IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1

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Macrophages (MΦs) colonize tissues during inflammation in two distinct ways: recruitment of monocyte precursors and proliferation of resident cells. We recently revealed a major role for IL-4 in the proliferative expansion of resident MΦs during a Th2-biased tissue nematode infection. We now show that proliferation of MΦs during intestinal as well as tissue nematode infection is restricted to sites of IL-4 production and requires MΦ–intrinsic IL-4R signaling. However, both IL-4Rα–dependent and –independent mechanisms contributed to MΦ proliferation during nematode infections. IL-4Rα–independent proliferation was controlled by a rise in local CSF-1 levels, but IL-4Rα expression conferred a competitive advantage with higher and more sustained proliferation and increased accumulation of IL-4Rα+ compared with IL-4Rα– cells. Mechanistically, this occurred by conversion of IL-4Rα+ MΦs from a CSF-1–dependent to –independent program of proliferation. Thus, IL-4 increases the relative density of tissue MΦs by overcoming the constraints mediated by the availability of CSF-1. Finally, although both elevated CSF1R and IL-4Rα signaling triggered proliferation above homeostatic levels, only CSF-1 led to the recruitment of monocytes and neutrophils. Thus, the IL-4 pathway of proliferation may have developed as an alternative to CSF-1 to increase resident MΦ numbers without coincident monocyte recruitment.

Abbreviations used: FSC, forward scatter; GI, gastrointestinal; Hp, Heligmosomoides polygyrus bakeri; Ls, Litomosoides sigmodontis; MΦ, macrophage; SSC, side scatter.
the CSF1R exhibit an extreme deficit in resident $\text{M} \phi$s in many tissues (Dai et al., 2002), and the same cells are ablated in a time-dependent manner after treatment with a blocking anti-CSF1R antibody (MacDonald et al., 2010). A proliferative signal through the CSF1R has been shown to maintain homeostatic numbers of resident peritoneal $\text{M} \phi$s in the steady-state (Davies et al., 2013) and mediate repopulation of resident lung and peritoneal $\text{M} \phi$s after acute inflammation or experimental depletion (Davies et al., 2013; Hashimoto et al., 2013). CSF1R signaling can also control in vivo proliferation of monocyte-derived $\text{M} \phi$s, required for the population of the growing myometrium in pregnancy (Tagliani et al., 2011) or maintenance of recruited cells during the resolution phase of sterile peritonitis (Davies et al., 2013).

The Th2 lymphokine IL-4 was first shown to regulate proliferation and accumulation of resident $\text{M} \phi$s in the context of filarial nematode infection. Moreover, serial administration of an rIL-4 complex (IL-4c) was sufficient to induce proliferation and accumulation of $\text{M} \phi$s throughout the body, including in the peritoneal cavity and liver (Jenkins et al., 2011), lung, and spleen (unpublished data), and to drive proliferation of recruited monocyte-derived cells (Jenkins et al., 2011). These studies did not reveal whether the actions of IL-4 were direct or indirect.

Akt signaling is required for CSF-1–mediated proliferation (Smith et al., 2000; Irvine et al., 2006; Huynh et al., 2011), and we have recently shown that intact Akt signaling is critically important for in situ $\text{M} \phi$ proliferation in response to IL-4 (Rückerl et al., 2012). However, IL-4 receptor (IL-4R) signaling does not effectively activate Akt in $\text{M} \phi$s in vitro despite phosphorylating PKB (Heller et al., 2008), thus raising the possibility that IL-4 acts via CSF1R signaling to induce Akt-dependent $\text{M} \phi$ proliferation. Indeed, many important parallels exist between IL-4 and CSF-1–activated $\text{M} \phi$s. For example, both IL-4 and CSF-1 promote a suppressive and a pro-repair phenotype in $\text{M} \phi$s (Alikhan et al., 2011). Furthermore, the transcription factors c-Myc and KLF-4 are critical for both the “alternative activation” state induced in $\text{M} \phi$s by engagement of the IL-4R (Liao et al., 2011; Pello et al., 2012) and CSF-1–dependent proliferation that occurs in the absence of Maf B and c-Maf (Aziz et al., 2009). Thus, understanding the relationship between CSF-1 and IL-4 is important, not least because several groups have used CSF-1 to generate human “M2” $\text{M} \phi$s (Verreck et al., 2004; Martinez et al., 2006; Fleetwood et al., 2007), which are often considered highly parallel to alternatively activated $\text{M} \phi$s driven by IL-4.

This study seeks to determine the contribution of CSF-1 to IL-4–driven $\text{M} \phi$ proliferation and alternative activation. Alternatively activated $\text{M} \phi$s are a distinguishing feature of inflammation driven by helminth infections and allergy but may also appear in cold-stressed adipose tissue (Nguyen et al., 2011; Karp and Murray, 2012), certain immunogenic tumors (DeNardo et al., 2009; Linde et al., 2012), and even the steady-state (Wu et al., 2011). Using direct delivery of IL-4 and Th2-biased infection models, we demonstrate that IL-4–mediated proliferation requires $\text{M} \phi$-intrinsic IL-4Rα signaling that is entirely independent of the CSF1R. However, these experiments revealed a significant contribution of IL-4Rα–independent, CSF1R–dependent $\text{M} \phi$ proliferation during nematode infection. We further demonstrate that IL-4Rα expression confers a major competitive advantage to $\text{M} \phi$s, such that IL-4Rα$^+$ cells rapidly outcompete those lacking receptor expression.

