



Interleukin-2 family cytokines IL-9 and IL-21 differentially regulate innate and adaptive type 2 immunity in asthma

Fabian Bick, MSc, Claudia M. Brenis Gómez, MD, Inés Lammens, MD, Justine Van Moorlegghem, BSc, Caroline De Wolf, BSc, Sam Dupont, BSc, Laure Dumoutier, PhD, Neal P. Smith, MSc, Alexandra-Chloé Villani, PhD, Robin Browaeys, PhD, Jehan Alladina, MD, Alexis M. Haring, BSc, Benjamin D. Medoff, MD, Josalyn L. Cho, MD, René Bigirimana, PhD, Joao Vieira, MSc, Hamida Hammad, PhD, Christophe Blanchetot, PhD, Martijn J. Schuijs, PhD, Bart N. Lambrecht, MD PhD

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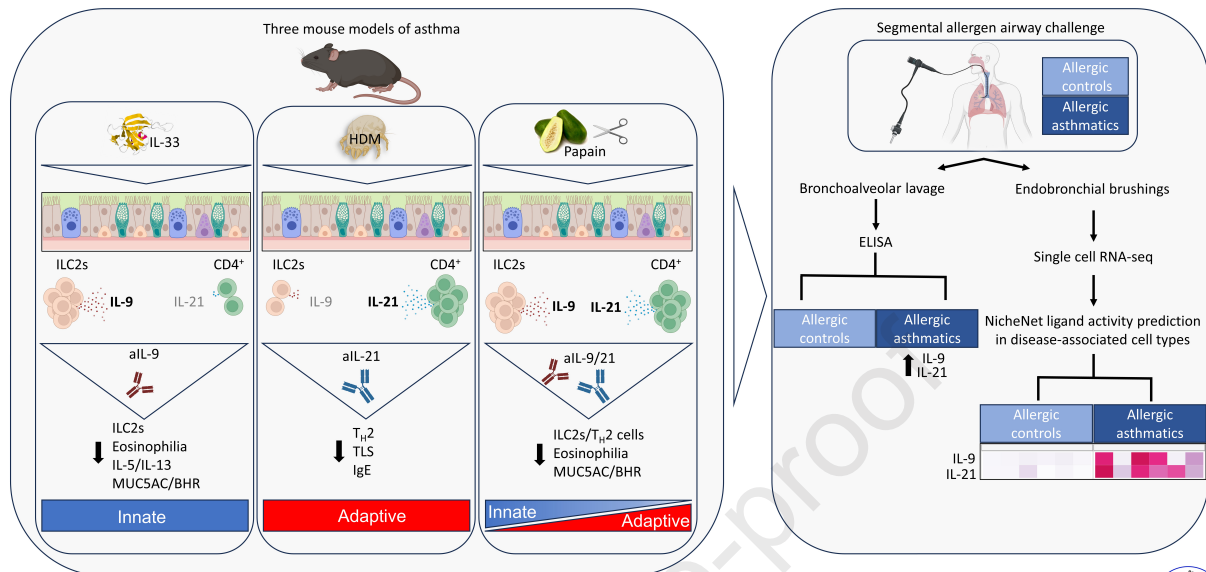
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IL-33, Interleukin-33; HDM, House dust mite; ILC2, type 2 innate lymphoid cell; T<sub>H</sub>2, type 2 helper T cell; BHR, bronchial hyperresponsiveness; TLS, tertiary lymphoid structure; IgE, Immunoglobulin E; BAL, bronchoalveolar lavage



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Fabian Bick, MSc,<sup>1,2,3,4</sup> Claudia M. Brenis Gómez, MD,<sup>2,3,4</sup> Inés Lammens, MD,<sup>2,3</sup> Justine Van Moorleghe, BSc,<sup>2,3</sup> Caroline De Wolf, BSc,<sup>2,3</sup> Sam Dupont, BSc,<sup>2,3</sup> Laure Dumoutier, PhD,<sup>5</sup> Neal P. Smith, MSc,<sup>6,7,8</sup> Alexandra-Chloé Villani, PhD,<sup>6,7,8</sup> Robin Browaeys, PhD,<sup>9,10</sup> Jehan Alladina, MD,<sup>6,11</sup> Alexis M. Haring, BSc,<sup>6,11</sup> Benjamin D. Medoff, MD,<sup>6,11</sup> Josalyn L. Cho, MD,<sup>12</sup> René Bigirimana, PhD,<sup>1</sup> Joao Vieira, MSc,<sup>1</sup> Hamida Hammad, PhD,<sup>2,3</sup> Christophe Blanchetot, PhD,<sup>1\*</sup> Martijn J. Schuijs, PhD,<sup>2,3,4†</sup> Bart N. Lambrecht, MD PhD,<sup>2,3,13\*</sup>

<sup>1</sup>argenx BV, 9052 Zwijnaarde, Belgium

<sup>2</sup>Laboratory of Immunoregulation and Mucosal Immunology, VIB-UGent Center for Inflammation Research, Ghent, Belgium

<sup>3</sup>Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium

<sup>4</sup>Cancer Research Institute Ghent, Ghent, Belgium

<sup>5</sup>de Duve Institute, Université catholique de Louvain (UCLouvain), 1200 Brussels, Belgium

<sup>6</sup>Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

<sup>7</sup>Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA

<sup>8</sup>Massachusetts General Hospital Cancer Center, Boston, MA, USA

<sup>9</sup>Bioinformatics Expertise Unit, VIB Center for Inflammation Research, Ghent, Belgium

<sup>10</sup>Department of Applied Mathematics, Computer Science and Statistics, Ghent University, Ghent, Belgium

<sup>11</sup>Division of Pulmonary and Critical Care Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA.

<sup>12</sup>Division of Pulmonary, Critical Care and Occupational Medicine, University of Iowa Carver College of Medicine; Iowa City, IA, USA

<sup>13</sup>Department of Pulmonary Medicine, Erasmus University Medical Center Rotterdam, Rotterdam, Netherlands.

\*Shared senior authors who contributed equally to this work.

† Corresponding authors.

**Corresponding authors:** Martijn J. Schuijs, PhD, Laboratory of Immunoregulation and Mucosal Immunology, VIB-UGent Center for Inflammation Research, Ghent, Belgium, TN 0032-9331-37-45. E-Mail: [Martijn.schuijs@irc.vib-ugent.be](mailto:Martijn.schuijs@irc.vib-ugent.be) and Bart N. Lambrecht, MD, PhD, Laboratory of Immunoregulation and Mucosal Immunology, VIB-UGent Center for Inflammation Research, Ghent, Belgium, TN 0032-9331-36-07. E-Mail: [Bart.Lambrecht@irc.vib-ugent.be](mailto:Bart.Lambrecht@irc.vib-ugent.be)

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## Abstract

**Background:** Asthma is often accompanied by type 2 immunity rich in IL-4, IL-5 and IL-13 cytokines produced by  $T_H2$  lymphocytes or type 2 innate lymphoid cells (ILC2s). Interleukin-2 family cytokines play a key role in the differentiation, homeostasis and effector function of innate and adaptive lymphocytes.

**Objective:** IL-9 and IL-21 boost the activation and proliferation of  $T_H2$  and ILC2s, but the relative importance and potential synergism between these  $\gamma_c$  cytokines is currently unknown.

**Methods:** Using newly generated antibodies, we inhibited IL-9 and IL-21 alone or in combination, in various murine models of asthma. In a translational approach using segmental allergen challenge, we recently described elevated IL-9 levels in human allergic asthmatics in comparison to non-asthmatic controls. Here, we also measured IL-21 in both groups.

