminth infection (45, 56). Moreover, the response to schistosome egg antigen (SEA) is also abolished in the absence of DCs (45), consistent with identification of molecular constituents of SEA acting via DCs to induce the Th2 phenotype (11, 59).

Basophils have also been identified as participants in Th2 immunity, in general as amplifiers through production of IL-4 (22, 33, 65), and are indeed the main source of IL-4 in primary infection with the gastrointestinal nematode *Nippostrongylus brasiliensis* (65). In some models, including *Trichuris muris* infection, basophils have also been reported to be essential for initiation of Th2 responses (44, 58). In other systems, however, basophils appear to be less critical for inducing primary Th2 differentiation. Thus, IL-3 or IL-3 receptor (IL-3R)-deficient mice show intact Th2 responses to *N. brasiliensis* in the absence of basophil recruitment (24), and antibody-mediated basophil depletion does not diminish Th2 induction in infection (24, 45, 65). Likewise, in transgenic constructs of basophil-specific lineage ablation, normal Th2 responsiveness to *N. brasiliensis* is observed (39, 60). Nonetheless, basophils can significantly enhance Th2 immunity in settings such as infected tissue (64) and can promote rapid primary (60) and secondary (41) Th2 immunity to *N. brasiliensis*.

Additional innate immune populations are implicated in amplifying Th2 responses and/or conducting important functions in protecting against disease. Among these, alternatively activated macrophages (AAMs) are prominent in the pulmonary response to *N. brasiliensis* (36, 50) and mediate intestinal immunity to both

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N. brasiliensis and *H. polygyrus* infection (4, 70). AAM induction takes place even in RAG-deficient (27) and SCID mice (50), although T cell-derived IL-4 and IL-13 are required for alternative activation to be sustained (27). Eosinophils also contribute significant IL-4 production (68), which can directly induce AAMs (69), and act as effector cells in immunity against many tissue-migrating helminth larvae (3). In addition, an important new cell type is the nuocyte or innate lymphoid type 2 cell, which lacks B or T cell receptors and markers yet produces significant levels of IL-4, IL-5, and IL-13 (25, 35, 37, 46, 51).

In this study, we therefore set out to establish the relative importance of DCs and basophils in the generation of these diverse innate and adaptive type 2 responder populations. Studying both infection and immunization with nematode-secreted Th2-disposing antigens, we showed that DCs are indeed essential for the Th2 response, while basophils are not required for this activity, in either setting. Critically, however, the depletion of DCs or basophils did not compromise expansion of AAMs, eosinophils, or innate type 2 lymphoid cells. These data suggest that multiple populations of innate cells act autonomously by adopting a type 2 program under the conditions of helminth infection, perhaps reflecting the imperative to evolve redundant immune mechanisms to protect against parasite invasion (2).

MATERIALS AND METHODS

Mice, parasites, and antigens. Wild-type BALB/c and C57BL/6 mice and CD11c.DOG mice on the C57BL/6 background, which express human diphtheria toxin receptor and ovalbumin amino acids (aa) 140 to 386 under the control of the CD11c promoter (19, 20), were bred and maintained in a specific-pathogen-free facility at the University of Edinburgh. Mice were injected subcutaneously (s.c.) with 250 *N. brasiliensis* infective third-stage larvae (L3) or with 200 *H. polygyrus bakeri* (7, 30) L3 using a gavage tube, or with 50 μg NES into the hind foot, and lymph node (LN) cells were recovered 5 to 7 days later. Excretory-secretory antigens from adult *H. polygyrus* (HES) and *N. brasiliensis* (NES) were prepared as previously described (18, 21).

***In vivo* depletion of dendritic cells and basophils.** CD11c.DOG mice were depleted of $CD11c^{high}$ dendritic cells by intraperitoneal (i.p.) injection of 8 ng/g diphtheria toxin daily from day −1 to 6 after infection (19, 20, 45). Efficacy of depletion was assessed by flow cytometry of splenocytes and Liberase-digested mesenteric LN cells (MLNC) (see Fig. 1A and B). BALB/c mice were depleted of basophils by i.p. injection of 10 μg MAR-1 antibody or Armenian hamster IgG isotype control (eBiosciences) on days 0, 1, and 2 postinfection. Cheek blood was taken on day 4 postinfection to assess basophil depletion by flow cytometry.

Bone marrow-derived DCs and basophils. DCs and basophils were generated *in vitro* from femoral bone marrow cells in the presence of 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 5 ng/ml IL-3, respectively, replenishing the medium at days 3, 6, and 8 for DCs and days 3 and 8 for basophils, before harvesting on day 10. Basophils were purified by flow sorting as DX-5^+ c-kit^- Gr-1^-. Cells were incubated for 18 h in medium containing 20 μg/ml antigen and 5 ng/ml GM-CSF or 5 ng/ml IL-3. DCs were >85% $CD11c^+$ major histocompatibility complex (MHC) class II^+, whereas basophils were >95% $CD49b^+$ c-kit^- and expressed high levels of FcεR1 but no detectable MHC class II following culture. Cells were then washed in phosphate-buffered saline (PBS) and transferred into the hind foot at 2.5×10^5 cells per foot. Popliteal LNs (PLNs) were harvested at day 5 posttransfer and antigen-specific restimulations performed in Ex-Vivo medium containing penicillin-streptomycin and L-glutamine.