**RESULTS**

**IL-4–dependent proliferation does not require the CSF1R**

We used delivery of IL-4c as a reductionist approach to investigate whether IL-4 acts via the CSF1R to drive expansion of resident serous cavity $\text{M} \phi$s. Ki67 expression was used to determine the frequency of all F4/80$^{\text{high}}$ $\text{M} \phi$s in cycle, as described previously (Jenkins et al., 2011), whereas a 3-h BrdU pulse before necropsy or high level of Ki67 expression (Ki67$^{\text{high}}$) was used to identify cells in S phase (Fig. S1 A; Landberg et al., 1990). Intracellular staining for RELMα and/or Ym1 was used as a marker of alternative activation. Consistent with the established role of CSF-1 in regulating steady-state $\text{M} \phi$ levels (Davies et al., 2013), proliferation observed in control PBS-treated mice was completely blocked by treatment with anti-CSF1R mAb (Fig. 1 A). In contrast, neither elevated proliferation nor marker induction by IL-4c was affected by antibody treatment (Fig. 1 A). The only influence of blocking CSF1R on IL-4c treatment was to reduce the final $\text{M} \phi$ number (Fig. 1 A). Daily oral gavage of GW2580, an inhibitor of the CSF1R tyrosine kinase, also had no effect on IL-4–induced $\text{M} \phi$ proliferation or alternative activation (Fig. 1 B). Furthermore, Csf1r gene expression was significantly reduced in FACS-purified peritoneal $\text{M} \phi$s 24 h after injection of IL-4c (Fig. 1 C). As expected, because the ligand is cleared by receptor-mediated endocytosis (Hume and MacDonald, 2012), CSF1R blockade resulted in elevated levels of CSF-1 in the tissue and bloodstream (Fig. 1 D), thereby confirming the effectiveness of the antibody. However, CSF-1 production was not increased in response to IL-4 (Fig. 1 D). Thus, in the context of IL-4 delivery, there was no evidence of CSF1R involvement in proliferation and alternative activation.

**IL-4-driven proliferation requires $\text{M} \phi$-intrinsic IL-4Rα signaling**

We next addressed the possibility that IL-4 signals directly to $\text{M} \phi$s to induce proliferation. We used LysM$^{\text{cre}}$II4na$^{-/-}$ mice to delete the IL-4Rα chain on myeloid cells including $\text{M} \phi$s and neutrophils (Herbert et al., 2004). Injection of IL-4c into LysM$^{\text{cre}}$II4na$^{-/-}$ mice resulted in elevated proliferation of F4/80$^{\text{high}}$ $\text{M} \phi$s in the cavities as well as elevated frequencies of alternatively activated $\text{M} \phi$s (Fig. 2 A). Nevertheless, $\text{M} \phi$ proliferation was lower in LysM$^{\text{cre}}$II4na$^{-/-}$ mice than in II4na$^{-/-}$ controls (pleural, $P < 0.01$; peritoneal, $P < 0.001$; Fig. 2 B). Co-staining for RELMα and BrdU (Fig. 2 B) revealed that RELMα$^+$ $\text{M} \phi$s from LysM$^{\text{cre}}$II4na$^{-/-}$ mice underwent significantly greater levels of proliferation than RELMα$^-$ cells. Because RELMα expression is a known marker of IL-4Rα engagement on $\text{M} \phi$s (Jenkins and Allen, 2010) and LysM-Cre is relatively inefficient (Hume, 2011), the data...
suggestion that IL-4-dependent proliferation predominantly occurs in the subset of MΦs that retain the IL-4R in LysM<sup>+</sup> Il4a<sup>−/−</sup> animals.

To confirm this conclusion, Il4a<sup>−/−Cd45.1<sup>+/+Cd45.2<sup>−/−</sup>C57BL/6 mice were lethally irradiated and transplanted with a 50:50 mix of BM from Il4a<sup>−/−Cd45.1<sup>+/+Cd45.2<sup>−/−</sup> and Il4a<sup>−/−Cd45.2<sup>−/−</sup>Cd45.1<sup>+/+</sup></sup> mice, so that cells derived from Il4a<sup>−/−</sup>BMM could be distinguished from WT by a lack of CD45.1 expression. Blood-borne monocytes (Fig. 2 C) and other myeloid populations (not depicted) exhibited roughly equal proportions of CD45.1<sup>+</sup> and CD45.1<sup>−</sup> cells 8 wk after reconstitution. This extends earlier data showing the key mediator of IL-4R signaling, STAT6, has no intrinsic role in steady-state proliferation or survival of hematopoietic stem cells despite their ubiquitous IL-4Rα<sup>+</sup> expression (Bunting et al., 2004).

Likewise, similar frequencies of Il4a<sup>−/+</sup> and Il4a<sup>−/−</sup> cells were detected in the resident F4/80<sup>high</sup> pleural and peritoneal cavity MΦ populations in control mice treated with PBS (Fig. 2 D and not depicted). In contrast, Il4a<sup>−/−</sup>F4/80<sup>high</sup> MΦs greatly outnumbered Il4a<sup>−/−</sup> cells in the pleural and peritoneal cavities of mice treated with IL-4c. Indeed, only the IL-4Rα<sup>+</sup> population increased in number after treatment with IL-4c (Fig. 2 D and not depicted), consistent with increased S-phase BrdU<sup>+</sup> cells (Fig. 2 E) and Ki67<sup>+</sup> cells (not depicted) being observed only in the CD45.1<sup>+</sup> IL-4Rα<sup>+</sup> cells. Notably, steady-state levels of proliferation in PBS-treated controls did not differ between Il4a<sup>−/+</sup> and Il4a<sup>−/−</sup> cells, suggesting IL-4 is only important in an inflammatory context. Intracellular staining for RELMα (not depicted) and Ym1 (Fig. 2 E) in these experiments confirmed an absolute requirement for intrinsic IL-4Rα signaling to up-regulate production of these archetypal alternative activation markers under this reductionist condition.

