Concerted IL-25R and IL-4Rα signaling drive innate type 2 effector immunity for optimal helminth expulsion

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Running Title: IL-25 in late-acting immunity
Interleukin 25 (IL-25) is a major 'alarmin' cytokine, capable of initiating and amplifying the type 2 immune response to helminth parasites. However its role in the later effector phase of clearing chronic infection remains unclear. The helminth *Heligmosomoides polygyrus* establishes long-term infections in susceptible C57BL/6 mice, but is slowly expelled in BALB/c mice from day 14 onwards. We noted that IL-25R (*Il17rb*)-deficient BALB/c mice were unable to expel parasites despite type 2 immune activation comparable to the wild-type. We then established that in C57BL/6 mice, IL-25 administered late in infection (days 14-17) drove immunity. Moreover when IL-25 and IL-4 were delivered to *Rag1*-deficient mice, the combination resulted in near complete expulsion of the parasite, even following administration of an anti-CD90 antibody to deplete innate lymphoid cells (ILCs). Hence, effective anti-helminth immunity during chronic infection requires an innate effector cell population that is synergistically activated by the combination of IL-4Rα and IL-25R signaling.
Introduction

Type-2 immunity is generated by the immune system in response to a range of environmental challenges from helminth worm parasites, ectoparasites and allergens (Hammad and Lambrecht, 2015, Harris and Loke, 2017). In the absence of any vaccines, gastrointestinal helminths continue to infect many hundreds of millions of people in less-affluent countries (Pullan et al., 2014). Type 2 immunity to parasites is critically dependent upon pathways driven through and co-ordinated by IL-4Ra signaling (Urban et al., 1998, Voehringer et al., 2006). IL-4Ra activation can result in a multitude of immune outcomes (Allen and Maizels, 2011, Patel et al., 2009), however, few other components of immunity have been defined as either necessary or sufficient for worm expulsion.

The epithelial-derived ‘alarmin’ cytokines, including IL-25, IL-33 and TSLP elicit and amplify type 2 immunity through the action of group 2 innate lymphoid cells (ILC2s) (Koyasu and Moro, 2012, McKenzie et al., 2014, Ziegler and Artis, 2010). There are however important distinctions in both the modes of action of these cytokines, and in the cells, bearing receptors for each mediator. Thus, ligation of the heterodimeric TSLP receptor stimulates dendritic cells to favor a Th2 cell response, and activates innate type 2 population (Ito et al., 2005, Siracusa et al., 2011, Soumelis et al., 2002, Ziegler et al., 2013); IL-25 receptors (heterodimers of IL-17RA and IL-17RB chains) are expressed on a number of tissues and cell types and increase IL-4 expression by Th2 cells (Angkasekwinai et al., 2007); and receptors for IL-33 (an Interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2) are broadly expressed in many tissues and induce type 2 cytokine expression by ILC2s and T cells (Cayrol and Girard, 2018, Schmitz et al., 2005).

Although IL-25 (originally termed IL-17E) shares structural similarity with other IL-17 family members it acts in an opposing manner to IL-17A, by inducing a suite of Type 2 responses (Kim et al., 2002, Pan et al., 2001) and preventing IL-17A-dependent autoimmune disease (Kleinschek et al., 2007). IL-25 is highly expressed within the tuft cells of the gastrointestinal tract (Gerbe et al., 2016, von Moltke et al., 2016), but is also produced by a range of core immune populations, including T cells and granulocytes. IL-25 plays an important role in inducing allergic inflammation (Barlow...
et al., 2012, Petersen et al., 2014) and in driving tissue remodeling and fibrosis
(Gregory et al., 2013, Hams et al., 2014)).

A number of laboratories have reported that IL-25 is induced by infection with, and
required for immunity to, the nematode *Nippostrongylus brasiliensis*, a rat parasite that
briefly infects immune-competent mice (Fallon et al., 2006, Fort et al., 2001, Hurst et
al., 2002, Zhao et al., 2010), as well as the nematodes *Heligmosomoides polygyrus*
(Pei et al., 2016), *Trichuris muris* (Owyang et al., 2006) and *Trichinella spiralis*
(Angkasekwinai et al., 2013). IL-25 may be more important in activation of innate than
adaptive immunity, as IL-25-deficient mice mounted normal Th2 responses following
*N. brasiliensis* infection (Mearns et al., 2014), while ILCs grown in vitro in the
presence of IL-25 and IL-33 were able to stimulate expulsion of this parasite upon
adoptive transfer to susceptible IL-25R-deficient (*Il17rb<sup>-/-</sup>*) mice (Neill et al., 2010).

