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Intestinal helminth infection promotes IL-5- and CD4⁺ T cell-dependent immunity in the lung against migrating parasitesKara J. Filbey¹, Mali Camberis¹, Jodie Chandler¹, Rufus Turner², Anthony J. Kettle², Ramon M. Eichenberger³, Paul Giacomini³ and Graham Le Gros¹

The ability of helminths to manipulate the immune system of their hosts to ensure their own survival is often credited with affecting responses to other pathogens. We undertook co-infection experiments in mice to determine how infection with the intestinal helminth *Heligmosomoides polygyrus* affected the parasitological, immunological and physiological outcomes of a primary infection with a distinct species of helminth; the lung migratory parasite *Nippostrongylus brasiliensis*. We found that migrating *N. brasiliensis* larvae were killed in the lungs of *H. polygyrus*-infected mice by a process involving IL-33-activated CD4⁺ T cells that released IL-5 and recruited activated eosinophils. The lung pathology normally associated with *N. brasiliensis* larval migration was also reduced. Importantly, lung immunity remained intact in mice cleared of prior *H. polygyrus* infection and also occurred during infection with another entirely enteric helminth, *Trichuris muris*. This study identifies a cross-mucosal immune mechanism by which intestinal helminths may protect their hosts against co-infection by a different parasite at a distal site, via circulation of activated CD4⁺ T cells that can be triggered to release effector cytokines and mount inflammatory responses by tissue damage-associated alarmins, such as IL-33.

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INTRODUCTION

Co-infection is considered the norm in human and wild animal populations.^{1,2} Helminth infections are often observed to have a negative impact on the host with respect to the control of bacterial, viral and protozoan infections through the skewing of host immune responses toward a Th2 phenotype, and the induction of regulatory mechanisms that can limit a protective Th1 response.³ However, some studies of helminth co-infection have demonstrated beneficial outcomes for the host in terms of reduced parasite burden and pathology. For example, infection with the trematode parasite *Schistosoma mansoni* can enhance immunity to the gastrointestinal nematode *Trichuris muris*.⁴ Similarly, infection with another helminth that has both lung and gut migratory phases, *Ascaris suum*, affords enhanced protection against *Nippostrongylus brasiliensis*, which traverses a similar migratory path through the host.⁵

Heligmosomoides polygyrus is a nematode parasite that establishes stable, long-lasting infection in laboratory mice with little apparent impact on the overall physiology of its host. Interestingly, although infection is restricted to the small intestine, the effects of *H. polygyrus* (Hp) on the immune system are systemic and profound, with potent induction of both Th2, and regulatory, cells and cytokines.^{6,7} Consistent with this, chronic Hp infection can reduce the immunopathology associated with *Schistosoma* eggs in the liver via the production of regulatory cytokines.⁸ Despite the supportive evidence for the ability of helminths, including Hp, to promote cross-protective

immunity to subsequent infection with distinct helminth pathogens, the immunological mechanisms of how this occurs are unclear.

N. brasiliensis (Nb) is a model of acute, migratory helminth infection. Within hours of entry through the skin, Nb stage 3 larvae migrate via the blood stream to the lung and through to the alveolar space, causing local tissue damage and haemorrhage.⁹ Maturing larvae are then transported up the airways and are swallowed to take up residence in the small intestine for a further 4–5 days to allow adult reproduction before eventual expulsion from day 7 post-infection.¹⁰ The lung is the site at which the protective response to Nb infection is most robustly manifested^{11,12} with immune-mediated larval damage key to limiting infections^{11,13} and type 2 cytokine-producing CD4⁺ T cells central players.^{12,14}

In the present study, we employed Hp infection to examine how infection with this intestinally confined parasite influences systemic and localised immunity to a distinct species of helminth, and the molecular and cellular mechanisms responsible for this cross-mucosal immune regulation. We found that intestinal Hp infection lead to a significant increase in immune-mediated killing of Nb larvae within the lung. This enhanced protection was associated with IL-33-mediated activation of CD4⁺ Th2 cells, which orchestrated IL-5-mediated eosinophil-dependent larval killing. These findings highlight a previously unrecognised mechanism by which intestinal helminths influence systemic immunity to extraneous pathogens.

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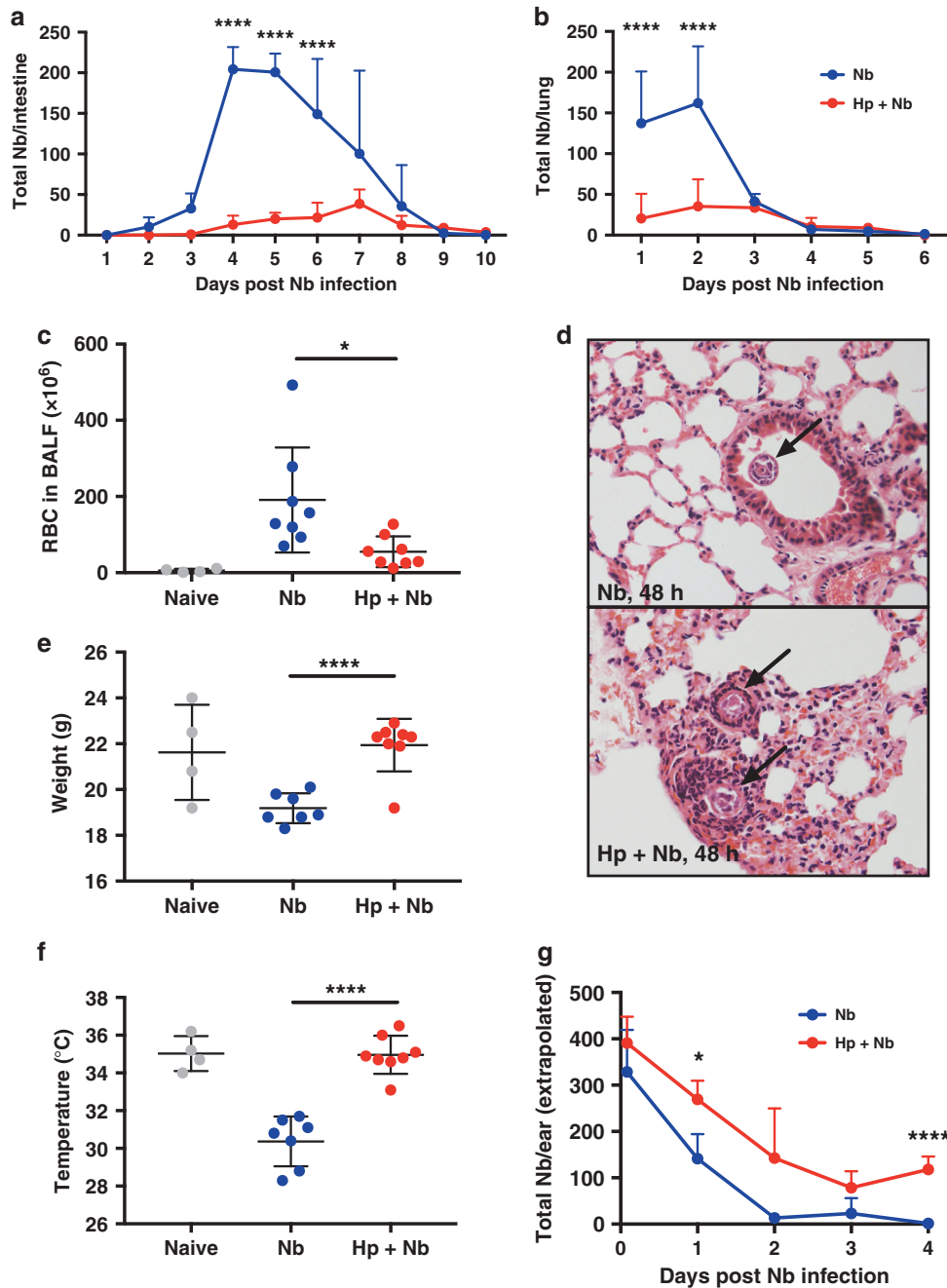


