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Interleukin 9 fate reporter reveals induction of innate IL-9 response in lung inflammation

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Abstract

Interleukin 9 (IL-9) is a cytokine implicated in lung inflammation, but its cellular origin and function remain unclear. Here we describe a reporter mouse strain designed to fate map cells that have activated IL-9. We show that during papain-induced lung inflammation IL-9 production was largely restricted to innate lymphoid cells (ILC). IL-9 production by ILC was dependent on IL-2 from adaptive immune cells and was rapidly lost in favor of other cytokines, such as IL-13 and IL-5. Blockade of IL-9 production via neutralizing antibodies substantially reduced IL-13 and IL-5, suggesting that ILC provide the missing link between the well-established functions of IL-9 on the regulation of T_H2 cytokines and responses.

Introduction

Interleukin 9 (IL-9) is a pleiotropic cytokine that is expressed at elevated levels in the lungs of asthmatic patients[1,2]. Blocking antibodies are currently in clinical trials as potential therapies for atopic disease[3]. Similar results have been obtained in mouse models, where specific over-expression of IL-9 in lungs results in the induction of an asthma-like phenotype[6-8] and blockage of IL-9 signalling reduces airway inflammation[4,5]. An important function attributed to IL-9 in lung physiology is the induction of mucus production, goblet cell hyperplasia and other features of airway remodelling[9,10], functions that were also attributed to IL-13[11] as well as IL-5 via the regulation of eosinophils[12]. IL-9 is also involved in protective immunity to helminth infections, indicated by the enhanced kinetics of worm expulsion seen in IL-9 transgenic mice[13,14] and the susceptibility to helminth infection upon IL-9 depletion[15].

The cellular source of IL-9 in the context of airway inflammation has been mainly attributed to T cells[16-18]. Activated $CD4^+$ T cells from the T helper cell 2 subset (T_H2) were believed to comprise the majority of IL-9 producing cells. However, substantial IL-9 production is induced in $CD4^+$ T cells differentiating *in vitro* in the presence of IL-4 and TGF-β, but not in the context of IL-4 alone[19]. Thus, IL-9 is not a T_H2 cytokine. In addition to T cells, eosinophils and mast cells also produce IL-9[20-22].

Novel cellular sources for the secretion of T_H2-type cytokines have been recently discovered. These cell types show striking similarities to lymphoid tissue inducer cells (LTi cells), do not express known lineage markers, are responsive to both IL-25 and IL-33 and play a protective role during helminth infections[23-29]. Such lineage negative (Lin^-) cells display some LTi-like properties, such as IL-7 receptor expression, but lack CD4 and Rorγt expression and have a different cytokine expression profile. Therefore, they were either termed natural helper cells (NHCs)[28], nuocytes[27], innate helper type 2 (Ih2) cells[29] or multipotent progenitors (MPPs)[26]. Nuocytes and MPPs reside in mesenteric lymph nodes and spleen, while NHCs were found in the fat associated lymphoid tissue and Ih2 cells are dispersed throughout the body, with the highest numbers recovered from the liver. This subsets of newly identified Lin^- cells, or innate lymphoid cells type 2 (ILC2s)[30], are characterised by the secretion of high amounts of the T_H2 cytokines IL-5, IL-6 and IL-13 after induction with IL-25 or IL-33, which is strongly indicative of a potential involvement in airway inflammation.

Here we show the induction of IL-9 producing ILC identified by an IL-9 specific reporter in a model of papain-induced airway inflammation. ILC were the major source of IL-9 and IL-9 production was transient and dependent on IL-2 from adaptive immune cells. While IL-9 expression waned quickly, ILC continued to produce IL-13 and IL-5. IL-9 was found to facilitate IL-5 and IL-13 production from ILC, while neutralisation of IL-9 reduced the levels of IL-5 and IL-13 after papain challenge. Our findings indicate a previously unrecognized mechanism for the induction of IL-9 from ILC and a potential involvement of

IL-9 in allergic lung diseases via the promotion of IL-5 and IL-13 production in ILC.

Results

The IL-9 fate reporter mice

Despite the demonstration that a subset of *in vitro* generated $CD4^+$ T cells can secrete IL-9, the cell types producing this cytokine *in vivo*, under physiological conditions remain unidentified. This is partially due to the technical difficulties associated with *ex vivo* intracellular staining for IL-9. We generated an IL-9 fate reporter BAC transgenic mice that expresses the Cre recombinase under the control of the endogenous IL-9 locus (*IL9*^{Cre} mice) (**Supplementary Fig. 1**). To visualize the Cre activity we bred the *IL9*^{Cre} mice with reporter mice expressing enhanced yellow fluorescent protein (eYFP) under the control of the endogenous Rosa 26 promoter (termed *R26R*^{eYFP}). In *IL9*^{Cre}*R26R*^{eYFP} mice the fluorescent reporter permanently labels cells that had expressed the IL-9 gene, irrespective of the current production status for this cytokine. Analysis of tissues from non-immune *IL9*^{Cre}*R26R*^{eYFP} reporter mice did not reveal any $eYFP^+$ cells in the hematopoietic compartment of peripheral lymph nodes, mesenteric lymph nodes, spleen, lung, liver, skin and lamina propria of small and large intestine (**Supplementary Fig. 2**).

In vitro stimulation of FACS purified naïve $CD4^+$ T cells with TGFβ and IL-4 generated a population of 'T_H9' cells that were detectable by intracellular staining for IL-9 as well as eYFP expression (**Supplementary Fig. 3a**). In line with recently published reports[31], IL-9 expression in T cell cultures was

transient. No eYFP induction occured under T_H1, T_H2 or iT_{reg} conditions, albeit a proportion of eYFP producing cells was induced under T_H17 conditions (**Supplementary Fig. 3b**). However, only about 10% of the cells detected by intracellular cytokine staining had turned on the eYFP gene, suggesting incomplete reporting of IL-9 expressing cells. Our data show that induction of Cre transcripts from the BAC construct was considerably lower than the induction of endogenous IL-9 transcripts. This may add to the observed under-reporting of the BAC transgene in this reporter line (**Supplementary Fig.3c)**. Despite this limitation, *IL9*^{Cre}*R26R*^{eYFP} mice comprise a useful tool for the detection of IL-9 production *in vivo*.

IL-9 is expressed during papain-induced airway inflammation

Because IL-9 expression has been linked to lung inflammation, we studied the IL-9 expression pattern in *IL9*^{Cre}*R26R*^{eYFP} mice in a setting of airway inflammation. Papain, a protease with adjuvant activity that leads to asthma-like responses[32] induces a strong T_H2 phenotype upon subcutaneous injection[33] and has been used to induce experimental airway inflammation[34,35]. Intranasal challenge of *IL9*^{Cre}*R26R*^{eYFP} reporter mice (**Fig. 1a**) with papain induces a robust T_H2-type lung inflammation as monitored by cell influx in the lungs (Fig. 1b) and cytokine production in the airways (Fig.1c). Notably, papain induced airway inflammation resulted in IL-9 expression in the lung (Fig. 1c) and analysis of reporter mice revealed that most, if not all, eYFP expression was found in non-T cells (Fig. 1d). Further phenotyping of these eYFP$^+$ cells showed that they were not related to any defined haematopoietic

lineage (**Fig. 1e**). No eYFP$^+$ Lin$^-$ cells were recovered from the lungs of mice challenged with PBS as control (**Fig. 1f**).

To address the phenotypic resemblance of the eYFP$^+$ cells that accumulated in papain-induced inflammation with the recently identified natural helper cells, Ih2 cells, nuocytes or MPPs cells[26-29], we further characterized their surface expression profile. Although eYFP$^+$ Lin$^-$ cells did not exactly resemble any of the reported cell types, they shared the expression of Thy1.2 and CD45 and the majority also showed expression of the IL-33 receptor (IL-33R), while expression of Sca-1, major histocompatibility complex (MHC) class II and CD25 were more heterogeneously distributed (**Fig. 1g**). We did not detect substantial IL-7R or c-Kit expression in eYFP$^+$ cells directly after papain challenge (data not shown). Hereafter these cells will be referred to as innate lymphoid cells (ILC) [36]. Thus, our data identified ILC as the major source of IL-9 in this model of lung inflammation.

Transient IL-9 but sustained IL-5 and IL-13 production in ILCs

We next examined the cytokine expression profile from eYFP$^+$ ILC by intracellular cytokine staining 24 h after the last papain challenge. Following a short 2.5h pulse with PdBU and ionomycin, all eYFP$^+$ ILC expressed IL-13 and the majority expressed IL-5, but only a minority were producing IL-4 (**Fig. 2a**). However, no IL-9 expression could be recalled in these cells by intracellular cytokine staining (**Fig. 2a**). IL-9 expression in CD4$^+$ T cells *in vitro* suggested short-lived IL-9 production. To investigate whether IL-9 expression from ILC is similarly curtailed in papain-mediated airway inflammation, we performed a kinetic analysis of cytokine secretion after papain re-challenge in

wild-type C57B6 mice. The percentage of IL-9$^+$ ILC recovered from the lungs of wild-type mice after an acute papain challenge changed rapidly over time, with peak expression observed 6 to 12 h after challenge, and a rapid decline by 24 h (**Fig. 2b,c**). Analysis of IL-9 protein expression at 6, 12 or 24 h after papain challenge supported the assumption of transient IL-9 expression, with peak protein expression levels observed after 12 h (Fig. 2d). ILC isolated from reporter mice showed eYFP expression co-localized with intracellular IL-9, if analysed 12 h after papain re-challenge (**Supplementary Fig. 4**). Thus, ILC induced in papain lung inflammation rapidly lose IL-9 protein expression in favour of IL-5 and IL-13 expression.

