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The cytokine IL-9 was discovered more than 20 yr ago and described as a T cell and mast cell growth factor produced by T cell clones (Uyttenhove et al., 1988; Hültner et al., 1989; Schmitt et al., 1989). Subsequently, IL-9 was shown to promote the survival of a variety of different cell types in addition to T cells (Hültner et al., 1990; Gounni et al., 2000; Fontaine et al., 2008; Elyaman et al., 2009). Until recently, Th2 cells were thought to be the dominant source of IL-9 and the function of IL-9 was mainly studied in the context of Th2 type responses in airway inflammation and helminth infections (Godfraind et al., 1998; Townsend et al., 2000; McMillan et al., 2002; Temann et al., 2002). IL-9 blocking antibodies were shown to ameliorate lung inflammation (Cheng et al., 2002; Kearley et al., 2011) and are currently in clinical trials for the treatment of patients with asthma (Parker et al., 2011). The paradigm that Th2 cells are the dominant source of IL-9 was challenged when it became apparent that naive CD4+ T cells cultured in the presence of TGF-β and IL-4 initiate high IL-9 expression without coexpression of IL-4, suggesting the existence of a dedicated subset of IL-9–producing T cells (Dardalhon et al., 2008; Veldhoen et al., 2008; Angkasekwinai et al., 2010; Chang et al., 2010; Staudt et al., 2010). Subsequently, the generation of an IL-9–specific reporter mouse strain enabled the study of IL-9–producing cell types in vivo and revealed that in a model of lung inflammation IL-9 is produced by innate lymphoid cells (ILCs) and not T cells (Wilhelm et al., 2011). IL-9 production in ILCs was transient...
but important for the maintenance of IL-5 and IL-13 in ILCs. Such type 2 cytokine-producing ILCs (ILC2s; Spits and Di Santo, 2011) were first described as a population of IL-5– and IL-13–producing non-B/non-T cells (Fort et al., 2001; Hurst et al., 2002; Fallon et al., 2006; Voehringer et al., 2006) and later shown to play a role in helminth infection via IL-13 expression (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010). In addition, important functions were ascribed to such cells in the context of influenza infection (Chang et al., 2011; Monticelli et al., 2011) and airway hyperactivity in mice (Barlow et al., 2012) and humans (Mjösgberg et al., 2011). However, although the contribution of ILC2s to host immunity against helminths in the gut is well established (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010), the function of ILC2s in helminth-related immune responses in the lung remains unknown. ILC2s are marked by expression of the IL-33R (Moro et al., 2010; Neill et al., 2010; Price et al., 2010), as well as the common γ chain (γc) cytokine receptors for IL-2 and IL-7 (Moro et al., 2010; Neill et al., 2010). Interestingly, gene expression array analyses have demonstrated that the receptor for IL-9, another member of the γc receptor family, is also expressed in ILC2s and differentiates them from Th2 cells (Price et al., 2010) and ROR-γt+ ILCs (Hoyle et al., 2012). However, the function of IL-9R expression for ILC2 biology has not been addressed so far.

Here we show that the production of IL-5, IL-13, and amphiregulin during infection with Nippostrongylus brasiliensis in the lung depends on ILC2s and their expression of IL-9R. The ability to signal via the IL-9R was crucial for the survival of ILC2s, but not Th2 cells. The absence of IL-9 signaling in IL-9R–deficient mice resulted in reduced lung ILC2 numbers and, consequently, diminished repair of lung damage in the chronic phase after helminth-induced lung injury despite the presence of an intact Th2 cell response. Thus, we identify IL-9 as a crucial autocrine amplifier of ILC2 function and survival.

RESULTS
IL-9 expression in the lung during N. brasiliensis infection
The larval stage of N. brasiliensis travels from the skin to the lung, where it exerts substantial tissue damage before reaching the gut where a protective immune response leads to worm expulsion in immunocompetent mice (Camberis et al., 2003; Harvie et al., 2010). Although IL-9–deficient mice on a mixed background display unimpaired N. brasiliensis expulsion, an involvement of IL-9 in helminth-induced lung inflammation was not addressed (Townsend et al., 2010) and ROR-γt+ ILCs (Hoyle et al., 2012). Here we show that the production of IL-5, IL-13, and amphiregulin during infection with N. brasiliensis in the lung depends on ILC2s and their expression of IL-9R. The ability to signal via the IL-9R was crucial for the survival of ILC2s, but not Th2 cells. The absence of IL-9 signaling in IL-9R–deficient mice resulted in reduced lung ILC2 numbers and, consequently, diminished repair of lung damage in the chronic phase after helminth-induced lung injury despite the presence of an intact Th2 cell response. Thus, we identify IL-9 as a crucial autocrine amplifier of ILC2 function and survival.

ILC2 accumulation in the lung tissue depends on IL-9R signaling
High expression of the IL-9R on ILC2s residing in the gut-associated lymphoid tissue has been described previously (Price et al., 2010; Hoyle et al., 2012). To address whether lung ILCs have the propensity to respond to IL-9, we assessed IL-9R expression, as monitored by eYFP expression, in eYFP+ T cells and eYFP+ ILCs sorted from the same samples, eYFP+ ILCs showed higher levels of Il9 mRNA transcripts than eYFP+ T cells, suggesting that IL-9–expressing ILCs not only outnumber IL-9–expressing T cells, but also express more IL-9 on a cellular level.

