

Received Date : 19-May-2015

Accepted Date : 21-Jul-2015

Article type : Original Article: Experimental Allergy and Immunology

Editor : Hans-Uwe Simon

T cells are the critical source of IL-4/IL-13 in a mouse model of allergic asthma

Katharina Oeser¹, Joachim Maxeiner², Cornelia Symowski¹, Michael Stassen², David Voehringer¹

¹Department of Infection Biology, University Hospital Erlangen at the Friedrich-Alexander University Erlangen-Nuremberg (FAU), 91054 Erlangen, Germany

²Asthma Core Facility, The Research Center Immunotherapy (FZI) at the University Hospital Mainz, 55131 Mainz, Germany

Short title: Allergic asthma depends on T cell-derived IL-4/IL-13

Key words: Asthma, basophils, eosinophils, ILC2, Th2 cells

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.12705

This article is protected by copyright. All rights reserved.

Correspondance: David Voehringer, PhD
University Hospital Erlangen
Department of Infection Biology
Friedrich-Alexander University Erlangen-Nuremberg (FAU)
91054 Erlangen
Germany
David.voehringer@uk-erlangen.de
Phone +49(0)9131-8532735
Fax +49(0)9131-8532733

Abstract

Background: IL-4 and IL-13 play a crucial role during allergic asthma. Both cytokines can be produced by T cells and a variety of cell types of the innate immune system. The relative contribution of T cell-derived versus innate IL-4/IL-13 for allergic inflammation and airway hyperreactivity remains unclear.

Methods: We compared the severity of OVA/alum-induced allergic lung inflammation in WT BALB/c mice to mice that lack expression of IL-4/IL-13 only in T cells (4-13Tko) or in all cell types (4-13ko).

Results: T cell-derived IL-4/IL-13 was required for IgG1 and IgE production, recruitment of eosinophils and basophils to the lung, goblet cell hyperplasia, expression of Muc5ac, Clca3, and RELM β , differentiation of alternatively activated macrophages, and airway hyperreactivity. Interestingly, ILC2 recruitment to the lung occurred independently of T cell-derived IL-4/IL-13 but was diminished in the absence of IL-4/IL-13 from all cell types. Thus, the number of IL-4/IL-13-competent ILC2s did not correlate with the severity of lung pathology.

Conclusions: Th2 cells appear to be the critical IL-4/IL-13-expressing cell type for induction of allergic airway inflammation and airway hyperreactivity. The translational perspective of our results indicates that inhibition or reprogramming of Th2 cells may be very effective for treatment of allergic asthma.

Introduction

Allergic asthma is a chronic inflammatory disease of the lung which can be elicited by a large variety of environmental allergens. The prevalence of allergic asthma has increased over the past decades in westernized countries and afflicts about 10% of the population in the United States¹. During the sensitization phase allergens initiate a so-called type 2 immune response by inducing the differentiation of Th2 cells, but the underlying mechanisms are still poorly understood². Interestingly, type 2 immune responses are also elicited by parasitic intestinal worms which often migrate through the lung during their lifecycle. Allergens and migrating helminths promote rapid release of IL-25, IL-33, and TSLP from lung epithelial cells which leads to accumulation of Th2 cells and several cell types of the innate immune system including eosinophils, basophils, mast cells, and ILC2 cells in the lung³. These cells produce IL-4 and IL-13 which are functionally closely related cytokines that play a crucial and largely overlapping role during the sensitization and effector phase of allergic asthma⁴.

IL-4 can activate two different receptors: the type I IL-4 receptor which is expressed mainly on hematopoietic cells and composed of the IL-4R α chain together with the common gamma chain (γ_c), or the type II IL-4 receptor which is expressed mainly on non-hematopoietic cells and composed of the IL-4R α chain and the IL-13R α 1 chain^{5,6}. IL-13 binds to the type II receptor but also to a second IL-13-specific receptor named IL-13R α 2. Signaling through the type I and type II receptors is largely mediated by STAT6 which gets phosphorylated by Janus kinases and translocates to the nucleus where it serves as transcription factor. In allergic asthma STAT6 is required for class switch recombination to IgE in B cells⁷, activation of goblet cells and smooth muscle cells in the lung, expression of eotaxins (CCL11/CCL24) and TARC (CCL17) for recruitment of effector cells, differentiation of alternatively activated macrophages and airway hyperreactivity (AHR)⁸. STAT6-regulated target genes also include

Resistin-like molecule (RELM) β which has inflammatory and remodeling activity in the murine lung⁹, the mucin Muc5ac which is secreted by lung epithelial cells and contributes to AHR¹⁰, and the Ca²⁺-activated chloride channel Clca3 (also named Gob5)¹¹.

Studies over the past several years revealed that IL-4/IL-13 from cells of the innate immune system can have a profound function for orchestration of type 2 immune responses in the lung and intestine. For example, IL-4/IL-13 from ILC2s and basophils was found to protect against the gastrointestinal helminth *Nippostrongylus brasiliensis* while T cell-derived IL-4/IL-13 was not required for protective immunity^{12,13}. Furthermore, basophil-derived IL-4 and ILC2-derived IL-13 was reported to orchestrate type 2 immunity in the lung after exposure to the allergenic protease papain^{14,15}. Given the critical role of IL-4/IL-13 for allergic inflammation it is important to dissect the relative contribution of T cells and innate cell types as source of these cytokines for lung pathology.

To address this point, we compared OVA/alum-induced allergic lung inflammation in wild-type BALB/c mice to mice that lack expression of IL-4/IL-13 only in T cells (4-13Tko) or in all cell types (4-13ko). We found that selective deletion of IL-4/IL-13 in T cells prevented OVA/alum-induced IgG1 and IgE production, expression of the chemokines eotaxin-1 (CCL11) and TARC (CCL17) in the lung, recruitment of eosinophils and basophils to the lung, differentiation of alternatively activated macrophages, goblet cell hyperplasia and AHR. However, ILC2 accumulation in the lung was only reduced when IL-4/IL-13 expression was absent from all cell types. These results demonstrate that T cells are the critical source of IL-4/IL-13 for orchestration of allergic airway inflammation in this model and accumulation of ILC2s does not correlate with severity of inflammation or AHR.

Materials and Methods

Mice

BALB/c mice were originally obtained from Charles River (Sulzfeld, Germany). IL-4/IL-13-deficient mice (4-13ko)¹⁶ backcrossed to BALB/c background were kindly provided by A.N. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). Mice with a loxP-flanked IL-4/IL-13 allele¹⁷ were crossed to CD4Cre mice¹⁸ to generate mice with selective deletion of both cytokines in T cells (4-13Tko mice) and backcrossed for more than 10 generations to BALB/c background. All mice were housed according to institutional guidelines and used between 6 and 13 weeks of age. The animal experiments were approved by the Regierung von Mittelfranken and performed in accordance with the German animal protection law and the EU guidelines 86/809.

OVA-induced allergic lung inflammation

OVA-induced acute allergic lung inflammation was elicited by i.p. sensitization with 50 µg Chicken-Ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO) emulsified in 200 µl Imject Alum (Thermo Fisher Scientific, Waltham, MA) on day 0 and day 7, followed by three i.n. challenges with 200 µg OVA dissolved in 50 µl PBS on day 14, 15, and 16. In some experiments the challenges were performed by inhalation with aerosols of OVA (1 % w/v solution in PBS). Control mice received only Alum or PBS. Mice were sacrificed on day 17.

Restimulations of tLN cells and ELISAs

Tracheal lymph nodes (tLNs) were mechanically disrupted in a 70 µm nylon strainer (Becton Dickinson, Franklin Lakes, NJ) to obtain single cell suspensions. 2x10⁶ cells per well were plated on a 24-well plate and restimulated with phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, 40 ng/ml) and Ionomycin (Sigma-Aldrich, 1 µg/ml). After 24 hours, IL-4, IL-5, IL-13, and IFN-γ concentrations in the supernatant were detected by standard ELISA. IL-4

was measured with the anti-IL-4 mAbs 11B11 for coating and BVD6-24G2 (BioLegend, San Diego, CA) for detection. IL-5, IL-13, and IFN- γ were measured with ELISA kits from BD Biosciences (San Jose, CA) and PeproTech (Rocky Hill, NJ).