IL-9 expression in ILCs is induced by IL-2

To test the capacity of ILC to up-regulate IL-9 expression upon stimulation with Toll-like receptor (TLR) ligands, we cultured MACS purified ILC cells (defined by the depletion of cells expressing the lineage markers TCRβ, TCR$\gamma\delta$, CD11c, CD11b, Gr-1, CD19, DX5, NK1.1 and Ter119) and Lin$^+$ cells isolated from papain challenged wild-type mice (**Supplementary Fig. 5a**) in the presence of PdBU and ionononomycin or TLR ligands. None of the tested TLR-ligands was able to induce IL-9 production in ILC or in Lin$^+$ cells, whereas overnight stimulation with PdBU and ionomycin (rather than a 2.5 h stimulation) induced IL-9 protein expression in ILC (**Supplementary Fig. 5b**). This suggested that IL-9 can be induced or re-induced in ILC upon extended stimulation or in response to factors expressed by other cell types upon PdBU and ionomycin stimulation.

IL-25, IL-33 or thymic stromal lymphopoietin (TSLP) or the combination of these cytokines did not induce substantial IL-9 production from ILC isolated from papain challenged mice (**Supplementary Fig. 5c**). However, culture of ILC in the presence of IL-2 resulted in IL-9 protein levels similar to those seen after stimulation with PdBU and ionomycin (Fig. 3a). CD25, the high affinity chain of the IL-2 receptor, is expressed on a proportion of $eYFP^+$ ILC and ILC responded to IL-2 stimulation with IL-9 production. To test if CD25 expression can be used to identify ILC poised for expression of IL-9 in wild-type B6 mice, we sorted $CD25^+$ ILC and $CD25^-$ ILC and tested their IL-2-induced IL-9 protein production in comparison to that of total $CD4^+$ T cells from papain-challenged mice (**Fig.3b**). IL-2-mediated IL-9 production was mainly found in $CD25^+$ ILC, suggesting an important role of IL-2 signalling for the production of IL-9 from ILC (**Fig. 3b**). No IL-9 production was detectable from $CD4^+$ T cells. IL-9 expression in $eYFP^+$ ILC isolated from *IL9*^{Cre}*R26R*^{eYFP} reporter mice was likewise dependent on IL-2 (**Fig. 3c**). Analysis of cytokine mRNA expression comparing lung-infiltrating unstimulated total $CD4^+$ T cells (set as reference standard) with $CD25^+$ or $CD25^-$ ILC from wild-type mice or $eYFP^+$ ILC showed substantially increased expression of T_H2-type cytokines, such as IL-5, IL-6 and IL-13, in $CD25^+$ ILC and especially in $eYFP^+$ ILC from the IL-9 reporter mice (**Fig. 3d**), emphasising their activated state. Expression of IL-9 was increased in $CD25^+$ ILC, in comparison to total $CD4^+$ T cells or $CD25^-$ ILC. IL-9 mRNA was even higher in $eYFP^+$ ILC from reporter mice, suggesting the eYFP specificity for reporting IL-9 expression. These data indicate the dependency of IL-9 production on the availability of IL-2.

IL-33, but not IL-25, induces IL-9 competent ILCs

Although neither IL-25 nor IL-33 were able to induce IL-9 production from ILC, previous analysis did not test the capacity of these cytokines to induce recruitment of ILC and the potential for subsequent IL-9 induction following exposure to IL-2. To test whether IL-25 and IL-33-induced ILC were able to produce IL-9 upon IL-2 stimulation we intranasally treated wild-type mice with IL-25 or IL-33. Intranasal challenge with IL-33 was much more potent than IL-25 treatment in inducing the accumulation of ILC in the lung (**Fig. 4a, b**). Although IL-25 challenge resulted in the induction or recruitment of ILC producing IL-13 (Fig 4a and c), this was again higher after IL-33 application. However, while no IL-9-positive cells were detected by intracellular cytokine staining, IL-33 induced high IL-9 production from ILC upon IL-2 stimulation (Fig. 4d). Additionally, IL-33-primed ILC were also more potent producers of IL-5 and IL-13 than IL-25-primed ILC (Fig. 4d). IL-33 was also more potent to induce $eYFP^+$ ILC upon intranasal administration (Fig. 4e) and such ILC expressed IL-13 and displayed similar surface markers as the $eYFP^+$ ILC generated during papain-induced lung inflammation (**Supplementary Fig. 6**). In order to compare IL-33 and papain-induced ILC with other ILC2 cell types, we investigated the induction of eYFP expression in nuocytes in the mesenteric lymph nodes after intraperitoneal injection of IL-25 and IL-33, NHCs in the FALT and Ih2 cells in the liver. IL-33, but not IL-25 administration induced a small percentage of $eYFP^+$ nuocytes, while no substantial NHCs or Ih2 cells were detected (**Supplementary Fig. 7**). Thus, the induction of ILC competent to produce IL-9 is mainly driven by IL-33.

IL-9 expression requires IL-2 from adaptive immune cells

ILC are present in *Rag1*$^{-/-}$ mice, which lack T and B cells, but not in mice lacking the common gamma chain (*IL2rg*$^{-/-}$ mice)[27-29]. To investigate whether the population of IL-9-expressing cells is independent of RAG expression but dependent on signalling via the common gamma chain we analysed IL-9 expression in the lungs of papain-challenged wild-type, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice. IL-9 production was reduced by 10-fold in the lungs of *Rag1*$^{-/-}$ mice as compared to wild-type mice and was absent in *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice (**Fig. 5a**). IL-5 and IL-13 protein expression was also reduced in *Rag1*$^{-/-}$ mice, in agreement with previous reports that IL-13-expressing cells in the mesenteric lymph nodes were not maintained during helminth infection in *Rag1*$^{-/-}$ mice[27]. However, isolation of ILC from the lungs of papain-challenged wild-type, *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice, followed by overnight stimulation with IL-2, resulted in similar IL-9 protein expression from ILC of wild-type and *Rag1*$^{-/-}$(**Fig. 5b**). In contrast ILC isolated from *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice failed to produce IL-9 upon IL-2 stimulation (**Fig. 5b**). These results suggest that IL-9 production in ILCs upon papain challenge *in vivo* is dependent on the presence of adaptive immune cells, although the intrinsic capacity of ILCs to produce IL-9 is still intact in *Rag1*$^{-/-}$ mice.

To address if IL-2 is important for IL-9 expression upon papain challenge *in vivo* we treated wild-type mice with neutralising IL-2-specific antibodies 30 min before the last papain challenge (**Fig. 5c**). IL-2 neutralization resulted in significant reduction of IL-9 expression in papain-treated mice, but not in isotype treated control mice (**Fig 5d**). Other T_H2 cytokines like IL-4, IL-5, IL-6

and IL-13 were not affected by IL-2 neutralisation, at least at the early timepoint of 18 h.

To further investigate if reduced IL-9 protein expression in *Rag1*$^{-/-}$ mice is caused by a lack of IL-2, papain-challenged *Rag1*$^{-/-}$ mice were treated intranasally with IL-2 or PBS 3 h after the last papain re-challenge (**Fig. 5e**). IL-2, but not PBS treatment, restored IL-9 expression in *Rag1*$^{-/-}$ mice to wild-type levels (**Fig. 5f**). Intranasal application of IL-2 alone, without papain challenge, did not result in IL-9 production (**Fig 5f**). Total ILC numbers in *Rag1*$^{-/-}$ mice were only slightly, but not significantly reduced in comparison to wild-type mice and IL-2 application did not result in elevated numbers of ILC (**Fig. 5g**). These results suggest that IL-9 protein production by ILC is critically dependent on the provision of IL-2 by adaptive immune cells.

ILC are the major source of IL-9 in papain-induced inflammation

To investigate whether ILC are the critical producers of IL-9 during papain-induced lung inflammation we performed intracellular cytokine staining for IL-9 and IL-13 in ILC and CD4$^+$ T cells. The majority of intracellular IL-9 was expressed in ILC, although we detected a minor contribution from CD4$^+$ T cells (**Fig. 6a, b**). While similar numbers of ILC and CD4$^+$ T cells are recovered from the lungs of papain-challenged mice (**Fig. 6c**), IL-9 and IL-13 expression stems almost exclusively from ILC (**Fig. 6d**).

To further explore the relative contribution of ILC or CD4$^+$ T cells to IL-9 production in papain-challenged mice, we adoptively transferred either ILC or

$CD4^+$ T cells wild-type sorted from the lung homogenate of papain-challenged intra-tracheally into *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ hosts. In this setting, only transferred cells are able to respond to IL-2 and produce IL-9. Transfer was followed by papain and IL-2 challenge. Whereas very little IL-9, IL-5 and IL-13 were detectable after $CD4^+$ T cell transfer, ILC transfer resulted in substantially elevated levels of IL-9, IL-5 and IL-13 (**Fig. 6e**). In contrast, sensitisation and challenge of *IL9*^{Cre}*R26R*^{eYFP} mice with ovalbumin, a model in which T cells were reported to be the major producers of IL-9[16-18], resulted in eYFP expression largely restricted to $CD4^+$ T cells rather than ILC (**Supplementary Fig.8**).