Further analysis confirmed that eYFP+Thy1.2+Lin− cells were marked by the surface expression of CD25, IL-7Rα (CD127), IL-33R (T1/ST2), ICOS (inducible T cell co-stimulator), e-Kit (tyrosine protein kinase kit), Sca-1 (stem cell antigen-1) and the production of high amounts of IL-13 and IL-5, but little IL-4, IL-17A, or IFN-γ (not depicted). Furthermore, eYFP+Thy1.2+Lin− cells sorted from the lungs of infected mice expressed high levels of the ILC2-related transcription factors Rora (retinoid acid receptor–related orphan receptor α; Wong et al., 2012) and Gata3 (Hoyle et al., 2012; Liang et al., 2012) but showed no expression of Rorc (not depicted), a transcription factor of IL-17A– and IL-22–producing ILCs (Spits and Di Santo, 2011). Thus, ILC2s represent the dominant IL-9–producing cell type in the lung during infection with N. brasiliensis.
Next, we wanted to address whether the observed reduction of the total ILC population was caused by a specific reduction of ILC2s. Flow cytometric analysis of the ILC lineage-defining transcription factors GATA3 and RORγt in the Thy1.2+Lin-ILC population revealed that after N. brasiliensis infection the vast majority (>80%) in the lung were GATA3+ ILC2s, whereas RORγt+ ILC3s represented only a minor fraction (Fig. 2 D). Importantly, absence of the IL-9R resulted in a specific reduction of the GATA3+ ILC2s in the lung, whereas the small RORγt+ ILC3 population remained unchanged (Fig. 2, D and E).

The expression of IL-9R has been described on ILC2 precursors in the bone marrow and ILC2s in the lamina propria of the intestine of naive mice (Hoyler et al., 2012), and observed an increase of total Lin-Thy1.2+ ILCs, which were homogeneously marked by expression of Thy1.2, in the lung tissue from day 6 on after N. brasiliensis infection (Fig. 2, B and C). In Il9r−/− mice, we observed a significant reduction of ILC numbers at days 9 and 12 after the infection, as compared with their WT counterparts, that was confined to the lung and not observed in the draining mediastinal LNs (MDLNs; Fig. 2, B and C; and not depicted). Importantly, the absolute numbers of CD4+ T cells in the lung were similar in Il9r−/− and WT mice throughout the time course of the infection (Fig. 2 C). The increase in CD4+ T cell numbers in the MDLNs was comparable in Il9r−/− mice and WT mice between days 2 and 9 and significantly higher in Il9r−/− mice at day 12 (not depicted).

Figure 1. IL-9 expression in the lung during N. brasiliensis infection. (A) Flow cytometry of lung and MDLN cells from Il9CreR26R26ReYFP mice at days 2, 6, 12, and 20 after N. brasiliensis infection as well as from naive Il9CreR26R26ReYFP controls. Plots are gated for CD45+ lymphoid cells. Numbers represent percentage of eYFP-Thy1.2+ cells of total cells gated. (B and C) Absolute numbers of eYFP-Thy1.2+ cells in lungs [B] and MDLNs [C] at the respective time points (n = 3–4 per time point; **, P < 0.01; ***, P < 0.001). (D) IL-9 protein concentration in the lungs of N. brasiliensis–infected WT mice and in naive controls (day 0; n ≥ 4 per time point; *, P < 0.05; **, P < 0.01). (E) Flow cytometry of lung cells from Il9CreR26R26ReYFP mice stained for CD45, Thy1.2, CD4, and lineage markers (Lin, including CD3, CD4, CD8, CD11b, CD11c, CD19, CD49b, TCR-β, TCR-γ, NK1.1, GR-1, and Ter119) at day 12 after N. brasiliensis infection. Plots are gated for CD45-Thy1.2+ lymphoid cells, and numbers indicate the percentage of cells in each quadrant. (F) Ratio of eYFP+ ILCs and eYFP+CD4+ T cells at various time points after the infection (n = 3–4 per time point). (G) Absolute number of eYFP+ ILCs and eYFP+CD4+ T cells in lungs and MDLNs of Il9CreR26R26ReYFP mice at day 12 after N. brasiliensis infection (n = 4 per group; **, P = 0.003 vs. lung CD4). (H) Quantitative RT-PCR analysis of Il9 transcripts in eYFP+ ILCs and eYFP+CD4+ T cells, sorted by flow cytometry, from the lungs of Il9CreR26R26ReYFP mice at day 12 of N. brasiliensis infection (n = 3). Paired samples sorted from the same mice are connected with a line. mRNA expression was normalized to Hprt1 (encoding hypoxanthine guanine phosphoribosyltransferase). Data represent two independent experiments with similar results. Bars show mean values ± SEM.
we observed IL-9R expression in lung ILC2s of naive mice (Fig. 2 A), raising the question of whether this receptor is required for ILC2 maintenance in steady-state. However, the analysis of Sca-1+ GATA3+Lin− cells in the lung, small intestine, and bone marrow of naive WT and Il9r−/− mice revealed similar numbers of these ILC2/ILC2 precursor populations (not depicted), indicating that the IL-9R is dispensable for ILC2 maintenance in the steady-state.