Fig. 1 Migrating *N. brasiliensis* larvae are killed in the lungs of mice infected with *H. polygyrus*. 550 *N. brasiliensis* iL3 were injected subcutaneously into female C57BL/6 mice infected with 200 *H. polygyrus* iL3 by oral gavage 14 days previously (Hp + Nb, red), or into naive mice (Nb, blue). **a** Viable adult Nb were enumerated from the small intestine (results combined from three independent experiments, *t* tests are for each time point). **b** At the time points indicated, viable Nb larvae from whole lung tissue were enumerated (results combined from three independent experiments, *t* tests are for each time point). **c** RBC were enumerated in the BALF at 48 h post Nb infection (results combined from two independent experiments). **d** Representative H&E stained sections of lung from 48 h post Nb infection. Cross-sections of larvae are indicated with an arrow. At 48 h post Nb infection mice were **e** weighed and **f** their temperature was taken with a rectal thermometer (results combined from two independent experiments). **g** Estimated number of Nb larvae trapped in the skin 2 h, and each day, after intradermal injection of 550 L3 (results are representative of two independent experiments). A *t* test was used for all comparisons. * = *p* < 0.05, **** = *p* < 0.0001

RESULTS

Migrating *N. brasiliensis* larvae are killed in the lungs of mice infected with *H. polygyrus*.

Hp is an entirely gut-dwelling helminth that induces robust local and systemic type 2 responses by 14 days post-infection.^{15,16} We challenged day 14 Hp-infected mice with a subcutaneous inoculation of 550 (Nb) L3 larvae. In a primary infection of naive

mice, 30–40% of the injected Nb larvae can be recovered from the intestinal lumen as live adult worms at days 4–5 (Fig. 1a), and, strikingly, in co-infected mice numbers were reduced by almost 90% (Fig. 1a). Interestingly, the same significant decrease in viable Nb larvae recovered from co-infected mice was noted when Hp infection was extended to 21, 28 and 35 days prior to Nb infection (Supp. Figure 1).

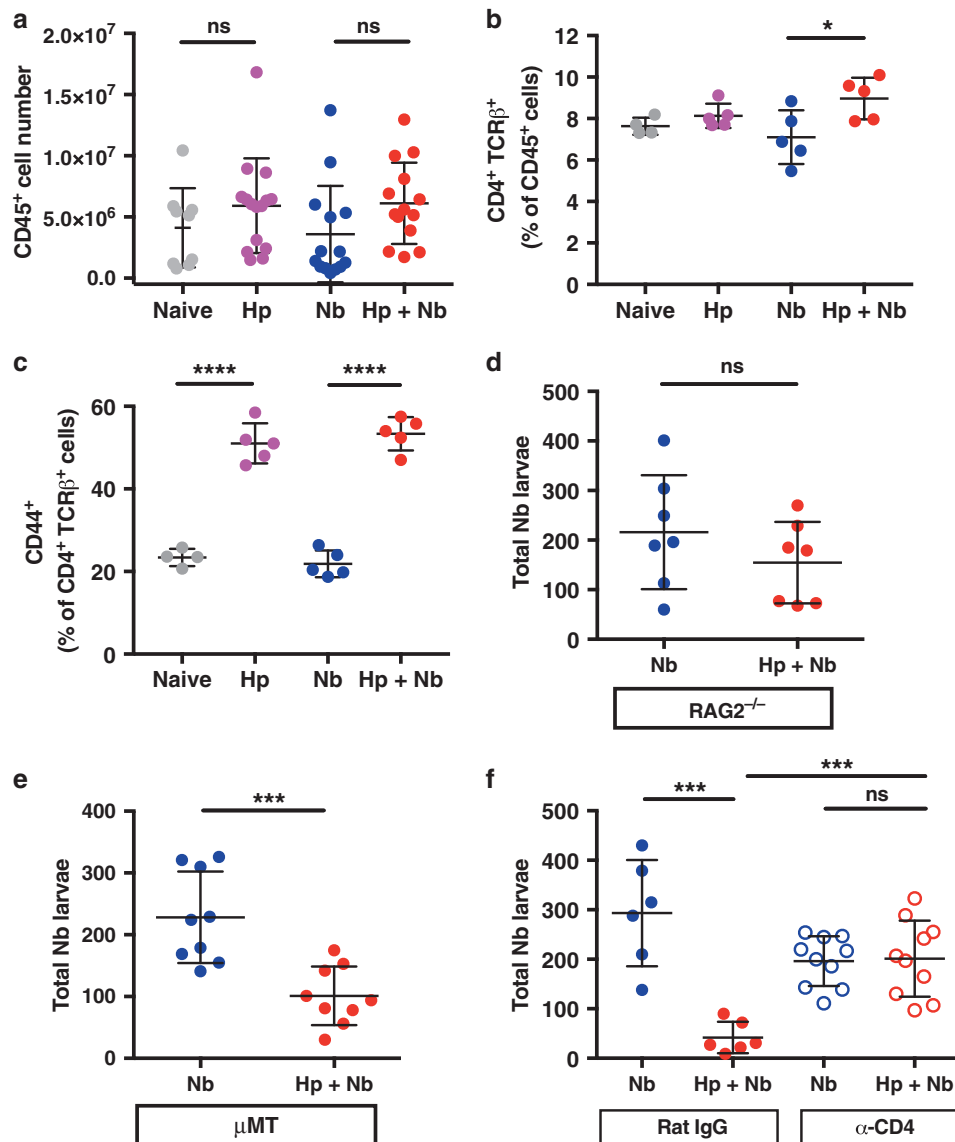


Fig. 2 Killing of *N. brasiliensis* larvae in the lung is dependent on CD4⁺ T cells. 550 *N. brasiliensis* iL3 were injected subcutaneously into female C57BL/6 mice infected with 200 *H. polygyrus* iL3 by oral gavage 14 days previously. **a** Total number of CD45⁺ cells in whole lung tissue after digestion (results combined from four independent experiments). **b** Proportion of CD45⁺ cells that are CD4⁺ TCR-β⁺ in the lung tissue at 48 h post Nb infection (representative of three independent experiments). **c** Proportion of CD4⁺ TCR-β⁺ cells that are CD44⁺ in the lung tissue at 48 h post Nb infection (representative of two independent experiments). **d** Total Nb larvae in the lung tissue at 48 h post Nb infection in RAG2^{-/-} mice (results are combined from two independent experiments). **e** Total Nb larvae in the lung tissue at 48 h post Nb infection in μMT mice (results are combined from two independent experiments). **f** Total Nb larvae in the lung tissue of mice treated with rat IgG isotype control, or anti-CD4 throughout prior Hp infection (results are combined from two independent experiments). A *t* test was used for all comparisons. ns = not statistically significant, ** = *p* < 0.01, *** = *p* < 0.001, **** = *p* < 0.0001

As numbers of Nb larvae entering the gut were already significantly diminished in Hp-infected mice, we decided to look at pre-gut stages of the parasite migratory route. The numbers of live Nb larvae recovered from the lung tissue of naive mice peaked at 48 h post infection (Fig. 1b) as previously documented.¹¹ Infection with Nb leads to significant tissue damage at sites where larvae migrate from the capillaries and interstitial tissues into the alveolar spaces of the lung, and significant numbers of red blood cells (RBC) are detected in the bronchoalveolar lavage fluid (BALF) (Fig. 1c). In contrast, in mice infected with Hp, the viability of Nb larvae that migrated to the lungs was significantly reduced, with fewer than 5% of the original inoculum remaining alive after 48 h

