



Research Article

Th2 cell-derived IL-4/IL-13 promote ILC2 accumulation in the lung by ILC2-intrinsic STAT6 signaling in mice

Cornelia Symowski and David Voehringer 

Department of Infection Biology, University Hospital Erlangen, Friedrich Alexander University Erlangen-Nuremberg (FAU), 91054, Erlangen, Germany

Infection of mice with the gastrointestinal helminth *Nippostrongylus brasiliensis* elicits profound local proliferation and accumulation of type 2 innate lymphoid cells (ILC2s) in the lung. The regulation of ILC2 proliferation and accumulation in the lung is poorly understood. Using T cell-specific IL-4/IL-13-deficient mice, we demonstrate that IL-4/IL-13 secretion from Th2 cells promotes proliferation and expansion of the ILC2 population in the lung of *N. brasiliensis*-infected mice. Competitive mixed BM chimeras containing normal and STAT6-deficient ILC2s further indicated that ILC2s have to respond directly to IL-4/IL-13 for this effect while STAT6 is not required for IL-13 production in ILC2s. In addition, expression of a constitutively active form of STAT6 in ILC2s was sufficient to promote their proliferation in uninfected mice. The expression of MHC class II in ILC2s appeared to be enhanced by STAT6 signaling supporting the concept that Th2 cells and ILC2s can communicate in an antigen-dependent manner resulting in a Th2-regulated accumulation of ILC2s in the lung during an acute type 2 immune response. Based on our observations, targeting the STAT6 pathway in ILC2s could help to develop new treatments to dampen ILC2 proliferation in the lung and thereby ameliorate ILC2-mediated allergic inflammation.

Keywords: Allergy · Asthma · Helminths · ILC2 homeostasis · Type 2 immunity



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The cytokines IL-4 and IL-13 expressed by a variety of different cell types, including type 2 T helper (Th2) cells, follicular T helper (T_{FH}) cells, basophils, mast cells, and innate lymphoid cells type 2 (ILC2), are critical to mediate most of the immunological aspects at the onset and effector phase of type 2 immunity. Type 2 immune responses are associated with eosinophilia, the accumulation of Th2 cells, basophils, and ILC2s in tissues, elevated serum IgE levels, hyperplasia of goblets cells, polarization of alternative activated macrophages (AAM), and activation of epithelial and

smooth-muscle cells [1]. IL-4 and IL-13 both bind to the shared type II IL-4 receptor, which is a heterodimer consisting of IL-4 receptor α (IL-4R α) and IL-13 receptor α 1 (IL-13R α 1). In addition, IL-4 can bind to the type I IL-4 receptor made up of the IL-4R α and the common gamma chain (γ c). Both receptors can activate the Jak/STAT signaling cascade [2–4]. The STAT6 plays an important role in the immune system leading to the transcription of genes in different cell types and thereby promotes Th2 differentiation, Ig class switching to IgE, secretion of chemokines like CCL17 (TARC), CCL11 (eotaxin 1), and CCL24 (eotaxin 2), differentiation of AAMs, GC formation, and antigen presentation by B cells [5, 6].

Gastrointestinal helminth infection represents one of the most widespread chronic human parasitoses and several mouse models

Correspondence: Prof. David Voehringer
e-mail: david.voehringer@uk-erlangen.de

serve as well-established tools to study helminth-induced type 2 immune responses and cytokine regulation [7, 8]. Mice infected with the rodent nematode *Nippostrongylus brasiliensis* (*Nb*) have revealed that the protective anti-helminth immunity is largely dependent on IL-4/IL-13-induced STAT6 activation [9, 10]. Using T cell-specific IL-4/IL-13-deficient mice (4-13Tko), we previously showed that IL-4/IL-13 produced by cells of the innate immune system is required for effective type 2 immune responses against *Nb* [11]. Transfer experiments of in vitro expanded ILC2s into *Nb*-infected IL-4/IL-13-deficient mice revealed that ILC2s, which are potent producers of IL-13, can efficiently promote worm expulsion during primary *Nb* infection [12]. Although T cell-derived IL-4/IL-13 is dispensable, it is well established that T cells are still required for efficient worm clearance under physiological conditions [12–15]. Numerous studies showed that ILC2s and Th2 cells collaborate during type 2 immune responses to establish protective immunity, either in a direct cell–cell contact-dependent manner via interaction of costimulatory molecules or through cytokines [16]. Others have reported that T cells promote ILC2 proliferation and IL-13 production in an MHC class II-dependent manner [17]. Interestingly, it has been described that ILC2s in *Nb*-infected STAT6-deficient mice show reduced cytokine production [18]. However, the role of STAT6 in ILC2s with regard to their proliferation, homeostasis, and effector function is still an open question.

Here, we show that *Nb*-infected T cell-specific IL-4/IL-13-deficient mice exhibit decreased ILC2 numbers in the lung compared to WT mice indicating that T cell-derived IL-4 or IL-13 promotes ILC2 accumulation in the lung. Analysis of mixed BM chimeras composed of WT and IL-4/IL-13-unresponsive STAT6-deficient ILC2s revealed a requirement of STAT6 in ILC2s for proliferation and accumulation in the lung but not in the lymph nodes. Interestingly, ILC2-intrinsic STAT6 was dispensable for IL-13 production by ILC2s. Furthermore, we observed that MHCII expression on ILC2s is reduced in the absence of STAT6 supporting the concept that ILC2s can act back on T cells in a positive feedback loop promoting the Th2 response as previously reported [17, 19].

Results

T cell-derived IL-4/IL-13 promote expansion of ILC2s after *Nippostrongylus brasiliensis* infection

We thought to dissect the relative contribution of Th2 cell-derived cytokines on ILC2 proliferation and expansion in vivo since previous studies have shown that ILC2s and Th2 cells can functionally interact in a reciprocal manner [17, 20, 21]. Therefore, we compared mice lacking IL-4/IL-13 only in T cells (4-13Tko), with complete IL-4/IL-13-deficient mice (4-13ko) and WT mice. ILC2s were identified by flow cytometry based on their expression of the IL-33 receptor (T1/ST2), ICOS, KLRG1, CD90, IL-7R α , and GATA3 (Supporting Information Fig. 1A and B). We observed no differences in the frequency and total cell number of ILC2s in the lung and the draining mesenteric lymph nodes (mLNs) of these

three groups of mice at steady state (Fig. 1A). To induce type 2 inflammation in the lung, mice were infected s.c. with *Nb* larvae, which migrate through the lung within the first few days of infection and translocate to the intestine by day 3, where they develop into mature adults and produce eggs, which are expelled in the feces [22]. When mice were analyzed on days 5, 7, and 9 after infection, we observed that the frequency and total cell number of ILC2s steadily increased in the lung of infected WT mice and this response was significantly reduced in 4-13Tko and 4-13ko mice (Fig. 1B). In contrast to the lung, the percentages of ILC2s in the mLNs were similar in *Nb*-infected WT and 4-13Tko mice and only slightly reduced in the complete IL-4/IL-13-deficient mice (Supporting Information Fig. 1C). To address whether the reduced number of ILC2s in 4-13Tko and 4-13ko mice is due to decreased ILC2 proliferation in the lung, we injected ethynyl deoxyuridine (EdU), a thymidine nucleoside analogue, into the mice 6 h before the analysis. Indeed, the percentage of EdU⁺ ILC2s was significantly reduced in 4-13Tko and 4-13ko mice in comparison to WT mice at day 5 after *Nb* infection, indicating that actively proliferating EdU⁺ ILC2s were directly or indirectly dependent on T cell-derived IL-4/IL-13 in the early phase of *Nb* infection (Fig. 1C and D). Furthermore, the proliferation rate went back to low levels by day 9 after infection and no differences were observed between all three strains of mice at these time points (Fig. 1C).

KLRG1^{high} ST2[−] “inflammatory” ILC2s (iILC2s) have been shown to expand in response to IL-25 and also transiently appear in low numbers in the lung after *Nb* infection [23]. Although we found no iILC2s in the lungs of uninfected mice, their frequency and total number increased on day 5 after *Nb* infection and this effect was promoted by T cell-derived IL-4/IL-13 (Fig. 1E). EdU incorporation analysis further revealed that the proliferation rate of iILC2s was about sevenfold higher as compared to ST2⁺ ILC2s in all three strains of mice but the significant difference between WT, 4-13Tko, and 4-13ko mice was also observed in this population (Fig. 1E).

These findings indicate that IL-4 or IL-13 from T cells contributes to proliferation of ILC2s in the lung, which leads to ILC2 accumulation during *Nb* infection.

ILC2-intrinsic STAT6 signaling is important for ILC2 accumulation in the lung

Since ILC2s express the receptor subunits IL-4R α and IL-13R α 1, they can respond to IL-4 and IL-13 directly [24–26]. We therefore decided to analyze whether ILC2 accumulation in the lung is dependent on direct IL-4/IL-13-induced STAT6 activation in ILC2s. For that reason, we generated mixed bone marrow chimeras (MBMCs) in which equal numbers of BM cells from congenic WT (Ly5.1⁺ Thy1.2⁺) and STAT6^{−/−} (Ly5.2⁺ Thy1.2⁺) mice were transplanted into lethally irradiated WT mice (Thy1.1⁺) (Fig. 2A). This setting allowed us to study WT ILC2s and STAT6-deficient ILC2s in the same cellular environment, and any defects in proliferation or effector function of the STAT6-deficient ILC2s would therefore indicate a cell-intrinsic requirement for STAT6