Cytokine production by ILC2s in the lung depends on IL-9R signaling on hematopoietic cells

To investigate the role of IL-9R expression for the function of ILC2 in the lung, we assessed the production of their hallmark cytokines IL-5 and IL-13 in Il9r−/− mice at day 12 after N. brasiliensis infection. The percentages and absolute numbers of IL-5− and IL-13−producing ILC2s were strongly reduced in the lung of helminth-infected Il9r−/− mice at days 6–12, whereas the IL-4, IL-5, and IL-13 production by CD4+ T cells was largely uncompromised, with only a minor decrease of IL-5+ T cells at day 9 (Fig. 3, A and B). Although CD4+ T cells outnumbered ILC2s in the lung of Il9r−/− mice, protein levels of IL-5 and IL-13 in the lung were significantly reduced at days 6, 9, and 12 after the infection (Fig. 3 C). In contrast, IL-4 production was unchanged during the course of N. brasiliensis infection in Il9r−/− mice (Fig. 3 C), further indicating that the Th2 cell response was not affected by the absence of IL-9R signaling. Furthermore, numbers of the other T helper cell subsets, γδ T, NK, and CD8+ T cells, remained unchanged in the lungs of Il9r−/− mice (not depicted).

To investigate whether IL-9 signaling on hematopoietic cells is important for maintaining ILC2s in the lung, we transferred bone marrow from either WT or Il9r−/− mice into irradiated CD45.1+Rag1−/− mice, waited 6–8 wk for reconstitution, and infected them with N. brasiliensis. At day 12 after infection, IL-5− and IL-13−producing CD45.1− negative donor ILC2s were reduced in chimeras containing a hematopoietic compartment deficient for the IL-9R (Fig. 3, D and E). In contrast, the few radioresistant CD45.1+ ILC2s remaining from the host showed similar IL-5 and IL-13 production, regardless of the bone marrow genotype. These data suggest that the maintenance of cytokine-producing ILC2s in the lung of N. brasiliensis−infected mice depends on their intrinsic ability to respond to IL-9.

Rapid worm expulsion depends on IL-9R signaling on hematopoietic cells

To determine the influence of IL-9R signaling on antihelminth immunity in the gut, we assessed egg production and intestinal worm burden in WT and Il9r−/− mice at different time points after N. brasiliensis infection (Fig. 4, A and B). Interestingly, we found increased fecal egg counts and worm numbers in the Il9r−/− mice at days 6–9. The worm burden at day 3, in contrast, was similar between both groups, indicating that the worm passage of N. brasiliensis was unperturbed in Il9r−/− mice and similar numbers of worms reach the intestine. However, altered kinetics of worm release from the lung in the Il9r−/− mice as a possible reason for increased intestinal worm burdens at later time points cannot be excluded. Worm counts in the irradiated Rag1−/− mice reconstituted with WT or Il9r−/− bone marrow (Fig. 3, D and E) showed that the absence of IL-9R on hematopoietic cells was sufficient to...
for prolonged microbleeding (Marsland et al., 2008), in the alveolar space of Il9r−/− mice at day 12 (Fig. 5, C and D). Additionally, the emphysema-like tissue damage that is characterized by bullae formation and destruction of the regular tissue structure and develops at later stages after N. brasiliensis infection (Marsland et al., 2008) was dramatically increased in Il9r−/− mice at days 12 and 24 after infection (Fig. 5, E and F).

Most importantly, these histopathological differences translated to a functional reduction of the lung capacity at later stages after the infection, as demonstrated by a reduced baseline tidal volume in Il9r−/− mice from day 12 onwards (Fig. 5 G). These data show that ILC2-derived IL-9 promotes damage repair and thereby ameliorates emphysema formation at chronic stages after helminth-induced lung injury.

ILCs promote lung tissue repair in Rag−/− mice after N. brasiliensis infection

As ILCs have been linked to epithelial regeneration in the lung after influenza infection (Monticelli et al., 2011), we wanted to investigate the impact of IL-9R signaling and ILC2s for initiation and repair of helminth-induced lung injury, we assessed lung damage parameters in WT and Il9r−/− mice at different stages after N. brasiliensis infection. The degree of acute lung hemorrhage and neutrophil infiltration was similar in WT and Il9r−/− mice, as indicated by equal numbers of erythrocytes and neutrophils in the bronchoalveolar lavage fluid at day 2 of the infection (Fig. 5, A and B). Although the resolution of major alveolar hemorrhage appeared to be normal in Il9r−/− mice (Fig. 5 A), we observed a strong increase in hemophagocytosis of erythrocytes and neutrophils in the bronchoalveolar lavage fluid at day 2 of the infection (Fig. 5, A and B). Therefore, hemophagocytic macrophages were observed in the lungs of Il9r−/− mice (Fig. 5 A), we observed a strong increase in hemophagocytosis of erythrocytes and neutrophils in the bronchoalveolar lavage fluid at day 2 of the infection (Fig. 5, A and B). Therefore, hemophagocytic macrophages were observed in the lungs of Il9r−/− mice (Fig. 5 A), we observed a strong increase in hemophagocytosis of erythrocytes and neutrophils in the bronchoalveolar lavage fluid at day 2 of the infection (Fig. 5, A and B).

Figure 3. Cytokine production by ILC2s in the lung depends on IL-9R signaling. (A) Flow cytometry of total lung cells from N. brasiliensis–infected mice (day 12) restimulated with phorbol 12,13-dibutyrate and ionomycin for 2.5 h and stained intracellularly for IL-4, IL-5, and IL-13. Plots are gated for Thy1.2−Lin− ILCs (left) and CD4+ T cells (right). Numbers indicate the percentage of cells in each quadrant. (B) Absolute number of IL-5−, IL-13−, and IL-4−positive ILC2s and CD4+ T cells in WT and Il9r−/− mice at days 6–12 of the infection (n = 3–8 per group; *, P < 0.05; **, P < 0.005; ***, P < 0.0001). Data in B and C are representative of at least two independent experiments with similar results. (C) Cytokine concentrations in the lungs of WT and Il9r−/− mice at days 6, 9, and 12 of the infection and in naive controls (Con; n = 3–7 per group; *, P < 0.05; **, P < 0.01; ***, P = 0.0006). Data in B and C are representative of at least two independent experiments with similar results (n = 3–8 per group). (D) Flow cytometry of total lung cells from N. brasiliensis–infected bone marrow chimera at day 12 restimulated with phorbol 12,13-dibutyrate and ionomycin for 2.5 h and stained intracellularly for IL-5 and IL-13. Plots are gated for Thy1.2−Lin− ILCs. Numbers in quadrants indicate percentage of cytokine-positive cells in the CD45.1− donor and CD45.1+ recipient ILC subset. (E) Absolute number of cytokine-positive CD45.1− donor ILCs in the lungs of the respective mice (***, P < 0.0001). Data represent two independent experiments with similar results (n = 4–6). Bars show mean values ± SEM.