Thus, ILC and not $CD4^+$ T cells are the major producers of IL-9, 5 and 13 in this particular model.

IL-9 alters cytokine expression and phenotype of ILC

To investigate the potential physiological consequences of IL-9 expression during papain-induced airway inflammation, we treated wild-type mice with neutralising IL-9-specific antibodies 30 minutes before the last papain challenge (**Fig. 7a**). The number and composition of cells infiltrating the lungs and airways of papain-challenged mice remained unchanged upon IL-9 neutralisation at this stage (data not shown) and the total number of ILC did not change significantly (**Fig. 7b,c**). However, we observed a drastic phenotypic change in the ILC population three days after papain challenge and IL-9 neutralisation. While ILC from papain-challenged mice treated with isotype control antibodies displayed high levels of CD25 and Sca-1, ILC from IL-9 neutralised mice showed a substantial reduction in $CD25^+$ $Sca\text{-}1^+$ double positive cells (**Fig. 7b,d**). The IL-9 receptor (IL-9R) was reported to be

expressed on Ih2 cells[29]. To address if ILC recovered from the lung of papain-challenged mice expressed the IL-9R, we sorted $CD25^+$ ILC and tested IL-9R expression compared to B cells, known to express the IL-9R, and naïve T cells that do not express the receptor. $CD25^+$ ILC showed IL-9R expression similar to B cells (**Fig. 7e**), implying that IL-9 could directly affect ILC. To investigate if exposure to IL-9 is able to change the cytokine profile of ILC, we cultured sorted $CD25^+$ ILC in the presence or absence of IL-9. Addition of IL-9 markedly increased the ILC production of IL-5, IL-6 and IL-13 (**Fig. 7f**), suggesting that IL-9 could provide an additional activation signal important for cytokine expression. To address if IL-9 has a similar impact on IL-5 and IL-13 expression *in vivo*, we analysed the lung homogenate and broncheo-alveolar lavage fluid (BALF) of papain-challenged mice treated with neutralising IL-9-specific or isotype control antibodies three days after challenge and IL-9 neutralisation. In IL-9-neutralized mice, IL-5 and IL-13 expression was reduced to less than half of the levels recovered from isotype control treated mice, indicating that IL-9 indeed has an additive effect on IL-5 and IL-13 expression *in vivo* (**Fig. 7g**). Thus, it appears that IL-9 has a feedback effect on ILC, resulting in increased production of IL-5 and IL-13.

Discussion

Several studies have implicated IL-9 in lung inflammation, although the cell type responsible was not identified due to technical difficulties related to intracellular staining for IL-9. In order to address the cellular source of IL-9 production *in vivo*, we generated an IL-9 fate reporter strain. Initial *in vitro* tests on T cell differentiation established faithful reporter induction under 'T_H9' conditions.

Even under T_H9 conditions only about 10% of the IL-9 producers were marked by eYFP, suggesting incomplete reporter activity. There are several potential reasons for this limitation. Firstly, it is possible that *in vitro* conditions for T_H9 induction are not efficient, similar to what was seen in the IL-17 Cre fate reporter[37] that effectively induces eYPF in T_H17 cells generated *in vivo*, but only marks about 30% of *in vitro* generated T_H17 cells. The second possibility is that incomplete reporting could be due to the BAC transgenic construct itself. The IRES-Cherry sequence contained in the construct was disabled due to a premature translational start site in the IRES sequence possibly diminishing translation from the ATG in the cherry gene. It is possible that the presence of a dysfunctional DNA sequence in the construct interferes with reporter induction. Indeed comparative analysis of transcripts for Cre *vs* endogenous IL-9 transcripts showed substantially lower Cre induction, pointing to transcriptional dysregulaton of the BAC transgene. However, we did not observe aberrant eYFP expression in a wide range of cell types, so despite the possibility that not all IL-9 producers were labelled by eYFP, we nevertheless could identify a proportion of such cells using this reporter.

Four types of ILC displaying similar surface markers and high amounts of the T_H2 cytokines IL-5 and IL-1 have recently been described[26-29]. The $eYFP^+$ ILC identified in our reporter system shared Thy1.2 surface marker expression with nuocytes, NHCs, MPPs and Ih2 cells and IL-33R and Sca-1 with nuocytes, NHCs and MPPs. Like nuocytes they expressed MHC class II and shared CD25 expression with NHCs. The majority of $eYFP^+$ ILC produced IL-5 and IL-13 but not IL-4 upon restimulation. Although $eYFP^+$ ILC displayed alterations in surface marker expression compared with previously identified ILC types, we cannot exclude that the cells we identified are identical or similar to NHCs, MPPs or nuocytes. We rather propose that changes in surface marker expression reflect activation status and probably tissue distribution.

In contrast to the reported induction of the ILC cytokines IL-5, IL-6 and IL-13 by IL-25 or IL-33, IL-9 was not directly induced by the latter two cytokines, but instead depended on IL-2. However, IL-33- but not IL-25- priming either recruited or induced the ILC population for IL-2 triggered IL-9 production. It was previously reported that IL-9 induction in human T cells is dependent on IL-2[38,39] ; however, we did not find similar effects in sorted mouse T cells from papain-challenged mice. Because in most studies T cell purity was assessed by CD2 staining and IL-9 was measured by ELISA, the possibility of an ILC contamination was not excluded.

While ILC are present in papain-treated *Rag1*$^{-/-}$ mice, IL-9 expression was considerably reduced compared to papain-treated wild-type animals, but could be restored by IL-2 application. This suggests that cells absent in *Rag1*$^{-}$

$^{/-}$ mice, such as T cells or NKT cells, could be the most important source of IL-2 to support IL-9 production by ILC.

IL-9 expression influenced the expression of other ILC cytokines, since ILC, like Ih2 cells, express high levels of IL-9R transcripts[29] and neutralisation of IL-9 in the context of papain challenge led to dramatic surface phenotype changes in ILC with reduction of both CD25 and Sca-1. ILC displaying CD25 showed higher expression levels of IL-5, IL-6 and IL-13 than CD25^{-} ILC, suggesting that CD25 expression, like in T cells, could reflect activation. Sca-1 expression is upregulated upon inflammation on hematopoietic stem cells[40] and increased Sca-1 expression on ILC could likewise indicate inflammatory activation. Neutralisation of IL-9 during papain-induced lung inflammation resulted in reduced levels of IL-13 and IL-5 when analysed 3 days after challenge. Since maintenance of IL-5 and IL-13 are linked to IL-9 and IL-9 induction requires IL-2, lack of IL-2 signaling might also affect the amounts of IL-5 and IL-13. Although blockade of IL-2 resulted in reduction of IL-9 early after papain challenge (18h), there was no effect on IL-5 and IL-13 at this time point. IL-5 and IL-13 expression might be affected at later timepoints. Our data might explain the observation that although nuocytes are initially induced and accumulate in the mesenteric lymph nodes of *N. brasiliensis* infected *Rag1*$^{-/-}$ mice, their numbers are not maintained in the absence of T cells[27]. In this system nuocytes were defined by acute reporting of IL-13 expression. We find reduced IL-13 expression, but not reduced numbers of ILC in the lungs of papain-challenged *Rag1*$^{-/-}$ mice. In contrast to an acute cytokine reporter, the advantage of the fate reporter is to allow detection of cells even after loss of

cytokine expression. Thus, IL-2 mediated IL-9 expression might be the missing link for sustained IL-13 expression in nuocytes and ILC in general. Culture of NHC with IL-2 alone or in combination with IL-33 promotes IL-13 expression[28], further strengthening IL-2 as the most likely connection to the adaptive immune system.

Spontaneous IL-9 induced airway inflammation is observed in IL-9 transgenic mice[6-8], but is ablated if IL-9 transgenic mice are backcrossed onto an IL-13 deficient background[6], indicating that IL-9 acts via expression of IL-13. Also IL-13 producing ILC have been described to cause lung pathology during influenza infection; however, the involvement of IL-9 was not investigated in this study[41].

IL-9 effects on intestinal mucosa were reported to be IL-13 dependent[42]. Furthermore blocking IL-5 in IL-9 transgenic mice reduced eosinophil recruitment[8], suggesting that IL-5 production in this spontaneous airway inflammation is also linked with IL-9. In agreement with these observations our data show increased IL-13 and IL-5 production in ILC upon IL-9 stimulation, suggesting that IL-9 mediated airway inflammation may be the result of enhanced IL-13 and IL-5 expression from ILC. It appears that IL-9 production has to be curtailed and tightly regulated to prevent lung pathology. Thus, understanding the mechanism of transient IL-9 regulation might prove essential to recognize the pathological function of IL-9 during airway inflammation. Taken together, our data suggest plasticity and a highly dynamic cytokine network in ILC.

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Figure legends

Figure 1: Papain induced eYFP^{+} cells do not express any lineage markers.

