Molecular Regulation of Group 2 Innate Lymphoid Cells

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To my parents who thought this day would never come.

Abstract

Mucosal surfaces lining the respiratory tract are constantly exposed to potentially pathogenic environmental insults. Here, the mucosal immune system comprises a complex integrated network of innate and adaptive immune cells as well as stromal populations that function in a coordinated manner to ensure efficient host protection as well as maintain barrier integrity and homeostasis. Group 2 innate lymphoid cells (ILC2s) are the predominant helper-like ILC population in the lung and are key regulators of early type 2 immune responses and pulmonary homeostasis. ILC2s are activated upon tissue injury by alarmins such as interleukin (IL)-33 and IL-25, rapidly proliferate and produce type 2 signature cytokines that mediate immunity to parasitic helminths and lung tissue repair. On the other hand, dysregulated ILC2 function is associated with type 2 immunopathologies and atopic diseases such as asthma and allergic airway inflammation. Thus, ILC2s must be tightly controlled in order to mount protective host defense responses yet avoid excessive activation and immunopathology. Despite rapid developments in the field of ILC2 biology, how ILC2s are controlled and maintained during homeostasis and activation remains incompletely understood. Hence, the overarching goal of this thesis was to decipher novel molecular mechanisms of ILC2 regulation and validate the physiological importance of our findings during allergic airway inflammation and lung infection.

Using transcriptomic analysis of isolated ILC2s at steady state and after IL-33-mediated activation in the absence and presence of known and potential regulatory units we identified novel mechanisms of transcriptional as well as peptide- and interferon-mediated ILC2 regulation. More specifically, we demonstrated that the NF-kB transcription factor c-Rel plays an essential role in promoting ILC2 effector functions following IL-33-mediated activation. We identified c-Rel target genes and further validated these findings in *in vivo* models of allergic airway inflammation.

Work by our lab and others recently showed that type I interferon (IFN-I) inhibits ILC2 effector functions and we now further established that IFN-I stimulation specifically suppresses ILC2-intrinsic production of the chemokine CCL1 and expression of its cognate receptor CCR8, important mediators of ILC2 maintenance and tissue migration during allergic airway inflammation.

In addition, we showed that the peptide adrenomedullin which is preferentially expressed in the murine lung under homeostatic conditions inhibited effector functions of pulmonary ILC2s in a cAMP-dependent manner, thereby identifying a novel mechanism of ILC2 regulation.

Collectively, the data presented in this thesis reveal novel avenues of ILC2 regulation during homeostasis as well as allergic airway inflammation and point out new potential therapeutic targets for the treatment of atopic diseases.

Résumé

Les muqueuses qui tapissent les voies respiratoires sont en tout temps exposées à de nombreuses agressions qui risquent de s'avérer pathogéniques. Unique à cet environnement, le système immunitaire des muqueuses se démarque par son réseau complexe et fortement intégré de cellules immunitaires innées et adaptatives qui, se rejoignant à certaines populations de cellules stromales, peuvent toutes agir en concert afin de bien protéger l'hôte et de maintenir l'intégrité et l'homéostasie des surfaces barrières. Les cellules lymphoïdes innées du groupe 2 (CLI2), qui représentent la principale population CLI de type auxiliaire dans les poumons, assurent la régulation des réponses immunitaires précoces de type 2 et de l'homéostasie pulmonaire. Une lésion tissulaire déclenchera aussitôt la production d'alarmines (les interleukines (IL)-33 et IL-25) pour activer les CLI2, permettant aux CLI2 de proliférer rapidement et de produire à leur tour les cytokines caractéristiques d'une réponse de type 2 qui offrent une immunité aux helminthes parasitiques et permettent au tissu pulmonaire de se réparer. D'autre part, un dysfonctionnement des CLI2 peut induire des pathologies immunitaires de type 2 de même que des maladies atopiques telles l'asthme ou l'inflammation allergique des voies respiratoires. Ainsi, les CLI2 font l'objet d'une étroite régulation qui assure la défense de l'hôte sans pour autant engendrer une trop forte activation immunologique ou une pathologie délétère. Malgré une évolution rapide dans le domaine de la biologie des CLI2, les mécanismes qui gouvernent l'homéostasie et l'activation des CLI2 ne sont pas totalement connus. Par conséquent, l'objectif principal de cette thèse était d'élucider et de découvrir de nouvelles voies moléculaires de régulation des CLI2, et de confirmer l'importance de leur potentiel rôle physiologique dans le contexte d'une infection pulmonaire ou de l'inflammation allergique des voies respiratoires.

À partir de populations homogènes de CLI2 isolées et récoltées en état de repos ou en état d'activation à l'IL-33, et suite à leur stimulation ou non avec divers agents régulateurs connus ou présumés, une pleine analyse transcriptomique nous a permis d'identifier de nouveaux mécanismes liés à la régulation des CLI2 qui agissent à l'aide de peptides ou d'interférons, mais également au niveau transcriptionnel. Plus particulièrement, nous avons démontré que le facteur de transcription c-Rel, appartenant à la famille NF-kB, joue un rôle essentiel pour promouvoir les fonctions effectrices des CLI2 activées à l'IL-33, au moyen de nombreux gènes cibles dont l'expression dépend directement et spécifiquement de c-Rel. Nous

avons validé ces mécanismes novateurs en poursuivant une étude *in vivo* dans un modèle expérimental d'inflammation allergique des voies respiratoires.

De récents travaux effectués dans notre laboratoire et par d'autres groupes ont révélé que l'interféron de type I (IFN-I) agit pour inhiber les fonctions effectrices des CLI2, et nous avons en outre établi qu'une stimulation à l'IFN-I puisse réprimer la production intrinsèque aux CLI2 du chimiokine CCL1, de même que l'expression du récepteur CCR8 qui reconnaît de façon spécifique le facteur CCL1, tous deux intervenants cruciaux dans la maintenance et la migration tissulaire des CLI2 lors d'une inflammation allergique pulmonaire.

De plus, nous démontrons que l'adrénomédulline, un peptide exprimé de façon préférentielle sous conditions homéostatiques dans les poumons murins, peut contrer les fonctions effectrices des CLI2 pulmonaires au moyen de la voie cAMP, ce qui constitue un nouveau procédé distinct de la régulation des CLI2.

Collectivement, les données présentées dans cette thèse dévoilent plusieurs mécanismes novateurs qui régulent les CLI2 dans le contexte de l'inflammation allergique des voies respiratoires, et ensemble nous dirigent vers de nouvelles cibles thérapeutiques pouvant améliorer le traitement des maladies atopiques.

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List of Abbreviations

A. alternata…*Alternaria alternata*

AD…atopic dermatitis

ADM…adrenomedullin

aGVHD…acute graft versus host disease

AhR…aryl hydrocarbon receptor

ANOVA…analysis of variance

AP-1…activator protein 1

APC…antigen-presenting cell

AREG…amphiregulin

ASC…adventitial stromal cell

ASPC…adipose stem and progenitor cell

ATAC-seq…Assay for Transposase-Accessible Chromatin using sequencing

ATP…adenosine 5'-triphosphate

BALF…bronchoalveolar lavage fluid

Bcl11b…B cell leukemia 11b

BCR…B cell receptor

BSA…bovine serum albumin

cAMP…cyclic adenosine monophosphate

CCL…chemokine (C-C motif) ligand

CCR…chemokine (C-C motif) receptor

CD…cluster of differentiation

cDC…conventional dendritic cell

cDNA…complementary DNA

CF…cystic fibrosis

CHILP…common helper ILC progenitor

ChIP…chromatin immunoprecipitation

CLA…cutaneous leukocyte-adhesion antigen

CLP…common lymphoid progenitor

CLR…C-type lectin receptor

CLR…calcitonin receptor-like receptor

cNK…conventional NK cell

COPD…chronic obstructive pulmonary disease

CRS…chronic rhinosinusitis

CRSsNP…chronic rhinosinusitis without nasal polyps

CRSwNP…chronic rhinosinusitis with nasal polyps

CRTH2…chemoattractant receptor expressed on Th2 cells

CSR…class switch recombination

CTL…cytotoxic T lymphocyte

DAMP…damage-associated molecular pattern

DC…dendritic cell

DHT…5α-dihydrotestosterone

DNA…deoxyribonucleic acid

DSS…dextran sodium sulfate

EAE…experimental autoimmune encephalomyelitis

EGFR…epidermal growth factor receptor

EILP…early innate lymphoid progenitor

ELISA…enzyme-linked immunosorbent assay

Eomes…eomesodermin

ETS1… avian erythroblastosis virus E26 homolog-1

FACS…fluorescence-activated cell sorting

FALC…fat-associated lymphoid cluster

GABA…gamma-aminobutyric acid

γc…common γ chain

GALT…gut-associated lymphoid tissue

GATA3…GATA binding protein 3

GCRP…calcitonin gene-related peptide

G-CSF…granulocyte colony-stimulating factor

Gfi1…growth factor independent 1

GILP…global innate lymphoid cell progenitor

GITR…glucocorticoid-induced tumor necrosis factor receptor

GM-CSF…granulocyte-macrophage colony-stimulating factor

GWAS…genome-wide association study

HDM…house dust mite

HMGB1…high mobility group box 1

H. polygyrus…*Heligmosomoides polygyrus*

HSC…hematopoietic stem cell

HSP…heat shock protein

IAV…influenza A virus

ICAM-1…intercellular adhesion molecule-1 (ICAM-1)

ICOS…inducible T cell co-stimulator

Id2…inhibitor of DNA-binding 2

IFN…interferon

IFN-I…type 1 interferon

IFN-II…type 2 interferon

IFN-III…type 3 interferon

Ig…immunoglobulin

Ih2…innate helper type 2 cells

iILC2…inflammatory group 2 innate lymphoid cell

IKK…IκB kinase

IL-…interleukin-

IL-1RAcP…IL-1 receptor accessory protein

IRAK1…IL-1 receptor-associated kinase 1

IRAK4…IL-1 receptor-associated kinase 4

ILC…innate lymphoid cell

ILC1…group 1 innate lymphoid cell

ILC2…group 2 innate lymphoid cell

ILC3…group 3 innate lymphoid cell

ILCP…innate lymphoid cell precursor

IRF9…interferon regulatory factor 9

ISG…interferon-stimulated gene

ISGF3…interferon-stimulated gene factor 3

IUIS…International Union of Immunological Societies

LN…lymph node

αLP…$\alpha_4\beta_7^+$ lymphoid progenitor

LRR…leucine-rich repeat

LSK…lineage$^-$Sca-1$^+$c-kit$^+$

LTα…lymphotoxin α

LTE_4…leukotriene E_4

LTiP…lymphoid tissue inducer progenitor

MAPK…mitogen-activated protein kinase

MBL…mannose-binding lectin

mesLN…mesenteric lymph node

MHC-II…major histocompatibility complex class II

medLN…mediastinal lymph node

mRNA…messenger ribonucleic acid

mSC…mesenchymal stromal cell

MyD88…myeloid differentiation factor 8

NFIL3…nuclear factor IL-3 induced

NF-κB…nuclear factor kappa-light-chain-enhancer of activated B cells

N. brasiliensis…*Nippostrongylus brasiliensis*

nILC2…natural group 2 innate lymphoid cell

NKP…natural killer cell precursor

NLR… nucleotide oligomerization domain-like receptor

NMU…neuromedin-U

NOD…nucleotide oligomerization domain

NP…nasal polyp

OVA…ovalbumin

PAMP…pathogen-associated molecular patterns

PBS…phosphate-buffered saline

PBMC…peripheral blood mononuclear cell

PCR…polymerase chain reaction

PD-1…programmed cell death-1

PGD_2…prostaglandin D_2

PLZF… promyelocytic leukemia zinc finger

PRR…pattern-recognition receptor

qRT-PCR…quantitative real-time polymerase chain reaction

RAG… recombination-activating gene

RBC…red blood cell

RIG-I…retinoic-acid inducible gene-I

RLR… retinoic-acid inducible gene-I-like receptors

RNA…ribonucleic acid

RSV…respiratory syncytial virus

RORα…retinoic acid receptor-related orphan nuclear receptor α

RORγt… retinoic acid receptor-related orphan nuclear receptor γt

RPMI…Roswell Park Memorial Institute

rRNA…ribosomal ribonucleic acid

S1P…sphingosine-1 phosphate

SCF…stem cell factor

SCID…severe combined immunodeficiency

SNPs…single nucleotide polymorphisms

ST2…suppression of tumorigenicity 2

STAT…signal transducer and activator of transcription

T1D…type 1 diabetes

T-bet…T box protein expressed in T cells

T_c…cytotoxic T cell

TCF-1…T cell factor 1

Tconv…conventional T cell

TCR…T cell receptor

TGF-β…transforming growth factor β

Th…helper T cell

Tigit…T cell immunoreceptor with Ig and ITIM domains

TNF-α…tumor necrosis factor α

TOX…thymocyte selection-associated high mobility group box protein

TLR…Toll-like receptor

TRAF6…TNF receptor-associated factor 6

Treg…regulatory T cell

VAT…visceral adipose tissue

VIP…vasoactive intestinal peptide

WAT…white adipose tissue

WAT-MSC… white adipose tissue-resident multipotent stromal cell

WT…wild-type

Contributions to Original Scientific Knowledge

Chapter 2: The NF-κB transcription factor c-Rel drives group 2 innate lymphoid cell effector functions and allergic airway inflammation. (manuscript in preparation)

We demonstrated that the NF-κB transcription factor c-Rel is induced and translocates to the nucleus upon IL-33-mediated activation of murine ILC2s. We further identified c-Rel target genes by ChIP-Seq and differentially regulated genes in the absence of c-Rel by RNA-Seq comparing wild-type and c-Rel-deficient ILC2s. Through this combined approach we identified the co-stimulatory ligand 4-1BBL to be directly regulated by c-Rel and induced on ILC2s upon activation with IL-33 *ex vivo*. We validated these findings *in vivo* showing that 4-1BBL expression is upregulated on ILC2s during IL-33-mediated allergic airway inflammation and allergic lung disease while its cognate receptor 4-1BB was induced on conventional and regulatory T cells to potentially mediate downstream effect. We hereby identified c-Rel as an essential transcription factor for the exertion of ILC2 effector functions.

Chapter 3: Type I interferon limits ILC2 effector functions via suppression of the CCR8-CCL1/CCL8 axis. (manuscript in preparation)

The aim of this chapter was to decipher mechanisms of type 1 interferon-mediated ILC2 inhibition during allergic airway inflammation and influenza virus infection. We established that IFN-β treatment of isolated lung ILC2s results in downregulation of surface CCR8, a chemokine receptor important for ILC2 tissue migration during allergic airway inflammation as well as ILC2 maintenance. Furthermore, IFN-I stimulation inhibited ILC2-intrinsic production of the CCR8 ligand CCL1 after *ex vivo* activation. CCR8 as well as CCL1 have previously been shown play a key role in mediating ILC2 effector functions further underlining the importance of this finding. We additionally show that IFN-I specifically targets the CCR8-CCL1/CCL8 axis in a mouse model of influenza A virus infection as well as in an IL-33-mediated allergic airway inflammation model, potentially impairing ILC2 mobility and effector functions. We thereby unraveled a novel mechanism of IFN-I mediated ILC2 perturbation.

Chapter 4: Adrenomedullin negatively regulates IL-33-mediated group 2 innate lymphoid cell responses. (manuscript in preparation)

Here we showed that ILC2s express both adrenomedullin receptor chain transcripts and that stimulation with adrenomedullin peptide restrains effector functions of lung ILC2s *ex vivo* in a cAMP-dependent manner, thereby identifying adrenomedullin as a novel negative regulator of ILC2 function.

Contributions of Authors

All contributing authors are designated by their initials and their individual contributions are listed.

Chapter 2: The NF-κB transcription factor c-Rel drives group 2 innate lymphoid cell effector functions and allergic airway inflammation. *(manuscript in preparation)*

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B.C.M. performed *in vivo* allergic airway inflammation models and *ex vivo* cell culture experiments as well as data analysis. C.U.D. assisted with *in vivo* experiments. M.M. conducted cellular fractionation and western blots. L.R. performed histological scoring. S.M.V., T.H.W. and S.G. provided critical reagents and mouse strains. D.L. performed analysis of RNA-seq and ChIP-seq datasets. B.C.M. and J.H.F designed experiments. B.C.M wrote the manuscript and J.H.F provided edits.

Chapter 3: Type I interferon limits ILC2 effector functions via suppression of the CCR8-CCL1/CCL8 axis. (manuscript in preparation)

Barbara C. Mindt, Jérémy Postat, Claudia U. Duerr, Judith N. Mandl, David Langlais, Jörg H. Fritz

B.C.M. performed mouse *in vivo* experiments (allergic airway inflammation models and influenza A virus infection) and the majority of *ex vivo* assays. J.P. conducted and imaged *ex vivo* ILC2 migration assays. C.U.D. prepared and titrated influenza virus stocks and assisted with *in vivo* experiments. D.L. performed analysis of RNA-seq datasets. B.C.M. and J.H.F designed experiments and B.C.M. wrote the manuscript. J.H.F provided edits to the manuscript.

Chapter 4: Adrenomedullin negatively regulates IL-33-mediated group 2 innate lymphoid cell responses. (manuscript in preparation)

Barbara C. Mindt, Alfredo Martínez, David Langlais, Jörg H. Fritz

B.C.M. performed mouse *in vivo* experiments and *ex vivo* cell culture experiments. A.M. provided reagents and mouse strains. D.L. performed analysis of RNA-seq datasets. B.C.M. and J.H.F designed experiments. B.C.M. wrote the manuscript and J.H.F and A.M. provided edits.

Chapter 1: General Introduction

In this chapter I will outline the general features and concepts of immune responses as well as more specifically the biology of group 2 innate lymphoid cells and their functional role and regulation at homeostasis and during immune challenge.

1.1. The immune system

The human body is in continuous contact with its surroundings and thereby constantly exposed to environmental insults. The immune system evolved to defend the host against these ubiquitous potentially pathogenic insults and maintain homeostasis. In mammals, the immune system comprises a complex integrated network where different cell populations and molecules function in a coordinated manner to ensure efficient host protection. The immune response to a homeostatic perturbation consists of four consecutive steps starting with (1) the sensing of the insult by respective immune receptors and (2) relaying those signals to communicate the nature and the extent of the challenge. Based on these cues, an appropriate effector response is initiated (3) to eradicate the underlying insult followed by (4) the resolving stage where collateral tissue damage is repaired and expanded immune cells contract again to return to a state of homeostasis. If any of these response phases is dysregulated, pathologies such as autoimmune disorders and allergies can arise as a consequence. Thus, immune responses must be tightly controlled to mount protective host responses yet avoid excessive activation and immunopathology.

The defense against microbes and innocuous agents is mediated by two distinct but highly interconnected arms of the immune system, the early broadly specific innate immune response and the subsequent highly specific adaptive response which are described in more detail below.

1.1.1. Innate and adaptive immunity – an overview

Human-environment interactions constantly take place at the skin and mucosal surfaces such as the gastrointestinal and respiratory tract. The main function of the innate immune system is to block the entry of pathogenic microbes at these sites, inhibit their replication to contain them and eradicate them if possible. The innate response thereby comprises the first line of defense against microbial encounters. It consists of mechanical barriers such as the skin and the epithelial linings of the mucosa which can secrete mucus and antimicrobial molecules to provide an additional biochemical layer of protection. If cells breach the host's mechanical and chemical protective barriers, they are first encountered by tissue-resident innate immune cells including phagocytes such as macrophages and dendritic cells (DCs) as well as neutrophils and cytotoxic natural killer (NK) cells (*Figure 1*). Activated granulocytes such as neutrophils, eosinophils, mast cells and basophils additionally aid in the innate response by degranulating and thereby releasing enzymes, toxic substances and immune mediators (*Figure 1*). In addition, a novel family of innate effector cells, helper-like group 1, group 2 and group 3 innate lymphoid cells, ILC1, ILC2 and ILC3, respectively, have been identified and shown to play key roles in inflammation and immunity, especially at barrier surfaces (*Figure 1*). If a microbe manages to circumvent the cell-mediated innate response in the tissue and enters the bloodstream it can be recognized by circulating innate immune proteins including complement system components such as C1q, mannose-binding lectin (MBL) and ficolin, that can confer additional protection (*Figure 1*). Ultimately, depending on the type of perturbation, members of the innate immune system will initiate an inflammatory or antiviral response to provide an initial defense against the underlying insult.

Innate immune cells are activated by the recognition of pathogen-associated molecular patterns (PAMPs) through so-called pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic-acid inducible gene-I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs). PAMPs are conserved structures of microbial origin such as bacterial cell wall components that are shared by a variety of microbes. The cognate PRRs are germline-encoded receptors present on the cell surface in association with the cell membrane or can be located intracellularly within endosomal membranes or the cytoplasm. In addition, secreted PRRs can be found in blood and interstitial fluids. The concept of innate immune cells expressing PRRs that recognize conserved bacterial products was first introduced by the late Charles Janeway in 1989 and revolutionized the field of

immunology[1]. In addition to PAMPs and first proposed in 1994 by Polly Matzinger in her "Danger Theory", it is now acknowledged that innate immune cells can also recognize endogenous molecules, termed damage-associated molecular patterns (DAMPs), that are released upon tissue injury caused either mechanically, by an invading microorganism or particle or as a result of inflammation[2,3]. DAMPs include metabolites like uric acid and high concentrations of adenosine 5'-triphosphate (ATP), heat shock proteins (HSPs), nucleosomes as well as so-called alarmins such as interleukin (IL)-33, high mobility group box 1 (HMGB1), IL-1α and S100 proteins[4]. Once activated, innate immune cells will initiate an antiviral or inflammatory defense program involving production and secretion of immune mediators such as cytokines and chemokines to activate and recruit additional immune cells. Inflammation involves the recruitment of leukocytes, mainly phagocytes such as neutrophils and monocytes, as well as blood proteins to the affected tissue. Recruited cells subsequently get activated in the target tissue in order to eliminate the causative insult. In the case of phagocytes, they will ingest microbes and dead cells and neutralize them in intracellular vesicles in order to clear the infection. Antiviral responses on the other hand comprise the acquisition of an antiviral state within cells to render them resistant to viral infection as well as involve the destruction of infected cells by cytotoxic NK cells.

Many pathogens have developed strategies to evade clearance by the innate immune system and require the more potent adaptive immune response for elimination. In contrast to the innate response, the adaptive response is highly specific and can distinguish between a large number of microbial as well as non-microbial agents. This specificity is achieved by utilizing antigen receptors generated by somatic recombination. While components of the innate immune system such as PRRs can be found throughout the whole animal kingdom as well as in plants, the adaptive immune system evolved around 500 million years ago in jawed fish with the emergence of the recombination-activating gene (RAG) transposon whose gene product mediates the somatic recombination of antigen receptors[5]. In addition, a primitive non-RAG-based adaptive immune system in jawless fish such as lampreys and hagfish has arisen by convergent evolution with leucine-rich repeat (LRR) based lymphocyte receptors[6].

Adaptive immunity can be subdivided into cell-mediated immunity which is conferred by subsets of activated T lymphocytes and humoral immunity mediated by antibodies that are generated by activated B lymphocytes upon recognition of cognate antigen (*Figure 1*). Naïve adaptive lymphocytes that exhibit specificity for a large number of antigens are already present at low frequencies in the body independently of exposure and circulate to maximize the potential of microbe recognition. While B cells can directly respond to native antigens using their B cell receptor (BCR), T lymphocytes are only able to recognize processed antigen displayed on an antigen presenting cell (APC). Professional APCs such as DCs, which can be found in epithelial linings as well as connective tissues and lymphoid organs, constantly sample their environment for potential threats. If they encounter a microbe, they capture it, digest its proteins into peptides and display them on their surface in complex with a member of the major histocompatibility complex (MHC) family. To enhance the chances of encounter of a specific adaptive immune cell with cognate antigen from a peripheral anatomical location, activated APCs migrate to the nearest draining lymph node (LN) or spleen to display their antigenic peptide-MHC cargo to circulating T lymphocytes. When a naïve T cell recognizes its cognate peptide-MHC complex on an APC with its T cell receptor (TCR) and receives additional stimulation by costimulatory molecules expressed on the activated APC, it starts to proliferate and differentiates into an effector T lymphocyte. Here, $CD8^+$ T cells proliferate and differentiate into cytotoxic T lymphocytes (CTLs) that kill cells infected with virus or harbouring intracellular bacteria thereby eliminating the pathogenic reservoir. On the contrary, $CD4^+$ helper T cells expand and differentiate into effector cells that mediate their functions mainly by cytokine secretion. They produce the growth factor IL-2 upon activation to aid their proliferation and can either stay in the LN to stimulate B cell responses or migrate to the site of infection where they further exert their effector functions upon cognate antigen encounter. Here, they can produce cytokines that activate tissue B cells or innate immune cells such as phagocytes and NK cells to boost their effector potential or to recruit additional immune cells to the site of insult.

B cells can either become activated T cell-independently by non-protein antigens such as lipids and polysaccharides or in a T cell-dependent manner by protein antigens which is followed by proliferation and differentiation into antibody-secreting effectors, so-called plasma cells. The generated immunoglobulin (Ig) antibodies have the same specificity as the BCR of the original cell with five functionally different Ig isotypes in mammals: IgA, IgD, IgE, IgG and IgM. While

non-protein antigens mainly stimulate production of IgM antibodies, class switch recombination (CSR) to and production of IgA, IgG and IgE antibodies is primarily induced by protein antigens in a T cell-dependent fashion. In addition to CSR, helper T cells aid B cells in producing antibodies of higher antigen affinity, so-called affinity maturation, to enhance the quality of the humoral response. Depending on their isotype, antibodies serve different functions. Both IgM and IgG can activate the complement system to promote phagocytosis and elimination of microbes while IgG can additionally coat microbes directly to tag them for uptake by phagocytic cells. IgA is the dominant Ig isotype in mucosal settings and promotes immune exclusion at these sites by neutralizing luminal microbes within the respiratory and gastrointestinal tract while IgE mediates allergic responses.

