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MHC class II–dependent basophil–CD4+ T cell interactions promote T_H2 cytokine–dependent immunity

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Dendritic cells can prime naive CD4+ T cells; however, here we demonstrate that dendritic cell–mediated priming was insufficient for the development of T helper type 2 cell–dependent immunity. We identify basophils as a dominant cell population that coexpressed major histocompatibility complex class II and interleukin 4 message after helminth infection. Basophilia was promoted by thymic stromal lymphopoietin, and depletion of basophils impaired immunity to helminth infection. Basophils promoted antigen-specific CD4+ T cell proliferation and interleukin 4 production *in vitro*, and transfer of basophils augmented the population expansion of helminth-responsive CD4+ T cells *in vivo*. Collectively, our studies suggest that major histocompatibility complex class II–dependent interactions between basophils and CD4+ T cells promote T helper type 2 cytokine responses and immunity to helminth infection.

Since the demonstration of specification of CD4+ helper T cell fates[1], substantial advances have been made in delineating the regulatory mechanisms that promote distinct modules of CD4+ T cell–dependent immunity and inflammation[2]. The differentiation of T helper type 2 cells (T_H2 cells) is dependent on interleukin 4 receptor (IL-4R) and the transcription factors STAT6 and GATA-3, and their 'signature' cytokine profile is characterized by expression of IL-4 (A001262), IL-5, IL-9 and IL-13 (ref. 3). The hallmarks of T_H2 cytokine–dependent inflammation at barrier surfaces such as the skin, airway and intestine include the recruitment of CD4+ T_H2 cells, eosinophils, mast cells and basophils, coupled with goblet cell hyperplasia, mucus production and greater smooth muscle contractility[4]. Type 2 inflammatory responses are required for immunity and tissue repair after exposure to helminth parasites. However, T_H2 cytokine responses can also promote the pathological changes found in the context of asthma and allergic diseases[5].

Although the sequelae of type 2 immunity and inflammation in peripheral tissues are well characterized, the innate responses that promote T_H2 cell development, including the nature of the antigen-presenting cell (APC) involved, the host–microbial receptor–ligand interactions and the APC-derived factors needed to initiate and sustain T_H2 cell differentiation remain less well defined[6]. Dendritic cells (DCs) are the only APC thought to prime naive T cells, and the present paradigm suggests that recognition of conserved pathogen-associated molecular patterns through distinct pattern-recognition receptors expressed on DCs promote the appropriate pathogen-specific CD4+ helper T cell responses[7]. Activation of DCs can result in higher surface expression of major histocompatibility complex (MHC) class II and costimulatory molecules such as CD40, CD80 and CD86, as well as the expression of factors that can shape the nature of the developing adaptive immune response[8]. However, the critical DC-derived signals responsible for driving T_H2 cell responses *in vivo* remain undefined[9]. *In vitro* studies have indicated that the requirements for DC-mediated T_H2 differentiation include differences in expression of the Notch ligand Jagged[10] and upregulation of the costimulatory molecules CD40 (ref. 11) and OX40L[12]. However, whether these pathways are sufficient for DCs to promote the differentiation of CD4+ T_H2 cells *in vivo* is unclear.

The recruitment and activation of mast cells, eosinophils and basophils are hallmarks of T_H2 cytokine–dependent inflammation in peripheral tissues, and earlier studies have suggested that these granulocyte populations may function as accessory cells in the initiation of CD4+ T_H2 cell responses. For example, mast cells, eosinophils and basophils are able to produce and secrete IL-4 from intracellular stores, which indicates these populations are potential early sources of IL-4 that could promote the differentiation of CD4+ T_H2 cells[13–15]. In

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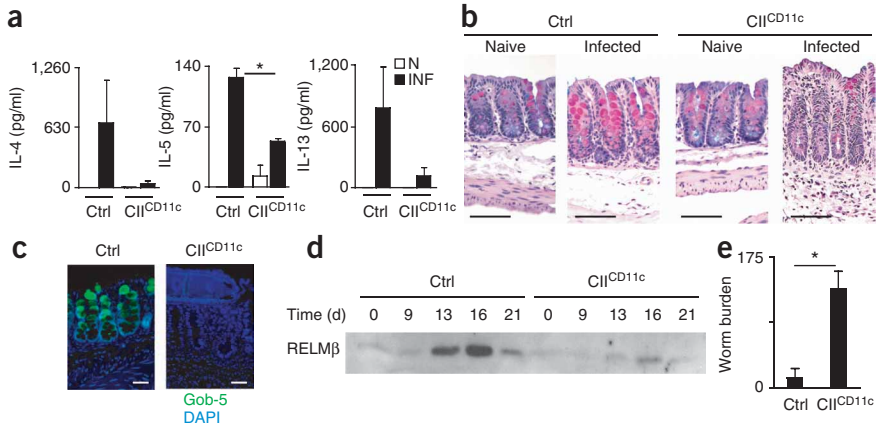

Figure 1 Restriction of MHC class II expression to CD11c$^+$ DC is insufficient to promote type 2 immunity to intestinal helminth infection. Analysis of littermate control mice (Ctrl) and MHC II^{CD11c} mice (CII^{CD11c}) on day 21 after no infection or infection with *T. muris* eggs. (**a**) Enzyme-linked immunosorbent assay (ELISA) of IL-4, IL-5, and IL-13 in supernatants of mLN cells obtained from naive (N) and infected (INF) mice and cultured *in vitro* for 48 h with anti-CD3 and anti-CD28. *$P = 0.003$ (Student's *t*-test). (**b**) Cecal sections from naive and infected mice, stained with Alcian blue–periodic acid Schiff reagent for the detection of mucins. Scale bars, 100 μm; original magnification ×20. (**c**) Cecal sections from infected mice, stained by immunofluorescence for the goblet cell marker Gob-5 (green) and with the DNA-intercalating dye DAPI (blue). Scale bars, 50 μm. (**d**) Immunoblot analysis of protein extracted from pooled fecal pellets of mice, collected on days 0–21 after infection (above lanes), to assess luminal secretion of RELMβ. (**e**) Cecal worm burdens in infected mice, assessed by microscopy. *$P = 0.0006$ (Student's *t*-test). Data are representative of three independent experiments with three to five mice per group (mean and s.e.m. in **a**,**e**).

addition, mast cells and eosinophils can express MHC class II, and eosinophils have been suggested to be potential APCs in both airway inflammation and helminth infection[16–18]. Basophil frequencies are higher after exposure to allergens and helminth parasites, and published work has demonstrated that basophils are a dominant source of IL-4 and IL-13 after helminth infection and contribute to protective immunity[19–21]. Although basophilia is a common feature of T_H2 cytokine–mediated inflammation, little is known about how these cells are activated and recruited to peripheral tissues. A conserved mechanism for basophil-mediated recognition of parasite products and allergens through protease-dependent activation has been proposed[22]. In that study, basophils were recruited to the draining lymph node early after allergen exposure and were essential for the generation of T_H2 cytokine responses elicited after papain immunization. However, the potential accessory cell functions of basophils during the development of CD4$^+$ T_H2 cells remain unknown. Collectively, the inability of DCs to express IL-4 and the lack of defined mechanisms by which DCs promote T_H2 cell differentiation provoked a reassessment of the relative contribution of DCs in promoting T_H2 cytokine responses *in vivo*.