Due to the rapid nature of expulsion of *N. brasiliensis*, it is difficult to separate the role
of IL-25 in the initial drive towards Th2 responsiveness and any subsequent activation
of effector mechanisms. We therefore studied chronic infection with the nematode *H.
polygyrus*, in which induction over the first 7-10 days can be analysed separately from
the subsequent expulsion phase: in so doing, we report that IL-25 is in fact redundant
for Th2 initiation, but acts during the later phase of effector expulsion against this
helminth parasite. Such a downstream role is consistent with recent reports
demonstrating a role for IL-25 in protective immunity to a secondary infection with *H.
polygyrus*, following drug clearance of a primary infection (Pei, et al., 2016), or
vaccination with parasite secreted antigens (Hewitson et al., 2015). More broadly, a
critical role for IL-25 in a late-stage immune response is also evident in airway
inflammation models, in which it plays a central role in stimulating inflammatory
myeloid cells and promoting airway remodeling (Gregory, et al., 2013, Petersen et al.,
2012), or in driving allergic reactions to airway challenge in previously sensitized mice
(Ballantyne et al., 2007, Cayrol and Girard, 2018).
Results

IL-25R is redundant for generation of adaptive type-2 responses following chronic helminth infection

To investigate the role of IL-25R in generation of innate and adaptive type-2 responses following chronic parasite infection, we used mice deficient in the IL-25-specific receptor subunit \textit{Il17rb} (Neill, \textit{et al.}, 2010). We evaluated the outcome of \textit{H. polygyrus} infection in mice of the BALB/c genetic background, which are partially resistant and able to expel most adult worms by day 28 following infection (Filbey \textit{et al.}, 2014, Reynolds \textit{et al.}, 2014). At day 14 post-infection, shortly after adult worms have matured, \textit{Il17rb\textsuperscript{-/-}} mice exhibited significantly increased egg production (Fig. 1 A) but equivalent adult worm burdens (Fig. 1 B) to the IL-25-sufficient wild-type BALB/c. By day 28, the \textit{Il17rb\textsuperscript{-/-}} genotype was unable to reduce adult worm or egg burdens (Fig. 1 C, D).

A number of cell types have been shown to express IL-25R and contribute to type-2 inflammation, including ILCs and multi-potent progenitor type 2 cells (Huang \textit{et al.}, 2015, Saenz \textit{et al.}, 2013), myeloid cells (Dolgachev \textit{et al.}, 2009, Petersen, \textit{et al.}, 2012), NKT cells (Stock \textit{et al.}, 2009, Terashima \textit{et al.}, 2008) and eosinophils (Kim, \textit{et al.}, 2002). We first took the approach of analysing individual cell types within BALB/c and \textit{Il17rb\textsuperscript{-/-}} mice and found equivalent increases in total and IL-13-expressing ILCs in the MLN (Fig. 1 E, F) in both strains at day 14 post-infection, and similar increases in the number of Siglec-F\textsuperscript{-}CD11b\textsuperscript{+}Ly6C/G\textsuperscript{+} myeloid cells in the peritoneal lavage (PL) (Fig. 1 G). However, we noted significantly reduced proportions of Siglec-F\textsuperscript{+} eosinophils and resistin-like molecule (RELM)-\textalpha\textsuperscript{+} macrophages in the PL of \textit{Il17rb\textsuperscript{-/-}} mice at steady-state and during the acute phase of infection (day 7). From day 14 when parasites are in the intestinal lumen and infection enters chronicity, proportions of both cell types increased closer to levels found in wild-type mice (Fig. 1 H, I). We also evaluated typical type-2 cytokine production (IL-4, IL-5 and IL-13) in response to restimulation with parasite antigen (HES); this was found to be equivalent in \textit{H.polygyrus}-infected BALB/c and \textit{Il17rb\textsuperscript{-/-}} mice (Fig. 1 J-L). Intracellular cytokine staining of MLN CD4\textsuperscript{+} T cell populations showed no significant difference in
expression of IL-4 (Fig. 1 M), IL-5 (Fig. 1 N) or IL-13 (Fig. 1 O) between 
*H. polygyrus*-infected BALB/c and *Il17rb<sup>−/−</sup>* mice.

**Effective clearance of adult worms requires IL-25R within the hematopoietic immune compartment.**

During inflammation, expression of IL-25R has also been reported on antigen presenting cells (Gratchev et al., 2004), memory T cells (Wang et al., 2007), eosinophils (Tang et al., 2014) and human vascular endothelial cells (Wang et al., 2012) as well as human fibroblasts (Gregory, et al., 2013). In addition, intestinal smooth muscle hypercontractility is compromised in IL-25-deficient helminth-infected mice (Pei, et al., 2016). Thus non-hematopoietic or hematopoietic cell expression of IL-25R might contribute to parasite expulsion in BALB/c mice. To assess this possibility, bone marrow chimeras were generated, infected with *H. polygyrus* and analysed 28 days later for egg and worm burden. Control chimeras reflected the phenotypes of intact mice as *Il17rb<sup>−/−</sup>* reconstituted with *Il17rb<sup>−/−</sup>* bone-marrow had significantly higher worm burdens than BALB/c reconstituted with BALB/c bone-marrow (Fig. 2 A). Efficient adult worm expulsion and decreased egg burden were evident in mice lacking IL-25R on the non-hematopoietic compartment (*Il17rb<sup>−/−</sup>* reconstituted with BALB/c bone-marrow), however, delayed worm expulsion and increased egg burden occurred in mice lacking IL-25R within the hematopoietic immune compartment (Fig. 2 A, B).