Another hallmark of the adaptive response is the generation of immunological memory conferred by long-lived B and T memory cells which mediate a more rapid and potentiated response when encountering the same antigen at a later time point and is successfully exploited with vaccination strategies. Importantly, innate and adaptive immune responses are highly interconnected and act in concert, with the innate immune response stimulating the adaptive response and *vice versa* the adaptive response potentiating the innate response.

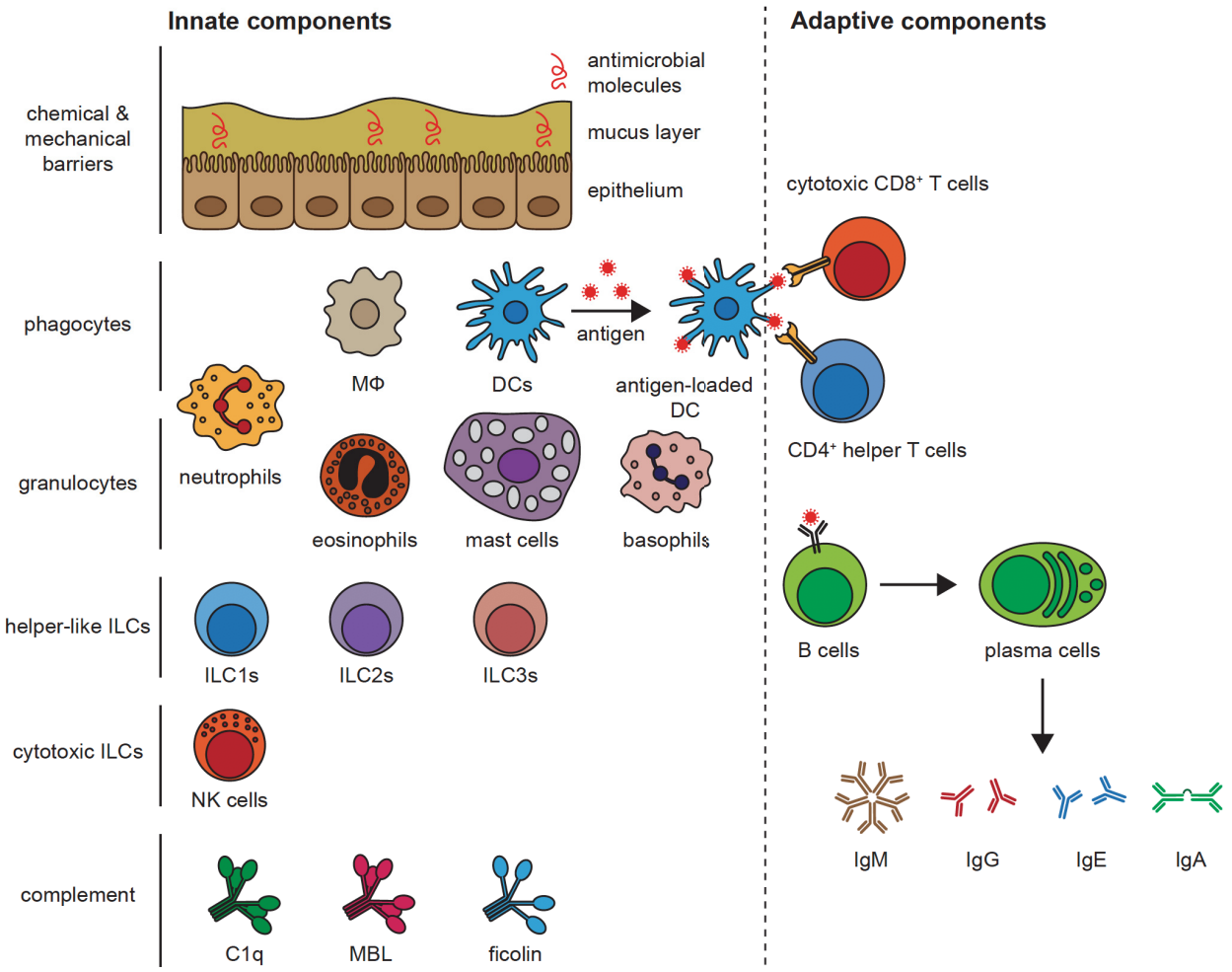

Figure 1. Cellular and molecular components of innate and adaptive immunity.

Mechanical barriers such as mucosal epithelial linings secrete mucus and/or antimicrobial molecules to provide physical and chemical protection against invading microbes. If these barriers are breached, tissue-resident innate immune cells such as macrophages, DCs as well cytotoxic and helper-like ILC family members and granulocytes will initiate inflammatory or antiviral effector responses to contain and eliminate the causative insult. If clearance by tissue-resident cells is evaded and the microbe enters the blood stream it can be recognized and ultimately eradicated by circulating proteins such as C1q, MBL and ficolin that activate the complement cascade. If innate immunity fails to eliminate the underlying insult, professional antigen-presenting cells such as DCs display antigen in complex with respective MHC molecules to $CD8^+$ and $CD4^+$ T lymphocytes resulting in their activation and initiation of effector functions. In addition, activated B cells mediate antigen-specific humoral immune responses by producing antibodies of IgM, IgG, IgE or IgA isotypes. Abbreviations: MΦ, macrophages; DCs, dendritic cells; ILC1s, ILC2s, ILC3s, group 1/2/3 innate lymphoid cells; NK cells, natural killer cells; MBL, mannose-binding lectin; Ig, immunoglobulin.

Besides innate and adaptive immunity, where classification is based on specificity of the involved immune cells and the kinetics of the response, a second system of immune categorization exists. Here, immune responses are broadly grouped into three main categories - type 1, type 2 and type 3 – based on the phenotype of the response which can be initiated by adaptive T helper cells as well as ILCs.

1.2. Innate lymphoid cells (ILCs) - early mediators of immunity

Innate lymphoid cells (ILCs) are lymphocytes that lack the expression of rearranged antigen receptors and get activated by environmental cues such as cytokines and alarmins provided by tissue-resident cells rather than antigen directly. ILCs exhibit striking functional similarities to T lymphocytes and can be perceived as their innate counterparts. While mounting an efficient T cell response can take several days due to the necessity of T cells to undergo clonal expansion, ILCs can act very early upon immune challenge due to the nature of their activating signals. They are predominantly tissue-resident, are present at relatively low numbers at steady state at various anatomical locations like the spleen, thymus, adipose tissue, and meninges and are enriched at barrier surfaces such as the lung, small intestine and skin[7-15]. Upon activation, they rapidly proliferate and secrete large amounts of their respective signature cytokines. ILCs and ILC-derived cytokines have been implicated in numerous physiological and pathophysiological processes including the regulation of inflammatory responses, orchestration of immune responses to pathogens, commensals and innocuous agents, amplification of antigen-specific adaptive immune responses but also in the maintenance of tissue homeostasis, repair and remodeling, as well as the integration of neuronal cues and whole body metabolism.

In accordance with the International Union of Immunological Societies (IUIS) and based on their development, expression of key transcription factors which regulate production of signature cytokines and respective biological functions, ILCs can be classified into five subsets – cytotoxic NK cells, non-cytotoxic helper-like ILC1, ILC2, ILC3 and lymphoid tissue inducer (LTi) cells which promote lymphoid tissue organogenesis during fetal development[16]. A unified nomenclature[17] defines group 1 ILCs (ILC1) as ILCs expressing *Tbx21* (encoding T box protein expressed in T cells, T-bet) and producing IFN-γ upon activation[18-21], ILC2s as expressing high levels of *Gata3* (encoding GATA binding protein 3, GATA-3) and *Rora* (encoding retinoic acid

receptor-related orphan nuclear receptor α, RORα) and producing IL-5 and IL-13[7-9,22] and group 3 ILCs (ILC3s) as ILCs that express *Rorc* (encoding retinoic acid receptor-related orphan receptor γ, RORγt) and secrete IL-17A and IL-22[23-26]. Furthermore, Id3-dependent IL-10-producing ILCs (ILCregs) have been described and may represent an additional helper-like ILC subset with similar functions to regulatory T cells (Tregs)[27]. However, ILC2 have also been shown to be significant producers of IL-10[28,29], as such it remains to be determined whether ILCregs exert physiological significance *in vivo*. Importantly, each ILC subtype exhibits phenotypical diversity based on its anatomical location and microenvironment as well as a degree of plasticity.

A landmark publication by Tim Mossman and Robert Coffman in 1986 described that naïve murine and human $CD4^+$ T helper cells can polarize into functionally different T_H1 and T_H2 subsets during activation in response to distinct microenvironmental cues[30-32]. The corresponding type 1 and type 2 responses are specifically tailored to the nature of the underlying immune challenge and can be distinguished by the types of innate and adaptive immune cells involved, their cytokine and effector molecule profiles as well as the response-associated Ig isotypes produced. In 2005, a third subset of polarized helper T cells, termed T_H17 cells, was discovered and found to mediate so-called type 3 immune responses[33-36] together with T_H22 cells which were identified shortly after in 2009[37-39]. It is now established that all three responses can also be triggered by helper-like ILC populations. As mentioned previously, ILCs exhibit striking functional similarities to T lymphocytes and can be perceived as their innate counterparts with cNK cells mirroring cytotoxic $CD8^+$ T cells and non-cytotoxic helper-like ILC subsets closely mimicking respective $CD4^+$ T_H1, T_H2 and $T_H17/22$ cells (*Figure 2*).

Type 1 responses are initiated to defend the host against viruses and intracellular bacteria as well as against tumors and are characterized by a milieu that is skewed towards enhancing cytotoxic effector functions to eradicate infected cells and tumors. Type 1 immunity is orchestrated by IFN-γ-, tumor necrosis factor (TNF)-α- and lymphotoxin (LT)α-secreting T-bet^+ innate cNK cells, ILC1s, natural killer T (NKT) cells and γδ T cells as well as adaptive $CD4^+$ T_H1 cells and cytotoxic $CD8^+$ T cells[40,41]. Innate and adaptive type 1 immune responses are promoted and sustained by the myeloid cell-derived pro-inflammatory cytokines IL-12, IL-15 and IL-18 and ultimately result in the enhancement of cytotoxic functions of cNK and $CD8^+$ T cells as well as activation of macrophages and production of antibodies of the IgG2 and IgG3 isotypes. Besides

T-bet, which is the key driver for type 1 differentiation, additional signature transcription factors such as STAT4 and Eomesodermin (Eomes) are important for mounting an efficient type 1 response[42]. Dysregulated type 1 responses have been implicated in a variety of autoimmune and chronic inflammatory disorders including type 1 diabetes (T1D) and Crohn's disease[43].

Type 3 responses are mediated by RORγt$^+$ IL-17 and IL-22 producing innate ILC3s[23-26], NKT cells[44] and γδ T cells[45,46] as well as adaptive T_H17[33-36] and T_H22 cells[37-39] to counter infections with fungi and extracellular bacteria but also play an important role in tissue homeostasis and organogenesis. Innate and adaptive type 3 responses are promoted by the cytokines IL-1β, IL-18 and IL-23 which are produced by myeloid cells upon activation[35,47,48]. In addition, IL-6 and TGF-β have been shown to be implicated in type 17 cell development[49-51]. Secretion of IL-17 and IL-22 results in the activation of mononuclear phagocytes, production of pro-inflammatory cytokines and chemokines and neutrophil recruitment as well as activation of antimicrobial peptide production by epithelial cells to promote the resolution of infection[52-55]. While RORγt is the master transcription factor driving ILC3 and T_H17 development, aryl hydrocarbon receptor (AhR) is particularly important for IL-22 production in both ILC3 and T_H22 cells[56-60]. In addition, STAT3 is essential for T_H17 lineage specification[61]. Dysregulation of type 3 immunity is implicated in autoimmune pathologies such as psoriasis as well as the multiple sclerosis model experimental autoimmune encephalomyelitis (EAE) and Crohn's disease[42].

Type 2 immune responses evolved to provide protection against extracellular parasites including helminths but they also mediate responses to innocuous agents such as allergens as well as tissue remodeling. Type 2 immunity is characterized by the secretion of the type 2 signature cytokines IL-4, IL-5, IL-9 and IL-13 by innate ILC2s, NKT cells and adaptive T_H2 lymphocytes which mediate activation of mast cells, eosinophils and basophils as well as B cell class switch recombination to IgE and IgG1[52]. ILC2s can additionally secrete the epidermal-like growth factor amphiregulin (AREG) to promote tissue repair and remodelling[13,62]. Type 2 effector responses are dependent on the expression of the master regulator of type 2 immunity, GATA3. Importantly, polarization of activated T cells towards a T_H2 phenotype requires IL-4-dependent activation of STAT6 which induces GATA3 expression[63-65]. However, the role of STAT6 in ILC2s remains controversial. In contrast to T cells, ILC2 become activated by alarmins such as IL-33 released upon tissue injury. Pathologies associated with dysregulated type 2 immune responses,

commonly termed atopic diseases, include allergic asthma, chronic rhinosinusitis (CRS), atopic dermatitis (AD) and anaphylactic allergic reactions[66].

In addition, type 1, type 2 and type 3 responses can be negatively regulated by the anti-inflammatory cytokines IL-10 and TGF-β produced by regulatory T cells (Tregs), regulatory ILCs (ILCregs) as well as myeloid and innate lymphoid cell populations[27,67,68].

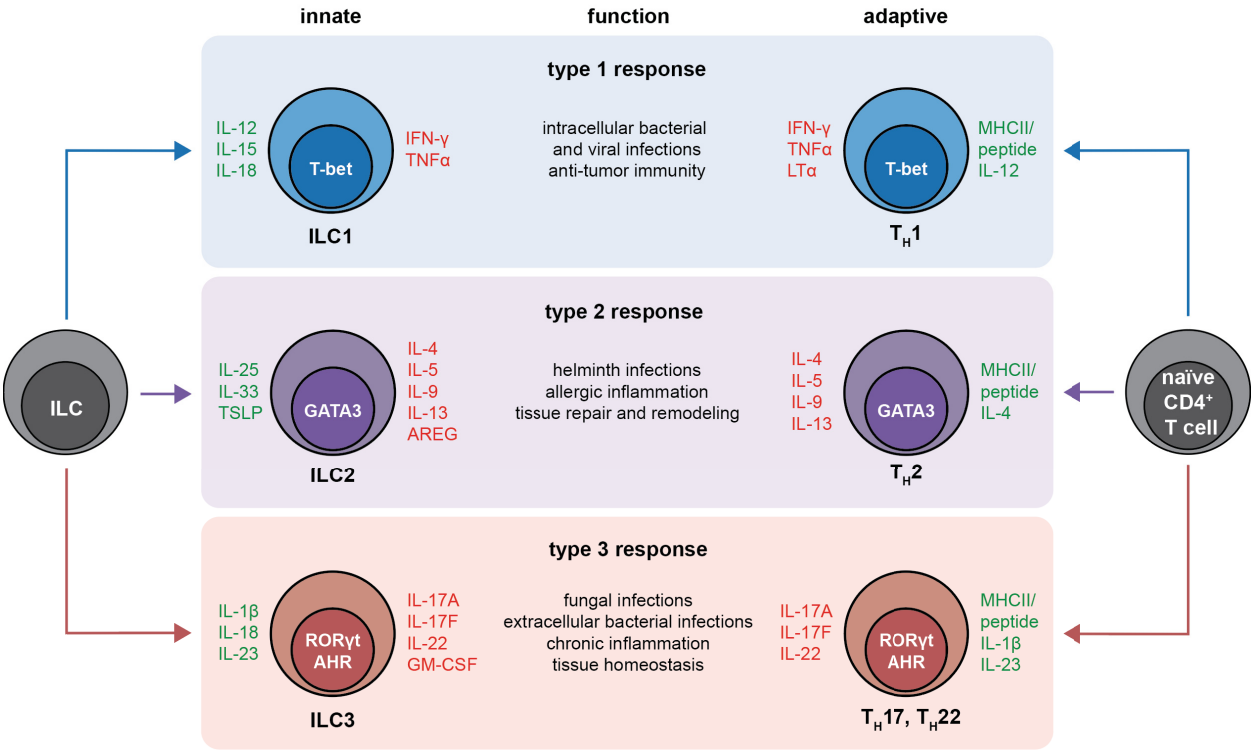

Figure 2. Helper-like innate lymphoid cells exhibit functional similarities to adaptive helper T cell subsets.
To initiate type 1, type 2 or type 3 immune responses, ILC1s, ILC2s or ILC3s are activated directly by respective pro-inflammatory mediators and alarmins (green) and rapidly produce signature effector cytokines (red). Adaptive naïve $CD4^+$ helper T cells require presentation of cognate peptide-antigen in complex with MHCII in addition to subset-defining polarizing cytokines (green) to differentiate into T_H1, T_H2 or T_H17 effector cells and secrete signature cytokines (red). T-bet dependent ILC1 mirror T_H1 functions while GATA3-dependent ILC2 and RORγt-depending ILC3 can be seen as the innate counterpart to T_H2 and T_H17 cells, respectively. Abbreviations: ILC, innate lymphoid cell; T-bet, T box protein expressed in T cells; GATA3, GATA-binding protein 3; RORγt, retinoic acid receptor-related orphan receptor γ; AhR, aryl hydrocarbon receptor; IL-, interleukin-; TSLP, thymic stromal lymphopoietin; AREG, amphiregulin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; TNFα, tumor necrosis factor α; LTα, lymphotoxin α; MHCII, major histocompatibility complex class II.

1.3. Group 2 innate lymphoid cells – novel players in allergy and atopic disease

1.3.1. The discovery and rise of group 2 innate lymphoid cells

The notion of an innate cell population producing type 2 signature cytokines emerged as early as 2001 with the discovery of the alarmin IL-25 and the identification of a splenic lineage-negative population secreting IL-5 and IL-13 after *in vitro* IL-25 stimulation[69]. The same study further showed that systemic *in vivo* administration of IL-25 in mice induces eosinophilia and mucus hyperproduction in the lung and digestive tract in a dose-dependent manner through induction of IL-5 and IL-13. Importantly, a similar phenotype was observed in RAG2-deficient animals lacking adaptive immune cells. Further supporting evidence was published in 2006 by the Locksley and McKenzie groups. The Locksley lab observed that innate rather than T cell-derived IL-4 and IL-13 of hematopoietic non-eosinophilic origin were required for worm expulsion following *Nippostrongylus brasiliensis* infection, a model for human hookworm infection, and that parasite migration was essential to mount an efficient anti-helminth response[70]. Again, using *N. brasiliensis* infection as a model, the McKenzie group additionally showed that an IL-25-dependent non-T/non-B cell c-Kit^{+}FcεR1^{-} population that produced IL-4, IL-5 and IL-13 was induced during infection and that *Il25*$^{-/-}$ mice failed to efficiently clear the parasite. Strikingly, administration of IL-25 accelerated worm expulsion independently of adaptive immunity in *Rag1*$^{-/-}$ animals, which was dependent on downstream induction of type 2 cytokines[71]. Another study in 2008 by the Nakanishi group demonstrated that, similar to IL-25, intranasal (i.n.) administration of the alarmin IL-33 induced airway hyperreactivity and goblet cell hyperplasia in RAG2-deficient mice further supporting the notion of an alarmin-induced innate non-B/non-T source of type 2 signature cytokines[72]. These innate type 2 cytokine producers were finally identified in mice in 2010 independently by three different groups and initially termed “natural helper cells”, “nuocytes” or “innate helper type 2 (Ih2) cells” and subsequently renamed to “group 2 innate lymphoid cells”[7-9].

The Koyasu group identified “natural helper cells” in fat-associated lymphoid clusters (FALCs) of the mesentery[7]. They defined this population as Lin^{-}c-Kit^{+}Sca-1^{+}IL-7Rα^{+}IL-33R^{+} lymphoid cells that also expressed high levels of CD25 and proliferated in response to IL-2 *in vitro*. In addition, they observed that differentiation of natural helper cells was mediated by IL-

7 due to their absence in common γ chain-deficient mice (*Il2rg*$^{-/-}$) as well as *Il7*$^{-/-}$ mice. IL-7 and stem cell factor (SCF) promoted natural helper cell survival and while little IFN-γ and no IL-17 could be detected in culture supernatants, high levels of the type 2 signature cytokines IL-5 and IL-13 could be measured after *in vitro* stimulation with IL-33 and IL-2 in combination with IL-25, supporting their potential role as innate type 2 cells. Furthermore, systemic IL-33 administration and IL-33 released after *N. brasiliensis* infection induced type 2 cytokine production by natural helper cells as well as goblet cell hyperplasia *in vivo*.

Using *Il13*-eGFP reporter mice, the McKenzie lab described an inducible eGFP^{+} population in the mesenteric LN (mesLN), intestine and spleen of mice after systemic administration of IL-25 or IL-33[9]. The majority of these cells, termed nuocytes, were Lin^{-} T1/ST2^{+} (IL-33R) and IL17BR^{+} (IL-25R) indicating that they can directly respond to IL-25 and IL-33. They also expressed high levels of inducible T cell co-stimulator (ICOS), IL-7Rα, MHC-II as well as Sca-1 and c-Kit, in line with the observations by the Koyasu group. Nuocytes were induced upon *N. brasiliensis* infection and mice deficient in IL-25R signaling (*Il17rb*$^{-/-}$) exhibited severely impaired eosinophil recruitment and worm clearance as well as antigen-specific adaptive T cell immunity which were all restored upon adoptive transfer of wild-type (WT) nuocytes. Importantly, nuocytes represented the major *Il13* expressing population early after infection with *N. brasiliensis* and when WT nuocytes were transferred into *Il4*$^{-/-}$*Il13*$^{-/-}$ mice they efficiently induced worm expulsion indicating that nuocyte-derived IL-13 is essential for parasite clearance. The authors further showed that purified nuocytes stimulated with IL-7 and IL-33 *in vitro* produced substantial amounts of the type 2 signature cytokines IL-5 and IL-13 as well as IL-6, IL-10 and GM-CSF.

In addition to these reports, the Locksley lab utilized IL-4 and IL-13 reporter mice to track type 2 cytokine producing lineage-negative innate cells, which they designated “innate helper type 2 (Ih2) cells”, that arose during infection with *N. brasiliensis* as well as after systemic administration of IL-25 and IL-33. In accordance with the other two groups they observed that Lin^{-}c^{-}kit^{+} Ih2 cells were the main innate IL-13 producers after IL-25 administration as well as helminth infection. While a substantial amount of Ih2 cells also produced IL-5, no protein production of IL-4 could be observed despite high levels of mRNA expression. They furthermore showed that Ih2 cells were prevalent in many organs at steady state, including the liver, spleen, mesLN, bone marrow (BM), peritoneum and lung and that

they expanded in all analyzed organs upon systemic administration of IL-25 or IL-33 as well as *N. brasiliensis* infection.

In 2011, one year after the discovery of mouse ILC2s, human ILC2s were identified in adult lung as well as adult and fetal gut and nasal polyps by the Spits group[22]. They were defined as $Lin^{-}CD127^{+}CD161^{+}$ cells that express the CRTH2 receptor and were further shown to produce IL-13 upon *in vitro* stimulation with IL-25 or IL-33.

Since their identification, extensive research resulted in rapid advancements in the field of ILC2 biology with a steady incline in relevant publications over the past years and in our understanding on how their effector mechanisms regulate homeostasis, anti-helminth immunity and atopic disease.

1.3.2. ILC2 development and ontogeny

1.3.2.1. Overview of ILC2 development

Like all other hematopoietic lineages, ILCs are initially generated from multipotent hematopoietic stem cells (HSCs) in primary lymphoid organs and, similar to T cells, ILC progenitors differentiate into specific ILC lineages via developmental intermediates that progressively lose alternative lineage potential (*Figure 3*). Common murine ILC progenitors that can generate all ILC lineages *in vitro* and *in vivo* following transfer into lymphopenic mice have been identified in the mouse fetal liver and gut as well as adult bone marrow[73-78]. Like adaptive B and T lymphocytes, ILCs develop from the $Lin^{-}CD127^{+}Flt3^{+}\alpha_4\beta_7^{-}c\text{-}kit^{int}$ common lymphoid progenitor (CLP) generated from HSCs via lymphopoiesis and more specifically arise from the $Lin^{-}Id2^{+}CXCR6^{+}IL\text{-}7R\alpha^{+}\alpha_4\beta_7^{+}$ α-lymphoid progenitor (αLP) and/or the $Lin^{-}Id2^{low/-}IL\text{-}7R\alpha^{low}\alpha_4\beta_7^{+}$ early innate lymphoid progenitor (EILP), commonly referred to as common ILC progenitors (GILPs)[76,78-81]. While αLP as well as EILP lost the potential to differentiate into T or B cells, both progenitor populations can generate NK cells as well as all helper-like ILC lineages[76,79-81]. However, their precursor-progeny relationship or if they represent two distinct yet complementary developmental pathways remains elusive. The EILP itself undergoes two successive stages of development, from the specified EILP (sEILP), which can give rise to DCs as well as ILCs, to the committed EILP (cEILP), which has been suggested to be the first uniquely determined ILC progenitor[82]. The EILP/αLP subsequently differentiates to the $CD25^{-}Id2^{+}IL\text{-}7R\alpha^{+}\alpha_4\beta_7^{+}$

common helper ILC progenitor (CHILP) that maintains the potential to generate all helper ILC lineages but lacks the ability to give rise to NK cells which in turn develop from NK1.1^{-}CD122^{+} NK progenitors (NKP)[77,83]. The CHILP population is not homogenous and can be stratified based on the expression of the transcription factor promyelocytic leukemia zinc finger (PLZF) into PD-1^{+}PLZF^{+} ILC precursors (ILCPs) that can generate ILC1s, ILC2s and CCR6^{+} ILC3s as well as NK1.1^{+}DX5^{-} NK-like cells and a PLZF^{-} population that gives rise to the lymphoid tissue inducer progenitors (LTiPs) that subsequently develop into LTi cells[76,77,80,84]. ILCPs exhibit a phase of multi-lineage priming, co-expressing genes for different ILC lineages and it was proposed that the ultimate commitment to one lineage over another is mediated by environmental as well as internal signals that tune down alternative developmental programs[78].