In this study, we demonstrate that DC-restricted expression of MHC class II was insufficient for the generation of protective CD4$^+$ T_H2 cytokine–dependent immunity to the gastrointestinal helminth *Trichuris muris*. We identify basophils as a dominant accessory cell population that expressed *Il4* message and MHC class II. *In vitro* studies show that basophils could promote MHC class II–dependent antigen-specific proliferation of CD4$^+$ T cells and differentiation of T_H2 cells. Depletion of basophils *in vivo* resulted in impaired protective immunity to *T. muris*, whereas adoptive transfer of primary wild-type basophils augmented the proliferation of CD4$^+$ T cells in response to the injection of *Schistosoma mansoni* eggs. Our studies collectively suggest a previously unrecognized function for basophils in MHC class II–dependent cognate interactions with CD4$^+$ T cells that promote parasite-specific T_H2 cytokine responses and host protective immunity.

RESULTS

CD11c-restricted MHC II is insufficient for type 2 immunity

To determine whether antigen presentation by CD11c$^+$ DCs was sufficient to promote CD4$^+$ T_H2 cell–dependent immunity *in vivo*, we infected mice in which MHC class II expression is restricted to CD11c$^+$ cells (MHC II^{CD11c} mice; **Supplementary Fig. 1** online) with the intestinal helminth parasite *T. muris*. Expulsion of *T. muris* and protective immunity is dependent on CD4$^+$ T_H2 cells, whereas parasite-specific production of interferon-γ (IFN-γ) promotes chronic infection[23–25]. *T. muris* infection provides a well-characterized *in vivo* model of T_H2 cytokine–dependent immunity that offers a functional 'readout' of the magnitude of the host T_H2 cytokine response. As MHC II^{CD11c} mice lack MHC class II expression on the thymic epithelium[26] and therefore are unable to positively select CD4$^+$ T cells in the thymus, we gave mice fetal thymic grafts 8 weeks before infection to provide an endogenous CD4$^+$ T cell population. After infection with *T. muris*, littermate control mice developed pathogen-induced T_H2 cytokine responses characterized by production of IL-4, IL-5 and IL-13 by mesenteric lymph node (mLN) cells (**Fig. 1a**). In contrast, MHC II^{CD11c} mice showed minimal infection-induced production of T_H2 cytokines (**Fig. 1a**). Histological analysis of intestinal tissues in infected control mice showed hallmarks of type 2 inflammation, including goblet cell hyperplasia and mucin production (**Fig. 1b**) and expression of Gob5 (also known as mCLCA3; **Fig. 1c**), a goblet cell–specific marker regulated by T_H2 cytokines and associated with type 2 inflammation[27]. Consistent with lower production of T_H2 cytokines, infected MHC II^{CD11c} mice had a notable absence of goblet cells and goblet cell–derived proteins (**Fig. 1b**,**c**). T_H2 cytokine–dependent expression and luminal secretion of goblet cell–derived resistin-like molecule-β (RELMβ) in resistant mice provides a noninvasive indicator of the kinetics of T_H2 cytokine responses in the intestinal microenvironment[28]. As reported before[28], luminal RELMβ protein peaked in resistant control mice between days 12 and 18 after infection, coincident with worm expulsion (**Fig. 1d**), whereas luminal secretion of RELMβ in infected MHC II^{CD11c} mice was much lower in magnitude (**Fig. 1d**). Associated with a polarized T_H2 cytokine response, littermate control mice also had higher titers of immunoglobulin G1 (IgG1) and IgE (data not shown). However, as MHC II^{CD11c} mice lack MHC class II expression on B cells[26], we detected no antigen-specific class-switched antibody in infected MHC II^{CD11c} mice (data not shown). Critically, the defect in T_H2 cytokine responses in MHC II^{CD11c} mice resulted in susceptibility to infection in mice on a normally genetically resistant background (**Fig. 1e**). These data collectively demonstrate that restriction of MHC class II–dependent antigen presentation to CD11c$^+$ cells was insufficient to promote CD4$^+$ T_H2 cell–dependent immunity after intestinal helminth infection.

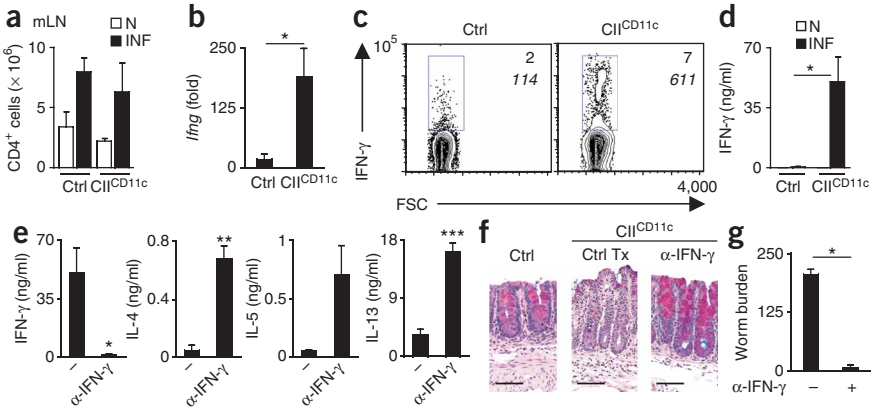

Figure 2 Blockade of IFN-γ in MHC II^{CD11c} mice restores T_H2 cytokine–dependent immunity to *T. muris* infection. (**a**) Total number of mLN CD4$^+$ T cells from naive and *T. muris*–infected littermate control and MHC II^{CD11c} mice, assessed by flow cytometry. Data are representative of three independent experiments with three to five mice per group (mean and s.e.m. of three mice per group). (**b**) Quantitative real-time PCR analysis of *Ifng* mRNA expression in mLNs from infected mice, presented as a 'fold increase' relative to expression in cells from naive control mice. $*P = 0.03$ (Student's *t*-test). Data are representative of three experiments (mean and s.e.m.). (**c**) Flow cytometry of intracellular IFN-γ staining of mLN cells from infected control and MHC II^{CD11c} mice, gated on CD4$^+$ T cells. Numbers adjacent to outlined areas indicate frequency of cells that produced IFN-γ (bold) or amount of IFN-γ per cell (mean fluorescence intensity; italics) for cells in the gated area. Data are representative of three independent experiments with three to five mice per group. (**d**) ELISA of IFN-γ secretion by mLN cells isolated from naive and infected mice. $*P = 0.03$ (Student's *t*-test). Data are representative of three experiments (mean and s.e.m.). (**e**) ELISA of cytokine production by mLN cells isolated from infected MHC II^{CD11c} mice given control treatment (–) or treatment with anti-IFN-γ (α-IFN-γ). $*P = 0.03$; $**P = 0.002$; and $***P = 0.001$ (Student's *t*-test). Data are representative of one experiment (mean and s.e.m. of three to four mice per group). (**f**) Cecal sections from infected littermate control mice or from infected MHC II^{CD11c} mice after control treatment (Ctrl Tx) or treatment with anti-IFN-γ, stained for mucins with Alcian blue–periodic acid Schiff. Scale bars, 100 μm. Results are representative of one experiment with three to four mice per group. (**g**) Worm burdens at day 21 after infection of MHC II^{CD11c} mice given control treatment (–) or treatment with anti-IFN-γ (+). $*P < 0.001$ (Student's *t*-test). Data are representative of one experiment (mean and s.e.m. of three to four mice per group).