**Effective clearance of adult worms in immune-deficient mice requires IL-25R and IL-4Rα signaling through the innate immune compartment**

To test whether stimulation of IL-25R within the innate immune compartment mediates adult worm expulsion and whether this is enhanced following IL-4Rα signaling, immune-deficient *Rag1<sup>−/−</sup>* mice were infected with *H. polygyrus* and injected with recombinant IL-25 late in infection (d14-17) and/or a complex of rIL-4:anti-IL-4 (IL-4C) on days 13, 16 and 19 post-infection (Fig. 3 A). IL-4C exerted significant but modest reductions in egg counts (44%) and adult worm burden (34%) in *Rag1<sup>−/−</sup>* mice but did not completely expel adult worms or eliminate egg production (Fig. 3 B, C). Administration of IL-25 alone to *Rag1<sup>−/−</sup>* mice elicited a downward trend in adult worm numbers, which did not attain statistical significance in two experimental repeats.
However when both cytokines were combined, egg production was reduced by 95% and the adult worm burden also greatly reduced (by 87%).

ILC2s may have a role in promoting acquired type-2 immune responses by activation of CD4+ T cell responses through expression of OX40L, MHC class II and PD-L1 (Drake et al., 2014, Mirchandani et al., 2014, Oliphant et al., 2014, Schwartz et al., 2017) or by promoting dendritic cell migration to draining lymph nodes following IL-13 production (Halim et al., 2014). Sustained activation of ILCs drives immunity to N. brasiliensis infection (Bouchery et al., 2015), however in H. polygyrus infection the transfer of activated ILC2s had only a limited effect on worm establishment (Pelly et al., 2017). In addition, IL-25 is able to induce type-2 inflammation and goblet cell hyperplasia in the small intestine, independently of ILCs (Saenz, et al., 2013). To test whether ILCs contribute to efficient worm expulsion in immune-compromised mice following co-administration of IL-25 and IL-4C, we treated H. polygyrus infected Rag1−/− mice with both cytokines and isotype or anti-CD90.2 (Thy1.2) antibodies. Administration of anti-CD90.2 antibody significantly reduced the number of CD45^Lin^- ILCs (Fig. 3 D, E) as well as the number of CD45^Lin^-ST2^- and CD45^Lin^-ST2^+ ILCs (Fig. 3 F, G) in the PL of H. polygyrus-infected Rag1−/− mice treated with IL-25 and IL-4C. However, immunity was fully intact in recipients of this antibody, with significantly decreased parasite egg and worm burden in ILC-depleted mice (Fig. 3 H, I).

**Stimulation of IL-25R induces adult worm clearance late, but not early, in infection**

The late manifestation of the IIL17rb−/− phenotype in H. polygyrus infection could reflect a requirement either for initial IL-25 to generate an effector response that is only active after day 14, or for a later IL-25R-driven pathway that is invoked once the Th2 response is generated. To evaluate the relative importance of IL-25R engagement during different phases of infection, we made use of the more susceptible C57BL/6 strain and delivered exogenous rIL-25 early (day 1-4) and late (day 14-17) post-infection (Fig. 4 A). Egg counts were equivalent at day 14 post-infection before delivery of IL-25 late (Fig. 4 B) and were significantly reduced in recipients of IL-25 early (66%; p=0.0013) or late (64%; p=0.0071) compared to PBS controls by day 28 post-infection (Fig. 4 C). Although early IL-25 administration also induced a modest
reduction in adult worm burdens, this did not reach significance in two repeated
experiments. In contrast, adult worm burden was significantly reduced in recipients of
IL-25 late (66%; p=0.0016) at day 28 post-infection (Fig. 4 D). Both CD4⁺ T cell IL-4
and IL-13 responses (Fig. 4 E-F), and the total number of ILCs (Fig. 4 G) were not
significantly altered by late IL-25 injection. The number of IL-13⁺ ILCs (ILC2s) was
not significantly increased in the MLN at day 18 H. polygyrus infection but was
significantly increased in infected mice following the administration of IL-25 (Fig. 4
H), however, these remained a small proportion of the total ILCs within the MLN

Monocytes and eosinophils require IL-25R and IL-4Rα signaling for a maximal
type-2 response.

Monocytes are important regulators of wound repair following Th2 inflammation and
are activated to proliferate within the tissue site in response to IL-4Rα signaling, as
confirmed in H. polygyrus-infected mice (Jenkins et al., 2013). These cells also
respond to the alarmins IL-25 and IL-33 to promote type-2 cytokine production and
alternative activation, where adoptive transfer of IL-33-activated macrophages has
been reported to induce worm expulsion in mice with chronic H. polygyrus infection
(Yang et al., 2013). Eosinophils also respond to IL-25 and are thought to play a role in
limiting Th2 responses following H. polygyrus infection (Strandmark et al., 2017).