to investigate whether a reduction of lung ILCs, as observed in the Il9r−/− mice, can lead to impaired lung damage repair after N. brasiliensis infection. To address this question, we used an anti-Thy1.2 antibody to deplete ILCs in N. brasiliensis–infected Rag1−/− mice. In these experiments, ILCs were identified by flow cytometry for lineage markers and IL-7R in the lungs of WT and Il9r−/− mice at days 3, 9, and 12 after N. brasiliensis infection (Fig. 6, A and B). Anti-Thy1.2 antibody treatment resulted in a ∼50% reduction in ILC numbers at day 12 after N. brasiliensis infection (Fig. 6, A and B). In line with the hypothesis that ILC reduction leads to impaired lung tissue restoration, we observed a significant increase in the emphysema-like lung damage in ILC-depleted Rag1−/− mice as compared with the isotype-treated controls (Fig. 6 C). Thus, ILCs contribute to damage repair after helminth-induced lung injury.

IL-9 signaling promotes eosinophil recruitment and alternative activation of macrophages after N. brasiliensis infection

Eosinophil recruitment is a hallmark of type 2 responses in the lung, and IL-33–induced ILC2 expansion has been shown to contribute to lung eosinophilia in Strongyloides venezuelensis–induced lung inflammation (Yasuda et al., 2012). Furthermore, it has been shown recently that eosinophils promote tissue regeneration after muscle injury (Heredia et al., 2013). In line with the reduced ILC2 numbers and IL-5 levels, we found significantly reduced eosinophil numbers (identified as CD11b+Ly6G−SiglecF−CD11c+) in the lungs of Il9r−/− mice at days 6–12 after N. brasiliensis infection (Fig. 7, A and B). Because histological analysis at day 12 after infection suggested differences in the macrophage populations (Fig. 5, C and D), we also quantified alveolar and conventional macrophages in the lungs of WT in Il9r−/− mice after N. brasiliensis infection (Fig. 7, A and C–E). Interestingly, we found increased numbers of CD11b+Ly6G−SiglecF−CD11c+ alveolar macrophages at days 9 and 12 after N. brasiliensis infection (Fig. 7 C), probably representing the increase in heme-laden macrophages observed in the Prussian blue staining. The total number of SiglecF−Ly6G−CD11b+F4/80+ conventional macrophages, in contrast, was unaltered in Il9r−/− mice at days 6–12 after N. brasiliensis infection (Fig. 7, D and E), as was the relation of Ly6C+ inflammatory monocytes to Ly6C− resident macrophages (Jenkins et al., 2011) at these time points (not depicted). To address the potential influence of IL-9R signaling on the activation status of conventional macrophages, we sorted SiglecF−Ly6G−CD11b+F4/80+ macrophages from the lungs of WT and Il9r−/− mice, whereas Arg1 (Arginase 1) and Chi3l3 (Ym-1) levels were also lower, but the reduction failed to reach statistical significance. Importantly, the Il9r mRNA levels in lung macrophages were very low (close to the detection limit; not depicted), indicating that the effect of IL-9R deficiency on macrophage activation status is indirect, probably via the reduced IL-13 levels found in the lungs of Il9r−/− mice. Next, we addressed the abundance and function of goblet cells and mast cells in Il9r−/− mice by histological analysis and expression analysis of goblet cell– and mast cell–related transcripts in total lung RNA extracts (Fig. 7, G–K). These analyses showed similar goblet cell hyperplasia and mast cell accumulation in WT and Il9r−/− mice at days 12 and 9 (not depicted) and revealed only a modest reduction of the mucin Muc5ac and the mast cell protease Mpt1 in Il9r−/− mice that did not reach statistical significance (Fig. 7, G–K). Furthermore, the mRNA expression of the matrix metalloproteinases MMP12 and MMP13, which play a role in tissue remodeling, was induced similarly in WT and Il9r−/− mice (Fig. 7 L). Collectively, these data indicate that IL-9 signaling, most likely by promoting ILC2 accumulation and enhancing production of IL-5 and IL-13 by ILC2s, can influence the function of eosinophils and alternatively activated macrophages that contribute to damage repair mechanisms in the lung.