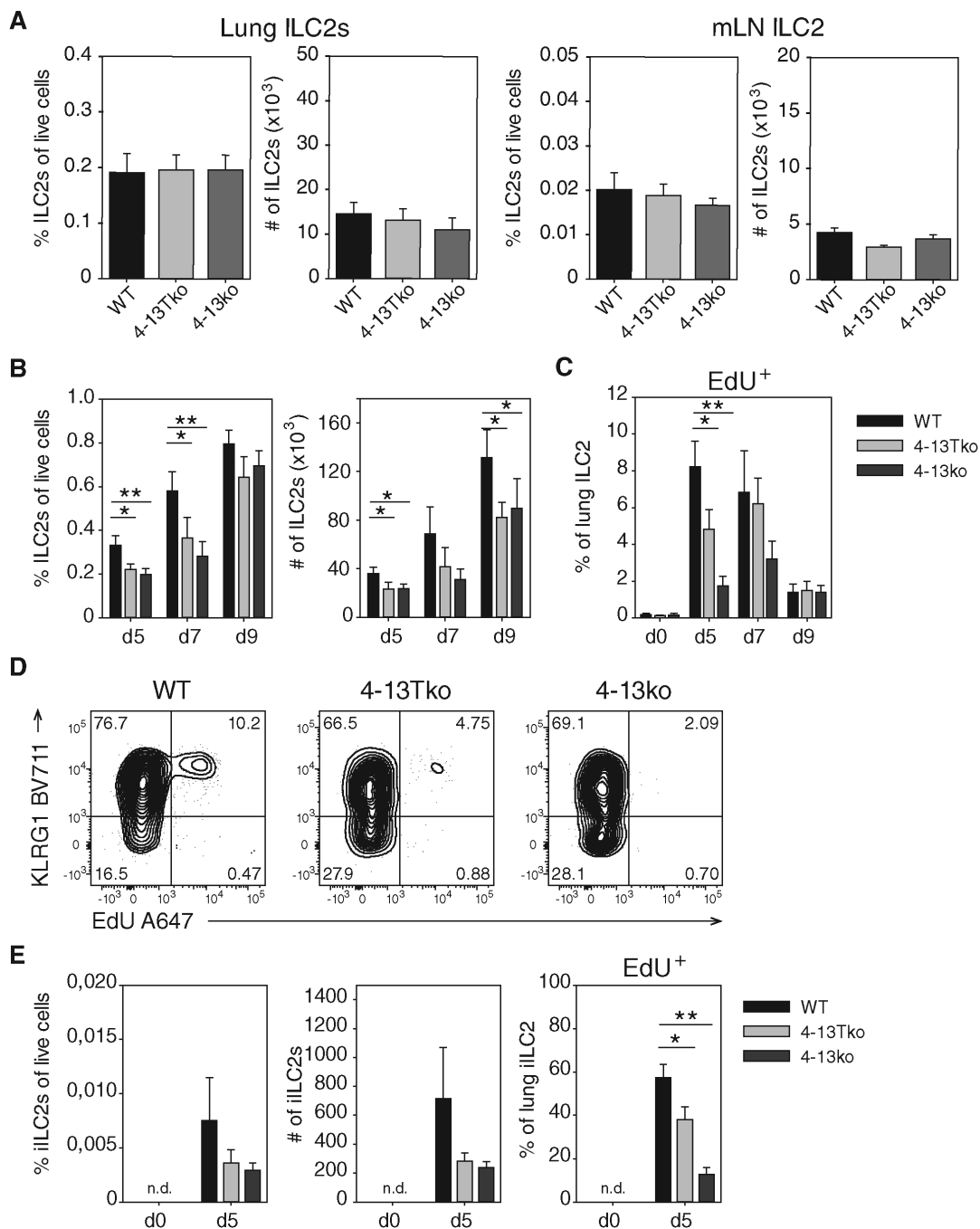


Figure 1. T-cell derived IL-4/IL-13-regulated expansion of ILC2s after *Nippostrongylus brasiliensis* infection. (A) ILC2s of indicated tissues from naïve WT (black), 4-13Tko (gray), and 4-13ko (dark gray) mice were analyzed by flow cytometry. Graphs show percentages and total numbers of Lin⁻ ST2⁺ ICOS⁺ KLRG1⁺ ILC2s (Supporting Information Fig. S1). Lin⁺ cells include CD3e⁺ CD4⁺ CD8e⁺ CD11b⁺ CD11c⁺ FcεR1⁺ B220⁺ Gr1⁺ Ter119⁺ NK1.1⁺ Nkp46⁺ cells. Bars show the mean + SD of three individual mice per group and are representative of two independent experiments. (B) Frequency (left panel) and total numbers (right panel) of lung ILC2s from WT (black), 4-13Tko (gray), and 4-13ko (dark gray) mice were analyzed at day 5, 7, and 9 after *Nb* infection. Bars show the mean + SEM from three to six individual mice per group and are representative of three independent experiments. (C) ILC2 proliferation was measured via flow cytometric analysis of EdU incorporation. WT (black), 4-13Tko (gray), and 4-13ko (dark gray) mice were treated with EdU (i.v.) 6 h before analysis. The percentage of proliferating EdU⁺ ILC2s was analyzed at indicated time points of infection. Bars show mean + SEM from three individual mice per group and are representative of three independent experiments. (D) Representative flow cytometry dot plots of EdU staining of LIN⁻ CD90⁺ ST2⁺ ICOS⁺ ILC2s in the lung of WT, 4-13Tko, and 4-13ko mice at day 5 after *Nb* infection. (E) Frequency (left panel), total numbers (middle panel) and proliferation of lung inflammatory iILC2s from WT (black), 4-13Tko (gray), and 4-13ko (dark gray) mice were analyzed at steady-state and day 5 after *Nb* infection. Bars show the mean + SD from three to four individual mice per group and are representative of two independent experiments except day 0 where data of one experiment is shown. * $p \leq 0.05$; ** $p \leq 0.01$, unpaired two-tailed Student's *t*-test.

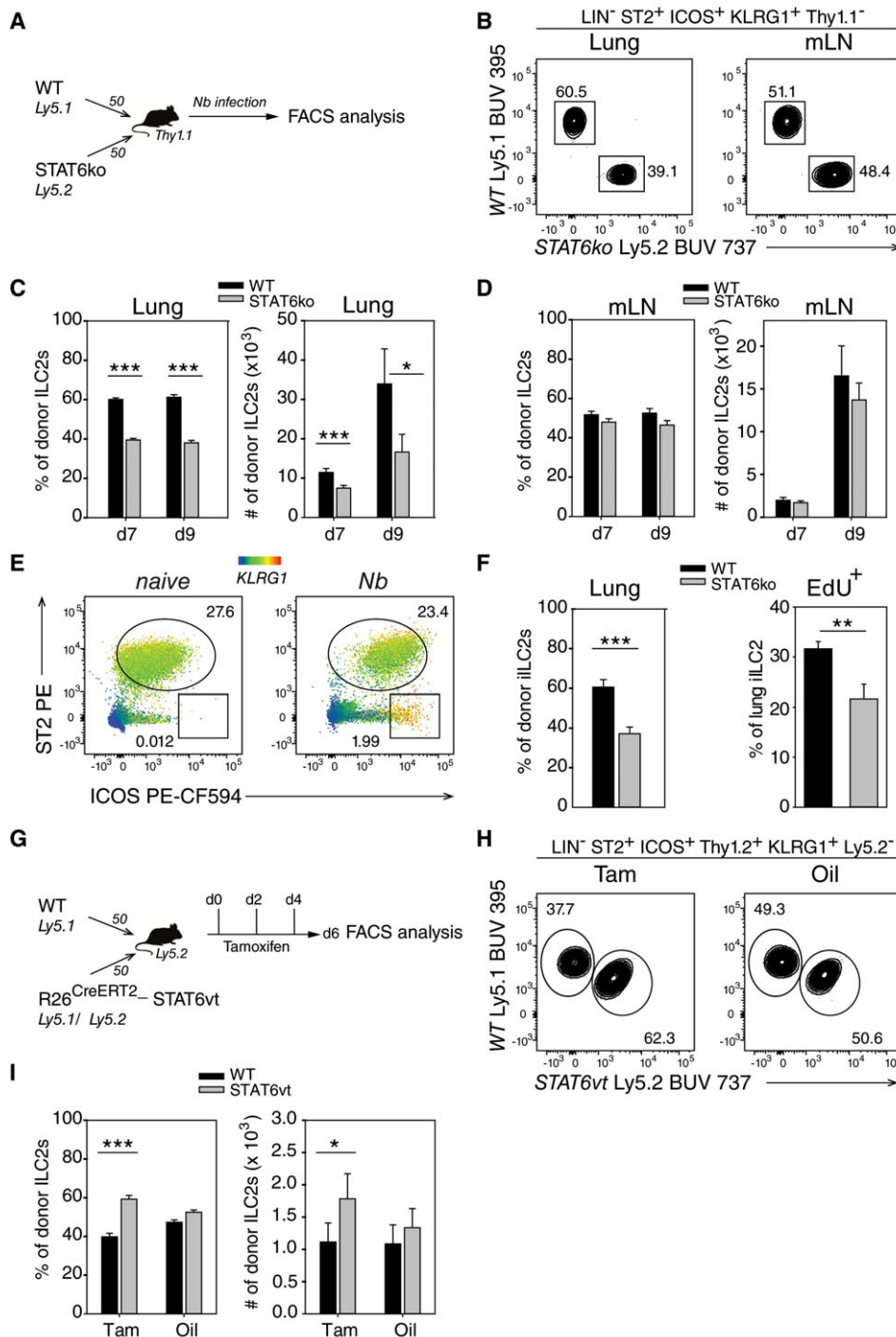


Figure 2. ILC2-intrinsic STAT6 signaling is important for ILC2 accumulation in the lung. (A) Mixed BM chimeras (MBMC) that received cells from Ly5.1⁺ WT and Ly5.2⁺ STAT6^{-/-} mice were analyzed by flow cytometry at days 7 and 9 after infection with Nb. (B) Displayed are representative dot plots of indicated tissues from (Ly5.1⁺) WT and (Ly5.2⁺) STAT6^{-/-} donor (Thy1.1⁻) ILC2s. Panels (C) and (D) show the frequency (left panels) and total numbers (right panels) of donor ILC2s in the lung (C) and in the mLN (D) of MBMCs. (E) Representative 3D dot plots show the lung LIN⁻ CD90⁺ ST2⁻ KLRG1^{high} inflammatory ILC2s (iILC2) indicated by the heat map (Blue: low expression; Red: high expression) and (F) the frequency and proliferation of donor iILC2 at day 7 after Nb infection in MBMCs. Bars show the mean + SEM of three to four individual mice per group and are representative of three independent experiments. (G) MBMCs that received cells from WT (Ly5.1⁺) and R26^{CreERT2}-STAT6vt (tamoxifen-inducible constitutively activated STAT6) (Ly5.1/Ly5.2⁺) were analyzed after treating three times with tamoxifen. (H) Displayed are representative dot plots of donor ILC2s after treatment with tamoxifen or oil as a control. (I) Bars show the mean + SEM frequency (left panels) and total numbers (right panels) of four individual WT (black) and STAT6vt (gray) mice. Data are representative of two independent experiments. **p* ≤ 0.05; ****p* ≤ 0.001, unpaired two-tailed Student's *t*-test.