Serum ELISAs

Serum IgE levels were determined with purified anti-IgE (clone R35-72, BD Biosciences, Heidelberg, Germany) for coating and biotinylated anti-IgE (clone R35-118, BD Biosciences) for detection. Total IgG1 and IgG2a concentrations were determined using a commercial ELISA kit (SouthernBiotech, Birmingham, AL). For measurement of OVA-specific antibody titers 50 μ g/ml OVA were used for coating, followed by similar protocols as used for measurement of total antibody isotypes.

Flow cytometry

Lung samples were digested with Collagenase Type VIII (10 μ g/ml; Sigma-Aldrich) for 30 minutes at 37°C. Afterwards, lungs were mechanically disrupted in a 70 μ m nylon strainer (Becton Dickinson) to obtain single cell suspensions. Lung and blood cells were washed with FACS buffer (PBS, 2 % FCS) and incubated with anti-CD16/CD32 blocking antibody (clone 2.4G2; BioXcell, West Lebanon, NH) for 5 minutes at room temperature followed by staining with antibody mixtures at 4°C. The following antibodies were used for surface staining:

FITC-labeled anti-CD4 (clone L3T4), PerCP-Cy5.5-labeled anti-CD4 (clone RM4-5), APC-eFluor 780-labeled anti-CD4 (clone RM4-5), FITC-labeled anti-CD90.2 (clone 53-2.1), PerCP-CY5.5-labeled anti-CD11c (clone N418), biotinylated anti-CD3 (clone 145-2C11), anti-CD11b (clone M1/70), anti-CD45R (clone RA3-6B2), anti-Fc ϵ RI (clone MARI), and anti-Ter119 (TER-119) were purchased from eBioscience. PE-labeled anti-CD200R3 (clone Ba13) and Alexa Fluor 647-labeled anti-CD49b (clone HM α 2) were purchased from BioLegend. Brilliant Violet-labeled anti-Siglec-F (clone E50-2440) was purchased from BD

Biosciences. FITC-labeled anti-CD90.1 (clone His51) and APC-labeled anti-ICOS (clone 7E.17G9) were purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany). To detect biotinylated antibodies, APC-eFluor 780-labeled streptavidin (eBiosciences) and PE-labeled streptavidin (Southern Biotech) was used.

Biotinylated anti-RELM α (PeproTech) was used for intracellular staining after cells had been fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions.

Dead cells were excluded by staining with DAPI (4,6 diamidino-2-phenylindole; Sigma-Aldrich), fixable viability dye APC-eFluor 780, or fixable viability dye APC-eFluor 506 (both eBioscience). Samples were acquired on FACSCanto II (BD Biosciences) or MACSQuant Analyzer (Miltenyi Biotec). Doublets were excluded by electronic gating. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Quantitative RT-PCR

RNA was extracted from lung and tLN samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany). 1 μ g total RNA was used for first-strand cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen - Life Technologies, Carlsbad, CA) and oligo(dT) primers. Polymerase chain reaction (PCR) was performed on the 7900HT Fast Real-Time PCR system (Applied Biosystems-Life Technologies, Carlsbad, CA) or the CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with 5-fold diluted cDNA templates using SYBR Green I (Qiagen) and the following primer sequences:

Porphobilinogen deaminase (PBGD) for 5'-TGGTTGTTCACTCCCTGAAGG-3', PBGD rev 5'-AAAGACAACAGCATCACAAGGGT-3',

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) for 5'-

GTTGGATACAGGCCAGACTTTGTT-3', HPRT rev 5'-

GAGGGTAGGCTGGCCTATAGGCT-3', IL-4 for 5'-ACTTGAGAGAGATCATCGGCA-

3', IL-4 rev 5'-AGCTCCATGAGAACACTAGAGTT-3', IL-5 for 5'-
AGGATGCTTCTGCACTTGAG3', IL-5 rev 5'-TCTGTACTCATCACACCAAG-3',
IL-13 for 5'-GCAGTCCTGGCTCTTGCTTG-3', IL-13 rev 5'-
TGCTTTGTGTAGCTGAGCAG-3', IFN- γ for 5'-ACGCTACACACTGCATCTTG-3', IFN-
 γ rev 5'-TCGGATGAGCTCATTGAATG-3', Eotaxin-1/CCL11 for 5'-
GAATCACCAACAACAGATGCAC-3', Eotaxin-1/CCL11 rev 5'-
ATCCTGGACCCACTTCTTCTT-3', TARC/CCL17 for 5'-
GAATGGCCCCCTTTGAAGTAA-3', TARC/CCL17 rev 5'-TGCTTCTGGGGACTTTTCTG-
3', Muc5ac for 5'-CTGTGACATTATCCCATAAGCCC-3', Muc5ac rev 5'-
AAGGGGTATAGCTGGCCTGA-3', Clca3 for 5'-AAGCAGCTGTGCTTCAGCAG-3',
Clca3 rev 5'-CAGATTGGACTTATCCACAG-3', RELM β forward 5'-
AAGCCTACACTGTGTTTCCTTTT-3', RELM β reverse 5'-
GCTTCCTTGATCCTTTGATCCAC-3', RELM α forward 5'-
CCATAGAGAGATTATCGTGGA-3', RELM α reverse 5'-
TGGTCGAGTCAACGAGTAAG-3'.

PBGD and HPRT served as reference genes.

PCR conditions were the following: 15 min initial denaturation at 95°C, 40 cycles with 30 seconds of denaturation at 95°C, 30 seconds of annealing at 58°C or 50°C, and 45 seconds of elongation at 72°C.

Immunofluorescence and Periodic Acid Schiff staining

For immunofluorescence staining, cryosections (6 μ m thick) of paraformaldehyde-fixed lung samples were pre-treated with sodium citrate buffer (10 mM sodium - citrate, 0.05 % Tween 20, pH 6.0) for 10 minutes at 90°C. Sections were then blocked with TNB-buffer (TSA amplification kit, Perkin Elmer) supplemented with 1 % rat serum, 1 % mouse serum and 40 μ g/ml CD16/32-blocking antibody and stained with rabbit anti-mouse RELM β

antibody (PeproTech) or with polyclonal rabbit anti-mouse Ym1/Chi3l3 (StemCell Technologies) followed by Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were counterstained with DAPI. Furthermore, lung samples were fixed in 10 % formalin, dehydrated and mounted in paraffin. Tissue cross sections were stained with hematoxylin/eosin and periodic acid-schiff (PAS; Sigma-Aldrich) according to manufacturer's instructions. PAS-positive epithelial cells were quantified per millimeter of basement membrane in 5–6 representative fields of each lung specimen by two experienced individuals in a double-blinded setting using light microscopy. Pictures were acquired on Axio Vert.A1 (Carl Zeiss, Jena, Germany). Image analysis of immunofluorescence was performed using the LSM image browser.

Measurement of Airway Hyperresponsiveness

To assess airway hyperresponsiveness (AHR), an invasive whole-body plethysmography was performed by the Asthma Core Facility, Mainz, Germany, using a resistance/compliance isovolumetric analyzer according to the manufacturer's instructions (Buxco Electronics, Inc., BioSystem XA for Windows) as previously described¹⁹. On day 17 the airway resistance measurements were performed on anesthetized, intubated, and mechanically ventilated mice in response to increasing doses (0-50mg/ml) of inhaled methacholine.

Statistical analysis

Statistics were calculated by Student's *t* test using SigmaPlot software (Systat Software Inc., San Jose, CA).

Results

Selective deletion of IL-4/IL-13 in T cells also impairs IL-5 production during OVA-induced allergic airway inflammation.