Intact T_H1 differentiation in MHC II^{CD11c} mice

The extensive physical and biochemical barriers between antigenic material in the enteric space and lymphocytes in the underlying lymphoid follicles and lamina propria of the intestine create unique challenges in antigen sampling and presentation[29]. Therefore, the impaired T_H2 cytokine responses in *T. muris*–infected MHC II^{CD11c} mice may indicate that additional APCs are required for either the sampling of *T. muris* antigens or the provision of signals necessary for the priming, proliferation and differentiation of pathogen-specific CD4$^+$ T cells. However, after *T. muris* infection, both littermate control mice and MHC II^{CD11c} mice showed an infection-induced increase in total CD4$^+$ T cell numbers in the draining mLNs (**Fig. 2a**), which suggests that DC-restricted antigen presentation was sufficient for promoting the proliferation of CD4$^+$ T cells after infection. To determine whether CD4$^+$ T cells in infected MHC II^{CD11c} mice were unresponsive or had received signals for alternative differentiation, we isolated mRNA from mLNs of naive and infected control and MHC II^{CD11c} mice and analyzed the expression of *Il10*, *Il17*, and *Ifng* to assess the magnitude of regulatory T cell, IL-17-producing T helper cell and T_H1 cell responses. Although there was little to no induction of the expression of *Il10* and *Il17* in infected control and MHC II^{CD11c} mice (**Supplementary Fig. 2** online), *Ifng* mRNA was selectively and significantly induced in infected MHC II^{CD11c} mice relative to its expression in control mice (**Fig. 2b**). Consistent with higher *Ifng* mRNA expression, the frequency of mLN CD4$^+$ T cells that produced IFN-γ as well as the amount of IFN-γ made per cell were greater in infected MHC II^{CD11c} mice than in control mice (**Fig. 2c**). Secretion of IFN-γ was also significantly greater after *in vitro* stimulation of mLN cells isolated from infected MHC II^{CD11c} mice than in control mice (**Fig. 2d**). Thus, after intestinal infection, cognate interactions between antigen-specific CD4$^+$ T cells and CD11c$^+$ DCs alone were sufficient to promote the priming and population expansion of CD4$^+$ T cells as well as to provide signals necessary for T_H1 differentiation but were insufficient for the development of T_H2 cytokine–dependent immunity. These data suggested that CD11c$^+$ cells may not be required for T_H2 cytokine–dependent immunity. To determine the relative contribution of CD11c$^+$ cells in immunity to *T. muris*, we used the CD11c–diphtheria toxin receptor (CD11c-DTR) mouse model, in which delivery of diphtheria toxin to CD11c-DTR mice results in the selective apoptosis of CD11c$^+$ cells, whereas similar administration to littermate controls has no effect. To avoid the toxicity associated with long-term treatment of intact CD11c-DTR mice with diphtheria toxin, we used bone marrow chimeras created by the transfer of wild-type or CD11c-DTR donor bone marrow into wild-type recipients. Although transient depletion of CD11c$^+$ cells (**Supplementary Fig. 3a** online) throughout the course of *T. muris* infection resulted in significantly fewer mLN CD4$^+$ T cells ($P = 0.01$; **Supplementary Fig. 3b**), there was no effect on the production of T_H2 cytokines or worm burden (**Supplementary Fig. 3c,d**). Together these data suggest that CD11c$^+$ cells may not be essential for protective immunity to *T. muris* and that another APC may be required for the development of T_H2 cytokine–dependent immunity *in vivo*.

To determine whether alterations in the cytokine milieu could restore immunity in MHC II^{CD11c} mice, we treated *T. muris*–infected MHC II^{CD11c} mice with a monoclonal blocking antibody to IFN-γ (anti-IFN-γ) during the course of infection. Consistent with findings reported above (**Figs. 1a** and **2d**), stimulated T cells isolated from the mLNs of infected MHC II^{CD11c} mice showed a robust IFN-γ response with low concentrations of IL-4, IL-5 and IL-13 (**Fig. 2e**). Associated with the lack of T_H2 cytokines, infected, control-treated MHC II^{CD11c} mice showed lower goblet cell responses and susceptibility to *T. muris* infection (**Fig. 2f,g**). Treatment of MHC II^{CD11c} mice with anti-IFN-γ resulted in significantly lower IFN-γ production and the emergence of a T_H2 cytokine response characterized by significantly greater production of IL-4, IL-5 and IL-13 by mLN cells, goblet cell hyperplasia and recovery of immunity to infection (**Fig. 2e–g**). These data collectively suggest that after blockade of a nonprotective T_H1 cytokine response, CD11c$^+$ cells alone can provide the antigen-specific interactions needed to drive CD4$^+$ T_H2 cell differentiation and protective immunity. However, in the presence of endogenous IFN-γ signals, non-DC populations are required for the development of protective T_H2 cytokine responses *in vivo*.

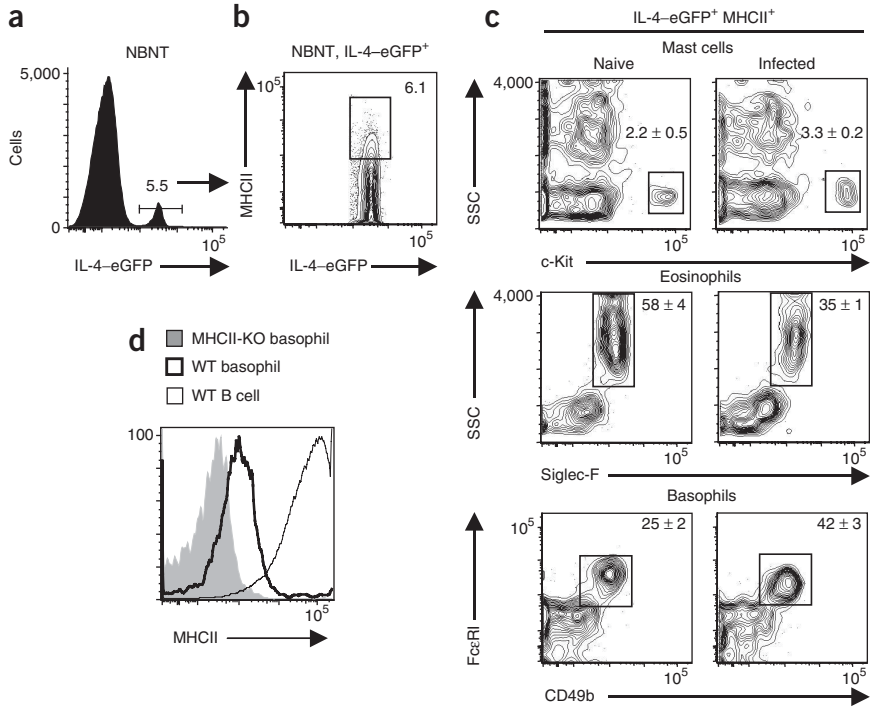

Figure 3 FcεRI$^+$CD49b$^+$ basophils coexpress MHC class II and IL-4–eGFP. Flow cytometry of splenocytes isolated from naive and *T. muris*–infected 4get mice at day 14 after infection. (**a**) Identification of IL-4–eGFP$^+$ cells with a non–B, non–T cell gate (CD3$^-$B220$^-$CD19$^-$; NBNT). (**b**) Analysis of the coexpression of MHC class II (MHCII) and IL-4–eGFP by the cells identified in **a**. (**c**) Expression of c-Kit, Siglec-F, FcεRI and CD49b by IL-4–eGFP$^+$, MHC class II–positive (MHCII$^+$) non–B, non–T cells. (**d**) MHC class II expression on MHC class II–deficient (MHCII-KO) basophils, wild-type (WT) basophils and wild-type B cells isolated from naive mice. Numbers above bracketed line (**a**) or adjacent to outlined areas (**b**,**c**) indicate percent cells in gate. Data are representative two experiments with three mice per group (mean ± s.e.m. in **c**).