**Both IL-4Rα-dependent and -independent pathways contribute to proliferation during nematode infection**

The Th2 cytokines IL-4 and IL-13 can both signal via the IL-4Rα to drive alternative activation (Gordon and Martinez, 2010). In our previous study, nematode-associated increased MΦ proliferation was not entirely absent in Il4<sup>−/−</sup> mice (Jenkins et al., 2011), suggesting a possible contribution from IL-13. To assess the potential of IL-13 to induce proliferation, mice were injected with IL-13 complexed with a neutralizing anti-IL-13 mAb (CSF1R), rat IgG (RigG), or PBS on days 0 and 2. The proportion of F4/80<sup>high</sup> pleural MΦs positive for Ki67, Ki67<sup>high</sup>, and total MΦ numbers were determined by flow cytometry on day 2 after the last injection. Graphs depict individual data for four mice/group. (B) As in A, but on day 3 after daily oral gavage with vehicle control or GW2580 on days 0–3. (C) Mice were injected with a single dose of PBS or IL-4c, and the expression of CSF1R mRNA was determined 24 h later in FACS-purified F4/80<sup>high</sup> peritoneal MΦs. ***, P < 0.001 determined by two-tailed Student’s t test. Individual data for five mice/group are shown. (D) CSF1 levels in pleural lavage fluid and serum from mice in A determined by ELISA. All data are representative of two to three separate experiments, with the same results observed in the peritoneal cavity. ([A–D] Horizontal bars indicate mean values.)
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by IL-4c versus Ls infection revealed key differences: IL-4c induced marker expression in peritoneal and pleural MΦs before detectable proliferation (Fig. 3 C and Fig. S1 B), whereas during infection proliferation preceded marker expression (Fig. 3 D). The data indicate that IL-4Rα-independent

(Fig. 3 B), and worm burdens did not significantly differ between strains at this stage of infection (not depicted). Hence, mechanisms independent of IL-4Rα contribute to elevated MΦ self-renewal during nematode infection. Comparison of the kinetics of alternative activation and proliferation stimulated

Figure 2. MΦ-intrinsic IL-4Rα signaling is essential for proliferation and alternative activation triggered by IL-4c. (A) LysMcreIl4ra–/lox BALB/c mice were injected i.p. on days 0 and 2 with PBS or IL-4c, and peritoneal lavage cells were analyzed on day 4 by flow cytometry for BrdU incorporation or Ki67, RELMα, and Ym1 versus F4/80 expression. Representative flow cytograms gated on F4/80+ peritoneal MΦs with frequencies depicting the mean ± SEM of four mice per group. (B) Il4ra–/lox (Het), LysMcreIl4ra–/lox (Lys), or Il4ra−/− (−/−) BALB/c were treated with PBS (open) or IL-4c (closed) as in A, and BrdU incorporation by F4/80high pleural MΦs was determined on day 3 together with the frequency of BrdU+ cells in RELMα-positive (+) or negative (−) F4/80high MΦs from the IL-4c–treated LysMcreIl4ra–/lox group. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 determined by ANOVA (left) or paired Student’s t test (right). Data are representative of four independent experiments inclusive of data in A, with three to four mice/group. (C) BL/6 Il4ra+/+Cd45.1+/+Cd45.2+ mice were lethally irradiated and reconstituted with a 50:50 mix of Il4ra+/+Cd45.1+/+Cd45.2+ and Il4ra−/−Cd45.1−/−Cd45.2+/+ congenic BM cells. The frequency of blood monocytes (gated as side scatter [SSC]lowCD11b+CD115+) derived from each BM was determined by analysis of CD45.1 and CD45.2 expression 8 wk later. A representative flow cytogram of CD45.1 and CD45.2 expression on blood monocytes is shown together with a graph depicting the frequency of CD45.1+ and CD45.1− monocytes in all blood leukocytes from 10 individual mice. (D) Mice from C were subsequently injected i.p. twice, 2 d apart, with PBS or IL-4c, and pleural lavage cells were analyzed 2 d after the last injection for CD45.1 and CD45.2 expression. Representative flow cytograms gated on all live F4/80hi MΦs were shown together with a graph depicting the total number of Il4ra+/+ Cd45.1+/+Cd45.2+ and Il4ra−/− Cd45.1−/−Cd45.2+/+ pleural MΦs from each group, with individual data for five mice/group presented. (E) Representative flow cytograms depicting CD45.1 expression versus BrdU incorporation or Ym1 expression gated on single F4/80hiCD19− pleural MΦs from mice in D, and graphs showing the proportion of Il4ra+/+Cd45.1+/+ (closed circles) and Il4ra−/−Cd45.1−/− (open circles) F4/80hiMΦs positive for BrdU or Ym1 for individual mice. (C–E) Data are representative of two experiments. (B–E) Horizontal bars indicate mean values.
proliferation occurs early during infection with subsequent peak proliferation driven by IL-4.

Of note, proliferation induced by a single IL-4c dose subsided after 4 d, whereas alternative activation markers were sustained (Fig. 3 C and Fig. S1 B). After two sequential IL-4c doses at days 0 and 2, proliferation remained evident at day 4 (Fig. 1 A). We therefore titrated the dose of IL-4c. As expected, proliferative activity required high doses of IL-4.
whereas induction of RELMα was maximal at the lowest dose tested (Fig. 3 E). Thus, detection of alternative activation markers in the tissues is not synonymous with proliferation, as marker expression may only indicate prior or low-level exposure to IL-4.

**IL-4 switches MΦs to CSF-1–independent proliferation**

We used CSF1R blockade to assess the potential contribution of CSF1R signaling to the IL-4Rα–independent proliferation that occurs during Ls infection. Mice were treated with anti-CSF1R mAb between days 8 and 10 to coincide with the approximate onset of alternative activation (Fig. 3 D) and when IL-4R–independent proliferation is detectable (Fig. 3 B). CSF1R blockade led to a significant reduction in infection-induced proliferation of pleural MΦs (Fig. 4 A), whereas alternative activation (Fig. 4 A), Th2 responses, and worm burden (not depicted) were unaffected. However, the response in the infected group appeared bimodal, with 13 of 20 anti-CSF1R–treated mice showing almost complete inhibition of proliferation (Fig. 4 A, black squares), whereas the remaining animals were similar to controls (Fig. 4 A, gray squares). The anti-CSF1R–treated infected animals in which high-level proliferation remained evident showed the highest frequencies of Ym1+ MΦs (Fig. 4 A, gray squares), and indeed the frequency of MΦs able to proliferate in the presence of anti-CSF1R correlated positively with Ym1+ MΦs (Spearman r = 0.84, P < 0.0001). The data suggest that as the strength of Th2 response and corresponding IL-4Rα signaling increased, the dependence on CSF1R signaling for MΦs to proliferate and accumulate during tissue nematode infection declined. In other words, IL-4 can substitute for CSF-1.

