Innate immunological function of $T_H2$ cells in vivo

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Type 2 helper T cells ($T_H2$ cells) produce interleukin 13 (IL-13) when stimulated by papain or house dust mite extract (HDM) and induce eosinophilic inflammation. This innate response is dependent on IL-33 but not $T$ cell antigen receptors (TCRs).

While type 2 innate lymphoid cells (ILC2 cells) are the dominant innate producers of IL-13 in naive mice, we found here that helminth-infected mice had more ILC2 cells compared to uninfected mice, and these cells became major mediators of innate type 2 responses. $T_H2$ cells made important contributions to HDM-induced antigen-nonspecific eosinophilic inflammation and protected mice recovering from infection with Ascaris suum against subsequent infection with the phylogenetically distant nematode Nippostrongylus brasiliensis. Our findings reveal a previously unappreciated role for effector $T_H2$ cells during TCR-independent innate-like immune responses.

Host immunity is composed of the nonspecific innate immune system and the specific adaptive immune system$^{1,2}$. Innate lymphoid cells (ILCs) are lymphocyte-like cells of the innate immune system that lack $T$ cell or B cell antigen receptors; they produce robust effector cytokine responses early in infection and often contribute to the resolution of such infections$^3$. CD4$^+$ T lymphocytes respond in an antigen-specific manner to infectious agents and can release the same set of cytokines that are produced by ILCs$^4$. In vitro–primed CD4$^+$ T cells of the Th1, Th2 and Th17 subsets of helper T cells produce many of their signature cytokines not only in response to their cognate antigen but also in response to certain inducing cytokines$^5$, in much the same manner that ILCs respond, and thus these constitute ‘innate’ immune responses by cells of the adaptive immune system.

The inducing cytokine requirements for the production of signature cytokines by CD4$^+$ helper T cells are stimulation with a member of the interleukin 1 (IL-1) family and an activator of the STAT family of transcription factors$^6$. For Th1 cells, the member of the IL-1 family is IL-18 and the STAT-activating cytokine is IL-12, an activator of STAT4; for Th17 cells, the pair is IL-1β plus IL-23, an activator of STAT3. ILCs use similar stimuli to produce their effector cytokines. For type 2 ILCs (ILC2 cells), which express the transcription factor GATA-3 and produce the type 2 cytokines IL-13 and IL-5, IL-33 is a principal stimulant, and TSLP can enhance that response.

The ability of memory-phenotype CD4$^+$ T cells to mount innate-like cytokine production in response to cytokine stimulation raises the question about the relative contributions of ILCs and CD4$^+$ helper T cells to innate-like cytokine responses. We sought to test this in models of ILC2 responses and $T_H2$ responses. $T_H2$ cells are very rare in naive mice, so it would be anticipated that ILC2 cells would dominate innate cytokine responses in such mice. The relative importance of the two cell types could be very different in mice that have mounted vigorous type 2 immune responses and that have relatively large numbers of memory-phenotype $T_H2$ cells.

To assess the relative importance of expanded ILC2 and $T_H2$ cell populations in early innate cytokine responses, we made use of 4C13R dual-reporter mice$^6$. In these mice, IL-4 production is reported by expression of the cyan fluorescent protein AmCyan, and production of IL-13 is reported by expression of the red fluorescent protein DsRed; this allows determination of in situ production of IL-4 and IL-13 without ex vivo stimulation. We found that $T_H2$ cells were able to produce IL-13 in vivo in response to the combination of IL-33 and a STAT5 activator and that ovalbumin (ova)–specific (OT-II) $T_H2$ cells produced IL-33-dependent IL-13 when challenged intratracheally with papain.

In mice recovering from infection with Nippostrongylus brasiliensis, $T_H2$ cells outnumbered ILC2 cells. These N. brasiliensis–induced $T_H2$ cells responded to challenge with papain or house dust mite extract (HDM) by producing IL-13 and mediating type 2 inflammatory responses, independently of stimulation via the $T$ cell antigen receptor (TCR). $T_H2$ cells induced in response to infection with the clade III parasitic nematode Ascaris suum contributed to host protection against the phylogenetically distant clade V nematode N. brasiliensis$^6$. Together these results indicated that $T_H2$ cells functioned actively in innate immunological defense, promoting type II inflammation and protecting the mucosal barrier. The relative importance of $T_H2$ cells and ILC2 cells in innate immunity depended largely on the relative abundance of the two cell types and therefore varied in naive mice versus antigen-experienced mice.

RESULTS

Response of transferred $T_H2$ cells to cytokine administration

The expression of DsRed or AmCyan in the 4C13R dual-reporter mice was a faithful indicator of the production of IL-13 or IL-4, respectively (Supplementary Fig. 1a–c). We prepared OVA–specific CD4$^+$ $T_H2$ cells

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from naive T cells from OT-II 4C13R mice and injected the cells intravenously into C57BL/6 (B6) host mice that we then challenged intratracheally with either cytokines or OVA. At 24 h after the final challenge with cytokines, 26% of the OT-II cells from the lungs of host mice expressed AmCyan (IL-4) and 12% expressed DsRed (IL-13) (Fig. 1). The administration of IL-33 and IL-7 led 1% of the cells to express AmCyan and 9% to express DsRed; the mean fluorescence intensity of DsRed expression induced by the cytokines was similar to that induced by OVA (Fig. 1). The administration of IL-33 or IL-7 alone caused only a modest increase (IL-33) or no increase (IL-7) in DsRed cells relative to their abundance after challenge with PBS (Fig. 1). The administration of IL-33 and TSLP similarly induced substantial DsRed expression but no AmCyan expression (Supplementary Fig. 2a,b). Thus, adoptively transferred T\(_{\text{H}2}\) cells were able to respond to the administration of IL-33 plus the STAT5 activator IL-7 to TSLP to produce IL-13.