signaling. During analysis of these MBMCs, we used B cells as a reference population and observed equal frequencies of B220⁺ B cells from both donor populations in different tissues (Supporting Information Fig. 2A and B). However, within the ILC2 compartment, we observed that IL-4/IL-13-unresponsive STAT6-deficient ILC2s have a competitive disadvantage compared to WT ILC2s in the lung of Nb-infected mice (Fig. 2B and C). This intrinsic effect was predominantly observed at a later time point of infection (Fig. 2C). Interestingly, we found no difference in the percentage and total

cell count of STAT6-deficient and WT ILC2s in the mLNs of Nb-infected mice excluding a systemic competitive disadvantage of STAT6-deficient ILC2s (Fig. 2B and D). The STAT6-independent ILC2 proliferation in mLNs indicates that ILC2 proliferation in lymphoid tissues might be regulated by other factors that remain to be identified.

The MBMCs further revealed that iILC2s from STAT6-deficient donors were significantly reduced at day 7 after Nb infection and this effect was most likely due to decreased proliferation (Fig. 2E

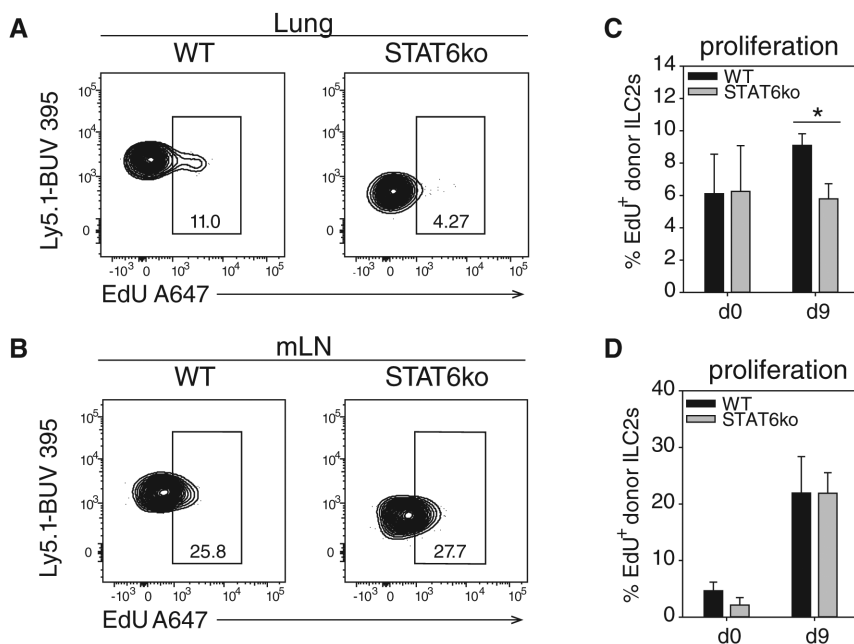


Figure 3. STAT6-dependent ILC2 proliferation in the lung. (A) Displayed are representative dot plots of proliferating donor-derived EdU⁺ WT (Ly5.1⁺) ILC2s and STAT6^{-/-} (Ly5.2⁺) ILC2s (LIN⁻ ST2⁺ ICOS⁺ KLRG1⁺ Thy1.1⁻) in the lung and (B) mLN of MBMCs (see Fig. 2A) infected with *Nb* (day 9). (C) The percentage of EdU⁺ WT (black) and STAT6^{-/-} (gray) ILC2s (Thy1.1⁻) was analyzed at indicated time points of infection in the lung and (D) mLN of MBMCs (see Fig. 2A). Bars show the mean + SEM of four individual mice per group and are representative of two independent experiments. **p* ≤ 0.05, unpaired two-tailed Student's *t*-test.

and F). Our competitive setting indicates that IL-4/IL-13 signaling via STAT6 is important for iILC2 proliferation and perhaps migration into the lung because it has been described that iILC2s are circulating cells that originate from ILC2s residing in the gut [27]. We also found significantly more WT ILC2s in the colon but not the lamina propria of the small intestine in the MBMCs (Supporting Information Fig. 2C).

To further investigate whether expression of a constitutively active version of STAT6 (STAT6vt) within ILC2s would promote their proliferation in the lung of uninfected mice, we generated competitive MBMCs in which irradiated Ly5.2⁺ recipient mice were reconstituted with 50% BM from WT (Ly5.1⁺) and 50% BM from R26^{CreERT2}-STAT6vt mice (Ly5.1/Ly5.2⁺) where STAT6vt can be inducibly expressed by tamoxifen injection (Fig. 2G). After tamoxifen administration, the percentage and total cell count of donor ILC2s expressing STAT6vt were increased compared to WT ILC2s in the lung while MBMCs exposed to oil (solvent control) showed equal distribution of both donor ILC2 populations (Fig. 2I and H).

We conclude from these experiments that lung ILC2s respond directly to IL-4/IL-13 and STAT6-regulated genes in ILC2s promote their accumulation in the lung during helminth infection.

STAT6-dependent ILC2 proliferation in the lung

To dissect the role of ILC2-intrinsic STAT6 on ILC2 proliferation, the MBMCs (see Fig. 2A) were injected intraperitoneally with EdU. Analysis of MBMCs before *Nb* infection (d0) revealed a relatively high baseline ILC2 proliferation rate in the lung (Fig. 3B) when compared to normal mice (Fig. 1C) suggesting that irradiation-induced effects may enhance ILC2 proliferation in steady-state situation independently of STAT6 signaling. However, we observed a significant reduction in the percentage of EdU⁺ STAT6-deficient

ILC2s (Ly5.1⁻) compared to WT ILC2s (Ly5.1⁺) at day 9 after *Nb* infection. Noteworthy, this effect was only restricted to cells in the lung (Fig. 3A and C). In the mLNs, we observed a similar level of proliferation of both donor ILC2 populations (Fig. 3B and D). The difference between lung and mLN might be due to different microenvironments in these tissues with yet to be identified factors that promote ILC2 proliferation in the mLN in a STAT6-independent manner.

All ILCs differentiate from Id2-expressing common helper-like ILC progenitor (CHILP) in the BM [28]. To further exclude that STAT6 plays an important role during ILC2 development in the BM, we analyzed the frequency of Lin⁻ FLT3⁻ CD25⁻ CD127⁺ α4β7⁺ CHILPs in the MBMC mice (Supporting Information Fig. 3A). We found no difference in the percentages of CHILPs between WT (Ly5.1⁺) and STAT6-deficient (Ly5.2⁺) cells at steady state and at day 7 after *Nb* infection (Supporting Information Fig. 3B), indicating that ILC2 development is not dependent on STAT6.

Overall, the competitive MBMC setting revealed that ILC2-intrinsic STAT6 regulates ILC2 proliferation during helminth infection in the lung but not mLN and also has no impact on ILC2 precursor populations in the BM.

STAT6 promotes expression of chemotactic receptors on ILC2s

Besides the effect of STAT6 on ILC2 proliferation, we further determined the role of STAT6 for expression of chemotactic receptors. We focused on the chemokine receptors CCR4 and CCR8, which have been shown to be expressed in Th2 cells in a STAT6-dependent manner [29]. In addition, we analyzed expression of the prostaglandin D2 receptor CRTH2 that promotes ILC2 accumulation in the lung of *Nb*-infected mice and chemotaxis of human ILC2s [30, 31]. We isolated ILC2s from the lungs of *Nb*-infected

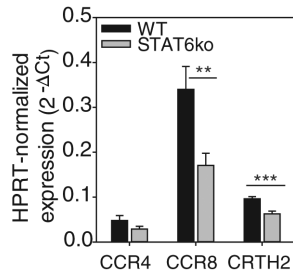


Figure 4. STAT6-dependent expression of chemotactic receptors. Quantitative RT-PCR analysis of ex vivo expanded WT (black) and STAT6^{-/-} (gray) ILC2s for expression of indicated chemotactic receptors. Bars show the mean + SEM of two independent experiments with triplicates from ILC2 cultures generated from five to ten pooled samples of each strain. ***p* ≤ 0.01, ****p* ≤ 0.001, unpaired two-tailed Student's *t*-test

WT and STAT6^{-/-} mice, expanded them in vitro for 7 days with medium containing IL-2, IL-7, IL-25, and IL-33, and determined expression of CCR4, CCR8, and CRTH2 by quantitative RT-PCR. CCR8 and CRTH2 were expressed at a significantly higher level in WT as compared to STAT6^{-/-} ILC2s (Fig. 4). This suggests that STAT6 may also regulate migration of ILC2s to the lung during inflammatory conditions, although the relative contribution of such an effect remains to be demonstrated in future experiments.

STAT6 is not required for the production and secretion of Th2 cytokines by ILC2

We further investigated whether STAT6 is required in ILC2s for production of IL-5 and IL-13 as it was implicated by comparing ILC2 cytokine production in WT and STAT6^{-/-} mice [18]. Therefore, we measured the concentration of IL-5 and IL-13 in the supernatants of in vitro expanded ILC2s sorted ex vivo from lungs of *Nb*-infected WT and STAT6^{-/-} mice and observed comparable cytokine levels in both cultures (Fig. 5A).