(a) Number of cells in the lungs of *IL9*^{Cre}*R26R*^{eYFP} mice challenged with papain (black bars) and PBS (white bars) on days 0, 3, 6, 14 and analysed 20 h after the last re-challenge (see scheme **Supplementary Fig.9**). (**b)** Cytokine concentration in the broncheo-alveolar lavage fluid (BALF) of mice immunized with papain (black bars) or PBS (white bars) as in **a**. **(c,d)** Flow cytometry of total lung cells from papain challenged *IL9*^{Cre}*R26R*^{eYFP} mice as in **a** stained for CD4 (**c**) or TCRβ, CD8α, TCRγδ, CD19, Nkp46, Gr-1, CD11c, SiglecF and CD11b (**d)** and assessed for eYFP expression. (**e)** Absolute number of eYFP^{+} ILC in the lung of papain challenged *IL9*^{Cre}*R26R*^{eYFP} mice. **g)** Flow cytometry of CD45, Thy1.2, IL33R, CD25, MHC II , Sca-1 and eYFP expression in papain-challenged *IL9*^{Cre}*R26R*^{eYFP} mice. Data represent at least three independent experiments with 3 mice in each experimental group (mean ±SEM in **b, c, f**). DCs, dendritic cells; Eos, Eosinophils: Mϕ, Macrophages: Neu, Neutrophils.

Figure 2: IL-9 expression by ILC is transient

a) Flow cytometry of lung cells isolated from *IL9*^{Cre}*R26R*^{eYFP} mice challenged

with papain or PBS challenged 24 h after the last papain re-challenge. Cells were stimulated with PdBU and ionomycin for 2.5 h, stained for CD4 and assessed for eYFP and intracellular IL-4, IL-5, IL-9 and IL-13 in the $CD4^-$ lymphocyte fraction. **b)** Flow cytometry of IL-9 and IL-13 expression in ILC (Lin^- $Thy1.2^+$), analyzed either before the last papain re-challenge or 6, 12 and 24 h after the last papain challenge (see scheme **Supplementary Fig.9)**. **c)** Frequencies of $IL\text{-}9^+$ ILC analyzed in the lung on indicated time point after papain re-challenge (0 timepoint reflects frequencies before the last papain re-challenge) ** p=0.009 and **d)** IL-9 cytokine concentration in the lung homogenate. ** p=0.004. Numbers in quadrants (**a, b**) indicate percent cells in each. Data represents at least three (**a**) or two (**b,c,d**) independent experiments with 3 mice in each experimental group (mean ±SEM in **c,d**).

Figure 3: IL-9 in ILC is induced by IL-2 but not IL-25, IL-33 or TSLP

a) IL-9 cytokine concentration in the supernatant of MACS sorted ILC (black bars) and cells expressing lineage markers (Lin^+, white bars) isolated from the lungs of papain challenged $IL9^{Cre}R26R^{eYFP}$ mice and stimulated *in vitro* overnight with P+I, IL-2, IL-25, IL-33 or TSLP. **b)** Concentration of IL-9 in the supernatant of sorted $CD4^+$ cells (grey bars), $CD25^-$ ILC (white bars) $CD25^+$ ILC black bars) and **c)** sorted $eYFP^+$ ILC cultured overnight as in **a**. **d)** Quantitative PCR analysis of the expression of transcripts for IL-4 (*Il4*), IL-5 I*il5*), IL-6 (*Il6*), IL-9 (*Il9*) and IL-13 (*Il13*) in sorted $CD4^+$, $CD25^-$ ILC, $CD25^+$ ILC, $eYFP^+$ ILC. mRNA expression was normalized to the expression of *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase) and is presented relative to $CD4^+$ expression levels. Data represents at least three

(**a,b,c**) or two (**d**) independent experiments (mean ±SEM in **a,b.c**). P+I: stimulated with PdBU and ionomycin; ctrl: un-stimulated.

Figure 4: Intranasal challenge with IL-33 induces ILC poised for IL-9 production

a) Flow cytometry of lung cells isolated from B6 mice challenged intranasally with PBS, IL-25 or IL-33, stained for surface lineage markers CD4, CD8α, TCRβ, TCRγδ, CD19, Nkp46, Gr-1, CD11c, Ter-119, CD11b (lineage) and Thy-1.2. (upper panel) and lung cells restimulated with PdBU and ionomycin for 2.5 h, stained for intracellular IL-9 and IL-13 and gated on ILC (lower panel). **b)** Absolute number of ILC (Lin^- $Thy1.2^+$ cells) in the lung and **c)** frequencies of IL-13^+ ILC analyzed in the lung after the indicated treatments. **d)** Cytokine concentration in the supernatant of MACS sorted ILC isolated from PBS, IL-25 or IL-33 treated mice stimulated *in vitro* with IL-2 overnight. **e)** Flow cytometry of lung cells isolated from *IL9*^{Cre}*R26R*^{eYFP} mice treated as in **a),** stained for lineage markers and Thy1.2 (upper panel) and assessed for eYFP and CD45 expression in ILC (lower panel). Numbers in gates or quadrants (**a,e**) indicate percent cells in each. Data represents at least two independent experiments with 3 mice in each experimental group (mean ±SEM in **b,c,d,e**).

Figure 5: IL-9 expression in ILC is dependent on IL-2 and the adaptive immune system

a) Cytokine concentration in the lung homogenate of papain challenged wt (black bars) $Rag1^{-/-}$ (white bars) and *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ (grey bars) mice 18 h

after the last papain re-challenge ***p=0.0001, **p=0.001 **b)** Concentration of IL-9 in the supernatant of MACS sorted ILC isolated from papain challenged wt *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice stimulated *in vitro* with IL-2 overnight. **c)** Cytokine concentration in the lung homogenate of papain immunized B6 mice treated with neutralising IL-2-specific (white bars) or IgG isotype control (black bars) antibody 18 h after challenge *p=0.03. **d)** IL-9 protein concentration in the lung homogenate of papain challenged wt (black bars) and *Rag1*$^{-/-}$ mice (white bars) analyzed 18 h after the last papain re-challenge. Where indicated papain challenged or control (PBS challenged only) *Rag1*$^{-/-}$ mice were treated intranasally with 500 ng recombinant mouse IL-2 3 h after the last papain re-challenge. **e)** Absolute number of ILC in the lung of papain challenged wt and papain challenged *Rag1*$^{-/-}$ mice left untreated or treated with intranasal IL-2. (treatment regimens are show in **Supplementary Fig.9).** Data represents two independent experiments with four mice in each experimental group (mean ±SEM).

Figure 6. ILC are the major source of IL-9

a) Flow cytometry of lung cells isolated from papain challenged B6 mice 12 h after papain re-challenge, restimulated with PdBU and ionomycin for 2.5 h and stained for lineage markers and intracellular IL-9 and IL-13. **b)** Intracellular IL-9 and IL-13 expression in gated CD4^{+} cells or ILC. **c)** Absolute number of ILC and CD4^{+} cells and **d)** IL9^{+}IL13^{+} ILC or CD4^{+} cells in the lungs of papain challenged mice 12 h after re-challenge. **e)** Flow cytometry of lung cells isolated from *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice (no transfer) or *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice transferred with CD4^{+} cells or ILC and challenged with papain and IL-2 (see

scheme **Supplementary Fig.9)**. Lung cells were stained for CD45 and Thy1.2 (left panel), restimulated with PdBU and ionomycin for 2.5 h, stained for intracellular IL-9 and IL-13 and gated on $CD45^+$ $Thy1.2^+$ cells (middle panel). Right panel: Absolute number of $IL\text{-}9^+IL\text{-}13^+$ cells (p= 0.0238). **f)** Cytokine concentration in the lung homogenate of papain and IL-2 challenged *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice (white bars) or papain and IL-2 challenged *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice transferred with $CD4^+$ cells (grey bars) or ILC (black bars) 15 h after papain challenge. Numbers in gates or quadrants **(a, b, e)** indicate percent cells in each. Data represents at least two independent experiments with 3 mice in each experimental group (mean ±SEM in **c, d, e** and **f).**

Figure 7. IL-9 promotes cytokine expression from ILC

a) Flow cytometry of lung cells isolated from papain challenged mice, treated with isotype control or neutralising IL-9-specific antibodies 30 min before the last papain re-challenge, stained for lineage markers and Thy1.2 (upper panel) and gated on ILC and stained for Sca-1 and CD25 (lower panel). **b)** Absolute number of ILC in the lung and **e)** absolute number of $Sca\text{-}1^+$ $CD25^+$ ILC in the lung of papain challenged mice 3 days after re-challenge and antibody treatment **p=0.001. **d)** Quantitative PCR analysis of the expression of transcripts for IL-9R in sorted naïve T cells and B and $CD25^+$ ILC isolated from the lungs of papain challenged mice. mRNA expression is presented relative to the expression of *Hprt1*. **f)** Cytokine concentration in the supernatant of sorted $CD25^+$ ILC stimulated with or without IL-9 *in vitro* overnight *p=0.04, **p=0.009 **g)** Cytokine concentration in the lung homogenate and BALF of papain challenged wt mice treated with IL-9-specific

or isotype control antibodies 3 days after the last papain re-challenge **p=0.002, ***p=0.0002. Numbers in gates indicate percent cells in each. (Treatment regimes are shown in **Supplementary Fig.9).**

.Data represents at least two (**e)** or three independent experiments with four mice (**b,c,d,g**) in each experimental group or three groups of pooled cells from two mice (**f**) (mean ±SEM in **c,d,f,g**).