**Results:** IL-9 played a central role in controlling innate IL-33 induced lung inflammation by promoting proliferation and activation of ILC2s, in an IL-21 independent manner. Conversely, chronic house dust mite induced airway inflammation, mainly driven by adaptive immunity, was solely dependent on IL-21, that controlled  $T_H2$  activation, eosinophilia, total serum IgE and formation of tertiary lymphoid structures. In a model of innate on adaptive immunity driven by papain allergen, a clear synergy was found between both pathways, since combined anti-IL-9 or anti-IL-21 blockade was superior in reducing key asthma features. In human bronchoalveolar lavage (BAL) samples we measured elevated IL-21 protein within the allergic asthmatic group, compared with the allergic control group. We also found increased *IL21R* transcripts and predicted IL-21 ligand activity in various disease-associated cell subsets.

**Conclusion:** IL-9 and IL-21 play important and non-redundant roles in allergic asthma by boosting ILC2s and  $T_H2$  cells, revealing a dual IL-9 and IL-21 targeting strategy as a new and testable approach.

## Clinical implications

IL-9 and IL-21 are master regulators of allergic asthma, by boosting  $T_H2$  and ILC2s. Our data indicate that dual blockade may be a promising avenue for the treatment of asthma.

## Capsule summary

IL-9 and IL-21 are master regulators of innate and adaptive type 2 immunity, making dual blockade a novel and promising approach in asthma.

## Key words

asthma, monoclonal antibodies, innate, adaptive, type 2 immunity, IL-9, IL-21

## Abbreviations

BAL: Bronchoalveolar lavage; BHR: Bronchial hyperresponsiveness; CH1, constant heavy-chain domain1; CL, constant light-chain domain; CSR, class switch recombination; ECM, extracellular



matrix; GCM: Goblet cell metaplasia; HDM, house dust mite; IgE, Immunoglobulin E; IL-,  
Interleukin-; ILC2s, type 2 innate lymphoid cells; IMs, interstitial macrophages; MNPs,  
mononuclear phagocytes; Muc5ac, mucin 5ac; PBL, peripheral blood lymphocytes;  
PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; pT<sub>H</sub>2, pathogenic type 2 helper T cell;  
SAC, segmental allergen challenge; Spdef, SAM pointed domain-containing Ets transcription  
factor; SPR, surface plasmon resonance; STAT, signal transducer and activator of transcription;  
T1/ST2, IL-33R, Interleukin 1 receptor-like 1; T<sub>FH</sub>, follicular helper T cell; TGF $\beta$ 1, transforming  
growth factor beta 1; T<sub>H</sub>2, type 2 helper T cell; TLS, tertiary lymphoid structure; Treg, regulatory  
T cell; TSLP, thymic stromal lymphopoietin; VH, variable heavy-chain domain; VL, variable light-  
chain domain

## Introduction

Asthma is a chronic inflammatory disease of the airways(1) affecting close to 300 million people(2). In around 4% of patients suffering from severe refractory asthma, the disease cannot be adequately controlled with standard of care treatments, and increasingly treatment with biologic therapy is required(3). Even though many patients respond well to these state-of-the-art therapies directed against type 2 cytokines like IL-4, IL-13, IL-5, or TSLP, a considerable number of patients still have persistent symptoms, recurrent exacerbations, and lung function impairment(4). To address this unmet clinical need, development of novel biological therapies targeting other cytokines could be a valuable option.

IL-2 family cytokines like IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 play a fundamental role in the life cycle of immunocytes, ranging from early differentiation from precursors to homeostatic control of peripheral pools and to effector function(5). They all signal via a common signaling chain, the gamma common chain or  $\gamma_c$ , that activates STAT signaling intermediates and combines with a unique cytokine specific receptor alpha subunit, that relays some of the specificity of signaling(5, 6). IL-9 is a pleiotropic cytokine(6), predominantly produced by CD4<sup>+</sup> helper T cells (T<sub>H</sub>) and by type 2 innate lymphoid cells (ILC2s), but to some extent also by mast cells(7). Stimulation of naïve CD4<sup>+</sup> T cells in the presence of IL-4 and TGF $\beta$ 1 *in vitro* leads to CD4<sup>+</sup> T cells producing IL-9 in the absence of other T<sub>H</sub>2 or T<sub>H</sub>1 cytokines, which led to the concept of T<sub>H</sub>9 cells as being a unique subset of T<sub>H</sub> cells(8-10). This idea has however been challenged since inception, because a unique lineage defining transcription factor has never been found, therefore T<sub>H</sub>9 cells might represent a subset of activated T<sub>H</sub>2 cells(11). In the context of asthma, IL-9 is particularly interesting, since increased levels of this cytokine were found following segmental allergen challenge to the lungs of asthmatics, and IL-9 genetic polymorphisms and serum levels track with bronchial hyperresponsiveness (BHR) in patients with severe airway inflammation(12-17).

How exactly IL-9 boosts asthma is currently still unclear. Airway overexpression of IL-9 in mice leads to mast cell expansion, mucus overproduction and goblet cell metaplasia, and to airway eosinophilia, and these features are absent when IL-9 or its receptor are inactivated(18-22). Initially, these effects were thought to result from the direct action of IL-9 on target cells expressing IL-9R, but it was later found that alterations in the epithelium were indirect and mediated via IL-13(23-25). In the lung, IL-9 can also activate interstitial macrophages (IMs) and epithelial cells to produce chemokines that recruit eosinophils and T<sub>H</sub>2 cells to the airways, although it is hard to detect IL-9R and *Il9r* mRNA expression on lung IMs, therefore these effects might be indirect(26, 27). The main direct biological effects of IL-9 in asthma *in vivo* are thought to occur via autocrine effects on innate and adaptive type 2 lymphocytes that produce IL-9 and react to it by increasing glycolysis and subsequent cytokine production in a PPAR $\gamma$ -dependent manner in humans and mice(28-31). Indeed, tissue resident ILC2s are a major source of IL-9, express the IL-9R and autocrine IL-9 leads to the expansion and production of IL-5 and IL-13 by these cells(32-35). Likewise, when pathogenic (p)T<sub>H</sub>2 cells are repeatedly stimulated with antigen, they start producing IL-9 which is required for expansion and activation of the resident memory T<sub>H</sub>2 cells in the lungs and recall responses to inhaled allergens, leading to airway eosinophilia, mucus production and BHR(36, 37).

Another poorly studied IL-2 family cytokine in asthma is IL-21(38). Numerous studies have implicated a role for IL-21 in driving type 2 immunity, predominantly by inducing alternative activation of macrophages(39-41), and suppression of allergen-specific regulatory T cells (Tregs)(42, 43), making IL-21 another pleiotropic  $\gamma_c$  cytokine acting on multiple cell types involved

139 in asthma. Although IL-21 is abundantly produced by T<sub>FH</sub> cells in the B cell area, a previous study  
140 from our lab showed in a house dust mite (HDM) driven asthma model, that non-T<sub>H</sub>2 resident  
141 helper CD4<sup>+</sup> T cells produced copious amounts of IL-21 in the lung(44). The paracrine release  
142 of IL-21 by these helper T cells led to upregulation of the IL-33 receptor T1/ST2 on effector pT<sub>H</sub>2  
143 cells(45) and ILC2s, leading to enhanced production of IL-5 and IL-13.

144 These studies revealed that two closely related IL-2 family cytokines stimulate effector functions  
145 of innate and adaptive type 2 lymphocytes. It is currently unknown if these type 2 immunity  
146 promoting pathways act in a redundant or synergistic manner, and we tested this in various  
147 models of asthma and validated key findings in a segmental allergen challenge model in human  
148 asthmatics.

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## Methods

### Llama immunization and Fab library construction

Two llamas housed outdoors according to French animal welfare legislation, were immunized intramuscularly weekly over six weeks with recombinant mouse IL-9 (R&D Systems). The proteins were administered mixed with incomplete Freund adjuvant (Sigma) with a dose of 100ug for the first two weeks and 50ug for the last four weeks. For antibody discovery the proprietary SIMPLE Antibody™ platform from argenx (Zwijnaarde, Belgium) was used as previously described(46). Briefly, five days after the last injection PBLs were isolated from the llamas blood and were used for total RNA isolation. Total RNA was used for random primed reverse transcription into cDNA which was subsequently used with specific primers to amplify genes encoding for the llama VH-CH1 and VL-CL domains (kappa and lambda) regions. Afterwards, amplified DNA was cloned into a proprietary phagemid vector.