The specific contribution of T cell-derived IL-4/IL-13 for OVA-induced allergic airway inflammation is poorly defined. To address this issue we analyzed the response in wild-type mice in comparison to mice that lack IL-4/IL-13 only in T cells (4-13Tko) or in all cell types (4-13ko). These three groups of mice on BALB/c genetic background were sensitized on day 0 and 7 by intraperitoneal injection of chicken ovalbumin emulsified with alum adjuvant (OVA/alum), followed by three intranasal administrations of 200 µg OVA on day 14, 15 and 17 before mice were analyzed on day 17. First, we measured IL-4, IL-5 and IL-13 expression from mediastinal lymph nodes samples that had been restimulated with PMA/Ionomycin for 24 hours. Analysis of cytokine concentrations in the supernatants revealed that cells from wild-type mice secreted large amounts of all three cytokines while this response was missing in 4-13Tko and 4-13ko mice (Fig. 1). The same result was obtained when we analyzed cytokine mRNA levels by quantitative RT-PCR (Fig. S1). This indicates that Th2 cells are indeed the major source of these cytokines in the draining lymph nodes at the challenge phase of the response.

Th2 cells are critical and innate IL-4/IL-13-producing cells are not sufficient for serum IgE and IgG1 responses.

IL-4 and to some extent IL-13 promote class switch recombination in B cells to IgG1 and IgE isotypes. Although it is well established that cognate T-B interaction and local presence of IL-4/IL-13 promotes the IgG1/IgE response, it is not clear whether secretion of IL-4/IL-13 from cells of the innate immune system (eosinophils, basophils, mast cells, ILC2s) can also contribute to this type of humoral immune response. Therefore, we determined the antibody

Accepted Article

response in the serum of wild-type, 4-13Tko and 4-13ko mice by OVA-specific and total isotype ELISA. The IgE and IgG1 levels were reduced and IgG2a levels increased to similar levels in 4-13Tko and 4-13ko when compared to wild-type mice (Fig. 2). This illustrates that innate IL-4/IL-13 expressing cells are not sufficient to provide the relevant switch signal to B cells.

Mobilization and recruitment of effector cells to the lung during allergic airway inflammation.

Recruitment of effector cells to the lung during type 2 immune responses is largely dependent on IL-4- or IL-13-induced activation of endothelial cells and secretion of the chemokines Ccl11 (Eotaxin-1) and Ccl17 (TARC). To determine the relative contribution of IL-4/IL-13 from T cells for this process we analyzed the frequency and total numbers of eosinophil, basophils and ILC2s in the lung and blood after OVA challenge. In the lung, eosinophils and basophils were significantly reduced in 4-13Tko and 4-13ko mice as compared to wild-type mice (Fig. 3A-C). In contrast, very few ILC2s were recruited to the lungs and their numbers were only reduced in 4-13ko mice. In the blood, wild-type and 4-13Tko mice showed no difference in the level of eosinophils, basophils or ILC2s while 4-13ko mice showed reduced levels of eosinophils and ILC2s (Fig. 3B and Fig. S2). The impaired recruitment of eosinophils and basophils was consistent with a lower expression of *Ccl11* and *Ccl17* in 4-13Tko and 4-13ko as compared to wild-type mice (Fig. 3D).

T cell-derived IL-4/IL-13 is required for goblet cell hyperplasia and induction of RELM β , Muc5ac, and Clca3 expression in the lung.

In addition to promoting effector cell recruitment to the lung, IL-4 and IL-13 also activate lung epithelial cells to release effector molecules and promote goblet cell hyperplasia. By staining lung tissue sections with periodic acid Schiff (PAS) we observed that goblet cell

hyperplasia was directly or indirectly dependent on T cell-derived IL-4/IL-13 (Fig. 4A). Furthermore, immunofluorescence staining demonstrated the requirement of T cell-derived IL-4/IL-13 for expression of RELM β which has been described to promote airway remodeling by activation of fibroblast differentiation and proliferation⁹ (Fig. 4B). Consistent with this histological analysis we observed that mRNA levels of *Relm β* were only increased in wild-type mice (Fig. 4C). Similarly, expression of two other IL-4/IL-13-regulated genes, namely *Mucin 5ac*, a mucin type that appears to be critical for airway hyperreactivity¹⁰ and the goblet cell-associated Ca²⁺-activated chloride channel *Clca3/Gob-5*¹¹ was dependent on T cell-derived IL-4/IL-13 (Fig. 4C).

T cell-derived IL-4/IL-13 is required for accumulation of alternatively activated macrophages (AAMs) in the lung during allergic inflammation.

In addition to promoting recruitment of pro-inflammatory effector cells and activation of epithelial cells, IL-4 and IL-13 can also confer immunosuppressive effects by inducing the differentiation of AAMs. These cells produce numerous immunomodulatory molecules including RELM α , Arg1 and PD-L2 which have been shown to inhibit T cells. Therefore, we compared the frequency and total number of AAMs in the lung of wild-type, 4-13Tko and 4-13ko mice by flow cytometric analysis of single cell suspensions from the lung. Since macrophages in the lung express high levels of CD11c and rather low levels of F4/80 we stained for CD11c in combination with intracellular RELM α (Fig. 5A) or surface PD-L2 (Fig. 5C). We further determined the *Relm- α* mRNA expression in lung tissue by quantitative RT-PCR (Fig. 5B). In addition, we performed immunofluorescence stainings for the AAM-associated marker Ym1/Chi3l3 (Fig. 5D). These analyses revealed that only wild-type mice showed increased AAM levels in the lung indicating that T cell-derived IL-4/IL-13 is required for AAM accumulation.

Airway hyperreactivity (AHR) is dependent on T cell-derived IL-4/IL-13.

Allergic lung inflammation results in massive changes of lung physiology. IL-4 and IL-13 are potent activators of smooth muscle cells located around the airways. To determine the relative contribution of T cell-derived IL-4/IL-13 for AHR we compared the response in wild-type, 4-13Tko and 4-13ko mice. For each strain of mice one group was sensitized on days 0 and 7 with OVA/alum i.p. and another group was injected with PBS i.p. On days 14, 15 and 16 all mice were exposed to aerosolized OVA and AHR was measured on day 17 by whole-body plethysmography with increasing doses of methacholine. OVA-sensitized wild-type mice showed increased airway resistance as compared to OVA-sensitized 4-13Tko and 4-13ko mice (Fig. 6A). The weak airway resistance in 4-13Tko and 4-13ko mice was not dependent on OVA-sensitization since it was also observed in PBS-injected control mice (Fig. 6B). This result demonstrates that T cell-derived IL-4/IL-13 is critical for the AHR response.

Discussion

We used here conditional IL-4/IL-13-deficient mice to determine the contribution of T cells as source of these cytokines for induction of allergic airway inflammation. By comparing wild-type mice to mice that either lack IL-4/IL-13 only in T cells or in all cell types we could show that all major parameters of allergic lung inflammation (effector cell recruitment, goblet cell hyperplasia, AHR) were indeed dependent on T cell-derived IL-4/IL-13. Using an adoptive transfer system of IL-4/IL-13-deficient T cells into Rag-deficient mice we previously found that effector cell recruitment and AHR could also be observed in this setting, although the response was rather weak compared to transfer of WT T cells²⁰. However, the absence of B and T cells in the recipient mice might have contributed to this weak inflammatory response. The use of 4-13Tko mice avoids the transfer of T cells and therefore serves as a more physiologic system. In addition, we included here 4-13ko mice to uncover potential contributions of innate IL-4/IL-13 for the inflammatory response.

Accepted Article

In previous studies we analyzed protective immunity against helminths in 4-13Tko mice. There, we found that during infection with *Schistosoma mansoni* T cell-derived IL-4/IL-13 was required for granuloma formation around eggs and prevention of fatal infection²¹. In contrast, innate IL-4/IL-13 was required and sufficient for protective immunity against *Nippostrongylus brasiliensis*²². In both infection models, T cell-derived IL-4/IL-13 was required for IgE production and for induction of AAMs. As we show here, both findings also apply to the OVA/alum model of allergic lung inflammation.