Increased expression of ILC2–derived amphiregulin after N. brasiliensis infection depends on IL-9 signaling

In addition to IL-9, IL-13, and IL-5, ILC2s produce the epidermal growth factor family member amphiregulin and thereby promote the regeneration of bronchiolar epithelium after influenza infection in Rag1−/− mice (Monticelli et al., 2011). To explore a potential role of ILC2-derived amphiregulin after N. brasiliensis–induced lung injury, we purified IL9Cre+YFP+ lung ILC2s at day 12 after the infection and assessed amphiregulin mRNA expression by quantitative RT-PCR. Indeed, we found high expression of amphiregulin mRNA in these lung ILC2s, whereas amphiregulin expression...
in **IL9Cre**eYFP⁺ T cells isolated from the same **IL9CreR26R2YFP** reporter mice was significantly lower (Fig. 8 A). Comparison of transcript levels between **IL9Cre**eYFP⁺ ILC2s and **IL4-GFP**⁺ Th2 cells from **IL4-GFP** (4get) mice, as well as eosinophils and macrophages all sorted from the lung at day 12 after infection showed the highest amphiregulin expression in ILC2s (Fig. 8 A). Interestingly, the RT-PCR analysis of ILC2s, sorted from the lung at day 12 after infection, indicated that **II9r⁻/⁻** ILCs from infected mice expressed similar levels of amphiregulin mRNA as their WT counterparts (Fig. 8 B). However, the reduction of ILC2 numbers in **II9r⁻/⁻** mice at day 12 after *N. brasiliensis* infection resulted in strongly reduced total *N. brasiliensis* infection in the lungs (Fig. 8 C). Thus, ILC2s appear to be the major source of amphiregulin in the infected lung and might use this mediator to promote tissue repair after *N. brasiliensis*–induced lung injury.

**IL-9 is dispensable for ILC proliferation**

One possible mechanism of how IL-9 might promote ILC2 accumulation in the lung is by enhancing their proliferation in *N. brasiliensis*-infected mice. We therefore assessed Ki67
expression in lung ILC2s, as a marker for cells that are in active phases of the cell cycle. As expected, the percentage of Ki67+ ILC2s in the lungs of helminth-infected mice increased over time (Fig. 9, A and B), indicating an accumulation of activated cells. Comparison of the numbers of Ki67+ ILC2s in WT and Il9r−/− mice at day 12 after infection demonstrated a reduction of Ki67+ ILC2s in the absence of IL-9R signaling (Fig. 9 C). However, the 5′-ethynyl-2′-deoxyuridine (EdU) incorporation rate analyzed 2 h after i.v. injection of the substance, as a direct indicator of actual in situ proliferation, was similar in ILC2s from the lung and Thy1.2+Lin− cells in the MDLNs and bone marrow of WT and Il9r−/− mice at the peak of IL-9 expression (day 9; Fig. 9, D and E). Interestingly, Thy1.2+Lin− ILCs in the MDLNs, being ~50% GATA3+ ILC2s (Fig. 2 D), showed a much higher EdU incorporation rate than lung ILC2s irrespective of IL-9R expression (Fig. 9, D and E). This suggests that ILC2 expansion in the later phase of the infection might take place in the draining LNs, followed by recruitment of the cells to the target organ. Thy1.2+Lin− cells in the bone marrow, which were ~80% GATA3+ ILC2/ILC2 precursors (not depicted; Hoyler et al., 2012), showed a baseline proliferation rate that was not changed after N. brasiliensis infection. These data indicate that proliferation of ILC2s in response to N. brasiliensis infection is not compromised by the lack of IL-9R signaling.

**IL-9 protects ILC2s from apoptosis**

To investigate a direct effect of IL-9 on ILC2s, we isolated ILC2s from the lungs of N. brasiliensis–infected mice at day 12 after the infection and cultured them in the presence or absence of IL-9. Supplementing the culture medium with IL-9 resulted in strongly increased production of IL-5 and IL-13 in WT but not in Il9r−/−deficient ILC2s (Fig. 10 A). As we had previously hypothesized IL-9 might mediate biological functions by promoting the survival of a variety of cell types (Wilhelm et al., 2012), we investigated the possibility that enhanced cytokine expression by cultured ILC2s in the presence of IL-9 could be a consequence of their increased IL-9–mediated survival. To address this issue, we assessed the amount of live cells in the ILC2 cultures by Annexin V and 7-amino-actinomycin D (7AAD) staining. The highly activated ILC2 population isolated at the height of lung inflammation at day 12 showed a large proportion of apoptotic cells after 2 d of culture, indicating the strong predisposition of this cell population to undergo apoptosis. The presence of IL-9 in the culture medium, however, was able to rescue a significant proportion of ILC2s from apoptotic cell death (Fig. 10, B and C). Additionally, the addition of IL-9 resulted in an increased forward scatter profile of ILC2s, indicating an activated blast stage (Fig. 10 C). To assess the number of apoptotic cells in the lung in vivo, we stained tissue sections for expression of the apoptosis marker cleaved caspase-3. Indeed, N. brasiliensis–infected Il9r−/− mice showed increased numbers of cleaved caspase-3–positive lymphoid cells in the lung infiltrates (Fig. 10, E and F). To further explore potential mechanisms of how IL-9 might protect ILC2s from apoptotic cell death in vivo, we purified ILC2s from the lungs of WT and Il9r−/− mice at the peak of IL-9 expression (day 9) and assessed mRNA expression of antiapoptotic proteins that have previously been linked to IL-9–mediated survival in different cell types in vitro (Richard et al., 1999; Rebollo et al., 2000; Fontaine et al., 2008). Whereas BCL2 and BCLXL expression were unchanged, we found a significant reduction of BCL3 expression in Il9r−/− ILCs (Fig. 10 G), a survival factor which has been shown to be directly regulated by IL-9 signaling (Richard et al., 1999). Expression of BCL2, which is induced by γ cytokines such as IL-7 and IL-2 (Deng and Podack, 1993; von Freeden-Jeffry et al., 1997) was unaffected in line with unimpaired surface expression of IL-7R and CD25 on Il9r−/− ILC2s (Fig. 10 H). Collectively, these data suggest that IL-9 is an autocrine factor that promotes ILC2 survival by inducing the expression of the antiapoptotic protein BCL3, thereby maintaining their functional activity in vivo.