Flow cytometry and LN cytokine assays. MLNs were removed into Hanks balanced salt solution (HBSS) before being digested in 250 μg/ml Liberase TL (Roche) for 30 min at 37°C in a shaking incubator with the addition of 0.02 M EDTA (pH 7.3) for the final 5 min. MLNs were then washed and homogenized in HBSS and centrifuged at 400 × *g* for 5 min before being resuspended in fluorescence-activated cell sorter (FACS) buffer (0.5% bovine serum albumin [BSA], 0.05% sodium azide, 1× PBS). The left lobe of the lung was prepared similarly by dissection into small pieces in PBS containing 250 μg/ml Liberase and 80 U/ml DNase I (Sigma). Following digestion and homogenization, the cell suspension was treated with red cell lysis buffer, and cells were washed, counted, and stained for flow cytometry. Cells were stained with surface and lineage markers as follows: phycoerythrin (PE)-conjugated Siglec F (clone E50-2440; BD Pharmingen), PE-Cy7-conjugated F4/80 (clone BM8; eBioscience), allophycocyanin (APC)-conjugated CD11c (clone N418; eBioscience), fluorescein isothiocyanate (FITC)-conjugated MHC class II (clone M5/114.15.2; BioLegend), and Pacific Blue-conjugated CD11b (clone M1/70; Biolegend) antibodies. Lung samples were then fixed with 1× Foxp3 fixation buffer (eBioscience) before intracellular staining was performed with antibodies to murine Resistin (RELM-α) at 2 μg/ml (Peprotech) and 0.72 μg/ml biotinylated antibody to mouse chitinase 3-like 3/ECF-L (Ym-1; R&D) in Foxp3 permeabilization buffer before a secondary stain with 0.67 μg/ml Zenon rabbit IgG Alexa-Fluor 488 (Invitrogen) and 1/200 streptavidin-peridinin chlorophyll protein (PerCP) (Biolegend). Staining was compared to that for isotype controls of pooled naïve and infected samples.

Cheek blood cells were sensitized with 12 μg/ml recombinant murine IgE (Pharmingen) and then stained using a combination of 1/125 biotin anti-mouse IgE (Pharmingen) and 1/200 streptavidin-APC (Biolegend) with FITC-conjugated CD3 (clone 17A2; Biolegend), PE-Cy7-conjugated CD49b (clone DX5; eBioscience), PerCP-conjugated B220 (clone RA3-6B2; Biolegend), and PE-conjugated CD117 (c-kit) (clone ACK2; Biolegend) antibodies. Red blood cells were lysed using 1× BD FACS red cell lysing solution before acquisition.

For intracellular staining, 6×10^6 cells/well were plated in a 24-well plate with 0.5 μg/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin for 1 h before addition of 10 μg/ml brefeldin A, which was left for a further 3 h. Cells were then washed and blocked by resuspension in FACS buffer containing FcR block for 15 min. After washing, cells were incubated with 1/200 anti-CD8–FITC and anti-CD4–PerCP for 20 min, washed again, and then fixed for 20 min 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-gamma interferon (IFN-γ)–APC, anti-IL-4–PE, anti-IL-10–APC, anti-IL-13–APC, or the relevant isotype control for 20 min in Perm buffer. After another wash in Perm buffer, samples were resuspended in FACS buffer and analyzed by flow cytometry using a Becton Dickinson Canto or LSR-II flow cytometer.

For antigen-specific restimulation, 10^6 mesenteric or popliteal lymph node cells per well were plated in the presence of medium, 5 μg/ml NES, or 1 μg/ml HES for 72 h at 37°C with 5% CO_2 before centrifuging at 400 × *g* for 5 min and freezing the supernatants at −20°C, which were then analyzed for IFN-γ, IL-4, IL-5, IL-10, IL-13, and IL-17 by commercially available enzyme-linked immunosorbent assay (ELISA) (BD Pharmingen).

RT-PCR. Lung and intestinal tissues were prepared for reverse transcriptase PCR (RT-PCR) by immersion in TRIzol (Invitrogen), and RNA extraction was performed following the manufacturer's protocol, transcribing 1 μg RNA using Moloney murine leukemia virus (MMLV) RT (Stratagene). Ym-1, RELM-α, RELM-β, arginase-1, and IL-13 mRNA levels were measured by real-time PCR using a Roche Light Cycler real-time PCR machine (47). The hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was used as the reference gene. Light Cycler PCR amplifications were carried out in 10-μl mixtures containing 4 μl cDNA, 0.3 μM primers, and 2× Light Cycler-DNA SYBR green I mix (Roche). PCR was performed using the following conditions: 5 min of denaturation at 95°C, 10 s of annealing of primers at 60°C, and 20 s of elongation at 72°C, for 50 cycles. The fluorescent DNA binding dye SYBR green