Basophils express MHC class II and *Il4* mRNA

In addition to DCs, macrophages and B cells are 'professional' APCs involved in the development of adaptive CD4$^+$ T cell–dependent immunity. However, depletion of macrophages in mice by treatment with clodronate-loaded liposomes had no effect on cytokine-dependent inflammation or worm expulsion (**Supplementary Fig. 4a–c** online). Published work has demonstrated that adoptive transfer of CD4$^+$ T cells alone into mice lacking both B cells and T cells is sufficient to restore immunity to *T. muris*[30]. Mice deficient in B cells (μMT mice) also showed intact T_H2 cytokine–dependent goblet cell responses and protective immunity (**Supplementary Fig. 4d–f**). Collectively, these data suggest that although macrophages and B cells may contribute to immunity to *T. muris* in a physiological setting, they do not have essential nonredundant functions in host protective immunity. We therefore focused on identifying cells of the innate immune system that could both express MHC class II and provide an innate source of IL-4 after *T. muris* infection. IL-4–eGFP (4get) reporter mice have been used to track emerging CD4$^+$ T_H2 responses after *T. muris* infection[31]. These mice have an internal ribosomal entry site–enhanced green fluorescent protein (eGFP) element in the *Il4* locus that allows direct *ex vivo* analysis of cells able to express IL-4 (ref. 32). We used 4get mice to identify non–B, non–T cells that coexpressed *Il4* mRNA and MHC class II molecules. By gating on non–B, non–T cells, we identified an IL-4–eGFP$^+$ cell population (**Fig. 3a**) that expressed MHC class II (**Fig. 3b**). Published studies have shown that mast cells and eosinophils can express MHC class II (refs. 16–18) and are able to produce IL-4 (refs. 13,14). However, we did not find classical mast cells (c-Kit$^+$ side-scatter high (SSC^{hi})) after infection with *T. muris*, and frequencies of Siglec-F$^+$SSC^{hi} eosinophils were lower after infection (**Fig. 3c**). In contrast, CD49b$^+$ FcεRI$^+$ basophils emerged as a dominant cell type that expressed both *Il4* mRNA and MHC class II after *T. muris* infection (**Fig. 3c**), consistently comprising 40% of IL-4–eGFP$^+$ MHC class II–positive cells. Although their MHC class II expression was not as high as that in 'professional' APCs such as B cells, basophils had intermediate expression of MHC class II relative to that of MHC class II–deficient basophils (**Fig. 3d**). Although there have been reports of MHC class II expression on eosinophils[17,18], this is the first report to our knowledge demonstrating MHC class II expression on mouse basophils, and this result suggests a potential accessory cell function for this cell population during helminth infection.

Basophil depletion impairs immunity

To determine whether basophils are involved in the development of T_H2 cytokine–dependent protective immunity, we infected wild-type C57BL/6 mice with *T. muris* and treated them with either control imunoglobulin or a monoclonal antibody (MAR-1) to the receptor FcεRI. Studies have demonstrated efficient depletion of basophils for up to 10 d after intraperitoneal injection of MAR-1 (ref. 33), and in mice treated with MAR-1, we found 90% depletion of basophils at day 21 after infection (**Fig. 4a**). Depletion of basophils in infected mice resulted in lower expression of *Il4* mRNA (**Fig. 4b**), much less T_H2 cytokine–dependent goblet cell hyperplasia (**Fig. 4c**) and less luminal secretion of RELMβ in the intestine (**Fig. 4d**). Loss of basophils and impaired T_H2 cytokine responses were associated with impaired expulsion of *T. muris* (**Fig. 4e**). These data collectively support the idea of a function for basophils in the development of protective type 2 immunity to intestinal helminth infection.

Thymic stromal lymphopoietin selectively elicits basophils

Essential functions have been identified for the intestinal epithelial cell (IEC)-derived cytokines IL-25 (ref. 34) and thymic stromal lymphopoietin (TSLP; A002363)[27] in the development of T_H2 cytokine–dependent immunity to *T. muris*. In addition, IEC-derived IL-33 has been shown to promote T_H2 cytokine responses and worm expulsion[35], and several studies have demonstrated that treatment with IL-33 can directly stimulate cytokine and chemokine production from basophils and mast cells *in vitro*[36–38]. To determine whether IL-25, IL-33 or TSLP contributed to basophil responses *in vivo*, we injected 4get mice with recombinant IL-25, IL-33 or TSLP and examined peripheral basophil responses by flow cytometry. As reported before[39], IL-25 treatment elicited a population of IL-4–eGFP$^+$SSC^{hi} cells (**Fig. 5a**). Treatment with IL-33 also resulted in a higher frequency of IL-4–eGFP$^+$SSC^{hi} cells (**Fig. 5a**). However, phenotypic analysis of these IL-4–eGFP$^+$ cells showed that there were two distinct cell

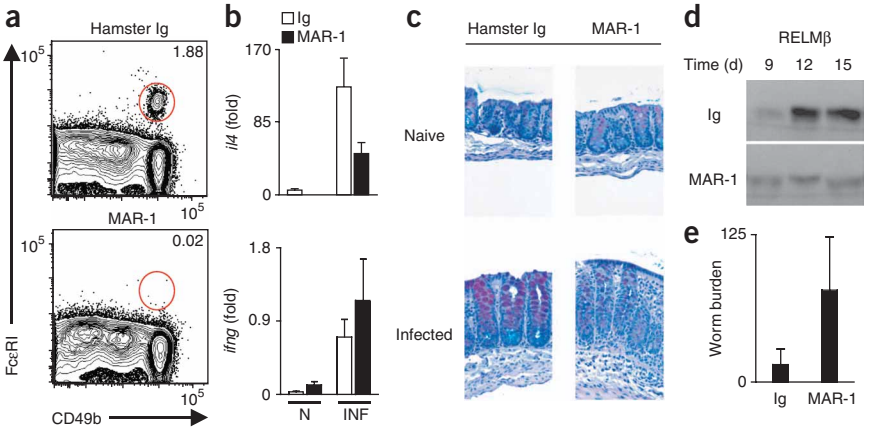

Figure 4 Depletion of FcεRI$^+$ cells *in vivo* results in impaired immunity to *T. muris* infection. (**a**) Flow cytometry of splenic basophils from wild-type mice treated with control immunoglobulin (Hamster Ig) or MAR-1 at day 21 after infection, gated on CD3$^-$B220$^-$CD19$^-$ non–B, non–T cells. Numbers adjacent to outlined areas indicate percent cells in each gate. (**b**) Real-time quantitative PCR analysis of colon tissue from naive and infected mice treated with control immunoglobulin (Ig) or MAR-1, assessed at day 21 after infection, presented as 'fold increase' relative to expression in naive mice treated with control immunoglobulin. (**c**) Cecal sections from naive and infected mice treated with control immunoglobulin (Hamster Ig) or MAR-1, assessed at day 21 after infection, stained for mucins with Alcian blue–periodic acid Schiff. Original magnification, ×20. (**d**) Immunoblot analysis of RELMβ in protein extracted on days 9, 12 and 15 after infection (above lanes) from pooled fecal pellets of mice treated with control immunoglobulin (Ig) or MAR-1. (**e**) Cecal worm burdens at day 21 after infection in mice treated with control immunoglobulin (Ig) or MAR-1. Data are representative of two independent experiments with three to four mice per group (error bars (**b**,**e**), s.e.m.).

populations selectively elicited by each cytokine. Treatment with IL-25 resulted in greater frequencies of a non–B, non–T cell c-Kit$^+$ mast cell–like population, whereas treatment with IL-33 led to a greater frequency of CCR3$^+$ eosinophils (**Supplementary Fig. 5** online). Although administration of TSLP also resulted in a threefold greater number of IL-4–eGFP$^+$ cells than did treatment with PBS (**Fig. 5a**), unlike IL-25 and IL-33, TSLP selectively elicited CD49b$^+$FcεRI$^+$ basophils (**Fig. 5b**). These data suggest that whereas IEC-derived IL-25, IL-33 and TSLP promote the expansion of diverse innate cell populations able to produce IL-4, only TSLP promotes basophil population expansion.