### Phage selection for anti-mouse IL-9 Fab generation

Electrocompetent *Escherichia coli* TG1 cells (Lucigen) were transformed with the generated phagemid to create one kappa and one lambda Fab-expressing phage library per immunized llama. Briefly, a titration of recombinant mouse IL-9 was coated on Maxisorp plates and exposed to the produced phages. Three rounds of panning were performed to enrich for mouse IL-9 specific binders. Fab expression was induced using IPTG and Fab-containing periplasmic fractions of the bacteria were used for screening by surface plasmon resonance (SPR) using a Biacore 3000 device (GE Healthcare).

### mAb production, purification and characterization

GeneArt DNA fragments (Thermo Scientific) encoding the VH and VL sequences of the respective neutralizing antibodies were cloned into separate expression vectors encoding either constant heavy chain or light chain regions, respectively (ImmunoPrecise Antibodies). The heavy chain vectors comprised either human IgG1 or murine IgG1 CH1-CH2-CH3 domains, both including the N297A mutation that abrogates binding to all Fc-gamma receptors. Light chain vectors comprised either kappa or lambda constant regions. Antibody production was done by transient co-transfection of HEK293 cells using the corresponding heavy and light chain vector pairs. Six days post transfection, antibodies were purified endotoxin-free by affinity chromatography using MabSelect SuRe LX protein A resin (Cytiva) and size exclusion chromatography (SEC) to confirm purity and monomericity.

### IL-9 bioassay

Fab clones showing the lowest dissociation constants on SPR were produced in full IgG format before being screened in vitro for cytokine neutralization as previously described(47).

### IL-21 bioassay

IL-21 sensitive human Mino cells (lymphoblasts, ATCC) were cultured at 37°C with 5% (vol/vol) CO<sub>2</sub> in RPMI 1640 growth medium (Gibco) containing 10% (vol/vol) heat-inactivated FCS (Sigma), 1x Pen-strep and 1x GlutaMAX (Gibco). Briefly, growth medium containing 1x10<sup>5</sup> cells was added per well containing a fixed concentration of mouse IL-21 and a titration of the respective antibody. Cells were incubated for 2 days before adding fresh growth medium containing 1x alamarBlue cell viability reagent (Invitrogen). After two more days of incubation, absorbance<sub>570-600</sub> was measured using a spectrophotometer.

### Production of anti-mouse IL-21 neutralizing antibody

VH and VL sequences of the anti-mouse IL-21 surrogate antibody described in patent US 2017/0043014 A1 were cloned into the expression vectors mentioned above and used for antibody production.

### Mouse models

IL9<sup>-/-</sup> mice were a kind gift from Laure Dumoutier, de Duve institute Brussels. 6-8 week old female C57BL/6J wild-type mice were ordered from Janvier (France). On day of sensitization or challenges, mice were lightly anesthetized using 4% isoflurane (vol/vol in air). For the innate IL-33 model, mice were challenged with 150ng of murine IL-33 (BioLegend) intratracheally for three consecutive days. 200µg of the respective

antibodies were injected 4 hours prior to the challenges. On day 4, mice were sacrificed. For the acute adaptive model (papain), mice were sensitized intranasally on day 0 and 1 with 10µg of papain extract (Merck) and were challenged intranasally on day 14 and day 21. 100µg per antibody were injected on days 14, 16, 18 and 21. On day 22, mice were sacrificed. In the chronic model (HDM), mice were sensitized with 1µg of HDM on day 0 and challenged with 10µg of HDM (ALK) on days 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31, 34, 36 and 38. 100µg of mAbs were injected on days 6, 10, 13, 17, 20, 24, 27, 31, 36 and 38. Mice were sacrificed on day 42. Unless otherwise stated, mAb treatments were administered intraperitoneally and mice were sacrificed with an overdose of pentobarbital.

### Collection and analysis of sera, bronchoalveolar lavage fluid and lungs

Mice were terminally bled by cutting one of the inguinal veins and blood was collected, centrifuged and serum was stored at -20°C for further analysis. Bronchoalveolar lavage (BAL) was performed three times with EDTA-containing ice-cold PBS into the cannulated trachea with a total volume of 1ml. Cells situated in the BAL were pelleted by centrifugation into a staining plate and the supernatant was stored for further analysis by ELISA to determine concentrations of inflammatory cytokines. To quantify levels of IL-9 and IL-21 protein in the BAL samples, original matching capture and detection antibody clones discovered by phage display were cloned, purified and used for ELISA purposes. For quantification of all other cytokines, the used ELISA kits are mentioned in the supplementary material. All lung lobes were isolated. The superior and accessory lobe were snap frozen in liquid nitrogen for the purpose of lung homogenate generation and qPCR analysis, respectively. The left lobe was collected for histology. The remaining lung lobes were digested using DNase type 1 (Sigma) and collagenase type 1 (Gibco). Digested lungs were filtered through a 70µm cell strainer and underwent red blood cell lysis before being transferred onto a staining plate for further analysis by flow cytometry. Serum was collected by tail vein puncture and total IgE levels were measured using ELISA (BD Biosciences).

### Histology and confocal microscopy

Left lung lobes were fixed in 4% PFA overnight at room temperature and afterwards embedded in paraffin. For confocal microscopy, tissue sections were de-paraffinized and antigen retrieval was performed for 2 hours in citrate buffer in a benchtop oven. After permeabilization using 0.01% triton, tissue sections were blocked with 2% BSA before being stained with the respective antibodies against CD3, B220 and GL7 (Suppl. material).

### RNA extraction, cDNA generation and qPCR

Accessory lung lobes were collected during dissection and immediately snap frozen in liquid nitrogen and stored at -80°C upon further processing. Later, lobes were thawed and homogenized using a TissueLyser (Qiagen) and RNA was isolated using the TriPure Isolation Reagent (Roche) and reverse transcribed into cDNA using the SensiFAST cDNA Synthesis kit (Bioline). qPCR was performed using SYBR green using a LightCycler 480 device (Roche). qPCR analysis was performed using qBase+ (Biogazelle).

### FlexiVent experiments

Measurement of mouse lung function was done using the flexiVent system (SCIREQ), as previously described(48).

### Statistical analysis

If not stated elsewhere, data are represented as means ± SEM. Statistical significance between groups was calculated with ordinary one-way ANOVA with Sidak's multiple comparisons test using GraphPad Prism software(v9.4.0; GraphPad Software, La Jolla, Calif). Differences between groups were considered significant at P values of .05 or less, .01 or less, .001 or less, and .0001 or less versus the group of mice challenged with either IL-33, HDM or papain and treated with an irrelevant mIgG1 N297A antibody.

### Human cytokine quantification

Cytokines were analyzed by R&D Systems ELISA (IL-21) using 30-fold concentrated BAL supernatant. BAL was concentrated in Amicon Ultra-15 centrifugal filter units (EMD Millipore, catalog no. UFC9100) according

to the manufacturer's instructions. Cytokines were measured on a Luminex 200 and analyzed using xPONENT v3.1 (Luminex Corp).

### Bioinformatic analysis

The Alladina and Smith *et al.* single cell dataset was downloaded from the GEO accession website (GSE193816). It was converted from H5AD file format to a Seurat object using the SeuratDisk package. Seurat package (v4.4.0) was used for further analysis. DE genes were calculated using the FindMarkers and FindAllMarkers function. Dotplots and featureplots were made using the built-in functions in the Seurat package. NicheNet ligand activity and ligand-target predictions were performed with the multinichenetr package (v1.0.3) as described in the "detailed\_analysis\_steps\_empirical\_pvalues" tutorial(49). The analysis was conducted with default parameters, except for min\_cells = 5, with AA.Ag-(AA.Ctrl+AC.Ag+AC.Ctrl)/3 as contrast definition for the underlying DE analysis. This Ctrl group was created by pooling both Dil and Bln samples to maintain more cells per cell type/sample combination.