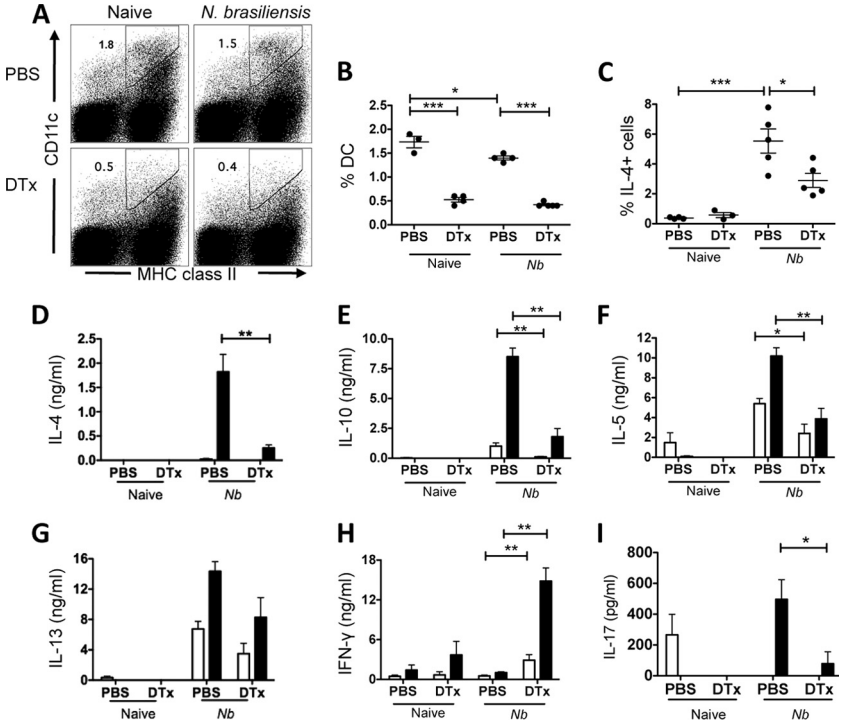

FIG 1 CD11c$^+$ cell depletion ablates adaptive Th2 responsiveness. CD11c.DOG mice were given 8 ng/g DTx (or PBS as a control) daily from day 1 to 6 of mouse-adapted *N. brasiliensis* (*Nb*) infection, and MLNC were harvested at day 7 postinfection. (A and B) DC depletion in the mesenteric lymph node (MLN) as staining for CD11c and MHC class II, shown as a representative bivariant plot scaled to 210,000 events (A) and a graphical summary of all data (B). (C) Intracellular IL-4 expression by MLN CD4$^+$ T cells in PBS- and DTx-treated uninfected and infected (*Nb*) mice. (D to I) Cytokine release by cultured MLNC stimulated with NES (black bars) or medium (open bars), in PBS- and DTx-treated uninfected and infected (*Nb*) mice, assayed for IL-4 (D), IL-10 (E), IL-5 (F), IL-13 (G), IFN-γ (H), and IL-17 (I). Data presented are means ± standard errors (SE) for LNC from 3 to 5 individual mice per group and are representative of two similar experiments. Statistically significant differences are shown as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

(Roche) was monitored after each cycle. Expression levels were estimated using the absolute quantitation method by comparison to a standard curve generated from a pool of all samples appropriately diluted. Relative expression of the gene of interest was then calculated as the ratio to expression of a housekeeping gene that remained unaltered after treatment.

Statistical analysis. Data were assessed for equal variance and log transformed if they did not fit this criterion. Statistical analyses used paired *t* tests or nonparametric Mann-Whitney U tests if the data were not normally distributed.

RESULTS

We have previously reported that depletion of CD11c-expressing cells in helminth-infected mice severely disrupts an adaptive Th2 response, as measured by IL-4 and IL-13 production by CD4$^+$ T cells, to infection with *S. mansoni* (45) or *H. polygyrus* (56). Because recent studies have identified a strong type 2 innate cell response (including for subsets such as AAMs, eosinophils, and innate lymphoid cells) in infections with *N. brasiliensis*, we first tested whether innate and adaptive type 2 responses were intact in DC-depleted mice infected with this parasite. Depletion, measured the day following final DTx administration, was >85% of total CD11c^{high} cells (Fig. 1A and B).

As expected from our previous data using *S. mansoni* and *H. polygyrus* infections, CD11c^{high} cell depletion significantly ablated the Th2 response to *N. brasiliensis*, as measured by polyclonal CD4$^+$ IL-4$^+$ cell percentages (Fig. 1C) as well as the antigen-specific IL-4 and IL-10 cytokine response from MLNC cultured *in vitro* with *N. brasiliensis* excretory-secretory antigen (NES) (Fig. 1D and E). IL-5 secretion by MLNC from infected mice *in vitro* could be observed at two levels: a constitutive response, which did not require the presence of NES antigen, and an antigen-dependent element. The latter was more severely diminished in mice following DC depletion (Fig. 1F). Interestingly, there was a trend, but not a significant one, for reduced production of antigen-specific IL-13 following depletion of CD11c$^+$ cells in *N. brasiliensis*-infected mice (Fig. 1G), while a sharp increase in the Th1 (IFN-γ) profile was observed following DC depletion (Fig. 1H), perhaps reflecting the loss of antigen-specific IL-10 production in the CD11c^{high} DC-depleted mice. While no significant change in Foxp3$^+$ Treg numbers is observed in *N. brasiliensis* infection (data not shown), we also found that the IL-17 recall response of MLNC to NES was significantly depressed in CD11c^{high} DC-depleted mice (Fig. 1I).