CSF-1 is increased in the circulation and local lesions in many infections, chronic inflammation, and malignancy (Chitu and Stanley, 2006; Hume and MacDonald, 2012). We therefore measured levels of cytokine in the pleural lavage fluid and serum. There was a modest increase in accumulation of CSF-1 in the pleural cavity of infected anti-CSF1R–treated mice compared with naive controls, but no increase in the serum (Fig. 4 B). No correlation was found between the level of pleural CSF-1 and frequency of BrdU+ MΦs in anti-CSF1R–treated infected mice (Spearman r = 0.1398, P = 0.5565), consistent with IL-4R signaling controlling CSF-1–independent proliferation in these mice directly rather than via elevation of CSF-1.

**IL-4Rα expression provides a competitive advantage to MΦs during nematode infection**

We next infected Il4ra+/+ Cd45.1+; Il4ra−/− Cd45.1null mixed BM chimeric mice with Ls to investigate the relevance of MΦ–intrinsic IL-4Rα signaling in a setting in which IL-4Rα–independent proliferation can occur. In contrast to the highly restricted response seen in these mice after IL-4c injection (Fig. 2, D and E), increased proliferation of both Il4ra−/− and Il4ra+/− pleural F4/80high MΦs was evident by day 10 after Ls infection (Fig. 5 A). However, the frequency of cycling cells was significantly greater in the IL-4Rα− population in all infected mice at this time (P < 0.001), and only in these cells did heightened proliferation remain evident at day 16 (Fig. 5 A). In contrast, production of Ym1 (Fig. 5 A) and RELMα (not depicted) during infection was wholly dependent on MΦ–intrinsic IL-4Rα signaling. Consistent with the pattern of proliferation, both Il4ra−/− and Il4ra+/− populations significantly increased in number in the pleural cavity by day 10 after infection (P < 0.01), yet by day 16 the IL-4Rα− MΦs, although still elevated in number (P < 0.05), were significantly outnumbered by the IL-4Rα+ cells (Fig. 5 B; P < 0.05). The selective advantage provided by the IL-4Rα was only observed in the F4/80high MΦ population and not in other myeloid cells (Fig. 5 C), Thus, although confirming the existence of an IL-4Rα–dependent pathway of proliferation, these data show that direct IL-4Rα signaling to MΦs conveys a competitive advantage during infection at least in part by enhancing entry into cell cycle. These findings explain the emergence of
Figure 5. IL-4Rα signaling to MΦs provides a competitive advantage during tissue nematode infection. (A) BL/6 Il4ra+/+Cd45.1+Cd45.2+ mice were lethally irradiated and reconstituted with a 50:50 mix of Il4ra+/+Cd45.1+Cd45.2+ and Il4ra−/−Cd45.1nullCd45.2+/+ congenic BM cells over 8 wk. Mice were infected with Ls, after which BrdU incorporation or Ym1 expression versus expression of CD45.1 was determined on single F4/80highCD19low pleural MΦs at days 10 and 16 after infection. Representative flow cytograms are shown, whereas graphs depict the proportion of IL-4Rα+CD45.1+ (closed...
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RELMα⁺ pleural MΦs in MΦ-specific IL-4Rα–deficient LysM⁺/Il4ra⁻/⁺lox mice during the later phase of Ls infection (Fig. 5 D). Indeed, by day 60 after infection, RELMα and YM1 expression by MΦs is indistinguishable between WT and LysM⁺/Il4ra⁻/lox mice (not depicted). IL-4Rα expression on MΦs in the LysM⁺/Il4ra⁻/lox mice was confirmed by surface staining (Fig. 5 D). Thus, IL-4Rα expression can give MΦs a competitive advantage during a chronic Th2 inflammatory setting such as helminth infection.

Proliferation during infection requires adaptive immunity and is localized to the infection site

We have previously established using Rag1⁻/⁻ mice that IL-4–driven MΦ proliferation per se does not require adaptive immunity (Jenkins et al., 2011). In the context of chronic nematode infection, however, MΦ accumulation requires Th2 cells and MHC class II (Loke et al., 2007). We thus assessed the requirement for adaptive immunity in MΦ proliferation during infection. There was a dramatic reduction in the number of F4/80⁺ MΦs in nematode-infected Rag1⁻/⁻ mice compared with WT controls, matched by a complete failure of the MΦs to divide or up-regulate alternative activation markers (Fig. 5 E). Consistent with a need for cognate antigen-specific interaction, proliferation and alternative activation were localized to the site of parasite infection in WT animals, with significantly (P < 0.05) elevated responses observed in the pleural but not the peritoneal cavity of infected mice (Fig. 5 F).

IL-4–dependent MΦ proliferation, alternative activation, and competitive advantage are features of intestinal nematode infection