(Fig. 1b), and a significantly lower number of RBC appearing in the BALF (Fig. 1c). Normally, naive mice do not mount an inflammatory response against Nb larvae as they migrate through the lungs (Fig. 1d, top panel). However, in mice infected with Hp, striking immune cell foci surrounding the Nb larvae in the lung parenchyma were apparent at 48 h post infection (Fig. 1d, bottom panel). The histological appearance of the cellular foci closely resembled those that form in Nb re-infection models.¹²

The reduction in numbers of viable Nb larvae detected in Hp-infected mice correlated with the absence of transient weight loss and drop in body temperature normally seen at 48 h post infection with Nb (Fig. 1e, f). Furthermore, prior Hp infection

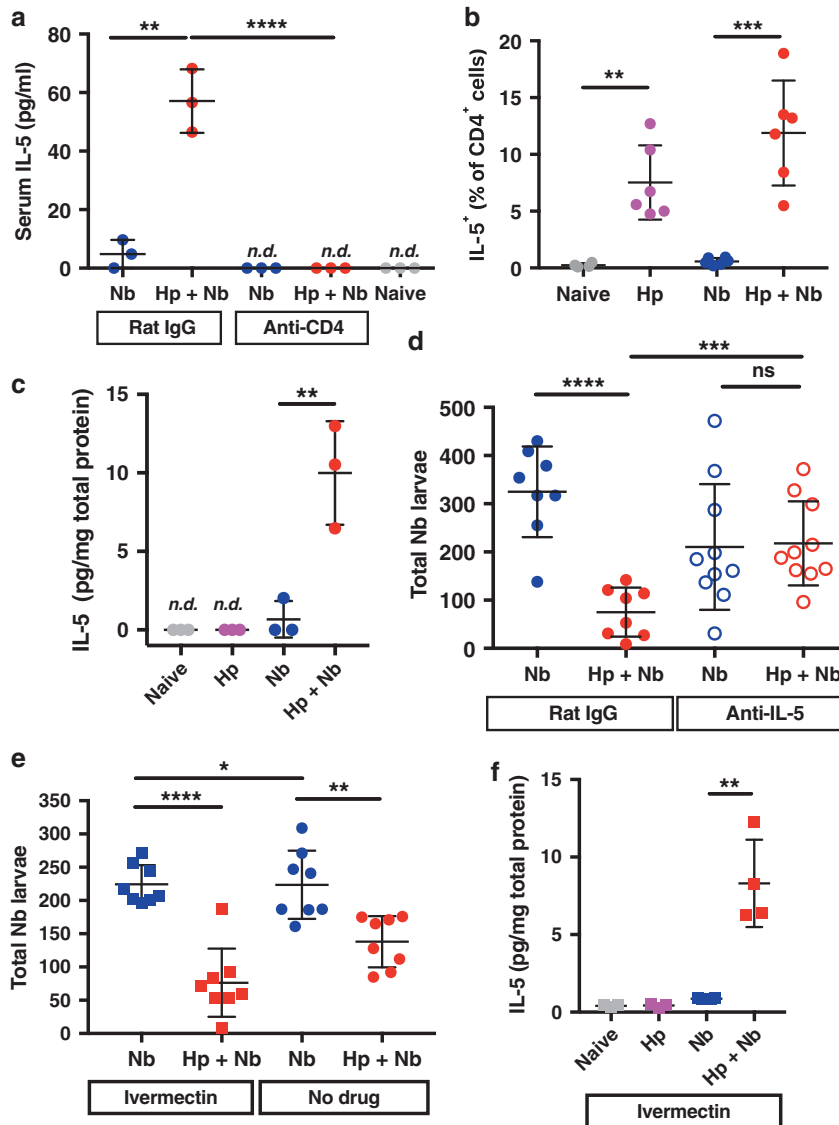


Fig. 3 Control of migrating *N. brasiliensis* larvae is dependent on IL-5 and is intact after clearance of *H. polygyrus*. 550 *N. brasiliensis* iL3 were injected subcutaneously into female C57BL/6 mice infected with 200 *H. polygyrus* iL3 by oral gavage 14 days previously. **a** Serum IL-5 levels (pg/ml) measured by LegendPlex in a subset of mice from Fig. 2f. **b** Proportion of lung CD4⁺ cells that are IL-5⁺ by ICCS at 48 h post infection with Nb (results are combined from two independent experiments). **c** Amount of IL-5 in lung homogenate taken 48 h post Nb infection, measured with LegendPlex bead array, and normalised to total protein level in the sample (results are representative of three independent experiments). **d** Total Nb larvae in the lung tissue of mice treated with rat IgG isotype control, or anti-IL-5 throughout prior Hp infection (results are combined from two independent experiments). **e** C57BL/6 mice were infected with 200 Hp iL3, and after 2 weeks given 2 consecutive doses of ivermectin to clear the infection. Hp-infected, drug-treated and untreated mice were infected with 550 Nb and larvae enumerated in the lung 48 h later (results are combined from two independent experiments). **f** IL-5 in the lung homogenate of ivermectin treated mice was measured and normalised to total protein levels (results are combined from two independent experiments). A t test was used for all comparisons. ns = not statistically significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. n.d., not detected

protected mice against the Nb-induced emphysema-like deterioration normally observed in the lung tissue several weeks after infection is cleared¹⁷ (Supp. Figure 2).

Finally, as numbers of Nb larvae entering the lung were already reduced in co-infected mice at 24 h post infection, we used a DNA-based quantification strategy to assess whether protection was occurring at pre-lung stages. As has been described in secondary infection,¹⁸ we detected increased levels of Nb larval DNA in the skin after intradermal injection in co-infected mice, translating to higher numbers of larvae still trapped in the skin tissue up to 3 days longer than in mice infected with Nb alone (Fig. 1g). This indicates that Hp instigates a systemic response that

is protective at more than one site and larvae are trapped in the skin by, as yet, unknown mechanisms.

In summary, infection of mice with Hp appears to arm the host immune system to be able to respond to acute challenge infection by Nb and significantly reduced the larval viability and immunopathology normally associated with larval migration through tissues. Given that the lung is the major site of immune-mediated protection in mice re-infected with Nb^{11,12} and having detected a cellular immune response around Nb larvae in the lungs of co-infected mice, we decided to focus further investigations on the effect of Hp infection on host lung tissues.

Reduction of *N. brasiliensis* viability in *H. polygyrus*-infected mice is dependent on CD4⁺ T cells.

CD4⁺ T cells are instrumental in protective immune responses against Nb, although they do not normally appear in the lung in significant numbers until 9–14 days after a primary infection.¹⁹ Hp infection did not significantly increase the number of CD45⁺ immune cells that could be isolated from lung tissue compared with naive or Nb L3-infected mice, or from mice infected with Hp and challenged 48 h later with Nb L3 (Fig. 2a). However, Hp infection modestly, but significantly, increased CD4⁺ TCR-β⁺ cell proportions in the lung of co-infected mice (Fig. 2b). Strikingly, a significant proportion of the CD4⁺ cells appearing in the lung in Hp-infected mice were CD44⁺ indicating that they had acquired an 'activated' phenotype (Fig. 2c), consistent with the notion that these T cells had been primed in the gut by Hp and had subsequently migrated to the lung.²⁰

To determine what role CD4⁺ T cells played in Hp-induced reduction in viability of Nb larvae, we performed the same co-infection protocol in recombination-activating gene (RAG)2^{-/-} mice, which lack mature T and B lymphocytes. Compared with the significant reduction in viable Nb L3 numbers seen in Hp-infected wild-type mice, Hp did not afford protection against Nb in RAG2^{-/-} (Fig. 2d), indicating that either CD4⁺ T cells or B cells were important. Our observation that Hp was still able to significantly diminish Nb viability in μMT mice (Fig. 2e) and in mice unable to mount antibody responses against helminth antigens (Tg (IghelMD4)) (Supp. Figure 3A), demonstrated that neither B cells or antibodies were involved. To more specifically focus on the role of CD4⁺ T cells, we used a monoclonal antibody depletion regime to ablate CD4⁺ T cells throughout the period of Hp infection, before infecting mice with Nb. Hp-infected mice that had received anti-CD4 were unable to mount a protective response against Nb L3 (Fig. 2f). Furthermore, killing of Nb L3 did not occur in Hp-infected P25 T-cell receptor (TCR)-transgenic mice (Supp. Figure 3B), in which the TCR cannot bind to parasite antigens, suggesting that CD4⁺ T cells require activation through their TCR at some point during Hp infection for their subsequent involvement in the Nb-killing process.