The general model of ILC development which implies that the cNK cell lineage follows a distinct developmental pathway that diverges before the CHILP stage has been challenged by two recent publications[85,86]. Here, utilizing a novel more sensitive Id2^{RFP} reporter mouse model over the previously used Id2^{GFP} animals, the DiSanto group showed that the PLZF^{+} ILCP population exhibits a large degree of heterogeneity and harbors functional precursors for both cNK and helper-like ILC populations that can be stratified by the expression of *Zbtb16* (encoding PLZF) and *Bcl11b* thereby redefining the ILCP as a multipotent common ILC precursor (CILCP)[85]. The McKenzie lab obtained similar results, identifying ILCPs that retain some NK lineage potential by utilizing polychromic reporter mice that allow tracing the simultaneous expression of the key ILC transcription factors Id2, GATA3, RORα, RORγt and Bcl11b[86]. These very recent developments highlight that, with the continuous advancement of technologies and resources at hand, our understanding of ILC development is still relatively limited and more comprehensive experiments are needed to understand the detailed underlying mechanisms. Hereafter, I will discuss the transcription factors that regulate and control murine ILC development with a focus on ILC2s.

1.3.2.2 Transcriptional regulation of early ILC development

Transcription factors can either enhance or inhibit the expression of target genes during development and thereby promote specific lineages at the expense of others. While the exact transcriptional hierarchy and essential targets of each transcription factor required for ILC development still remain elusive, it is becoming increasingly evident that many transcriptional regulators that are involved in the generation of T lymphocytes are also associated with ILC development, again underlining the close relationship between these populations. Early ILC differentiation is highly controlled by the coordinated expression of the lineage-determining transcription factors nuclear factor IL-3 induced (NFIL3), thymocyte selection-associated high mobility group box protein (TOX), T cell factor 1 (TCF-1) and inhibitor of DNA-binding 2 (Id2) as well as exogenous tissue-specific stimuli such as Notch ligands.

The basic leucine zipper transcription factor NFIL3 has been shown to be the earliest critical initiator of ILC lineage commitment by driving the transition of the CLP towards the αLP. NFIL3 is expressed from the CLP stage on and while *Nfil3*$^{-/-}$ mice exhibited normal numbers of CLPs they harbored significantly lower numbers of αLPs as well as a severe reduction in more committed ILC progenitors such as the CHILP and ILC2P and consequently all mature ILC subsets[79,87-89]. Mechanistically, *Nfil3*$^{-/-}$ CLPs exhibited reduced expression of TOX, a critical regulator of T cell differentiation, and NFIL3 was shown to directly bind to the *Tox* promoter[79]. Furthermore, restoration of TOX expression in *Nfil3*$^{-/-}$ Lin-Sca-1^{+}c-kit^{+} (LSK) stem cells and subsequent adoptive transfer into irradiated recipient mice increased numbers of cNK, ILC1, ILC2 as well as ILC3 when compared to animals that received control *Nfil3*$^{-/-}$ LSK cells. This data indicates that NFIL3 induces TOX expression that in turn drives ILC development downstream of NFIL3. An additional study showed that NFIL3 can also directly regulate the expression of Id2 at the CHILP stage and downstream PLZF expression[89]. Here, transduction of *Nfil3*$^{-/-}$ ILC precursors with Id2 rescued numbers of helper-like ILCs *in vivo* thereby indicating that NFIL3 may act via different mechanisms depending on the stage of ILC development. Taken together, NFIL3 drives ILC lineage commitment of the αLP by inducing the expression of the key ILC transcription factors TOX and Id2.

Two additional studies further highlighted the role of TOX during early ILC development demonstrating that *Tox*$^{-/-}$ mice harboured normal numbers of CLPs but exhibited greatly reduced numbers of CHILPs and ILCPs and thus lacked all mature ILC subsets[90,91].

Finally, a more recent publication identified EILPs as the stage of developmental block in the absence of TOX with *Tox*$^{-/-}$ mice exhibiting severely reduced numbers of EILPs[92]. Mechanistically, the expression of the Notch target *Tcf7,* encoding TCF-1, a critical factor in T lineage specification and commitment, was diminished in *Tox*$^{-/-}$ mice as were the TCF-1 target genes *Gata3* and *Bcl11b*[91,93,94]. The authors therefore proposed that TOX might relay Notch signaling to induce TCF-1 expression and further commitment to the ILC lineage. TCF-1 in turn is highly expressed in EILPs and, while dispensable for the generation of sEILPs, is essential for the development of cEILPs[82]. In accordance, mice lacking TCF-1 (*Tcf7*$^{-/-}$) exhibited reduced numbers of all mature ILC subsets as well as CHILPs and NKPs indicating an essential role for TCF-1 in early ILC development[95,96]. Both studies further showed that TCF-1 acts downstream of Notch signaling during ILC2 and ILC3 development. In addition, forced expression of TCF-1 in LSK cells expressing the pan-Notch inhibitor dominant-negative Mastermind-like 1 (dnMAML) partially rescued their potential to give rise to ILC2 *in vivo*[95]. Furthermore, TCF-1 regulates *Il7r* expression directly as well as expression of *Il2ra* and *Il17rb* in a GATA3-dependent manner[95]. Taken together, early steps of ILC development involve the transcription factors NFIL3, TOX and TCF-1 with NFIL3 inducing expression of TOX, which in turn regulates expression of TCF-1 to drive ILC lineage commitment.

Another essential regulator for ILC development, the helix-loop helix transcription factor Id2, is induced immediately downstream of the CLP and is constitutively expressed in all mature ILC subsets[7,77,78,80,97-99]. Id2 is a member of the Id family of transcriptional repressors that lack DNA-binding domains but can form heterodimers with E protein family transcription factors such as E2A, E2-2 and HEB and thereby functionally inactivate them[100]. E proteins are indispensable for the development of T and B lymphocytes and regulate genes involved in lineage specification and commitment as well as rearrangement of antigen receptors[101]. Mechanistically, a recent study showed that thymic E2A and Id2 levels are inversely correlated in ILC precursors and that E2A and HEB synergistically enforce T cell lineage commitment by inducing a T cell-specific enhancer repertoire while suppressing an ILC signature-biased regulome[102]. In accordance, using transgenic mice that ectopically express the Id family member Id1 under the *Lck* promoter, thereby facilitating inhibition of E proteins led to expansion of ILC2 in thymus, lung and other organs[103]. Thus, Id2 upregulation can generally be regarded as a marker for the establishment of ILC fate and restriction of T and B potential.

To this end, Id2-reporter mice are widely used to track ILCs and analyze their development. Besides NFIL3, which can bind to the Id2 promotor in CHILPs, the environmental or transcriptional cues that drive Id2 expression early during ILC development have not yet been characterized.

The transition from the EILP/αLP to the CHILP in the bone marrow might be at least partly regulated by the transcription factor avian erythroblastosis virus E26 homolog-1 (ETS1). Using mice that carry a conditional deletion of *Ets1* in *Il7ra*$^{+}$ cells, a recent study demonstrated that while CHILPs can be generated in these mice, ETS1 is essential for CHILP fitness and its ability to generate ILC2s by sustaining expression of Id2[104].

Expression of the type 2 transcription factor GATA3 increases with the developmental progression of the CLP to mature ILC2s. However, GATA3-deficiency only modestly affected EILP numbers. In contrast, GATA3 was shown to be essential for the generation of CHILPs and consequently all mature helper-like ILC subsets, while being dispensable for the development of cNK cells[75,77,92,105-110]. Hence, GATA3 is not only the transcriptional master regulator of ILC2 generation but an essential driver of common helper-like ILC development in general.

Using reporter and/or fate-mapping approaches it was originally proposed that expression of PLZF distinguishes the ILCP from the CHILP subpopulation that gives rise to LTi cells[76,77]. PLZF was further shown to be co-expressed with GATA3 in ILCPs and while ILC2Ps and mature ILC2s retained and upregulated GATA3 expression, PLZF was only transiently expressed and absent in mature ILCs[76]. Conversely, a recent study using *Zbtb16*-tdTomato reporter mice found high PLZF expression in all CHILPs as well as LTi cells, ILC1s and ILC3s but not in mature ILC2s[86]. In each case, the precise drivers of PLZF expression as well as its targets and function remain elusive and require further investigation.

1.3.2.3. Transcriptional regulation of ILC2 lineage commitment

In addition to the aforementioned transcription factors that drive the development of ILC progenitors, it is well established that differentiation of the ILCP via the ILC2P to functionally mature ILC2s involves the key transcription factors GATA3, RORα, TCF-1, B cell leukemia 11B (Bcl11b) and growth factor independent 1 (Gfi1)[75,95,105,106,111-116]. Besides GATA3, the transcriptional master regulator of type 2 immunity, that is able to directly induce expression of the type 2 signature cytokines IL-5 and IL-13 as well as the ligand-binding subunit of the IL-33 receptor (suppression of tumorigenicity 2, ST2), the precise mechanisms of how the above-mentioned transcriptional regulators promote ILC2 differentiation are not yet fully characterized[75,108].

TCF-1 can induce expression of ILC2 signature genes *Gata3*, *Bcl11b* and *Il17rb*, therefore, high expression of TCF-1 might predispose to ILC2 differentiation but its precise role in ILC2 development remains yet to be identified[95].

While RORα, a transcription factor of the nuclear hormone receptor superfamily, is highly expressed in all ILC subsets, except NK cells, "staggerer" mice harbouring a deletion within the *Rora* gene that results in a truncated non-functional gene product, specifically exhibited a reduction in ILC2 numbers and remaining ILC2s failed to respond to challenge with IL-25 or IL-33 and clear *N. brasiliensis* infection[86,111,112,117,118]. While induction of RORα has been suggested to involve GATA3, the precise mechanisms that govern RORα expression, its target genes and ILC2-intrinsic functions remain elusive[106].

The zinc finger transcription factor Gfi1 is essential for T lymphopoiesis, controls IL-2-mediated T_H2 expansion and promotes polarization of T_H2 cells by antagonizing TGF-β-induced T_H17 and Treg cell development[119-122]. During ILC2 development, Gfi1 expression gradually increases (CLP < ILC2P < mature ILC2s) and is positively correlated with ST2 expression[105]. Similar to T cells, it was found that Gfi1 controls ILC2 development as absence of Gfi1 resulted in reduced frequency of bone marrow ILC2Ps and mature lung ILC2s[105]. Notably, the remaining lung ILC2s failed to expand in response to IL-33, helminth infection or allergen challenge, indicating an additional role for Gfi1 in ILC2 activation. Mechanistically, the authors showed that *Gfi1*$^{-/-}$ ILC2s exhibited reduced expression of *Il1rl1* and corresponding surface ST2 as well as *Gata3* and *Il5* transcript expression which were further shown to be

directly regulated by Gfi1. On the other hand, genes associated with type 3 inflammation such as *Rorc*, *Il23r*, *Il17a* and *Il17f* were de-repressed and ILC2s lacking Gfi1 co-expressed IL-13, IL-17A and IL-17F. These data clearly demonstrate that Gfi1 activates type 2 while repressing type 3 signature genes, thereby maintaining ILC2 lineage commitment[105]. Interestingly, the histone methyltransferase G9a can interact with Gfi1 directly and was shown to be required for ILC2 development[123,124]. Absence of G9a was associated with the expression of type 3 signature genes in ILC2 and the repressive activity of Gfi1 in ILC2 may therefore be mediated by G9a.

Another zinc finger transcription factor, Bcl11b, can either act as a transcriptional repressor or activator and is important for T cell differentiation and lineage commitment[125-128]. In ILC development, Bcl11b expression in ILCPs in conjunction with high expression of GATA3 is associated with ILC2 lineage commitment and defines an ILC2 progenitor population that lost the ability to give rise to other helper ILC subsets[86,114,115]. Mature peripheral *Bcl11b*$^{-/-}$ ILC2s exhibited reduced expression of critical ILC2 lineage factors ST2, GATA3 and RORα while levels of RORγt and IL-23R, normally expressed in ILC3, were increased. In accordance with increased receptor expression, *Bcl11b*$^{-/-}$ ILC2s acquired responsiveness to IL-23 and produced type 3 cytokines IL-17 and IL-22 upon stimulation while they lost their ability to respond to IL-33[113]. Importantly, Bcl11b-deficient ILC2s exhibited a significant reduction in Gfi1 expression. Moreover, Bcl11b was found to bind directly to the Gfi1 promoter region and transduction of *Bcl11b*$^{-/-}$ ILC2s with Gfi1 resulted in upregulation of GATA3 and ST2 as well as a reduction in RORγt and IL-23R expression. It was therefore proposed that Bcl11b acts upstream of Gfi1 and maintains it expression[113].

Altogether, despite considerable progress, the detailed mechanisms that control the induction of specific transcriptional programs that govern ILC2 development are just starting to be revealed and will require yet more comprehensive studies.

1.3.2.4. Regulation of ILC2 development by environmental factors

All lymphocytes, except for IL-15-dependent cNK cells require the cytokine IL-7 for development and differentiation[129,130]. As mentioned earlier, IL-7 is indispensable for ILC2 development with ILC2s being absent in mice deficient in the IL-7R signaling component common gamma chain (γc) (*Il2rg*$^{-/-}$) or IL-7 (*Il7*$^{-/-}$)[7]. However, development of the αLP, CHILP and ILCP is taking place independently of IL-7R or other cytokines that signal via γc, as numbers of these progenitors remained unaffected in *Il2rg*$^{-/-}$ mice, indicating that IL-7 might be required at later stages of ILC development[75,92,131]. Interestingly, it has been shown recently that ILC2s can also develop in an IL-7-independent manner. Here, residual ILC2s can persist in the small intestinal lamina propria (siLP) of *Il7ra*$^{-/-}$ mice due to IL-15 partially compensating for the loss of IL-7 signals[131]. In humans, cNK cells and all helper-like ILCs were absent in patients with severe combined immunodeficiency (SCID) carrying mutations within the gene encoding for γc which indicates differential usage of IL-7 or other γc cytokines between mouse and humans[132].

Previous studies have demonstrated that signaling via the cell surface receptor Notch is essential for T lineage commitment with high Notch amounts favoring the generation of αβ T cells while low levels result in generation of γδ T cells[133,134]. While the Notch pathway was shown to be essential for ILC2 differentiation *in vitro*, partly by inducing TCF-1, the physiological role of Notch signaling in ILC2 generation *in vivo* remains elusive[20,95,111,135,136]. A recent observation proposed that Notch signal strength is a critical determinant in the decision of T cell versus ILC2 fate in the fetal liver and that generation of ILC2s requires short exposition of CLPs to intermediary Notch amounts in combination with high amounts of IL-7. In contrast, high and continuous exposition to Notch favored T cell commitment[137,138]. The proposed role for IL-7 in early fetal liver ILC development is conflicting with the fact that *Il2rg*$^{-/-}$ mice exhibit no apparent difference in numbers of αLPs, CHILPs or ILCPs in the bone marrow, suggesting differential environmental requirements of fetal liver and bone marrow progenitors[92]. However, the necessity for IL-7 for fetal liver ILC development was demonstrated in an *in vitro* system and thus might not be representative of physiological processes *in vivo*. Since a STAT5 inhibitor abrogated ILC2 differentiation in this system it might as well be possible that other STAT5 activators that signal independently of γc drive ILC2 differentiation in the fetal liver *in vivo*. In

summary, environmental signals inducing Notch and STAT5 signaling play important roles in the development of ILC2s in both bone marrow and fetal liver but the detailed kinetic still remains ill-defined.

Human ILC development is less well characterized, but precursors capable of generating helper-like ILC1s, ILC2s, ILC3s, and NK cells, similar to the murine GILP, have been observed in cord and adult blood, fetal liver and secondary lymphoid organs[139,140]. A restricted human CHILP has not yet been identified and further work is required to elucidate the developmental trajectory for ILC2 in humans.

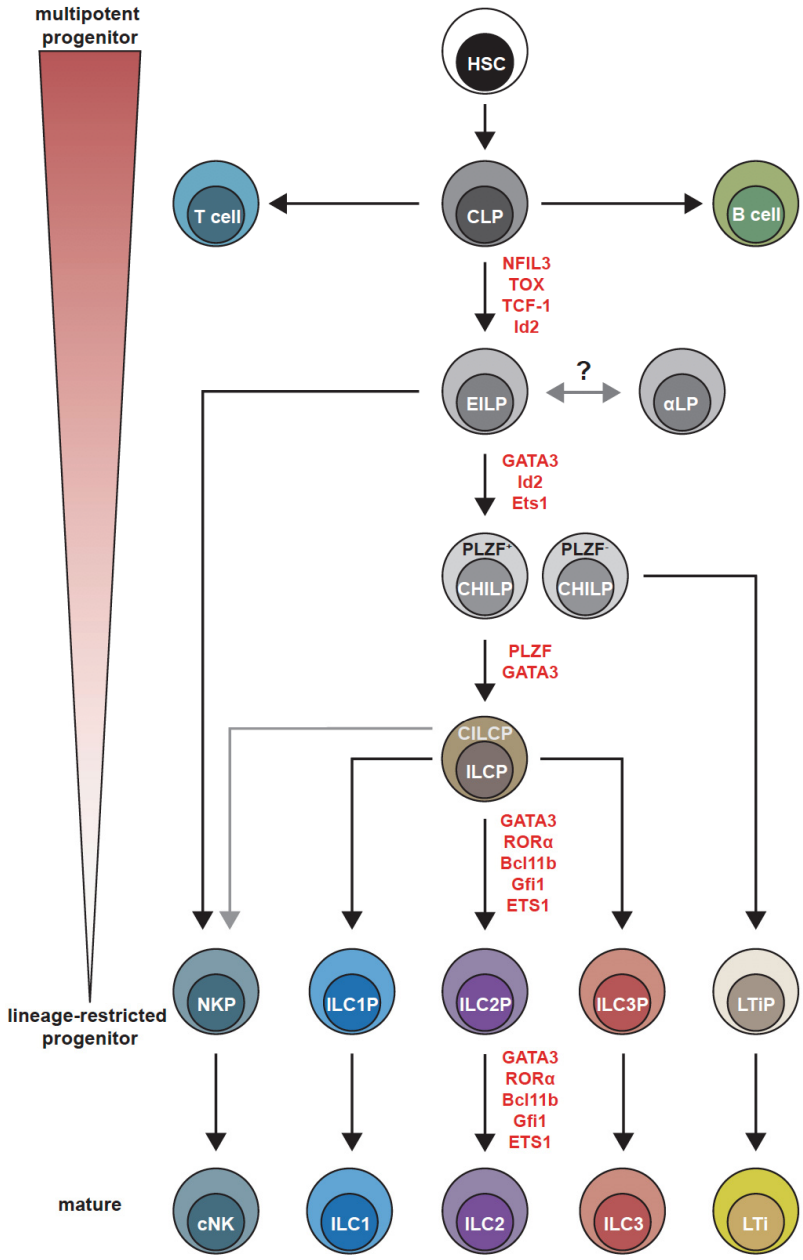

Figure 3. Regulation of murine ILC2 development.

Arrows indicate precursor-progeny relationship. Transcription factors that are essential for subsequent stages of ILC2 development are depicted in red. Abbreviations: HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; EILP, early innate lymphoid progenitor; αLP,$\alpha_4\beta_7^+$ lymphoid progenitor; CHILP, common helper ILC progenitor; ILCP, ILC precursor; NKP, NK cell precursor; cNK, conventional NK cell; ILC1/2/3P, group 1/2/3 innate lymphoid cell precursor; ILC1/2/3, group 1/2/3 innate lymphoid cell.

1.3.2.5. ILC2 ontogeny

ILC2 progenitors develop in the mouse during embryogenesis in the fetal liver[73,78] as well as during adult life in the bone marrow[75-77]. They subsequently home to peripheral tissues where they differentiate into mature effector cells in response to respective stimuli[73]. Within peripheral tissues ILC2s are maintained and controlled by environmental signals that mediate cellular homeostasis and effector functions. The generation of ILC2s follows similar developmental trajectories in both the embryo and adult mice where EILPs-αLPs, CHILPs, ILCPs and ILC2Ps with similar phenotypes and developmental potential have been identified.

In addition, emerging data suggests that thymic progenitors, generally associated with T lymphocyte development, can adopt an innate fate and differentiate into ILC2s[102,111,136,141]. The decision towards the ILC over a T cell fate is dependent on the balance between E proteins and Id transcription factor expression with E2A and HEB suppressing the induction of ILC2s whereas Id proteins promote ILC2 development[102]. In addition, Notch ligands were proposed to regulate thymic ILC2 development[138]. Functionally competent ILC2s have been found in human and mouse thymus where they gradually increase after birth to become the dominant thymic ILC population[102,111,136,141]. Furthermore, ILC2s can be generated *in vitro* through culture of thymic progenitors with IL-7 and IL-33 indicating that in addition to transcriptional control, environmental signals are important in establishing thymic ILC2 development[111,136]. However, the physiological relevance and function of thymic ILC2s is still relatively poorly understood. It has been recently demonstrated that iNKT cell-derived IL-4 and IL-13 act on thymic stromal cells and thereby regulate emigration of mature thymocytes. Hence, it has been suggested that ILC2s may represent an additional local source of type 2 cytokines to contribute to this process[141,142].

Besides bone marrow, fetal liver and thymus, multipotent ILCPs were characterized in the murine fetal intestine and ILC2Ps were described in the fetal mesentery, intestine and lung[73,80,138]. Moreover, CHILPs and ILC2Ps were identified in the neonatal spleen as well as neonatal lung in higher frequencies than in the bone marrow and it was thus proposed that the spleen and lung represent major sites of ILC2 generation shortly after birth with a subsequent shift to the bone marrow[143,144].

Importantly, in humans, a heterogeneous population of unipotent and multipotent ILCPs has been characterized in cord and adult blood as well as fetal liver and adult lung and tonsils[139].

These $Lin^{-}CD7^{+}CD127^{+}CD117^{+}$ ILCPs expressed transcription factors such as ID2, GATA3, TOX, TCF-1 and PLZF that are associated with early mouse ILC development and were able to generate tissue-resident ILC2s and other ILC subsets *in vitro* and after adoptive transfer to immunodeficient humanized mice. Due to the presence of multi-potent ILCPs in human tissues it was suggested that tissue ILC differentiation ('ILC-poiesis') might be a mechanism to replenish ILCs locally during homeostasis as well as after infection and inflammation.

A recent study identified circulating multipotent ILCPs in the peripheral blood of mice that were distinguishable from tissue-resident ILCs by expression of the secondary lymphoid organ homing receptor CD62L[145]. However, parabiosis experiments, in which the circular system of congenically marked mice is surgically conjoined, demonstrated minimal replacement of tissue ILC2s by peripheral blood cells under homeostatic conditions[146-148]. These data, in addition to slow turnover rates of tissue ILC2s, support the notion that mouse ILC2s are generally long-lived tissue-resident cells that are maintained during adulthood independently from bloodborne progenitors[10,146-149]. Thus, to what extent and under which conditions tissue-resident ILC2s are replenished by circulating immature precursors is still poorly understood.

Extrahepatic ILC2Ps and immature ILC2s were also characterized in the fetal mesentery, intestine and lung[138]. In addition, using *Arg1*^{YFP} reporter mice, a heterogenous population of multi- and uni-potent progenitors was identified in fetal intestinal tissue that was able to give rise to ILC2s and other helper-like ILC lineages[73,150]. It was therefore proposed that ILC progenitors originating from the fetal liver might seed peripheral tissues prenatally and that ILCs found in adult mice stem from fetally-generated precursors.

Follow-up work by the same group used complementary *Arg1*- and *Id2*-driven fate-mapping approaches in combination with transcriptomic profiling to decipher ILC2 ontogeny and turnover during prenatal, neonatal and adult stages in order to determine ILC2 origins and lifespans in different tissues[151]. In this seminal study the authors identified three chronologically distinct origins of tissue-resident ILC2s: (1) fetally-derived ILC2s that seed tissues before birth, (2) postnatally-derived ILC2s that develop from birth through weaning and (3) adult-derived ILC2s that slowly dilute out the former two groups. Furthermore, they demonstrated that homeostatic turnover of ILC2s in adult mice by *de novo* generation was tissue-specific and dynamic with slow replacement of ILC2s in lung, fat and small intestine and more rapid turnover and continuous expansion of ILC2 pools in skin and bone marrow (*Figure 4*). They

thereby further confirmed the general designation that adult tissue ILC2s are stable long-lived cells. In addition and in accordance with previous reports[10,148,152-154], the authors observed acute expansion and activation of ILC2s in peripheral tissues shortly after birth and further demonstrated that this neonatal wave of ILC2 expansion in lung, small intestine, skin and bone marrow was dependent on IL-7R signaling. Similar to adaptive lymphocytes, the authors showed that *Nr4a1* expression can serve as a proxy for the activation status of ILC2s and using *Nr4a1*-GFP mice they observed an increase in reporter expression during the early postnatal period in lung, small intestine and skin ILC2s which was maintained into adulthood but was nearly absent in fetal liver and BM ILC2s. This induction coincided with ILC2 proliferation and postnatal expression of effector cytokines such as IL-5 and thereby indicates that tissue ILC2s undergo a phase of activation shortly after birth, lasting until early adulthood. Moreover, single-cell RNA-sequencing analysis of early postnatal (2 weeks) and adult (8 – 14 weeks) ILC2s revealed that cells from the same tissue clustered together, indicating that tissue-specific gene expression signatures in ILC2s are established already early during the postnatal period and persist throughout life[155].