Oral infection of BALB/c mice with the gastrointestinal (GI) nematode Heligmosomoides polygyrus bakeri (Hp) leads to invasion of the submucosa of the duodenum where the worms undergo developmental maturation. Despite restriction of the worms to the GI tract, there is systemic dissemination of Th2 cells with selectivity for the peritoneal cavity (Mohrs et al., 2005). In Hp-infected mice, there was a striking increase in proliferation of F4/80⁺Hp⁺ resident MΦs by day 7 after infection in the peritoneal (Fig. 6 A) but not the peritoneal cavity (not depicted), consistent with the localized MΦ proliferation observed in our Ls model. Infection led to a two- to threefold increase in cells in the peritoneal lavage, exclusively within the F4/80⁺Hp⁺ MΦ population. As expected for a Th2 setting, these MΦs expressed RELMα and Ym1 (Fig. 6 A). As in the Ls model, Il4⁻/⁻ mice had a reduced response, although a significant increase in BrdU⁺ (P < 0.001), Ki67⁺ (P < 0.001), and total MΦs (P < 0.01) remained (Fig. 6 B). A minor population of Ym1⁺ MΦs was also detected in infected Il4⁻/⁻ mice, suggesting a limited influence of IL-13 (Fig. 6 B). Blockade of CSF1R signaling demonstrated a CSF1R–dependent component to the proliferative but not the alternative activation response (Fig. 6 C), and thus as in the Ls model, both CSF1R and IL-4Rα signaling also contribute to MΦ proliferation in this model. We also reexamined the relative importance of cell-autonomous IL–4R signaling in the Hp model. The majority of F4/80⁺Hp⁺ MΦs, but not other myeloid cell populations, were IL-4Rα⁺ by day 14 after infection of Il4ra⁻/⁻Cd45.1⁻: Il4ra⁻/⁻/Cd45.1⁻/lox mixed BM chimeric mice, despite earlier accumulation of both IL-4Rα⁺ and IL-4Rα⁻ cells (Fig. 6 D). This competitive advantage was confirmed using LysM⁺/Il4ra⁻/lox mice infected with Hp, in which the proportion of RELMα⁺ positive cells increased from 4% in naive mice (not depicted) to 25% at day 14 and 70% by day 28 (Fig. 6 E), by which point >50% of the MΦs expressed IL-4Rα detectable by flow cytometry (not depicted). Infection with a GI nematode thus led to a MΦ response in the peritoneal cavity that mirrored the pleural cavity during infection with the filarial worm Ls. In both models, CSF1R and IL-4Rα contributed independently to proliferation.

Differing consequences of CSF-1– versus IL-4–induced inflammation

Because both IL-4 and CSF-1 can induce local MΦ proliferation, we sought to compare the MΦ elicited by the two stimuli. To assess the contribution of CSF-1 to cellular dynamics in the serous cavity, we used a new reagent, Fc–CSF-1 designed for stable in vivo delivery of CSF-1. We compared Fc–CSF-1 with IL-4c at similar molar doses that induced maximal levels of proliferation with both reagents (280% Ki67⁺ by 48 h; not depicted). Despite near identical levels of MΦ proliferation at 24 h, only IL-4c induced RELMα (Fig. 7 A) or Ym1 (not depicted) production. Furthermore, Fc–CSF-1
Figure 6. IL-4–dependent proliferation occurs during GI nematode infection and provides a competitive advantage to IL-4Rα+ MΦs. (A) BALB/c mice were infected orally with Hp, and peritoneal lavage cells were assessed at day 7. Representative flow cytograms of all peritoneal cells showing gates and frequencies for all or BrdU+, Ki67+, RELMα+ or Ym1+, F4/80High MΦs. Data are representative of five experiments. (B) BALB/c (WT) or Il4−/− (−/−) mice were infected with Hp (closed symbols) or left naive (open symbols), and the total peritoneal F4/80High MΦs and the proportion

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induced the recruitment of inflammatory cells including neutrophils and Ly-6C⁺ monocytes (Fig. 7 B), consistent with its recently described ability to drive proinflammatory chemokine production (Taghian et al., 2011) and acute monocytopenia (Ulrich et al., 1990). In striking contrast, no inflammatory recruitment was seen with IL-4c, consistent with the ability of IL-4 to actively down-regulate proinflammatory chemokines (Thomas et al., 2012). Thus although CSF-1 and IL-4 both induce MΦ proliferation in the tissues, they have otherwise markedly different consequences. Additionally, these data demonstrate that elevated CSF-1 signaling is sufficient to stimulate heightened proliferation (Fig. 7 A) and accumulation (not depicted) of resident MΦs in vivo without need for additional signals that occur during infection/inflammation and provide some explanation for the elevated peritoneal MΦ numbers seen in the original study of systemic CSF-1 treatment of mice (Hume et al., 1988).

DISCUSSION
We previously demonstrated the requirement for IL-4 to achieve maximal proliferation of MΦs during nematode infection and an absolute requirement for IL-4Rα for MΦ proliferation after IL-4c delivery (Jenkins et al., 2011). Our working hypothesis was that IL-4 acted indirectly via an intermediate cell, to stimulate production of a MΦ mitogenic factor, such as CSF-1, similar to the process by which a vitamin D3 analogue acts on keratinocytes to stimulate Langerhans cell proliferation (Chorro et al., 2009). This hypothesis was supported by our preliminary data in which IL-4-driven proliferation was observed in mice apparently lacking IL-4Rα on MΦs. However, using mixed BM chimeric mice, we demonstrate that MΦ-intrinsic IL-4Rα signaling is required for MΦ proliferation in response to endogenous or exogenous IL-4. Furthermore, IL-4-mediated proliferation was entirely dependent on CSF1R signaling, in contrast to the homeostatic proliferation of CβR-resident MΦs, which is completely dependent on CSF-1 (Fig. 1, A and B; and Fig. 4 A; Davies et al., 2013). Treatment with IL-4c did not increase production of CSF-1 in either serum or tissues, consistent with an intrinsic and CSF-1-dependent effect of IL-4 on MΦs. Thus, it would appear that IL-4Rα signaling to MΦs allows their substantial outgrowth above normal tissue levels at least in part by switching them to a CSF-1-dependent program of proliferation.

Nevertheless, IL-4R⁺ MΦs remain reliant on CSF-1 for survival after IL-4-driven expansion, as indicated by the reduced numbers observed after CSF1R blockade. Seemingly at odds with such a requirement, we found that IL-4 treatment was accompanied by a sharp down-regulation in MΦ transcription of the CSF1R. However, lower levels of CSF1R signaling are required for MΦ survival than are needed for their proliferation (Tushinski et al., 1982). CSF-1 is likely present in the steady-state serous cavities in greater abundance than required for MΦ survival given the evident homeostatic CSF-1-dependent proliferation in these sites (Figs. 1 and 4; Davies et al., 2013). We suggest that IL-4-mediated down-regulation of CSF1R reduces local CSF-1 consumption by individual cells, thereby maintaining sufficient tissue CSF-1 levels to allow survival of the expanding MΦ population. Such a model would explain how the basal levels of proliferation and total number of IL-4Rα⁻ MΦs in mixed BM chimeras appeared to be maintained at near normal steady-state levels after treatment with IL-4c (Fig. 2 E) despite the large outgrowth of IL-4Rα⁺ cells and without a corresponding increase in CSF-1 production (Fig. 1 D). Together, our data suggest that IL-4 and CSF-1 are entirely distinct in terms of proliferation although CSF-1 is still critical for survival in the context of IL-4 expansion.