Basophils promote the differentiation of CD4$^+$ T_H2 cells

The demonstration that depletion of basophils resulted in impaired immunity to *T. muris* (**Fig. 4**), coupled with the coexpression of MHC class II and *Il4* mRNA (**Fig. 3**), suggested that they may also participate in MHC class II–dependent cognate interactions with CD4$^+$ T cells to promote T_H2 differentiation. To determine whether basophils could present antigen, we adopted an *in vitro* coculture system in which we activated antigen-pulsed purified basophils with recombinant IL-3 to provide survival signals and promote IL-4 production and cultured them together with purified ovalbumin (OVA)-specific DO11.10 CD4$^+$ T cells labeled with the cytosolic dye CFSE. Sorted basophils showed characteristic multilobed nuclei and expressed both MHC class II and IL-4–eGFP (**Fig. 6a**). Although we detected minimal proliferation in the absence of OVA peptide, approximately 75% of CD4$^+$ T cells cultured in the presence of antigen-pulsed basophils had diluted CFSE, consistent with proliferation (**Fig. 6b**). Basophil-induced proliferation of CD4$^+$ T cells was dependent on MHC class II expression, as the addition of a blocking antibody to MHC class II abrogated these responses (**Fig. 6b**). To determine whether basophils could influence the differentiation of CD4$^+$ T_H2 cells after antigen-specific stimulation of T cells, we did intracellular cytokine staining for IL-4 (**Fig. 6b**) and measured IL-4 in supernatants of cocultured cells (**Fig. 6c**). Supernatants of basophils and T cells cultured together in the absence of antigen contained basal amounts of IL-4 (**Fig. 6c**), and after the addition of OVA peptide, there was two- to threefold more IL-4, an enhancement that was abrogated in the presence of anti–MHC class II (**Fig. 6c**). Therefore, MHC class II–dependent cognate interactions between basophils and CD4$^+$ T cells can promote antigen-specific T_H2 differentiation *in vitro*.

S. mansoni eggs recruit basophils to lymph nodes

We sought to determine whether the recruitment of IL-4–eGFP$^+$ MHC class II–positive basophils was unique to *T. muris* infection or whether that was a common event after exposure to other helminth parasites. For this, we used footpad injection of *S. mansoni* eggs, which results in acute and synchronous T_H2 cytokine responses in the draining popliteal lymph node (pLN). Robust proliferation of CD4$^+$ T cells has been demonstrated after egg injection, with over 40% of pLN CD4$^+$ T cells becoming positive for the thymidine analog BrdU

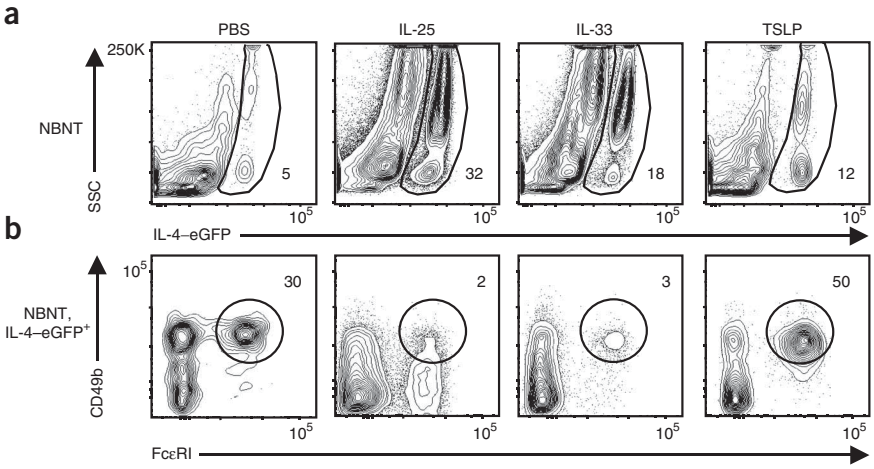

Figure 5 TSLP treatment selectively increases basophil frequencies *in vivo*. Flow cytometry of basophil frequencies in blood of mice treated daily for 4 d with recombinant IL-25, IL-33 or TSLP. (**a**) Expression of IL-4–eGFP by non–B, non–T cells from the peripheral blood. (**b**) Basophil frequencies in IL-4–eGFP$^+$ non–B, non–T cell populations. Numbers adjacent to outlined areas indicate percent among total cells of gated population. Data are representative of at least two independent experiments with three mice per group.

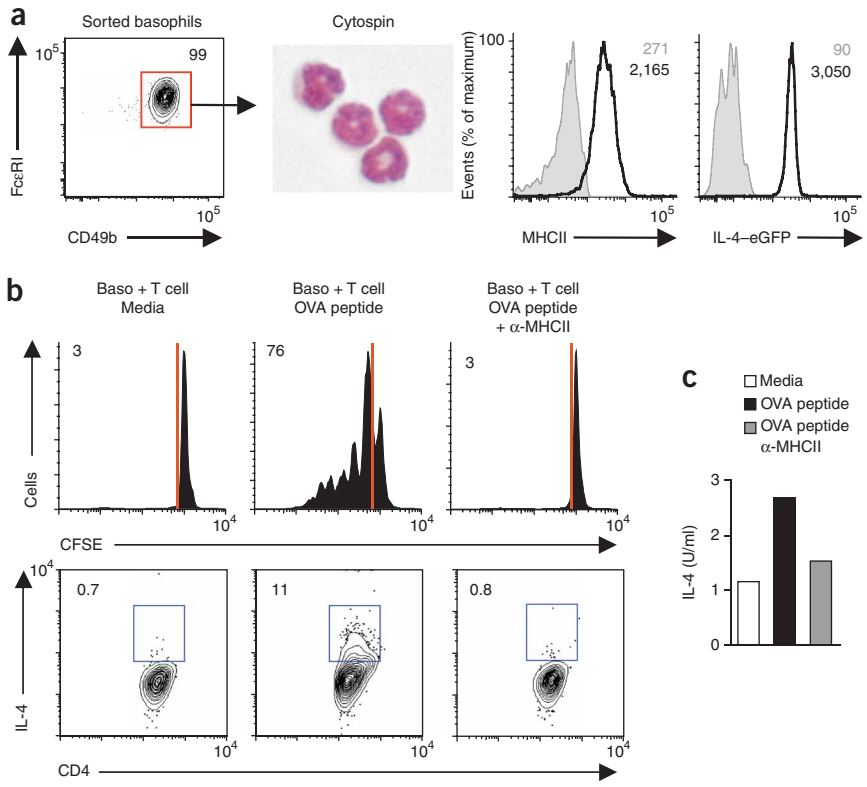

Figure 6 Basophils promote MHC class II–dependent antigen-specific CD4$^+$ T cell proliferation and T_H2 cytokine production *in vitro*. (**a**) Flow cytometry (left) and DiffQuick staining (middle) of sorted TSLP-elicited basophils; number adjacent to outlined area (left) indicates percent FcεRI$^+$CD49b$^+$ cells. Right, expression of MHC class II and IL-4–eGFP (solid lines) on sorted TSLP-elicited basophils; shaded histograms, fluorescence minus one (FMO) controls. Numbers in plots indicate mean fluorescence intensity. (**b**) CFSE dilution (top) by DO11.10 CD4$^+$ T cells after 4 d of culture together with basophils (Baso) in media alone or with OVA peptide or OVA peptide plus MHC class II–blocking antibody M5114 (α-MHCII). Below, frequency of IL-4$^+$CD4$^+$ T cells (numbers adjacent to outlined areas), assessed by intracellular cytokine staining. Numbers in plots indicate percent CFSE^{lo} cells (divided cells, left of orange vertical line, top) or percent IL-4$^+$CD4$^+$ cells (below). (**c**) ELISA of IL-4 in supernatants of basophil–CD4$^+$ T cell cocultures treated as described in **b**. Data are representative of four independent experiments with results of five to ten mice pooled per experiment (**a**) or are representative of two independent experiments (**b**,**c**).