The importance of the perinatal period for the establishment of peripheral ILC2 niches has already been recognized by previous studies. Here it was shown that initial seeding of the lungs with ILC2s occurs in an IL-33-dependent manner within the first two weeks after birth and is associated with a wave of pulmonary IL-33 expression that might be induced by the onset of breathing[152-154]. Importantly, IL-33 can induce egress of ILC2Ps from the BM and IL-33R-deficient mice (*St2*$^{-/-}$) exhibited progressive accumulation of ILC2Ps in the BM, coinciding with reduced numbers of ILC2s in the lungs in the perinatal period[156]. These data suggest that initial seeding of peripheral tissues might be initiated by BM ILC2Ps in an IL-33-dependent manner. However, the precise contributions of BM progenitors versus precursors from other sites towards perinatal tissue seeding still need to be addressed. Nevertheless, as ILC2 expansion and activation occurs systemically in neonatal mice, albeit with different kinetics in different tissues, a common yet unidentified niche factor might be involved in shaping the general ILC2 effector repertoire.

In summary, ILC2s are first generated during fetal hematopoiesis and distributed into peripheral tissues during a perinatal window. Once in the tissue, ILC2s undergo rapid expansion, and acquire specific transcriptomic signatures associated with their tissue of residence.

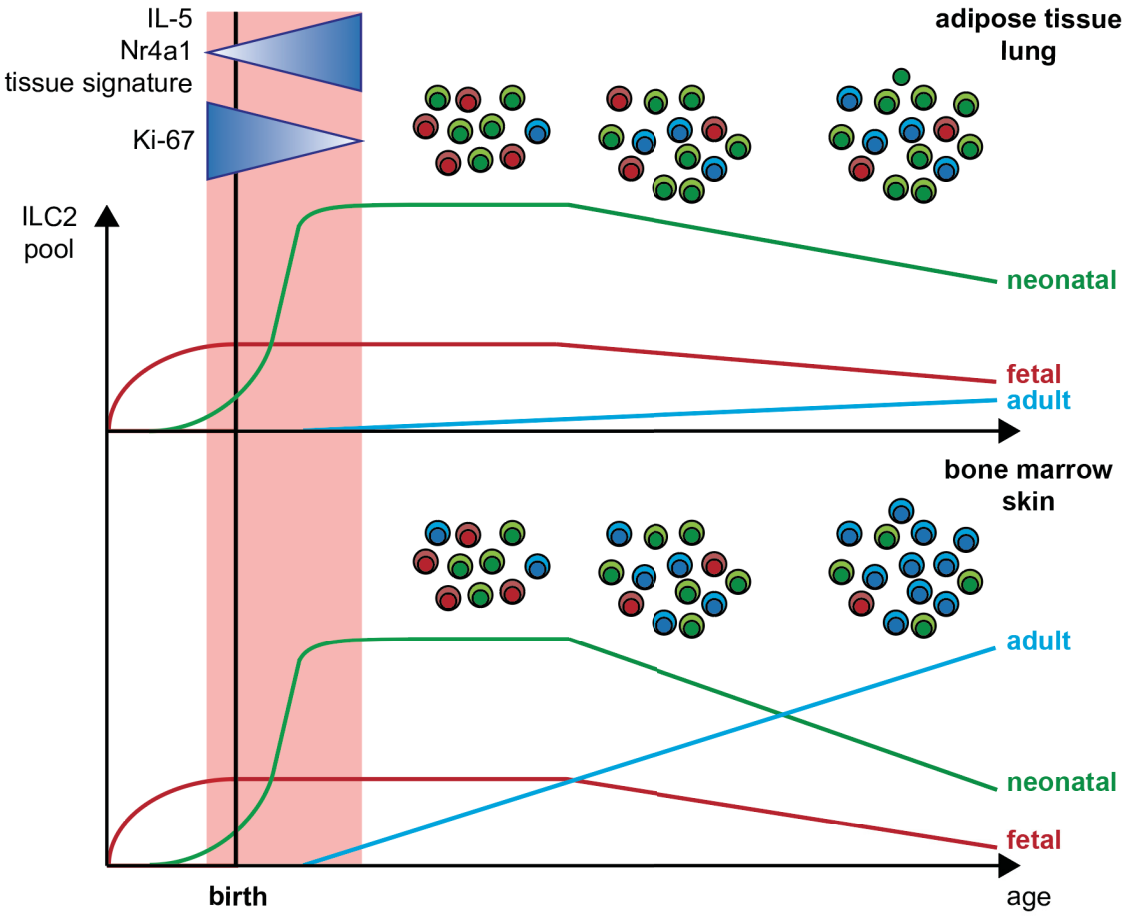

Figure 4. ILC2s differentiate by layered ontogeny.

Peripheral ILC2 pools in adult mice are comprised of cells of fetal, postnatal and adult origin. Fetal and perinatal tissue ILC2s are diluted over time by tissue-derived adult ILC2s with slow replacement in lung and adipose tissue and higher turnover in bone marrow and skin. Abbreviations: IL-5, interleukin-5; Nr4a1, nuclear receptor subfamily 4 group A member 1; ILC2, group 2 innate lymphoid cell. Adapted from[151].

1.3.3. Location, location, location – anatomical niches of ILC2s, tissue-specific heterogeneity and plasticity

1.3.3.1. Anatomical ILC2 niches and maintenance of ILC2s in peripheral tissues

ILC2s are present at diverse anatomical locations throughout the body in mice and humans but are predominantly localized at barrier surfaces which provides them with a strategic advantage as early responders to tissue perturbation. In mice, ILC2s have been found at multiple sites of the gastrointestinal tract including the gastric mucosa[157] as well as small intestinal[9] and colonic lamina propria[158]. Additionally, they have been identified within the respiratory tract in lung tissue[8] and the nasal mucosa[159], as well as in the skin[10,14] and uterine wall[10,160]. Moreover, murine ILC2s were also described at lymphoid and non-lymphoid 'non-barrier' sites including mesenteric FALCs[7], visceral adipose tissue (VAT)[161,162], mesLNs[8,9], meninges[12], spleen[8,9], heart[10], kidney[10] and liver[8]. Similarly, human ILC2s were characterized at barrier surfaces including the lung and nasal passages[22,163] and are the predominant ILC subset in the skin[163,164]. They were additionally found in metabolic tissue, fetal and adult intestine, mesLN as well as in the adult circulation[22,163,165]. Despite their extensive characterization in peripheral tissues, the developmental stage at which ILC2 precursors emigrate from the bone marrow or fetal liver to seed peripheral tissues as well as the signals and receptors that drive this egress and direct their positioning within target tissues remain largely elusive.

Once in the peripheral tissue, ILC2s locally expand and acquire effector as well as tissue-specific phenotypes in response to local environmental cues such as survival factors and activating cytokines[151]. Within the tissue they are maintained in specialized microanatomical stromal niches that support their differentiation and maturation and facilitate interaction with other immune as well as non-hematopoietic cells. These ILC2 niches have been recently described in detail in mucosal as well as non-mucosal mouse tissues[166]. Here, ILC2s localized to perivascular adventitial cuff regions in the lung as well as non-barrier tissues such as brain meninges, perigonadal and mesenteric adipose tissue, spleen, pancreas, liver and uterus[166]. Within these anatomically conserved niches, ILC2s reside in close proximity to Treg cells, DCs and lymphatic vessels and are sustained by IL-33- and TSLP-producing mesenchymal fibroblast-like adventitial stromal cells (ASCs). Importantly, IL-5$^+$ ILC2s already localized to CD45$^-$CD31$^-$Sca-1$^+$PDGFRα$^+$ ASCs in developing lung cuffs shortly after birth by postnatal day 2, indicating that strategic positioning of

ILC2s in peripheral tissues begins early in life. Furthermore, and in support of *in vivo* observations, ASCs were able to promote ILC2 proliferation and type 2 cytokine production *in vitro* in a TSLP-dependent manner. Importantly, production of other STAT5 activators, such as IL-7, was not observed in ASCs. Depletion of ASCs resulted in impaired induction of anti-helminth immunity in response to *N. brasiliensis* infection and implies an additional role for ASC-derived IL-33 in the induction of ILC2 effector functions. Notably, ILC2-derived IL-13 increased IL-33 expression in ASCs, indicating that bi-directional ILC2-ASC signaling might shape ILC2 tissue niches for long-term support and maintenance of stable ILC2 tissue pools[166].

Similar observations were made by other groups, identifying mesenchymal stromal cells as the predominant IL-33-expressing population in adipose tissue under homeostatic conditions in mice and humans[167-169]. IL-33-competent $CD45^{-}PDGFR\alpha^{+}$ white adipose tissue-resident multipotent stromal cells (WAT-MSCs) were able to sustain ILC2 populations within adipose tissue in mice[167]. In addition to IL-33, WAT-MSCs expressed intercellular adhesion molecule-1 (ICAM-1) which interacts with its ligand LFA-1 on the ILC2 surface to further promote proliferation and IL-5 production[167]. Similar to observations made with ASCs, ILC2-derived IL-13 enhanced IL-33 expression within WAT-MSCs and might serve as a positive feedback loop for ILC2 maintenance in adipose tissue[167]. On the contrary, WAT-MSCs did not produce TSLP or other STAT5 activators, implying either tissue-specific differences in ILC2 requirements and/or the presence of alternative sources of survival cytokines[167]. $CD45^{-}CD31^{-}Sca\text{-}1^{+}PDGFR\alpha^{+}$ stromal cells, designated adipose stem and progenitor cells (ASPCs) or mesenchymal stromal cells (mSCs), exhibited similar phenotypes[168,169]. Like WAT-MSCs, ASPCs were able to induce type 2 cytokine production in ILC2s in an ST2-dependent manner *in vitro* as well as *in vivo* and created an immunological milieu that sustains normal tissue homeostasis and beneficially impacts metabolism in obesity-induced type 2 diabetes[168]. A similar subset of mesenchymal $CD45^{-}Sca\text{-}1^{+}$ stromal cells was identified as the main source of IL-33 in mouse pancreatic islets. Here, endogenous IL-33 was required for maintaining islet-resident ILC2s, type 2 cytokine production as well as beta-cell insulin secretion in diabetes models[170]. Moreover, it has been described that terminal differentiation of mouse embryonic ILC precursors to mature ILC2s in the mesentery is supported by IL-33-expressing $CD45^{-}CD31^{-}PDGFR\alpha^{+}gp38^{+}$ cells, suggesting that mesenchymal cells may be involved in shaping ILC2 development[138].

In summary, mesenchymal stromal cells appear to be the predominant endogenous source of IL-33 within an anatomically conserved ILC2 tissue niche. Here they can promote type 2 cytokine production as well as expansion of ILC2s during development, homeostasis and immune perturbations. The precise role of the ILC2 tissue niche in ILC2 development in the periphery as well as postnatal positioning within tissues still remains elusive and further studies are needed to address these questions. In addition to ILC2s, the adventitial niche hosts a diverse array of immune populations such as DCs, macrophages and T cells as well as non-hematopoietic specialized stromal and endothelial cells and is also innervated by peripheral nerves[171]. It would therefore be important to explore whether additional factors including STAT5 activators are generated by these distinct cell populations and contribute to ILC2 maintenance and function at steady state and during immune challenge. Additionally, since adventitial cuffs are rich in neurons, it would be of interest to investigate whether neuropeptides or other neuron-derived mediators regulate ILC2 maintenance and function within the peripheral tissue niche.

1.3.3.2. ILC2 tissue-specific phenotypes

While previous studies have already reported that ILC2s can vary in phenotype depending on their activation state and/or tissue localization, recent advances in high-resolution and high-dimensional technologies such as single-cell RNA sequencing (scRNA-seq) analysis and mass cytometry revealed an unexpected degree of ILC2 heterogeneity in murine as well as human tissues[29,144,155,165,172]. Here, scRNA-seq analysis of the mouse small intestinal lamina propria identified four different ILC2 clusters based on expression of *Gata3* and *Klrg1*[29]. Moreover, analysis of ILC2s in various human tissues via mass cytometry revealed extensive heterogeneity based on their tissue of origin as well as interindividual variability[172]. Strikingly, mouse ILC2s from the same tissue clustered together during scRNA-seq analysis even in the absence of signaling via the main activating cytokines IL-33, IL-25 and TSLP[155]. This implies that the microenvironment imprints a tissue-defining transcriptomic signature on ILC2s that remains unaffected by the absence of canonical activating signaling.

Below I will broadly summarize molecular markers commonly used to define ILC2s in mice and humans and briefly discuss tissue-specific differential expression of activating receptors and other surface molecules.

Murine helper-like ILCs can generally be identified across tissues as $CD45^+$ cells that express Thy-1 (CD90) and IL-7Rα (CD127). They additionally lack expression of lineage markers associated with T cells (CD3, CD5, TCRβ, TCRγδ), B cells (CD19, B220), NK cells (NK1.1, DX5) as well as myeloid cells (Ly6G, Ly6C, CD11b, CD11c, FcεRI) and are thus designated lineage-negative (Lin^-)[7-9]. Human ILCs are similarly characterized as $Lin^-CD45^+CD127^+$ cells and can be differentiated from other cell types by expression of the lymphocyte antigen CD7[22,139,173]. To identify human ILC2s, lineage staining typically includes markers to exclude hematopoietic progenitor cells (CD34), T cells (CD3, CD5, TCRβ, TCRγδ), B cells (CD19), NK cells (CD56, CD94) and myeloid cells (CD1a, CD11b, CD11c, CD14, CD16, CD123, BDCA, FcεRI)[22,139,172-175]. To further distinguish ILC2s from other ILCs, subset-defining and in some cases tissue-specific markers need to be employed.

In addition to the absence of lineage markers and expression of CD45, CD90 and CD127, murine ILC2 have been defined as IL-$2R\alpha^+$ (CD25) and $GATA3^+$[176]. They further express Sca-1, CD44 and varying levels of c-kit (CD117)[176]. Major ILC2-activating signals include the cytokines IL-25, IL-33, TSLP and IL-18 which modulate basal as well as ILC2 effector activity in accordance with tissue-specific differential receptor expression[144,155]. The IL-33 receptor chain ST2 was considered a classical marker to identify tissue-resident ILC2s but recent studies have shown that ST2 expression varies according to the tissue of origin and the state of activation. While lung and adipose tissue ILC2s express high levels of ST2, ILC2s within the skin lack expression and activated 'inflammatory' ILC2s (iILC2s) and memory ILC2s also downregulate ST2 expression upon activation by IL-25[177,178]. On the other hand, small intestinal murine ILC2s constitutively express IL-25R (IL-17RB) and depend on IL-25 for cytokine production and function while lung ILC2s express very low levels[179-181]. Conversely, skin ILC2s exhibit low expression of both ST2 and IL-25R but instead express high levels of IL-18R and are accordingly activated predominantly by IL-18 at steady state and during allergic skin inflammation[155]. Notably, small subsets of IL-$18R^+$ ILC2s have been identified in bone marrow as well as lung[155]. In addition to their differential expression of activating receptors, pulmonary and small intestinal ILC2s differ in *Il5* and *Il13* transcript expression patterns[10]. Using respective reporter mice, *Il5* expression was observed under homeostatic conditions in both tissues while *Il13* was constitutively expressed in small intestinal ILC2 with transcripts in pulmonary ILC2s only detectable after activation[10]. Murine ILC2s also express inducible T-cell co-stimulator (ICOS) and ICOS ligand (ICOSL)

whose interaction promote survival and cytokine production of ILC2s[182]. Moreover, constitutive as well as activation-induced expression of other co-stimulatory ligands and receptors including DR3, GITR and OX40L on ILC2s have been reported and a subpopulation of activated $MHCII^+$ antigen-presenting ILC2s was identified as well[183-188]. Activated ILC2s also express the inhibitory receptors programmed cell death 1 (PD-1) and the E-cadherin receptor KLRG1 which act as negative regulators[189,190].

Human blood and tissue ILC2s have been identified by the simultaneous expression of the C-type lectin-like receptor CD161 (KLRB1) and the prostaglandin D2 receptor chemoattractant receptor expressed on Th2 cells (CRTH2)[22]. They can be additionally characterized by the expression of IL-25R (IL-17RB), KLRG1, differential levels of c-kit as well as high expression of GATA3[22,172,191,192]. Importantly, GATA3 is also expressed by ILC3s and should therefore only be used in combination with other markers for the identification of human ILC2s. As opposed to mouse ILC2s, ICOS expression is mainly limited to mucosal tissue-derived human ILC2s and a subset of cord blood ILC2s[172]. While ST2 has been used to characterize tissue-resident human ILC2s, peripheral blood ILC2s lack ST2 expression[13,163,193]. Notably, a recent report described expression of IL-18R on human ILC2s from various tissues and peripheral blood ILC2s produced type 2 signature cytokines upon IL-18 stimulation[172]. When compared to ILC2s isolated from nasal polyps of chronic rhinosinusitis patients, pulmonary $Lin^-CD45^+CD127^+$ ILC2s exhibited differential expression of CRTH2 and c-kit[191]. In addition, ILC2s downregulate CRTH2 expression upon entry into the tissue environment from the blood and since CRTH2 was recently implied in ILC2 accumulation within murine and human lungs this may dictate ILC2 tissue localization and function[194].

Altogether, these data support the idea that ILC2s adopt tissue-specific phenotypes in response to their local environment to tailor their functional capacities to the environment they reside in. This may involve alteration of their receptivity to various activating signals, such as tissue-specific cytokines, growth factors, hormones, or neurotransmitters. However, the underlying tissue-specific signals and mechanisms that drive this diversity remain to be further detailed.

1.3.3.3. ILC2 plasticity

The term 'plasticity', also referred to as 'trans-differentiation', describes how differentiated cells can acquire new characteristics in response to polarizing signals in their microenvironment and has been observed in various cell types and anatomical niches. Fate-mapping and adoptive transfer studies in mice, as well as observations made in patients suffering from inflammatory conditions suggest that ILC2s exhibit considerable plasticity and can adapt their functional programs to changes in tissue environment (*Figure 5*)[195]. This conversion is generally induced by modulation of lineage-determining transcription factors and cytokine receptors that drive the differential phenotypes and functional capacities[195]. Therefore, ILC2 plasticity may be essential to shape and calibrate effective responses to different types of basal as well as pathogenic stimuli.

ILC2 to ILC3 plasticity

The first study demonstrating that ILC2s exhibit functional plasticity showed that systemic injection of IL-25 or helminth infection promoted the generation of a KLRG1^{hi}IL-25R^{+}ST2^{-} ILC2 subset that produced IL-17 in addition to type 2 signature cytokines[178]. These so-called 'inflammatory' ILC2s (iILC2s) co-expressed intermediate amounts of RORγt and high levels of GATA3 which explains their capacity to produce both, IL-13 as well as IL-17[178,196]. In accordance with their dual type 2 and type 3 cytokine expression profile, iILC2s were able to limit worm burden upon *N. brasiliensis* infection and conferred partial protection against the IL-17-sensitive fungal pathogen *Candida albicans*[178]. Tuft cell-derived IL-25 in the small intestine was shown to induce the generation of iILC2s from IL-25R^{+} lamina propria-resident precursors constitutively [148,181]. As opposed to IL-33-responsive 'natural' KLRG1^{int}IL-25RB^{-}ST2^{+}ILC2s (nILC2s) which are restricted to GATA3 expression and to the production of type 2 signature cytokines, iILC2s exhibited migratory potential with the ability to circulate[148]. Importantly, during *in vitro* culture in the presence of the survival cytokines IL-2 and IL-7, iILC2s quickly upregulated ST2 and acquired responsiveness to IL-33 while losing IL-25 responsiveness, an effect that was even more pronounced when IL-33 was added to the culture[178]. Furthermore, iILC2s gradually gave rise to nILC2s in the lung after adoptive transfer indicating that they are of transient nature and might revert to nILC2s in order to restore homeostasis after inflammation[178].

The reverse process, namely conversion of mature nILC2s to an ILC3-like iILC2 phenotype, has also been described by several studies in mice and humans. This transition was proposed to be dependent on Notch signaling which induces expression of RORγt in mouse nILC2s[196]. It still remains unclear to what extent iILC2s are generated from nILC2s and thereby represent different activation states of the same plastic cell type, or if they develop from a progenitor population and constitute a different ILC2 subset. This could be addressed by transferring nILC2s from inducible transcription factor fate mapping mice into ILC2-deficient recipient mice followed by IL-25 administration or helminth infection.

In humans, trans-differentiation of ILC2s to IL-17-producing cells was observed in nasal polyps of cystic fibrosis (CF) patients and was induced by epithelium-derived IL-1β, IL-23, and TGF-β, established T_H17-skewing cytokines[197]. IL-17-competent ILC2s exhibited higher expression of RORγt and downregulated ILC2 signature genes including *GATA3*, *IL1RL1, IL5* and *IL13* upon *in vitro* stimulation with a combination of IL-1β, IL-23, and TGF-β. RORγt upregulation and conversion to this ILC3-like phenotype was inhibited by vitamin D3 which is associated with poor lung function in asthma patients and insufficiency is common in CF patients. Importantly, converted ILC2s contributed to disease pathology by inducing epithelial IL-8 production and neutrophil recruitment which mediate inflammation in nasal polyps of CF patients[197].

In addition, when human skin ILCs where stimulated with *C. albicans* hyphae in the presence of dermal cells, IL-17 was produced by activated ILC2s rather than ILC3s[198]. Moreover, naive cord blood and adult peripheral blood ILC2s exhibited similar plasticity in response to *C. albicans*. Consistent with previous reports, an ILC2 to ILC3-like conversion was dependent on, in this case, dermal stromal cell-derived IL-1β, IL-23 and TGF-β and could be induced *in vitro* by a combination thereof[197,198]. Again, trans-differentiation of ILC2s was dependent on RORγt induction and GATA3 downregulation and could be inhibited and partially reverted by IL-4[198]. The authors also provided some correlative data implying that IL-17-producing ILC3s that are enriched in skin lesions of psoriasis patients might originate from ILC2s further supporting the idea that ILC2 to ILC3 conversion plays a role in pathogenicity during chronic inflammatory conditions.

Importantly, two functionally distinct $CRTH2^+$ ILC2 subsets in adult peripheral blood were identified based on c-Kit expression[198,199]. These subsets exhibited differential requirements for

conversion to RORγt$^+$ ILC3-like cells with CCR6$^+$c-Kit$^+$ ILC2s readily producing IL-17 in response to IL-1β and IL-23, whereas c-Kit$^-$ ILC2s additionally required TGF-β[198,199]. These data indicate that lower c-Kit expression might be associated with a more polarized, less plastic ILC2 phenotype.

Finally, ST2$^+$ IL-17-producing ILC2s have been reported that were highly pathogenic in IL-33-driven models of allergic airway inflammation[200]. Here, induction of IL-17 was not facilitated by RORγt but AHR and synergistically promoted by IL-33 and leukotrienes LTC_4 and LTD_4.

ILC2 to ILC1 plasticity

Both mouse and human ILC2s can adapt an ILC1-like phenotype in response to the IL-1 family cytokines IL-1β and IL-12, characterized by T-bet and IL-12R expression as well as secretion of IFN-γ[163,173,201,202]. On the contrary, these 'ex-ILC2s' exhibited reduced levels of ST2 and GATA3. Importantly, IL-12-mediated polarization of human ILC2s into ILC1s was prevented by addition of IL-4 indicating that a type 2 skewed microenvironment can maintain ILC2 function and prevent trans-differentiation[163]. When stimulated with IL-18, another IL-1 family member, mouse but not human ILC2s additionally produced IFN-γ and upregulated T-bet and IL-18R[202]. Mechanistically, converted ILC2s exhibited abnormal epigenetic changes characterized by the simultaneous transcriptional accessibility of IFN-γ, IL-5 and IL-13 loci[201]. Since the conversion of ILC2s into ILC1s requires inflammatory mediators of the IL-1 family, it was proposed that inflammatory conditions associated with elevated levels of these cytokines might promote this conversion *in vivo*. Indeed, IFN-γ-producing ex-ILC2s were found in lungs of mice infected with influenza virus[202]. Moreover, intestinal samples from Crohn's disease patients[173] and blood samples of patients with chronic obstructive pulmonary disease (COPD) showed increased proportions of ILC1s over ILC2s, and ILC1 levels positively correlated with disease severity[163,202]. Infections, inflammation and autoimmunity all elicit production of inflammatory cytokines in the affected tissues and therefore could potentially induce ILC2 to ILC1 plasticity, which may contribute to disease severity.

ILC2 to ILC2$_{10}$ plasticity

Lastly, a subset of IL-10-producing ILC2s, termed ILC2$_{10}$, was identified in the mouse lung after intranasal administration of the allergen papain or systemic IL-33 challenge and represented a large portion of the total IL-10 producing lung population[28]. IL-10 secretion can be induced *in vitro* via stimulation of ILC2s with IL-2, and IL-10 production was shown to be significantly increased if IL-2 was added in combination with retinoic acid, an established inducer of Treg cells[28,203]. In addition, systemic administration of IL-2 in complex with an anti-IL-2-monoclonal antibody (IL-2c), which has been shown to greatly enhance biological IL-2 activity, induced *in vivo* expansion of lung ILC2$_{10}$[28,204]. Moreover, systemic co-administration of IL-2c and IL-33 resulted in reduced lung eosinophilia in *Rag1*$^{-/-}$ mice suggesting an immunomodulatory role of ILC2$_{10}$. Importantly, while ILC2$_{10}$ upregulated Id3, the proposed defining transcription factor of ILCregs, and downregulated genes associated with IL-33-mediated ILC2 activation, they maintained GATA3 expression[28]. This implies that ILC2$_{10}$ are an alternatively activated ILC2 subpopulation rather than a distinct ILC subset. It was therefore suggested that ILC2$_{10}$ might be generated as a byproduct of highly inflammatory conditions and that ILC2$_{10}$-derived IL-10 may play a role in dampening inflammation. To this end it would be of interest if a similar ILC2 subset exists in humans suffering from chronic inflammatory diseases at mucosal sites.