During nematode infection, our data suggest that CSF-1 plays an early role in MΦ proliferation but is superseded by IL-4 presumably upon entry of Th2 cells. Supporting this, CSF1R blockade during infection almost completely inhibited proliferation in all WT animals except those exhibiting the highest levels of IL-4 exposure, as indicated by the percentage of cells expressing alternative activation markers (Fig. 4 A). Furthermore, time course analysis during infection demonstrated that proliferation occurred before, but only peaked after, the onset of alternative activation (Fig. 3 D), whereas more sustained and higher proliferation was achieved by IL-4Rα⁺ than IL-4Rα⁻ cells in infected mixed BM chimeric mice (Fig. 5 A). Lastly, the restriction of proliferation and MΦ accumulation to the infection site, and the absence of any such response in Rag1⁻/⁻ animals (Fig. 5, E and F), mirrors our previous findings that MΦs fail to accumulate in a related nematode infection in the absence of Th2 cells (Loke et al., 2007). IL-4 may also contribute to elevated tissue MΦ density during infection through additional cell-intrinsic mechanisms, for example, by reducing reliance on local glucose levels (Vats et al., 2006), inhibiting migration from the tissue
(Thomas et al., 2012), or protecting against apoptosis, as occurs in other leukocytes (Würster et al., 2002). Although the mixed Il-4ra<sup>+/+</sup>:Il-4ra<sup>-/-</sup> BM mice do not reflect the natural distribution of IL-4R expression in vivo, they are critically important for understanding how cell-intrinsic IL-4R signaling augments MΦ numbers during nematode infection. Furthermore, the data generated using these mice may be relevant to circumstances in which differential IL-4R expression does occur (Wermeling et al., 2013).

RELMα and Ym1 were used here as surrogate markers of IL-4R-experienced MΦs. Elsewhere, Ym1 expression has been documented in MΦs from LysM<sup>cre</sup>Il4ra<sup>-/-</sup> mice in the liver granulomas surrounding schistosome eggs and peritoneal cavity after injection of schistosome eggs (Dewals et al., 2010). Because Ym1 expression is reduced significantly by in vivo IL-10 blockade, it was concluded that IL-10 induces MΦ Ym1 expression in these settings independently of IL-4 (Dewals et al., 2010). However, we observed a large proportion of serous cavity MΦs in LysM<sup>cre</sup>Il4ra<sup>-/-</sup> mice expressing Ym1 and RELMα in response to IL-4c injection and during infection with Ls or Hp, with co-staining confirming IL-4Ra expression. Moreover, expression of both Ym1 and RELMα was exclusively restricted to IL-4Ra<sup>+</sup> MΦs in IL-4–treated or nematode-infected mixed BM chimeric mice despite abundant IL-10 detectable in the pleural lavage fluid during infection (not depicted). It would seem that the selective advantage of IL-4Rα expression leads to accrual of a minor non-gene–deleted population, which could account for the Ym1<sup>+</sup> cells observed in LysM<sup>cre</sup>Il4ra<sup>-/-</sup>/lox mice during schistosome infection (Dewals et al., 2010). Alternatively, MΦs elicited by schistosome eggs may differ qualitatively from those found during nematode infection and IL-4c injection, being capable of Ym1 production in response to IL-10. In this respect, schistosome eggs elicit CCR2-dependent inflammatory MΦs (Chensue et al., 1996; Lu et al., 1998), contrasting with the resident-derived cells in our systems (Jenkins et al., 2011). Furthermore, Il-4ra<sup>-/-</sup> and LysM<sup>cre</sup>Il4ra<sup>-/-</sup> mice show no defect in overall MΦ numbers elicited to schistosome granulomas (Dewals et al., 2010), perhaps because of the proinflammatory nature of the eggs. Therefore, the balance of proliferation versus recruitment in Th2 settings almost certainly depends on integration of multiple inflammatory signals. Of relevance, variation of IL-4c dose and delivery timing suggested that proliferation may only be apparent in environments in which critical IL-4 thresholds are sustained.
Steady-state proliferation in naïve animals was not influenced by IL-4Rα signaling in mixed BM chimeric mice, LysM<sup>cre</sup>Il-4ra<sup>−/−</sup> mice, or in global IL-4Rα-deficient animals, demonstrating that IL-4 mediates density of cavity MΦs only under conditions of inflammation and not homeostasis. IL-4Rα signaling also had little or no bearing on the wholly CSF-1–dependent elevated proliferation of resident peritoneal MΦs that occurs during resolution of microbial-induced peritonitis (Davies et al., 2013). In this process, however, proliferation acts simply to restore the MΦ population to its original level after depletion during the acute stage of inflammation (Davies et al., 2011) and is therefore akin to homeostatic maintenance rather than an inflammatory process. In keeping with this, the levels of CSF-1 did not differ between naïve and inflammatory-challenged mice in this study (Davies et al., 2013). Elevated levels of circulating CSF-1 do occur in many disease states, and administration of CSF-1 can greatly increase tissue MΦ numbers in rodents and primates (Hume and MacDonald, 2012), but it is unclear whether this increase results from the outgrowth of resident MΦs or recruitment of new monocyte-derived cells. Using a recombinant Fc–CSF-1 fusion protein, we demonstrate that CSF-1 efficiently stimulates proliferation of resident peritoneal MΦs (Fig. 7 A). Furthermore, during nematode infection, we detected an approximate doubling in the number of IL-4Rα–deficient MΦs in mixed BM chimeras (Fig. 5 A) and in global Il4ra<sup>−/−</sup> mice (Fig. 3 B), mirroring the twofold increase in local CSF-1 production (Fig. 4 B). Elevated CSF-1 can therefore act during an inflammatory episode to increase local numbers of resident tissue MΦs and could play a more significant role in expansion of resident MΦs in pathologies in which much higher CSF-1 levels are observed (Hamilton, 2008).