Online Methods:

Mice. For visualization of Cre-mediated recombination, IL9Cre mice were backcrossed to C57Bl/6.Rosa26eYFP mice generating *IL9*^{Cre}*R26R*^{eYFP} mice. The reporter mice used in this study had been backcrossed to C57Bl/6 (B6) mice for at least six generations. C57Bl/6, Rag1-deficient mice (Rag1$^{-/-}$) and *Rag2-IL2rg*-deficient mice (*Rag2*$^{-/-}$ *IL2rg*$^{-/-}$) were bred in our animal facility and all mice were kept under specified pathogen free conditions. All animal experiments were done according to institutional guidelines (NIMR Ethical Review panel) and Home Office regulations.

Induction of papain induced airway inflammation. C57Bl/6, *Rag1*$^{-/-}$, *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ or IL9Cre were anaesthetized with isoflurane and exposed intranasally to 25 µg papain (Calbiochem) in 50 µL PBS on day 0, 3 and 6. Seven days later mice were re-challenged intranasally with 25 µg papain in PBS. In some experiments papain challenged mice were treated i.v. with 75 µg neutralising IL-2-specific antibodies (JES6-5H4) 30 minutes before papain re-challenge. Control animals received IgG isotype control antibodies. 18-24 h after the last challenge bronchoalveolar lavage was performed by flushing out the lung with 0.5 ml PBS via a tracheal cannula. Lung homogenate was obtained by meshing diced lung fragments through a 40µm cell strainer and the cell free supernatant was stored at -20˚C for cytokine analysis. Isolated cells were further purified using a 37.5% percoll gradient. Cells were stained for surface antigens or stimulated with PdBU/ionomycin for 2.5 h if intracellular cytokine staining was performed.

IL-2 administration in Rag1$^{-/-}$ mice. 4 h after the last papain or PBS challenge Rag1$^{-/-}$ mice were intranasally treated with 500 ng recombinant mouse IL-2 (R&D systems) in 35µl PBS. Control animals received PBS only.

Intranasal IL-25 and IL-33 administration. Wt B6 mice were intranasally challenged with 500ng recombinant mouse IL-25 (eBioscience) or recombinant mouse IL-33 (Biolegend) on day 0, 1 and 3. Control animals received PBS only. Mice were euthanized on day 4 and lung cells isolated for analysis.

Administration of neutralising IL-9-specific antibodies. Papain challenged mice were injected i.v. with 25 µg of neutralising IL-9-specifc antibody (MM9C1) 30 minutes before the last papain re-challenge. IgG isotype control antibodies were delivered to control animals. Three days after treatment with IL-9-specific antibodies, mice were culled, lung cells isolated and cell free lung homogenate analysed for cytokine expression.

Isolation and culture of ILC and Lin$^+$ cells form the lungs of papain challenged mice. Lung cells isolated from papain challenged mice were purified by negative sorting on an AutoMACS Pro (Miltenyi) with a combination of monoclonal antibodies against CD4, CD8α CD11b, CD11c, CD49b, TCR-β, TCR-$\gamma\delta$, CD19, Ter119, Nk1.1 and Gr-1 coupled to APC followed by incubation with APC-specific magnetic microbeads (Miltenyi). MACS purified ILC or Lin$^+$ were cultured in 96-well plates at the adjusted

density of $5x10^3$ cells in 30 µl culture medium overnight in the presence of PdBU and ionomycin (both at 500 ng/ml). In some experiments cells were cultured in the presence of LPS (100 ng/ml), CpG (10 µg/ml), PolyI:C (2 µg/ml), Pam_3CSK_4 (10 µg/ml) or R848 (10 µg/ml). Cells were also stimulated with IL-2 (40 ng/ml), IL-25 (50 ng/ml), IL-33 (50 ng/ml) or TSLP (50 ng/ml). Cytokine production in the cell free supernatant was measured by using a bead based cytokine detection assay (FlowCytomix (Bender Medsystems) or CBA (BD Biosciences) for IL-9).

Fluorescence-activated cell sorting and culture of $CD4^+$, $CD25^+/CD25^-$ ILC and $eYFP^+$ ILC. $CD4^+$ cells were sorted by flow cytometry from total lung cells isolated from papain challenged mice based on the expression of the CD4 surface marker. $CD25^+/CD25^-$ ILC were sorted on the absence of CD4, CD8α, CD11b, CD11c, DX5, CD49b, TCR-β, TCR-γδ, CD19, Ter119, NK1.1 and Gr-1 but expression of Thy1.2 and absence or presence of CD25 respectively. $EYFP^+$ ILC were sorted from total lungs of papain challenged *IL9*^{Cre}*R26R*^{eYFP} mice on the expression of Thy1.2 and eYFP but absence of CD4. $CD4^+$, $CD25^+/CD25^-$ ILC and $eYFP^+$ ILC were cultured in the presence of IL-2, IL-25, IL-33 and TSLP overnight at the adjusted density of $5x10^3$ in 30µl culture volume.

Intratracheal transfer of cells*:* ILC and $CD4^+$ T cells were sorted from lungs of papain challenged mice. $1x10^5$ ILC or $CD4^+$ T cells, respectively were intratracheally transferred to *Rag2*$^{-/-}$ *IL2rg*$^{-/-}$ mice. 30 min later, mice were intranasally challenged with 25 µg of papain followed by 500 ng recombinant

mouse IL-2 2-3 hours post papain challenge. 15 h after papain challenge mice were sacrificed and lung cells stained for surface antigens and stimulated with PdBU/ionomycin for 2.5 h for intracellular cytokine staining. Lung homogenate was analysed for cytokine production.

***Real time PCR*.** $CD4^+$, $CD25^+/CD25^-$ ILC and $eYFP^+$ ILC were sorted by flow cytometry and RNA was extracted using Trizol and reverse transcribed with Omniscript (Qiagen) according to the manufacturer's protocol. The cDNA served as template for the amplification of genes of interest and the housekeeping gene (*Hprt1*) by real-time PCR, using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA), universal PCR Master Mix (Applied Biosystems, Warrington, UK) and the ABI-PRISM 7900 Sequence detection system (Applied Biosystems, Foster City, CA). Target gene expression was calculated using the comparative method for relative quantification upon normalisation to *Hprt* gene expression.

Statistical analysis. A two-tailed Student's *t*-test was used for all statistical analysis. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