Control of migrating *N. brasiliensis* L3 is dependent on IL-5 and is intact after clearance of *H. polygyrus*.

A cytokine bead array was used to determine which type 2 cytokines were released systemically into the circulation during Hp infection. We found high levels of IL-5 in the serum of Hp-infected mice protected against challenge with Nb larvae, and this was completely ablated in mice that had been treated with the monoclonal anti-CD4 antibody (Fig. 3a), illustrating that CD4⁺ T cells are a key IL-5-producing population during Hp infection. Intracellular staining for IL-5 revealed a significant proportion of the Hp-induced CD4⁺ T cells in the lung had the potential to secrete IL-5 (Fig. 3b and Supp. Figure 4), and that the majority of these IL-5⁺ T cells were also CD44⁺ (Supp. Figure 4C), suggesting that antigen encounter had induced cytokine expression in these cells. Interestingly, upon assessing IL-5 protein levels in lung homogenate samples, this cytokine was only found to be released into the tissue in Hp-infected mice challenged with Nb larvae, and not in those infected with Hp alone (Fig. 3c). This intriguing result indicates that although T cells induced by Hp infection have the capacity to make IL-5, only those 're-stimulated' by the subsequent challenge with Nb L3 actually release it into the immediate tissue environment. To ascertain the role of IL-5 in Hp-induced protection against Nb, we blocked IL-5 with a monoclonal antibody (TRFK5) throughout the period of Hp infection. This regimen had the same striking effect as depleting CD4⁺ T cells, and killing of Nb larvae in Hp-infected mice was completely blocked (Fig. 3d).

Several publications have described IL-5-producing memory T cells that can contribute to pathogenesis and inflammation^{21,22}

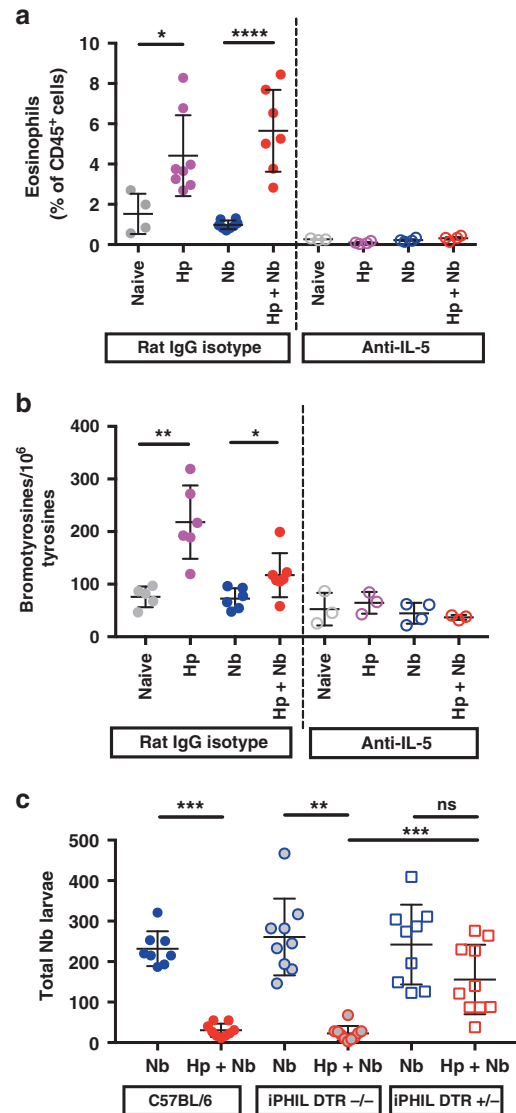


Fig. 4 Eosinophils are key for the control of migrating *N. brasiliensis* larvae. 550 *N. brasiliensis* iL3 were injected subcutaneously into female C57BL/6 mice infected with 200 *H. polygyrus* iL3 by oral gavage 14 days previously. **a** Proportion of CD45⁺ cells that are eosinophils (SiglecF⁺ CD11c⁺) in lung tissue at 48 h post Nb infection, in mice treated with anti-IL-5 or rat IgG isotype control throughout prior Hp infection (results are combined from two independent experiments). **b** Quantification of bromotyrosine residues per 10⁶ tyrosines in proteins from BALF taken from mice 48 hours post Nb infection (results are combined from two independent experiments). **c** 20 ng/g diphtheria toxin was injected intraperitoneally every 3 days into iPHIL mice heterozygous for the diphtheria toxin receptor (DTR[±], squares) or their homozygous negative littermates (-/-, grey-filled circles), or C57BL/6 controls (coloured circles), starting at day 2 of Hp infection (or the equivalent in uninfected mice), up to and including day 14, when Nb was injected (combined from two independent experiments). A *t* test was used for all comparisons. ns = not statistically significant, * = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001, **** = *p* < 0.0001

and lung-resident CD4⁺ T cells have been shown to be sufficient for protection against Nb re-infection.²³ We set out to determine if killing of migrating Nb larvae could still occur after clearance of Hp adult worms from the gut by administration of the anthelmintic drug ivermectin. One month after clearance of Hp, mice were infected with Nb. Significant Nb killing still occurred in the absence