**OT-II T\(_{\text{H}2}\) cells respond to papain by producing IL-13**

Papain has been reported to induce the production of both IL-33 and TSLP by epithelial cells\(^8\). We sought to determine whether in vivo-generated T\(_{\text{H}2}\) cells would respond to papain challenge by producing IL-13 and, if they responded, whether the response would be independent of major histocompatibility complex (MHC) class II. We obtained naive OVA-specific CD4\(^+\) T cells from OT-II 4C13R mice and injected the cells intravenously into B6 recipients that we then infected with *N. brasiliensis* third-stage larvae (L3) and, at the same time, immunized with endotoxin-free OVA. The host mice received an intratracheal OVA ‘boost’ 5 d later. At 25 d after the infection with *N. brasiliensis* and priming with OVA, we challenged the mice intratracheally with PBS or endotoxin-free OVA once or with PBS or papain for 3 consecutive days, in the presence or absence of antibody to MHC class II (anti–MHC class II), and analyzed lung cells 24 h later. In response to OVA challenge, ~19% of the OT-II cells expressed AmCyan and ~9% expressed DsRed (Fig. 2). Treatment with anti–MHC class II diminished DsRed expression to basal amounts and substantially inhibited AmCyan expression in OVA-challenged mice. In response to challenge with papain, 8% of OT-II cells expressed DsRed; this frequency was not affected by treatment with anti–MHC class II. Papain did not induce AmCyan expression.

Since the diminution in the expression of DsRed and AmCyan in the group treated with anti–MHC class II and challenged with OVA might conceivably have been caused by depletion of MHC class II-expressing cells rather than by blockade of TCR stimulation, we did a similar experiment with recipient mice deficient in the common \(\gamma\)-chain of the Fc receptor, in which anti–MHC class II would be less likely to interfere with other cell types. Treatment with anti–MHC class II inhibited responses to OVA in these mice (Supplementary Fig. 3), which indicated that the inhibition of antigen-driven responses mediated by anti–MHC class II was probably not caused by the depletion of antigen-presenting cells. Thus, in vivo–generated OVA-specific OT-II T\(_{\text{H}2}\) cells responded to papain by producing IL-13 but not IL-4, and such cytokine production was independent of MHC class II.

**Papain-induced IL-13 production requires IL-33**

We obtained naive OVA-specific CD4\(^+\) T cells from OT-II 4C13R mice and injected the cells intravenously into IL-33-deficient or wild-type recipients. Papain has been reported to induce the production of both IL-33 and TSLP by epithelial cells\(^8\). We sought to determine whether in vivo-generated T\(_{\text{H}2}\) cells would respond to papain challenge by producing IL-13 and, if they responded, whether the response would be independent of major histocompatibility complex (MHC) class II. We obtained naive OVA-specific CD4\(^+\) T cells from OT-II 4C13R mice and injected the cells intravenously into B6 recipients that we then infected with *N. brasiliensis* third-stage larvae (L3) and, at the same time, immunized with endotoxin-free OVA. The host mice received an intratracheal OVA ‘boost’ 5 d later. At 25 d after the infection with *N. brasiliensis* and priming with OVA, we challenged the mice intratracheally with PBS or endotoxin-free OVA once or with PBS or papain for 3 consecutive days, in the presence or absence of antibody to MHC class II (anti–MHC class II), and analyzed lung cells 24 h later. In response to OVA challenge, ~19% of the OT-II cells expressed AmCyan and ~9% expressed DsRed (Fig. 2). Treatment with anti–MHC class II diminished DsRed expression to basal amounts and substantially inhibited AmCyan expression in OVA-challenged mice. In response to challenge with papain, 8% of OT-II cells expressed DsRed; this frequency was not affected by treatment with anti–MHC class II. Papain did not induce AmCyan expression.

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Figure 3  IL-33 acts directly on in vivo–generated T\(_{H2}\) cells and causes them to produce IL-13 in response to papain. (a) Expression of DsRed and AmCyan by donor OT-1 TI T\(_{H2}\) cells in lungs from wild-type (WT) or IL-33-deficient (Il1rl1\(^{-/-}\)) recipient mice given intravenous injection of 0.5 x 10\(^6\) naive CD4\(^+\) cells from OT-II CD11.13R mice, then immunized with N. brasiliensis and OVA and challenged with PBS, OVA or papain (as in Fig. 2a), with intravenous administration of IL-33 (150 ng) on days 1 and 5 of infection and analysis 72 h after OVA challenge or 24 h after the final papain challenge. (b) Frequency of DsRed\(^+\) (IL-13\(^+\)) cells (top) and AmCyan\(^+\) (IL-4\(^+\)) cells (bottom) among donor OT-II T\(_{H2}\) cells in lungs from wild-type (WT) or IL-33-deficient (Il1rl1\(^{-/-}\)) DO11.10 mice given intravenous injection of 0.5 x 10\(^6\) CD4\(^+\) T cells sorted from Il1rl1\(^{-/-}\)CD90.1\(^{-/-}\)CD90.2\(^{+/+}\) DO11.10 mice or Il1rl1\(^{-/-}\)CD90.1\(^{-/-}\)CD90.2\(^{+/+}\) DO11.10 mice were cultured for three rounds under TH2 conditions with OVA peptide and antigen-presenting cells, then were cultured for 7 d in IL-7-containing medium; T\(_{H2}\) cells were mixed (1:1 ratio) and injected intravenously into wild-type (CD90.1\(^{-/-}\)CD90.2\(^{+/+}\)) BALB/c recipients, followed 24 h later by intratracheal challenge of the hosts for 3 consecutive days with PBS, IL-33 (150 ng) pus IL-7 (100 ng), papain (25 µg) or heat-inactivated papain (25 µg) or once with endotoxin-free OVA (100 µg), with isolation of lungs and preparation of single-cell suspensions 24 h after challenge with PBS, IL-33, IL-7 or papain or 4 h after challenge with OVA, followed by sorting of donor T\(_{H2}\) cells from the suspensions. (d,e) Real-time PCR analysis of Il13 mRNA (d) and Il4 mRNA (e), presented relative to their abundance in wild-type donor cells from PBS-treated recipients, set as 1. *P < 0.01 (two-tailed Student’s t-test). Data are representative of two independent experiments (a,b) or one experiment (d,e) with two to five mice per group (mean and s.d. in d,e).