Memory ILC2s

Similar to cytokine-elicited memory NK cells, ILC2s displaying antigen-independent memory functions were described after IL-33- and allergen-induced allergic airway inflammation[205,206]. Here it was observed that pulmonary ILC2 numbers remained higher than in naïve mice for up to one year after the resolution of inflammation and that some previously activated ILC2s became long-lived cells. Strikingly, when these mice were re-challenged with an allergen unrelated to the one previously used, they exhibited elevated airway inflammation characterized by increased mucus production and eosinophilia when compared to challenged naïve mice[205]. This exacerbated response was attributed to excessive induction of activation-experienced primed ILC2s, which were shown to produce elevated amounts of type 2 cytokines in comparison to their naïve counterparts. This suggests that challenge-experienced ILC2s are able to acquire a so-called 'memory' phenotype in order to mount a more potent response upon re-activation. Interestingly, the conversion of naïve into primed memory ILC2s was accompanied by a conversion from IL-33

responsiveness to IL-25 responsiveness[205]. Future experiments will have to define the detailed molecular mechanisms underlying the generation of memory ILC2s and whether this critical observation can be translated to atopic diseases in humans.

Exhausted ILC2s

Similar to exhausted T cells which have been described under conditions of continuous antigen stimulation such as chronic viral infections or cancer, hyporesponsive ILC2s were identified in mice after severe allergic airway inflammation[207,208]. These 'exhausted-like' ILC2s expressed high levels of IL-10, T cell immunoreceptor with Ig and ITIM domains (Tigit), the inhibitory receptor PD-1, KLRG1 and glucocorticoid-induced tumor necrosis factor receptor (GITR), and as such it was proposed that they play a role in the alleviation of chronic allergy symptoms[207]. If these exhausted-like ILC2s are identical to IL-10-producing $ILC2_{10}$s that arise after severe allergic inflammation remains to be clarified[28]. Moreover, it needs to be determined whether a similar population exists in patients suffering from chronic inflammatory atopic diseases. Interestingly, in addition to PD-1 expression, exhausted human T cells exhibit low expression of CD127 and a $CD127^{-}/CD127^{lo}$ ILC2 population in asthmatic patients has been described[209-211]. Whether this population exhibits other exhaustion-associated features such as PD-1 expression and/or hypo-responsiveness would definitely be of interest.

The ability of ILC2s to dynamically acquire differential functional phenotypes and even reconvert thereafter enables them to adapt to changes in their microenvironment and may be essential to shape their responses to diverse pathogenic stimuli. ILC2s are predominantly tissue-resident under homeostatic conditions and the relative distribution of helper-like ILC subsets varies greatly between peripheral tissues. Therefore, functional plasticity and adaptation of a differential cytokine expression profile may enable innate responses to a wide array of pathogens even in the absence of the ILC subset that is prototypically associated with these types of responses. Plastic ILC2s are potent sources of type 1 and type 3 cytokines in respective inflammatory human conditions and were shown to actively contribute to disease pathology (*Figure 5*). Therefore, understanding the mechanisms that drive and regulate functional ILC2 plasticity during disease progression is of great importance and may reveal novel therapeutic targets and biomarkers.

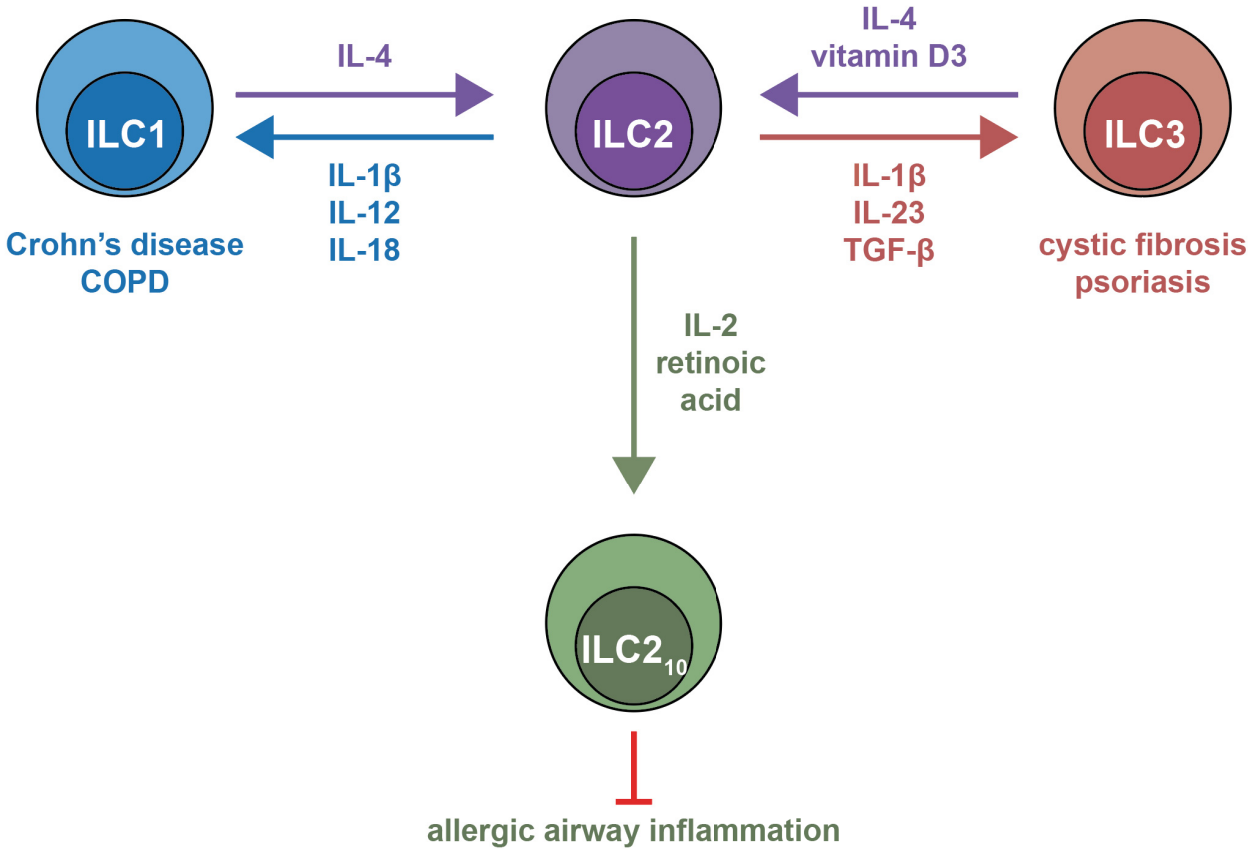

Figure 5. Functional ILC2 plasticity and implications in chronic inflammatory disease.

Human and mouse ILC2s trans-differentiate into ILC1s in response to IL-1β and IL-12 (as well as IL-18 in mouse) and secrete IFN-γ. IFN-γ-producing ILC2s have been described in Crohn's disease and COPD patients. Conversely, IL-1β in combination with IL-23 can induce an ILC3-like phenotype and IL-17 production in ILC2. Here, the less plastic human c-Kit$^-$ ILC2s require TGF-β in addition to IL-1β and IL-23. Differentiation of ILC2s into ILC1s and ILC3s can be reversed by IL-4 and in the case of ILC3s inhibited by vitamin D3 as well. IL-17-generating ILC2s have been described in psoriasis lesions and nasal polyps of cystic fibrosis patients. Moreover, ILC2s can produce IL-10 in response to IL-2 and retinoic acid and dampen allergic airway inflammation. Abbreviations: COPD, chronic obstructive pulmonary disease; ILC1, group 1 innate lymphoid cell; ILC2, group 2 innate lymphoid cell; ILC3, group 3 innate lymphoid cell; IL-, interleukin-; TGF-β, transforming growth factor beta.

1.3.3.2. ILC2 trafficking

Efficient immune protection is provided by recruitment of immune cells from primary and secondary lymphoid organs as well as the blood to the site of immune challenge. In addition, tissue-resident cells that are strategically positioned within a defined cellular network might migrate locally in response to tissue perturbation. As mentioned previously, studies using parabiotic animals revealed that ILC2s are predominantly tissue-resident at steady state and presumably self-maintained via local expansion and/or development from ILC2 progenitors *in situ* within peripheral tissues[146-148,151,212]. Consistent with these studies, low engraftment of donor ILC2s in host tissues has been observed after hematopoietic stem cell transplantation[132]. However, circulating ILC precursor populations have been described in the blood of both mice and humans and emerging evidence suggests that under certain circumstances, ILC2s can migrate from systemic sites to specific tissues in order to concentrate where they are needed and exert their effector functions[139,145].

Interorgan trafficking of ILC2s

ILC2 migration to different tissues was first observed in mice following intraperitoneal administration of IL-25 as well as infection with the migratory helminth *N. brasiliensis*[148]. Here, IL-25 treatment and helminth infection elicited induction of intestinal IL-25-responsive iILC2s that exited the lamina propria via the lymphatics and entered the blood circulation. Once in the blood, iILC2s migrated to and infiltrated the lung and other distal sites where they contributed to anti-helminth defense and tissue repair[148]. Similar to adaptive lymphocytes, egress from the tissue to the blood was mediated by activation-induced upregulation of sphingosine-1 phosphate (S1P) receptors on ILC2s and S1P-mediated chemotaxis[148,213]. Strikingly, when the S1P receptor agonist fingolimod (FTY720) was administered prior to infection of *Rag1*$^{-/-}$ mice with *N. brasiliensis*, 80% of the animals succumbed to infection in this typically chronic infection model. Mortality and helminth-associated tissue damage were largely prevented by replenishing the circulating iILC2 pool via intravenous transfer, further supporting their importance in anti-helminth defense[148]. CD69 expression antagonizes S1P signaling through downmodulation of cell surface S1P receptors[214]. Consistently, intestinal ILC2s expressed substantial levels of CD69 while peripheral iILC2s were CD69^{lo}[148,213]. Intriguingly, ILC2s are the predominant mature ILC subset in human peripheral blood and multiple sclerosis patients treated with FTY720

exhibited decreased numbers of circulating ILC2s when compared to treatment-naive control patients, indicating similar migratory mechanisms in this setting[215,216]. Furthermore, consistent with observations made in mice, CD69 expression on human ILC2s was restricted to tissue ILC2s while it was not observed on cord or adult peripheral blood ILC2s[172].

A recent study further expanded on the tissue origin of activation-induced blood ILC2s following helminth infection. Using a combination of fate-mapping approaches, cytokine receptor profiling and cytokine-deficient mice, it was revealed that peripheral blood ILC2 origins were temporally homogenous during the course of *N. brasiliensis* infection[213]. Rather than being exclusively derived from the small intestine, as previously proposed[148], the authors observed that small intestinal ILC2s enter the blood first but are subsequently replaced by a second wave of lung-derived ILC2s[213]. It was suggested that tissue egress of ILC2s after helminth infection was driven by niche extrusion as a result of excessive proliferation of the ILC2 population thereby exceeding a certain tissue carrying capacity[213].

In addition to migration of mature peripheral ILC2s, the egress of bone marrow ILC2Ps and ILC2s was investigated upon systemic challenge with the ILC2-activating cytokine IL-33, intranasal allergen administration or radiation-induced tissue disruption[156,217]. It was observed that *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice exhibited significantly increased frequencies and numbers of ILC2Ps in the bone marrow at steady-state and lower ILC2 numbers in peripheral tissues including lung, skin and mesLN when compared to control mice[156]. Notably, the rate of ILC2P *de novo* generation remained unaltered in the absence of IL-33 signaling indicating that IL-33 promotes BM egress rather than ILC2P development or fitness[156]. Furthermore, systemic administration of IL-33 induced egress of ILC2Ps from the bone marrow via an ST2-dependent and ILC2P-intrinsic mechanism[156]. Mechanistically, IL-33 negatively regulated CXCR4 expression on ILC2Ps, a chemokine receptor known for mediating retention of leukocytes in the BM[156]. Furthermore, radiation-induced tissue disruption in a parabiotic model resulted in the generation of an empty-niche effect and elicited marked reseeding to lungs, skin and mesLNs by circulating ILC2s[156]. Moreover, systemic administration of IL-33 augmented ILC2 trafficking to the lungs in this model. Importantly, intranasal challenge with the fungal aeroallergen *A. alternata* promoted ILC2P egress from the BM in an IL-33 dependent manner[156]. Egress of ILC2s from the bone marrow and subsequent recruitment to the lung was reported in the same model and lung accumulation of BM ILC2s was dependent on β_2 integrin adhesion receptor expression on

ILC2s[217]. Collectively, these findings suggest a key role for IL-33 in BM egress of ILC2Ps to facilitate initial tissue seeding and repopulation of ILC2 niches after tissue disruption. In addition to ST2, BM egress and entry into the circulation of mouse ILCPs and ILC2Ps was partially dependent on the expression of the chemokine receptor CXCR6[218].

In addition to CXCR6, ILC2 progenitors and mature ILC2s express a variety of other chemokine receptors that mediate homing to peripheral tissues, recruitment to inflammatory sites as well as local migration. Chemokine receptors are expressed in a tissue-specific fashion and expression can change with activation status. It has been demonstrated that ILC2s express CCR9, CXCR4, and CXCR6, which appear to be functionally involved in tissue homing[14,156,219,220]. The gut homing receptor CCR9 was also shown to be highly expressed on ILC2Ps and iILC2s[75,148,219]. In addition, similar to T_H2 cells, mouse and human ILC2s were described to express CCR1, CCR4 and CCR8[22,75,118,172,221,222]. Moreover, human ILC2s additionally expressed CCR6, regardless of their tissue of origin[172]. Furthermore, human skin ILC2s express the skin-homing receptors cutaneous leukocyte-adhesion antigen (CLA) and CCR10[223].

As opposed to S1PR, which mediates tissue egress of ILC2s, the prostaglandin D_2 (PGD_2) receptor CRTH2 has been reported to promote accumulation of peripheral blood ILC2s within the lungs of mice upon systemic IL-33 treatment and helminth infection[194,224]. In addition, stimulation of human ILC2s with PGD_2 mediated *in vitro* ILC2 migration and activation in a CRTH2-dependent manner suggesting that CRTH2 is an important chemoattractant receptor in both mice and humans[225,226]. Notably, another bioactive lipid, leukotriene E_4 (LTE_4) was also capable of mediating human ILC2 migration *in vitro* [227].

ILC2s have been identified in mesLN, mediastinal (medLN) and brachial (bLN) as well as various other lymph nodes in mice under steady state conditions and upon immune perturbation[205,228-230]. While most lymph node ILC2s were tissue-resident, the use of *Kaede* photoconvertible mice allowed for the identification of a small subset of migratory ILC2s at steady-state in the brachial and mesLNs[228,229]. Migratory ILC2s were further shown to constitutively traffic from the small intestine to the mesLN[229]. Intriguingly, ILC2s reside in the interfollicular space of LNs and distinct ILC2s subsets were described that express MHC-II or CD1a and are capable of presenting peptide or lipid antigens to T cells, respectively[188,231,232].

However, whether migratory LN ILC2s possess antigen-presenting capacity and their physiological relevance still remain elusive.

Collectively, under certain circumstances and at distinct anatomical locations, ILC2s may adapt a migratory phenotype and similar to adaptive lymphocytes may be deployed to distal sites to mediate host protection. However, precise mechanisms and pathways that govern ILC2 tissue accumulation and migration between tissues upon type 2 inflammation and during homeostasis are not fully defined.

Local migration of ILC2s within tissues

Due to their low abundance in tissues, ILC2s need to be strategically positioned to provide efficient protection following immune challenge. This can be achieved by local migration in response to environmental cues to increase the concentration of cytokines at the site of perturbation.

Using IL-13-eGFP mice, lung intravital microscopy and *ex vivo* cultured live lung slices, a recent study revealed that pulmonary ILC2s are highly dynamic, exhibit ameboid-like movement and accumulate in the lung peribronchial and perivascular spaces upon intranasal administration of IL-33 or *A. alternata*[221]. Interestingly, pulmonary ILC2s exhibited significantly higher motility than $CD4^+$ T cells after IL-33 administration. The majority of ILC2s highly expressed the T_H2-associated chemokine receptors CCR1 and CCR8 *in vivo* and expression was further upregulated upon intranasal IL-33 challenge[221]. Furthermore, the CCR8 ligands CCL1 and CCL8 were induced during allergic airway inflammation and ILC2s co-localized with macrophage-derived CCL8 deposits in the peribronchial region. Strikingly, blocking of the CCR8-CCL1/CCL8 axis with a CCR8 blocking antibody significantly impaired ILC2 migration and accumulation within the peribronchial space following pulmonary IL-33 challenge[221]. Importantly, CCL8 also promoted migration of human peripheral blood ILC2s *in vitro* suggesting that similar migratory mechanisms may be employed[221].

Consistent with the above described observations, another study demonstrated high expression of CCR8 on pulmonary ILC2s at steady state as well as after IL-25 and IL-33 administration or *N. brasiliensis* infection. Interestingly, IL-25-elicited iILC2s exhibited reduced CCR8 expression when compared to IL-33-induced nILC2s[222]. Despite CCR8 being highly expressed, ILC2s failed to migrate towards CCL8 *in vitro* in a transwell chemotaxis

assay employed in this study[222]. In addition, ILC2s did not migrate towards CCL1 and a selective synthetic CCR8 agonist, but instead exhibited motility towards the CCR4 ligands CCL17 and CCL22[222]. To analyze the role of CCR8 and CCR4 on ILC2 homing capacity to the lung *in vivo*, papain-challenged recipient mice were transferred with fluorescently labeled ILC2s from WT, *Ccr8*$^{-/-}$ or *Ccr4*$^{-/-}$ mice followed by light-sheet microscopy. In accordance with their previous data, CCR4-deficient ILC2s failed to efficiently home to the inflamed lung as opposed to WT and *Ccr8*$^{-/-}$ ILC2s[222]. However, the absence of migratory capacity of ILC2s towards CCL8 *in vitro* is in stark contrast with previously observations[221]. This discrepancy could be explained by the use of expanded splenic and mesLN ILC2s[222] which may exhibit altered expression of chemokine receptors as a consequence of continuous activation during the expansion process. They thereby may not be able to relay CCR8 signals to the same extent as the fresh pulmonary ILC2s used in the previous study[221]. Since these expanded ILC2s were also used for *in vivo* transfer experiments it is difficult to perceive whether CCR8 is ydispensable for systemic migration of ILC2s to the lung from the circulation, or if ILC2-intrinsic CCR8 signaling is impaired. In addition, it is possible that CCR8 may exclusively play a role in local tissue migration. Future experiments will have to clarify the overlapping and complementary roles of CCR8 and CCR4 for ILC2 migration to and within airways.

Despite these recent advances, the detailed cell-intrinsic and environmental guidance cues governing ILC2 tissue distribution and migration during homeostasis, allergic inflammation or helminth infection remain poorly defined. Here, it would be of interest to explore whether bioactive lipids, such as PGD_2, and LTE_4, which are both induced upon allergic inflammation and have been shown to mediate ILC2 migration, also play a role in intratissue migration of ILC2s *in vivo*. Additionally, investigations of ILC2 motility within other ILC2-rich barrier tissues such as the small intestine would add important novel insights to ILC2 biology.

1.3.4. The ILC2 regulatory network

ILC2s are highly versatile and in addition to activating signals provided by alarmins, can integrate dietary[60,233-235], metabolic[161,236,237], neural[232,238-245], and hormonal[246-249] cues to mediate type 2 immune responses and/or tissue restorative programs (*Figure 7*). To this end, ILC2s express a broad array of cell surface and intracellular receptors that enable them to sense changes in their tissue microenvironment and modulate their gene and protein expression program accordingly to initiate respective effector mechanisms. Recent progress made in understanding the regulation of ILC2s at steady state and upon immune perturbation is summarized below.

1.3.4.1. Activating cytokines

ILC2-activating cytokines of the IL-1 cytokine family as well as IL-25 activate NF-κB and MAPK signaling pathways upon binding to their cognate receptors on ILC2s and thereby trigger production of effector cytokines. As discussed earlier, expression of activating receptors on ILC2s can vary based on their anatomical location as well as their activation status and optimal activation may require additional cues by co-stimulatory cytokines or molecules.

IL-33

The IL-1 cytokine family member IL-33 is arguably the most potent activating cytokine of pulmonary mouse and human ILC2s[250]. IL-33-mediated ILC2 activation results in rapid production of type 2 signature cytokines including IL-5, IL-9, IL-13, GM-CSF and AREG and IL-33-deficient mice exhibited substantial protection from type 2 inflammation[250]. IL-33 serves as an alarmin at barrier surfaces such as the lung and intestine, where it induces type 2 inflammation upon tissue damage[251]. Under homeostatic conditions IL-33 is constitutively expressed in the nuclei of epithelial and endothelial cells[252,253] and expression can be further induced in immune cell populations such as DCs, mast cells and macrophages under certain inflammatory conditions[254-257]. Functional IL-33 is released upon necrotic or necroptotic cell death following tissue damage, while it is inactivated by caspase-3- and caspase-7-mediated proteolytic cleavage during apoptotic cell death[258]. Despite lacking a conventional signal sequence, release from living cells was reported during allergic airway inflammation and may be induced by extracellular ATP[259]. IL-33 signals through the heterodimeric IL-33 receptor, composed of the ligand-binding

ST2 chain and the IL-1 receptor accessory protein (IL-1RAcP) signaling chain, which is shared with other members of the IL-1 family[251]. While IL-1RAcP is broadly expressed on hematopoietic cells, expression of ST2 is mainly restricted to immune cells that are associated with type 2 and regulatory immune functions, including T_H2 cells, a subset of Treg cells, mast cells and ILC2s[258]. Binding of IL-33 to ST2 induces dimerization with IL-1RAcP which in turn facilitates the recruitment of the cytosolic adaptor protein myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinases 1 (IRAK1) and 4 (IRAK4) as well as the ubiquitin ligase TNF receptor-associated factor 6 (TRAF6)[260] (*Figure 6*). TRAF6 activates the IκB kinase (IKK) complex, which phosphorylates inhibitor of nuclear factor kappa B (IκBα) and thereby enables nuclear translocation of NF-κB transcription factors[260]. In addition, TRAF6 triggers the mitogen-activated protein kinase (MAPK) signaling pathway, resulting in activation of the transcription factor activator protein 1 (AP-1)[260]. Low concentration of tissue IL-33 and basal activation of ILC2s and other $ST2^+$ resident cell populations was proposed to play an important role in sustaining physiological processes such as adipose tissue metabolism and thermogenesis[161,168,237,251]. Accordingly, circulating IL-33 levels in humans are negatively correlated with body mass index and adiposity[261] and genetic variations in the IL-33 gene have been previously linked to the development of obesity in humans[262,263]. In addition, constitutive expression of IL-33 by stromal cells was demonstrated to be critical for the maintenance of ILC2s within their respective tissue niche[166]. However, excessive IL-33 release, as observed upon exposure to allergens, noxious chemicals, viral or helminth infections, results in elevated activation of ILC2s, allergic inflammation and type 2 immunopathologies in mice. Accordingly, increased plasma concentrations of IL-33 have been observed in atopic human diseases, including asthma[264-266], atopic dermatitis[267] and allergic rhinitis[268]. Moreover, several genetic studies and large-scale genome-wide association studies (GWASs) further link the IL-33/ST2 axis to asthma susceptibility[269-273]. In many of these diseases, ILC2s are thought to contribute to the establishment of inflammation, highlighting the importance of IL-33 as a mediator of type 2 immune responses[251]. Nevertheless, $ST2^-$ ILC2 populations, such as iILC2s and $CD127^-$ unconventional ILC2s, are also induced upon helminth infection and/or allergic airway inflammation, underlining the importance of additional IL-33/ST2-independent avenues of ILC2 activation[178,274].

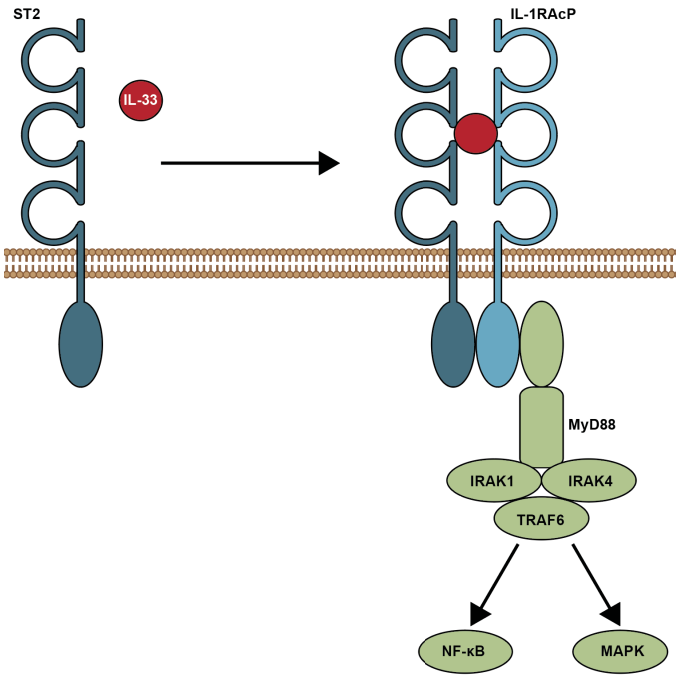

Figure 6. IL-33 signals via IL-33R to activate NF-κB and MAPK signaling pathways.