In addition, quantitative RT-PCR analysis of ex vivo-isolated ILC2s confirmed that IL-5 and IL-13 production is independent of STAT6, while we also excluded the possibility that sorted Lin⁻ ST2⁺ CD90⁺ ILC2s from lungs of *Nb*-infected mice would be contaminated with IFN- γ producing ILC1s and IL-17 producing ILC3s (Fig. 5B). Although it has been described that ILC2s transcribe and produce small amounts of IL-4 [32, 33], we were not able to detect any IL-4 by ELISA or RT-PCR (Fig. 5B and data not shown). To verify that STAT6 is not crucial for cytokine production by ILC2s in vivo, we infected WT/STAT6^{-/-} MBMCs (see Fig. 2A) with *Nb* and measured IL-13 production after brief re-stimulation with PMA and ionomycin. Intracellular staining of IL-13 in WT and STAT6-deficient ILC2s showed that the expression level (MFI) and the percentage of IL-13 was elevated during helminth infection, however, lack of STAT6 in donor ILC2s had no impact on cytokine expression and production in lung and mLN (Fig. 5C and D).

This indicates that, unlike in conventional Th2 cells, STAT6 does not regulate type 2 cytokine production in ILC2s during steady-state conditions and after helminth infection.

STAT6-dependent MHC class II expression on ILC2s in the lung

Several studies demonstrated that ILC2s can regulate Th2 function and augment IL-13 production under certain conditions via cytokine secretion or in a MHC class II- or PD-L1-dependent manner [17, 34, 35]. Since we could show that cytokine production by ILC2s was independent of STAT6, we thought to investigate whether ILC2-intrinsic STAT6 is involved in a positive feedback loop by upregulating PD-L1 or MHC class II to control adaptive Th2 cell function, which then leads to accumulation of ILC2s. However, the expression of PD-L1 was equal in WT and STAT6-deficient donor ILC2s of *Nb*-infected MBMCs (Fig. 6A), indicating that STAT6 does not upregulate PD-L1 on lung ILC2s to interact with PD-1 expressing T cells (Fig. 6A and B). Several cell surface molecules like MHC class II are known to be upregulated on lymphocytes in response to IL-4 [36]. Since others have reported that MHC class II-expressing ILC2s interact with antigen-specific T cells during protective immunity to helminth infection [17], we decided to investigate whether IL-4/IL-13 directly regulate MHC class II expression on ILC2s. Therefore, we analyzed ex vivo expanded lung ILC2s from WT and STAT6-deficient *Nb*-infected mice and observed that MHC class II was downregulated in STAT6-deficient ILC2s as compared to WT ILC2s (Fig. 6C). Additionally, we examined the MHC class II expression on ILC2s of the WT/STAT6^{-/-} MBMCs in vivo at steady-state conditions and after *Nb* infection. This competitive setting revealed that the MFI of MHCII and the percentage of MHCII⁺ ILC2s lacking STAT6 (Ly5.2⁺) were significantly reduced compared to IL-4/IL-13-responsive ILC2s (Ly5.1⁺) at day 9 after *Nb* infection (Fig. 6D and E). In uninfected lungs of MBMC mice, we observed no differences of MHC class II expression on ILC2s (Fig. 6D). These data indicate that MHC class II expression by ILC2s was induced by IL-4/IL-13/STAT6 signaling during type 2 inflammation.

Discussion

In this study, we found a new mechanism by which Th2 cells interact with ILC2s and regulate their proliferation in the lung in a STAT6-dependent manner during protective immunity against helminth infection. ILC2s expand early during *Nb* infection in both the lung and in the draining mLN in concert with early Th2 cell differentiation. However, the mechanisms that lead to accumulation of ILC2s in tissues are not completely understood. Recruitment of effector cells to the lung during type 2 immune responses is largely driven by IL-4- or IL-13-mediated mechanisms. We have previously shown that IL-4/IL-13 from T cells promotes recruitment of innate immune cells including eosinophils and basophils into the lung after *Nb* infection [11]. Recent reports showed that IL-4 secreted from basophils has a pivotal role to induce ILC2 proliferation in lung and skin inflammation models, indicating that ILC2s have the potential to respond to IL-4 [25, 26]. ILC2 proliferation in a papain-induced model of allergic lung inflammation has further been shown to be promoted by T cell-derived IL-2

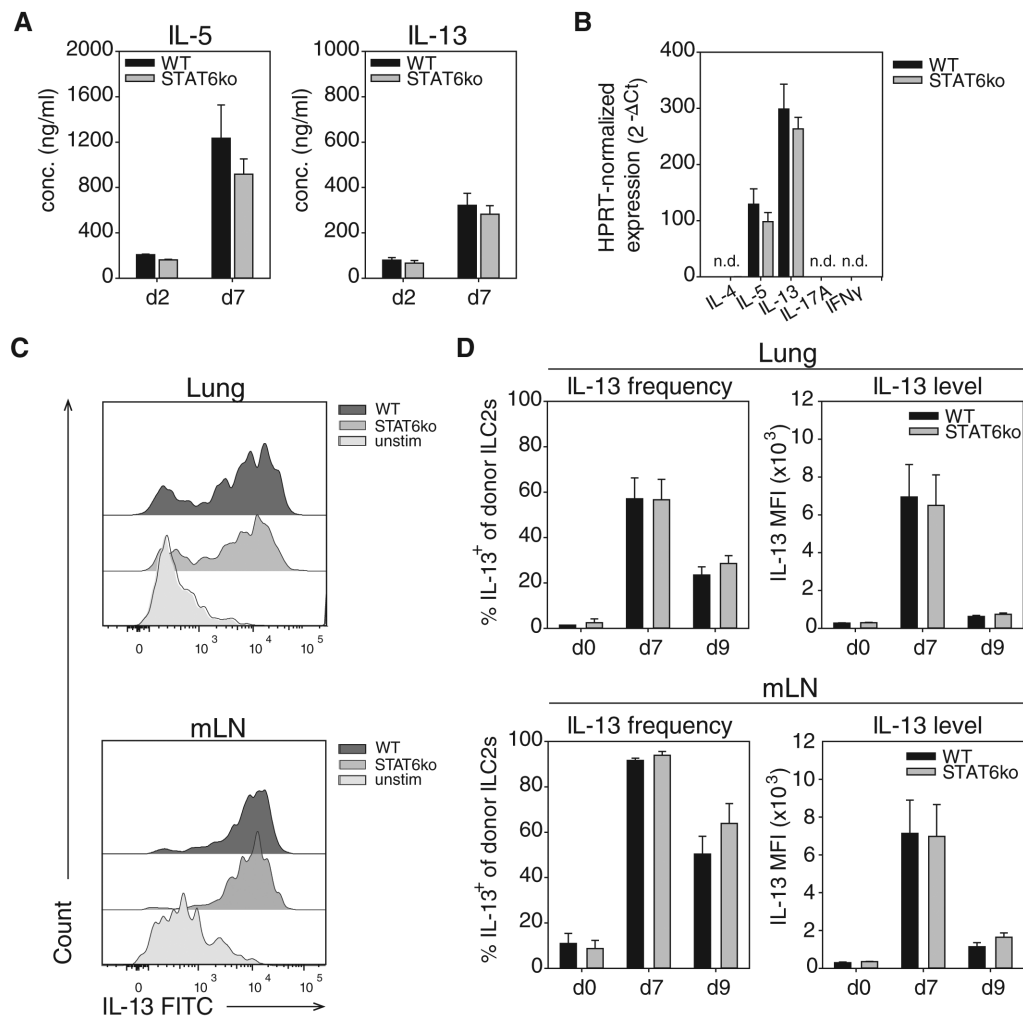


Figure 5. STAT6 is not required for the production and secretion of Th2 cytokines by ILC2. (A) The supernatants from ex vivo expanded WT (black) and STAT6^{-/-} (gray) ILC2s were analyzed on days 2 and 7 after cultivation with IL-2, IL-7, IL-25, IL-33 for indicated cytokines by ELISA. Bars show the mean + SEM of three individual mice per group and are representative of two independent experiments. (B) Quantitative RT-PCR analysis of ex vivo expanded WT (black) and STAT6^{-/-} (gray) ILC2s for expression of indicated cytokines. Bars show the mean + SEM of two experiments performed in triplicates. (C) MBMCs (see Fig. 2A) were infected with *Nb* and cytokine production was measured after 4 h restimulation with phorbol-12-myristate-13-acetate (PMA) and ionomycin. Representative histograms show intracellular IL-13 staining in donor LIN⁻ ST2⁺ ICOS⁺ KLRG1⁺ Thy1.1⁻ ILC2 in the lung (top panel) and mLN (bottom panel) of MBMC at day 7 after helminth infection (WT: black; STAT6^{-/-}: dark gray; unstimulated control: gray). (D) Percentage (left panels) and the MFI (right panels) of IL-13 among donor ILC2 in the lung (top panel), mLNs (bottom panel) of MBMC (see Fig. 2A). Bars show the mean + SEM of three individual mice per group and are representative of two independent experiments.

and autocrine IL-9 signaling [37]. Although ILC2s are present in Rag1^{-/-} mice, it is described that CD4⁺ T cells are required for the maintenance of ILC2-induced immunity to *Nb* [17, 21, 37]. Here, we show that T cell-derived IL-4/IL-13 directly promote ILC2 responses by inducing proliferation of ILC2s, which leads to their accumulation in the lung. This is consistent with the role of IL-4 in supporting proliferation of other lymphoid cell types such as T cells or B cells [36, 38]. Future experiments will have to further decipher, whether T cell-derived IL-13 or IL-4 is the potential cytokine directly activating ILC2s. So far, in a papain-induced lung inflammation model, ILC2s showed lower expression of the IL-13 receptor, which suggests that ILC2s more likely respond to IL-4 [25].