### Human ethics

This study was a nonrandomized, nonblinded mechanistic study with the primary aim of comparing the airway mucosal response to allergen challenge in AAs and ACs. Volunteers were screened for eligibility with a full medical history, baseline spirometry and methacholine challenge, and allergen skin testing. Detailed inclusion and exclusion criteria has been previously published (Sci Immunol. 2023 May 12; 8(83): eabq6352). Briefly, male and female participants between 18 and 50 years old with a clinical history of allergic rhinitis and/or conjunctivitis to HDM and/or cat hair and a positive skin prick test to the same allergen were enrolled. AAs additionally had a clinical history of mild asthma, baseline FEV1  $\geq$  75% of the predicted value, and positive methacholine challenge testing defined as a provocative concentration causing a 20% drop in FEV1 (PC20)  $<16$  mg/ml. Participants could not be on systemic steroids for 6 weeks before the study, and inhaled corticosteroids were held for 2 weeks before the study procedure. All participants provided written informed consent before testing and sample collection. The study protocol was approved by the Massachusetts General Brigham Institutional Review Board (IRB# 2007P001050) and registered at clinicaltrials.gov (NCT00595491).

### Animal ethics

All mouse experiments were approved by the local ethics committee of the VIB-UGent Center for Inflammation Research Center (EC2021-046 and EC2024-014) and were in accordance with Belgian animal protection law.



## Results

### Generation of potent neutralizing antibodies against murine IL-9 and IL-21

Using our proprietary antibody discovery platform that is based on active immunization of llamas(50), we generated a panel of antibodies against murine IL-9. After immunization, PBLs were isolated from two llamas for total RNA extraction followed by reverse transcription into cDNA. Genes encoding for the llama VH-CH1 regions and VL-CL domains ( $\kappa$  and  $\lambda$ ) were amplified by PCR using specific primers and afterwards cloned into suitable phagemid vectors for immune library construction. After phage production and several rounds of panning, we discovered a potent neutralizing antibody targeting murine IL-9 (Fig 1A.). We also made a neutralizing IL-21 antibody using variable domain sequences from public data(51) and confirmed function (Fig 1B). We reformatted both monoclonals into the aglycosylated mouse IgG1-N297A backbone, allowing us to study the consequence of IL-9 and or IL-21 inhibition in the absence of Fc-mediated effector functions (Fig 1C). Both antibodies bound to their respective target with nanomolar affinity as determined by 1:1 binding kinetics using surface plasmon resonance (SPR), resulting in picomolar neutralization potencies in cell-based bioassays either sensitive for mouse IL-9 or mouse IL-21 (Fig 1, D-E, Suppl. Fig 1A).

### IL-9 blockade ameliorates IL-33 induced airway inflammation by suppressing ILC2s

To study the role of IL-9 and IL-21 in type 2 airway inflammation, we repeatedly administered the epithelial alarmin IL-33 to induce innate eosinophilic airway inflammation(52) (Fig 2A). Mice were treated with the IL-9- and IL-21mAbs either as monotherapy or in combination, and a group of mice also received irrelevant isotype control. First, we measured IL-9 and IL-21 levels in the bronchoalveolar lavage (BAL) of IL-33 challenged and isotype control treated mice to confirm induction of the cytokines of interest within this model. Following IL-33 challenge, both IL-9 and IL-21 were elevated significantly in comparison to PBS treated mice with an average increase of 23-fold and 2.5-fold, respectively (Fig 2B). Flow cytometric analysis of cells in the BAL demonstrated a strong influx of eosinophils, neutrophils, T1/ST2<sup>+</sup> T<sub>H</sub>2 lymphocytes, and Sirp $\alpha$ <sup>+</sup> cDC2s in the IL-33 treated group, compared with the PBS treated group (Fig 2C, Suppl. Fig 1B). A similar effect was observed in single lung cell suspensions (Fig 2D, Suppl. Fig 2 and 4). This IL-33 induced response was significantly inhibited by the IL-9mAb, whereas IL-21 blockade was without effect and the combination overall behaved similar to the IL-9mAb monotherapy (Fig 2C-D). In support of the reduced immunocyte influx, BAL fluid concentration of IL-5, IL-6, IL-13 and the eosinophil chemoattractants CCL11 and CCL24, were all blunted by IL-9mAb, but not by IL21mAb (Fig 2E). Recently, arginase expressing IMs were shown to mediate IL-9's proallergic effects(26), and we observed an IL-33 induced increase of these CD163<sup>+</sup> Arg1<sup>+</sup> IMs, that were reduced upon IL-9 blockade (Fig 2F).

Given the short time frame of IL-33 administration, and lack of specific antigen, it is generally assumed that this model depends on the activation of innate ILC2s, that express the IL-33 receptor T1/ST2(52). In support, we observed an increase in ILC2s in BAL fluid and lungs of mice receiving IL-33 (Fig 2C-D). Since IL-9 can act as an autocrine growth and activation cytokine for ILC2s, induced by many stimuli, and IL-21 also affects the activation of ILC2s(44) we measured the proliferation and activation state of ILC2s by staining for Ki-67 and IL-13. The IL-33 induced proliferation and IL-13 protein expression of ILC2s were significantly reduced in the IL-9mAb and combined IL9/21mAb treated group, whereas IL-21mAb was without effect (Fig 3A). Using Red5 mice, that report for IL-5 expression through tdTomato production(53), we found that the majority of IL-5 positive cells were indeed ILC2s (Fig 3B, Suppl. Fig 3), and that IL-33 administration



increased *Il5* promoter activity in ILC2s, an effect that was blunted in IL-9mAb treated mice, concomitant with reduced eosinophilia (Fig 3B). We subsequently compared the potency of IL-9mAb with genetic deficiency of the entire receptor in *Il9r<sup>-/-</sup>* mice. In *Il9r<sup>-/-</sup>* mice, IL-33 failed to induce as much proliferation and IL-13 cytokine production in ILC2s as in wild type mice, and the magnitude of this reduction was as strong as in IL-9mAb treated mice. Thus, the IL-9 antibody achieved full blockade of this pathway and remaining IL-33 induced inflammatory effects act independently of the IL-9 axis (Fig 3C). We also assessed the tissue alterations induced by IL-33 and found that peribronchial and perivascular inflammatory lesions were reduced in IL-9mAb and *Il9r<sup>-/-</sup>* mice (Fig 3D). Goblet cell metaplasia (GCM) and mucus overproduction are important aspects of type 2 immunity, and to assess this quantitatively, we measured lung mRNA transcripts of the hallmark genes *Muc5ac* and *Spdef*, known to regulate GCM(54, 55). The relative expression of *Muc5ac* was significantly reduced in mice receiving IL-9mAb, whereas *Spdef* was also reduced by IL-21mAb (Fig 3E). Another important hallmark of asthma is BHR, characterized by the sensitivity of the airways to react to bronchoconstrictor agents such as methacholine. We observed increased airway resistance in IL-33 challenged mice treated with the isotype control antibody in comparison to PBS treated mice and this effect was significantly reduced in mice treated with IL-9mAb (Fig 3F). Collectively, these data suggest that the alarmin IL-33 induces type 2 immunity via IL-9 and activation of ILC2s and that IL-21 is dispensable in this setting.