**DISCUSSION**

For many years IL-9 was considered to be a cytokine produced by T cells and involved in Th2 responses. IL-9 acts on a wide spectrum of hematopoietic and nonhematopoietic cell types (Goswami and Kaplan, 2011); however, its exact function remained elusive. Several publications provided evidence that in the context of type 2 responses, IL-9 promotes IL-5 and IL-13 production (at that time attributed mainly to Th2 cells) in the lung (Temann, Ray, and Flavell, 2002) and gut-associated lymphoid tissue (Fallon et al., 2000). This suggested an indirect action of IL-9 via promotion of other cytokines.
Indeed, the spontaneous airway inflammation observed in mice with transgenic overexpression of IL-9 was found to be independent of IL-9R expression in nonhematopoietic cells (Steenwinckel et al., 2007), suggesting that the role of IL-9 might be that of a regulatory cytokine rather than a direct effector cytokine. This is further illustrated by the fact that the pulmonary phenotype of Il9 transgenic mice is abolished if these mice are crossed to an IL-13–deficient background (Steenwinckel et al., 2007; Temann et al., 2007). Strikingly, pulmonary inflammation and IL-13 production in Il9 transgenic mice were not abrogated, but in contrast even enhanced on a T cell– and B cell–deficient background (Temann et al., 2007), strongly suggesting that an innate cell type and not Th2 cells is one of the major targets of IL-9.

Recently, the generation of an IL-9 fate reporter mouse strain (IL9creR26R<sup>YFP</sup> mice) enabled us to identify ILC2s as potent producers of IL-9 in vivo in a model of papain-induced lung inflammation (Wilhelm et al., 2011). We could further show that IL-5 and IL-13 expression in ILC2s is regulated by IL-9, albeit the underlying mechanism and the functional importance of ILC2-derived IL-9 was not addressed (Wilhelm et al., 2011).

Figure 7. IL-9 signaling promotes eosinophil recruitment and alternative activation of macrophages. (A and D) Flow cytometry plots of lung cells from WT and Il9<sup>r<sup>−/−</sup></sup> mice at day 9 after N. brasiliensis infection gated on CD11b<sup>+</sup>Ly6G<sup>−</sup> cells (A) and Ly6G<sup>−</sup>SiglecF<sup>−</sup> cells (D). Numbers represent the percentage of SiglecF<sup>−</sup>CD11c<sup>−</sup> eosinophils (A), SiglecF<sup>−</sup>CD11c<sup>−</sup> alveolar macrophages (A), and conventional CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages/monocytes (D). (B, C, and E). Absolute numbers of eosinophils (B), alveolar macrophages (C), and macrophages/monocytes (E) at days 6–12 in the lung of WT and Il9<sup>r<sup>−/−</sup></sup> mice (n = 3 per group; *, P = 0.01; **, P = 0.003; ***, P < 0.004). (F) Quantitative RT-PCR analysis of mRNA transcripts in conventional lung macrophages, sorted by flow cytometry, from the lungs of WT and Il9<sup>r<sup>−/−</sup></sup> mice at day 12 of N. brasiliensis infection (n = 5; **, P = 0.003). (G) Representative periodic acid–Schiff staining of lung sections in WT and Il9<sup>r<sup>−/−</sup></sup> mice at day 12 of the infection. (H) Quantitative RT-PCR analysis of goblet cell–related transcripts in total lung RNA samples at day 12 after infection of WT and Il9<sup>r<sup>−/−</sup></sup> mice and in naive controls (Con). (I and J) Representative immunohistochemical staining for mast cell tryptase (I) and quantification of mast cell tryptase<sup>+</sup> cells (J) in WT and Il9<sup>r<sup>−/−</sup></sup> mice at day 12 of the infection. The inset shows granular mast cell tryptase staining at a higher magnification. Bars: (G) 100 µm; (I) 25 µm. (K and L) Quantitative RT-PCR analysis of mRNA transcripts in total lung RNA samples at day 12 after infection of WT and Il9<sup>r<sup>−/−</sup></sup> mice and in naive controls (Con; H–L: n = 8–9 for day 12, n = 3 for controls). mRNA expression was normalized to Hprt1 (encoding hypoxanthine guanine phosphoribosyltransferase). Data represent at least two independent experiments with similar results. Bars show mean values ± SEM.
Here we show, using a model of *N. brasiliensis* infection in mice, that IL-9 is an ILC2-derived cytokine that critically amplifies the function of IL-13– and IL-5–producing ILC2s by promoting their survival and activation in a positive autocrine feedback loop. Furthermore, we show for the first time that a reduction of lung ILC2s in *Il9r*−/− mice leads to reduced levels of IL-13, IL-5, and amphiregulin, reduced eosinophil recruitment, and alternative activation of macrophages and consequently to impaired lung tissue repair, even though the Th2 response in these mice is intact.

In contrast to the IL-7R (Hoyler et al., 2012), expression of IL-9R was dispensable for maintenance of ILC2s in naive mice, indicating that IL-9 provides a survival signal only for activated ILC2s. Here we identify the antipapoptotic protein BCL3 as a potential mediator for IL-9–mediated protection of activated ILC2s from apoptosis. Interestingly, BCL3 has been described to depend directly on IL-9–mediated Jak/STAT signaling but was not induced by IL-2 (Richard et al., 1999). Furthermore, BCL3 has been shown to promote the survival of T cells in vitro (Rebollo et al., 2000; Bauer et al., 2006). In contrast to BCL3, the expression of the antipapoptotic factor BCL2 that is induced by other γ receptor cytokines like IL-2 and IL-7 (Deng and Podack, 1993; von Freeden-Jeffry et al., 1997) was not changed in *Il9r*−/− ILC2s, indicating that IL-9 might use a survival pathway distinct from the other γ receptor cytokines in vivo.