Competitive mixed BM chimeras composed of WT and IL-4/IL-13-unresponsive STAT6-deficient ILC2 further revealed a requirement of STAT6 in ILC2s for the IL-4/IL-13-dependent proliferation and local accumulation in the lung. No difference was observed in peripheral lymph nodes arguing against a systemic effect of STAT6 in ILC2s. Indeed, expression of a constitutively active version of STAT6 (STAT6vt) in ILC2s was sufficient for ILC2 expansion in uninfected lung. These data are consistent with an earlier study where STAT6 controls ILC2 proliferation after airway challenges with the fungal allergen *Alternaria*, although it remained unclear whether STAT6 was required within ILC2s [39]. Previous studies have documented that the T cell-derived cytokine IL-2 is important to promote ILC2 proliferation and IL-13 production, and

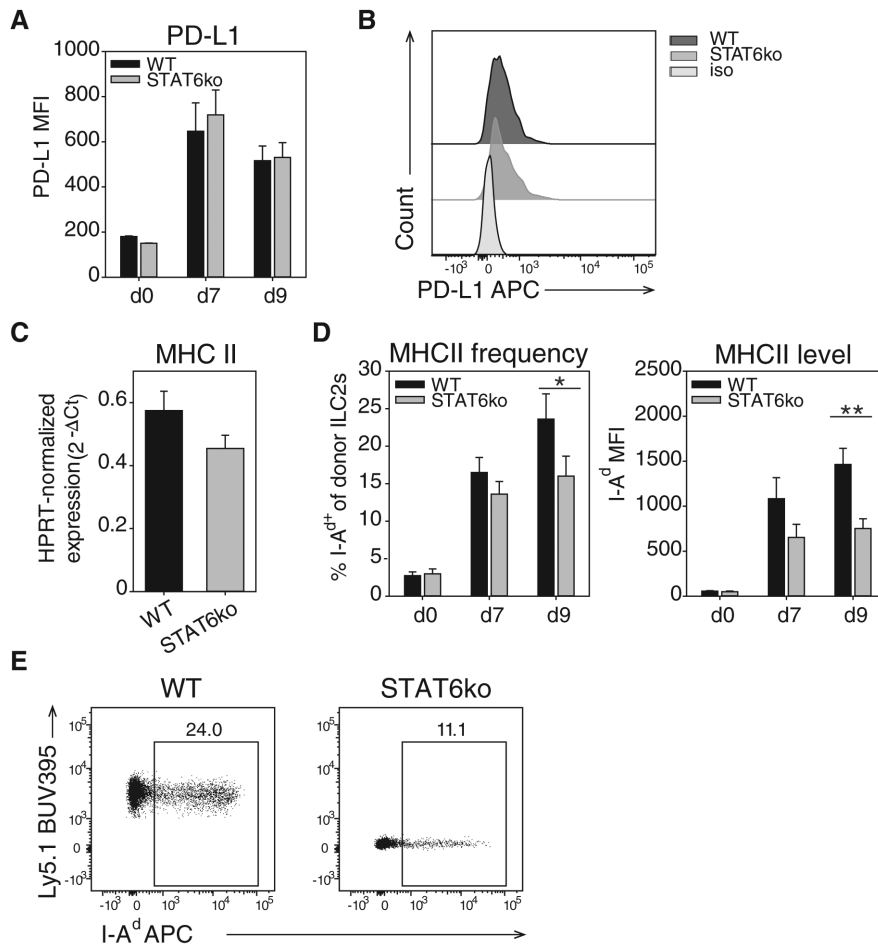


Figure 6. STAT6-dependent MHCII expression on ILC2s in the lung. (A) MFI of PD-L1 among donor (Thy1.1⁺) WT (black) and STAT6^{-/-} (gray) ILC2s in the lung of naïve and *Nb*-infected MBMCs (see Fig. 2A). (B) Histogram of PD-L1 staining of WT (Ly5.1⁺) ILC2s and STAT6^{-/-} (Ly5.2⁺) ILC2s (LIN⁻ ST2⁺ ICOS⁺ KLRG1⁺ Thy1.1⁺) in the lung at day 7 after *Nb* infection. (C) Quantitative RT-PCR analysis of ex vivo expanded WT (black) and STAT6^{-/-} (gray) ILC2s for MHCII (H2-Aa) expression and normalized to HPRT. (D) Percentage (left panel) and the MFI (right panel) of MHCII among donor (Thy1.1⁺) WT (black) and STAT6^{-/-} (gray) ILC2s in the lung of naïve and *Nb* infected MBMCs (see Fig. 2A). (E) Displayed are representative dot plots of MHCII⁺ WT (Ly5.1⁺) ILC2s and STAT6^{-/-} (Ly5.2⁺) ILC2s (LIN⁻ ST2⁺ ICOS⁺ KLRG1⁺ Thy1.1⁺) in the lung at day 9 after *Nb* infection in the competitive setting. Bars show the mean + SEM of two to three individual mice per group and are representative of two independent experiments. **p* ≤ 0.05; ***p* ≤ 0.01, unpaired two-tailed Student's *t*-test.

subsequent upregulation of CD25 (IL-2R) expression [17, 21, 40]. In Th2 cells, CD25 is regulated via STAT6 [41], however, real-time PCR analysis revealed equal CD25 expression level in STAT6-deficient ILC2s and WT ILC2s (data not shown), suggesting that IL-2 is not the major driver for ILC2 proliferation in the lung.

In addition, it was previously reported that ILC2s from *Nb*-infected STAT6^{-/-} mice produce less IL-13 as compared to WT mice [18]. In contrast, we could prove that in vitro and in vivo ILC2 production of Th2 cytokines occurred independent of STAT6. The results indicate that indirect effects from STAT6 in other cell types in vivo may be contributing to regulation of IL-13 expression in ILC2s.

Parabiosis experiments in an unchallenged setting revealed that ILC2s are long-lived cells and are locally maintained as tissue-resident cells in lymphoid and nonlymphoid organs. However, in the context of prolonged parasitic infection or allergic lung inflammation, ILC2s can infiltrate to the sites of inflammation [42, 43]. In addition, inflammatory ILC2s (iILC2s), which were proposed to be transient progenitors of natural ILC2s (nILC2s) in the lung, are described to migrate from the intestine to the lung [27]. Since we could show that STAT6-deficient iILC2s have a competitive disadvantage in the lung, we currently cannot exclude that STAT6 might be involved in regulation of recruitment of iILC2s to the

lung. Moreover, ILC2s express various chemotactic receptors and we found a STAT6-dependent upregulation of CCR8 and CCR2 expression in ILC2s. This indicates that ILC2-intrinsic STAT6 could also be involved in orchestrating trafficking between tissues by regulating expression of chemotactic receptors. However, to determine the role of STAT6 in ILC2 recruitment, further studies are required including global analysis of STAT6-regulated genes in ILC2s.

In particular, recent work has demonstrated that ILC2s are important in building a bridge between innate and adaptive immune response through MHC class II interaction in an antigen-specific manner to orchestrate inflammation [17, 19, 40]. Interestingly, we showed that MHC class II expression in lung ILC2s is induced by STAT6 signaling, suggesting that ILC2-intrinsic STAT6 is involved in a positive feedback loop by promoting MHC class II expression to control adaptive Th2 cell function, which then leads to proliferation and expansion of ILC2s. This feedback may explain why ILC2 numbers are still increasing after helminth larvae already left the lung. Since it is known that ILC2s not only act as alarmin sensors and that these cells can cooperate with CD4⁺ T cells to ensure efficient long-term immunity by maintaining AAM macrophages [21], it is important to understand complex mechanisms that control ILC2 population size in the lung following *Nb* infection.

In conclusion, although ILC2s can be activated directly by epithelial cell-derived alarmins, lung ILC2s respond to T cell-derived IL-4/IL-13, leading to activation of STAT6 signaling. ILC2-intrinsic STAT6 signaling regulates ILC2 number and proliferation in the lung, but not production of IL-5 and IL-13. In the context of type 2 inflammation, it appears that reciprocal coordinated interaction between Th2 cells and ILC2s is important for facilitating local ILC2 accumulation in the lung to orchestrate protective immunity against helminth infection.

Materials and Methods

Mice

IL-4/IL-13-deficient mice (4-13ko) were kindly provided by A.N. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK) [22]. Mice with a loxP-flanked IL-4/IL-13 allele [44] were crossed to CD4Cre mice [45] to generate mice with selective deletion of both cytokines in T cells (4-13Tko mice) and backcrossed for more than ten generations to BALB/c background. STAT6^{-/-} mice [46] and Thy1.1 congenic mice were described previously and were used on a 4get/BALB/c background [47]. Rosa26^{LSL-STAT6vt} mice (STAT6vt mice) were generated by inserting a constitutively active version of STAT6 with a C-terminal FLAG-tag (STAT6vt) [48] behind a loxP-flanked Stop cassette into the genomic Rosa26 locus by standard ES cell technology and pBigT/pROSA26-PA targeting vector system [49]. STAT6vt mice were bred to R26^{CreERT2} (C57BL/6-Gt(ROSA)26Sor^{tm9(Cre/ESR1)Arte}) mice [50] to generate experimental R26^{CreERT2}-STAT6vt mice. All mice were housed according to institutional guidelines and used between 6 and 12 weeks of age. All animal experiments were performed in accordance with German animal protection law and European Union guidelines 86/809 and were approved by the Federal Government of Lower Franconia.

Helminth infections

Infectious third-stage *Nb* larvae were recovered from the cultured feces of infected rats and washed extensively in sterile 0.9% saline (37°C). 500 viable larvae (L3) were inoculated subcutaneously. Mice were supplemented with antibiotics-containing water (2 g/L neomycin sulfate, 100 mg/L polymyxin B; Sigma-Aldrich, St Louis, MO) for the first 5 days after infection. Mice were analyzed at indicated time points after infection.

Generation of MBMCs

For generation of MBMCs, BM cells were obtained from tibia and femur of 4get/STAT6^{-/-} (Ly5.2⁺ congenic) or R26^{CreERT2}-STAT6vt (Ly5.1/Ly5.2 congenic) mice and mixed at a 1:1 ratio with BM cells from WT mice (Ly5.1⁺ congenic). A total of

2×10⁶ cells were injected into the tail vein of lethally irradiated recipient (9 Gy) 4get-Thy1.1-BALB/c (Ly5.2⁺ congenic) or (11Gy) C57BL/6 (Ly5.2⁺ congenic) mice. Mice were treated with antibiotic-containing drinking water for 8 weeks after reconstitution.