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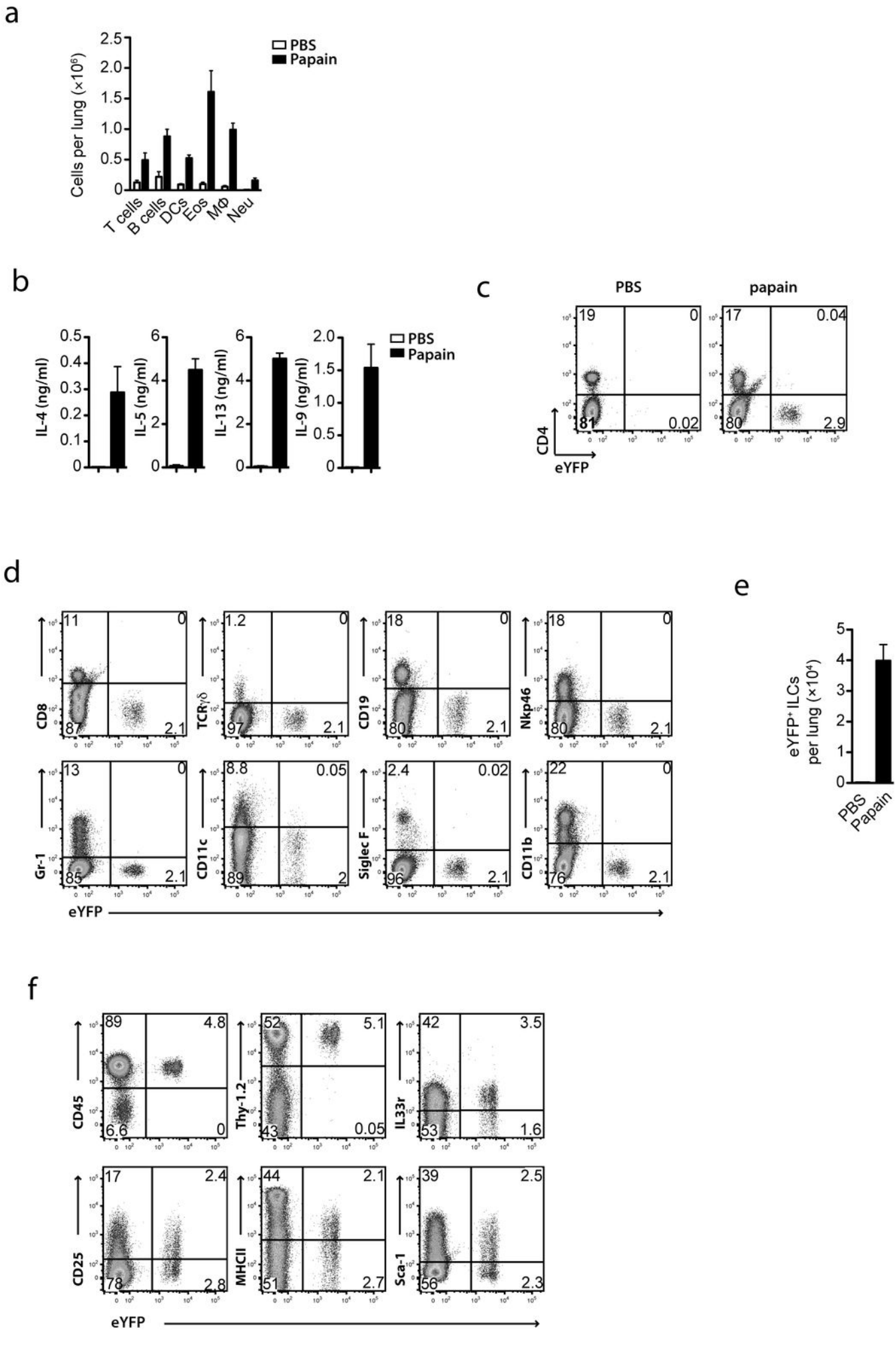
Figure 1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Papain
Cells per lung (×10⁶)
T cells
B cells
DCs
Eos
MΦ
Neu
b
IL-4 (ng/ml)
IL-5 (ng/ml)
IL-13 (ng/ml)
IL-9 (ng/ml)
PBS
Papain
c
PBS
papain
CD4
eYFP
d
CD8
TCRγδ
CD19
Nkp46
Gr-1
CD11c
SiglecF
CD11b
eYFP
e
eYFP⁺ ILCs per lung (×10⁴)
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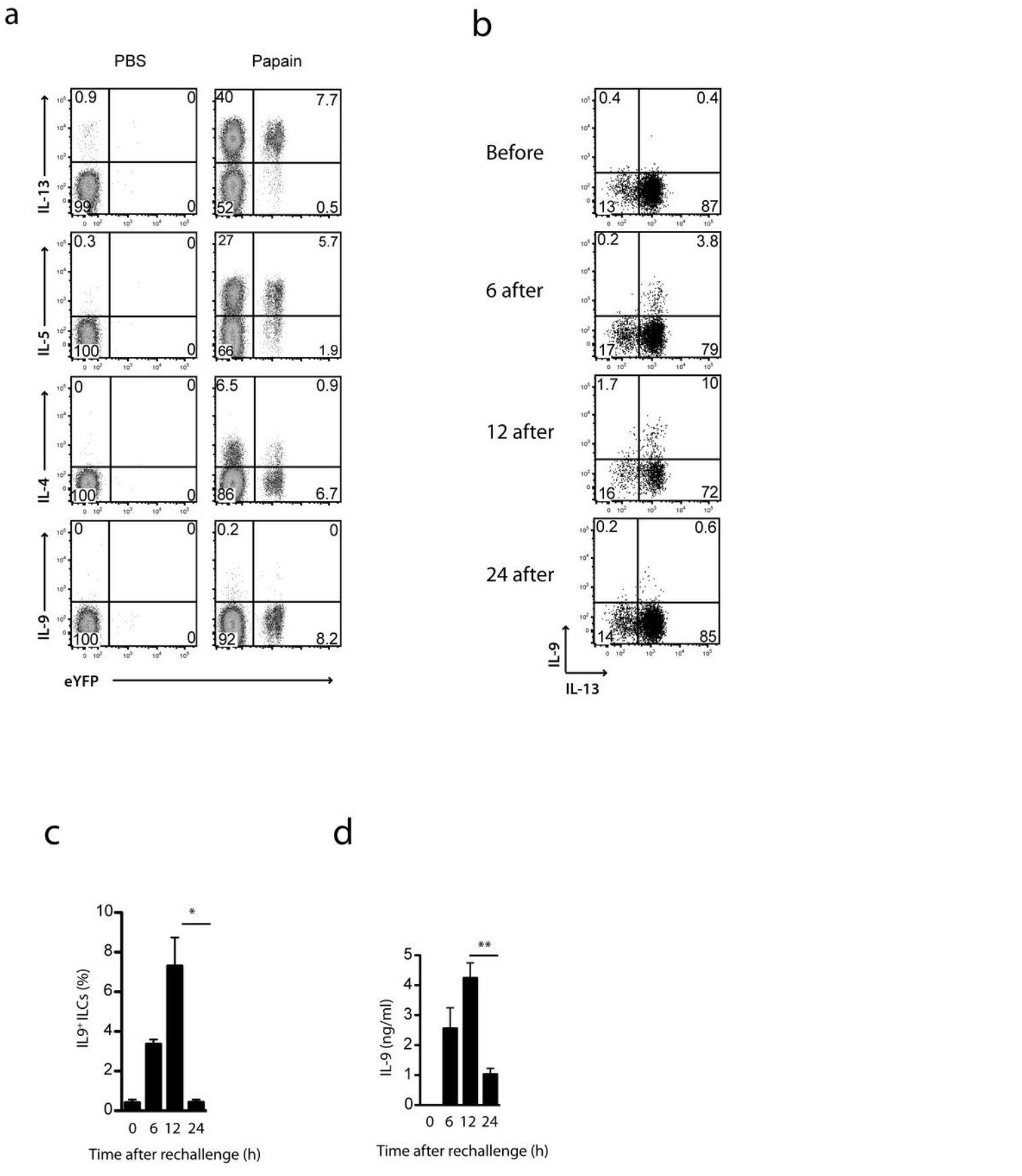
Figure 2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0.3
0
100
0
27
5.7
66
1.9
0
0
100
0
6.5
0.9
86
6.7
0
0
100
0
0.2
0
92
8.2
b
Before
6 after
12 after
24 after
0.4
0.4
13
87
0.2
3.8
17
79
1.7
10
16
72
0.2
0.6
14
85
IL-9
IL-13
c
IL9+ ILCs (%)
*
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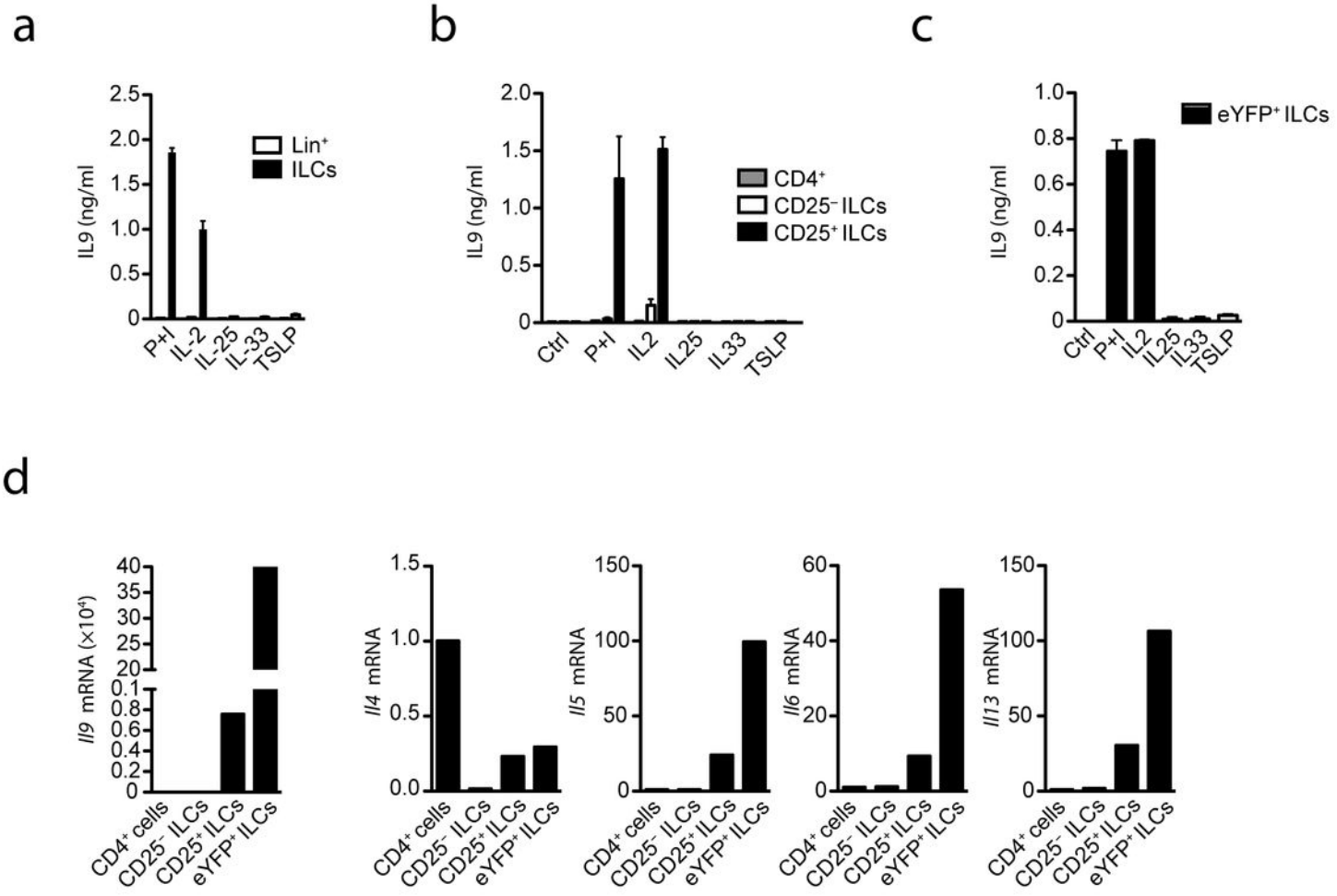
Time after rechallenge (h)
d
IL-9 (ng/ml)
**
0 6 12 24
Time after rechallenge (h)