Binding of IL-33 to the ligand-binding domain of the IL-33R, ST2, leads to heterodimerization with IL-1RAcP and recruitment of the intracellular adaptor protein MyD88, the receptor-associated kinases IRAK1 and IRAK4 as well as the ubiquitin ligase TRAF6. Signal transduction ultimately results in activation of NF-κB and MAPK pathways which mediate ILC2 survival, proliferation and type 2 cytokine production. Abbreviations: ST2, suppression of tumorigenicity 2; IL-, interleukin-; IL-1RAcP, IL-1 receptor accessory protein; MyD88, myeloid differentiation factor 88; IRAK, IL-1 receptor-associated kinase; TRAF6, TNF receptor-associated factor 6; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase.

IL-18 and IL-1β

In addition to IL-33, the IL-1 family members IL-18 and IL-1β have been reported to activate ILC2 effector functions. IL-18 signals via the IL-18R, composed of the IL-18Rα and IL-18Rβ chains whereas IL-1 binds to the IL-1R, comprised of the IL-1R1 chain and IL-1RAcP[275]. Similar to IL-33, binding of IL-18 and IL-1β to their cognate receptors leads to the recruitment of MyD88 and downstream activation of NF-κB and MAPK pathways.[275]. ILC2s constitutively expressing IL-18Rα were initially identified by mass cytometry in human mucosal as well as non-mucosal tissues[172]. While IL-18 is generally implicated in promoting type 1 signature cytokine production upon receptor binding, it was observed that peripheral human blood ILC2s produced type 2

signature cytokines, including IL-4, IL-5 and IL-13, in response to IL-18 stimulation[172]. IL-18R$^+$ ILC2s were subsequently described in murine skin and to a lesser extent in lung tissue and bone marrow[155]. Mouse skin and lung ILC2s produced significantly higher amounts of IL-13 when stimulated *in vitro* with TSLP in the presence of IL-18 than when subjected to TSLP alone[155]. In addition, intradermal injection of IL-18 resulted in markedly increased IL-13 expression by skin and lung ILC2s *in vivo*[155]. Importantly, while total ILC2 numbers were comparable to control mice, IL-18-deficient animals exhibited significantly reduced IL-5 and IL-13 expression in skin ILC2s at steady state, implying a non-redundant role of IL-18 as a basal ILC2 activator[155]. Furthermore, decreased numbers of activated skin ILC2s and eosinophils were observed in *Il18*$^{-/-}$ mice in a mouse model of atopic-like inflammation[155]. These findings suggest that in addition to homeostatic ILC2 activation, IL-18 also promotes ILC2 effector responses during atopic dermatitis. Recent work using Rorα lineage tracer mice also identified the presence of a small IL-18Rα$^+$ST2$^-$ subset in the lung that expressed intermediate levels of the prototypical ILC2 markers GATA3, KLRG1 and CD25 and expanded but did not produce cytokines upon intranasal IL-18 treatment[144]. It was further shown that this progenitor population can give rise to ILC2s, is present already in the lungs of neonatal mice already and may comprise a major source of lung ILC2s[144].

Recent studies demonstrated that IL-1β is an additional potent activator of human ILC2s. In the presence of IL-2, IL-1β was able to elicit proliferation and type 2 cytokine production of peripheral blood and tonsil ILC2s to a similar extent as IL-33 in combination with IL-2[163,201]. Notably, IL-1α exhibited comparable effects on ILC2 proliferation[201]. Additionally, as discussed in detail above, priming of ILC2s by IL-1β was critical for IL-12- and IL-23-induced ILC2 trans-differentiation[163,197,198,201].

IL-25

IL-25, also known as IL-17E, is a member of the IL-17 cytokine family and, unlike other IL-17 cytokines, a strong inducer of type 2 immune responses. In the small intestine chemosensory epithelial cells, termed tuft cells, constitutively express IL-25 and thereby sustain ILC2 homeostasis in the lamina propria as well as induce production of IL-13[179-181]. ILC2-derived IL-13 in turn feeds forward on epithelial crypt progenitors to induce differentiation and expansion of tuft cells as well as goblet cells and thereby promotes small intestinal remodeling[179-181]. IL-25 production and the tuft cell-ILC2 circuit can be further induced by protist-derived succinate as

well as helminth infection[233,234]. Moreover, IL-25 expression is initiated in lung epithelial cells upon allergen exposure and can act via an autocrine feed-forward mechanism to amplify production of IL-25, IL-33 and TSLP, thus further potentiating the allergic response[276-278]. IL-25 signals via the heterodimeric IL-25 receptor complex, composed of the ligand-binding IL-17RB chain and the IL-17RA signaling chain. IL-25 binding to its receptor leads to the recruitment of the adaptor molecule Act1 which in turn associates with TRAF6 and mediates downstream activation of NF-κB and MAPK signaling pathways[279]. As mentioned previously, expression of IL-25R and thereby responsiveness of ILC2s to IL-25 varies substantially depending on the tissue of residence. While murine lung and adipose tissue ILC2s express low levels of IL-25R, small intestinal ILC2s constitutively express IL-17RB and depend on IL-25 for cytokine production and effector functions[177,179-181]. In addition, type 2 inflammation-induced migratory iILC2s and memory ILC2s express IL-25R and downregulate ST2 expression upon activation by IL-25[178,205]. IL-25R signaling in ILC2s drives proliferation and type 2 signature cytokine production and promotes allergic inflammation and anti-helminth immunity[279]. Importantly, increased expression of IL-25 and IL-17RB transcripts was detected in the bronchial mucosa of asthmatic patients[277]. In addition, elevated expression of IL-25 transcripts in bronchial biopsies from asthmatic patients were correlated with enhanced airway hyper-responsiveness, eosinophilia, subepithelial thickening as well as high serum IgE levels and type 2 cytokine expression[280]. Thus, it was proposed that IL-25 may contribute to the pathogenesis of this ‘T2-high’ asthma subtype[280].

While activating cytokines provide the initial cue for ILC2 activation, additional signals that promote survival, proliferation and further amplify type 2 cytokine production are required for optimal ILC2 effector responses and will be discussed further below.

1.3.4.2. Costimulatory cytokines

Costimulatory ILC2 cytokines act in concert with activating cytokines to promote ILC2 function and are critical for ILC2 survival, development and maintenance. They include the γ_c cytokine family members IL-2, IL-4, IL-7 and IL-9 as well as TSLP which trigger activation of the JAK/STAT pathway upon binding to their respective receptors.

IL-2

IL-2 exerts a wide spectrum of activities and modulates both immune homeostasis and activation[281]. The primary source of IL-2 are T cells which rapidly secrete IL-2 upon antigen-dependent activation[281]. IL-2 exerts its functions by binding to the heterotrimeric IL-2R which is comprised of the ligand-binding IL-2Rα (CD25) chain as well as the signaling chains IL-2Rβ (CD122) and γ_c (CD132)[281]. IL-2-binding to IL-2R triggers downstream activation of the JAK/STAT pathway and results in phosphorylation of particularly STAT5 but also, albeit to a lesser extent, STAT1 and STAT3[279]. ILC2s constitutively express all three IL-2R components and IL-2 has been shown to be critical for survival, proliferation and sustained activation[8,9,279,282]. While IL-2 itself induced little proliferation and production of effector cytokines from ILC2s, synergy with IL-33 or IL-25 augmented rapid expansion and potent type 2 cytokine production[22,147,283]. This is consistent with other type 2 immune populations such as T_H2 cells and basophils which require both STAT5 and NF-κB activation to initiate optimal effector responses[282].

IL-7

IL-7 is mainly produced by non-hematopoietic cells such as epithelial cells and stromal cells and mediates its functions by binding to the heterodimeric IL-7R composed of the IL-7Rα (CD127) chain and γ_c which are both constitutively expressed by ILC2s[130]. Similar to IL-2, downstream IL-7R signaling triggers JAK/STAT signaling and phosphorylation of STAT5 but also STAT1 and STAT3[130]. IL-7-mediated STAT5 activation was shown to promote ILC2 development, maturation, proliferation and survival[130]. Stimulation of murine and human ILC2s with IL-7 only yields low production of type 2 cytokines but IL-7 can act in concert with the activating cytokines IL-33 or IL-25 to amplify expression of effector cytokines[7,9,147,283,284].

TSLP

The alarmin TSLP is a critical mediator of allergic disease and type 2 inflammation and is predominantly produced by epithelial cells including keratinocytes as well as mesenchymal cells[285]. TSLP signals through a heterodimeric receptor composed of the IL-7Rα chain and TSLPR and signaling results in downstream activation of STAT5[286]. Similar to IL-7, *in vitro* stimulation of pulmonary ILC2s with TSLP alone does not elicit significant cytokine expression[283]. However, in combination with IL-33, TSLP synergistically promotes proliferation, survival as well as IL-5 and IL-13 production in murine and human ILC2s. Importantly, in addition to IL-33, expression of TSLP by adventitial stromal cells within the ILC2 tissue niche promotes ILC2 accumulation and maintenance[166].

Notably, while initially sensitive to corticosteroids, TSLP induced dexamethasone resistance in murine ILC2s *in vitro* as well as *in vivo*, which could be counteracted by treatment with a STAT5 inhibitor[287]. Mechanistically, STAT5 activation resulted in expression of the anti-apoptotic protein Bcl-xL in mouse ILC2s and triggering of the MAPK signaling cascade in human ILC2s, thereby providing strong survival signals that overcome corticosteroid-mediated ILC2 inhibition[287,288]. Besides TSLP, the STAT5-activating cytokines IL-2, IL-7 and to a lesser extent IL-9 also decreased corticosteroid sensitivity of ILC2s upon *in vitro* stimulation[287]. Treatment with inhaled corticosteroids is key in the management of asthma and most asthmatic patients can be well controlled with a low-to-moderate dose thereof. However, 5 to 10% of asthmatics display so-called 'severe refractory asthma' and are unresponsive to corticosteroid drugs which represents a major clinical problem[289]. Importantly, TSLP is overexpressed in the airways of severe asthmatics and is considered a biomarker for severe refractory asthma[289,290]. Moreover, several large-scale genome-wide association studies linked polymorphisms in the TSLP gene with increased susceptibility to asthma and allergic rhinitis[289]. Treatment of individuals suffering from mild allergic asthma with the neutralizing anti-TSLP antibody tezepelumab resulted in reduced allergen-induced bronchoconstriction as well as blood and sputum eosinophilia[291] and patients with moderate-to-severe poorly controlled asthma displayed significantly lower rates of asthma exacerbations and a progressive decrease in serum IgE levels upon treatment[292]. Collectively, these findings implicate TSLP as a key player in the pathogenesis of asthmatic disease and suggest that TSLP may at least partially contribute to disease progression by its action on ILC2s.

IL-9

IL-9 promotes survival of a variety of cells including T cells and mast cells[293]. Primary sources of tissue IL-9 are $CD4^+$ T cells, NKT cells, mast cells and, importantly, activated ILC2s[293]. IL-9 signals via the IL-9 receptor which is composed of the ligand specific IL-9R subunit as well as γ_c[293]. Engagement and subsequent signal transduction of IL-9R results in activation of STAT5 but also STAT1 and STAT3[293]. It was shown early on that ILC2s constitutively express high levels of IL-9R and that IL-9 can act in an autocrine manner to promote ILC2 function[8,294,295]. IL-9 production by ILC2s was initially described using IL-9 fate mapping mice during papain-induced allergic airway inflammation[294]. Here, lung ILC2s were identified as the major IL-9-expressing population upon challenge[294]. IL-9 production by ILC2s was shown to be IL-33-dependent and was further elevated by IL-2 provided *in vivo* by adaptive immune cells[294]. In addition to IL-2 and IL-7, TSLP can synergize with IL-33 to promote IL-9 expression in ILC2s *in vitro*[283]. Mechanistically, ILC2-derived IL-9 production was dependent on IRF4 *in vitro* as well as *in vivo* following *N. brasiliensis* infection[283]. Direct stimulation of sort-purified ILC2s with IL-9 resulted in enhanced type 2 cytokine production whereas diminished IL-5 and IL-13 expression in IL-9-deficient ILC2s could be rescued by the addition of exogenous IL-9[283,294]. Moreover, it was demonstrated that IL-9 augments ILC2 survival by initiating expression of the anti-apoptotic protein BCL3[295]. Taken together, these findings demonstrate that IL-9 feeds back on ILC2s to promote their survival and function. Consistently, neutralization of IL-9 upon intranasal papain challenge led to a significant decrease in IL-5 and IL-13 expression *in vivo*[294]. Furthermore, it was observed that ILC2s are the dominant source of IL-9 in the lungs after *N. brasiliensis* infection and that IL-9 signaling was critical for accumulation of ILC2s, ILC2-intrinsic type 2 cytokine production, worm expulsion and tissue repair[283,295]. Cumulatively, these findings clearly show that activated ILC2s are a potent source of IL-9 during allergic airway inflammation and helminth infection and that IL-9 acts in an autocrine manner *in vitro* as well as *in vivo* to promote ILC2 survival and cytokine production. Importantly, both IL-9 and IL-9R expression are associated with an asthma-like phenotype in mice and humans, highlighting the critical role of IL-9 signaling during allergic airway inflammation[296-299]. Notably, *Il9*-deficient mice developed a chronic phenotype upon antigen-induced arthritis as opposed to WT mice that spontaneously resolve inflammation in this model[300]. Here, IL-9-mediated ILC2 induction resulted in expression of the co-stimulatory ligands GITRL and ICOSL, Treg activation and resolution of arthritis[300]. Thus, IL-

9-initiated boost of ILC2 functions can promote inflammation in acute models of allergic airway inflammation or infection but can also contribute to the resolution of inflammation and return to homeostasis in the context of chronic disease.

IL-4

The type 2 signature cytokine IL-4 is critical for the polarization of T_H2 cells and induction of IgE class switch recombination in B cells. IL-4 is mainly produced by basophils, eosinophils, mast cells, T cells and NKT cells and mediates its effects by binding to the IL-4R composed of the IL-4Rα chain and γ_c[64]. IL-4R signaling triggers the JAK/STAT pathway and downstream STAT6 activation[301]. IL-4Rα can also heterodimerize with IL-13Rα1 and this receptor complex can bind both IL-4 and IL-13[301]. Murine ILC2s express IL-4Rα and *in vitro* stimulation with IL-4 has been shown to promote ILC2 proliferation and type 2 cytokine production alone and in synergy with IL-33, thereby further amplifying ILC2 functions[302,303]. Moreover, basophil-derived IL-4 promoted ILC2 accumulation and enhanced expression of IL-5, IL-9 and IL-13 in lung ILC2s upon papain-induced allergic airway inflammation as well as in cutaneous ILC2s in an atopic dermatitis model[302,303]. In addition, since eosinophils are a major source of IL-4, crosstalk between ILC2 and eosinophils was proposed to amplify type 2 inflammation in chronic rhinosinusitis[163].

1.3.4.3. Suppressive cytokines

Suppressive cytokines, including IL-10, IL-27, TGF-β as well as type I and type II interferons, restrain ILC2 proliferation and activation and thereby play key roles in maintaining homeostasis and protecting the host from excessive type 2 inflammation and associated immunopathologies.

Type I interferons, type II interferons and IL-27

Type I interferons (IFN-I; IFN-α, IFN-β) signal through the IFN-α/β receptor comprised of the IFNAR1 and IFNAR2 chains. Engagement of IFNAR results in activation of STAT1 and STAT2 which associate with interferon regulatory factor 9 (IRF9) to form the interferon-stimulated gene factor 3 (ISGF3) complex. ISGF3 further mediates transcription by binding to response elements of interferon stimulated genes (ISGs)[279]. Type II interferon (IFN-II; IFN- γ)

binds to the IFNGR consisting of IFNGR1 and IFNGR2 chains, leading to STAT1 activation, homodimerization and ISG expression[279]. IL-27, a member of the IL-12 cytokine family, signals via the IL-27 receptor which is composed of gp130 and IL-27Rα and mediates downstream activation of STAT1 and STAT3[279].

It has been recently demonstrated by several groups that IFN-I, IFN-II as well as IL-27 can potently inhibit proliferation, survival and type 2 cytokine production of murine as well as human ILC2s[147,177,304-309]. IFNs and IL-27 mediated their suppressive action by activation of STAT1 and IL-27 has been shown to additionally restrain ILC2s via STAT3 activation[147,304-306,308]. Moreover, IFN-I was shown to require ISGF3 to restrain ILC2 activation[304]. IFNs are strongly induced upon viral infection and mice deficient in IFN-I signaling exhibited increased numbers of pulmonary ILC2s and excessive type 2 immunopathology following infection with influenza A virus (IAV)[304]. Consistently, IFN-I and IFN-II receptor-deficient mice showed enhanced type 2 immune responses upon *H. polygyrus*[304] or *N. brasiliensis*[147] infection, respectively, while exogenous treatment with IFN-γ or IFN-γ overexpression suppressed *N. brasiliensis*-induced ILC2 accumulation and activation[147,177]. Moreover, intranasal administration of IFN-α, IFN-β or IFN-γ restrained IL-33-mediated allergic airway inflammation in an ILC2-intrinsic manner, and IFN-γ also suppressed *A. alternata*-induced asthmatic symptoms when co-administered[147,304,306]. Similar findings have been reported in another context where endogenous IFN-γ, induced upon ageing or high-fat diet, restrained adipose tissue ILC2 function[177]. Collectively, these data indicate that endogenous IFNs suppress ILC2 function and excessive type 2 immune responses and thereby prevent type 2 immunopathologies and chronic allergic inflammation. Importantly, single nucleotide polymorphisms (SNPs) within interferon genes and respective signaling pathway components, such as IFNG, STAT1, STAT2 and IRF1, are associated with increased asthma susceptibility[310-312]. Furthermore, lower levels of IFN-I have been reported in asthmatic individuals and treatment with IFN-I dramatically improved disease symptoms of patients suffering from severe asthma[313,314]. This suggests that defective endogenous IFN-signaling and concomitant increased ILC2 functions may contribute to asthma pathogenesis. Hence, promoting IFN-signaling may be a promising therapeutic strategy for the management of type 2 immunopathologies. In addition to its suppressive capacities on ILC2s *in vitro*, IL-27 was shown to suppress ILC2 function *in vivo* during *N. brasiliensis* infection and allergen-induced lung inflammation[147,305].

Thus, therapeutic modulation of the IL-27/IL-27R axis may have beneficial effects regarding the management of atopic diseases.

IL-10 and TGF-β

The immunosuppressive cytokines IL-10 and TGF-β are produced by Tregs, ILCregs and other immune cells and were shown to differentially inhibit ILC2 functions. Murine ILC2s constitutively express IL-10Rα and it was observed that IL-10 dampens type 2 cytokine production in naive and IL-33-activated ILC2s, while it failed to inhibit cytokine secretion by ILC2s cultured with IL-2 or IL-2 in combination with IL-25[147,315,316]. These findings suggest that IL-10-mediated suppression of murine ILC2s can vary based on the type of activating stimulus. Expression of IL-10R chains was also described on human blood, tonsil and nasal polyp ILC2s[317,318]. Here, IL-10 stimulation was shown to inhibit IL-5 and IL-13 production from IL-33-activated human ILC2s[317,318]. Interestingly, IL-10 expression is significantly impaired in patients suffering from allergic asthma and rhinitis and lack of IL-10 may lead to increased ILC2 activation and thus contribute to disease pathology[319,320].

Stimulation of naive murine ILC2s with TGF-β impaired production of type 2 cytokines but did not affect proliferation or survival[316]. In addition, it was reported that TGF-β inhibited expression of IL-4, IL-5 and IL-13 by activated human ILC2s while upregulating IL-9[317]. However, another study observed no effect of TGF-β on human ILC2 cytokine production[318]. Further investigations are required to decipher the precise role of TGF-β on ILC2 activation and its physiological functions during allergic inflammation.

1.3.4.4. Costimulatory molecules

Similar to T cells, ILC2s express co-stimulatory receptors that have been shown to modulate ILC2 function in disease settings as well as during homeostasis[183-186,321,322].

ICOS/ICOSL. Murine and human ILC2s constitutively express ICOS independently of their tissue location and mice deficient in either ICOS or its ligand (ICOSL) exhibited reduced numbers of ILC2s in the lung as well as small intestine[182,323-326]. Furthermore, deficiency in ICOS-signaling led to decreased survival, type 2 cytokine production and STAT5

phosphorylation in lung ILC2s upon intranasal IL-33 administration[182,325]. Interestingly, it was reported that Tregs inhibit ILC2 function via the ICOS-ICOSL axis in the lung as well as VAT and that only iTregs but not nTregs mediate this suppressive function[177,316]. These data indicate that the ICOS-ICOSL pathway can either positively or negatively regulate ILC2 function, but the underlying regulatory mechanisms still remain elusive and require further investigation.

NKp30/B7-H6. In addition, expression of the activating NK cell receptor NKp30 was reported on human ILC2s and stimulation with the NKp30 ligand B7-H6 elicited rapid production of type 2 cytokines[327]. Interestingly, B7-H6 expression is elevated in the skin of atopic dermatitis patients and may directly sustain ILC2 activation[327].

Members of the tumor necrosis factor receptor superfamily (TNFRSF) and their cognate ligands (TNFSF) relay important co-stimulatory cues to T cells and other immune cells[328]. Several TNFRSFs and TNFSFs have been demonstrated to play key roles in ILC2 activation and effector responses as well as during homeostasis and are briefly discussed below.

GITR/GITR-L. The co-stimulatory receptor GITR (TNFRSF18) is expressed on T cell subsets and interaction with GITR ligand (GITR-L, TNFSF18) promotes T cell expansion and cytokine production *in vivo*[186]. Murine and human ILC2s express high levels of GITR and deficiency in GITR signaling resulted in impaired ILC2 proliferation, survival and type 2 cytokine production as well as attenuated allergic airway inflammation upon intranasal papain or IL-33 administration[185,186]. Mechanistically, GITR-signaling synergistically increased IL-33-elicited IL-9 induction which further promoted IL-5 and IL-13 production[185]. Importantly, IL-9 administration restored ILC2 functions during IL-33-mediated allergic airway inflammation in GITR-deficient animals[185]. A different study also reported that treatment with a GITR agonist induces type 2 cytokine production in activated murine VAT ILC2s and improves glucose tolerance and insulin sensitivity in a diet-induced obesity model[186]. Importantly, GITR co-stimulation also amplifies type 2 cytokine production in activated human ILC2s[185,186].

DR3/TL1A. Death receptor 3 (DR3, TNFRSF25) is highly expressed on T cells and activation by its cognate ligand TNF ligand-related molecule 1 (TL1A, TNFSF15) promotes T cell proliferation and dampens the suppressive capacity of Tregs[328]. Constitutive expression of DR3 was detected on both human and murine ILC2s and engagement with TL1A promoted expansion, survival and cytokine production independently of IL-25 or IL-33 activation[183,184]. In accordance with these findings, DR3-deficient mice failed to mount ILC2-dependent allergic airway inflammation in response to papain[183,184].

TNFR2/TNF-α.. Recent studies revealed that murine lung as well as human peripheral blood ILC2s selectively express TNFR2[329,330]. Moreover, stimulation with the cognate TNFR2 ligand TNF-α induced robust IL-5 and IL-13 production[329,330]. Blocking of the TNF/TNFR2 axis inhibited ILC2 survival and production of type 2 cytokines as well as ILC2-dependent pulmonary inflammation and airway hyper-reactivity *in vivo*[329]. Importantly, TNF-α is elevated in the airways of patients with severe asthma where it promotes bronchoconstriction and airway hyper-reactivity[329]. Thus, TNFR2 signaling may represent a novel therapeutic target for ILC2-dependent asthma.

RANK/RANK-L. Finally, it was demonstrated that human peripheral blood and nasal polyp ILC2s express receptor activator of NF-κB (RANK, TNFRSF11A) and treatment of ILC2s with an agonistic RANK antibody induced type 2 cytokine production[331]. Furthermore, expression of endogenous RANK ligand (RANK-L) was detected on nasal polyp T_H2 cells[331]. Notably, co-culture of nasal polyp-derived ILC2s and T_H2 cells enhanced IL-5 and IL-13 production and this effect was abrogated when RANK-L blocking antibody was added to the culture[331]. Importantly, elevated RANK-L expression was observed in nasal polyps and the RANK-RANK-L axis may therefore promote ILC2-mediated inflammation in patients with chronic rhinosinusitis with nasal polyps (CRSwNP)[331].

ILC2s also express several co-stimulatory cell surface receptors that contain intracellular tyrosine-based inhibitory motifs (ITIMs) which act as scaffolds for cytoplasmic tyrosine or lipid phosphatases that negatively regulate ILC2 activation and effector functions.