The ability of helminths to stimulate Th2 responses can be reproduced by certain helminth-derived products given in soluble form to naïve mice (11, 21, 59). Th2 responses can also be induced by adoptive transfer into naïve hosts of CD11c$^+$ bone marrow-derived DCs exposed to helminth molecules (5, 29), including NES (21). In view of recent reports that Th2 responses can be induced by basophils, even in the absence of DCs, we reevaluated the ability of NES-pulsed cells to drive Th2 differentiation *in vivo*. DCs and basophils were generated from murine bone marrow with GM-CSF or IL-3, respectively; basophils were flow sorted for CD49b$^+$ c-kit$^-$ Gr-1$^-$, and then both cell types were cultured overnight with medium or NES and adoptively transferred into

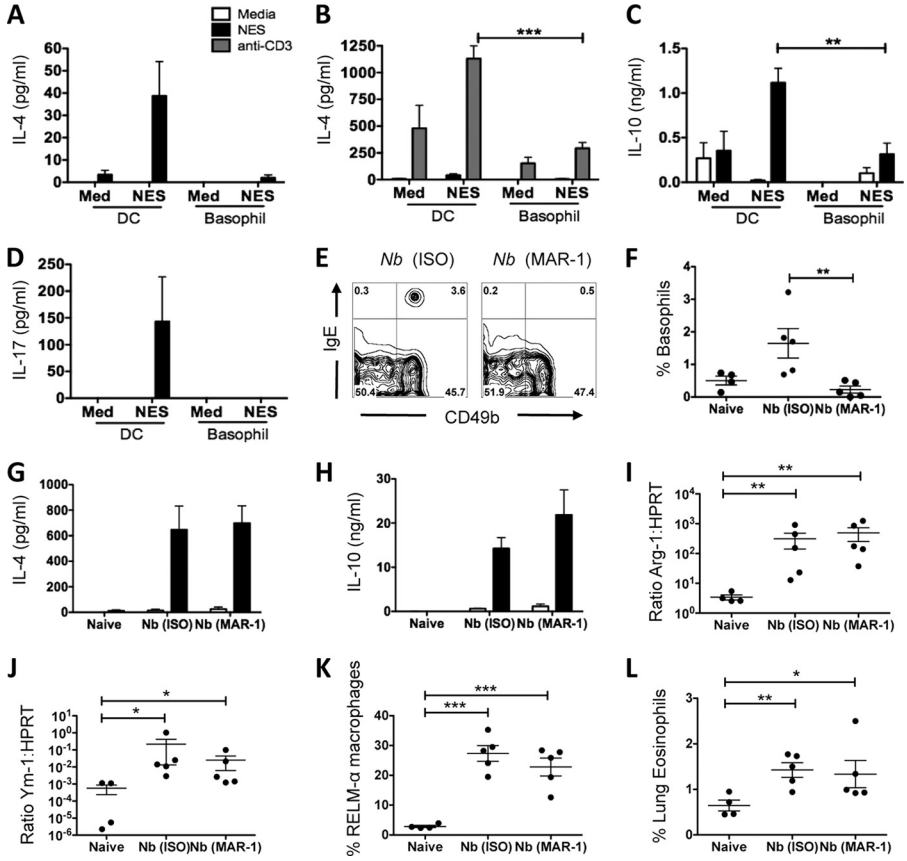

FIG 2 Basophils do not initiate or contribute to adaptive or innate type 2-associated parameters following primary *N. brasiliensis* infection. (A to D) Antigen-specific and polyclonal cytokine responses of popliteal LN at 5 days following transfer of bone marrow-derived DCs or basophils, pulsed with medium alone or with *N. brasiliensis* ES (NES) antigen. Cells were challenged *in vitro* with medium alone (open bars), 5 μg/ml NES (black bars), or 1 μg/ml anti-CD3 (gray bars) and supernatants collected after 72 h. (A) NES-specific IL-4 responses or medium-alone controls; (B) polyclonal IL-4 responses (NES responses are shown for comparison); (C) NES-specific IL-10 responses; (D) NES-specific IL-17 responses. (E and F) Depletion of peripheral blood $CD3^-$ $CD19^-$ IgE^+CD49b^+ c-kit^- basophils in naïve and 4-day *N. brasiliensis*-infected mice treated with isotype control or MAR-1 antibody. (G and H) NES-specific IL-4 and IL-10 production in response to medium (open bars) or NES (black bars) by MLNC from naïve, isotype-treated, and MAR-1-treated *N. brasiliensis*-infected (Nb) mice. (I and J) RT-PCR for arginase-1 and Ym-1 expression from lung homogenates from naïve, isotype-treated, and MAR-1-treated *N. brasiliensis*-infected (Nb) mice. (K) Intracellular RELM-α staining of $CD11b^+$ $F4/80^+$ $SiglecF^-$ AAMs from naïve, isotype-treated, and MAR-1-treated *N. brasiliensis*-infected (Nb) mice. (L) Eosinophil populations identified as $CD11b^+$ Siglec F^+ cells in the lungs of naïve, isotype-treated, and MAR-1-treated *N. brasiliensis*-infected (Nb) mice. For transfer experiments, data presented are means ± SE for LNC from 4 individual mice and are representative of two similar experiments. For infection experiments, MLNC and lungs were harvested at 7 days following infection. Data presented are means from 4 or 5 individual mice and are representative of two similar experiments. Statistically significant differences are shown as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

the hind feet of naïve mice. Five days following transfer, popliteal lymph node (PLN) cells were recovered and stimulated with NES antigen or anti-CD3 *in vitro*. While DC transfer induced strong antigen-specific and polyclonal Th2 responses in terms of IL-4 production, only low levels were observed following basophil transfer (Fig. 2A and B). Moreover, DCs induced strong antigen-specific release of IL-10 (Fig. 2C), which we have shown to be instrumental in establishing dominant Th2 responses in *N. brasiliensis* infection (6). NES-pulsed DCs also elicited low levels of antigen-specific IL-17 production (Fig. 2D), reflecting the underlying Th17 response evident during infection with this parasite. Hence, insofar as *in vitro*-differentiated DCs and basophils replicate the functions of their *in vivo* counterparts, transfer of antigen to the draining lymph node and induction of Th2 responses are accomplished primarily by DCs.