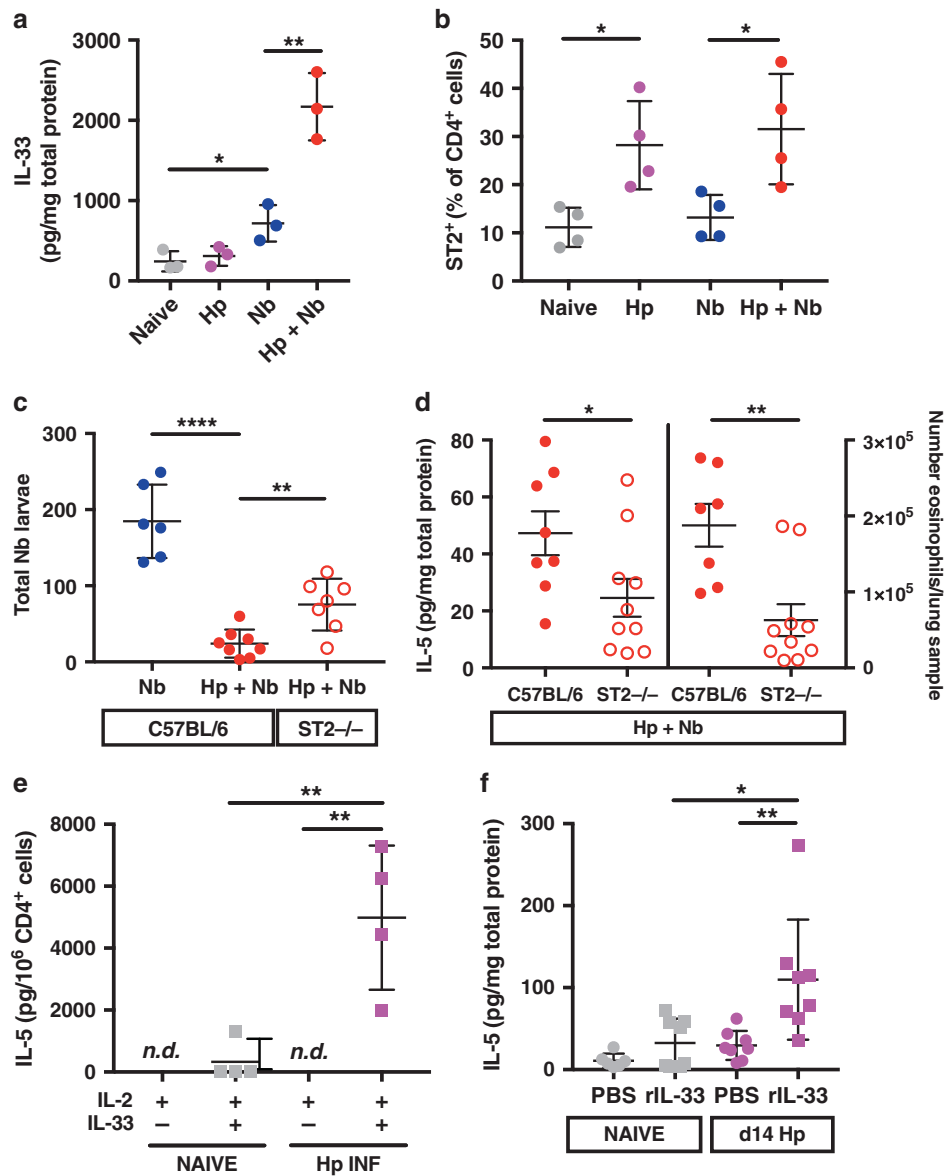


Fig. 5 IL-33 signalling through the ST2 receptor contributes to the control of *N. brasiliensis* larvae. 550 *N. brasiliensis* iL3 were injected subcutaneously into female C57BL/6 mice infected with 200 *H. polygyrus* iL3 by oral gavage 14 days previously. **a** Amount of IL-33 in lung homogenate taken 48 h post Nb infection, measured with LegendPlex bead array, and normalised to total protein level in the sample (results are representative of three independent experiments). **b** Proportion of CD4⁺ cells that express ST2 in lung tissue at 48 h post Nb infection (results are representative of two independent experiments). **c** Number of Nb larvae in the lung at d2 post infection in C57BL/6 or ST2^{-/-} mice, previously infected with Hp. **d** Amount of IL-5 in lung homogenate, and number of eosinophils in lung samples, taken 48 h post Nb infection in C57BL/6 and ST2^{-/-} mice infected with Hp 14 days previously (results are combined from two independent experiments). **e** 0.5 × 10⁶ Dynabead separated CD4⁺ T cells from MLN of naive or 14-day Hp-infected mice were cultured in IL-2-containing media with or without IL-33, for 3 days, and IL-5 was measured in the supernatant by ELISA (results are representative of two independent experiments). **f** Amount of IL-5 in lung homogenate taken 24 h post-rIL-33 intranasal administration, measured by ELISA, and normalised to total protein level in the sample (results are combined from two independent experiments). A *t* test was used for all comparisons. * = *p* < 0.05, ** = *p* < 0.01, **** = *p* < 0.0001

of Hp (Fig. 3e). Importantly, high levels of IL-5 could be detected in the lungs of mice that had been sterilised of Hp prior to Nb larval challenge, (Fig. 3f). This observation supports the view that IL-5⁺ CD4⁺ T cells can reside in the lung after Hp infection, and can be 'reactivated' to release IL-5 upon challenge with Nb larvae.

Eosinophils are key for the control of migrating *N. brasiliensis* larvae.

IL-5 is the major differentiation, maturation and accumulation factor for eosinophils^{24–26} cells that are a characteristic feature of Hp infection.²⁷ Eosinophils were completely ablated in the lung

tissue after neutralisation of IL-5 where killing of Nb larvae in the lung was inhibited (Fig. 4a). In control mice, Hp infection resulted in a significant increase in the proportion of eosinophils in the lung compared with naive animals and mice infected with Nb L3 only (Fig. 4a).

Bromotyrosine is formed in proteins when tyrosine residues are brominated by hypobromous acid, which is derived from the eosinophil peroxidase-dependent oxidation of bromide. It is an indicator of eosinophil activity.^{28,29} Levels of bromotyrosine residues on proteins in BALF taken from Hp- and co-infected mice were significantly greater than in those infected with Nb L3

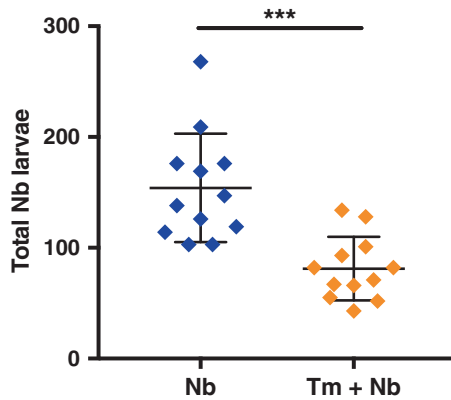


Fig. 6 *Trichuris muris* confers significant protection against migrating *N. brasiliensis* larvae. C57BL/6 mice were infected orally with 200 *T. muris* ova, and 35 days later infected subcutaneously with 550 Nb iL3 (Tm + Nb). Numbers were compared with mice infected with Nb alone (Nb). Larvae were enumerated in the lung 48 h post infection with Nb (results are combined from two experiments). A *t* test was used for comparisons. ***=*p* < 0.001

alone, indicating that not only are eosinophils present in the lung in greater numbers, but that they are more activated in mice infected with Hp (Fig. 4b). Notably, bromotyrosine levels were completely ablated upon treatment of mice with anti-IL-5 antibody, showing that eosinophils are the relevant source of this toxic mediator.

To assess the role of eosinophils directly, we used a highly specific inducible depletion method, involving the injection of diphtheria toxin (DTx) into iPHIL mice, which express the human diphtheria toxin receptor (DTR) within the start codon of the eosinophil peroxidase gene locus.³⁰ Importantly, littermates homozygous negative for the DTR (DTR^{-/-}) had exactly the same phenotype as wild-type C57BL/6 mice (both sets of mice were also administered DTx), and exhibited significant killing of lung migrating Nb L3 when infected with Hp (Fig. 4c). When eosinophils were depleted throughout the period of Hp infection in mice expressing the DTR (DTR[±]) (Supp. Figure 5A&B), killing of Nb L3 in the lung was significantly reduced (Fig. 4c).

IL-33 triggers IL-5 release by lung CD4⁺ T cells in *N. brasiliensis*-challenged mice.

As IL-5-competent CD4⁺ T cells do not release IL-5 into the lung until migrating Nb larvae appear (Fig. 3c, f), we sought to identify the mechanism that induces this cytokine release. Previous studies have shown that the alarmin IL-33 is rapidly released into the bronchoalveolar space after Nb infection³¹ and that IL-33 is vital to the development of robust type 2 immunity and expulsion of worms.^{31,32} IL-33 has also been shown to induce IL-5⁺ T cells in the lung.³³

IL-33 protein levels in the lung tissue were increased in Nb-infected animals compared with naive controls, and were threefold higher in the lungs of Hp-infected, Nb-challenged animals (Fig. 5a). Proportions of CD4⁺ T cells expressing the IL-33 receptor chain ST2 were also increased in mice infected with Hp compared with naive control mice or those challenged with Nb alone (Fig. 5b). We also observed that Hp-infected ST2-deficient mice³⁴ were unable to kill migrating Nb larvae to the same extent as wild-type mice (Fig. 5c). Significantly, this diminished protection correlated with a lower level of IL-5 protein detected in lung tissue by enzyme-linked immunosorbent assay (ELISA), and a lower number of lung eosinophils, in ST2-deficient mice infected with both parasites, compared with wild-type mice (Fig. 5d).