Analysis of the peritoneal lavage revealed striking changes within macrophage and
eosinophil populations following administration of IL-25 to H. polygyrus infected
mice. IL-25 injection significantly increased production of IL-13 by Siglec-F⁺CD11b⁺F4/80⁺ monocyte populations following administration of IL-25 to naïve and
H. polygyrus infected mice (Fig. 5 A, B). Administration of IL-25 also significantly
increased expression of the marker of alternative activation RELM-α by naïve
macrophages in vivo (in an environment with low IL-4/13 levels), however alternative
activation was further increased above and beyond this level in the setting of H.
polygyrus infection (Fig. 5 B, C). A similar pattern was seen for the number of
eosinophils in the PL (Fig. 5 D, E). The number of peritoneal monocytes was also
significantly increased in response to IL-25, similar to the setting of H. polygyrus
infection and a combination of infection and IL-25 (Fig. 5 F). In vitro treatment of
bone-marrow-derived macrophages with recombinant cytokines confirmed previous
reports that IL-4, but not IL-25, induced expression of the alternatively activated
macrophage marker RELM-α (Rizzo et al., 2012). However, when combined in vitro, we found a very marked synergy between IL-4 and IL-25 for increased macrophage RELM-α (Fig. 5 G, H), as well as Arginase-1 expression (Fig. 5 I, J); in each case far above that observed for either cytokine alone.

**IL-25R co-operates with IL-4Rα signaling to prime innate immunity for effective clearance of *H. polygyrus***.

It has long been known that IL-4Rα-mediated signaling is the pivotal component of the protective immune response to helminth infection, being required to generate the appropriate innate and adaptive type 2 cellular responses. To test whether IL-25-mediated promotion of immunity to primary *H. polygyrus* infection was entirely dependent upon IL-4Rα, or could mediate an IL-4Rα-independent mode of protection, we administered exogenous IL-25 from days 14-17 of infection to *Il4ra*-deficient mice (Fig. 6 A). Although late IL-25 injection was previously demonstrated to induce adult worm expulsion in C57BL/6 mice (Fig. 4 D), the worm burden of *Il4ra*-/- mice was unaffected by administration of IL-25 at this time-point (Fig. 6 B). In a similar manner to C57BL/6 mice (Fig 5 C and D) IL-25 injection significantly increased the percentage of eosinophils in the PL of *H. polygyrus* infected BALB/c mice by day 18 (Fig. 6 C). Within the BALB/c strain, 100% of macrophages within the PL expressed RELM-α by day 18 in *H. polygyrus* infected mice following administration of IL-25 or PBS control, compared to an average of 28% in naïve mice (Fig. 6 D). However, these responses were completely lacking in infected *Il4ra*-/- mice treated with IL-25 (Fig. 6 C, D), confirming that the IL-25 induced response is itself wholly dependent on IL-4Rα signaling.
Discussion

Generation of the Type 2 immune response, and protection from helminth parasite infection, requires sustained interaction and participation of both innate and adaptive immune cells (Allen and Maizels, 2011, Grencis, 2015, Harris and Loke, 2017, Van Dyken et al., 2016). Alarmins such as IL-25 are potent activators of Type 2 immunity, but as we report here, can play an even more important role in stimulating and mobilising effector mechanisms to expel infection. Specifically, we show that adaptive type 2 responses develop normally in the absence of IL-25R but that eosinophil and macrophage responses are impaired, which is likely to explain compromised helminth expulsion in IL25R-deficient mice.

Notably, by day 28 of *H. polygyrus* infection, the *Il17rb* \(^{-/-}\) genotype was unable to reduce adult worm or egg burdens, despite the expression of comparable adaptive Th2 response features such as IL-4 and IL-13 expression in CD4\(^+\) T cells. A requirement for IL-25/IL-25R signaling to expel *H. polygyrus* has previously been reported in other settings in which Th2 response levels appear unaffected, including both a vaccination model (Hewitson, et al., 2015), and following drug-abbreviated primary infection (Pei, et al., 2016). Similarly, it has been reported that the resolution of acute *N. brasiliensis* infection is delayed in *Il25* \(^{-/-}\) mice despite sufficient Th2 cell differentiation (Mearns, et al., 2014). These results therefore suggest that Th2 differentiation can occur independently of IL-25R signaling following helminth infection and reinforce the idea that innate type-2 responses can operate autonomously from adaptive type-2 responses to control parasite expulsion (Smith et al., 2012).

A wide range of cell types respond to IL-25, but we demonstrated by chimera experiments that parasite expulsion requires receptor expression on haematopoietic, rather than non-haematopoietic cells *in vivo*. As adaptive Th2 responses were comparable in IL-25-deficient and sufficient infected mice, we examined innate populations. In *N. brasiliensis* infections, intestinal tuft cell-derived IL-25 drives parasite expulsion and the activation of ILC2s in a positive feedback loop mediated by IL-13 (Gerbe, et al., 2016, von Moltke, et al., 2016). However, our data indicate that innate lymphoid cells may not be required for immunity to *H. polygyrus*, because anti-CD90 (Thy1) antibody depletion of these cells from *Rag1*-deficient mice did not affect the ability of IL-25 (in combination with IL-4R\(\alpha\) signaling) to expel *H. polygyrus*.
parasites. Through the use of ST2 staining we were able to demonstrate that both the resident Lin^ST2^CD90^{hi}, and the inflammatory Lin^ST2^CD90^{low} ILC populations recently described (Huang, et al., 2015) are depleted following administration of anti-CD90.2 antibody to H. polygyrus-infected mice given IL-25. It is possible that our regime of anti-CD90.2 antibody treatment spares a residual and essential ILC population, but genetic strategies to fully ablate ILCs by deletion of the common gamma chain receptor (γc) are confounded in our system because γc also forms part of the IL-4R.