### **IL-21 blockade ameliorates chronic HDM-induced airway inflammation by reducing adaptive immunity**

Because IL-33 is a prototype of innate immune system driven type 2 immunity, we next wanted to test a model of 5 week chronic allergen exposure driven by the relevant inhaled antigen HDM (Fig 4A), that leads to key features of asthma, accompanied by high levels of allergen-specific IgE and formation of tertiary lymphoid structures (TLS) rich in T cells and B cells, and therefore much more reflects an ongoing adaptive immune response. We measured IL-9 and IL-21 levels in the BAL of HDM sensitized and challenged mice from the isotype control group. In comparison to PBS sensitized and challenged mice, both IL-9 and IL-21 were significantly elevated with an average increase of 22-fold and 68-fold, respectively (Fig 4B). In this model, serum IgE levels peaked after three weeks and remained on that level until week 6, when all animals were sacrificed for analysis (Fig 4C). At week 6, mice receiving HDM showed significant increases in total cells, eosinophils, neutrophils, cDCs, T<sub>H</sub>2 cells, B cells and ILC2s in the BAL fluid and lung cells, accompanied by an increase in serum IgE, compared with mice receiving PBS (Fig 4C-F). It is known from *Il21r<sup>-/-</sup>* mice that IL-21 negatively regulates IgE class switch recombination (CSR) leading to increased levels of IgE(56). Interestingly however, groups receiving IL-21mAb showed significantly less serum IgE at week 6. We also looked at HDM specific IgE and IgG1 levels at week 6 and these followed the same pattern (Fig 4C-D). This was accompanied by reduced numbers of immunocytes recovered from the BAL and lungs, whereas the IL-9mAb alone did not have any effect and the combination overall behaved similar to the IL-21mAb monotherapy (Fig 4E-F). We could not confirm any changes within the arginase-expressing IMs in any of the treatment groups (Fig 4G), even though macrophages expressed IL-21R (data not shown). However, IL-21mAb-treated mice displayed significantly lower percentages and absolute numbers of pathogenic T1/ST2 positive GATA3<sup>+</sup> pT<sub>H</sub>2 cells, known to be key regulators of the HDM asthma model (Fig 4H, Suppl. Fig 5). Remarkably, whereas ILC2s were clearly expanded in this model, neither IL-9 nor IL-21 blockade was able to reduce ILC2s in the lungs, whereas in the BAL fluid, only IL-21 blockade reduced ILC2 numbers, an effect we previously also noticed when we studied *Il21r<sup>-/-</sup>* mice in a shorter HDM model(44).

In contrast to the IL-33 innate model, *Muc5ac* and *Spdef* mRNA transcripts were not induced in comparison to PBS treated mice (Suppl. Fig 6A), suggesting that this model was not accompanied by GCM at 6 weeks, a finding also supported by PAS staining (Suppl. Fig 6B). In accordance, we could not confirm BHR in HDM sensitized and challenged mice after methacholine challenge (data not shown). Instead, we observed highly organized cell infiltrates on the H&E stained tissue sections, surrounding the blood vessels and airways in the HDM exposed mice treated with the control antibody (Fig 4I). Based on the appearance and location of these infiltrates, we hypothesized that they were TLS rich in T cells and B cells, known to be induced upon chronic antigen stimulation(57). Using fluorescence microscopy, we confirmed that the structures were indeed TLS, containing T cells and B cells expressing the germinal center (GC) marker GL7 (Fig 4I). We quantified the relative occupied surface area per tissue section and also quantified the amount of contained cells within the TLS using the H&E stained slides. Mice receiving the IL-21mAb had less and smaller TLS in comparison to the isotype control treated group (Fig 4J), and this was not the case in the IL-9mAb treated group. Collectively, these results define a key role for IL-21 in maintaining a chronic adaptive type 2 response in a model of HDM-driven allergic airway inflammation, whereas IL-9 blockade is ineffective.

### **Synergistic IL-9 and IL-21 inhibition ameliorates papain-induced airway inflammation**

Since acute IL-33 induced innate inflammation required IL-9 but not IL-21, and chronic adaptive immune system activation with the HDM extract required IL-21 but not IL-9, we decided to test a model of mixed innate on adaptive inflammation and made use of a three week exposure model using the enzymatic allergen papain(58) (Fig 5A). Whereas papain clearly elicits adaptive CD4<sup>+</sup> T cells responses, with each administration to the airways, the enzymatic activity leads to IL-33 release and innate immune cell activation(59-61). Cytokine levels in BAL supernatant were measured by ELISA but were not detectable, most likely due to the proteolytic activity of the papain itself(62). Increased total serum IgE confirmed presence of a type 2 skewed immune response, peaking in week three (Fig 5B). We also assessed papain-specific IgE and IgG1 responses at week three, but did not observe significant differences after any of the treatments (Fig 5C).

Compared with PBS, papain administration led to influx of total cells, neutrophils, eosinophils, cDC2s, T<sub>H</sub>2 cells, B cells and ILC2s into the BAL and lungs (Fig 5D-E), and IL-9mAb monotherapy reduced eosinophilia, neutrophils and DCs in the BAL, while IL-21mAb monotherapy only affected eosinophil numbers significantly. On the other hand, total live cell numbers and neutrophil numbers were only reduced in the combined treatment group and, throughout the lowest levels of eosinophils, T cells, B cells and DCs were observed in the combined treatment group as well, indicating that IL-9 and IL-21 synergistically control key inflammatory events (Fig 5D-E). The same pattern was confirmed in the lungs (Fig 5E). Moreover, arginase expressing interstitial macrophages were not affected by either treatment (Fig 5F). We next looked at the relative impact on innate ILC2s or adaptive T<sub>H</sub>2 cells. T<sub>H</sub>2 numbers were approximately 2-fold higher than ILC2 numbers, suggesting that T<sub>H</sub>2 cells may be the main drivers in this model. However, ILC2s are known to be crucial for the induction of the adaptive immune response in the context of papain(58). Given that they were still present in high numbers even after three weeks, this model likely equally relies on ILC2s and T<sub>H</sub>2 cells. In contrast to the IL-33 model and HDM model, ILC2 numbers in the lung were only significantly reduced in the combined treatment group and not by monotherapy. We observed high numbers of T cells in the lung after papain administration and quantified the amount of T1/ST2<sup>+</sup>, GATA-3<sup>+</sup> pT<sub>H</sub>2 cells. While both monotherapies failed to reduce the frequency and absolute numbers of pT<sub>H</sub>2 cells, the combination significantly inhibited pT<sub>H</sub>2

cell accumulation (Fig 5G), suggesting a possible synergistic effect when targeting both cytokines simultaneously.

Furthermore, papain induced histological changes around the blood vessels and airways of mice (Fig 5H) that were reduced only with combined cytokine blockade, and the relative expression of *Muc5ac* and *Spdef* transcripts was significantly reduced in mice receiving combination therapy, showing that combination therapy was able to reduce GCM (Fig 5I). We also observed increased airway resistance in papain sensitized and challenged mice treated with the isotype control antibody in comparison to PBS treated mice. While the monotherapies showed trends towards reduction of BHR, only the combination of both protected the mice from BHR by significantly reducing airway resistance (Fig 5J), confirming a synergistic role for both IL-9 and IL-21 in this innate on adaptive type 2 asthma model.

### **Levels of IL-21 protein and its receptor transcript are increased in human allergic asthmatics, predicting ligand activity in multiple disease-associated cell subsets**

To translate our murine findings to human asthma, we opted for re-analysis of a dataset that we recently published and that made use of segmental allergen challenge (SAC) with specific allergen in allergic controls without asthma and allergic asthmatics(16). We found that the lung environment of allergic asthmatics after allergen challenge is altered by amplified type 2 signaling, extracellular matrix (ECM) remodeling and GCM, while allergic controls responded with upregulation of genes associated with antioxidant response, tissue repair and phagolysosomal clearance to resolve inflammation. The cellular composition of allergic asthmatics consisted of IL-9 expressing  $T_H2$  cells interacting with DC2s (CD1C) and immature inflammatory monocytes (CCR2) that failed to develop into pro-resolving macrophages(16). We also found that SAC induced increased IL-9 protein specifically in allergic asthmatics(16).