Following initiation, multiple cell types are involved in expanding and sustaining the type 2 response (2, 42). In particular, basophils can become sensitized by NES and release large quantities of IL-4 and IL-13 (40). Given the amounts of IL-4 released by polyclonal stimulation of recipient lymph node cells following transfer of NES-pulsed DCs (Fig. 2B), it is possible that host basophil activity could contribute to a similar function in response to primary helminth infection. We therefore assessed whether basophils promoted type 2 immunity later in the course of live infection in the contrasting model systems of *N. brasiliensis* (in which worm expulsion occurs within 6 to 10 days) and *H. polygyrus* (which establishes a long-lived chronic infection in susceptible mouse strains).

Following *N. brasiliensis* infection, basophilia is known to develop rapidly from day 3 following infection (33, 38, 54, 60, 65). Rather than use genetic models of basophil deficiency (39, 60), which may affect the baseline levels of innate populations, we chose to treat mice with the monoclonal antibody MAR-1, as described by other authors (10, 15, 24, 45, 57, 63), which eliminated >90% of circulating

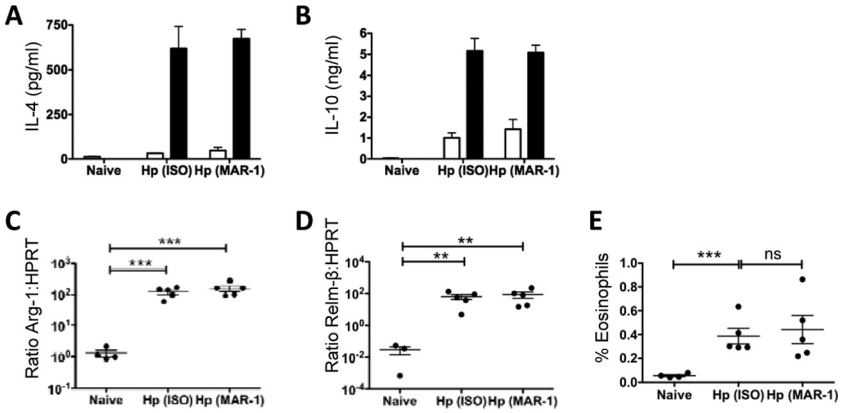

FIG 3 Basophil depletion does not diminish adaptive or innate type 2-associated parameters following *H. polygyrus* infection. (A and B) HES-specific IL-4 and IL-10 production in response to medium (open bars) or HES (black bars) by MLNC from naïve, isotype-treated, and MAR-1-treated *H. polygyrus*-infected (Hp) mice. (C and D) RT-PCR for arginase-1 and RELM-β expression in intestinal tissue from naïve, isotype-treated, and MAR-1-treated *H. polygyrus*-infected (Hp) mice. (E) Eosinophil populations in the mesenteric lymph nodes of isotype- and MAR-1-treated *H. polygyrus*-infected (Hp) mice. Mesenteric LNC and lungs were harvested at 7 days following infection. Data presented are means from 4 or 5 individual mice and are representative of two similar experiments. Statistically significant differences are shown as ** ($P < 0.01$) or *** ($P < 0.001$).

FcεR1$^+$ CD49b$^+$ basophils in blood (Fig. 2E and F). Consistent with previous reports, basophil-depleted *N. brasiliensis*-infected mice showed no loss of antigen-specific Th2 responsiveness, with strong IL-4, IL-10, and IL-13 production when MLNC were cultured *in vitro* with NES (Fig. 2G and H; see Fig. S1A and B in the supplemental material). In addition, the substantial generation and persistence of AAMs in the lung was unaffected by basophil depletion, with high levels of mRNA transcripts for arginase-1 (Fig. 2I) and Ym-1 (chitinase 3-like protein 3 [Chi3L3]) (Fig. 2J) in pulmonary tissue. In particular, we noted that following infection, some 20 to 30% of CD11b$^+$ CD11c$^-$ SiglecF$^-$ nonalveolar macrophages expressed the AAM product RELM-α by intracellular staining, which was not significantly reduced in the absence of basophils (Fig. 2K). Similarly, increased pulmonary (Fig. 2L) eosinophilia in *N. brasiliensis*-infected mice was observed irrespective of the basophil status of the host.

We next tested basophil depletion during infection with *H. polygyrus*, which normally survives for ≥10 weeks in C57BL/6 mice, generating a mixed Th2/Treg response (13, 31, 49). The results closely paralleled those observed in *N. brasiliensis* infection, with similar *in vivo* depletion (see Fig. S1C in the supplemental material) and indistinguishable IL-4, IL-10, and IL-13 responses to *H. polygyrus* ES (HES) challenge *in vitro* (Fig. 3A and B; see Fig. S1D in the supplemental material). The proportions of Th2 cytokine-synthesizing cells identified by intracellular staining were also unchanged in basophil-depleted mice (see Fig. S1E in the supplemental material). Because *H. polygyrus* remains in the gastrointestinal tract, AAM responses were measured in the intestine and showed marked elevation of arginase-1 (Fig. 3C) and RELM-α and Ym-1 (see Fig. S1F in the supplemental material) in both isotype- and MAR-1 antibody-treated mice. An important innate effector in immune defense against *H. polygyrus* is RELM-β, an IL-13- and STAT6-regulated product of mucosal epithelial cells (16, 34). RELM-β expression was found to be highly elevated in intestinal tissue in both basophil-depleted and control mice (Fig. 3D). Increases in eosinophilia in the draining lymph nodes following *H. polygyrus* infection were also unchanged following administration of MAR-1 antibody (Fig. 3E).