It has been postulated that the type 2 immune response induced by helminth infections, apart from being instrumental in effective antihelminth immunity, is also required for the wound-healing process that is critical for limiting the extensive tissue damage that these multicellular pathogens often cause while migrating through the host (Allen and Maizels, 2011). In line with this, the absence of the IL-4Rα chain, abolishing both IL-4 and IL-13 signaling, in mice with *N. brasiliensis* infection greatly impaired the resolution of the acute lung hemorrhage caused by this parasite in an early stage (day 4) of the infection (Chen et al., 2012). However, the cell type responsible for this early production of type 2 cytokines in response to the helminth infection was not identified in this study (Chen et al., 2012).

Apart from the acute lung injury observed in *N. brasiliensis*-infected mice, these mice develop chronic histopathological alterations of the lung tissue that resemble lung emphysema, a common end-stage of chronic obstructive pulmonary disease in humans (Marsland et al., 2008). Although previous studies described an accumulation of ILC2s in the lungs of *N. brasiliensis*-infected mice predominantly at later time points (after day 7; Price et al., 2010; Liang et al., 2012), the role of these cells in the chronic tissue remodeling process...
After the acute wound closure has not been addressed so far. In line with these studies, we observed a striking increase of ILC2s in the lung after day 6 of the infection compared with naive mice.

The involvement of ILC2s in maintaining lung tissue integrity had so far only been addressed in mice lacking an adaptive immune system (Monticelli et al., 2011). Therefore, the importance of ILC2s for total cytokine production and tissue repair in comparison with Th2 cells in immunocompetent mice remained unknown. A detailed characterization of ILC populations and T cell subsets in the lung and draining MDLNs of I9r−/− mice infected with N. brasiliensis revealed an organ- and cell type–specific reduction of lung ILC2s caused by their impaired survival in the absence of IL-9R signaling. Using this model, we demonstrate that, even though the acute resolution of alveolar hemorrhage appeared unimpaired in I9r−/− mice, the absence of IL-9R signaling, most likely caused by the reduction of the ILC2 population, resulted in increased emphysema formation and reduced lung function in the chronic stage after the infection. Furthermore, we show that treatment of Rag1−/− mice with a depleting α-Thy1.2 antibody partially reduces ILC numbers in the lungs after N. brasiliensis infection and leads to an increase in emphysematous pathology, supporting an important role for ILCs in damage repair after helminth-induced lung inflammation.

ILC2s produce IL-5, IL-9, IL-13, and potentially other mediators that may enhance damage repair by either directly acting on tissue-resident cells or by changing the abundance and/or activation status of other immune cells. We show here that, in line with reduced IL-5 and IL-13 levels, the absence of IL-9R signaling reduces eosinophil recruitment, increases the presence of alveolar macrophages, and impairs alternative activation of interstitial macrophages. As macrophages sorted from the lung did not express the IL-9R, this effect is likely to be mediated indirectly via the reduced IL-13. Both eosinophils and macrophages have recently been shown to promote tissue repair (Chen et al., 2012; Heredia et al., 2013), and macrophages are known to play an important role in emphysematous lung pathology after N. brasiliensis infection (Heitmann et al., 2012). However, it is possible that the slight reduction in the number of IL-5+ CD4+ T cells in I9r−/− animals contributes to the effect of IL-9R deficiency, reduced IL-5 levels, and eosinophil recruitment.

As an important part of the type 2 response, goblet cells and mast cells are two other potential target cell populations of ILC2-produced mediators. However, goblet cell hyperplasia...
and mast cell accumulation in the lung were only slightly reduced in Il9r−/− mice in the late phase after N. brasiliensis–induced injury, arguing against a major role of IL-9/ILC2 for these cell types in this model.

A previous study in mice lacking T and B cells (Monticelli et al., 2011) identified ILC2s as an important source of amphiregulin, a member of the epidermal growth factor family which promotes regeneration of the bronchial epithelium after acute virus–induced epithelial cell death. Amphiregulin is also produced by mouse Th2 cells (Zaiss et al., 2006), so that the relative contribution of ILC2s compared with Th2 to the production of this mediator, as well as its functional importance for lung repair in immunocompetent mice, remained unknown. Here we show in mice with a largely uncompromised immune system that ILC2s are an important source of amphiregulin in chronic lung inflammation and that, along with the reduction in ILC2s, the absence of IL-9R signaling prevents up-regulation of amphiregulin expression in the lung at later stages after helminth–induced lung injury. It is conceivable that ILC2s might use this mediator to promote tissue repair after N. brasiliensis–induced lung injury.

Interestingly, the deficiency in IL-4, IL-5, or IL-13 signaling alone appears to have no effect on N. brasiliensis–induced emphysema formation in BALB/c mice (Marsland et al., 2008). The relevance of this autocrine feedback loop that promotes ILC2 accumulation in the tissue might lie in a need to tightly control this potentially harmful cell population that can reduce in ILC2s, the absence of IL-9R signaling prevents up-regulation of amphiregulin expression in the lung at later stages after helminth–induced lung injury. It is conceivable that ILC2s might use this mediator to promote tissue repair after N. brasiliensis–induced lung injury.