We extended this scRNA-seq analysis and looked for *IL21* and *IL21R* transcripts, among B cells, T cells, epithelial cells, mononuclear phagocytes (MNPs), mast cells and NK cells (Fig 6A, Suppl. Fig 7). In line with the literature, IL-21R expression was confirmed in the MNP and lymphocyte subsets, whereas it was absent in mast cells and epithelial cells. Splitting the data on group level revealed increased IL-21R expression amongst the allergic asthmatics (AA, 6.2%) in comparison to the allergic controls (AC, 3.1%) (Fig 6B). It was hard to detect *IL21* transcript within the dataset (expressed only in 0.15% of T cells). However, in a series of new samples taken before and after SAC, we measured IL-21 protein concentration in BAL fluid by ELISA. At baseline, AA patients already had higher IL-21 than AC patients, and with SAC this difference became even more prominent (Fig 6C). Within the AA patients, there was a trend for SAC to induce higher IL-21, but this did not reach statistical significance. To understand which cellular subsets respond to IL-21 within AC and AA, we looked at frequencies of *IL21R* transcript on cell subset level for T cells and MNPs (Fig 6D). For the AA we observed elevated *IL21R* transcript predominantly in the CD4 T (*CD40LG*), CD4 Th1FNR (*ISG15*), Treg and Tgd (*TRDC*) subsets, while AC showed higher frequencies within the CD8 T (*EGR2*) compartment, suggesting ongoing dysregulated IL-21 signaling between the AC and AA groups. We observed similar results within the MNP compartment; IL-21R expression was increased within the AA in nearly all subsets including predominantly the DC1 (*CLEC9A*), DC2 (*CD1C*), MC1 (*CXCL10*) and MC4 (*CCR2*) subsets, while AC showed slightly increased expression in migratory DCs (migDC, *CCR7*) and plasmacytoid DCs (pDC; *TCF4*) (Fig 6D).

We leveraged the NicheNet cellular interaction prediction algorithm(63) to understand the consequence of IL-9 and IL-21 signaling on these cells. We aimed to predict ligands potentially

driving differential expression in AA after allergen challenge compared to AA before allergen challenge and AC before and after allergen challenge. Overall, we observed upregulatory ligand activity for both IL-9 and IL-21 within the T cell compartments, whereas IL-21 additionally showed ligand activity in B cells and several MNP subsets (Fig 6E). IL-21 was amongst the predicted top-ligands on cell subset level, responsible for upregulation of target genes in the CD8 T cell subset (*CLIC3*) in the AA (Suppl. Fig 8A). To focus our analysis, we looked in particular at the upregulated genes induced by IL-9 and IL-21 specifically in the defined disease associated cell subsets, including CD4 T cells (*CD40LG*), Treg (*FOXP3*),  $T_H2$  (*GATA3*), DC2 (*CD1C*) and MC4 (*CCR2*) (Suppl. Fig 8 and 9). This revealed that the predicted target genes of IL-9 and IL-21 overlapped only partially, with most target genes being induced by IL-21 rather than IL-9.

In the  $T_H2$  subset, we observed predicted upregulation of *GZMB*, *IL10*, *IL2RA*, *GADD45B* and *IRF4* in response to IL-9. (Suppl. Fig 8B). All of these genes were also predicted to be upregulated by IL-21, suggesting partial redundancy of IL-9 and IL-21 in driving  $T_H2$  responses. Genes specifically upregulated by IL-21 included *CEBPD*, *IL18RAP*, *SEMA7A* and *TNIP*, genes which are known to be involved in various aspects of asthma(64-66), and DC-dependent  $T_H2$  differentiation(67), respectively. Within the MNP subsets, IL-21 also had high predicted ligand activity. Genes like *PLGRKT*, *AQP3*, *FCER2* and *IGHE* were predicted to be upregulated in response to IL-21. Furthermore, several matrix metalloproteinases like MMP1, MMP3 and MMP10 were upregulated by IL-21, thereby contributing to asthma pathology through extracellular matrix (ECM) remodeling(68). The other disease-associated subset MC4 (*CCR2*) was also predicted to mainly respond to IL-21, showing upregulated expression of the genes involving MMP9 and MMP10, contributing to ECM remodeling just like DC2s. Together, these data confirm that both IL-9 and IL-21 protein expression and *IL21R* transcript is increased in AA and that a key transcriptome signature can be explained by the action of these cytokines.

## Discussion

The precise contribution of IL-9 in asthma has been very controversial, some mouse models showing clear reduction in key asthma features, and some showing no effect, when IL-9 was neutralized or genetically inactivated(21, 22, 26, 36). Similarly, despite clear genetic association between the *IL9* gene and BHR(14), a clinical trial in which IL-9 was neutralized in asthmatic patients using a humanized mAb showed a lack of efficacy(69), potentially explained by inadequate patient selection criteria. The idea that IL-9 is made by a unique  $T_H9$  subset has also been heavily debated, and there is a picture emerging that IL-9 production by CD4<sup>+</sup> T cells is only a transient state of  $T_H2$  cells that acquire the potential to become p $T_H2$  cells(11). These mouse and human data clearly indicate that the implication of IL-9 in type 2 immunity and asthma is highly context dependent, and the idea of targeting IL-9 as a novel target for therapy in asthma became less attractive over the years(7).

Renewed interest in IL-9 as a druggable cytokine was sparked by applying single-cell mRNA sequencing approaches, that allowed a much higher granularity and resolution of cellular phenotyping in human asthmatics. These studies confirmed the presence of a p $T_H2$  cell subset expressing high amounts of IL-9 in either lung or blood of asthmatics, and more importantly, the induction of IL-9 transcripts following allergen challenge to the lungs clearly distinguished allergic asthmatic patients from allergic non-asthmatic controls(15, 16). One of the major differences between allergic controls and allergic asthmatics is the presence of BHR in the latter, potentially explained by localization of mast cells in the airway smooth muscle layer(70, 71). In addition,



elevated numbers of epithelial mast cells have been shown to correlate with clinical severity(72), likely driven by an IL-13 dependent cross talk with the airway smooth muscle cells(73). Since IL-9 is a well-known growth factor for mast cells, IL-9 could be linked to BHR via mast cell effects. We looked for mast cells in our mouse models, but numbers were too low to address this hypothesis, even when we extended our analysis to BALB/c mice that are known to have higher baseline mast cell numbers. This suggests significant species differences and illustrates how important it is to also perform human translational studies, if a particular pathway cannot be addressed in mice.

A recent paper in a HDM-driven mouse model of asthma has also reignited the interest in IL-9, by showing that lung perivascular CD163<sup>+</sup> IMs express the IL-9R, and react to the cytokine by turning up arginase expression and producing chemokines that recruit innate and adaptive immune cells to the lungs(26). We did find these arginase-expressing CD163<sup>+</sup> IMs in all our asthma models, but strikingly, we saw no effect of IL-9 neutralization in the HDM and papain model, nor could we detect *Il9r* mRNA transcripts on IMs in mice or humans (data not shown). In the IL-33 induced model however, the Arg1<sup>+</sup> IMs were reduced by IL-9mAb. Although arginase can also be induced in macrophages by IL-21 (39) and we did observe IL-21R on murine and human macrophages, we did not see effects of IL-21 blockade on arginase induction. It could be that this IL-9-IM axis only comes into play when there is particularly high level of stimulation by IL-33 or other alarmins. Clearly more work is needed before we fully understand all the cellular pathways engaged by IL-9.