recipients. We infected the recipient mice with N. brasiliensis, immunized them with OVA and boosted them as in the experiment described above. To exclude the possibility of impairment of T\(_{H2}\) priming in IL-33-deficient recipients, we administered exogenous IL-33 (150 ng) intravenously on days 1 and 5 of infection. Intratracheal challenge with papain caused ~9% of OT-II CD11.13R cells in wild-type recipient mice to express DsRed, but less than 2% were DsRed\(^+\) in IL-33-deficient mice (Fig. 3a,b). OVA challenge–induced expression of AmCyan and DsRed was not significantly different in wild-type and IL-33-deficient recipients, which indicated no intrinsic defect in the priming of OT-II cells for development into T\(_{H2}\) cells in IL-33-deficient mice. We concluded that it was the absence of IL-33 at the time of challenge that was responsible for the lack of response of T\(_{H2}\) cells to papain in the IL-33-deficient recipient mice.

T\(_{H2}\) cells are the direct targets of IL-33

We sorted naive CD4\(^+\) cells from CD90.1\(^{-/-}\)CD90.2\(^{+/+}\) mice of the DO11.10 strain (with transgenic expression of an OVA-specific TCR) that were deficient in the IL-33 receptor Il1rl1\(^{-/-}\) DO11.10 mice and CD90.1\(^{-/-}\)CD90.2\(^{-/-}\) Il1rl1\(^{-/-}\) DO11.10 mice and primed the cells under T\(_{H2}\) conditions in vitro (Supplementary Fig. 4a,b). We mixed those T\(_{H2}\) cells at a ratio of 1:1 and transferred the mixture intravenously into BALB/c recipient mice (Fig. 3c). In recipients that had been challenged intratracheally with OVA, sorted Il1rl1\(^{-/-}\) DO11.10 T\(_{H2}\) cells and Il1rl1\(^{-/-}\) DO11.10 T\(_{H2}\) cells showed similar antigen-induced upregulation of Il4 and Il13 mRNA (Fig. 3d,e), which indicated no intrinsic defect in the T\(_{H2}\) differentiation of Il1rl1\(^{-/-}\) DO11.10 cells.

In recipients that had been challenged with IL-33 plus IL-7, Il1rl1\(^{-/-}\) T\(_{H2}\) cells had ~50-fold higher expression of Il13 mRNA than that of Il1rl1\(^{-/-}\) T\(_{H2}\) cells (Fig. 3d). Indeed, Il1rl1\(^{-/-}\) T\(_{H2}\) cells challenged with IL-33 plus IL-7 had no more Il13 mRNA than its abundance in such cells challenged with PBS (Fig. 3d). Similarly, treatment with papain resulted in a 140-fold induction of Il13 mRNA in Il1rl1\(^{-/-}\) T\(_{H2}\) cells but not in Il1rl1\(^{-/-}\) T\(_{H2}\) cells (Fig. 3d). Inactivated papain did not induce upregulation of Il13 mRNA (Fig. 3d). Upregulation of Il4 mRNA in response to IL-33 plus IL-7 or papain did not occur in T\(_{H2}\) cells from either donor (Fig. 3e). Thus, IL-33 and papain, through IL-33, acted directly on T\(_{H2}\) cells.

Analysis of endogenous polyclonal T\(_{H2}\) cells in vivo

We identified endogenous T\(_{H2}\) cells as CD4\(^+\)CD44\(^+\)Foxp3\(^-\)GATA-3+IL-33R\(^{+}\)CD127+Thy-1\(^+\) marked ILC2 cells (data not shown). Only a few hundred T\(_{H2}\) cells were detectable in the lungs of naive wild-type mice, while several thousand ILC2 cells were present (Fig. 4a,b). The number of T\(_{H2}\) cells increased during infection with parasitic N. brasiliensis larvae, which resulted in ~1 x 10\(^5\) lung T\(_{H2}\) cells at 13 d after inoculation. The number of ILC2 cells increased to a more modest degree, so that there were two- to fourfold more lung T\(_{H2}\) cells than ILC2 cells on day 13 after inoculation. The number of T\(_{H2}\) cells
and ILC2 cells decreased proportionally thereafter, but there were still significantly more T\(\text{H}2\) cells than ILC2 cells at 3–4 weeks after inoculation with \textit{N. brasiliensis}.