We then analyzed stimulation of innate immune cell populations following CD11c$^+$ cell depletion in *N. brasiliensis*-infected mice, given the high expression of this marker by dendritic cell and alveolar macrophage populations in the lung. Because *N. brasiliensis* traverses the lung when migrating from the skin site of infection to the small intestine, we first examined pulmonary cell suspensions and tested lung homogenates for quantitative PCR estimations of mRNA levels. Lung eosinophilia, which is known to be Th2 independent in this system (32, 52), was clearly undiminished in DTx-treated CD11c.DOG mice (Fig. 4A and B). In contrast, there was total loss of CD11c$^+$ alveolar macrophages, which express the highest levels of CD11c (Fig. 4A and C; see Fig. S2A in the supplemental material), as previously reported for an independently constructed CD11c.DOG transgenic mouse (66).

Despite the ablation of alveolar macrophages following DTx treatment, a population of CD11b$^+$ CD11c$^-$ SiglecF$^-$ macrophages expressing intermediate levels of F4/80 remained (Fig. 4D); this subset expresses high levels of RELM-α in *N. brasiliensis*-infected mice (Fig. 2G), even following depletion of CD11c$^+$ cells (Fig. 4E; see Fig. S2B and C in the supplemental material). Overall expression of the AAM genes for Ym-1 and RELM-α (Fig. 4F and G) and arginase-1 (see Fig. S2D in the supplemental material) increased in the lungs of infected mice irrespective of CD11c$^+$ cell depletion, showing that alveolar macrophages are not the only source of products of alternative activation during *N. brasiliensis* infection.

Similar evidence for unabated AAM development in the CD11c$^+$ cell-depleted setting was also obtained from the small intestine, in which comparable levels of Ym-1 were observed in both groups of infected mice (Fig. 5A). Moreover, this tissue showed similar expression of the epithelial cell product RELM-β associated with antihelminth immunity (16) in the presence or absence of CD11c$^+$ cells (Fig. 5B). In the MLNs, eosinophilia in response to infection was also unaltered by CD11c$^+$ cell depletion (Fig. 5C). Finally, we also investigated innate non-B, non-T cell sources of cytokines by gating on CD3$^-$ CD19$^-$ cells in infected and DTx-treated mice. We noted that while both IL-4$^+$ and IL-13$^+$ NBNT cell frequencies increased with *N. brasiliensis* infection, their numbers were not significantly reduced in CD11c$^+$