Single-cell tissue preparation

Lungs were perfused by injection of 10 mL cold PBS into the right ventricle of the heart. Immune cells were isolated from lung and lamina propria with the GentleMACS device using GentleMACS C tubes according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Percoll density gradient centrifugation was performed to remove debris. mLN's were mechanically dispersed using a 100 µm cell nylon strainer (Becton Dickinson, Franklin Lakes, NJ). Red blood cells were removed from all organs by ACK lysis (155 mM NH₄Cl, 9.99 mM KHCO₃, 0.10 mM EDTA). Cell suspensions were washed in FACS buffer (PBS, 2% fetal calf serum; Life technologies by Thermo Fisher Scientific, Darmstadt, Germany) and subjected to staining protocols for flow cytometry analysis.

Flow cytometry

Single cell suspensions were first incubated with rat antimouse CD16/CD32 antibodies (clone 2.4G2; BioXcell, West Lebanon, NH) to avoid unspecific staining. The following antibodies were applied for 30 min at 4°C: BioLegend (San Diego, CA, USA) mAbs: anti-CD3-PE/Cy7 (17A2), anti-CD4-PE/Cy7 (RM4-5), anti-CD11c-PE/Cy7 (N418), anti-CD127-PE/DazzleTM 594 (A7R34), anti-Ly5.2-Brilliant Violet 421TM (104), anti-CD3e-FITC (500A2), anti-ICOS-APC (C398.4A), anti-I-A^d-APC (AMS-32.1), anti-Thy1.2-APC (30-H12), anti-Thy1.2-PerCP/Cy5.5 (53-2.1), anti-B220-PerCP/Cy5.5 (RA3-6B2), anti-LPAM-1-PE (DATK32); Miltenyi mAbs: anti-NKp46-PE-Vio770 (REA815), anti-NK1.1-PE-Vio770 (PK136); BD Biosciences (Heidelberg, Germany) mAbs: anti-PD-L1(CD274)-APC (MIH5), anti-ICOS-PE-CF594 (C398.4A), anti-KLRG1-BV711 (2F1) anti-CD127-BV711 (SB/199), anti-Ly5.1-BUV395 (A20); anti-Ly5.2-BUV737 (104), MD Bioproducts (Egg b. Zürich, Switzerland): anti-ST2-PE (DJ8); eBioscience/Thermo Fisher Scientific mAbs: anti-CD11b-PE-Cy7 (M1/70) anti-FcεR1-PE-Cy7 (MAR-1), anti-B220-PE-C7 (RA3-6B2), anti-CD4-FITC (RM4-5), anti-CD8a-FITC (53-6.7), anti-B220-FITC (RA3-6B2), anti-Thy1.1-PerCP-Cy5.5 (HIS51), anti-Sca-1-PE (D7), anti-Ly5.2-PE (104), anti-CD25-APC-eFluor 780 (PC61.5), anti-Flt3-APC (A2F10), and biotinylated anti-CD3 (clone 145-2C11), anti-CD4 (GK1.5), anti-CD8a (53-6.7), anti-B220 (RA3-6B2), and anti-Gr-1 (RB6-8C5). To detect biotinylated antibodies, PE-Cy7-, APC-Cy7-, and FITC-labeled streptavidin was used. Live/dead cell exclusion was performed with Fixable Viability Dye eFluorTM 506 (Thermo Fisher Scientific). For staining of transcription factor, cells were fixed and permeabilized using the FoxP3 staining buffer kit (Thermo Fisher Scientific)

according to manufacturer's protocol and stained with the following antibodies: anti-GATA3-BUV395 (L50-823). For intracellular detection of cytokines, cells were stimulated with 50 ng/mL phorbol-12-myristate-13-acetate and 2 µg/mL ionomycin for 4 h. After 2 h, 10 µg/mL Brefeldin A (Sigma–Aldrich) was added. The cells were fixed with 4% para-formaldehyde (PFA, Carl Roth, Karlsruhe, Germany) at room temperature for 20 min, followed by adding permeabilization buffer (BioLegend) and stained with anti-IL-13 Alexa Fluor 488 (eBio13A) from eBioscience/Thermo Fisher Scientific. Samples were measured (see gating strategy in Supporting Information Fig. S1) on an LSRFortessa cell analyzer (BD Biosciences) and were analyzed with Flowjo (V10; TreeStar, Ashland, OR, USA) in accordance to the “Guidelines for the use of flow cytometry and cell sorting in immunological studies” [51].

EdU incorporation

After *Nb* infection, 6 h before analysis, mice were injected with 1 mg 5-ethynyl-2'-deoxyuridine (EdU) (Sigma–Aldrich) in 100 µL PBS into the tail vein. Single-cell suspensions from mLN and lungs were stained for surface antigens, washed with 1% BSA, and subjected to the Click-it Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific).

Tamoxifen treatment

Mice were injected three times every second day i.p. with 1 mg tamoxifen (Sigma–Aldrich) dissolved in 200 µL Miglyol (Caesar & Loretz GmbH, Hilden, Germany).

In vitro culture of ILC2 cells and ELISA

ILC2s were sorted by S3 Cell Sorter (Bio-Rad, Hercules, CA) from the lungs of day 7 *Nb*-infected WT or STAT6^{−/−} mice. ILC2s identified as lineage-negative, ST2⁺ CD90⁺ cells were subsequently expanded in high glucose medium (Dulbecco's modified Eagle's GlutaMAX medium (PanBiotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FCS), 50 µM β-mercaptoethanol, 10 mM nonessential amino acids, 1 mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin (all from Life technologies by Thermo Fisher Scientific), and 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer) stimulated with 10 ng/mL IL-2 (Biolegend), 10 ng/mL IL-7 (R&D Systems, Minneapolis, MN), 10 ng/mL IL-25 (Immunotools, Friesoythe, Germany), and 10 ng/mL IL-33 (R&D Systems) for 7 days. Cell-free supernatants were collected for ELISA at days 2 and 7 of ex vivo cultured cells. IL-5 and IL-13 concentrations were measured with an ELISA Development Kit (Peprotech, Hamburg, Germany). Alkaline phosphatase-coupled streptavidin and para-Nitrophenylphosphat substrate (both from Southern Biotec, Birmingham, AL) were used for detection.

Quantitative RT-PCR

Total RNA was extracted from sorted lung ILC2s at day 7 after *Nb* infection with the RNeasy Mini Kit (Qiagen, Hilden, Germany) using MinElute spin column. Then, 1 µg total RNA was used for first-strand complementary DNA synthesis using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and oligo(dT) primers. The resulting cDNA was used as template for quantitative PCR. Real-time PCR was performed with a CFX96 Connect Real-Time PCR Detection System (Bio-Rad Laboratories) with ten-fold diluted cDNA templates using SYBR Green I (Thermo Fisher Scientific) and the following primer sequences: IL-4 forward 5'-ACT TGA GAG AGA TCA TCG GCA -3'; IL-4 reverse 5'-AGC TCC ATG AGA ACA CTA GAG TT -3'; IL-5 forward 5'-AGG ATG CTT CTG CAC TTG AG -3'; IL-5 reverse 5'-TCT GTA CTC ATC ACA CCA AG -3'; IL-13 forward 5'-GCA GTC CTG GCT CTT GCT TG -3'; IL-13 reverse 5'-TGC TTT GTG TAG CTG AGC AG -3'; IL-17A forward 5'-ACT ACC TCA ACC GTT CCA CG -3'; IL-17A reverse 5'-CAC ACC CAC CAG CAT CTT CT -3'; IFN-γ forward 5'-ACG CTA CAC ACT GCA TCT TG -3'; IFN-γ reverse 5'-TCG GAT GAG CTC ATT GAA TG -3'; H2-Aa forward 5'-TGG GCA CCA TCT TCA TCA TTC -3'; H2-Aa reverse 5'-GGT CAC CCA GCA CAC CAC TT -3'; CCR4 forward 5'-AGA AAA TTC ATG CGA GGG AGA A -3'; CCR4 reverse 5'-TGT CAC TCA AGG GTC GTG TCA -3'; CCR8 forward 5'-TGA CCG ACT ACT ACC CTG ATT TCT T -3'; CCR8 reverse 5'-GCT GCC CCT GAG GAG GAA -3'; CRTH2 forward 5'-CCT GGT GGA AAA CGG ACT CAT C -3'; CRTH2 reverse 5'-GGA AGT AGG TGA AGA AAG GCA GG -3'. Normalized gene expression values were calculated as the ratio of expression of mRNA of interest to the expression of mRNA for Hprt (encoding hypoxanthine guanine phosphoribosyltransferase). HPRT1 forward 5'-GTT GGA TAC AGG CCA GAC TTT GTT-3', HPRT1 reverse 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'. PCR conditions were the following: 15 min initial denaturation at 95°C, 40 cycles with 30 s of denaturation at 95°C, 45 s of annealing at 58°C, and 45 s of elongation at 72°C.

Statistics

Statistical assessment of the acquired data was performed with SigmaPlot 12.3 software (Systat Software Inc., San Jose, CA). Data were analyzed using two-tailed Student's *t*-test for normally distributed data or Mann–Whitney *U*-test for nonnormally distributed data as indicated in the figure legends. *p*-Values <0.05 were considered statistically significant.

Acknowledgements: The authors thank S. Wirtz for critical technical advice, K. Castiglione and D. Döhler for technical support, and members of the Voehringer laboratory for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft

DFG as part of the SPP1937 Priority Program on ILC research (grant Vo944/8-1)

Conflict of Interest: The authors declare no commercial or financial conflict of interest.