We inoculated 4C13R mice with \textit{N. brasiliensis} and, 25 d later, challenged the mice with papain intratracheally for 3 consecutive days in the presence or absence of anti–MHC class II. In response to papain, 16% of T\(\text{H}2\) cells and 28% of ILC2 cells expressed DsRed; the administration of anti–MHC class II did not diminish the frequency of DsRed\(^+\) cells among either cell population (Fig. 4c). Although the proportion of DsRed-expressing T\(\text{H}2\) cells among total T\(\text{H}2\) cells was lower than those among ILC2 cells, the total number of T\(\text{H}2\) cells and of ILC2 cells in the lungs that produced IL-13 in response to papain was similar (Fig. 4d). Treatment with anti–MHC class II did not diminish the frequency of DsRed-expressing T\(\text{H}2\) or ILC2 cells (Fig. 4c,d). Thus, both endogenous lung-resident T\(\text{H}2\) cells and ILC2 cells responded to papain by producing IL-13.

HDM induces eosinophilic inflammation in pre-infected mice

There were few DsRed-expressing cells in the lungs of mice at 23 d after inoculation with \textit{N. brasiliensis} (Fig. 5a). Three daily challenges of uninoculated mice with HDM also led to very few DsRed-expressing cells. However, three challenges with HDM in mice recovering from infection with \textit{N. brasiliensis} resulted in substantial expression of DsRed, but not of AmCyan, by T\(\text{H}2\) cells and ILC2 cells. The number of DsRed-expressing T\(\text{H}2\) cell was similar to the number of DsRed-expressing ILC2 cells. Treatment with anti–MHC class II did not diminish DsRed expression by either cell population.

Three daily challenges of previously uninoculated mice with HDM caused an increase in the number of neutrophils (Gr1\(^+\)CD11b\(^+\) cells) but not that of eosinophils (SiglecF\(^+\)CD11\(\text{c}^-\) cells) in bronchoalveolar lavage (BAL) fluid, relative to the abundance of these cells in mice not challenged with HDM (Fig. 5b). BAL fluid from mice recovering from infection with \textit{N. brasiliensis} contained \(\sim 3 \times 10^4\) eosinophils, which represented \(\sim 20\%\) of BAL fluid cells; this reflected residual airway eosinophilia in mice recovering from a helminth infection. In such mice, HDM challenge induced a substantial cellular response dominated by eosinophils; the number of eosinophils in BAL fluid was \(\sim 2 \times 10^5\).

HDM challenge of mice recovering from infection with \textit{N. brasiliensis} caused massive lung eosinophilic and lymphocytic infiltration, bronchial and bronchiolar mucus metaplasia, and numerous large alveolar macrophages with eosinophilic crystals positive for the alternative activation marker Ym1 (Fig. 5c). We did not observe such pathological changes in the other groups. Thus, in contrast to the recruitment of neutrophils by HDM in unimmunized mice, three HDM challenges induced massive migration and recruitment of eosinophils into both the lung tissues and BAL fluid in mice previously exposed to parasites, and also induced allergic inflammatory responses.

**Mechanism of induced eosinophilic airway inflammation**

Treatment with anti–MHC class II did not diminish the number of eosinophils in BAL fluid or the degree of lung inflammation in HDM-challenged mice recovering from infection with \textit{N. brasiliensis} (Fig. 6a). To determine whether CD4\(^+\) T cells had an important role in the development of HDM-induced eosinophilic airway inflammation in mice recovering from infection with \textit{N. brasiliensis}, we rendered such mice deficient in CD4\(^+\) T cells by injecting a CD4-depleting antibody intravenously into the mice during the 3-day HDM challenge. Depletion of CD4\(^+\) T cells led to a significant reduction of \(-50\%\) in the number of eosinophils in BAL fluid (Fig. 6b).

To determine whether the HDM-induced eosinophilia was mediated via IL-33, we inoculated IL-33-deficient mice with \textit{N. brasiliensis} L3 and treated the mice with IL-33 on days 1 and 5 of infection, then challenged the mice with HDM 25 d later. In contrast to the marked eosinophilia in \textit{N. brasiliensis}-infected wild-type mice that received the HDM challenge, the number of eosinophils in BAL fluid recovered from IL-33-deficient \textit{N. brasiliensis}-infected mice remained low (Fig. 6c). The administration of IL-33 (100 ng) at the time of challenge with HDM restored the recruitment of eosinophils. Thus, HDM-induced eosinophilia was independent of MHC, was partially dependent on CD4\(^+\) T cells and was mediated via IL-33.

**Induction of eosinophilic inflammation by CD4\(^+\) T cells alone**

We injected in vitro–cultured OT-II T\(\text{H}2\) cells intravenously into mice deficient in the recombimase component RAG-2 and the common cytokine receptor \(\gamma\)-chain (Rag\(^2^-\)/Il2rg\(^-\)) mice, which lack
Figure 5 Short-term HDM challenge induces prompt eosinophilic airway inflammation in mice recovering from infection with *N. brasiliensis*. (a) Quantification of DsRed+ cells and AmCyan+ cells in the lungs of B6 mice infected with *N. brasiliensis* and then challenged with PBS 23 d later (NB + PBS), left uninfected and challenged with HDM (UI + HDM), or infected with *N. brasiliensis* and then challenged intratracheally 23 d later (daily for 3 consecutive days) with HDM (25 µg in PBS) alone (NB + HDM) or together with intravenous injection of anti–MHC class II (500 µg) on days 1 and 3 of HDM challenge (NB + HDM + α-MHCII), followed by analysis 24 h after the final challenge. (b) Frequency of eosinophils and neutrophils among live cells (left) and quantification of eosinophils and neutrophils (right) in BAL fluid of B6 mice left uninfected and treated with PBS (UI + PBS) or HDM (UI + HDM), or infected with *N. brasiliensis* and then challenged with PBS (NB + PBS) or challenged as in a with HDM (NB + HDM), followed by analysis timed as in a. (c) Microscopy of sections of lungs from mice treated as in b, stained with hematoxylin and eosin (top row), periodic acid–Schiff stain (bottom left) or Luna stain (bottom middle) or stained for Ym1 (bottom right); arrows indicate mucus metaplasia (top and bottom left), perivascular infiltration (top middle), alveolar infiltration (top right), eosinophils (bottom middle) or Ym1+ macrophages (bottom right). Original magnification, ×100. Each symbol (a,b) represents an individual mouse; small horizontal lines indicate the mean (ss.d.). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (two-tailed Student’s t-test). Data are representative of two (a,c) or three (b) independent experiments with three to four mice per group.