and 20% expressing IL-4–eGFP[40], which provides a powerful *in vivo* model for examining helminth-induced innate and adaptive responses. We delivered *S. mansoni* eggs into the footpads of 4get mice and collected pLNs at various times after injection. Transient recruitment of basophils to the draining pLN occurred by day 2 after injection, with an over 20-fold higher frequency (**Fig. 7a**) and number (**Fig. 7b**) that was absent by day 5 (data not shown). Sorted IL-4–eGFP$^+$ basophils from mice injected with *S. mansoni* eggs showed characteristic multilobular nuclei by cytospin analysis (**Fig. 7c**), were FcεRI$^+$ and expressed MHC class II, as assessed by both flow cytometry (**Fig. 7d**) and immunofluorescence (**Fig. 7e**). We next investigated whether helminth-elicited basophils could influence the proliferation of CD4$^+$ T cells *in vivo*. To address this, we adoptively transferred CFSE-labeled CD4$^+$ T cells into MHC II^{CD11c} mice. We then used the *S. mansoni* egg–injection model to assess whether adoptive transfer of basophils influenced helminth-induced proliferation of CD4$^+$ T cells in the draining pLN. We sorted wild-type basophils from mice injected with *S. mansoni* eggs and adoptively transferred the cells into naive MHC II^{CD11c} recipients. In the absence of additional antigen stimulation in recipient mice, transfer of

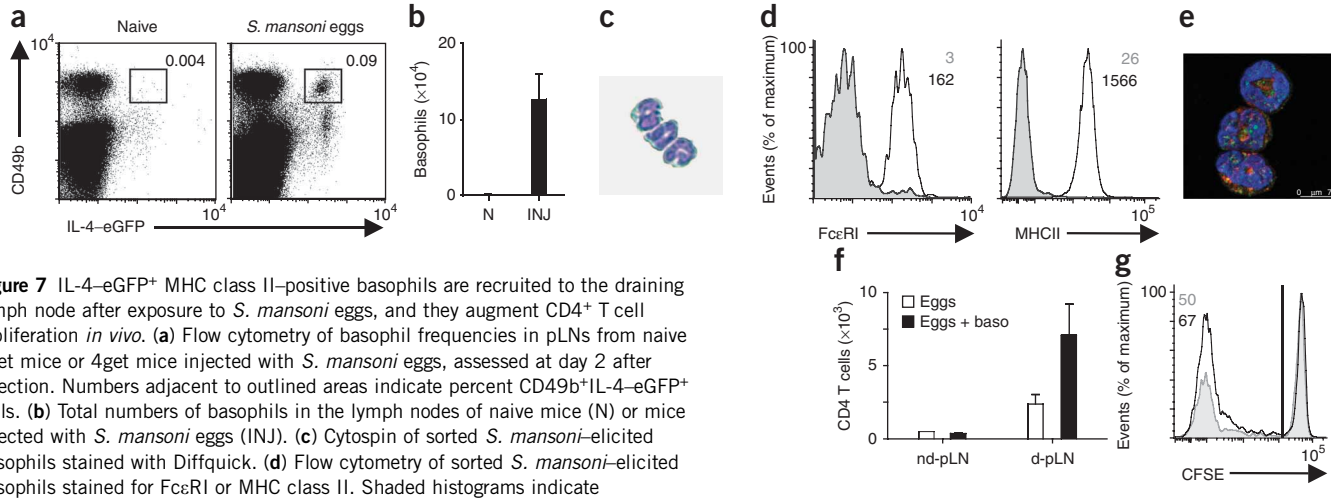

Figure 7 IL-4–eGFP$^+$ MHC class II–positive basophils are recruited to the draining lymph node after exposure to *S. mansoni* eggs, and they augment CD4$^+$ T cell proliferation *in vivo*. (**a**) Flow cytometry of basophil frequencies in pLNs from naive 4get mice or 4get mice injected with *S. mansoni* eggs, assessed at day 2 after injection. Numbers adjacent to outlined areas indicate percent CD49b$^+$IL-4–eGFP$^+$ cells. (**b**) Total numbers of basophils in the lymph nodes of naive mice (N) or mice injected with *S. mansoni* eggs (INJ). (**c**) Cytospin of sorted *S. mansoni*–elicited basophils stained with Diffquick. (**d**) Flow cytometry of sorted *S. mansoni*–elicited basophils stained for FcεRI or MHC class II. Shaded histograms indicate expression on CD3$^+$ cells; black lines are sorted basophils. Numbers in plots indicate mean fluorescence intensity. (**e**) Confocal microscopy of sorted *S. mansoni*–elicited basophils. Green, GFP; red, MHC class II; blue, DAPI. Original magnification, ×100. (**f**) Total CD4$^+$ cells in the nondraining pLN (nd-pLN) and draining pLN (d-pLN) of MHC CII^{CD11c} mice that received *S. mansoni* eggs alone (Eggs) or in combination with basophils (Eggs + baso). (**g**) Flow cytometry of CFSE dilution by CD4$^+$ T cells from MHC CII^{CD11c} mice that received *S. mansoni* eggs alone (shaded histogram) or with basophils (black line). Numbers in plots indicate percent CFSE^{lo} cells. Data are representative of five independent experiments with three to five mice per group (**a**–**e**) or one experiment with two mice per group (**f**,**g**; error bars (**b**,**f**), s.e.m.).

basophils alone did not induce the recruitment of antigen-specific T cells to the pLN (data not shown). We therefore challenged MHC II^{CD11c} mice that had received both T cells and basophils with *S. mansoni* eggs in the footpad. After egg injection, MHC II^{CD11c} mice that received eggs alone had four- to fivefold more CD4$^+$ T cells in the draining pLN than in the nondraining pLN (**Fig. 7f**), and 50% of pLN CD4$^+$ T cells were CFSE^{lo} (**Fig. 7g**). In contrast, proliferation of CD4$^+$ T cells was substantially augmented in MHC II^{CD11c} mice that had received activated basophils. At day 4 after delivery of *S. mansoni* eggs, there were over 14-fold more pLN CD4$^+$ T cells (**Fig. 7f**), and nearly 70% of the CD4$^+$ T cells were CFSE^{lo} (**Fig. 7g**). This proliferation was consistent with the magnitude of CD4$^+$ T cell responses reported before in egg-injected wild-type mice[40]. In addition, published studies have shown that unlike T cells adoptively transferred into recipient mice deficient in recombination-activating gene 1 or 2, donor CD4$^+$ T cells delivered into MHC II^{CD11c} mice do not undergo homeostatic proliferation, probably because MHC II^{CD11c} mice have a normal CD8$^+$ T cell compartment[26,41]. Collectively, these data demonstrate that MHC class II–positive basophils are rapidly recruited to the lymph node after exposure to helminth antigens and suggest potential 'cooperation' between basophils and DCs in the efficient population expansion of helminth-responsive CD4$^+$ T cells *in vivo*.