To confirm a role for IL-33 in the innate restimulation of Hp-induced T cells, we isolated CD4⁺ cells from the mesenteric lymph nodes (MLN) of naive and Hp-infected mice and cultured them

with recombinant (r)IL-33 in vitro. CD4⁺ T cells stimulated with rIL-33 released high levels of IL-5 into the culture supernatant, and cells from Hp-infected animals produced significantly higher levels than their naive counterparts (Fig. 5d). To translate this finding to an in vivo setting, we introduced rIL-33 intranasally to naive and d14 Hp-infected mice and measured the release of IL-5 in the lung homogenate. We found significant levels of IL-5 in the lungs of Hp-infected mice stimulated with rIL-33 compared with naive controls (Fig. 5e), suggesting that this alarmin could indeed be the innate stimulation needed for antigen-independent activation of lung-resident CD4⁺ T cells to release their IL-5 upon the appearance of Nb L3 into the lung.

T. muris also mediates protection against migratory *N. brasiliensis* larvae.

We sought to determine whether the effect of co-infection on Nb viability was specific to Hp or also occurred when mice were infected with another species of gastrointestinal nematode. We infected mice orally with 200 *T. muris* (Tm) ova, in accordance with previous studies characterising this high dose as an inducer of type 2 responses in C57BL/6 mice,³⁵ 35 days prior to Nb infection, and assessed Nb larval viability in the lung after 48 h. As was the case with Hp, significantly lower numbers of live Nb larvae were recovered from the lungs of Tm-infected mice compared with naive controls (Fig. 6). These findings suggest that a common mechanism for limiting host damage by a migratory pathogen, such as Nb, may be shared by long-lived gastrointestinal helminths, such as Hp and Tm.

DISCUSSION

By challenging Hp-infected mice with Nb larvae we sought to explore whether the immune regulatory mechanisms that arise as a consequence of long-lived gastrointestinal helminth infections affect host immune responses to other parasites at tissue sites separate from the gut. We found that Hp-infected mice mount a rapid and extremely robust immune response against lung migrating Nb larvae leading to a significant loss in viability of the larvae and a reduction in the pathology associated with their migration through the lung tissue. Although our findings contradict previous studies that have shown that Hp infection prolonged adult Nb survival and fecundity in the gut,^{36,37} others have demonstrated a reduction in the total number of Nb larvae entering the gut from the lung in mice co-infected with Hp,³⁸ suggesting a pre-gut protective phenomenon, similar to our findings. We also noted pre-lung protection in co-infected mice, illustrated by the detection of higher levels of Nb DNA in the skin of co-infected mice compared with mice intradermally injected with Nb without prior Hp infection. This suggests that a similar phenomenon may be occurring in the skin as occurs in secondary Nb infection, where larvae are trapped by immunological mechanisms,¹⁸ and further investigations are ongoing.

We found that the killing of migrating Nb larvae in Hp-infected mice is unaffected by a lack of B cells and helminth-specific antibodies, ruling out a role for humoral immunity. However, killing of Nb larvae is completely absent in TCR-transgenic mice that are unable to respond to Hp antigens, indicating that Hp-specific priming of T cells is required. When combined with our observation that Hp infection increases the proportion of CD44⁺ CD4⁺ T cells in the lung, our results suggest a mechanism by which Hp infection activates CD4⁺ T cells in the intestine, which circulate and redistribute around the body, including the lung, where they remain in a state of 'readiness' to respond to the assault of tissue damaging migratory larvae, such as Nb. Immune cross-talk and cellular trafficking between the lung and intestinal tissues has been documented previously^{39,40} and, of note, the systemic dissemination of Th2 cells after Hp infection has been demonstrated, with the lung highlighted as one 'hot-spot' for their

accumulation.¹⁵ How long these activated T cells can be maintained in the lung is unclear. Interestingly, when Hp infection was extended to 28, and even 35, days before Nb larvae were introduced, protection was still significant. This suggests that even in the more regulatory immune environment known to have been established by Hp at these later time points,⁶ the same effective protective mechanisms can act against Nb larvae.

There are several mechanisms by which a gut-dwelling helminth may influence systemic immunity. First, the dissemination of helminth secreted products into the circulation has been demonstrated during Hp infection,⁴¹ although whether specific proteins can enter distal tissues and activate cells *in situ* has not been reported. Second, a helminth could influence host systemic immunity via the microbiome, and infections with both Hp and Tm have been shown to alter the composition of the microbiota, which can have profound effects on immune responses throughout the body.⁴² For instance, a recent study showed that Hp infection could protect the host against respiratory syncytial virus infection in the lung in a process that was dependent on an intact microbiome.⁴³ Certainly, cross-mucosal immunity and protection from inflammation have been illustrated in a number of helminth models, including low-dose *T. muris* infection which, in converse to the Th2-inducing high dose which we employed in this study, induces interferon-gamma-producing CD4⁺ T cells in the distal lung microenvironment which protect the host from airway inflammation.⁴⁴ Intriguingly, our data suggest that an active gastrointestinal helminth infection may not be absolutely required to confer protection in the lung, as clearance of Hp had no effect on the level of protection seen against Nb. Thus, the systemic impact of the previous Hp infection appears to persist after the worm is eliminated from the host. The same is most likely true for the Tm experiments, as by day 35 post infection with this dose of Tm larvae, the majority of adult worms have been expelled from the gut.³⁵ Further investigation is underway to determine whether shared immunological mechanisms exist in both the Hp and the Tm models of co-infection with Nb.

The conclusion that reactivation of helminth-induced Th2 cells by a distinct parasite may not necessarily rely on antigen cross-reactivity at the TCR level has also been reported in a recent study demonstrating the 'innate' properties of CD4⁺ T cells responding to either house dust mite or papain by producing IL-33-dependent, TCR-independent IL-13.⁵ This study also showed that prior infection with an unrelated helminth, *A. suum*, protected against subsequent Nb infection by the same mechanism.⁵ Importantly, a role for IL-13 production by type 2 innate lymphoid cells (ILC2s) in this context was ruled out by the use of chimeric mice, which lack ILC2s, but were still protected. Although we did not have access to a mechanism for directly eliminating ILC2s to assess their direct contribution, our finding that Hp-induced killing of Nb larvae in the lung is lost in *Rag2*^{-/-} mice, in which ILC2s are still present, and the complete loss of IL-5 from the circulation upon depletion of CD4⁺ cells, indicates a key role for IL-5-producing CD4⁺ T cells in our model.

That polarised Th2 cells can be stimulated to release cytokines by the addition of IL-33 to cultures, and induce IL-5 and IL-13 gene expression in the lung when administered *in vivo*, has been previously reported.⁴⁵ The antigen-independent recruitment and activation of Th2 cells in the lung have also been demonstrated previously,⁴⁶ as has the 'reactivation' of resting CD4⁺ T cells by cytokines.⁴⁷ Indeed, lung-resident T cells are sufficient for protection in secondary Nb infection, without the need for recruitment of fresh cells from the lymph node.²³ Furthermore, Hp itself has recently been shown to induce local memory CD4⁺ T cells that can be re-stimulated by IL-33 in a TCR-independent manner to release IL-5 and IL-13.⁴⁸ Building on these results, we show that IL-33 can stimulate IL-5 release from Hp-induced CD4⁺ T cells in the lung, which leads to the subsequent activation of eosinophils that can contribute to killing of migrating Nb larvae.