Here, we focused on the autocrine and paracrine effects of IL-9 and the closely related cytokine IL-21 as stimulators of lymphocytes, with the hypothesis that the controversy on the role of IL-9 in asthma might be time dependent, model driven, and caused by redundancy between both cytokines. We found that IL-9 is a main amplifier of ILC2s, particularly in the acute onset, innate type 2 immune response driven by IL-33 or by the enzymatic allergen papain, known to cause IL-33 release and activation in the airways. Autocrine IL-9 derived from ILC2s subsequently drove ILC2 proliferation and effector cytokine production. Such a dependence on IL-9 might occur in the clinical setting too, when the damaged epithelium releases alarmins such as IL-33 in response to protease allergens, viral infection, or exposure to environmental pollutants. In asthmatics, this alarmin-driven acute activation likely occurs on a background of chronic airway inflammation in which the adaptive immune response and T<sub>H</sub>2 lymphocytes continuously produce cytokines to maintain inflammation. In such a chronic setting, acute IL-33 release by the damaged epithelium will likely also activate tissue resident T1/ST2 expressing pT<sub>H</sub>2 cells in a non-cognate manner, akin to ILC2s, again relying on autocrine IL-9 for initial expansion and activation(36, 45, 74). In our translational studies in human asthmatics, there was indeed a marked increase in IL-9 protein levels derived from T<sub>H</sub>2 cells following SAC, and this was not the case in allergic non-asthmatic controls(16). An autocrine loop is also in line with *IL9R* transcript predominantly being picked up in the T<sub>H</sub>2 cell compartment (Suppl. Fig 7F). There were no ILC2s recovered after SAC in human asthmatics, which did not allow us to model the effects of IL-9 on human ILC2s. In our chronic HDM model in mice we failed to find a role for IL-9, perhaps because this model is unable to elicit repeated IL-33 release in the airways with the low doses of allergen used to elicit the recall response(75). With chronic low grade HDM exposure, the epithelium and immune response gradually cool down, often leading to decreasing airway eosinophilia as HDM exposure is prolonged.

We also hypothesized that IL-21, a closely related IL-2 family cytokine, might take over some of the effects of IL-9 in boosting type 2 immunity. This hypothesis stemmed from previous work by us and others showing that IL-21 can promote effector functions in  $T_H2$  cells and ILC2s, very similar to what IL-9 is known for(42, 44). The interest in targeting IL-21 in allergic diseases like asthma has been met with some skepticism, since IL-21 is known to suppress IgE synthesis(56), and full-body *Il21r<sup>-/-</sup>* mice have a hyper IgE syndrome. Using temporary IL-21mAb treatment, we did not however detect significant increases of circulatory IgE in the serum of mice. Instead, using the chronic HDM model, we discovered that IL-21 acts as a master regulator of chronic type 2 inflammation. While IL-9 blockade did not significantly inhibit  $pT_H2$  cell numbers or key asthma features in the lung, anti-IL-21 monotherapy was sufficient to do so. Similarly, in the papain model, we found a clear benefit of neutralizing IL-21 on top of IL-9, suggesting that IL-21 can take over functions of IL-9, a conclusion also reached when we looked at the predicted gene signatures in human  $pT_H2$  cells and MNPs following allergen challenge.

We found formation of TLS in airways of HDM-treated mice, which were absent in the groups receiving IL-21 blockade. Even though  $T_{FH}$  derived IL-21 is known to be essential for germinal center responses in secondary lymphoid tissues(76, 77), it remains unknown whether IL-21 production is crucial for the formation of TLS. Resident helper cells ( $T_{RH}$ ) producing IL-21 have recently been described also in TLS formed after viral infection of the lung, form independently from  $T_{FH}$  cells, and control key aspects of humoral immunity(78). Future studies in our lab will address the functional role of TLS and downstream humoral responses in the chronic HDM model. While  $CD4^+$  T cells are most likely the main producers of IL-21, it remains less clear which cell types are the main responders to IL-21 signaling within this model. Besides T and B lymphocytes, also macrophages, monocytes and DCs could be responding to IL-21, since expression of IL-21R has been confirmed on those cell types(39-41). In our previous work in the HDM model, we described how IL-21R was needed for ILC2 activation, but we failed to reveal a cell-intrinsic role for IL-21R on these cells(44). Therefore, more detailed studies are required before we can conclude how IL-21 precisely controls type 2 immunity, exerts its synergistic role with IL-9, and contributes to the formation of TLS.

Interleukin-21 has so far been poorly investigated in human asthmatic samples. We found elevated IL-21R expression and evidence for downstream signaling within the allergic asthmatics in comparison to the allergic controls, predominantly within the T cell and MNP compartment. Moreover, we also detected elevated IL-21 protein in human BAL samples. While IL-21R expression was mainly found on  $CD8^+$  T cells within allergic controls, allergic asthmatics showed increased expression in Treg cells. Within the Tregs, IL-21 is predicted to induce FOXP3 expression itself and multiple downstream regulators of cytokine signaling like *SOCS3* and *CISH*, potentially dampening Treg responses, as previously reported in the mouse. While IL-21R signaling can directly induce apoptosis in Tregs(42), others have shown that their suppressive potential is dampened in presence of IL-21, thereby amplifying the  $T_H2$  response(40). Within the  $CD4^+$  T cell subset, IL-21 is also predicted to induce *SOCS3*, *CISH* and *SOCS1*, *IL-2RA* and *JAK3* indicating strong cytokine signaling activity. Interestingly, IL-21 also was predicted to upregulate *BATF3*, a transcription factor linked to  $T_H9$  differentiation(79) and *VDR* as well as *IFITM1*, which are linked to development of asthma(80) in children and exacerbating asthma by promoting the  $T_H2$  cell development(81). Using NicheNet analysis, we could predict strong IL-21 ligand activity also within the MNP compartments (cDC2 and MC4). Strikingly, all of these subsets are associated with having asthma after allergen challenge(16). While  $T_H2$  derived IL-13 most likely prevents incoming monocytes from developing into mature macrophages, thereby maintaining a



pro-inflammatory state, while also signaling in cDC2s(16), our results suggest that IL-21 could be another cytokine contributing to this phenotype. There were indeed clear signs that IL-21 upregulated the expression of gene associated with oxidative stress response and ECM remodeling like *MMP9* and *MMP10*, in line with murine data(82, 83).

In conclusion, this study leveraged experimental mouse models to unravel a synergistic cytokine axis involving IL-9 and IL-21 across innate and adaptive immunity, and our data following SAC in human asthmatics show that this is a likely scenario in clinical asthma. Further studies are now needed to investigate more closely which patients could benefit from single or dual IL-9 and IL-21 targeted therapy.

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## Figure legends

**Figure 1: Generation of potent neutralizing antibodies against murine IL-9 and IL-21.** **A**, Schematic representation of the anti-IL-9 antibody discovery workflow. **B**, Schematic representation of the anti-IL-21 antibody reformatting. **C**, Fc $\gamma$ R-binding ELISA results for antibodies reformatting into mIgG1-N297A backbone. **D**, Affinity of the used antibodies towards their target as determined by 1:1 fitting using SPR. **E**, Proliferation-based bioassay data showing the neutralizing effect of the antibodies in presence of either IL-9 or IL-21. Schemes shown in A and B were created with BioRender.com.

**Figure 2: IL-9 blockade broadly ameliorates IL-33 induced airway inflammation.** **A**, Experimental setup of IL-33 exposed C57BL/6J wild type, Red5<sup>-/-</sup> or IL-9R<sup>-/-</sup> mice treated with antibodies during challenge. **B + E**, Cytokine levels as measured by ELISA in the supernatant of BAL fluid recovered from IL-33 challenged mice. **C**, Cell counts recovered after BAL as determined by flow cytometry. T<sub>H</sub>2 cells are defined like CD4<sup>+</sup> and ST2<sup>+</sup>. **D + F**, Cell counts from enzymatically digested lungs as determined by flow cytometry.