Ascaris suum protects against *N. brasiliensis*

Next we sought to determine whether Th2 cells generated in response to infection with one helminth would produce innate IL-13 and offer protection during infection with a different helminth. We chose two evolutionarily distant nematodes, *Ascaris suum* and *N. brasiliensis*, for this study, to minimize antigen cross reactivity. *A. suum* larvae derived from oral inoculation of infective eggs optimally migrate to the lungs by day 7 and are immediately cleared from the intestine following further migration. We inoculated mice orally with *A. suum* eggs and then inoculated them subcutaneously with 500 *N. brasiliensis* L3 11 d later, or either inoculated mice orally with *A. suum* only or inoculated them subcutaneously with *N. brasiliensis* only, as control conditions. We detected no adult *N. brasiliensis* worms and no egg production in the intestine of such mice 8 d after inoculation with *N. brasiliensis*, which differed from the response seen in *N. brasiliensis*-infected naive mice (Fig. 7a). We also observed full protection in mice inoculated with *N. brasiliensis* 25 d after infection with *A. suum* (Supplementary Fig. 5). Thus, prior infection with *A. suum* resulted in prompt clearance of *N. brasiliensis*.

At 17 d after infection with *A. suum*, the number of both Th2 cells and ILC2 cells had greatly increased (Fig. 7b), indicative of a robust *A. suum*–induced type 2 response. Approximately 5% of the Th2 cells in these mice expressed DsRed and ~4% expressed AmCyan, and among these cells, some expressed both (Fig. 7c). ~7% of the ILC2 cells expressed DsRed and almost none expressed AmCyan (Fig. 7c). By day 6 of subsequent infection with *N. brasiliensis* (day 17 after inoculation with *A. suum*), the total number of Th2 cells had increased twofold (Fig. 7b); ~19% of these Th2 cells were DsRed+ (Fig. 7c). The increase in the frequency of DsRed+ Th2 cells was matched by an increase in the number of DsRed+ Th2 cells, from ~1 × 10^5 in mice that had recovered from *A. suum* infection to ~1 × 10^4 in such mice subsequently inoculated with *N. brasiliensis* L3 (Fig. 7d). Subsequent infection with *N. brasiliensis* did not increase the number of ILC2 cells, but the frequency of DsRed+ ILC2 cells increased to 50% and the number of DsRed+ ILC2 cells increased to ~25 × 10^3 (Fig. 7b–d). Thus, Th2 cells made up ~30%
of the total IL-13-producing cells in *N. brasiliensis*-challenged mice that had recovered from infection with *A. suum* (Fig. 7d).

In contrast to DsRed expression, AmCyan expression by T\(\text{H}2\) cells from mice recovering from infection with *A. suum* remained unchanged or increased only modestly upon challenge with *N. brasiliensis* (Fig. 7c), which suggested that the T\(\text{H}2\) cytokine production observed was not driven by engagement of the TCR but instead was mediated via IL-33 or IL-25. At 6 d after inoculation of previously uninfected mice with *N. brasiliensis*, the number of T\(\text{H}2\) cells and ILC2 cells was still low (Fig. 7b,c), which indicated that the increased number of cells and their cytokine production was dependent on the prior infection with *A. suum*.

**T\(\text{H}2\) cells partially protect against *N. brasiliensis* infection**

We injected *in vitro*-cultured OT-II T\(\text{H}2\) cells intravenously into *Rag2\(^{-/-}\)Il2rg\(^{-/-}\)* mice that we then inoculated with *N. brasiliensis*. At 8 d after inoculation, intestinal adult worm burden was significantly lower in mice that had received OT-II T\(\text{H}2\) cells than in control mice that did not receive T\(\text{H}2\) cells (Fig. 7e). Thus, OVA-specific T\(\text{H}2\) cells provided partial protection against infection with *N. brasiliensis*.

In wild-type mice infected with *N. brasiliensis* after recovery from infection with *A. suum*, endogenous IL-13-producing ILC2 cells out-numbered IL-13-producing T\(\text{H}2\) cells (Fig. 7d). We sought to determine whether the T\(\text{H}2\) cells made a substantial contribution to the innate protection against *N. brasiliensis* infection in mice previously infected with *A. suum*. We generated chimeras by injecting wild-type or ROR\(\alpha\)-deficient bone marrow (BM) cells into *Rag2\(^{-/-}\)Il2rg\(^{-/-}\)* recipient mice (Fig. 7f), as the transcription factor ROR\(\alpha\) has been reported to be critical for ILC2 development\(^*\). Both uninfected chimeras given ROR\(\alpha\)-deficient BM and those infected with *N. brasiliensis* without prior *A. suum* infection had very few ILC2 cells T (Supplementary Figs. 6a,b and 7a), consistent with a published report\(^*\). *In vitro*-primed T\(\text{H}2\) cells from chimeras given wild-type BM and those given ROR\(\alpha\)-deficient BM produced similar amounts of cytokines in response to stimulation with the phorbol ester PMA plus ionomycin or with IL-33 and IL-7 (Supplementary Fig. 8), which indicated that *in vitro* T\(\text{H}2\) differentiation was unimpaired in the absence of ROR\(\alpha\).