Interestingly, production of IL-5 was reduced in restimulated lymph node cells from 4-13Tko and 4-13ko mice. This was unexpected as we previously found no difference in IL-5 expression from in vitro differentiated Th2 cells of both mouse strains²². In vitro cultures contain large amounts of exogenous IL-4 which plays an important role for Th2 differentiation and hence IL-5 expression from T cells. In the model of allergic lung inflammation we used here, IL-4/IL-13 production from innate cell types or their numbers might be too low to promote Th2 differentiation with IL-5 expression as surrogate marker. Eosinophils, basophils and ILC2s can produce IL-4 and IL-13 but it is poorly understood whether they play an important role as source of these cytokines during allergic lung inflammation. Since 4-13Tko show reduced numbers of eosinophils and basophils in the lung after OVA/alum treatment it remains to be determined whether reduced pathology can be observed in a situation where T cells make normal IL-4/IL-13 while innate cell types do not. Eosinophil-deficient PHIL mice were shown to mount a weak inflammatory response²³ and adoptive transfer of eosinophils into PHIL mice promoted recruitment of effector Th2 cells to the lung²⁴. The importance of eosinophils for allergic lung inflammation seems to depend on the genetic background as diminished inflammation was only observed in eosinophil-deficient mice on C57BL/6 but not on BALB/c background^{25,26}. Potential differences regarding the pro-inflammatory roles of basophils and ILC2s on C57BL/6 and BALB/c backgrounds and in other models of allergic lung inflammation including house dust mite or fungal models are

This article is protected by copyright. All rights reserved.

currently unknown. Furthermore, the role of eosinophil-, basophil-, and ILC2-derived IL-4/IL-13 for pathology in different models of allergic lung inflammation remains to be determined.

Basophils produce large amounts of IL-4 but genetic ablation of basophils in two different strains of mice did not diminish Th2 polarization and lung inflammation in the OVA/alum model^{27,28}. Others reported that basophil-derived IL-4 is critical for IL-5 and IL-13 release from ILC2s which then promotes eosinophilia and mucus production during papain-induced lung inflammation¹⁴. Further studies are required to dissect potential differences regarding the role of basophils in different models of allergic lung inflammation. Anyhow, we observed impaired recruitment of eosinophils and basophils to the lung in the absence of IL-4/IL-13 from T cells. In contrast, ILC2s were recruited to the lung of WT and 4-13Tko mice with the same efficiency. This shows that in this model accumulation of ILC2s in the lung is not a predictive marker for disease severity as increased numbers of ILC2s in 4-13Tko mice did not result in recruitment of eosinophils and basophils, induction of goblet cell hyperplasia or AHR. These data indicate that innate IL-4/IL-13 is required and sufficient for ILC2 development the regulation of which is still poorly understood and requires further investigation.

Taken together, we demonstrate that IL-4/IL-13 from T cells accounts for all major aspects of the inflammatory response and airway hyperreactivity in the OVA/alum model of allergic lung inflammation on BALB/c background. Assuming that this finding can be translated to the human immune system, therapeutic interventions acting by inhibition or reprogramming of Th2 cells might also be very effective to prevent development and acerbation of human allergic asthma.

Acknowledgments

We thank Christian Schwartz, Kirstin Castiglione and Anne Hahn for technical assistance, and Manfred Kirsch and Lisa Gundel for animal husbandry. Part of this work was supported by the doctoral training program GRK1660 from the German Research Foundation (DFG).

Author contributions

KO and DV designed experiments and wrote the manuscript. KO, CS, JM and MS performed experiments.

Conflict of interests

The authors declare that they have no conflicts of interest.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. Cytokine mRNA expression in mediastinal lymph nodes.

Figure S2. Distinguishing eosinophils and macrophages by flow cytometry.

Figure S3. Gating strategy to detect ILC2s in the lung

References

- 1 Akinbami, L. J., Moorman, J. E. & Liu, X. Asthma prevalence, health care use, and mortality: United States, 2005-2009. *National health statistics reports*, 1-14 (2011).
- 2 Galli, S. J., Tsai, M. & Piliponsky, A. M. The development of allergic inflammation. *Nature* **454**, 445-454, doi:10.1038/nature07204 (2008).
- 3 Locksley, R. M. Asthma and allergic inflammation. *Cell* **140**, 777-783, doi:10.1016/j.cell.2010.03.004 (2010).

- 4 Chatila, T. A. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends in molecular medicine* **10**, 493-499, doi:10.1016/j.molmed.2004.08.004 (2004).
- 5 LaPorte, S. L. *et al.* Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell* **132**, 259-272, doi:10.1016/j.cell.2007.12.030 (2008).
- 6 Ramalingam, T. R. *et al.* Unique functions of the type II interleukin 4 receptor identified in mice lacking the interleukin 13 receptor alpha1 chain. *Nature immunology* **9**, 25-33, doi:10.1038/ni1544 (2008).
- 7 Linehan, L. A., Warren, W. D., Thompson, P. A., Grusby, M. J. & Berton, M. T. STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. *Journal of immunology* **161**, 302-310 (1998).
- 8 Walford, H. H. & Doherty, T. A. STAT6 and lung inflammation. *Jak-Stat* **2**, e25301, doi:10.4161/jkst.25301 (2013).
- 9 Mishra, A. *et al.* Resistin-like molecule-beta is an allergen-induced cytokine with inflammatory and remodeling activity in the murine lung. *American journal of physiology. Lung cellular and molecular physiology* **293**, L305-313, doi:10.1152/ajplung.00147.2007 (2007).
- 10 Evans, C. M. *et al.* The polymeric mucin Muc5ac is required for allergic airway hyperreactivity. *Nature communications* **6**, 6281, doi:10.1038/ncomms7281 (2015).
- 11 Nakanishi, A. *et al.* Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5175-5180, doi:10.1073/pnas.081510898 (2001).
- 12 Schwartz, C. *et al.* Basophil-mediated protection against gastrointestinal helminths requires IgE-induced cytokine secretion. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E5169-5177, doi:10.1073/pnas.1412663111 (2014).

- 13 Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367-1370, doi:10.1038/nature08900 (2010).
- 14 Motomura, Y. *et al.* Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation. *Immunity* **40**, 758-771, doi:10.1016/j.immuni.2014.04.013 (2014).
- 15 Halim, T. Y. *et al.* Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* **40**, 425-435, doi:10.1016/j.immuni.2014.01.011 (2014).
- 16 McKenzie, G. J., Fallon, P. G., Emson, C. L., Grencis, R. K. & McKenzie, A. N. Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. *The Journal of experimental medicine* **189**, 1565-1572 (1999).
- 17 Voehringer, D., Wu, D., Liang, H. E. & Locksley, R. M. Efficient generation of long-distance conditional alleles using recombineering and a dual selection strategy in replicate plates. *BMC biotechnology* **9**, 69, doi:10.1186/1472-6750-9-69 (2009).
- 18 Lee, P. P. *et al.* A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* **15**, 763-774 (2001).
- 19 Hausding, M. *et al.* Lung CD11c+ cells from mice deficient in Epstein-Barr virus-induced gene 3 (EBI-3) prevent airway hyper-responsiveness in experimental asthma. *European journal of immunology* **37**, 1663-1677, doi:10.1002/eji.200636675 (2007).
- 20 Voehringer, D., Reese, T. A., Huang, X., Shinkai, K. & Locksley, R. M. Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *The Journal of experimental medicine* **203**, 1435-1446, doi:10.1084/jem.20052448 (2006).
- 21 Schwartz, C., Oeser, K., Prazeres da Costa, C., Layland, L. E. & Voehringer, D. T cell-derived IL-4/IL-13 protects mice against fatal *Schistosoma mansoni* infection