Flow cytometry and cell sorting. To identify ILCs, isolated leukocytes were stained by using fluorochrome-coupled antibodies against CD45, Thy1.2, CD4, and a combination of lineage markers (Lin), including CD3, CD4, CD8, CD11b, CD11c, CD19, CD49b, TCR–β, TCR–γ, NK1.1, GR-1, and Ter119. For further characterization of ILC surface marker expression, antibodies against CD25, IL–7Rα (CD127), IL–33R (T1/ST2), ICOS, cKit, and Sca-1 were used. For characterization of macrophages and eosinophils, antibodies against CD11b, Ly6G, Ly6C, SiglecF, and CD11c were used. For cell culture experiments and real-time PCR, ILCs were sorted by flow cytometry based on the expression of Thy1.2 in the absence of all lineage markers. CD4+ T cells were sorted as CD4+Thy1.2Lin− cells, eosinophils were sorted as CD11b+Ly6G+SiglecF+CD11c−, and macrophages as Ly6G−SiglecF−CD11b+F4/80+Ly6C+ Sorting purity was typically >95%. Cultured ILCs were stained with fluorochrome-coupled Annexin V in Annexin V–binding buffer according to the manufacturer’s instruction (BioLegend), and dead cells were stained by addition of 7AAD (BioLegend). For intracellular cytokine staining, isolated leukocytes were restimulated with 0.5 μg/ml phorbol 12,13-dibutyrate and 0.5 μg/ml ionomycin in the presence of 1 μg/ml brefeldin A for 2.5 h, fixed with 3.8% formalin, permeabilized with 0.1% IGEPAL CA-630 (Sigma-Aldrich), and stained with combinations of fluorochrome-coupled antibodies against IL–4, IL–5, IL–13, IL–17A, and IFN–γ. Intracellular staining, using an antibody against Ki67, GATA3 (clone 50H12; Bio X Cell), and ROR–γt (clone B2D) was performed with the Transcription Factor Staining Buffer Set (eBiosciences) according to the manufacturer’s instruction. All samples were acquired on a LSRII flow cytometer (BD) and analyzed with FlowJo software (Tree Star).

EdU incorporation assay. EdU (1 mg per mouse) was injected i.v. 2 h before sacrificing the mice. Incorporation of EdU was assessed by using the Click-IT EdU Cell Proliferation Assay (Invitrogen) according to the manufacturer’s instruction.
mercaptopoethanol, 100 U/liter penicillin, and 100 mg/ml streptomycin at a concentration of 2 × 10⁶ per ml with or without 50 ng/ml IL-9 (R&D Systems) for 2 d.

**Cytokine measurements.** For cytokine measurements from lung homogenates, lungs were finely minced, supplemented with proteinase inhibitor (Complete; Roche) in 50 µl PBS, and spun over a 40-µm strainer, and the cell-free supernatant was collected. Cytokine concentrations in lung supernatants and in ILC culture supernatants were measured by using the bead-based cytokine detection assays FlowCytomix (eBioscience) or Cytometric Bead Array (BD) according to the manufacturers’ instructions. Amphiregulin protein content of lung supernatants and ILC culture supernatants was measured by ELISA according to the manufacturer’s instructions (R&D Systems).

**Histology.** After excision of the lungs, the left upper lobe was perfused with 500 µl neutral-buffered formalin (10%) via the main bronchus. The tissue was then fixed overnight in neutral-buffered formalin, washed in 75% ethanol, and embedded in paraffin. Lung sections were stained with Mason’s trichrome, Prussian blue, and periodic acid–Schiff according to standard laboratory procedures. Immunohistochemistry was performed by using antibodies against mast cell tryptase (clone EP847; Abcam) or cleaved caspase-3 (clone Asp175; Cell Signaling Technology), followed by development of the tissue sections with the ZytocChem Plus (AP) Polymer kit (Zytomed) according to the manufacturer’s instructions. Slides were scanned with a VS120 SL slide scanner (Olympus). Images were analyzed with the OlyVIA image viewer (Olympus) and Image software (National Institutes of Health). Emphysema-like damage was quantified by measuring the lung area affected by bullae formation with destruction of the regular tissue architecture and expressed as a percentage of total lung area of the section. Prussian blue–positive macrophages and mast cell tryptase–positive cells were counted manually in an area of at least 2.5 mm² and cleaved caspase-3–positive, non-epithelial, lymphoid cells were counted manually in an area of at least 10 mm² per lung section. Both were expressed as cell number per mm².

**Lung function measurement.** Baseline lung function parameters of conscious mice were obtained with a Buxco FinePointe System for noninvasive airway measurement (Buxco Research Systems) according to the manufacturer’s instructions.

**Real-time PCR.** RNA from sorted ILCs and CD4⁺ T cells was extracted by using the TRIzol Reagent (Life Technologies) and reversely transcribed with the Omniscript RT kit (Qiagen) according to the manufacturers’ instructions. TaqMan Gene Expression Assays in combination with the Universal PCR Master Mix and the ABI-Prism 7900 system (all Applied Biosystems) were used for quantification of the housekeeping gene (Hprt1) and the genes of interest. Target gene quantification was normalized to Hprt1 gene expression.

**Statistical analyses.** The Student’s t test was used for comparison between two groups. In case of three or more groups, one-way ANOVA was used, followed by a post hoc analysis with Bonferroni’s test for multiple comparisons. In case of three or more groups, one-way ANOVA was used, followed by a post hoc analysis with Bonferroni’s test for multiple comparisons. Statistical analyses were performed using GraphPad Prism. The authors have no competing financial interests.

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