There is a well-documented role for eosinophils in the killing of Nb larvae via adhesion and release of toxic mediators.^{49,50} One such toxic product is hypobromous acid. It is produced by eosinophil peroxidase, reacts rapidly with proteins and brominates tyrosine residues. Measurement of bromotyrosine in lung fluid or tissue gives a read-out of the activation state of eosinophils *in vivo*.⁵¹ The significantly higher levels of brominated tyrosine residues on proteins in BALF from Hp-infected mice give an indication that the eosinophils present in the lungs of these mice are highly activated and ready to participate in a protective response against the incoming Nb larvae.

Why might a gut-dwelling helminth, such as Hp or Tm, have evolved to induce such a strong systemic response? Perhaps the prevention of a possible competitor from arriving in the gut has beneficial consequences for the already established helminth. In recent years, much discussion has centred around the role of Th2 immunity as being primarily for tissue repair and maintenance, particularly in the context of helminth infections.⁵² Thus, a second intriguing possibility could be that the systemic type 2 response induced by Hp (and presumably Tm) in our studies could be an evolutionarily important safety mechanism to assure protection and tissue repair upon future insult, so that the host is kept alive. This would be especially pertinent in the context of a tissue-destructive hookworm infection, modelled here with Nb. Third, the detection of activated cells in the lung following an intestinally restricted infection could simply be a result of the high level of immunological communication between mucosal sites, especially the intestine and lung.³⁹

In conclusion, we reveal that long-lived infection by Hp or Tm species confers a state of innately regulated adaptive immunity involving the tissue damage-induced release of IL-33 that reactivates CD4⁺ T cells to release IL-5 and activate eosinophils at sites where another species of parasite migrates into the lung. This cross-mucosal response results in significant killing of migrating Nb larvae and also reduces the appearance of the pathological consequences of parasite migration in the lung. The implications of this host-protective relationship by parasites, for people living in environments where multiple species of helminth parasites are endemic, is important. The observation that previous infection is sufficient to confer protection to the host may have implications for treatment strategies and future studies.

MATERIALS AND METHODS

Mice

C57BL/6 and P25 TCR-transgenic mice were bred and maintained in the Biomedical Research Unit (BRU) at the Malaghan Institute of Medical Research (MIMR) or were purchased from the Animal Resources Centre (Australia) and held at James Cook University, Cairns, Australia for the *T. muris* experiments. *RAG2*^{-/-}, μ MT and MD4 BCR-transgenic mice were purchased from Jackson Laboratories and housed in the BRU only for the duration of the experiments listed. iPHIL mice (generously gifted from James Lee, Mayo Clinic, Scottsdale, Arizona, USA) and *ST2*^{-/-} mice (generously gifted from Scott Mueller, Doherty Institute, University of Melbourne, Melbourne, Australia) were bred and genotyped in the BRU at MIMR. iPHIL mice have the human DTR knocked-in at the endogenous start codon for the eosinophil peroxidase (*epx*) gene.³⁰ *DTR*^{+/+} heterozygotes and their *DTR*^{-/-} homozygous negative littermates were used for eosinophil depletion experiments.

All mice were over 6 weeks of age at the beginning of experiments, age and sex matched where appropriate, and experiments were always repeated at least twice, with data being combined from several experiments where possible. All experimental procedures were approved by Victoria University of Wellington, Wellington, New Zealand or the James Cook University animal ethics committee.

Helminth infections and enumeration

N. brasiliensis and *H. polygyrus* were maintained via passage through Lewis rats and C57BL/6 mice, respectively, and infective L3 larvae prepared from fecal cultures, as previously described.¹⁰ *T. muris* was maintained in genetically susceptible mouse strains as previously described.⁵³ For every co-infection experiment, 200 Hp L3 were given by oral gavage 2 weeks prior (day -14) to subcutaneous injection in the scruff of 550 Nb L3 in 200 µl phosphate-buffered saline (PBS). For the time course experiment (Supp. Figure 1) Hp was given at the time points stated prior to Nb infection. For skin enumeration, mice were lightly anaesthetised and 550 Nb larvae were intradermally injected in 50 µl PBS into the ear pinna, from where larvae can migrate effectively to the lung in a similar time-frame to subcutaneous injection.

Whole lung tissue was harvested, diced and placed in gauze suspended in a 50 ml tube filled with PBS, and incubated in a 37°C water bath overnight so that viable larvae could migrate along a temperature gradient to the bottom of the tube. (This method has been verified to give similar results to combining separate bronchoalveolar lavage and lung tissue larval counts). Small intestine was sliced along its length and hung over a parafilm strip in a PBS-filled 50 ml Falcon tube, and viable Nb and Hp adult worms were allowed to migrate in a similar fashion to the lung larvae. Larvae and adult worms were counted under a dissecting microscope.

Hp infection was cleared with two doses of ivermectin (Noromectin, Norbrook Laboratories Ltd, Newry, Northern Ireland) on days 14 and 15 given subcutaneously (200 µg in 200 µl PBS). Clearance was confirmed by checking fecal samples for eggs 7 days later. Mice were rested for 4 weeks before infection with Nb.

Mice were infected by oral gavage with 200 *T. muris* ova in PBS, 35 days prior to subcutaneous Nb infection, and viable Nb lung larval numbers assessed as above at 48 hours post infection.

Physiological measurements and in vivo treatments

Core temperature was measured with a digital rectal thermometer and mice were weighed on a standard bench-top weighing scale.

500 µg of anti-CD4 (GK1.5), anti-IL-5 (TRFK5) or rat IgG isotype control (all from BioXCell, West Lebanon, NH, USA) were given intraperitoneally (i.p.) in 200 µl of sterile PBS on days -15, -7, and 0 of the co-infection protocol. 300 ng of rIL-33 (R&D Systems, Minneapolis, MN, USA) was administered intranasally in 50 µl PBS, 14 days after Hp infection or to naive mice. 24 h later lungs were removed for homogenisation and cytokine measurements.

20 ng/g body weight of DTx in 200 µl sterile PBS was injected i.p. either throughout the co-infection protocol (on days -11, -8, -5, -2, 0) or just around Nb infection (days -1, 0 and +1). DTx was administered to both DTR^{+/-} and homozygous negative (DTR^{-/-}) littermates.

Real-time PCR quantification of Nb in the skin

DNA was extracted from ear tissue using DNeasy Blood and Tissue kit (Qiagen, Germany). Whole ears were excised and minced finely and bead-beaten for 2 × 2 minutes at 25 Hz in lysis buffer. Proteinase K was added per instructions, with an additional 20 µl of 1 M dithiothreitol (Sigma). Tissues were lysed at 56 °C on a shaking heat block overnight. 4 µl of 10 mg/ml RNAse A (Thermo Scientific, NZ) was added to each sample and incubated at room temperature for 30 minutes. The subsequent steps were followed as per manufacturers protocol and final DNA was eluted in 100 µl of elution buffer. The total DNA quantity and purity ratios were determined by NanoDrop spectrophotometer. DNA was diluted to 6.25 ng/µl for use. Standards were generated using naive whole ears and spiking in exactly 640, 320, 160, 80, 40, 20, 10 or 0 Nb L3 larvae. The Nb 18 S gene was used as an amplicon due to it giving the most consistent standard curves compared with other genes tested, with r² values of > 0.98 and gradients of -3.3, indicating

100% efficient PCR reactions. The mouse ApoB100 gene was used as an internal control to account for DNA input variances. A real-time ΔC_T PCR was performed using PowerUp SYBR Mastermix (Life Technologies, NZ). Primer sequences were as follows: Nb 18 S Forward 5'-GGATCTGAGTTACATGCAGTGGTTC-3'; Nb 18 S Reverse 5'-TCAAAGTAACTCGCTAGCCACT-3'; Mouse ApoB100 Forward 5'-CACGTGGGCTCCAGCATT-3'; Mouse ApoB100 Reverse 5'-TCACAGTCATTTCTGCCTTTG-3'. The 10 µl reaction contained 1X SYBR green Mastermix, 1 µM each primer and 4 µl template (25 ng total input). Standard curves were run on every plate, each template was run in duplicate for both Nb 18 S and mouse ApoB. Cycling conditions were as follows: 10 minutes at 95 °C followed by 40 cycles of: 15 seconds at 95 °C, 15 s at 60 °C, 15 s at 72 °C, followed by 10 minutes at 60 °C. ΔC_T values were calculated as Nb18S C_T - Mouse ApoB C_T and standards were plotted on a semi-log plot, where X was number of Nb larvae (on log scale) and Y was ΔC_T. Equation was generated by Prism as: Number of Nb = 10^{ΔC_T - Yintercept}/gradient, which was subsequently used to determine 'total Nb per ear'.