- independently of basophils. *Journal of immunology* **193**, 3590-3599, doi:10.4049/jimmunol.1401155 (2014).
- 22 Oeser, K., Schwartz, C. & Voehringer, D. Conditional IL-4/IL-13-deficient mice reveal a critical role of innate immune cells for protective immunity against gastrointestinal helminths. *Mucosal immunology*, doi:10.1038/mi.2014.101 (2014).
- 23 Lee, J. J. *et al.* Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* **305**, 1773-1776, doi:10.1126/science.1099472 (2004).
- 24 Jacobsen, E. A. *et al.* Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *The Journal of experimental medicine* **205**, 699-710, doi:10.1084/jem.20071840 (2008).
- 25 Humbles, A. A. *et al.* A critical role for eosinophils in allergic airways remodeling. *Science* **305**, 1776-1779, doi:10.1126/science.1100283 (2004).
- 26 Walsh, E. R. *et al.* Strain-specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma. *The Journal of experimental medicine* **205**, 1285-1292, doi:10.1084/jem.20071836 (2008).
- 27 Ohnmacht, C. *et al.* Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* **33**, 364-374, doi:10.1016/j.immuni.2010.08.011 (2010).
- 28 Sawaguchi, M. *et al.* Role of mast cells and basophils in IgE responses and in allergic airway hyperresponsiveness. *Journal of immunology* **188**, 1809-1818, doi:10.4049/jimmunol.1101746 (2012).

FIGURE LEGENDS

Figure 1. Reduced cytokine production in 4-13Tko and 4-13ko mice after OVA-induced allergic lung inflammation. , Wild-type (WT, hatched), 4-13Tko (grey) and 4-13ko (black) mice were subjected to OVA-induced allergic lung inflammation and supernatants from restimulated mediastinal lymph node cells were analyzed on day 3 after OVA challenge for indicated cytokines by ELISA. Bars show the mean+SD of three individual mice per group and are representative of three independent experiments. $\ast=P<0.05$ by two-tailed Student's t-test.

Figure 2. Innate IL-4/IL-13 is not sufficient for induction of OVA-specific IgE and IgG1 responses. Serum IgE, IgG1, and IgG2a levels from WT (hatched), 4-13Tko (grey) and 4-13ko (black) mice were determined by ELISA on day 3 after OVA challenge . A, Levels of OVA-specific antibodies. B, Total antibody concentrations. Bars show the mean+SD of three individual mice per group and are representative of four independent experiments. $\ast=P<0.05$, $\ast\ast=P<0.01$, $\ast\ast\ast=P<0.001$ by two-tailed Student's t-test.

Figure 3. IL-4/IL-13-regulated mobilization and tissue recruitment of basophils, eosinophils, and ILC2s. Lung and blood leukocytes from WT (hatched), 4-13Tko (grey) and 4-13ko (black) mice were analyzed on day 3 after OVA challenge. A, Representative flow cytometry dot plots of live singlet and autofluorescence-negative cells for the identification of basophils ($CD4^{-}CD49b^{+}CD200R3^{+}$, left panels), eosinophils ($CD4^{-}Siglec-F^{+}SSC^{hi}$, middle panels), and ILC2s ($CD4^{-}Lin^{-}ICOS^{+}CD90^{+}$, right panels) in WT mice. See also Figures S2 and S3. B, Percentages and C, total numbers of respective cell types. D, Quantitative RT-PCR analysis of total lung tissue for expression of Eotaxin-1 (*Ccl11*) and TARC (*Ccl17*). Bars show the mean+SD of three individual mice per group and are representative of four

independent experiments. $\ast=P<0.05$, $\ast\ast=P<0.01$, $\ast\ast\ast=P<0.001$ by two-tailed Student's t-test.

Figure 4. T cell-derived IL-4/IL-13 is required for activation of goblet cells in the lung during OVA-induced allergic lung inflammation. A, Lung samples of WT, 4-13Tko, and 4-13ko mice were stained on day 3 after OVA challenge with Periodic acid-Schiff (PAS) and PAS⁺ cells were quantified. B, Lung samples were stained with anti-RELM β (red) and DAPI (blue). Scale bars represent 20 μ m. C, Quantitative RT-PCR analysis for expression of *Relm β* , *Muc5ac* and *Clca3* from WT (hatched), 4-13Tko (grey), and 4-13ko (black) mice. Bars show the mean+SD of three individual mice per group and are representative of three independent experiments. $\ast=P<0.05$, $\ast\ast=P<0.01$ by two-tailed Student's t-test.

Figure 5. T cell-derived IL-4/IL-13 promotes accumulation of alternatively activated macrophages (AAM) in the lung during OVA-induced allergic lung inflammation. A, Lung cells were stained for surface CD11c and intracellular RELM α . Top panels show representative dot plots of CD11c⁺ RELM α ⁺ AAMs of indicated mice after gating out dead cells and eosinophils (Siglec-F+autofluorescence-). Gates were set accordingly to the corresponding isotype staining control. Bars show percentages (left panel) and total numbers (right panel) of CD11c⁺ RELM α ⁺ AAMs in lung samples. B, Quantitative RT-PCR analysis of lung tissue for expression of *Relm- α* . Bars show the mean+SD of three individual mice per group and are representative of three independent experiments. $\ast=P<0.05$, $\ast\ast=P<0.01$, $\ast\ast\ast=P<0.001$ by two-tailed Student's t-test. C, Dot plots show the expression of CD11c and PD-L2 after gating out dead cells and eosinophils (Siglec-F+autofluorescence-). Numbers indicate the mean+SD from three mice per group. D, Immunofluorescence staining for Ym1/Chi313 (red) in lung sections of indicated mice. Slides were counterstained with DAPI (blue). Scale bars represent 50 μ m.

Accepted Article

Figure 6. Airway hyperresponsiveness (AHR) is dependent on T cell-derived IL-4/IL-13.

AHR was assessed by measuring airway resistance following methacholine challenge during OVA-induced allergic lung inflammation. A, WT (●), 4-13Tko (▲) and 4-13ko (■) mice sensitized and challenged with OVA. B, Comparison of indicated OVA-sensitized mice (●) to non-sensitized (PBS) controls (▲) which both had been challenged with OVA. Graphs show the mean+SEM with eight mice per group pooled from two independent experiments.

*= $P < 0.05$ by two-tailed Student's t-test.