Although both IL-4 and CSF-1 elicted proliferation of resident MΦs, they differed fundamentally in other actions. In particular, injection of Fc–CSF-1 also induced recruitment of Ly6<sup>c</sup> monocytes and neutrophils, consistent with studies elsewhere (Lenda et al., 2003; Taghianli et al., 2011), suggesting elevated CSF-1 secretion may act as an emergency stopgap to rapidly fill the tissue MΦ compartment. In contrast, we saw no evidence of increased recruitment of inflammatory cells after IL-4c administration and previously observed recruitment of only low numbers during the early stages of L<sub>s</sub> infection despite the large increase in resident tissue MΦs (Jenkins et al., 2011). CSF1R signaling directly induces production of CCR2 ligands by tissue MΦs to stimulate recruitment of Ly6<sup>c</sup> monocytes (Taghianli et al., 2011), whereas IL-4 down-regulates MΦ production of CCL2, CCL7, and CCL3 during tissue nematode infection (Thomas et al., 2012). Although the finding that IL-4–mediated proliferation is CSF1R independent was at first unexpected, our data suggest that this mechanism may have developed as an alternative to a CSF-1 pathway to increase numbers of resident MΦs without coincident increase in monocyte recruitment. This could be seen as an additional tissue-protective function of IL-4 beyond induction of the immunoregulatory and pro-wound repair phenotype associated with alternative activation (Murray and Wynn, 2011).

In summary, the impact of IL-4 during infections is likely twofold: it increases numbers without the need for recruitment, whereas in the presence of recruitment it insures a noninflammatory environment by switching MΦs to an alternatively activated phenotype. Thus, the numerical advantage provided by MΦ IL-4Rα expression combined with the anti-inflammatory chemokine profile and alternative activation state ultimately leads to the development of a noninflammatory environment to contain worm infection and repair tissue regardless of MΦ source.

**MATERIALS AND METHODS**

**Mice.** BALB/c Il4<sup>−/−</sup> (Noben-Trauth et al., 1996), Il4ra<sup>−/−</sup>, LysM<sup>cre</sup>Il4ra<sup>−/−</sup>, and Il4ra<sup>−/−</sup> mice (Herbert et al., 2004), C57BL/6 Il4ra<sup>−/−</sup> and Rag1<sup>−/−</sup> mice, and WT controls were bred and maintained in specific pathogen–free facilities at the University of Edinburgh. All experiments were permitted under a Project License granted by the Home Office UK and were approved by the University of Edinburgh Ethical Review Process. Experimental mice were age and sex matched. C57BL/6 Il4ra<sup>−/−</sup> mice were generated by backcrossing from the BALB/c Il4ra<sup>−/−</sup> strain a minimum of nine times. Competitive mixed BM chimeric mice were created by lethally irradiating C57BL/6 C57D4.1<sup>+/−</sup> mice with 11.5 Gy γ radiation administered in two doses ~3 h apart, followed by i.v. injection of 5 × 10<sup>5</sup> BM cells depleted of mature T cells using CD90 microbeads (Miltenyi Biotec) and comprised of a 1:1 mix of cells from C57BL/6 C57D4.2<sup>Il4ra<sup>−/−</sup></sup> mice and C57BL/6 C57D4.1<sup>Il4ra<sup>−/−</sup></sup> mice. Chimeric animals were left for at least 8 wk before further experimental manipulation.

**Parasites and reagents.** H<sub>p</sub> and L<sub>s</sub> life cycles were maintained, and infective third-stage larvae (L<sub>3</sub>) were obtained as described elsewhere (Behnke and Wakelin, 1977; Le Goff et al., 2002). Mice were infected with 200 H<sub>p</sub> L<sub>3</sub> oral gavage or 25 L<sub>s</sub> L<sub>3</sub> s by s.c. injection. IL-4–anti–IL-4 mAb complex (IL-4c) was prepared as described previously (Finkelman et al., 1993), and Fc–CSF-1 was conjugated to Fc for increased stability in vivo. Extensive experiments in mice and pigs reveal that pig CSF-1 is equally active in mice (Gow et al., 2012). The presence of endotoxin-like activity in Fc–CSF-1 was tested in murine BM–derived MΦs. There was no detectable induction of the LPS-responsive TNF gene under conditions in which LPS induced the gene >1,000-fold. Mice were infected i.p. with 20 µg Fc–CSF-1 in PBS. Where specified, mice were given 1 mg BrdU i.p. 3 h before the experimental end point.

**Isolation of cells from the peritoneal and pleural cavity.** Mice were sacrificed by exsanguination via the brachial artery under terminal anesthesia. After sacrifice, pleural or peritoneal cavity exudate cells were obtained by washing of the cavity with lavage media comprised of RPMI 1640 containing 2 mM l-glutamine, 200 µM sodium pyruvate, 100 µg/ml streptomycin, and 2 mM EDTA (Invitrogen). The first 2 or 3 ml of lavage wash supernatant by oral gavage or 25 µg BrdU i.p. 3 h before the experimental end point.
saline solution containing 2 mM EDTA (Inovio). Cells were stained with LIVE/DEAD (Inovio). All samples were then blocked with 5 μg/ml anti-CD16/32 (2.4G2; produced in-house) and heat-inactivated normal mouse serum (1:20) in FACS buffer (0.5% BSA and 2 mM EDTA in Dulbecco’s PBS) before surface staining on ice with antibodies to F4/80 (BM8), Siglec-F (E50-2440), Ly-6C (AL-21 or HK1.4), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418), MHCII (MHCI/114.15.2), CD19 (eBio1D3), CD4 (GK1.5), CD3 (17A2), CD115 (AFS98), IL-4Ra (mIL4R-M1), CD45.1 (A20), or CD45.2 (10e; eBioscience or BD). Erythrocytes in blood samples were lysed using FACS Lyse solution (BD).