Tissue preparation

Blood was collected by brachial bleed, prior to BALF and lung collection, and kept at 4° overnight to clot. Blood was centrifuged and serum was collected and stored at -70°C.

Lung homogenate for measurement of cytokines was prepared from ¼ of the total lung mass, in 500 µl cell lysis buffer (Cell Signaling Technologies, Danvers, MA, USA) with 1:100 phenylmethylsulfonyl fluoride (Sigma-Aldrich, St Louis, MO, USA) added just before homogenization with a 5 mm stainless steel bead using a TissueLyser (Qiagen, Hilden, Germany). Tubes were centrifuged to remove debris from lysis supernatant, which was then stored at -20°C.

BALF was collected by flushing the airways, prior to lung removal, with three washes of 1 ml cold PBS. RBC were enumerated with a haemocytometer.

For flow cytometry, half the lung was minced into 2 ml digestion buffer (Dulbecco's Modified Eagle's Medium) (Thermo Fisher, Waltham, MA, USA) containing 2.4 mg/ml Collagenase 1 and 120 µg/ml DNase 1 (both from Sigma-Aldrich, St Louis, MO, USA) and put in a 37°C shaking incubator for 1 h. Tissue was then pushed through a 70 µm strainer to obtain a single cell suspension, RBC lysed (Sigma-Aldrich, St Louis, MO, USA) and viable cells counted with Trypan Blue.

Mesenteric lymph nodes were excised and mashed through a 70 µm strainer, RBC lysed and enumerated before CD4 separation.

CD4⁺ T cell separation for restimulation

CD4⁺ cells were separated from whole mesenteric lymph node preparations using the Dynabead FlowComp Mouse CD4 kit (Thermo Fisher, Waltham, MA, USA) as per manufacturer's instructions. For cytokine restimulation, 0.5 × 10⁶ CD4⁺ cells were stimulated for 4 days in 24-well plates in RPMI (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% PenStrep (both from Thermo Fisher, Waltham, MA, USA) and 20 U/ml rIL-2 (Peprotech, Rocky Hill, NJ, USA), with or without 50 ng/ml rIL-33 (R&D Systems, Minneapolis, MN, USA). Supernatants were collected and cellular debris removed before cytokine measurement.

Flow cytometry

1 × 10⁶ cells were washed twice with PBS in round-bottom 96-well plates and stained with 200 µl blue fluorescent LIVE/DEAD fixable dead cell stain (Thermo Fisher, Waltham, MA, USA) diluted in PBS, for 30 mins. Cells were then washed with PBS, and Fc receptors blocked (with 2.4G2), before staining for 20 mins with fluorochrome-labelled antibodies for surface antigens: CD45-PerCPCy5-5 (30.F11), CD4-BUV737 (GK1.5), TCRβ-BV605 (H57-597), SiglecF-PECF594 (E50-2440), CD11c-BUV395 (HL3),

CD11b-APC-Cy7 (M1/70)(all from BD, Franklin Lakes, NJ, USA), CD44-AF700 (IM7, Biolegend, San Diego, CA, USA), ST2-PerCP-eF710 (RMST2-2, Thermo Fisher, Waltham, MA, USA). Pre-gating for singlets and live cells was undertaken before analysis of other markers. Eosinophils were identified as CD45⁺ CD11b⁺ SiglecF⁺ CD11c⁻ distinguishing them from alveolar macrophages which were identified as CD45⁺ CD11b⁺ SiglecF⁺ CD11c⁺.

For intracellular IL-5 staining, 3×10^6 cells were stimulated with 50 ng/ml Phorbol 12-myristate-13-acetate, 1 µg/ml ionomycin and 10 µg/ml Brefeldin (all from Sigma-Aldrich, St Louis, MO, USA) for 4 h prior to surface staining. After surface staining, cells were permeabilised with Cytofix/Cytoperm (BD, Franklin Lakes, NJ, USA) for 20 mins before staining with IL-5-PE (TRFK5, BD, Franklin Lakes, NJ, USA) for a further 20 mins.

Cell suspensions were run on either the LSRII or Fortessa flow cytometers (BD) and data were analysed using FlowJo version 9.9.4 (Treestar, Ashland, OR, USA).

Lung sectioning and staining

Lung tissue was harvested directly into 10% formalin before embedding in paraffin. 10 µm sections were mounted onto slides and stained with haematoxylin and eosin (Thermo Fisher, Waltham, MA, USA) according to manufacturer's instructions. Pictures were taken using an Olympus BX51 compound microscope and DP73 camera.

Cytokine measurements

Cytokines were measured in lung homogenate (diluted 1:10 in PBS) using the LEGENDplex Mouse Th1/2 8-plex panel and the 13-plex Mouse Cytokine Panel 2 (Biolegend, San Diego, CA, USA), following the manufacturer's instructions. Data were collected on the LSRII flow cytometer and analysed using LEGENDplex Data Analysis Software. Cytokine or chemokine levels were normalised to total protein levels in the sample measured with a Bradford assay (Thermo Fisher, Waltham, MA, USA).

IL-5 was measured in neat serum samples and culture supernatants using the Mouse IL-5 ELISA Ready-SET-Go! kit (Thermo Fisher, Waltham, MA, USA).

Bromotyrosine measurements in BALF

BALF containing 40 µg protein (as measured by Bradford assay (Thermo Fisher, Waltham, MA, USA)) was used to measure bromotyrosine residues on proteins. BALF and isotope-labelled bromotyrosine standards were hydrolysed with 4 M methanesulfonic acid for 18 h at 110°C. Samples were then purified using solid phase extraction and run on a LCMS/MS (4000 QTrap, Thermo Fisher, Waltham, MA, USA) with multiple reaction monitoring and quantified against a standard curve containing the normal and stable isotopes as previously described.^{54,55}

Statistical analysis

All statistical analyses were done using GraphPad Prism version 7. All figures are presented with mean and error bars indicate standard deviation. Data from several similar experiments have been combined where possible, otherwise data are representative of several repeat experiments (indicated in legend). Groups were compared using a Student's *t* test. Statistically significant values are indicated as follows: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. ns = not statistically significant. nd = not detected.

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AUTHOR CONTRIBUTIONS

K.F. designed and performed the experiments and wrote the paper; M.C. assisted with experimental procedures and supervised the running of the parasite lifecycles; J. C. assisted with experimental procedures; R.T. and A.K. designed and undertook the bromotyrosine measurements in BALF; R.E. and P.G. designed and undertook the *T. muris* co-infection studies and G.L.G. contributed to the conception of the project, writing and discussion of this paper.

ADDITIONAL INFORMATION

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