References

- Finkelman, F. D., Shea-Donohue, T., Morris, S. C., Gildea, L., Strait, R., Madden, K. B., Schopf, L. et al., Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol. Rev.* 2004. **201**: 139–155.
- Takeda, K., Kishimoto, T. and Akira, S., STAT6: its role in interleukin 4-mediated biological functions. *J. Mol. Med.* 1997. **75**: 317–326.
- Hebenstreit, D., Wirnsberger, G., Horejs-Hoeck, J. and Duschl, A., Signaling mechanisms, interaction partners, and target genes of STAT6. *Cytokine Growth Factor Rev.* 2006. **17**: 173–188.
- LaPorte, S. L., Juo, Z. S., Vaclavikova, J., Colf, L. A., Qi, X., Heller, N. M., Keegan, A. D. et al., Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell* 2008. **132**: 259–272.
- Turqueti-Neves, A., Otte, M., Prazeres da Costa, O., Hopken, U. E., Lipp, M., Buch, T. and Voehringer, D., B-cell-intrinsic STAT6 signaling controls germinal center formation. *Eur. J. Immunol.* 2014. **44**: 2130–2138.
- Goenka, S. and Kaplan, M. H., Transcriptional regulation by STAT6. *Immunol. Res.* 2011. **50**: 87–96.
- Finkelman, F. D., Pearce, E. J., Urban, J. F., Jr. and Sher, A., Regulation and biological function of helminth-induced cytokine responses. *Immunol. Today* 1991. **12**: A62–66.
- Garside, P., Kennedy, M. W., Wakelin, D. and Lawrence, C. E., Immunopathology of intestinal helminth infection. *Parasite Immunol.* 2000. **22**: 605–612.
- Urban, J. F., Jr., Noben-Trauth, N., Donaldson, D. D., Madden, K. B., Morris, S. C., Collins, M. and Finkelman, F. D., IL-13, IL-4R α , and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity* 1998. **8**: 255–264.
- Schubart, C., Krljanac, B., Otte, M., Symowski, C., Martini, E., Gunther, C., Becker, C. et al., Selective expression of constitutively activated STAT6 in intestinal epithelial cells promotes differentiation of secretory cells and protection against helminths. *Mucosal Immunol.* 2018.
- Oeser, K., Schwartz, C. and Voehringer, D., Conditional IL-4/IL-13-deficient mice reveal a critical role of innate immune cells for protective immunity against gastrointestinal helminths. *Mucosal Immunol.* 2015. **8**: 672–682.
- Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K., Bucks, C. et al., Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 2010. **464**: 1367–1370.
- Katona, I. M., Urban, J. F., Jr. and Finkelman, F. D., The role of L3T4+ and Lyt-2+ T cells in the IgE response and immunity to *Nippostrongylus brasiliensis*. *J. Immunol.* 1988. **140**: 3206–3211.
- Voehringer, D., Reese, T. A., Huang, X., Shinkai, K. and Locksley, R. M., Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J. Exp. Med.* 2006. **203**: 1435–1446.
- Seidl, A., Panzer, M. and Voehringer, D., Protective immunity against the gastrointestinal nematode *Nippostrongylus brasiliensis* requires a broad T-cell receptor repertoire. *Immunology* 2011. **134**: 214–223.
- Symowski, C. and Voehringer, D., Interactions between innate lymphoid cells and cells of the innate and adaptive immune system. *Front. Immunol.* 2017. **8**: 1422.
- Oliphant, C. J., Hwang, Y. Y., Walker, J. A., Salimi, M., Wong, S. H., Brewer, J. M., Englezakis, A. et al., MHCII-mediated dialog between group 2 innate lymphoid cells and CD4(+) T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity* 2014. **41**: 283–295.
- Liang, H. E., Reinhardt, R. L., Bando, J. K., Sullivan, B. M., Ho, I. C. and Locksley, R. M., Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat. Immunol.* 2011. **13**: 58–66.
- Mirchandani, A. S., Besnard, A. G., Yip, E., Scott, C., Bain, C. C., Cerovic, V., Salmond, R. J. et al., Type 2 innate lymphoid cells drive CD4+ Th2 cell responses. *J. Immunol.* 2014. **192**: 2442–2448.
- Roediger, B., Kyle, R., Tay, S. S., Mitchell, A. J., Bolton, H. A., Guy, T. V., Tan, S. Y. et al., IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation. *J. Allergy Clin. Immunol.* 2015. **136**: 1653–1663 e1657.
- Bouchery, T., Kyle, R., Camberis, M., Shepherd, A., Filbey, K., Smith, A., Harvie, M. et al., ILC2s and T cells cooperate to ensure maintenance of M2 macrophages for lung immunity against hookworms. *Nat. Commun.* 2015. **6**: 6970.
- Bouchery, T., Volpe, B., Shah, K., Lebon, L., Filbey, K., LeGros, G. and Harris, N., The study of host immune responses elicited by the model murine hookworms *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Curr. Protoc. Mouse Biol.* 2017. **7**: 236–286.
- Huang, Y., Guo, L., Qiu, J., Chen, X., Hu-Li, J., Siebenlist, U., Williamson, P. R. et al., IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat. Immunol.* 2015. **16**: 161–169.
- Bando, J. K., Nussbaum, J. C., Liang, H. E. and Locksley, R. M., Type 2 innate lymphoid cells constitutively express arginase-I in the naive and inflamed lung. *J. Leukoc. Biol.* 2013. **94**: 877–884.
- Motomura, Y., Morita, H., Moro, K., Nakae, S., Artis, D., Endo, T. A., Kuroki, Y. et al., Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation. *Immunity* 2014. **40**: 758–771.
- Kim, B. S., Wang, K., Siracusa, M. C., Saenz, S. A., Brestoff, J. R., Monticelli, L. A., Noti, M. et al., Basophils promote innate lymphoid cell responses in inflamed skin. *J. Immunol.* 2014. **193**: 3717–3725.
- Huang, Y., Mao, K., Chen, X., Sun, M. A., Kawabe, T., Li, W., Usher, N. et al., S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science* 2018. **359**: 114–119.
- Klose, C. S. N., Flach, M., Mohle, L., Rogell, L., Hoyler, T., Ebert, K., Fabianke, C. et al., Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* 2014. **157**: 340–356.
- Zhang, S., Lukacs, N. W., Lawless, V. A., Kunkel, S. L. and Kaplan, M. H., Cutting edge: differential expression of chemokines in Th1 and Th2 cells is dependent on Stat6 but not Stat4. *J. Immunol.* 2000. **165**: 10–14.
- Wojno, E. D., Monticelli, L. A., Tran, S. V., Alenghat, T., Osborne, L. C., Thome, J. J., Willis, C. et al., The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol.* 2015. **8**: 1313–1323.
- Salimi, M., Stoger, L., Liu, W., Go, S., Pavord, I., Klenerman, P., Ogg, G. et al., Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. *J. Allergy Clin. Immunol.* 2017. **140**: 1090–1100 e1011.

- 32 Fallon, P. G., Ballantyne, S. J., Mangan, N. E., Barlow, J. L., Dasvarma, A., Hewett, D. R., McIlgorm, A. et al., Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J. Exp. Med.* 2006. **203**: 1105–1116.
- 33 Pelly, V. S., Kannan, Y., Coomes, S. M., Entwistle, L. J., Ruckerl, D., Seddon, B., MacDonald, A. S. et al., IL-4-producing ILC2s are required for the differentiation of TH2 cells following *Heligmosomoides polygyrus* infection. *Mucosal Immunol.* 2016. **9**: 1407–1417.
- 34 Halim, T. Y., Steer, C. A., Matha, L., Gold, M. J., Martinez-Gonzalez, I., McNagny, K. M., McKenzie, A. N. et al., Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 2014. **40**: 425–435.
- 35 Schwartz, C., Khan, A. R., Floudas, A., Saunders, S. P., Hams, E., Rodewald, H. R., McKenzie, A. N. J. et al., ILC2s regulate adaptive Th2 cell functions via PD-L1 checkpoint control. *J. Exp. Med.* 2017. **214**: 2507–2521.
- 36 Noelle, R., Krammer, P. H., Ohara, J., Uhr, J. W. and Vitetta, E. S., Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor. *Proc. Natl. Acad. Sci. USA* 1984. **81**: 6149–6153.
- 37 Wilhelm, C., Hirota, K., Stieglitz, B., Van Snick, J., Tolaini, M., Lahl, K., Sparwasser, T. et al., An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nat. Immunol.* 2011. **12**: 1071–1077.
- 38 Li-Weber, M. and Krammer, P. H., Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat. Rev. Immunol.* 2003. **3**: 534–543.
- 39 Doherty, T. A., Khorram, N., Chang, J. E., Kim, H. K., Rosenthal, P., Croft, M. and Broide, D. H., STAT6 regulates natural helper cell proliferation during lung inflammation initiated by *Alternaria*. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2012. **303**: L577–588.
- 40 Drake, L. Y., Iijima, K. and Kita, H., Group 2 innate lymphoid cells and CD4⁺ T cells cooperate to mediate type 2 immune response in mice. *Allergy* 2014. **69**: 1300–1307.
- 41 Kim, S. H., Gunst, K. V. and Sarvetnick, N., STAT4/6-dependent differential regulation of chemokine receptors. *Clin. Immunol.* 2006. **118**: 250–257.
- 42 Gasteiger, G., Fan, X., Dikiy, S., Lee, S. Y. and Rudensky, A. Y., Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science* 2015. **350**: 981–985.
- 43 Stier, M. T., Zhang, J., Goleniewska, K., Cephus, J. Y., Rusznak, M., Wu, L., Van Kaer, L. et al., IL-33 promotes the egress of group 2 innate lymphoid cells from the bone marrow. *J. Exp. Med.* 2018. **215**: 263–281.
- 44 Voehringer, D., Wu, D., Liang, H. E. and Locksley, R. M., Efficient generation of long-distance conditional alleles using recombineering and a dual selection strategy in replicate plates. *BMC Biotechnol.* 2009. **9**: 69.
- 45 Li, S., Bostick, J. W., Ye, J., Qiu, J., Zhang, B., Urban, J. F., Jr., Avram, D. et al., Aryl hydrocarbon receptor signaling cell intrinsically inhibits intestinal group 2 innate lymphoid cell function. *Immunity* 2018. **49**: 915–928 e915.
- 46 Shimoda, K., van Deursen, J., Sangster, M. Y., Sarawar, S. R., Carson, R. T., Tripp, R. A., Chu, C. et al., Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 1996. **380**: 630–633.
- 47 Schwartz, C., Oeser, K., Prazeres da Costa, C., Layland, L. E. and Voehringer, D., T cell-derived IL-4/IL-13 protects mice against fatal *Schistosoma mansoni* infection independently of basophils. *J. Immunol.* 2014. **193**: 3590–3599.
- 48 Osbourn, M., Soares, D. C., Vacca, F., Cohen, E. S., Scott, I. C., Gregory, W. F., Smyth, D. J. et al., HpARI protein secreted by a helminth parasite suppresses interleukin-33. *Immunity* 2017. **47**: 739–751 e735.
- 49 Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F., Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev. Biol.* 2001. **1**: 4.
- 50 Schwartz, C., Turqueti-Neves, A., Hartmann, S., Yu, P., Nimmerjahn, F. and Voehringer, D., Basophil-mediated protection against gastrointestinal helminths requires IgE-induced cytokine secretion. *Proc. Natl. Acad. Sci. USA* 2014. **111**: E5169–5177.
- 51 Cossarizza, A., Chang, H. D., Radbruch, A., Akdis, M., Andra, I., Annunziato, F., Bacher, P. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur. J. Immunol.* 2017. **47**: 1584–1797.