It has previously been noted that ILC2 numbers show only modest expansion in the MLN of C57BL/6 mice infected with H. polygyrus and that these can be considerably boosted following administration of IL-25 (Hepworth et al., 2012). In confirming this, our data also show an increase in total ILCs with H. polygyrus infection, of which only a small proportion are ILC2s. One explanation is that chronic H. polygyrus infection limits IL-25 and ILC2 expansion by eliciting the production of IL-1β (Zaiss et al., 2013). We have also demonstrated that H. polygyrus infection results in the preferential trafficking of LTi-like ILC3s to the MLN (Mackley et al., 2015), pointing again to a diminished role of ILC2s in immunity to this infection., while a recent study reported only modest reduction in adult H. polygyrus worm burdens in mice in whom ILC2 numbers were expanded fivefold with IL-2:anti-IL-2 complex (Pelly, et al., 2017).

In contrast, we uncover striking IL-25R-dependent modifications to macrophage and eosinophil populations following H. polygyrus infection, in two important respects. Firstly, the expansion of eosinophil and alternatively activated macrophage numbers seen following helminth infection is strikingly amplified by IL-25. Secondly, IL-25 was required for the expression of IL-13 within macrophages, in accordance with earlier publications (Fort, et al., 2001, Yang, et al., 2013). Macrophages may thus represent a major source of IL-13 in infected animals, suggesting an element of autologous stimulation through the IL-13-responsive IL-4Rα, which is required for macrophage alternative activation.

The alternative activation of macrophages is critical for immunity to H. polygyrus (Anthony et al., 2006), and we now show that IL-25, in addition to IL-4Rα signaling, is required both for optimal macrophage activation, and for parasite elimination. Thus,
for example, the two cytokines synergise dramatically to induce high levels of RELM-α. Consistent with established findings, macrophage activation by IL-25 cannot occur in the absence of IL-4Rα signaling (Kang et al., 2012). Interestingly, the production of IL-25 following nematode infection is itself dependent on IL-4Rα, mediated by IL-13 activation of STAT6 (Zhao, et al., 2010). Furthermore, upregulation of the IL-25 receptor Il17rb in the small intestine is STAT-6 dependent (Zhao, et al., 2010). We are currently investigating whether macrophage expression of the IL-25 receptor is specifically required for parasite expulsion in the presence of IL-4Rα signaling.

In summary, we demonstrate that the IL-25R is not required for generation of a sufficient Th2 response to helminth infection and that instead, it is required for late effector responses to fully resolve chronic infection. We identify the macrophage and the eosinophil as two prominent populations activated following IL-25 administration, with induction of IL-13 expression within macrophages that is likely, in turn, to drive the suite of intestinal epithelial mechanisms that lead to nematode expulsion (Cliffe et al., 2005, Maizels et al., 2012, Patel, et al., 2009). We also clearly demonstrate an enhancement in macrophage alternative activation, which plays a critical role in nematode immobilisation and killing (Hewitson, et al., 2015) and suggest that ILC2s may be redundant in driving IL-25R-dependent immunity to chronic helminth infection. These results now place IL-25 in the central role of mobilising innate effector cells other than ILCs, in the context of an IL-4/13-replete environment, to protect against chronic gastrointestinal helminth infection. Future work will elucidate in greater detail the range of responsive cells and their inter-relationship in the network that orchestrates parasite expulsion and immunity.
### Materials and Methods

#### Key Resources Table

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Mice

BALB/c, IL-25R<sup>−/−</sup> (Il17rb<sup>−/−</sup>) (Neill, et al., 2010), C57BL/6, Rag<sup>1</sup>−/− mice and Il4ra<sup>−/−</sup> mice were bred at the University of Edinburgh. All animal protocols adhered to the guidelines of the UK home office, complied with the Animals (Scientific Procedures) Act 1986, were approved by the Ethical Review Committees of the University of Edinburgh and the University of Glasgow, and were performed under the authority of the UK Home Office Project numbers 60/4105 and 70/8384.

Construction of bone marrow chimeras

BALB/c or Il17rb<sup>−/−</sup> mice were exposed to 11.5 Gy γ radiation administered in two doses before intravenous reconstitution with 2 million bone marrow cells from BALB/c or Il17rb<sup>−/−</sup> mice, which had been depleted of CD90<sup>+</sup> cells using CD90.2 microbeads (Miltenyi). Eight weeks post-transfer, recipients were infected with 200 H. polygyrus by gavage.