We inoculated the chimeras described above with *N. brasiliensis* L3 only or with *A. suum* eggs followed by *N. brasiliensis*. In chimeras given wild-type BM, previous infection with *A. suum* resulted in expulsion of *N. brasiliensis* by day 8 (Fig. 7f), consistent with the full protection observed in wild-type mice (Fig. 7a). Notably, in some chimeras given ROR\(\alpha\)-deficient BM, prior infection with *A. suum* led to the induction of as much as 7% ILC2 cells (Supplementary Fig. 7b), while similarly infected chimeras given wild-type BM had ILC2 cells ranging from 2% to 20% (Supplementary Fig. 7d). We assessed the worm burden in chimeras that were given ROR\(\alpha\)-deficient BM and had less than 3% ILC2 cells. In these mice, previous infection with *A. suum* led to a significant ~50% diminution in the intestinal *N. brasiliensis* worm burden, relative to the burden in their counterparts not previously infected with *A. suum*, and some completely eliminated the *N. brasiliensis* (Fig. 7f). The protection of such chimeras (given ROR\(\alpha\)-deficient BM, and having less than 3% ILC2 cells) was eliminated by treatment with anti-CD4 during inoculation with *N. brasiliensis* (Fig. 7f and Supplementary Fig. 7c). Thus, *A. suum*-generated T\(\text{H}2\) cells were sufficient to partially provide protection against infection with *N. brasiliensis*.

We also observed that infection with *N. brasiliensis* afforded protection against subsequent infection with *A. suum* by substantially reducing the number of migrating *A. suum* larvae detected in the lungs (Fig. 7g). Because of the dominance of T\(\text{H}2\) cells in *N. brasiliensis*-infected mice, this result strengthened the contention that innate action of T\(\text{H}2\) cells might have an important role in protection against infection with a phyogenetically distant helminth.
IL-33 is present in the nuclei of cells in epithelial barrier tissues and lymphoid organs and is released into the extracellular space as an alarmin after cell damage or cellular stress. TSLP is also an epithelium-derived cytokine. The combination of these two alarmins induces Th2 cells to produce IL-13, but not its closely related Th2 cytokine IL-4, in vitro. This suggests that in response to helminth invasion or allergen exposure, innate production of IL-13 is mounted by tissue-resident Th2 cells in response to IL-33 produced by epithelial cells and probably in response to TSLP produced by similar cells.

Although the closely linked Th2 cytokines IL-4 and IL-13 have many similarities, they have distinct physiological functions. IL-13 is essential for the expulsion of N. brasiliensis helminths, but IL-4 is not. Anti-IL-13 inhibits elicitation of airway hypersensitivity responses, but anti-IL-4 does not. Thus, the selective production of IL-13 from Th2 cells induced by IL-33 plus TSLP is consistent with the role of IL-13 as an effector molecule and suggests that effector helper T cells could, like ILCs, exert innate immunological functions especially at mucosal sites. To test this hypothesis, we used IL-4- and IL-13 dual-reporter mice, which allowed us to assess the in situ production of IL-4 and IL-13 without ex vivo stimulation, and focused on lung Th2 cells during airway inflammation and during defense against helminth infection.

Adoptively transferred, in vitro–primed, OVA-specific Th2 cells and in vivo–generated Th2 cells produced IL-13 in response to the administration of IL-33 and IL-7 or of papain, respectively. In the latter case, IL-13 production was not inhibited by a treatment with anti–MHC class II but was IL-33 dependent, which indicated that it was TCR independent but cytokine dependent. In unimmunized mice, there were very few Th2 cells but thousands of ILC2 cells, consistent with the dominant role of ILC2 cells during early type 2 responses in naive mice. After infection with N. brasiliensis, Th2 cells ‘preferentially’ differentiated and expanded their populations to an abundance that reached $1 \times 10^6$ by day 13 after inoculation, and thus they outnumbered ILC2 cells. After the expulsion of parasites, the number of Th2 cells and ILC2 cells decreased proportionally so that the frequency of Th2 cells remained greater than that of ILC2 cells at 4 weeks after infection. Thus, it might be anticipated that Th2 cells would have a more important role in cytokine-dependent cytokine production in mice recovering from responses that induce strong Th2 immunity than they would in naive mice.

Three daily exposures to HDM in mice recovering from infection with N. brasiliensis induced a notable TCR-independent eosinophilia in BAL fluid and lung tissues, whereas in unimmunized mice, such a challenge resulted in the accumulation of neutrophils in BAL fluid but no lung eosinophilic inflammation. Depletion of Th2 cells by treatment with anti-CD4 led to a substantial reduction in BAL fluid eosinophilia in mice recovering from infection with N. brasiliensis. BAL fluid eosinophilia was induced by HDM in mice lacking...
endogenous T cells and ILCs that had received in vitro–cultured OT-II T\(_2\) cells. Thus, innately activated T\(_2\) cells had an important role in the induction of eosinophilic airway inflammation in response to HDM exposure, and T\(_2\) cells alone were able to mediate such antigen-nonspecific airway inflammation.

The interplay of helmint infection and allergic disorders is complex\(^{21,22}\). While there are reports that prior helmint infection of humans can provide protection against allergic responses, there are many other reports that helmint infection can potentiate such inflammation\(^{23–26}\). Infection intensity and induced immunomodulation are thought to be the most important factors that account for such differential effects\(^{22,27–29}\). Intense and chronic helmint infections are associated with protection against allergic disease, whereas mild and transient infections have been linked to the potentiation of allergic responses.