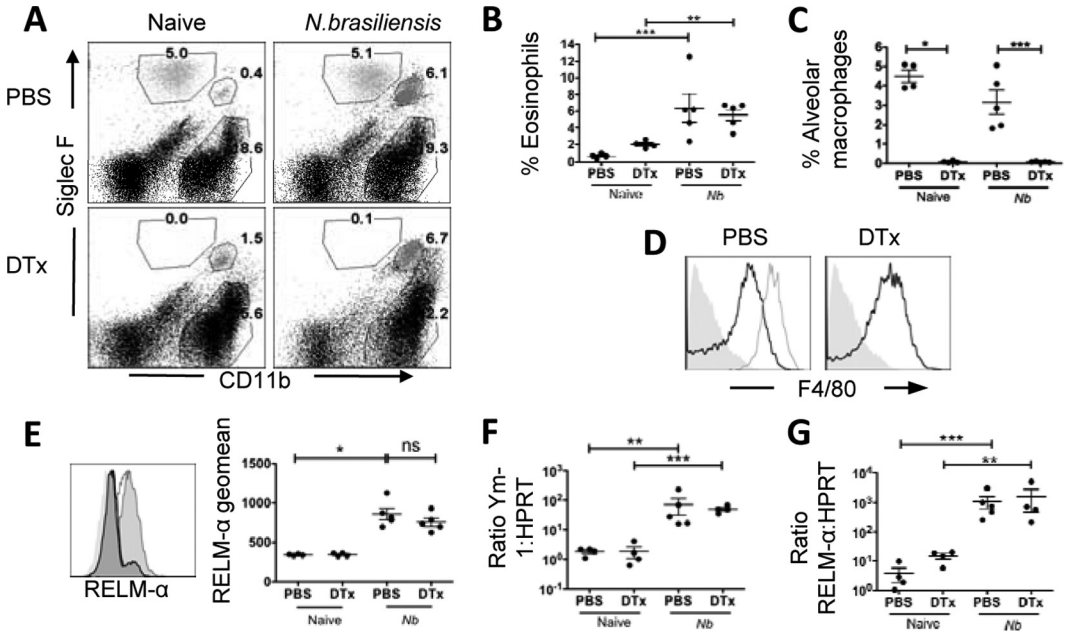

FIG 4 Depletion of CD11c$^+$ cells does not diminish type 2 innate responder populations. (A) Expression of Siglec F and CD11b by innate immune cells in lung homogenates from naïve and *N. brasiliensis*-infected CD11c.DOG mice given PBS or DTx. Alveolar macrophages (Siglec F$^+$ CD11b^{int} CD11c$^+$ F4/80$^+$) are shown as light gray, eosinophils (Siglec F^{+t} CD11b$^+$ CD11c^{in} F4/80^{int} SSC^{hi}) as dark gray, and nonalveolar macrophages (Siglec F$^-$ CD11b$^+$ F4/80$^+$) as black. A total of 20,000 events are shown for each analysis. (B and C) Lung eosinophils (B) and alveolar macrophages (C) in PBS- and DTx-treated uninfected and infected (*Nb*) mice. (D) F4/80 expression on alveolar (light gray line) and nonalveolar (black line) macrophages compared to an isotype control (filled light gray) in PBS- and DTx-treated *N. brasiliensis*-infected mice. (E) RELM-α expression by CD11b$^+$ Siglec F$^-$ F4/80$^+$ macrophages in PBS-treated naïve (filled dark gray histogram with black line) and *N. brasiliensis*-infected (filled light gray histogram with light gray line) mice along with DTx-treated groups (open histograms with corresponding lines) (left panel). The associated interstitial macrophage RELM-α staining intensity (right panel) is shown for all samples. (F and G) RT-PCR for Ym-1 (Chi3L3) (F) and RELM-α (G) mRNA expression in lung homogenates from PBS- and DTx-treated uninfected and infected (*Nb*) mice. Lungs were harvested at 7 days following infection. Data presented are means from 4 or 5 individual mice and are representative of two similar experiments. Statistically significant differences are shown as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

cell-depleted mice (Fig. 5D and E). Surface phenotyping revealed that the CD3$^-$ CD19$^-$ IL-13$^+$ cells were CD8$^-$, TCRβ$^-$, c-kit^{int}, CD90$^+$, T1/ST2$^+$, Sca-1$^+$ and CD4$^+$, similar to the case for innate helper cells or nuocytes (Fig. 5G).

DISCUSSION

Type 2 immunity integrates multiple components of both innate and adaptive natures, embracing both antigen-specific Th2 lymphocytes and a range of innate inducer and effector cell types (2, 42, 53, 68). Although a prominent question in recent years has been the nature of the innate cell type that drives Th2 differentiation (28, 42), a broader issue is how innate type 2 populations are themselves elicited. For adaptive T cell populations, Th2 induction can now be unequivocally attributed to the action of CD11c^{high} DCs, both because antigen-pulsed DCs are sufficient for Th2 induction and because DC depletion results in profound ablation of the adaptive Th2 response (15, 45). While an essential role for basophils in Th2 stimulation has been suggested (44, 58), our data and those of others (24, 45, 65) argue that this does not generally hold. Further, we are able to draw a clear distinction between DC-dependent induction of canonical Th2 cells and the responsiveness of innate type 2 populations, which require neither DCs nor basophils for their activation.

Our data identify three primary innate cell types, which are activated in the type 2 pathway whether or not the DC or basophil compartment is intact. Foremost among these are the AAMs, which arise rapidly and in great numbers following helminth infection (1, 26). We show that in both *N. brasiliensis* and *H. polygyrus* infections, and in both the lung and the intestinal tract, AAM expansion and maintenance dominate the macrophage compartment irrespective of CD11c$^+$ cell depletion. Likewise, although AAMs are activated through the IL-4R, they are unaffected by removal of IL-4-producing basophils from infected mice. Previously, it had been reported that alveolar macrophages express AAM markers within 48 h of *N. brasiliensis* infection (50) and that AAMs maintain their phenotype in a CD4$^+$ T cell-replete environment (27, 50). Interestingly, we show that the generation of lung AAMs occurs even when resident alveolar macrophages are efficiently deleted and that their numbers are undiminished even after 7 days in a DC- and Th2-depleted environment.

It is well established that AAMs can arise in lymphopenic environments such as RAG$^{-/-}$ and SCID mice, which have no adaptive immunity, and that they are prominent in the innate response to injury (27, 50). In general, adaptive Th2-derived IL-4/IL-13 is necessary to maintain the AAM population (27, 50), although eosinophil-derived IL-4 is sufficient for AAM induction in adipose tissue (69). Our studies indicate that innate IL-4, and particularly IL-13, is sufficiently robust in the absence of DCs to stimulate AAM development during helminth infection. One source of DC-independent IL-13 in infection may be the innate helper cell or nuocyte, which, interestingly, displays a CD4$^+$ phenotype in *N. brasiliensis* infection, implying that not all CD4$^+$ sources of type 2 cytokines are conventional T cells.