Parasites and Antigens

H. polygyrus bakeri was maintained and adult H. polygyrus HES was prepared as described elsewhere (Grainger et al., 2010, Johnston et al., 2015). Egg counts from individual mice were assessed by weighing the feces before dissolving in 2 ml saturated sodium chloride solution; egg counts were performed using a McMaster chamber and the average number of eggs/g feces calculated per sample. Mice were infected with 200 L3 stage H. polygyrus by gavage.

Preparation and administration of IL-4/anti-IL-4 complexes

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A pre-prepared complex of 5μg rIL-4 (Peprotech) and 25μg anti-IL-4 (clone 11B11; BioXcell, NH) was administered to mice i.p. (*Urban et al., 1995*).

**Depletion of ILCs**

*Rag1*−/− mice received 200μg anti-CD90.2/Thy1.2 (clone 30H12; BioXcell) or a rat IgG2b control (clone LTF-2; BioXcell) i.p. on days 12, 15, 18 and 21 post-infection.

**In vitro Ag-specific restimulation**

A single cell suspension was made of MLN before plating cells at 5x10^5/well in the presence of 2μg/ml HES and media alone for 72 h at 37°C/5% CO_2_. Supernatants were then harvested and analysed for IL-4, IL-5, IL-13 by commercially available ELISA (BD Pharmingen).

**Generation of bone marrow-derived macrophages**

Bone marrow was extracted from tibia and femurs of C57BL/6 mice. A single cell suspension was formed in 10ml of PBS by passing through a 23g needle, then filtered through a 100μm nylon cell strainer. Cells were plated at a density of 6 x 10^6 cells/plate on 90 cm Petri dishes in 10 ml cDMEM with 20% L929 media as a source of M-CSF and incubated at 37°C incubator with 5% CO_2_. A further 5 ml of cDMEM with 20% L929 was added on day 3. Differentiated macrophages were harvested on day 7 using 3 mM EDTA/10 mM glucose in PBS. Cells were washed in PBS, resuspended in cDMEM, plated at 2 x 10^5 cells/well in 96 well plates and stimulated with 10ng/ml IL-4, 200ng/ml IL-25 or a combination of both for 16 hours before analysis of the cells by flow cytometry.

**Flow Cytometry**

All flow cytometry was performed using Becton-Dickinson Canto or LSR-II flow cytometers. For innate cell surface phenotyping, PL or MLN were stained with a combination of antibodies to Siglec-F (E50-2440), CD11b (M1/70), F4/80 (BM8), Ly6G (1A8) and Ly6C (AL-21 or HK1.4). Following fixation and permeabilisation with the Foxp3 staining kit (eBioscience) cells underwent intracellular staining with RELM-α (RnD Systems) followed by zzenon anti-rabbit A647 (Invitrogen) and FITC-
conjugated anti-human Ki67 (BD Biosciences). For intracellular cytokine staining of monocytes, 0.5-1x10^6 PL cells were incubated with 10μg/ml Brefeldin A for 4 hours. Following cell surface staining as above, and fixation and permeabilization with Fix/Perm buffer (BD Pharmingen), cells underwent intracellular staining with anti-IL-13 (JES10-5A2). For intracellular staining of lymphocytes, MLNCs were incubated with 0.5μg/ml PMA and 1μg/ml ionomycin for 1 h before the addition of 10μg/ml Brefeldin A for a further 3 h. Staining was performed by re-suspending cells in a combination of Abs to CD4 (GK1.5), ICOS (DX29), and the following combination to define Lin−: CD3 (17A2), CD5 (53-7.3), CD8α (RPA-T8), CD49b (DX5), CD11c (HL3), F4/80 (BM8), CD19 (eBio1D3), Gr-1 (RB6-8C5), TCRβ (H57-597) and CD11b (M1/70).

**Statistical analysis**

Data were assessed for normality and equal variance and were log transformed if required; all data passed these criteria and an unpaired t test was used or, where more that three groups were tested, a parametric one-way ANOVA followed by Tukey’s multiple comparison test was used.

**Acknowledgements**

We thank Judi Allen and Dominik Rückerl (University of Manchester, UK) for Rag1−/− and II4ra−/− mice. We thank the Wellcome Trust for support through a Senior Investigator Award to RMM for SL (Ref 1061220), the European Commission for support through a Marie Skłodowska-Curie global fellowship to KAS, an Edinburgh Clinical Academic Track (ECAT) Studentship for FV, and for core support from the Wellcome Trust through the Wellcome Centre for Molecular Parasitology (Ref 104111).
References