PD-1/PD-L1/PD-L2. PD-1 is an inhibitory cell surface receptor that functions as a major checkpoint in T cell activation[332]. PD-1 is upregulated upon T cell activation and co-stimulation by its ligands PD-L1 or PD-L2 results in suppression of proliferation and effector functions and/or adaptation of a regulatory phenotype[332]. High PD-1 expression has been described on ILCPs and PD-1 is subsequently expressed in a tissue- and activation-dependent manner on mature ILC2s, and was shown to inhibit ILC2 effector functions during infection, allergic airway inflammation, obesity and cancer[84,189,333,334]. PD-1-deficient mice exhibited increased numbers of ILC2s in the lung and small intestine and PD-1 expression was upregulated upon IL-33-elicited ILC2 activation[189]. Moreover, loss of PD-1 or blocking of PD-1 signaling during *N. brasiliensis* infection or intranasal IL-33 administration resulted in increased ILC2 expansion and cytokine production as well as significantly reduced worm burden[189,190]. ILC2s are critical in maintaining adipose tissue homeostasis and limit adiposity by promoting a T_H2-prone environment and beige fat biogenesis[161,236,237]. Accordingly, ILC2s are dysregulated and present at lower numbers in the adipose tissues of obese mice[236]. It was shown that decreased adipose tissue ILC2 function correlated with upregulation of surface PD-1 in obese mice and PD-1 blockade partially restored ILC2 function and tissue homeostasis[333]. Furthermore, PD-L1 expression on M1 macrophages in adipose tissue was increased in obese mice and M1-mediated suppression of ILC2s in an *in vitro* co-culture system was ameliorated in the presence of PD-1 blocking[333]. Lastly, PD-L1 and PD-L2 are expressed on many tumors and a recent report showed that ILC2s infiltrate pancreatic ductal adenocarcinomas (PDACs) in humans and respective mouse models[334]. Furthermore, tumor ILC2 numbers and *IL33* transcript expression positively correlated with long-term survival in both mice and humans[334]. It was also shown that PD-1 inhibits tumor ILC2 intrinsic effector functions and that IL-33-mediated ILC2 activation in combination with ILC2-intrinsic disruption of PD-1 signaling promotes tumor control[334]. ILC2s may therefore partially contribute to the efficacy of anti-PD-1 therapy in human cancers.

KLRG1/E-cadherin. Like PD-1, the C-type lectin receptor KLRG1 is an immune checkpoint receptor that inhibits the activity of T cells and NK cells upon ligation[335]. KLRG1 is upregulated on IL-25- or IL-33-activated ILC2s and interaction with its ligand E-cadherin on epithelial cells was shown to suppress ILC2 proliferation and cytokine secretion upon IL-25 or IL-33

stimulation[223]. Interestingly, loss of E-cadherin on human lung epithelial cells is correlated with increased asthma severity[336].

Taken together, these findings establish co-stimulatory molecules as important positive and negative modulators of ILC2 function.

1.3.4.5. Lipid mediators

Bioactive lipids generated from arachidonic acid such as prostaglandins (PG), cysteinyl leukotrienes (cystLT) and lipoxins (LX) are potent immunomodulators and detected at elevated concentrations in atopic patients[337-341].

Prostaglandins were shown to either positively or negatively regulate ILC2 functions[225,342-344]. As mentioned previously, PGD_2 binds to CRTH2 and induces human and mouse ILC2 chemotaxis and type 2 cytokine production[194,225,226,342]. Conversely, *in vitro* stimulation of murine and human tonsillar and blood ILC2s with PGE_2 inhibited GATA3 expression as well as IL-33-mediated proliferation and type 2 cytokine production in an E-type prostanoid receptor 4 (EP4) dependent manner[343,345]. In human ILC2s, PGE_2 also engages EP2 to mediate its suppressive effect[343]. Intranasal administration of PGE_2 or an EP4 agonist suppressed IL-33-elicited allergic airway inflammation and EP4-deficiency resulted in an exacerbated allergic response upon *A. alternata* challenge[345]. These findings indicate that endogenous PGE_2 actively modulates ILC2-mediated allergic airway inflammation. Similar to PGE_2, *in vitro* stimulation of murine lung and human blood ILC2s with the PGI_2 analog cicaprost restrained IL-33-initiated proliferation and production of IL-5 and IL-13 in a prostacyclin receptor (IP)-dependent manner[344]. Upon intranasal *A. alternata* administration, IP-deficient mice showed a significantly greater induction of total as well as $IL5^+$ and $IL\text{-}13^+$ ILC2s, accompanied by more severe eosinophilia and mucus production[344]. Moreover, administration of cicaprost dampened *A. alternata*-mediated ILC2 induction, type 2 cytokine production and allergic airway inflammation[344]. Taken together, these findings indicate that endogenous PGI_2 suppresses ILC2-mediated allergic airway inflammation upon allergen challenge and that treatment with a PGI_2 agonist may present an interesting treatment option for allergic respiratory diseases.

Cysteinyl leukotrienes are potent proinflammatory lipids, generated predominantly by mast cells and basophils, and key mediators of allergic inflammation and asthma pathogenesis[346]. Murine lung ILC2s highly express the leukotriene receptors CysLT1R and *in vitro* stimulation of ILC2s with LTC_4 and LTD_4 induced NFAT1 translocation and rapid production of type 2 cytokines[227,347-349]. Furthermore, intranasal administration of LTC_4, LTD_4 and LTE_4 elicited IL-5 and IL-13 production by lung ILC2s[347,348]. Moreover, lung ILC2 activation was severely diminished in the absence of leukotriene signaling upon intranasal chitin challenge or *N. brasiliensis* infection[348]. LTC_4 and LTD_4 were able to potentiate IL-33-mediated ILC2 activation *in vitro* as well as IL-33- and allergen-induced ILC2 activation and lung inflammation *in vivo* [347-349]. Importantly, CysLT1R expression was found on human peripheral blood ILC2s and was increased by ILC2s from atopic dermatitis patients[227]. *In vitro* stimulation with LTC_4, LTD_4 and especially LTE_4 induced migration, survival and production of type 2 cytokines in human ILC2s[227].

Lastly, lipoxins, which are generally considered as anti-inflammatory lipid mediators that promote the resolution of inflammation were shown to negatively regulate ILC2 activation[350,351]. Specifically, lipoxin A_4 (LXA_4) was shown to inhibit IL-13 production by PGD_2-activated human peripheral blood ILC2s[351]. Importantly, decreased LXA_4 generation and enhanced eosinophilia were reported in severe asthma and these observations are consistent with the potent inhibitory effect of LXA_4 on ILC2s and highlight a new putative mechanism of asthma pathogenesis[341,352].

Taken together, these findings indicate that prostaglandins, leukotrienes and lipoxins are potent modulators of ILC2 activation and that lipid-mediated ILC2 activation may contribute to allergic inflammation and asthma pathology.

1.3.4.6. Neuropeptides and neurotransmitters

Neuronal-derived bioactive molecules such as neuropeptides and neurotransmitters relay signals from the central nervous system (CNS) to the periphery and vice versa. It is becoming increasingly evident that these cues can also be integrated by immune cells and modulate their function to orchestrate tissue homeostasis and integrity. Intestinal and lung ILC2s express several neuropeptide and neurotransmitter receptors and are strategically positioned in close proximity to enteric neurons and within highly innervated regions in the lungs, respectively.

Specifically, ILC2s express receptors for catecholamine and acetylcholine neurotransmitters as well as for the neuropeptides vasoactive intestinal peptide (VIP), neuromedin U (NMU) and calcitonin gene-related peptide (CGRP) and concomitantly have been shown to directly respond to these cues[353].

Catecholamines

Catecholamines, including dopamine, epinephrine and norepinephrine, are monoamine neurotransmitters that are rapidly released by adrenal glands and adrenergic nerve fibers of the sympathetic nervous system upon emergency 'fight-or-flight' reactions and during exercise[354]. They signal via adrenergic receptors which can be either stimulatory or inhibitory[354]. Lung and intestinal-associated ILC2s express high levels of the inhibitory adrenergic receptor β2AR[232]. Notably, β2AR transcripts could also be detected in human lung and peripheral blood ILC2s[232]. ILC2s were found in close proximity to adrenergic neurons in the villi and submucosa of the small intestine. Upon infection with *N. brasiliensis*, β2AR-deficient ($\beta2AR^{-/-}$) mice exhibited increased numbers of total and $IL\text{-}13^{+}$ ILC2s as well as exaggerated eosinophilia, goblet cell hyperplasia and reduced worm burdens when compared to small intestines of WT mice[232]. These results suggest that the β2AR pathway negatively regulates ILC2 responses. Importantly, treatment of mice with the β2AR agonists clenbuterol or salmeterol, both widely used in the treatment of allergic asthma, during *N. brasiliensis* infection inhibited ILC2 responses and dampened type 2 inflammation[232]. Consistent with these findings, $\beta2AR^{-/-}$ mice exhibited increased frequencies of lung ILC2s in response to intranasal IL-33 or *A. alternata* administration and presence of β2AR agonists inhibited the exacerbated ILC2 response[232]. Importantly, β2AR stimulation intrinsically inhibited ILC2 proliferation and effector functions during helminth infection and IL-33-elicited allergic inflammation as well as in an *ex vivo* culture system of sort-purified ILC2s[232]. Collectively, these results reveal that signaling through β2AR negatively regulates ILC2 proliferation and effector functions. β2AR agonists are widely and successfully used to treat allergic asthma and, taken these results into account, their beneficial effects may be also mediated by dampening ILC2 responses in addition to inhibition of smooth muscle cell contraction and airway hyper-reactivity[355].

Acetylcholines

Acetylcholine neurotransmitters are produced by cholinergic nerves of the parasympathetic nervous system and signal via nicotinic or muscarinic acetylcholine receptors[354]. Lung ILC2s were shown to express the α7 nicotinic acetylcholine receptor (α7nAChR) under homeostatic conditions and expression was further upregulated upon intranasal treatment with IL-25 or IL-33[238]. *Ex vivo* stimulation of activated pulmonary ILC2s with an α7nAChR agonist resulted in dose-dependent suppression of type 2 cytokine production[238]. Intranasal administration of IL-33 or *A. alternata* in combination with α7nAChR agonist resulted in decreased AHR, eosinophilia and suppression of ILC2 effector functions compared to mice that received IL-33 or allergen alone[238]. Furthermore, α7nAChR agonist-mediated inhibition of IL-33-elicited allergic airway inflammation was abrogated in α7nAChR-deficient mice and the observed effects were shown to be ILC2-intrinsic[238]. Importantly, α7nAChR agonist also inhibited IL-5 and IL-13 production by activated human peripheral blood ILC2s *ex vivo*, and prevented IL-33-induced AHR in a humanized mouse model[238]. Altogether, these findings indicate a protective role of cholinergic signaling during asthma pathogenesis and imply that α7nAChR agonist may represent a novel therapeutic candidate for the management of inflammatory lung disease.

Neuromedin U (NMU)

The neuropeptide NMU signals via the G protein-coupled receptors NMUR1 and NMUR2. NMU is produced by cholinergic neurons in the small intestine, and transcript expression was also detected in sensory neurons that originate from the dorsal root ganglia and innervate the lung[239-241,356]. NMUR1 is selectively expressed on lung and intestinal ILC2s at steady state and during allergic inflammation, while NMUR2 expression was not detectable[239-241]. Notably, human peripheral blood as well as intestinal ILC2s showed enriched expression of NMUR1 transcripts over other immune cell populations[239,240]. It was shown that murine small intestinal ILC2s co-localize with NMU^+ cholinergic neurons and *in vitro* stimulation of intestinal ILC2s with NMU elicited rapid proliferation and production of type 2 cytokines in an NMUR1- and $G_{\alpha q}$-dependent manner[239,240]. NMUR1 activation further resulted in increased Ca^{2+} influx, calcineurin activation and translocation of NFAT to induce transcription of type 2 cytokines[239]. Furthermore, intraperitoneal injection of NMU induced small intestinal ILC2 expansion and activation, accompanied by goblet cell hyperplasia and increased mucus production[239,240]. Notably, intestinal

NMU expression was upregulated upon helminth infection and NMU delivery during infection with *N. brasiliensis* resulted in increased ILC2 numbers, eosinophilia and faster worm expulsion[239,240]. Interestingly, IL-33 and *N. brasiliensis* excretory/secretory products efficiently induced NMU expression in enteric neuron organoid cultures[239]. In addition to intestinal ILC2s, NMUR1 expression was reported on lung ILC2s and a majority of lung ILC2s are located in close proximity to *Nmu*-expressing afferent neurons[241]. Similar to intestinal ILC2s, *in vitro* stimulation of pulmonary ILC2s with NMU resulted in proliferation and rapid production of type 2 signature cytokines[239,241]. Importantly, intranasal administration of NMU induced ILC2 activation and allergic airway inflammation indicating that NMU can regulate ILC2s at multiple barrier sites[240,241]. Interestingly, asthmatic patients exhibited elevated NMU transcript levels in bronchial brushing samples and expression levels correlated with disease severity, indicating that NMU may also mediate ILC2 effector functions during allergic airway inflammation in humans[357].

Vasoactive intestinal peptide (VIP)

The neuropeptide VIP engages VPAC1 or VPAC2 receptors to exert its biological functions[358]. Notably, while intestinal ILC2s express transcripts for both receptors, lung ILC2s only express VPAC2 mRNA[10,244]. VIP has been shown to promote ILC2 functions upon allergic inflammation as well as during mucosal homeostasis. It was observed that intestinal ILC2s produce large amounts of IL-5 when cultured with VIP in the presence of IL-7 *in vitro*[10]. Furthermore, VIP has been shown to stimulate peripheral tissue ILC2s *in vivo* in response to circadian and metabolic cues[10]. Here, ILC2-derived IL-5 was crucial for basal eosinophil accumulation in the small intestine and lungs and thereby maintenance of barrier homeostasis[10]. Nociceptor-derived VIP was also shown to be instrumental in the generation of type 2 immune responses following HDM- and OVA-induced allergic airway inflammation[359]. It was observed that VIP was able to act on both $CD4^+$ T cells and ILC2s via VPAC2 to induce type 2 cytokine production[359]. ILC2-derived IL-5 in turn feeds back on neurons to induce VIP to further amplify the response[359]. Collectively, these findings demonstrate that VIP is a potent driver of adaptive and innate type 2 immunity.

Calcitonin gene-related peptide (CGRP)

Lung and intestinal ILC2s were shown to express transcripts of both chains of the CGRP receptor, calcitonin receptor-like receptor (CLR) as well as the receptor-activity modifying protein 1 (RAMP1)[242-245]. Innervated sensory lung epithelial cells, termed pulmonary neuroendocrine cells (PNECs), are a potent source of CGRP and it was demonstrated that ILC2s co-localize with PNEC clusters in the lung[242]. Following OVA challenge, mice with a specific deletion of the transcription factor ASCL1 in epithelial PNEC precursors and therefore ablation of PNECs, exhibited reduced numbers of pulmonary type 2 immune populations, including ILC2s[242]. Moreover, *in vitro* culture of isolated pulmonary ILC2s with CGRP in the presence of IL-33 or IL-25 resulted in increased production of IL-5[242]. Selective deletion of the CLR gene in ILC2s using *Il5*^{cre} mice did not affect lung ILC2 numbers but resulted in decreased recruitment of immune cells upon HDM-elicited allergic airway inflammation[242]. These findings indicated that CGRP may amplify effector functions of activated ILC2s. However, three recent studies reported overall inhibitory effects of CGRP on ILC2s[243-245].

Using scRNA-seq, Nagashima et al. identified a subset of lung ILC2s in *N. brasiliensis*-infected mice that co-expressed transcripts for CGRP and its receptor chains CLR and Ramp1, as well as IL-5[243]. Furthermore, it was observed that *in vitro* stimulation of ILC2s with CGRP in the presence of IL-33 or NMU promoted IL-5 while suppressing IL-13 production and ILC2 proliferation[243]. Mechanistically, CGRP exerted its inhibitory effect on ILC2s via $G_{\alpha s}$ and downstream induction of cAMP[243]. Importantly, CGRP administration dampened IL-33-elicited allergic airway inflammation *in vivo* and CGRP-deficient mice exhibited enhanced protection upon infection with *N. brasiliensis*[243]. Accordingly, mice lacking CGRP receptor signaling (*Ramp1*$^{-/-}$) within the hematopoietic compartment harboured higher ILC2 numbers and significantly less worm burden than receptor-sufficient mice following *N. brasiliensis* infection[243].

Wallrapp et al. reported CGRP expression in lung ILC2s upon IL-33-mediated activation, and it was further observed that CGRP inhibited ILC2 proliferation *in vitro* and promoted IL-33-mediated IL-5 production by lung ILC2s in the short term[244]. However, CGRP inhibited effector cytokine production during prolonged culture[244]. Mechanistically, CGRP treatment induced upregulation of genes associated with Treg function or negative regulation of effector T cell responses in ILC2s, such as inhibitory surface receptors PD-1 and Tim-3 and the transcription

factor FoxP3[244]. Importantly, intranasal administration of CGRP in combination with IL-33 dampened IL-33-mediated ILC2 expansion and airway inflammation[244].

Again, using scRNA-seq, Xu et al. observed that small intestinal KLRG1$^+$ ILC2s highly upregulated CGRP transcript expression upon oral OVA challenge of previously sensitized mice while CGRP receptor chains were expressed at steady state and downregulated upon inflammation[245]. In addition, this study identified enteric ChAT$^+$ neurons as main source of basal CGRP[245]. *In vitro* stimulation of intestinal ILC2s with CGRP inhibited IL-25-mediated proliferation in a cAMP-dependent manner but did not affect *Il5* induction[245]. Furthermore, intraperitoneal administration of CGRP suppressed IL-25-induced intestinal ILC2 expansion[245]. Importantly, CGRP-deficiency resulted in elevated numbers of intestinal ILC2s and consequently also tuft cells, indicating that basal CGRP signaling suppresses ILC2s and maintains homeostasis[245].

Interestingly, spinal cord injury induces expression of CGRP transcripts in meningeal ILC2s and CGRP has been implicated in regeneration of sensory neurons in the CNS after injury[12]. However, whether meningeal ILC2-derived CGRP exhibits similar functions remains to be investigated.

Altogether, these findings indicate that lung and small intestinal ILC2s can upregulate production of CGRP upon activation and while CGRP may promote IL-5 production in the short term it predominantly restrains ILC2 expansion and effector functions during helminth infection as well as allergic inflammation.

1.3.4.7. Endocrinal regulation

In general, men develop less pronounced adaptive immune responses compared to women and are therefore more susceptible to certain infectious diseases while at lower risk for autoimmunity[360]. This sex bias underlines the important role of estrogens, progesterone and androgens in immune regulation. Asthma is a prime example of an inflammatory disease with underlying sex bias. Sex hormones were implicated in the regulation of asthma-associated airway inflammation, smooth muscle contraction, airway mechanics and mucus production[361-363]. While childhood asthma is more prevalent in boys than girls, this ratio shifts with rising levels of sex hormones during adolescence, resulting in women being twice as prone to

developing adult-onset asthma by mid-life[362,364]. Similar observations regarding sex bias were made in mouse models of ovalbumin (OVA)- and house-dust-mite (HDM)-induced allergic airway inflammation[248,365]. ILC2s are critical in the initiation and orchestration of allergic lung inflammation. Importantly, allergic asthma patients exhibit higher frequencies of ILC2s within their peripheral blood mononuclear cells (PBMCs) when compared to healthy controls[284]. Furthermore, ILC2 numbers in the circulation were higher in asthmatic women than men and female ILC2s produced higher levels of IL-5 upon re-stimulation[247]. Consistently, substantially higher numbers of total as well as Ki-67$^+$ bone marrow ILC2Ps and mature ILC2s in lung, VAT and mesLNs were observed in female mice at steady state[248]. Mature male lung ILC2s also expressed higher levels of the inhibitory receptor KLRG1, while female ILC2s and ILC2Ps showed increased expression of CD25[247-249]. Furthermore, presence of the testosterone derivative 5α-dihydrotestosterone (DHT) inhibited *ex vivo* expansion of cultured ILC2Ps in response to IL-33 and this effect could be reversed by addition of the androgen receptor antagonist flutamide, suggesting that androgens directly suppress ILC2s[248]. Importantly, while intact male mice exhibited less severe allergic airway inflammation upon intranasal IL-33 treatment than their female counterparts, this difference was abolished upon castration, further highlighting a potential inhibitory role of endogenous androgens on ILC2s *in vivo*[248]. Differential CD25 expression might at least partly account for the sex-specific differences observed. Lung ILC2s from female mice exhibited enhanced proliferative capacity and cytokine production upon stimulation with IL-33 in the presence of IL-2 when compared to ILC2s from male or pre-pubescent mice of either sex[247]. Moreover, DHT administration to gonadectomized mice, which lack testosterone, decreased lung ILC2 numbers compared to gonadectomized mice treated with vehicle or sham-operated female mice[247]. ILC2s from DHT-treated mice also exhibited reduced CD25 surface expression and type 2 cytokine production after *ex vivo* re-stimulation[247]. Consistently, direct *ex vivo* stimulation of ILC2s with DHT suppressed production of IL-5 and IL-13 in a dose-dependent manner. Importantly, endogenous androgens also exhibited a protective role during allergic *A. alternata*-induced allergic airway inflammation with ILC2s from male mice exhibiting significantly diminished production of type 2 signature cytokines[247].

Estrogens and androgens act via the nuclear hormone receptors estrogen receptor (ER) α, ERβ and androgen receptor (AR), respectively, to modulate immune cell functions[366]. Androgen receptor transcripts were highly expressed in ILC2Ps and lung ILC2s whereas ERα

was preferentially expressed in uterine ILC2[118,246,248]. Consistent with these findings, ovariectomized mice exhibited markedly decreased numbers of uterine ILC2s while lung ILC2 counts remained unaffected[246]. Furthermore, treatment with 17β-estradiol increased uterine ILC2 numbers, whereas lung ILC2 numbers remained stable[246].

In addition, it was suggested that, based on intrinsic differences in gene expression, female ILC2s are more metabolically active and exhibit enhanced responsiveness to IL-33-mediated activation[367]. Altogether, these studies have demonstrated that, depending on the tissue, endogenous sex hormones differentially modulate ILC2s function during homeostasis as well as upon activation. However, the precise downstream targets of androgen and estrogen receptors in ILC2s and ILC2Ps remain to be investigated.

Obesity and insulin resistance are generally more prevalent in men than age-matched women and male mice are consistently more prone to metabolic disorders in high-fat diet-induced obesity models than their female counterparts[368]. As noted above, a reduction in ILC2s was observed in male VAT and ILC2s have been further shown to regulate thermogenesis from beige fat, prevent metabolic syndrome and insulin resistance as well as promote insulin secretion under diabetic conditions[161,170,236,237,248]. Thereby, sex hormone-mediated ILC2 regulation may also contribute to the sex-biased differences in metabolic homeostasis and disease susceptibility.

1.3.4.8. Bacterial-derived metabolites and dietary nutrients

ILC2s are enriched at barrier surfaces and thereby reside in close vicinity to commensal microorganisms. It was initially proposed that ILC2s are sustained independently from microbiota-derived signals, with their functional capacity and numbers in small intestine and lung being unaffected in germ-free mice[29,151]. However, in a recent study, markedly reduced numbers of stomach ILC2s were observed in germ-free mice[157]. It was further demonstrated that commensal-derived signals elicited IL-7 production in the stomach which was essential for ILC2 accumulation[157]. These findings highlight yet again the differential requirements of ILC2s in different tissues.

Short chain fatty acids (SCFAs), such as butyrate, are end products of dietary fiber fermentation by anaerobic bacteria and play an essential role in maintaining gut homeostasis by driving Treg differentiation[369,370]. *In vitro* stimulation of murine lung and human peripheral

blood ILC2s with butyrate directly suppressed type 2 cytokine production and oral or local administration of butyrate prior to and during intranasal *A. alternata* or IL-33 exposure inhibited ILC2 expansion and alleviated allergic airway inflammation in mice[371,372].

Active metabolites of vitamins A and D have long been known to exhibit immunomodulatory properties[373]. Retinoic acid (RA), an active metabolite of vitamin A, that binds to the intracellular retinoic acid receptor (RAR), has been shown to suppress ILC2s by downregulation of IL-7Rα[235]. Accordingly, vitamin A-deficient mice harboured higher numbers of ILC2s in the small intestinal lamina propria[235]. In contrast, in human ILC2s, RA was shown to act synergistically with IL-2 or IL-7 to promote *in vitro* production of IL-5 and IL-13 and expression of the gut homing integrin α4β7 whereas the active vitamin D metabolite 1,25D3 dampened RA-induced cytokine production and α4β7 expression[374]. These discrepancies in the response of human versus murine ILC2s to RA still require further investigation.

The aryl hydrocarbon receptor (AhR) is a cytosolic environmental sensor that translocates to the nucleus upon ligand binding[375]. Ahr responds to environmental toxins but can also sense endogenous and physiological ligands, such as dietary or microbial metabolites[375]. Intestinal murine ILC2s specifically express high levels of AhR and it was observed that AhR-deficient mice ($AhR^{-/-}$) harboured increased numbers and frequencies of total as well as $IL\text{-}5^{+}$ and $IL\text{-}13^{+}$ intestinal ILC2s[60]. Accordingly, $AhR^{-/-}$ mice exhibited enhanced ILC2 immunity and protection upon helminth infection, further supporting the suppressive effect of AhR on ILC2 functions[60]. However, endogenous ligands of AhR that may mediate ILC2 suppression as well as the role of AhR in human ILC2s still need to be identified.

1.3.4.9. Growth factors, chemokines and complement peptides

It has been demonstrated recently that murine and human ILC2s produce vascular endothelial growth factor (VEGF-A) upon activation and that VEGF-A production promoted airway hyper-reactivity upon *A. alternata*- and IL-33-mediated airway inflammation in mice[376]. Mouse ILC2s also expressed the VEGF-A receptor VEGFR2 and inhibition of signaling through VEGFR2 suppressed IL-13 production in ILC2s, indicating that ILC2-derived VEGF-A may act in an autocrine manner to promote ILC2 function[376]. Interestingly, VEGF-A production was higher

in ILC2s isolated from asthmatic patients and may thereby contribute to enhanced ILC2 functions and disease pathology[376].