Figure 3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IL33
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eYFP+ ILCs
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IL33
TSLP
d
Il9 mRNA (×10⁴)
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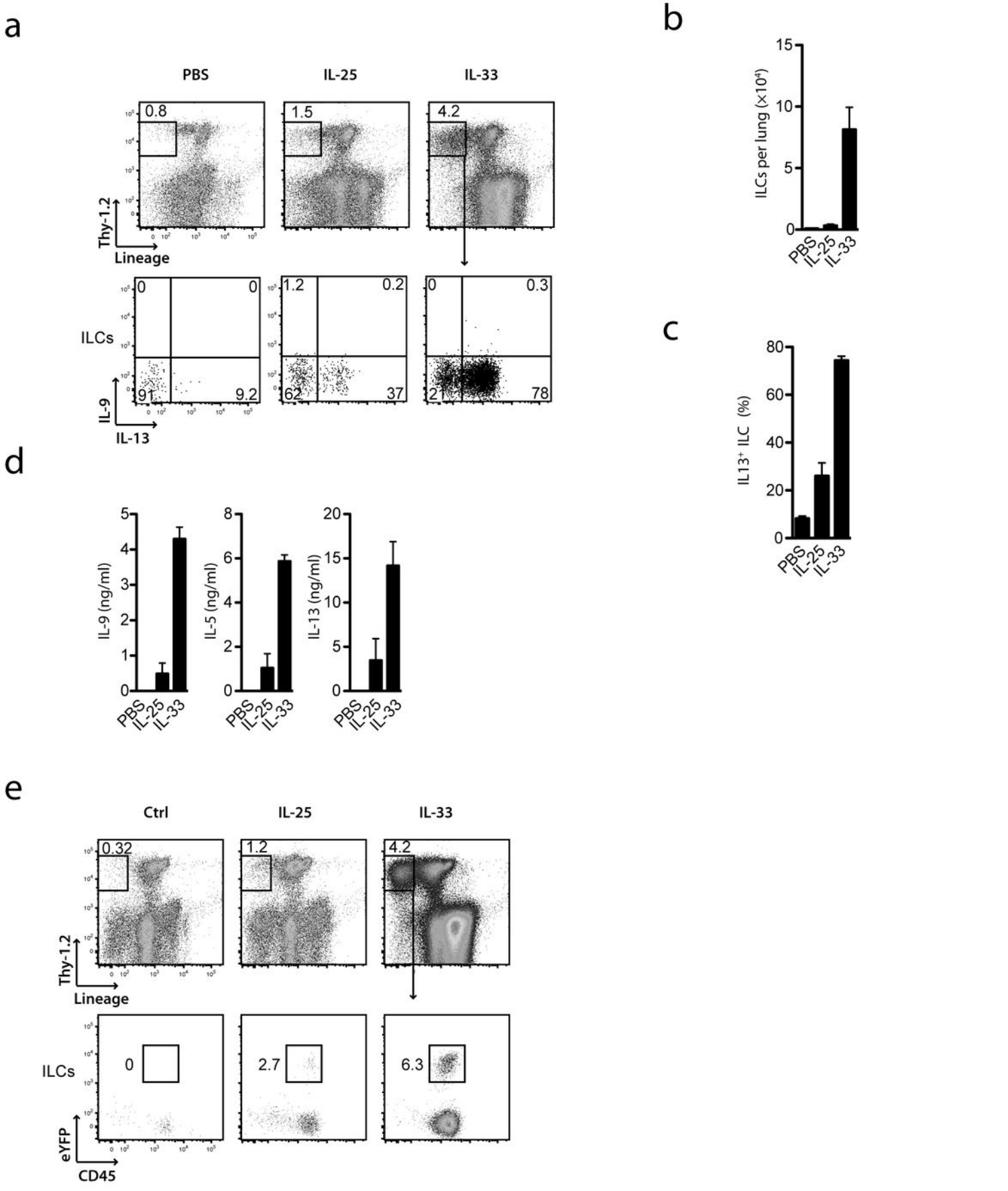
CD25+ ILCs
eYFP+ ILCs

Figure 4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0
0.3
91
9.2
62
37
2
78
IL-9
IL-13
b
ILCs per lung (×10⁴)
15
10
5
0
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IL-25
IL-33
c
IL13⁺ ILC (%)
80
60
40
20
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IL-25
IL-33
d
IL-9 (ng/ml)
IL-5 (ng/ml)
IL-13 (ng/ml)
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IL-25
IL-33
e
Ctrl
IL-25
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Lineage
ILCs
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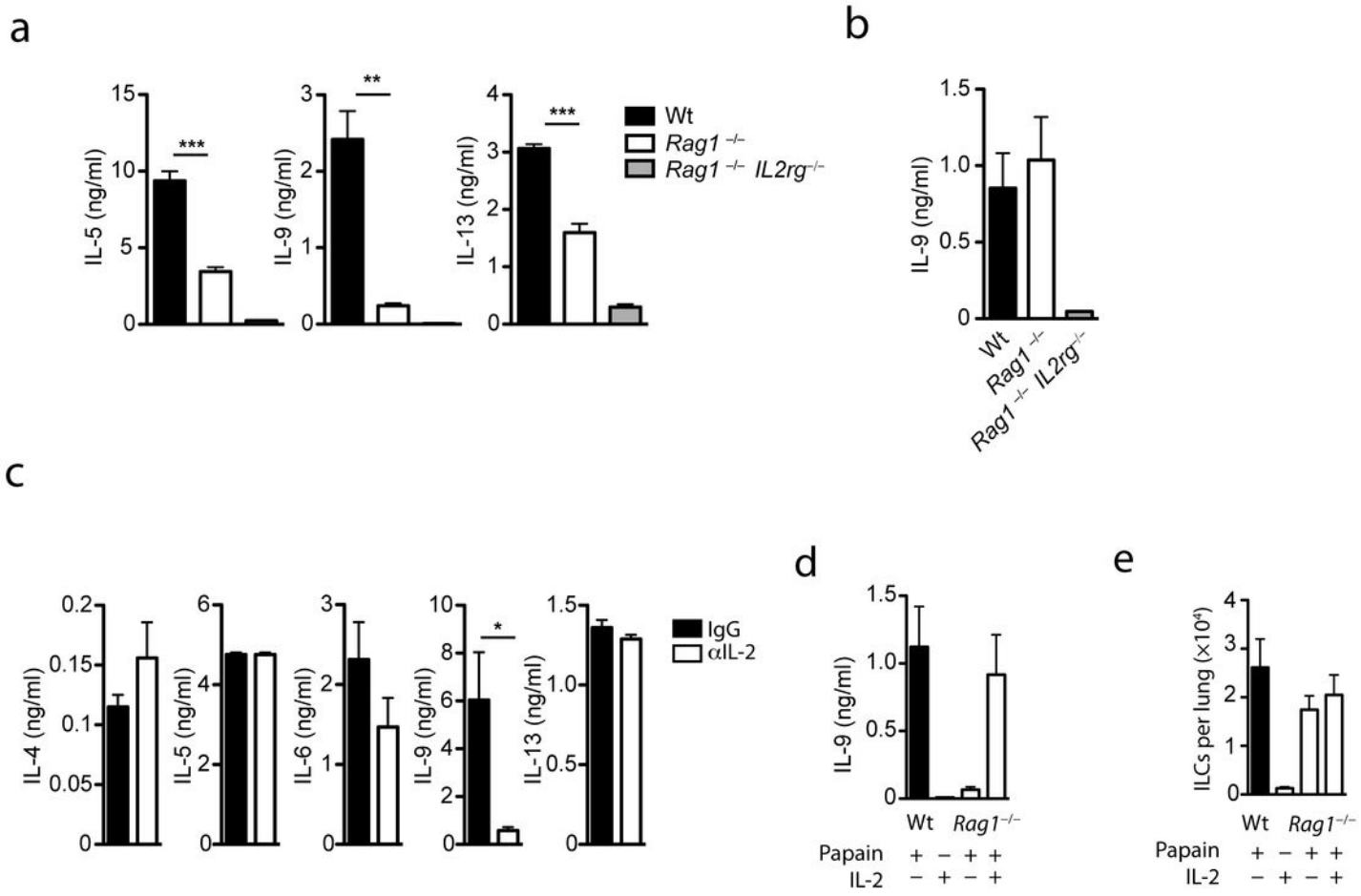
eYFP
CD45

Figure 5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IL-5 (ng/ml)
IL-9 (ng/ml)
IL-13 (ng/ml)
***
**
***
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b
IL-9 (ng/ml)
Wt
Rag1 −/−
Rag1 −/− IL2rg−/−
c
IL-4 (ng/ml)
IL-5 (ng/ml)
IL-6 (ng/ml)
IL-9 (ng/ml)
IL-13 (ng/ml)
*
IgG
αIL-2
d
IL-9 (ng/ml)
Wt
Rag1−/−
Papain + − + +
IL-2 − + − +
e
ILCs per lung (×10⁴)
Wt
Rag1−/−
Papain + − + +
IL-2 − + − +