Detection of intracellular RLMx, Ym1, K67, and BrdU was performed directly ex vivo. Cells were stained for surface markers then fixed and permeabilized using FoxP3 staining buffer set (eBioscience). For BrdU staining, cells were incubated first with or without DNase for 30 min at 37°C. Cells were then stained with biotinylated goat anti-Ym1 (R&D Systems), purified polyclonal rabbit anti-RLMx (PeproTech), or directly labeled mAbs to K67 (B57) or anti-Brdu (B44 or Bio20a; BD or BioLegend), followed by Zenon anti-rabbit reagent (Invitrogen) or streptavidin-conjugated fluorochromes (BioLegend). Expression of Ym1, RLMx, and K67 was determined relative to appropriate polyclonal or monoclonal isotype control, whereas incorporation of BrdU was determined relative to staining on non-DNase-treated cells. Analysis of proliferation and alternative activation was performed on single live cells, determined using LIVE/DEAD and forward scatter (FSC) width (FSC-W) versus FSC area (FSC-A), respectively, and subsequently gated on CD19− cells to remove B cell–MΦ doublets. Analysis for calculation of total F4/80+ MΦs was performed on all cells. Samples were acquired using FACS LSRII or FACSCanto II using FACS Diva software (BD) and analyzed with Flowjo version 9 software (Tree Star).

**Analysis of MΦs from competitive mixed BM chimeras.** MΦs derived from Cd45.1 iLuca+ B+ appeared to have a greater tendency to form doublets with Cd19+ B− cells after injection of IL-4c than cells derived from Cd45.1 iLuca+ B− BM, as judged by the high level of F4/80 expression on B cell–MΦ doublets (Fig. S2 A), a marker which is known to be up-regulated by IL-4R signaling (Jenkins et al., 2011). If doublets were excluded from analysis, such bias in doublet formation would distort the ratio of WT to iLuca+ MΦs. Thus, cells were treated for 5 min with Accumax cell aggregate dissociation medium (eBioscience) immediately before fixation, and population frequency analysis was performed without a single cell gate applied. To further prevent bias induced by the few remaining doublets, the gate for CD45.1+ CD45.2− cells was set to exclude events that deviated from the expected linear relationship of CD45.1 and CD45.2 on this population (Fig. S2 B, R1).

Analysis of proliferation and alternative activation in these experiments was performed on CD19− F4/80+ MΦs gated on single cells, using FSC−W versus FSC−A, to minimize the potential for false positives (Fig. S2 B, R2). Because of a minor bias toward more WT than iLuca+ MΦs in naive chimeras, one-way ANOVA was used to analyze differences in cell numbers after infection, whereas paired statistical tests were used to analyze rates of proliferation and alternative activation, to better account for the variation in overall level of proliferation between mice.

**CSF1R experiments.** Mice were injected i.p. with 0.5 mg anti-CSF1R mAb (clone AFS98), purified rat IgG control antibody, or PBS vehicle control either concurrent with IL-4c injections or on day 8 after Li infection or day 6 after Hp infection. AFS98 mAb and purified rat IgG control were produced in-house from cultured hybridoma cells or from naive rat serum, respectively. The e−fms kinase inhibitor GW2580 (LC Laboratories; Conway et al., 2005) was suspended in 0.5% hydroxypropylmethylcellulose and 0.1% Tween 20 using a Teflon glass homogenizer. Dosed control or 160 mg/kg GW2580 was administered daily by oral gavage from 1 h before initial dose of IL-4c on day 0 to 5 h before mice were culled on day 3. Previous pharmacokinetic analysis has shown this daily dose regimen is as effective as twice daily administration of 80 mg/kg and maintains a serum concentration of the drug over 24 h above that estimated to achieve therapeutic effect (Priceman et al., 2010). Analysis of CSF-1 in brachial arterial blood and pleural lavage fluid was performed according to the manufacturer’s instructions (PeproTech).

**MΦ purification and gene expression.** Gene expression analysis on MΦs from IL-4c− or PBS-treated mice was performed on mRNA for which we have previously published data (Ruckerl et al., 2012). In brief, 24 h after treatment of mice with PBS or IL-4c, peritoneal MΦs were sorted using a FACSAria cell sorter (BD) according to their expression of F4/80+, Siglec−F−, CD11b+, CD11c−, B220−, CD3− to purities >90%. Total RNA was then isolated using the miRNAeasy kit (Qiagen) according to the manufacturer’s instructions, measured using a NanoDrop (Thermo Fisher Scientific), and converted to cDNA with BioScript reverse transcription (Bioline) and p(dT)15 primers (Roche). Expression levels were quantified using Light-Cycler 480 SYBR Green I Master (Roche), with measurements performed on a LightCycler 480 (Roche). Expression of Csf1r (5′-CGAGGGGAGCTC-CAGCTACA–3′ and 5′-GACTGGAGAAGCCGACTG—CC–3′) was normalized against Gapdh (5′-ATGACATCAAGAAGGTGGTG–3′ and 5′-CATACAGGGAAATGGCTTG–3′).

**Statistics.** Data were log-transformed to achieve normal distribution and equal variance where required and then tested using one-way ANOVA or Student’s t test. Where equal variance or normal distribution was not achieved, Kruskal Wallace or Spearman correlation was used. Paired Student’s t test was used to determine differences between proliferation and alternative activation of CD45.1+ MΦs obtained from Li-infected competitive mixed BM chimeric mice. Statistics were performed using Prism 5 (GraphPad Software). Each data point represents one animal.

**Online supplemental material.** Fig. S1 shows that MΦs in S phase express the highest levels of K67, such that gating on K67− cells provides an accurate estimate of their frequency and provides representative flow cytograms of K67, RELMx, and Ym1 staining on peritoneal and pleural MΦs at various times after IL-4c injection. Fig. S2 shows the gating strategy used to determine frequency of MΦ subsets from competitive mixed BM chimeric experiments. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20121999/DC1.

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