Abbreviations: **AAM:** alternative activated macrophage · **EdU:** ethynyl deoxyuridine · **ILC:** innate lymphoid cell · **Nb:** *Nippostrongylus brasiliensis* · **T_{FH}:** follicular T helper

Full correspondence: Prof. David Voehringer, Department of Infection Biology, University Hospital Erlangen, Friedrich Alexander University Erlangen-Nuremberg (FAU), 91054 Erlangen, Germany.
e-mail: david.voehringer@uk-erlangen.de

The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.201948161>

Received: 21/2/2019

Revised: 17/4/2019

Accepted: 28/5/2019

Accepted article online: 29/5/2019

European Journal of Immunology

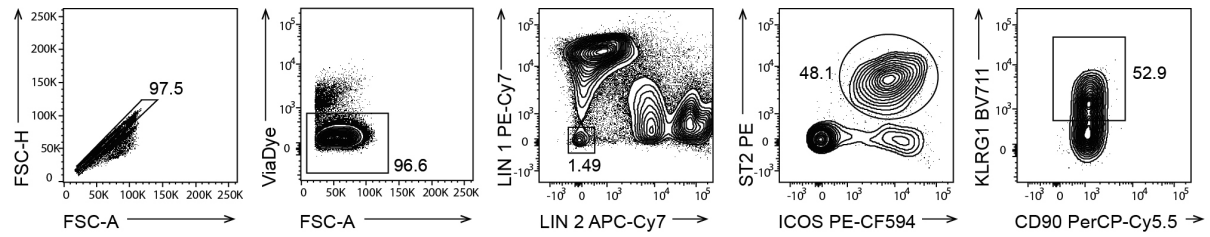
**Supporting Information
for**

DOI 10.1002/eji.201948161

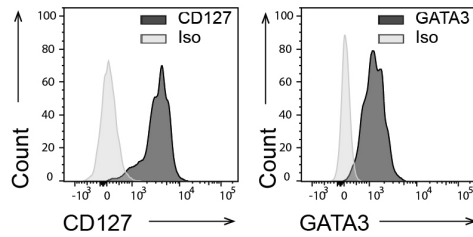
Cornelia Symowski and David Voehringer

**Th2 cell-derived IL-4/IL-13 promote ILC2 accumulation in the lung by
ILC2-intrinsic STAT6 signaling in mice**

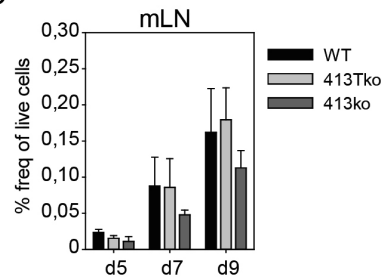
A



B

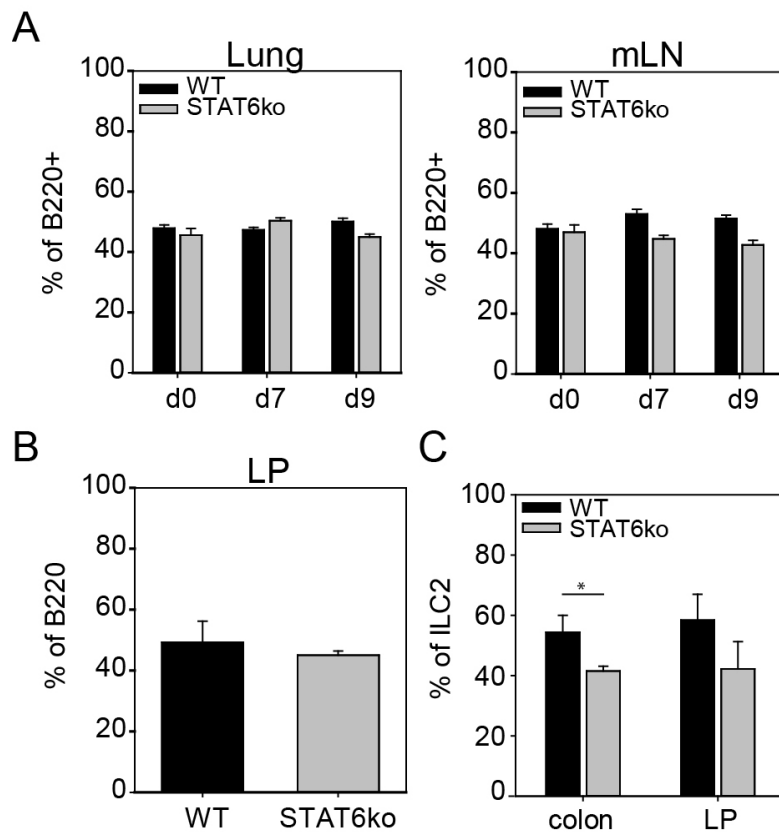


C



Supplemental figure 1

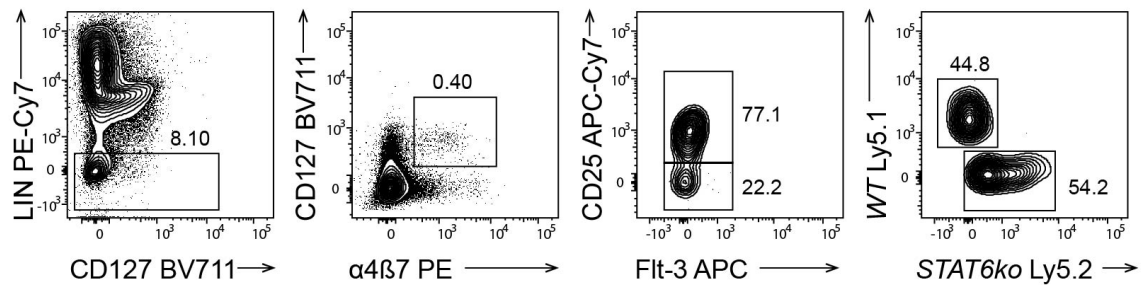
Flow cytometry gating strategies of ILC2s. (A) Panels represent gating strategy for lineage negative (LIN 1: $CD3\epsilon^+$ $CD4^+$ $CD8\alpha^+$ $B220^+$ $Gr1^+$ $Ter119^+$; LIN 2: $CD11b^+$ $CD11c^+$ $Fc\epsilon R1^+$ $NK1.1^+$ $Nkp46^+$) lung ILC2s gated for $ST2^+$ $ICOS^+$ $CD90^+$ $KLRG1^+$ after debris and doublets were excluded and lymphocytes selected. (B) Lin^- $ST2^+$ $ICOS^+$ $CD90^+$ $KLRG1^+$ ILC2s were analyzed for CD127 and GATA3 expression. Gates were set accordingly to the corresponding isotype staining control. (C) Frequency of mLN ILC2s from WT (black), 4-13Tko (gray), and 4-13ko (dark gray) mice were analyzed at d 5, 7 and 9 after *Nb* infection. Bars show the mean + s.e.m. three individual mice per group and are representative of two independent experiments.



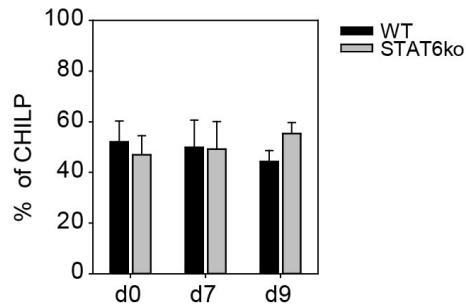
Supplemental figure 2

Distribution of reference population in different organs of MBMCs. (A) Frequency of B cells in the lung (left panel) and mLN (right panel) of MBMCs (see Fig. 2A) at steady state and at d 7 and 9 after infection with *Nb*. Bars show the mean + s.e.m. of three to four individual mice per group and are representative of three independent experiments. (B) Frequency of B cells in the lamina propria (LP) of naïve MBMCs. (C) Frequency of ILC2 ($\text{Lin}^- \text{Sca-1}^+ \text{CD90}^+ \text{KLRG1}^+$) in the colon and LP of naïve MBMCs. Bars show the mean + s.e.m. of two individual experiments with 3-6 mice per group. * $P < 0.05$ by two-tailed Student's t-test.

A



B



Supplemental figure 3

No difference of ILC2 progenitor cells in the bone marrow of MBMCs. (A) Gating strategy of WT (black) and STAT6^{-/-} (gray) Lin⁻ FLT3⁻ CD25⁻ CD127⁺ $\alpha 4\beta 7$ ⁺ common “helper-like” innate lymphoid cell progenitors (CHILP) in the bone marrow of naïve mice. (B) Frequency of donor CHILPs in the bone marrow of MBMCs (see Fig. 2A). Bars show the mean + s.e.m. of three individual mice per group and are representative of two independent experiments.