Figure Legends

Figure 1

IL-25R signaling is required for expulsion of *H. polygyrus* from BALB/c mice

*H. polygyrus*-infected BALB/c or *Il17rb*-deficient (IL25R−/−) BALB/c mice were analyzed at day 14 post-infection for fecal egg counts (A) and intestinal adult worm burden (B) or day 28 post-infection for fecal egg counts (C) and intestinal adult worm burden (D). MLN underwent intracellular cytokine staining (ICCS) to compare the number of Lin ICOS+ innate lymphoid cells (ILCs) (E) and IL-13+ ILC2s (F) by flow cytometry in the different naïve and day 14 infected genotypes. Peritoneal lavage cells (PL) were stained with Siglec-F, CD11b and Ly6G/C to compare the number of Siglec-F CD11b+Ly6G/C+ monocytes (G) in the different naïve and day 14 infected genotypes. Percentages of eosinophils (H) and RELMα+ alternatively-activated macrophages (I) were also determined over a 4-week infection timecourse. ELISA of supernatants from MLN incubated with media or 1 μg HES for 72 h was performed to compare IL-4 (J), IL-5 (K) and IL-13 (L) production in the different naïve and day 14 infected genotypes. ICCS of MLN allowed a comparison of the proportion of IL-4+, IL-5+ and IL-13+ CD4+ T cells by flow cytometry in the different naïve and day 14 infected genotypes (M-O). Results shown are one representative of 3 experiments with n≥4 mice/group (A-D), pooled data from 2 experiments with n≥4 mice/group (C-F, M-O) or one representative of 2 experiments with n≥4 mice/group (G-L). Data were analysed by unpaired *t* test or one way ANOVA, where * = p≤0.05, ** = p≤0.01, *** = p≤0.001 and ns = not significant. Error bars represent Standard Error of the Mean.
Figure 2

IL-25R signaling is required within the hematopoietic compartment for efficient expulsion

Bone marrow chimeras generated from BALB/c (WT) or Il17rb-deficient (KO) donor and BALB/c (WT) or Il17rb-deficient (KO) recipient mice were infected with *H. polygyrus* and intestinal adult worm burden (A) and fecal egg burden (B) performed at day 28 post-infection. Results shown are pooled data from 2 experiments performed with n≥3 mice/group, and data from all individual mice are presented. Data were analysed by unpaired *t* test, where * = p≤0.05, ** = p≤0.01, *** = p≤0.001 and ns = not significant. Error bars represent Standard Error of the Mean.

Figure 3

IL-25R signaling synergises with IL-4Ra within the innate immune compartment to facilitate efficient worm expulsion.

*H. polygyrus* infected *Rag1*−/− mice (RAG−/−) were given 0.4 µg recombinant IL-25 i.p. days 14-17 (late) post-infection with or without a complex of 5 µg rIL-4 and 25 µg anti-IL-4 (IL-4C) on days 13, 16 and 19 post-infection, according the schedule shown in (A). Mice were analyzed at 28 days post-infection for fecal egg burden (B) and intestinal adult worm burden (C). *H. polygyrus*-infected *Rag1*−/− mice were given IL-25 and IL-4C according to the same schedule, as well as 200 µg of anti-CD90.2/Th1.2 antibody or rat IgG2b control (days 12, 15, 18 and 21). The peritoneal lavage was analyzed at 28 days post-infection for CD45+lin− (CD3, CD5, CD8α, CD11c, CD19, DX5, F4/80, GR-1, TCRβ, CD11b), ICOS and ST2 staining by flow cytometry as shown (D) and the number of CD45+lin− (E), CD45+lin ST2− (F) and CD45+lin ST2+ (G) ILCs was determined. Mice were analysed at 28 days post-infection for fecal egg burden (H) and intestinal adult worm burden (I). Results shown are one representative of 2 experiments with n=4 mice/group (D-G), or pooled data from 2 experiments with n≥3 mice/group (B,C,H,I). Data were analysed by unpaired *t* test, where * = p≤0.05, ** = p≤0.01, *** = p≤0.001 and ns = not significant. Error bars represent Standard Error of the Mean.
**Figure 4**

**IL-25 induces protective immune responses in the late stage of infection**

*H. polygyrus* infected C57BL/6 mice were given 0.4 μg recombinant IL-25 i.p. at day 1-4 (early) or day 14-17 (late) post-infection according to the schedule shown in (A). Before administration of IL-25, intestinal egg burden was analysed in two groups at day 14 post-infection (B). Mice were then analysed at day 28 post-infection for fecal egg counts (C) and intestinal adult worm burden (D) following administration of IL-25 early or late. *H. polygyrus* infected C57BL/6 mice were given 0.4μg recombinant IL-25 i.p. at day 14-17 (late) post-infection. Day 18 post-infection, MLN underwent ICCS to compare the proportion of IL-4+CD4+ (E) and IL-13+CD4+ (F) T cells, as well as the number of ILCs (G) and Lin-IL-13+ ILC2s (H) by flow cytometry. Results shown are data pooled from 3 experiments with n≥3 mice/group (B-D), or are representative of 2 experiments with n=4 mice/group (E,F) or pooled from 2 experiments with n=4 mice/group (G, H). Data were analysed by unpaired t test, where * = p≤0.05,** = p≤0.01,*** = p≤0.001 and ns = not significant. Error bars represent Standard Error of the Mean.