**Figure 3: IL-9 blockade ameliorates IL-33 induced airway inflammation by suppressing ILC2s** **A**, MFIs of Ki-67 and IL-13 on ILC2s as determined by flow cytometry. **B**, Gating strategy shown for ILC2s in enzymatically digested lungs using Red5<sup>-/-</sup> mice. MFIs of IL-5 (tdTomato) on ILC2s and cell counts of eosinophils in the lungs as determined by flow cytometry. **C**, Comparison of ILC2 numbers and MFIs (Ki-67 and IL-13) from enzymatically digested lungs between C57BL/6J wild type and IL-9R<sup>-/-</sup> mice. Contour plots show frequencies representative for quantification. **D**, Representative histological comparison of hematoxylin-eosin stain from lungs after IL-33 challenge. **E**, Relative lung mRNA expression levels of Muc5ac and Spdef as determined by qRT-PCR. **F**, BHR measured in IL-33-challenged mice in response to increasing doses of methacholine using FlexiVent (SCIREQ). Data are representative of 3 independent experiments, with at least 5 mice per group, and are presented as means  $\pm$  SEM. ns, Not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001 versus mice treated with an irrelevant mIgG1 N297A antibody. AM, Alveolar macrophages; DCs, dendritic cells; cDC2s, conventional type 2 dendritic cells; IMs, interstitial macrophages.

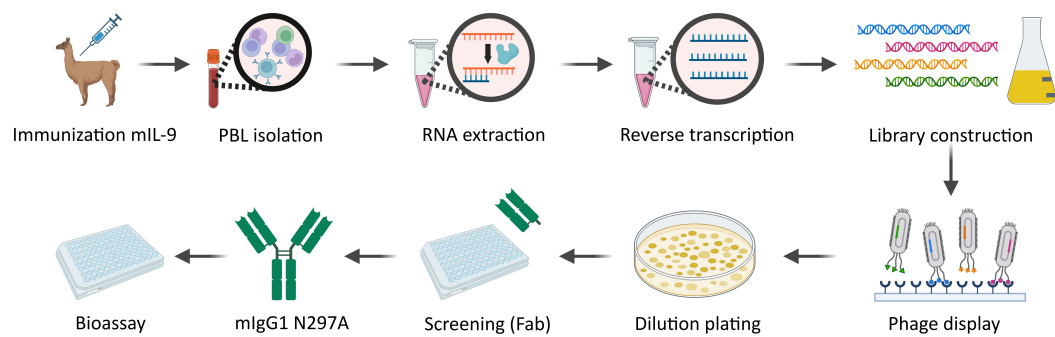
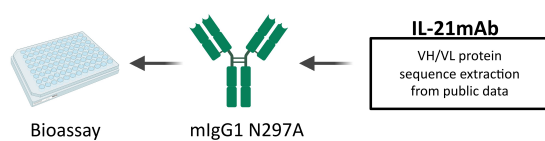
**Figure 4: IL-21 blockade ameliorates chronic HDM-induced airway inflammation by reducing adaptive immunity** **A**, Experimental setup of HDM exposed C57BL/6J wild type mice treated with antibodies during challenge. **B**, Cytokine levels as measured by ELISA in the supernatant of BAL fluid recovered from HDM sensitized and challenged mice **C**, Total IgE level time course as measured by ELISA in serum. **D**, HDM specific IgE and IgG1 levels at week six as measured by ELISA in serum. **E**, Cell counts recovered after BAL as determined by flow cytometry. T<sub>H</sub>2 cells are defined as CD4<sup>+</sup> ST2<sup>+</sup>. **F-H**, Cell counts from enzymatically digested lungs as determined by flow cytometry. Quantification of T<sub>H</sub>2 cells (CD4<sup>+</sup> ST2<sup>+</sup> GATA3<sup>+</sup>) as determined by flow cytometry. **I**, Representative histological comparison of hematoxylin-eosin stain from lungs after challenge as measured using brightfield microscopy. Squares indicate areas shown in the panel below, depicting confocal immune stainings against B220, GL7, DAPI and CD3e as measured by confocal microscopy. **J**, Quantification of TLS structures based on hematoxylin-eosin (H&E) stainings. Data are representative of 3 independent experiments, with at least 5 mice per group, and are presented as means  $\pm$  SEM. ns, Not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001 versus mice treated with an irrelevant mIgG1 N297A antibody. AM, Alveolar macrophages; DCs, dendritic cells; cDC2s, conventional type 2 dendritic cells; IMs, interstitial macrophages; TLS, tertiary lymphoid structure.

**Figure 5: Synergistic IL-9 and IL-21 inhibition ameliorates papain-induced airway inflammation.** **A**, Experimental setup of papain exposed C57BL/6J wild type mice treated with antibodies during challenge. **B-C**, Total IgE time course as measured by ELISA in serum. HDM specific IgE and IgG1 levels at week three as measured by ELISA in serum. **D**, Cell counts recovered from BAL as determined by flow cytometry. T<sub>H</sub>2 cells are defined as CD4<sup>+</sup> ST2<sup>+</sup>. **E-G**, Cell counts from enzymatically digested lungs as determined by flow cytometry. Representative contour plots of T<sub>H</sub>2 cells (CD4<sup>+</sup>, ST2<sup>+</sup>, GATA3<sup>+</sup>) as determined by flow cytometry and quantification thereof. **H**, Representative histological comparison of hematoxylin-eosin stain from lungs after challenge. **I**, Relative lung mRNA expression levels of Muc5ac and Spdef as determined by qPCR. **J**, BHR measured in papain-challenged mice in response to increasing doses of methacholine using FlexiVent (SCIREQ). Data are representative of 3 independent experiments, with at least 5 mice per group, and are presented as means  $\pm$  SEM. ns, Not significant. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, and \*\*\*\**P* < 0.0001 versus mice treated with an irrelevant mIgG1 N297A antibody. AM, Alveolar macrophages; DCs, dendritic cells; cDC2s, conventional type 2 dendritic cells; IMs, interstitial macrophages.

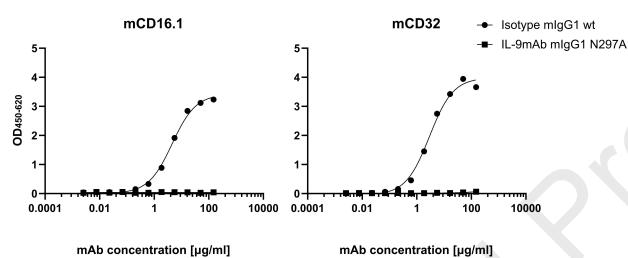
**Figure 6: Levels of IL-21 protein and its receptor transcript are increased in human allergic asthmatics, predicting ligand activity in multiple disease-associated cell subsets.** **A**, UMAP of a total of 52,152 cells recovered from 21 lower airway brushings. **B**, Depiction of IL-21R mRNA transcript expression (2,404 cells = 4.6% of total cells) **C**, IL-21 protein levels in allergic asthmatics and controls before and after segmental allergen challenge (SAC) with either diluent or allergen as determined by ELISA. **D**, IL-21R expression separated by group per cellular subset. **E**, predicted IL-9 and IL-21 ligand activities in the AA group. Upregulated target genes within the Treg and DC2 subsets are shown for representation. Black rectangles highlight cell subsets associated with having asthma. Target genes for

869 these subsets are shown in Suppl. Fig 8 and 9. In **c**, p-values were calculated using ordinary two-way ANOVA with  
870 Tukey's correction for multiple comparisons. ns, Not significant. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ .

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**A****B****D**

|          | $K_D$ [nM] | $K_{on}$ [ $M^{-1}s^{-1}$ ] | $K_{off}$ [ $s^{-1}$ ] |
|----------|------------|-----------------------------|------------------------|
| IL-9mAb  | 0.11       | $1.40E+06$                  | $1.47E-04$             |
| IL-21mAb | 1.30       | $1.22E+05$                  | $1.58E-04$             |

**C****E**