Figure 1

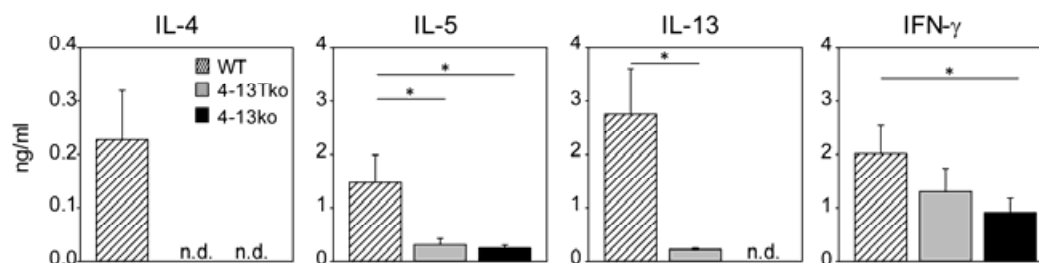


Figure 2

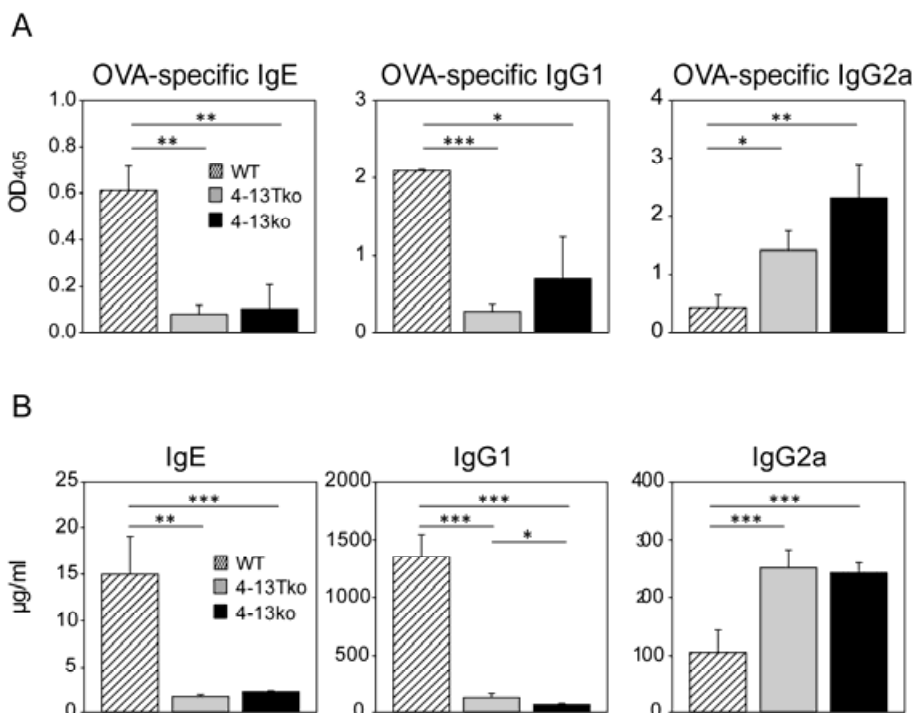


Figure 3

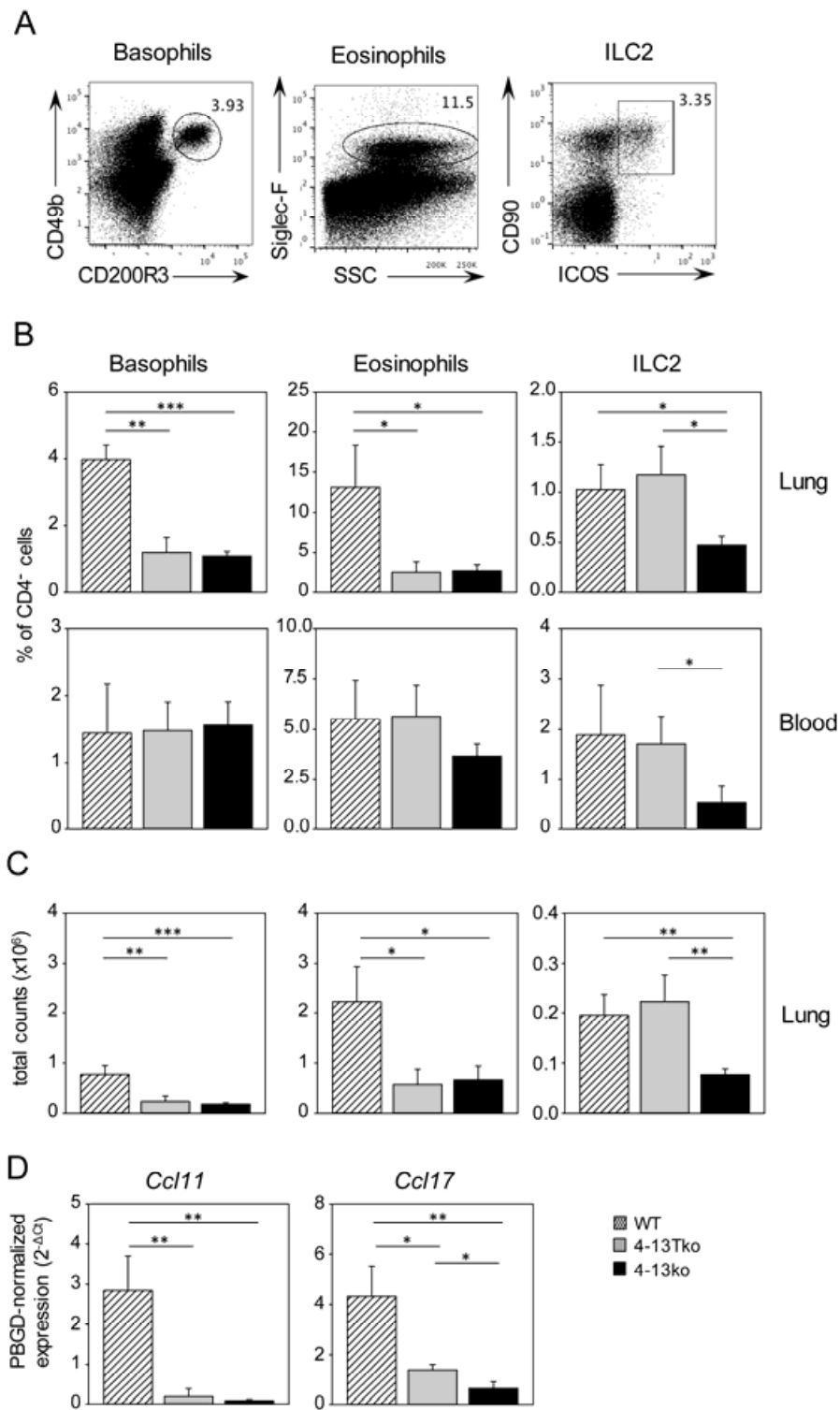


Figure 4

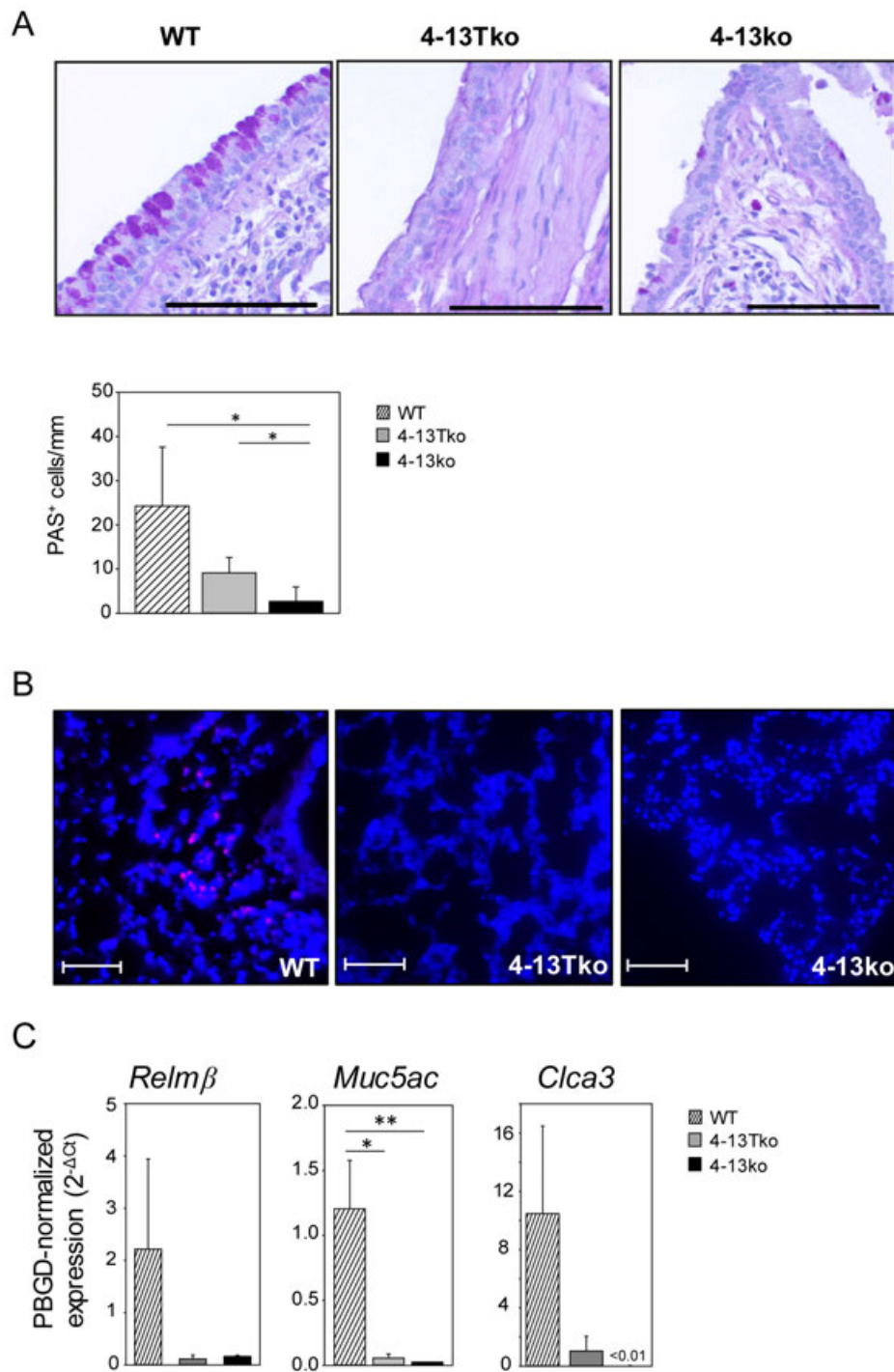


Figure 5

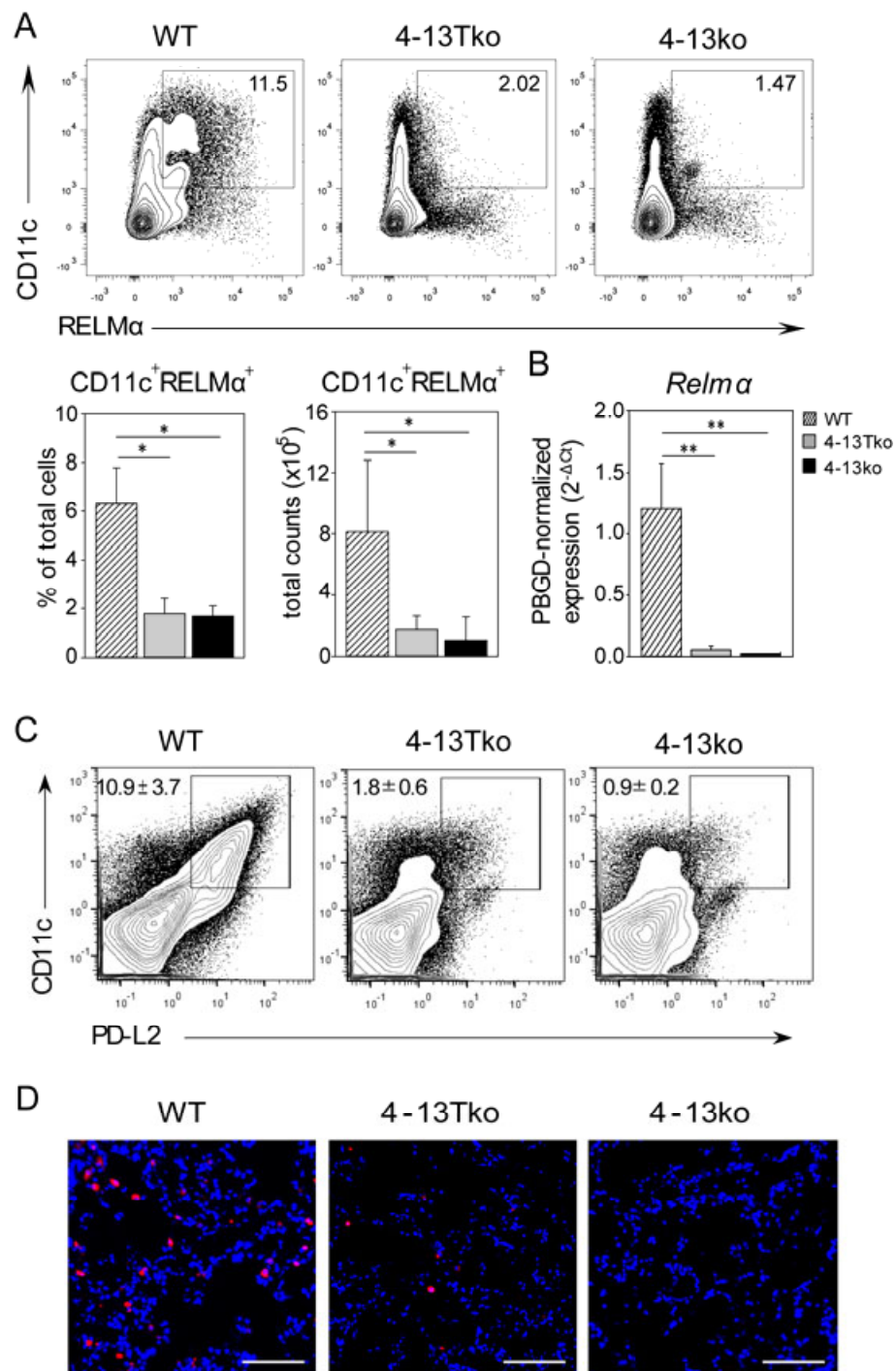
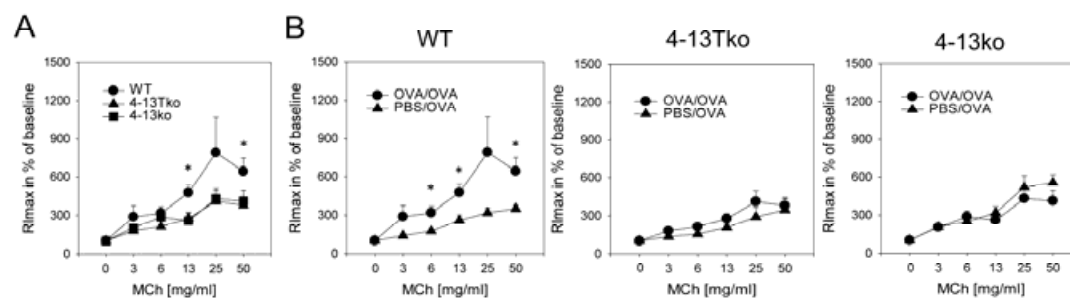


Figure 6



Supplemental Figure 1

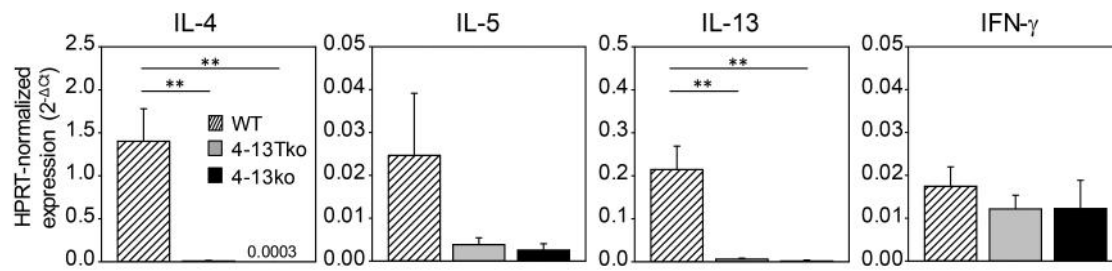


Fig. S1. Cytokine mRNA expression in mediastinal lymph nodes after OVA-induced allergic lung inflammation. WT (hatched), 4-13Tko (grey) and 4-13ko (black) mice were subjected to OVA-induced allergic lung inflammation. Cytokine mRNA levels in restimulated mediastinal lymph node samples were determined by quantitative RT-PCR on day 3 after challenge. Bars show the mean+SD of three individual mice per group and are representative of three independent experiments. **= $P < 0.01$ by two-tailed Student's t-test.