**Figure 5**

**IL-25 drives alternative activation and IL-13 expression of macrophages**

*H. polygyrus* infected C57BL/6 mice were given 0.4 μg recombinant IL-25 i.p. at day 14-17 (late) post-infection. At day 18 post-infection, PL cells were taken and stimulated with 10μg Brefeldin A to determine the expression intensity (geomean) of RELM-α (B, C) within Siglec-F+CD11b+F4/80+ monocytes by flow cytometry, as shown by the example flow cytometry plots in B and compared to fluorescence minus one (FMO) samples stained from the Hp:IL-25 group. Total Siglec-F+CD11b+ eosinophil (D) numbers were calculated from populations shown in the example flow cytometry plots (E), numbers of Siglec-F+CD11b+ (F) monocytes. Bone marrow-derived macrophages from C57BL/6 mice were generated *in vitro* and stimulated with 10 ng/ml IL-4, 200 ng/ml IL-25 or a combination of both for 16 hours before analysis of the percentage of RELM-α expression (G, H) and the mean fluorescence intensity (geometric mean) of Arginase-1 expression (I, J) within CD11b+F4/80+ cells by flow cytometry. Results shown are one representative of 2 experiments with n=4 mice/group.
(A-F, I, J) or pooled from 2 experiments with n=4 replicates/group (G, H). Data were analysed by unpaired t test, where * = p≤0.05, ** = p≤0.01, *** = p≤0.001 and ns = not significant. Error bars represent Standard Error of the Mean.

Figure 6

**IL-4Ra expression is required for activation of the immune system by IL-25.**

*H. polygyrus* infected *Il4ra*<sup>−/−</sup> mice (IL-4R<sup>−/−</sup>) were given 0.4 μg recombinant IL-25 i.p. days 14-17 (late) post-infection (A). Mice were analyzed at day 28 post-infection for intestinal adult worm burden (B). *H. polygyrus* infected BALB/c and *Il4ra*<sup>−/−</sup> mice (IL-4R<sup>−/−</sup>) were given 0.4 μg recombinant IL-25 i.p. days 14-17 (late) post-infection. Day 18 post-infection, PL cells were taken to determine the percentage of eosinophils (C) and percentage of RELM-α expression within Siglec-F<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages by flow cytometry (D). Results shown are one representative of 2 experiments with n≥4 mice/group (A-D). Data were analysed by unpaired t test, where * = p≤0.05, ** = p≤0.01, *** = p≤0.001 and ns = not significant. Error bars represent Standard Error of the Mean.
Smith et al., Figure 1

A. Egg burden d14

B. Worm burden d14

C. Egg burden d28

D. Worm burden d28

E. MLN number of ILCs (10^7)

F. MLN number of IL-13+ILCs

G. PL number of Siglec-F-CD11b+Ly6G/C+ (10^6)

H. PL % Eosinophils

I. PL % Relm-α within Siglec-F-CD11b+F4/80+

J. MLN IL-4 (pg/ml)

K. MLN IL-5 (pg/ml)

L. MLN IL-13 (pg/ml)

M. % IL-4+ within CD4+

N. % IL-5+ within CD4+

O. % IL-13+ within CD4+
A. 

\[ H.\text{polygyrus} \text{ infection (RAG}^{-/-}\text{)} \rightarrow \text{PBS IL-25 IL4C IL4C + IL-25} \rightarrow \text{d28 Harvest} \]

B. 

Egg burden d28

C. 

Worm burden d28

D. 

ISO aCD90.2 Lineage IL-25/IL-4C

E. 

PL CD45Lin number

F. 

PL CD45LinST2 number

G. 

PL CD45LinST2 number

H. 

Egg burden d28 (x10^3)

I. 

Worm burden d28
A. Schematic diagram showing the experimental setup:

- IL-25 Early: d1-4 i.p.
- IL-25 Late: d14-17 i.p.
- H. polygyrus infection (C57BL/6)
- d28 Harvest

B. Egg burden d14

- Prior to PBS
- Prior to IL-25 Late

C. Egg burden d28

- PBS
- IL-25 Early
- IL-25 Late

D. Worm Burden d28

- PBS
- IL-25 Early
- IL-25 Late

E. % IL-4+ within CD4+

- Naive
- Hp:PBS
- Hp:IL-25

F. % IL-13+ within CD4+

- Naive
- Hp:PBS
- Hp:IL-25

G. MLN number of ILCs (x10^7)

- Naive
- Hp:PBS
- Hp:IL-25

H. MLN number of IL-13+ILCs (x10^7)

- Naive
- Hp:PBS
- Hp:IL-25
Smith et al., Figure 5

A
PL IL-13 geomean within Siglec-F+CD11b+F4/80

B

C
PL Relm-α geomean within Siglec-F+CD11b+F4/80

D

E

F
PL Eosinophil number (x10^7)

G
% RELM-α+ within F4/80+CD11b+

H
FMO Unstimulated IL-25 IL-4 IL-4 + IL-25

I
Arginase-1 MFI within F4/80+CD11b+
Smith et al., Figure 6

**A**

*H. polygyrus* infection (IL-4Ra–/–) → IL-25 Late d14-17 i.p. → d28

**B**

Worm Burden d28

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**C**

d18 PL % Eosinophils

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**D**

d18 PL % Relm-α within Siglec-F+CD11b+F4/80+

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