While intense helmint infection causes strong T\(_2\) responses, it also results in the induction of regulatory T cells and M2 (alternatively activated) macrophages and production of the anti-inflammatory cytokine IL-10 (refs. 21,30–33). Such immunomodulation does not occur during mild helmint infection\(^{22,27}\). Under these circumstances (mild infection), the large number of resultant tissue-resident T\(_2\) cells and ILC2 cells could trigger more frequent and more severe allergic diseases and thus represent innate immunological memory. This could explain our observation that HDM induced robust eosinophilic airway inflammation in mice 3–4 weeks after inoculation with N. brasilensis but not in uninfected mice. N. brasilensis infection is self-curing, and parasites are expelled 10–12 d after inoculation of mice with infective L3.

We also investigated the importance of effector T\(_2\) cells in the innate defense against helmint infection. A previous infection with A. suum led to full protection against a subsequent infection with the evolutionarily distant helmint N. brasilensis; we observed complete clearance of N. brasilensis from the intestine as early as 6 d after exposure, far earlier than in mice not exposed to A. suum. Published studies have suggested that eosinophils\(^{34,35}\), basophils\(^{36}\) and macrophages\(^{37}\) have roles in protection during secondary infection with N. brasilensis. IL-13 is crucial for effective expulsion of N. brasilensis, as it coordinates various effector systems\(^{16}\). Here we determined the cellular sources of IL-13 production in mice 6 d after inoculation with N. brasilensis L3. Mice previously infected with A. suum demonstrated a marked increase in IL-13 production by both T\(_2\) cells and ILC2 cells. IL-13-producing lung-resident T\(_2\) cells made up to 30% of the total IL-13-producing cells. Adoptive transfer of OVA-specific T\(_2\) cells into mice lacking endogenous T cells, B cells and ILCs conferred partial protection against infection with N. brasilensis L3, which indicated that lung-resident T\(_2\) cells contributed to protection against helmint infection.

Collectively, our results indicated that both T\(_2\) cells and ILC2 cells expressed the receptor for IL-33 and responded to epithelium-derived IL-33 by producing the effector cytokine IL-13. Under physiological or pathological circumstances in which both T\(_2\) cells and ILC2 cells are present, their relative contributions to cytokine production, allergic inflammation and protection against subsequent helmint infection seem to be proportional to their numbers, the amount of GATA-3 they express and their level of IL-33 receptor. Thus, tissue-resident T\(_2\) cells can exert innate functions upon encountering the appropriate stimuli, and their innate responses can contribute to both airway inflammation and protection against helmint infection. Our results suggest that humans in areas in which polyparasitism from phylogenetically diverse helmints is endemic may display a substantial degree of protective T\(_2\) cell–based innate immunity.

### METHODS

Methods and any associated references are available in the online version of the paper.

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### AUTHOR CONTRIBUTIONS

J.G. designed, performed and interpreted experiments and wrote the manuscript; Y.H., X.C. and J.H.-L. assisted with some experiments; J.E.U. helped to design, perform and interpret experiments involving infection with A. suum and N. brasilensis, provided N. brasilensis, and read the manuscript; and W.E.P. designed and interpreted the experiments, wrote the manuscript and supervised the study.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Mice. The generation of the 4C13R mice on the B6 background has been described. OT-II 4C13R mice were generated by the breeding of OT-II B6 mice with 4C13R mice. H1rl1+/- CD90.1 CD90.2+ DO11.10 mice were generated by the crossing of CD90.2+ Rag2+/- DO11.10 mice to CD90.2+ IL-33-deficient mice. FcRγ-deficient mice, Rag2+/- Il2rg-/- recipient mice, CD90.2+ Rag2+/- DO11.10 mice, BALB/c mice and OT-II mice were from Taconic. BALB/c CD90.1+ mice were from Jackson Laboratory. BALB/c mice. IL-33-deficient mice were backcrossed to BALB/c for ten generations. IL-33-deficient mice10 on the B6 background were provided by J.-P. Girard. ROαR-deficient mice on the B6 background were from Jackson Laboratory. Heterozygous ROαR-deficient mice were used for breeding; pups were identified by tattooing of the plantar surface of the paws of 6-day-old neonates. Tail clipping and PCR were performed for genotyping. The primers used for genotyping were as follows: WT-F, 5′-TCTCCCTTCTGCTGAGCA-3′; WT-R, 5′-TTATTCACCAGGCGAA-3′; ROαR-deficient-F, 5′-GATTGAAAGCTGACTGTCCTC-3′; and ROαR-deficient-R, 5′-CGTTTGCGAACCTCACC3′. All mice were bred and/or maintained in a specific pathogen–free animal facility of the National Institute of Allergy and Infectious Diseases, and all animal experiments were performed under the approval of the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases (US National Institute of Health).

BM chimeras. ROαR-deficient mice and their wild-type littermates at 10 days of age were used as donor mice. Rag2+/- Il2rg-/- mice at 6–10 weeks of age were used as recipients. Recipient mice received sublethal irradiation (single dose of 450 rads) before transplantation of 1 x 105 whole BM cells from donor mice by retro-orbital injection on the same day. Mice were treated with trimethoprim (0.67 mg/ml) and sulfamethoxazole (0.13 mg/ml) via their drinking water for 5 weeks. Reconstitution was assessed 8 weeks later.