DISCUSSION

Basophils are rare circulating cells that make up less than 0.5% of total blood leukocytes yet are evolutionarily conserved across all vertebrate species and can accumulate in peripheral tissues in many settings associated with type 2 inflammation. Although basophils were first described 130 years ago[42], their scarcity, coupled with a paucity of reagents, has made it difficult to study their function *in vivo*. The availability of new reagents has allowed the identification of distinct nonredundant functions for basophils in augmenting CD4$^+$ T_H2 cytokine responses[22,43], in providing B cell help for IgE class-switch recombination and enhanced humoral immune responses[33,44], and in the initiation and maintenance of chronic allergic inflammation[45]. To those functions, the results of our study here add a previously unrecognized function for basophils as accessory cells that can promote the differentiation of CD4$^+$ T_H2 cells in part through MHC class II–dependent cognate interactions, as indicated by basophil–CD4$^+$ T cell coculture experiments. A critical question that emerges from these findings is where the functional basophil–T cell cognate interactions occur *in vivo*. Basophils are readily found in the blood and spleen but have been reported to be excluded from lymph nodes, where CD4$^+$ T cell priming probably takes place[46]. However, basophils have been shown to be transiently recruited to draining lymph nodes after allergen exposure[22]. In this study, we have demonstrated that basophils were rapidly recruited to the lymph node after exposure to *S. mansoni* eggs. Basophils that accumulated in lymph nodes expressed both MHC class II and *Il4* mRNA, which suggests that they are able to interact directly with naive T cells in peripheral lymphoid tissues. Consistent with involvement of basophils in the development of T_H2 cell responses, *in vivo* depletion of basophils resulted in impaired expression of T_H2 cytokines and host protective immunity, whereas adoptive transfer of basophils augmented helminth-induced proliferation of CD4$^+$ T cells.

In addition to their potential involvement in the initial priming of naive CD4$^+$ T cells in the lymph node, basophils may act as accessory cells at the site of inflammation, where activated T cells may require additional cognate interactions to promote or maintain T_H2 cell differentiation and effector function. In support of that idea, a published report of cytokine reporter mice has demonstrated that cytokine mRNA expression and cytokine protein expression are uncoupled after the priming and population expansion of naive T cells, which suggests that additional activation at the site of infection may be needed to 'license' effector function[47]. Depletion of basophils also suggests that these cells may provide chemotactic factors, either directly or indirectly, that are required for the recruitment of eosinophils to peripheral tissues[48]. Microarray analyses of basophils sorted from the lung during infection with *Nippostrongylus brasiliensis* has also shown high expression of the chemokines CCL3 (MIP1α), CCL4, (MIP1β), CCL6 (C10) and CCL17 (TARC), which supports the idea that basophils are involved in the recruitment of activated CD4$^+$ T cells to the site of infection[21]. Therefore, identifying the factors that regulate basophil proliferation and recruitment could be an important target for modulating early events in the generation of CD4$^+$ T_H2 cell–dependent immunity and inflammation.

Published reports have identified T cell–derived IL-3 as a critical cytokine for basophilia during intestinal helminth infection[49]. However, whether other cytokines derived from cells of the innate immune system contribute to early basophil responses is unclear. A critical function has been identified for IEC activation in the generation of protective T_H2 cytokine–dependent immunity to *T. muris*, and TSLP has been shown to be an important part of the IEC response required for immunity to infection[27,50]. Here we have shown that delivery of recombinant TSLP resulted in the selective accumulation of basophils in the periphery, which identifies a previously unappreciated function for TSLP in promoting basophilia. TSLP has been linked before to the promotion of type 2 inflammation in the skin and lung through effects on cells of both the innate and adaptive immune systems[51,52]. Although there was no analysis of basophil responses in the TSLP-transgenic mice used in those earlier studies, it is plausible that a component of the enhanced type 2 inflammation observed could have been a consequence of enhanced basophil responses.

In addition to TSLP, the IEC-derived cytokines IL-25 and IL-33 have also been linked to the promotion of type 2 inflammation, and IL-33 can directly activate basophils[34,36–38,53–56]. However, we have shown that these cytokines, although they were able to promote the accumulation of IL-4–eGFP$^+$ innate cells, did not promote basophilia *in vivo*. Instead, IL-25 promoted the proliferation and/or accumulation of c-Kit$^+$ cells, whereas IL-33 promoted peripheral eosinophilia. Although TSLP seems to have a selective effect on basophil responses, the influence of TSLP, IL-25 and IL-33 in combination with other stimuli, such as IL-3, IL-18, Toll-like receptor ligation and FcεRI crosslinking, on basophil cytokine production, recruitment to the lymph nodes and APC function remains to be determined.

In addition to IEC-derived cytokines, there is evidence to suggest that basophils can be directly activated by either helminth-derived products or allergens that may act as 'superallergens' to stimulate FcεRI crosslinking in a non–antigen-specific way[57]. For example, IPSEα1, a glycoprotein derived from *S. mansoni* eggs, has been shown to directly stimulate the production of IL-4 by basophils via an IgE-dependent but non–antigen-specific mechanism[58]. Thus, a combination of cytokines derived from nonhematopoietic cells and cells of the innate immune system, coupled with direct stimulation by helminth products or allergens, may act together to elicit basophil proliferation and activation *in vivo*.

In addition to a function for basophils in the MHC class II–dependent promotion of T_H2 differentiation and immunity to *T. muris* infection, a critical function for basophils has been found in the development of allergen-specific T_H2 cytokine responses. In those studies, allergen-stimulated basophils expressed MHC class II, the transcriptional coactivator CIITA, the invariant chain and costimulatory molecules and promoted allergen-specific CD4$^+$ T_H2 cell

differentiation (R. Medzhitov, personal communication). These findings collectively indicate that basophil-mediated recognition of allergens and helminth-derived products, coupled with their MHC class II–dependent promotion of T_H2 cell responses, may be an evolutionarily conserved pathway that serves a cardinal function in the development of type 2 inflammation at mucosal sites.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001262 and A002363.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/natureimmunology/.

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ONLINE METHODS

Mice and parasites. C57BL/6 mice and 'timed pregnant' female B6.SJL mice 6–8 weeks of age were from The Jackson Laboratories. MHC II^{CD11c} mice (also known as CD11c-Aβ^{b} mice; generated as described[26]), MHC class II–deficient (*H2-Ab1*$^{-/-}$) mice, 4get (IL-4eGFP; C.129-*IL4*^{tm1Lky}/J) mice (from M. Mohrs), B cell–deficient (μMT) mice, CD11c-DTR mice and DO11.10 mice were bred and housed in specific pathogen–free conditions at the University of Pennsylvania. Littermate control mice were 'sham grafted' and MHC II^{CD11c} mice were given subcutaneous thymic grafts from neonatal (0–2 d of age) B6.SJL mice at 4–6 weeks of age and then were allowed 8 weeks to reconstitute CD4^{+} T cells before experimental use. Bone marrow chimeras were generated by intravenous injection of 5 × 10^{6} bone marrow cells from wild-type or CD11c-DTR mice into irradiated (500 rads, twice) wild-type recipients. Recipient mice were given antibiotics for 2 weeks and were allowed 8 weeks to reconstitute. All experiments were accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania. *T. muris* was maintained in genetically susceptible mouse strains and eggs were collected as described[31]; mice were infected by oral gavage with 200–300 embryonated *T. muris* eggs. *S. mansoni* eggs were prepared as described[40]; mice were injected in the footpad with 2,500 eggs in 50 μl PBS.