Figure 6

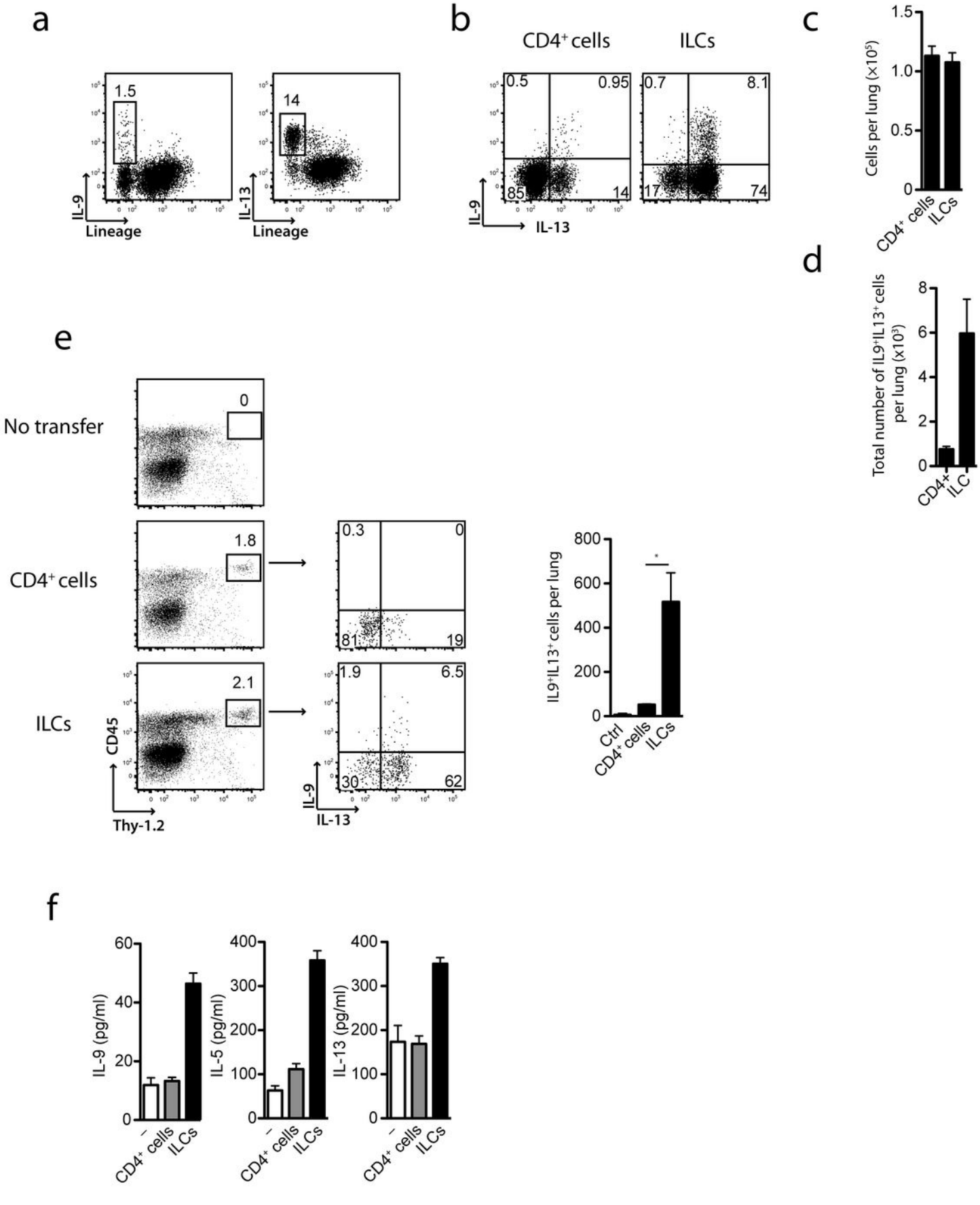
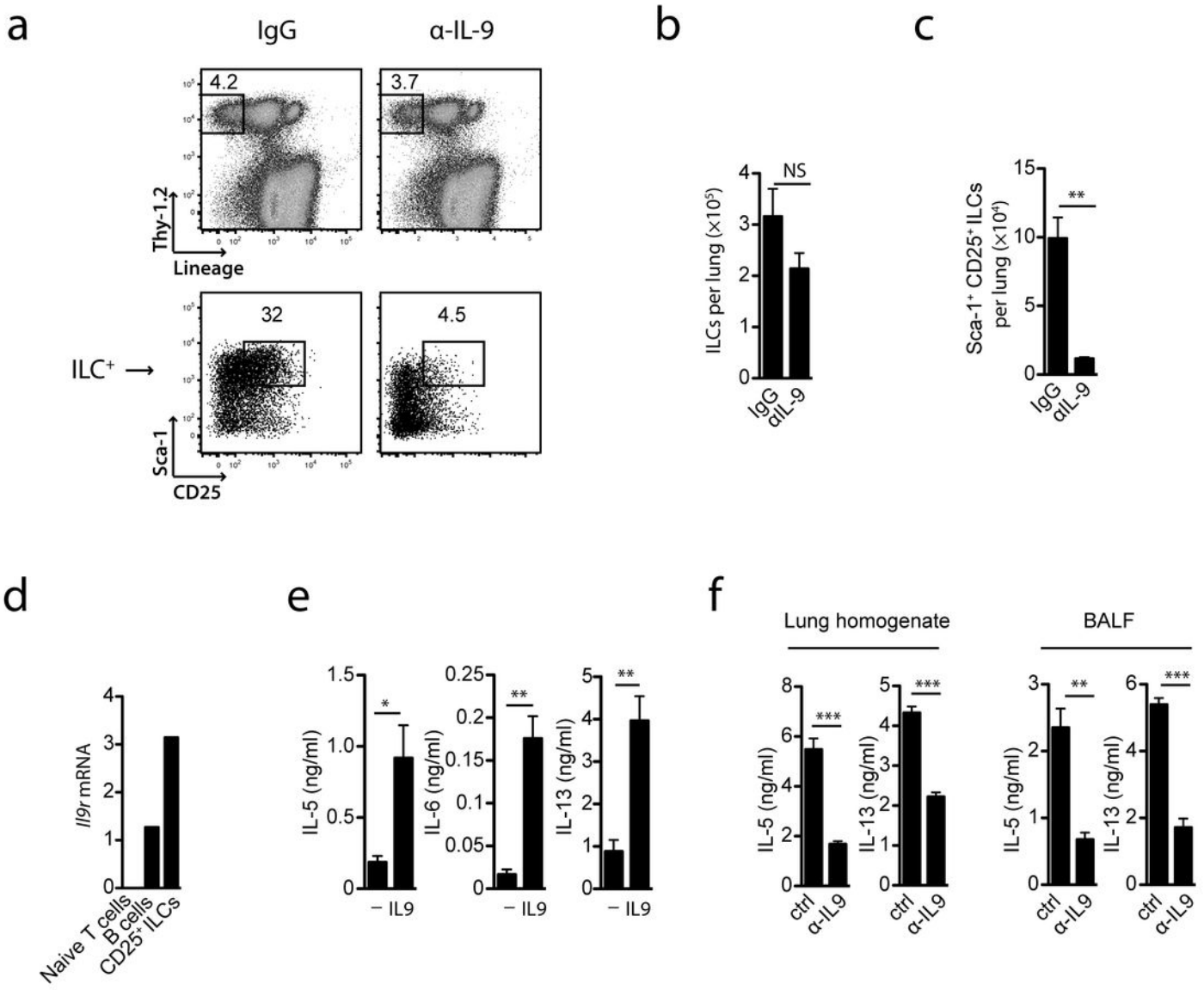

a
1.5
IL-9
Lineage
14
IL-13
Lineage
b
CD4+ cells
ILCs
0.5
0.95
85
14
0.7
8.1
17
74
IL-9
IL-13
c
Cells per lung (×10^5)
1.5
1.0
0.5
0
CD4+ cells
ILCs
d
Total number of IL9+IL13+ cells per lung (×10^3)
8
6
4
2
0
CD4+
ILC
e
No transfer
0
CD4+ cells
1.8
0.3
0
81
19
ILCs
2.1
1.9
6.5
30
62
CD45
Thy-1.2
IL-9
IL-13
IL9+IL13+ cells per lung
800
600
400
200
0
*
Ctrl
CD4+ cells
ILCs
f
IL-9 (pg/ml)
60
40
20
0
IL-5 (pg/ml)
400
300
200
100
0
IL-13 (pg/ml)
400
300
200
100
0
–
CD4+ cells
ILCs

Figure 7


a
IgG
α-IL-9
4.2
3.7
Thy-1.2
Lineage
ILC+
32
4.5
Sca-1
CD25
b
NS
ILCs per lung (×10⁵)
IgG
αIL-9
c
**
Sca-1+ CD25+ ILCs per lung (×10⁴)
IgG
αIL-9
d
Il9r mRNA
Naive T cells
B cells
CD25+ ILCs
e
*
**
**
IL-5 (ng/ml)
IL-6 (ng/ml)
IL-13 (ng/ml)
− IL9
− IL9
− IL9
f
Lung homogenate
BALF
***
***
**
***
IL-5 (ng/ml)
IL-13 (ng/ml)
IL-5 (ng/ml)
IL-13 (ng/ml)
ctrl
α-IL9
ctrl
α-IL9
ctrl
α-IL9
ctrl
α-IL9