Helmint infections. Mice at least 8 weeks of age were used for infection with N. brasiliensis and A. suum. 500 infective N. brasiliensis L3 were inoculated subcutaneously into the mice. Worm expulsion was determined by counting of adult worms in the small intestine on day 8 after inoculation. Eggs in fecal pellets were counted 8–10 d after inoculation in McMaster counting chambers. A suspension of embryonated A. suum eggs containing 1,500 infective larvae were inoculated orally into mice, and parasitic L3 were detected 8 days later in lungs after processing. Explanted lungs were finely minced with scissors and then suspended in tubes containing 8 ml of RPMI-1640 medium, followed by incubation for 3 h at a 37 °C water bath. The suspension was transferred to a six-well culture plate and parasitic L3 were counted using an inverted microscope with a 200× objective.

Adoptive cell transfer. 0.5 x 106 sorted Lin− CD25− CD45RBhi cells from OT-II 4C13R mice, 0.5 x 106 ILC2 cells, or 5 x 106 in vitro–cultured Tg21 cells that had been maintained for 10 d in IL-7 containing medium were used for transfer. After being extensively washed in PBS, cells were suspended in 200 µl of PBS and were injected intravenously into the retro-orbital sinuses of recipient mice.

Administration of cytokines, papain, HDM and antibodies. Mice were anesthetized with volatile isoflurane and were held on an intubation stand by having their upper incisors hooked onto it. Materials were dissolved in 40 µl of sterile PBS and were administered intratracheally into mice through a catheter inserted into the trachea. Reagents used were as follows: IL-33 (100 or 150 ng) (PeproTech), IL-7 (100 ng) (PeproTech), TSLP (150 ng) (R&D Systems), EndoFit Ovalbumin (100 µg) (InvivoGen), papain (25 µg) (Merck Millipore), and HMD extract (Dermatophagoides farinae) (25 µg) (Greer Laboratories). For inactivation of papain, papain was heated for 10 min at 100 °C.

In some experiments, 500 µg of anti-CD4 (GR1.5; BioXCell) or 500 µg of anti-MHC class II (Y-3P; BioXCell) was injected into mice via retro-orbital injection.

BAL fluid cytology. BAL was performed by instillation of 1 ml PBS into lung through a tracheal cannula, followed by gentle aspiration of the fluid. The lavage was repeated four times; BAL fluid from four washes was pooled and centrifuged (337 g at 4 °C for 5 min) for collection of the cells. Cells were stained and the cellular composition of BAL fluid was analyzed by flow cytometry. Neutrophils were identified as live SSC−CD3− B220− Gr-1− CD11b+ Siglec F−CD11c+ MHCI− cells. Eosinophils were identified as live SSC−CD3− B220− Gr-1− CD11b+ MHCI− cells.

In vitro culture of Tg21 and Tg17 cells. CD62L−CD44hi naive CD4+ T cells were sorted from OT-II, DO11.10 or B6 mice. Antigen-presenting cells (APCs) were purified from splenocytes by treatment for 45 min at 37 °C with anti-Thy-1.2 (Supplementary Table 1) and low-Tox M rabbit complement and were irradiated at 30 Gy. For Tg21 culture, CD4+ T cells were primed with OVA peptide (0.2 µM) or anti-CD3 (3 µg/ml; Supplementary Table 1) and anti-CD28 (3 µg/ml; Supplementary Table 1) and APCs in the presence of IL-2 (100 units/ml), IL-4 (1000 units/ml), anti-IL-12 (10 µg/ml; Supplementary Table 1) and anti-IFN-γ (10 µg/ml; Supplementary Table 1). After a 4-day priming period, Tg21 cells were washed and then were cultured in medium containing IL-7 (10 ng/ml). Cells were cultured under Tg21 conditions for three rounds of 4 d each. For Tg17 culture, CD4+ T cells were primed with anti-CD3 (3 µg/ml; Supplementary Table 1), anti-CD28 (3 µg/ml; Supplementary Table 1) and APCs in the presence of IL-6 (10 ng/ml) (PeproTech), TGF-β (10 ng/ml) (PeproTech), IL-1β (10 ng/ml) (PeproTech), anti-IL-4 (10 µg/ml; Supplementary Table 1), anti-IL-12 (10 µg/ml; Supplementary Table 1) and anti-IFN-γ (10 µg/ml; Supplementary Table 1).

In vitro ILC2 cells. IL-25 (200 ng) (R&D Systems) was administrated intraperitoneally to mice for 3 consecutive days. Mesenteric lymph nodes were collected, and Lin− KLRG1+ cells were sorted and then cultured in medium containing IL-33 (5 ng/ml) and IL-7 (5 ng/ml).

Isolation of lymphocytes from lung tissues. Mice were killed and their lungs were perfused with cold PBS. Afterward, lung tissue was dissected, the upper airway was ligatured, and the trachea was exposed and a ligature was placed around the tracheal wall with a 22-gauge needle. The lung was disected and then were stored for 24 h in neutral buffered formalin and then transferred into 70% ethanol until histologic processing was done. Embedding, sectioning and staining (with hematoxylin and eosin, luna stain, periodic acid–Schiff reagent, or Ym1) were performed by Histoserve. Mouse antibody to Ym1 (no antigen retrieval; 1:400 dilution; Supplementary Table 1) was used for Ym1 staining.
Quantitative PCR. Total RNA was isolated with an RNeasy mini kit (Qiagen); first-strand cDNA was prepared with Superscript III (Invitrogen). All PCR was performed on 7900HT sequence detection systems (Applied Biosystems). TaqMan universal PCR SuperMix and primer and probe sets for mouse Il4, Il13 and Gapdh were from Applied Biosystems.

Statistical analysis. Statistical significance was determined by application of a two-tailed Student's t-test.

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