Polyclonal T cell stimulation. Single-cell suspensions of mLNs were prepared in complete medium (DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 25 mM HEPES, pH 7.4, and 50 μM β-mercaptoethanol). The mLN cells were seeded in 48-well plates at a density of 2.5 × 10^{6} per well and were incubated for 48 h with media or 1 μg/ml of soluble anti-CD3 (145-2C11) and anti-CD28 (37-51) eBioscience). Cell-free supernatants were collected and cytokine production was determined by sandwich ELISA (all antibody pairs from eBioscience: AN-18 and R4-6A2 (anti-IFN-γ); 11B11 and BVD6-24G2 (anti-IL-4); TRFK5 and TRFK4 (anti-IL-5); and eBio13A and eBio 1316H (anti-IL-13)).

Immunoblot. Fecal protein was isolated as described[28]; 30 μg protein per sample was separated by SDS-PAGE and analyzed by immunoblot for RELMβ with polyclonal rabbit anti–mouse RELMβ (Peprotech).

Real-time PCR. RNA was isolated from intestinal tissues of mice by TRizol extraction (Invitrogen) and from mLN cells with RNEasy Spin Columns (Qiagen). Tissues were disrupted with a tissue homogenizer (TissueLyzer; Qiagen) and cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). Quantitative real-time PCR analysis of cDNA samples used commercial primer sets (*Il4*, QT00160678; *Ifng*, QT01038821; *Il10*, QT00106169; and *Il17a*, QT00103278; Qiagen) and SYBR Green. All reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Samples were normalized to naive controls unless otherwise stated.

Histology and immunofluorescence. Cecal tips were fixed in 4% (vol/vol) paraformaldehyde and were embedded in paraffin. Sections 5 μm in thickness were cut and were stained with hematoxylin and eosin or Alcian blue–periodic acid Schiff. Unstained sections prepared on immunoslides were stained for Gob5 by immunofluorescence as described[27]. Samples were deparaffinized by consecutive rinses in methanol and ethanol, then were boiled in citric acid buffer and stained overnight at 4 °C with anti-Gob5, followed by staining with carbocyanine-conjugated anti-goat (705-225-003; Jackson Immunoresearch). Sorted basophils were subjected to cytospin and were fixed overnight at 4 °C in 2% (vol/vol) paraformaldehyde. Slides were washed in PBS, then cells were made permeable in Triton-X, blocked with streptavidin and biotin and stained overnight at 4 °C with biotin–anti-MHC class II (M5114) and anti-GFP (14-6774-81; eBioscience). Slides were then washed with PBS and stained for 2 h at 25 °C with streptavidin-indocarbocyanine and carbocyanine-conjugated donkey anti-rabbit (711-165-152; Jackson Immunoresearch), then were washed again with PBS and nuclei were stained with DAPI (4,6-diamidino-2-phenylindole).

Neutralizing and depleting antibodies and recombinant cytokines. Neutralizing monoclonal anti-IFN-γ (XMG-6) was purified from ascites fluid (grown by Harlan Bioscience) by ammonium sulfate precipitation and was dialyzed against PBS. Mice were given 1 mg antibody intraperitoneally every 3–5 d during the course of infection, starting at day 0. Mice were depleted of basophils by intraperitoneal injection of 10 μg anti-FcεRI (MAR-1; eBioscience) on days 0, 1, 2 and days 10, 11, 12 after infection. Recombinant mouse IL-25 (4 μg/ml), IL-33 (20 μg/ml) and TSLP (0.1 mg/ml; all from R&D Systems) and 100 μl PBS were injected intraperitoneally once daily for 4 d.

Basophil isolation and CD4^{+} T cell coculture. CD4^{+} T cells were isolated from spleens by negative selection by incubation with hybridoma supernatants (anti-B220 (RAE), anti-Fcr (24G2), anti-CD8 (2.43) and anti–MHC class II (M5114); prepared 'in-house') followed by magnetic bead purification (Qiagen). To obtain purified basophils, blood, spleen and mLN cells were isolated from 4get mice injected intraperitoneally with 10 μg recombinant TSLP (R&D Systems) once daily for 4 d for enrichment of basophils, were positively selected for CD49b expression by purification on a MACS column (Milltenyi) and were stained with fluorochrome-conjugated monoclonal anti-B220 (RA3-6B2), anti-CD3ε (145-2C11), anti-c-Kit (2B8), anti-CD49b (HMα2) and anti-FcεRI (MAR-1; all from BD Biosciences and eBioscience). Basophils were sorted with a FACSAria (BD Bioscience) on the basis of negative staining for B220, CD3 and c-Kit, positive staining for CD49b and FcεRI, and expression of IL-4–eGFP. After purification, sorted basophils were resuspended at a density of 1 × 10^{5} cells per ml in complete medium; 100 μl of the basophils were used for cytospin and were stained by Diffquick for confirmation of cellular morphology. Between 5 × 10^{3} and 1 × 10^{4} basophils were cultured together with 2 × 10^{5} purified, CFSE (carboxyfluorescein diacetate succinimidyl ester)–labeled DO11.10 CD4^{+} T cells with recombinant IL-3 (10 ng/ml R&D Systems) in the presence or absence of OVA peptide (1 μg/ml) and blocking antibody to MHC class II (5 μg/ml; M5/114; eBioscience). After 4 d of culture, cells were stimulated for 4 h with phorbol 12-myristate 13-acetate, ionomycin and brefeldin A. Cells were pelleted for 5 min at 485*g*. Supernatants were collected for ELISA and cells were washed in flow cytometry buffer, incubated for 10 min at 4 °C with Fc Block (2.4G2 and rat IgG), stained with fluorochrome-conjugated monoclonal anti-CD4 (RM4-5; eBioscience) and fixed with 2% (vol/vol) paraformaldehyde. Cells were made permeable with 0.4% (wt/vol) saponin in flow cytometry buffer and were stained for intracellular cytokines with fluorochrome-conjugated monoclonal anti-IL-4 (11B11; eBioscience) and anti-IL-13 (eBio13A; eBioscience).

Adoptive transfer of basophils. C57BL/6 mice were injected with 2.5 × 10^{3} *S. mansoni* eggs in each footpad, then pLNs, spleen and blood were pooled 2 d later and basophils were purified by sequential CD49b enrichment and cell sorting as described above. Recipient MHC II^{CD11c} mice were given 1 × 10^{7} purified CFSE-labeled CD4^{+} T cells from naive C57BL/6 mice 1 d before egg injection. Sorted basophils were resuspended in a suspension of PBS plus *S. mansoni* eggs, and each recipient MHC II^{CD11c} mouse was given either 5 × 10^{4} basophils and 2.5 × 10^{3} *S. mansoni* eggs or 2.5 × 10^{3} *S. mansoni* eggs alone in the right footpad in a volume of 50 μl. Cells from draining and nondraining pLNs were isolated 4 d after egg injection, then were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry on a FACSCanto II (BD Biosciences).

Macrophage and DC depletion. Liposomes loaded with PBS or clodronate (Roche Diagnostics) were prepared as described[59] and 150 μl of liposomes were injected intravenously every 2 d during the course of infection. CD11c-DTR mice were depleted of DCs by intraperitoneal injection of diphtheria toxin (100 ng per mouse) every 3 d during the course of infection.

Statistics. Statistical significance was determined by the Student's *t*-test.

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