Supplemental Figure 2

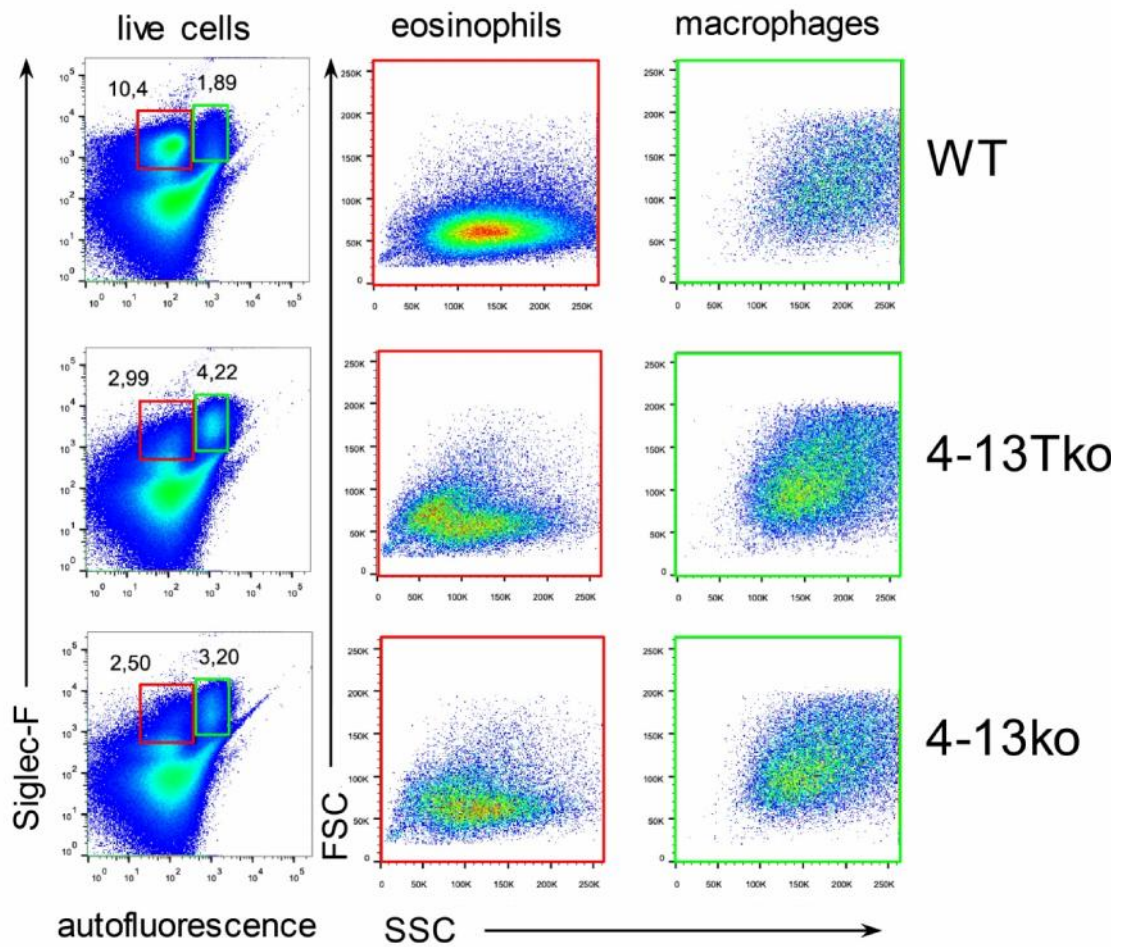


Figure S2. Eosinophils and macrophages can be distinguished by autofluorescence and FSC/SSC characteristics. Live cells of single cell suspensions from lung tissue of OVA/alum-treated mice were stained for Siglec-F and analyzed by flow cytometry. Eosinophils are Siglec-F⁺ and autofluorescence⁻ (red gate). Siglec-F⁺ macrophages are autofluorescence⁺ and appear larger (higher FSC, green gate).

Supplemental Figure 3

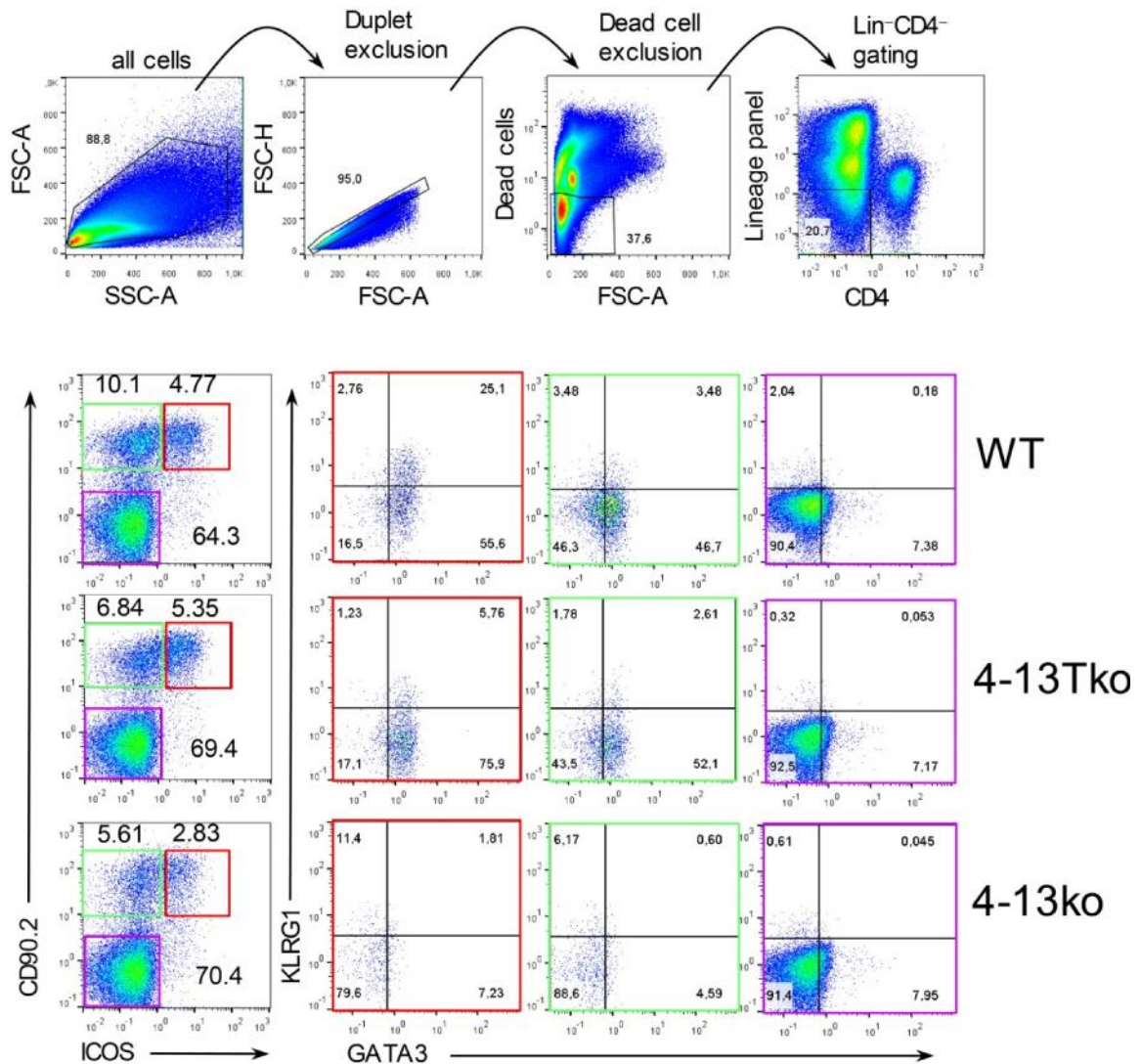


Figure S3. Gating strategy to detect ILC2s in the lung. The upper row of dot plots shows the gating of Lin-CD4⁻ cells which were then analyzed for expression of CD90, ICOS, KLRG1 and GATA3 in the lower panels of indicated mouse strains. The dot plots are representative of three mice per group.