Our data indicate that tissue and lymph node eosinophilia is

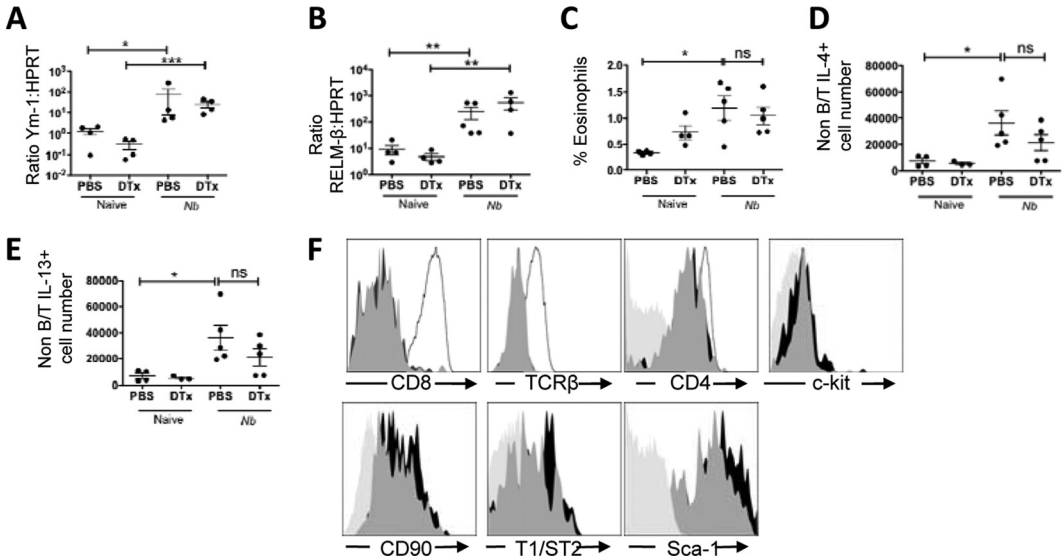

FIG 5 Innate type 2 responsiveness in the intestinal tract is not compromised by CD11c$^+$ cell depletion. (A and B) RT-PCR for Ym-1 (Chi3L3) (A) and RELM-β (B) mRNA expression in intestinal homogenates from PBS- and DTx-treated uninfected and infected (*Nb*) mice. (C) Eosinophilia (SSC^{high} cells) in the MLNs of PBS- and DTx-treated uninfected and infected (*Nb*) mice. (D and E) Intracellular IL-4 and IL-13 expression by CD3$^-$ CD19$^-$ NBNT cells in MLNs of PBS- and DTx-treated uninfected and infected (*Nb*) mice. (F) Surface phenotype of CD3$^-$ CD19$^-$ IL-13$^+$ NBNT cells in *N. brasiliensis*-infected mice given PBS (filled black) or DTx (filled dark gray) compared to a positive control (black line) or an isotype control (filled light gray). Data presented are means from 4 or 5 individual mice/group and are representative of two similar experiments; tissues from infected mice were harvested at day 7 postinfection. Statistically significant differences are shown as * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

also independent of basophils, supporting previous studies (39, 40, 60). As CD4$^+$ IL-5 responses were reduced by DC depletion, production of this cytokine by non-T cells may be sufficient to drive eosinophil development, as observed in earlier studies of T cell-deficient *nude* rodents infected with nematodes such as *Ascaris suum* (48) and *Toxocara canis* (62). Eosinophils are not required for Th2 generation (as shown in both *N. brasiliensis* [67] and *S. mansoni* [61]), although, as with basophils, their production of IL-4 can make a significant contribution to the pace and intensity of the Th2 response.

Despite the accumulating data showing that basophils are not necessary to raise the first alarm, and indeed are not well-equipped to do so due to their short life span (40) and absence of antigen presentation machinery (38), there are many examples in acquired helminth immunity and, more broadly, in allergy and IgG-mediated systemic anaphylaxis in which they play extremely important roles (22, 23). The recent construction of basophil reporter strains has suggested that this disparity may lie in the differential ability of basophils to interact with antigen-specific CD4$^+$ T cells in affected tissue but not in inflamed lymph nodes (23).

Reporter and lineage deletion strains offer elegant tools to analyze the contribution of key cell types to immunity, while the use of depleting antibodies allow experiments in a genetically unmanipulated host. While the MAR-1 antibody can raise neutrophil numbers (22) and partially deplete FCεR1$^+$ mast cell populations (35) depending on the route of administration, our data showing intact type 2 responsiveness are unlikely to be confounded by these effects. The long-term effects of MAR-1 administration and the individual contribution of mast cells to *H. polygyrus* fecundity, expulsion, and antibody formation are currently being explored. Similarly, while MAR-1 has also been reported to ablate FcεR1$^+$ inflammatory dendritic cells in lymph nodes draining the allergic airways (35), the unaltered Th2 response in MAR-1-treated mice suggests that inflammatory DCs do not contribute to Th2 induction in a number of helminth infection models.

The ability of helminths to stimulate Th2-driving pathways *in vivo* can be reproduced by pulsing DCs with helminth products *in vitro* (5, 29), as we have confirmed with NES. Interestingly, molecules from the strongly Th2-inducing *N. brasiliensis* and *S. mansoni* parasites show common effects such as suppression of Toll-like receptor (TLR)-stimulated IL-12p70 release (5, 8, 28). Importantly, in *H. polygyrus* infection, Th2 responsiveness is counterbalanced by a strong regulatory response and additional changes to DC function. Moreover, the DC subset which dominates in draining lymph nodes recovered from chronically infected mice can induce Foxp3$^+$ Tregs (56) and inhibit antibacterial immunity (9), while *H. polygyrus* ES (HES) treatment of DCs renders them less immunogenic when transferred into naïve hosts (55). Thus, while diverse helminth species show similar initiation of type 2 immunity, there are sharp contrasts in the subsequent course of infection as well as the functional phenotype of DC populations.

We have analyzed in this report the relative importance of two major innate immune populations in the initiation of type 2 immunity against helminth infection within both innate and adaptive settings. Our results conclusively show that the adaptive Th2 response in these systems is dependent upon CD11c^{high} DCs and that basophils are not required for this outcome. Our data also clearly highlight DC-independent pathways to mobilize innate type 2 immunity which operate autonomously during helminth infection. Such redundancy within the innate type 2 compartment may serve to safeguard the host from the range of helminth pathogens now known to interfere with normal DC function (12, 17, 56) and provide an alternative means to mobilize essential effector functions for protective immunity.

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