Another growth factor, granulocyte-colony stimulating factor (G-CSF) was found to act directly on pulmonary ILC2s during HDM-mediated allergic airway inflammation to promote ILC2 function[377]. It was shown that mouse and human ILC2s expressed G-CSF receptor transcripts and *in vitro* stimulation with G-CSF resulted in enhanced IL-5 and IL-13 production[377]. Moreover, *in vivo* co-administration of G-CSF in HDM-exposed animals further induced ILC2 type 2 cytokine production and led to elevated levels of IL-5 and IL-13 in the BALF thus highlighting a potential physiological role of G-CSF during ILC2-elicited allergic airway inflammation[377].

Beyond regulating migration and homing, several chemokines have been shown to promote proliferation and effector functions in target cells[222]. The chemokine CCL1 has recently been shown to be produced by murine as well as human ILC2s and expression was further increased upon activation[222]. Moreover, CCL1 acted as an autocrine ILC2-stimulating factor and potentiated proliferation, survival and IL-9 production *in vitro* in a CCR8-dependent manner. Furthermore, ILC2-intrinsic CCR8-deficiency resulted in impaired anti-helminth immunity upon *N. brasiliensis* infection, suggesting a critical role of CCR8 in ILC2-mediated type 2 immune responses[222].

Finally, it was demonstrated that the anaphylatoxin C3a is induced in mice in response to HDM and IL-33 and drives ILC2-dependent airway inflammation[378]. Specifically, it was shown that ILC2s highly express C3a receptor transcripts[378]. Moreover, stimulation with C3a induced type 2 cytokine production and facilitated ILC2 antigen-presentation, thereby promoting ILC2-T cell crosstalk and adaptive immunity[378]. Interestingly, C3a production was also initiated upon allergen exposure in humans and C3a levels are elevated in asthmatic patients and may thereby potentially contribute to ILC2-mediated disease pathology[379,380].

ILC2s are a potent source of type 2 cytokines and implicated in the development of various atopic diseases including asthma. Thus, ILC2s must be tightly regulated to ensure efficient host protection while preventing excessive activation that may result in type 2 immunopathologies. Over the past few years, multiple pathways have been described that modulate ILC2 effector functions and may therefore affect the pathogenesis of allergic diseases

(*Figure 7*). These findings provide key information for the design of novel therapeutic strategies to counteract asthma, atopic dermatitis and other allergic diseases.

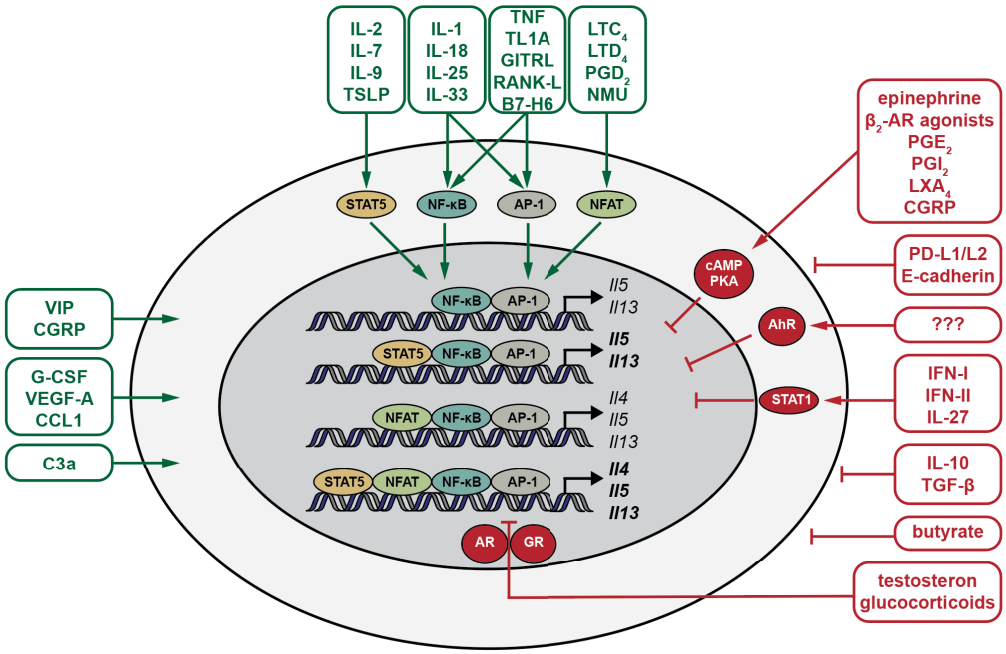

Figure 7. The ILC2 regulatory network.

ILC2s express a variety of extracellular and intracellular receptors to integrate activating (green) and inhibitory (red) cues from their environment to adapt their transcriptional program and functions accordingly. Activating signals from cytokines such as IL-33, IL-25, IL-1 and IL-18 as well as downstream signaling upon ligation of co-stimulatory receptors by TNF, TL1A, GITRL, RANK-L and B7-H6 result in activation NF-κB and AP-1 transcription factors which mediate survival, proliferation and type 2 cytokine expression. ILC2 functions can further be enhanced upon STAT5 activation by co-stimulatory cytokines such as IL-2, IL-7, IL-9 and TSLP. Moreover, translocation of NFAT is elicited upon stimulation with leukotriene and PGD_2 lipid mediators as well as the neuropeptide NMU and results in additional IL-4 production. Other neuropeptides, including VIP and CGRP, growth factors such as G-CSF and VEGF-A, the chemokine CCL1 and the complement protein C3a also promote ILC2 function. Conversely, the cytokines IL-10 and TGF-β as well as IFN-I and IFN-II suppress ILC2 effector functions. Additional inhibitory signals are provided by neurotransmitters, CGRP, the bioactive lipids PGE_2, PGI_2 and LXA_4 as well as ligation of the inhibitory surface receptors by E-cadherin and PD-L1/L2. Lastly, hormones such as testosterone and glucocorticoids and dietary nutrients including butyrate were shown to negatively regulate ILC2 activity. Abbreviations: VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related peptide; G-CSF, granulocyte-colony stimulating factor; VEGF-A, vascular endothelial growth factor A; CCL1, C-C motif chemokine ligand 1; IL-, interleukin-; TSLP, thymic stromal lymphopoietin; TNF, tumor necrosis factor; TL1A, TNF ligand-related molecule 1A; GITRL, glucocorticoid-induced tumor necrosis factor receptor ligand; RANK-L, receptor activator of NF-κB; LT, leukotriene; PG, prostaglandin; NMU, neuromedin U; LXA_4, lipoxin A_4; PD-L1/L2, programmed death ligand 1/ligand 2; IFN,

interferon; TGF-β, transforming growth factor β; STAT, signal transducer and activator of transcription; NF-κB, nuclear factor-κB; AP-1, activator protein 1; NFAT, nuclear factor of activated T cells; cAMP, cyclic adenosine monophosphate; Ahr, aryl hydrocarbon receptor; AR, androgen receptor; GR, glucocorticoid receptor. Adapted from [381].

1.3.5. ILC2s at the interface of innate and adaptive immunity

Besides initiating innate type 2 immune responses, it is becoming increasingly appreciated that ILC2s also play important roles in bridging innate and adaptive responses. ILC2s can modulate the quality and magnitude of adaptive responses via indirect interaction mediated through bystander cells as well as directly via soluble mediators or cell-cell interactions (*Figure 8*). The key role of ILC2s in orchestrating adaptive immune responses was highlighted by several studies demonstrating that the absence of ILC2s results in severely impaired T_H2 responses during allergic airway inflammation and helminth infection in mice[188,382].

Indirect regulation of adaptive responses through bystander cells. Upon allergen-induced allergic airway inflammation, ILC2-derived IL-13 was shown to be critical for the migration of activated lung DCs to the mediastinal lymph node to facilitate priming and polarization of naive $CD4^+$ T cells to T_H2 cells[382]. Moreover, it was suggested that IL-13 promotes lung DC migration, at least partially, by inducing EP_4 receptor expression on DCs and PGE_2 production by lung leukocytes[382]. Lung ILC2-derived IL-13 can also stimulate the production of the T_H2-attracting chemokine CCL17 by airway $CD11b^+CD103^-$ DCs which was demonstrated to be critical for the recruitment of memory T cell to the airways during secondary allergen challenge[383]. Furthermore, ILC2-derived IL-13 can induce polarization of macrophages to the alternatively-activated M2 phenotype which in turn mediate T_H2 cell differentiation and Treg effector functions[384,385].

Direct modulation of adaptive immunity via soluble mediators. ILC2s produce several cytokines that can directly regulate T or B cell responses (*Figure 8A*). Limited evidence suggests that ILC2-derived IL-4 may be important for stimulating T_H2 cell differentiation and restraining Treg responses during intestinal helminth infections or food allergy, respectively[386,387]. Moreover, FALC ILC2-derived IL-5 and IL-6 can act on B-1 cells to promote self-renewal as well as production of IgM and IgA[7,388].

Direct regulation of T cell immunity via antigen presentation. ILC2s can directly initiate adaptive type 2 responses by presenting antigens and/or providing co-stimulation to $CD4^+$ T cells and NKT cells (*Figure 8A*)[177,182,187,188,190,231,389]. MHC-II expression has been reported on murine ILC2s and MHC-II levels can be additionally augmented by ILC2 activation or trogocytosis of antigen-bearing MHC-II complexes from professional antigen-presenting cells[9,188,389]. Importantly, ILC2s were able to process and present antigens in complex with MHC-II to antigen-specific $CD4^+$ T cells to drive type 2 immune responses *in vitro* and *in vivo*[188,389]. Moreover, MHC-II^+ ILC2s from IL-33-treated mice also expressed the costimulatory molecules CD80 and CD86 to further stimulate T cell activation[188,389]. Besides promoting T_H2 cell effector functions, the ILC2-T cell crosstalk induces IL-2 production by T cells which feeds back on ILC2s to promote proliferation and IL-13 secretion[188,389]. In addition, it has been recently reported that skin ILC2s can present endogenous lipid antigens to T cells[231]. Using an *in vivo* human skin challenge model, it was observed that skin-derived ILC2s express CD1a which was further augmented by TSLP[231]. Moreover, ILC2s were able to generate and present endogenous lipid antigens via CD1a and thereby activate CD1a-responsive T cells[231]. Notably, stimulation with *Staphylococcus aureus*, which often colonizes skin lesions of atopic dermatitis patients, further enhanced endogenous lipid antigen presentation on ILC2s via CD1a and may contribute to the ILC2-mediated inflammatory response[231].

Direct regulation of T cell responses by costimulatory molecules. ILC2s express several costimulatory molecules that can directly modulate T cell function (*Figure 8B*). It was demonstrated that IL-33-mediated activation of ILC2s induces expression of the TNF superfamily ligand OX40L (TNFSF4) *in vitro* as well as *in vivo*[187]. Interaction of OX40L on ILC2s with its cognate receptor OX40, expressed on conventional T cells and Tregs, promoted T_H2 proliferation and effector responses as well as Treg expansion following allergic airway inflammation and *N. brasiliensis* infection[187]. In accordance, specific deletion of OX40L in ILC2s resulted in a significant reduction in antigen-specific T_H2 as well as Treg numbers, further highlighting the critical role of OX40L-mediated co-stimulation in the orchestration of adaptive T cell immunity[187]. ILC2s are further characterized by the expression of ICOS and interaction with ICOSL on Treg cells facilitates Treg accumulation in response to IL-33[177,182]. Finally, PD-L1 expression was upregulated on ILC2s upon *N. brasiliensis* infection and

conditional deletion of PD-L1 on ILC2s resulted in delayed worm expulsion[190]. Mechanistically, PD-L1$^+$ ILC2s interact with PD-1 on T_H2 cells during infection to promote their effector functions and thus further facilitate anti-helminth immunity[190].

Taken together, these findings suggest a key role for ILC2s in the orchestration of both humoral and T cell-mediated adaptive type 2 immune responses in addition to their function in initiating innate type 2 immunity.

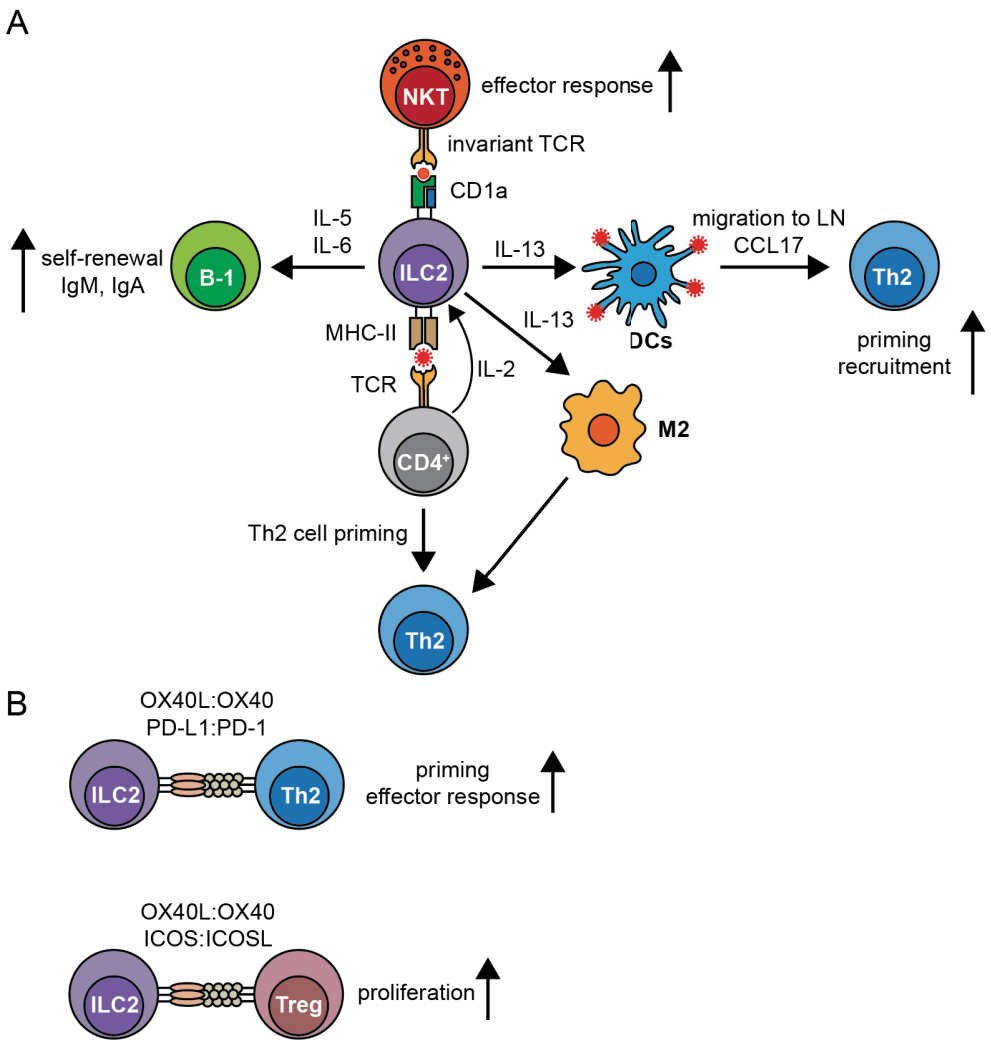

Figure 8. ILC2s modulate adaptive immune responses.

(**A**) ILC2s can regulate adaptive type 2 immune responses indirectly by activating bystander cells such as DCs and M2 macrophages via IL-13 which then promote the polarization and/or recruitment of T_H2 cells. Moreover, ILC2-derived IL-5 and IL-6 can directly activate B-1 B cells and ILC2s can directly present protein or lipid antigens to T cells via MHC-II or CD1a, respectively, thereby facilitating T_H2 polarization and function as well as NKT cell effector responses. (**B**) ILC2-mediated direct activation of adaptive T cell responses through costimulatory molecules. Abbreviations: Ig, immunoglobulin; IL-, interleukin-; NKT, natural killer T cell; ILC2, group 2 innate lymphoid cell; TCR, T cell receptor; LN, lymph node; CCL17, C-C motif chemokine ligand 17; DCs, dendritic cells; T_H2, type 2 helper T cell; M2, type 2 macrophage; PD-L1, programmed cell death 1 ligand 1; PD-1, programmed cell death 1; ICOS, inducible T cell costimulator; ICOSL, inducible T cell costimulator ligand; Treg, regulatory T cell.

1.3.6. ILC2-mediated immunity at barrier surfaces – effector cytokines and functions

Upon activation, ILC2s can produce a wide array of cytokines associated with allergic inflammation[16] but also metabolic and regenerative responses[66,390,391]. An important feature of ILC2s is their capacity to rapidly respond to environmental cues and induce transcription and synthesis of effector cytokines to mediate host defense and homeostasis. To this end, ILC2s adopt an epigenetically 'poised' state with lineage-specific chromatin landscapes diverging early during ILC development[392]. A seminal study published recently carried out Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) analysis to compare genome-wide chromatin accessibility of murine lung ILC2s and T_H2 cells, isolated from either naive or *N. brasiliensis* infected mice[392]. Strikingly, regulatory elements that control the expression of type 2 signature genes such as IL-5 and IL-13 were already selectively accessible in resting ILC2s and changed little upon infection[392]. On the contrary, identical regulatory regions in T_H2 cells were predominantly generated *de novo* after activation and closely resembled the ILC2 regulome thereafter[392]. Below I will briefly summarize main ILC2 effector cytokines and their functions with a focus on pulmonary immunity (*Figure 9*).

Amphiregulin

The epidermal-like growth factor amphiregulin (AREG) is a ligand of the epidermal growth factor receptor (EGFR) and as such can induce differentiation and proliferation of target cells to promote tissue repair[393]. It has been shown that murine and human ILC2s produce AREG upon stimulation with IL-33 or NMU and that ILC2-derived AREG promotes tissue homeostasis after influenza virus infection and dextran sodium sulfate (DSS)-induced colitis[13,62,223,239]. Following influenza A virus infection, ILC2s were induced in an IL-33-dependent manner in the lung parenchyma and depletion of ILC2s significantly impaired oxygen saturation, epithelial integrity and tissue remodeling following infection[13]. Importantly, adoptive transfer of ILC2 into ILC-depleted *Rag1*$^{-/-}$ mice restored oxygen saturation and lung function as well as tissue remodeling suggesting an ILC2-intrinsic role in promoting tissue repair and homeostasis[13]. The authors additionally observed that ILC2s produce AREG upon *in vitro* IL-33 stimulation and upon IAV infection *in vivo*. Furthermore, administration of AREG improved lung function as well as epithelial integrity and promoted lung homeostasis in ILC-depleted mice[13]. Collectively, these data indicate that ILC2-derived AREG may play an important role in restoring tissue homeostasis after IAV

infection. In a different study the same group further showed that ILC2-derived AREG can additionally mediate disease amelioration and tissue repair in a model of DSS-induced colitis[62]. Here, DSS exposure induced AREG$^+$ ILC2 in the mesLN and colonic lamina propria and mice deficient in AREG or AREG signaling exhibited more severe weight loss and colon pathology indicating that AREG signaling may dampen inflammation and promote tissue repair[62]. Importantly, systemic administration of IL-33 in parallel with DSS resulted in ILC2 expansion and a decrease in weight loss, restoration of crypt architecture and goblet cell responses in WT but not *Areg*$^{-/-}$ mice implying a role for the IL-33-AREG axis in tissue protection during colitis[62]. The authors further observed that adoptive transfer of activated WT ILC2 into *Areg*$^{-/-}$ mice was sufficient to improve intestinal damage and disease outcome highlighting the role of ILC2-derived AREG in the resolution of inflammation and tissue repair[62]. AREG transcript expression was also observed in human skin-resident ILC2s and was elevated in skin biopsies from atopic dermatitis lesions compared to non-affected skin[223]. Moreover, IL-33 but not IL-25, further elicited AREG production in human skin ILC2s[223]. Since AREG plays an important role in wound healing it would therefore be of interest to investigate whether ILC2-derived AREG is implicated in the maintenance of barrier integrity or barrier repair mechanisms in AD patients. ILC2-derived AREG was additionally shown to improve gastrointestinal barrier function in a model of acute graft versus host disease (aGVHD)[394] and displayed a renoprotective role in a model of ischemia-reperfusion injury[395]. Importantly, AREG promotes mucus production in human bronchial epithelial cells and asthmatic patients exhibit increased sputum levels of AREG correlating with eosinophil numbers[396,397]. However, whether ILC2s account for these elevated AREG levels in asthmatic individuals remains elusive.

IL-4

While murine ILC2s were reported to secrete no to very little IL-4 upon alarmin challenge, NFAT-mediated activation following exposure to lipid mediators or NMU potently induces IL-4 production[240,347,348]. Consistent with these findings, lung ILC2s did not produce IL-4 upon *N. brasiliensis* infection and rarely any IL-4 upon alarmin or OVA-induced allergic airway inflammation[323,398]. Nevertheless, IL-4 production by ILC2s was suggested to drive food allergy by blocking Treg function and to promote T_H2 differentiation during *Heligmosomoides polygyrus* infection[386,387]. As mentioned above, IL-4 can also act as an ILC2-stimulatory cytokine. Murine

ILC2s express IL-4Rα and addition of IL-4 to ILC2 cultured with IL-33 lead to a synergistic increase in ILC2 proliferation and type 2 cytokine production, further amplifying ILC2 functions[163,302,303].

IL-5

ILC2s constitutively secrete low levels of IL-5 and IL-5 production can be further induced upon ILC2 activation[10]. IL-5 signals through the heterodimeric IL-5 receptor composed of the ligand binding IL-5Rα chain and the common β chain (β_c) which is shared with receptors for IL-3 and GM-CSF. As mentioned previously, ILC2-derived IL-5 promotes self-renewal and antibody production by B-1 cells as well as IgE production by B-2 cells[7]. In addition, IL-5 acts on eosinophils and downstream signaling results in differentiation, activation and survival as well as expansion and trafficking of eosinophils from the bone marrow to the lung following allergen exposure[399,400]. It has been reported that ILC2-derived IL-5 is critical for eosinophil homeostasis as well as for eosinophil accumulation and effector functions upon parasitic helminth infection and allergic airway inflammation in mice[10,401]. Eosinophils are central effector cells in the propagation of allergic airway inflammation by releasing granule proteins including major basic protein, reactive oxygen species, cytokines, chemokines as well as bioactive lipids. Importantly, eosinophilia is a hallmark of allergic asthma where increased numbers of eosinophils are found in the circulation, airways and sputum of asthmatic patients and correlate with disease severity as well as the development of asthma exacerbations[402-409]. In accordance, IL-5 levels are increased in induced sputum and serum from allergic asthmatics compared to healthy controls and correlated with disease severity[410,411]. Furthermore, elevated levels of IL-5 were observed in induced sputum from patients experiencing acute asthma exacerbations[412]. IL-5 also contributes to the pathobiology of late-onset, non-allergic eosinophilic asthma[413]. Neutralization of IL-5 (mepoluzimab, reslizumab) or blockage of IL-5R (benralizumab) with humanized monoclonal antibodies reduced blood and sputum eosinophils as well as asthma exacerbation frequency[414-417]. However, since IL-5 can be produced by multiple cell types including T_H2 cells and NKT cells, the precise contribution of ILC2-derived IL-5 to asthma pathogenesis in humans remains elusive and remains to be further investigated.

IL-9

As mentioned earlier, ILC2s constitutively express high levels of IL-9R and IL-9 production is induced upon ILC2 activation and can act in an autocrine manner to promote ILC2 survival and cytokine production during allergic airway inflammation and helminth infection[8,283,294,295]. However, if ILC2-derived IL-9 also acts on other IL-9-responsive cells, such as T_H2 cells, remains to be investigated.

IL-13

Unlike IL-5 which is constitutively expressed by lung ILC2s, production of the type 2 signature cytokine IL-13 inducible upon ILC2 activation[8,10,74,382,401]. IL-13 signals through the heterodimeric IL-13 receptor (IL-13R) which consists of the ligand-specific IL-13Rα1 subunit and the IL-4Rα subunit, shared with IL-4[418]. Upon binding of IL-13, IL-13Rα1 dimerizes with IL-4Rα to trigger the JAK/STAT pathway resulting in phosphorylation, homodimerization and nuclear translocation of STAT6[418]. IL-13Rα1 and IL-4Rα chains are widely expressed at low levels on both hematopoietic and non-hematopoietic cells and IL-13 is thus able to exert a wide array of effector functions[418]. ILC2-derived IL-13 was shown to induce goblet cell hyperplasia, mucus production, smooth muscle contraction, airway hyper-reactivity and airway remodeling in models of allergen and cytokine-induced allergic airway inflammation[74,382,401]. ILC2s also represent the predominant early IL-13 source after helminth infection and ILC2-derived IL-13 mediates physiological changes such as intestinal goblet cell hyperplasia, mucus hyperproduction and smooth muscle cell hyper-contractibility that are critical for the so-called 'weep-and-sweep' response which promotes worm clearance[9,419]. Furthermore, tuft cell-derived IL-25 induces IL-13 production by ILC2s upon *N. brasiliensis* infection, which in turn acts on epithelial progenitors and biases their lineage commitment towards tuft cells as well as goblet cells[181]. ILC2-derived IL-13 has also been shown to induce a type 2 phenotype in macrophages as well as promote CCL17 production by DCs and migration to the mediastinal LN thereby shaping downstream adaptive immune responses[382-385]. Since IL-13 is involved in many of the pathophysiological features that characterize asthma, it is not surprising that multiple SNPs within the *IL13* gene as well as IL-13R subunits were found to be associated with asthma susceptibility and disease progression[420-424]. Accordingly, asthmatic patients exhibited higher levels of sputum IL-13 and the number of IL-13$^+$ ILC2s was significantly elevated in individuals with uncontrolled or partially-controlled asthma than in those from the

well-controlled group and healthy individuals[425,426]. Treatment of asthmatic patients with humanized monoclonal antibodies that neutralize IL-13 (tralokinumab, lebrikizumab) or block IL-4Rα (dupilumab) significantly improved lung function and reduced the rate of exacerbations[427-432].

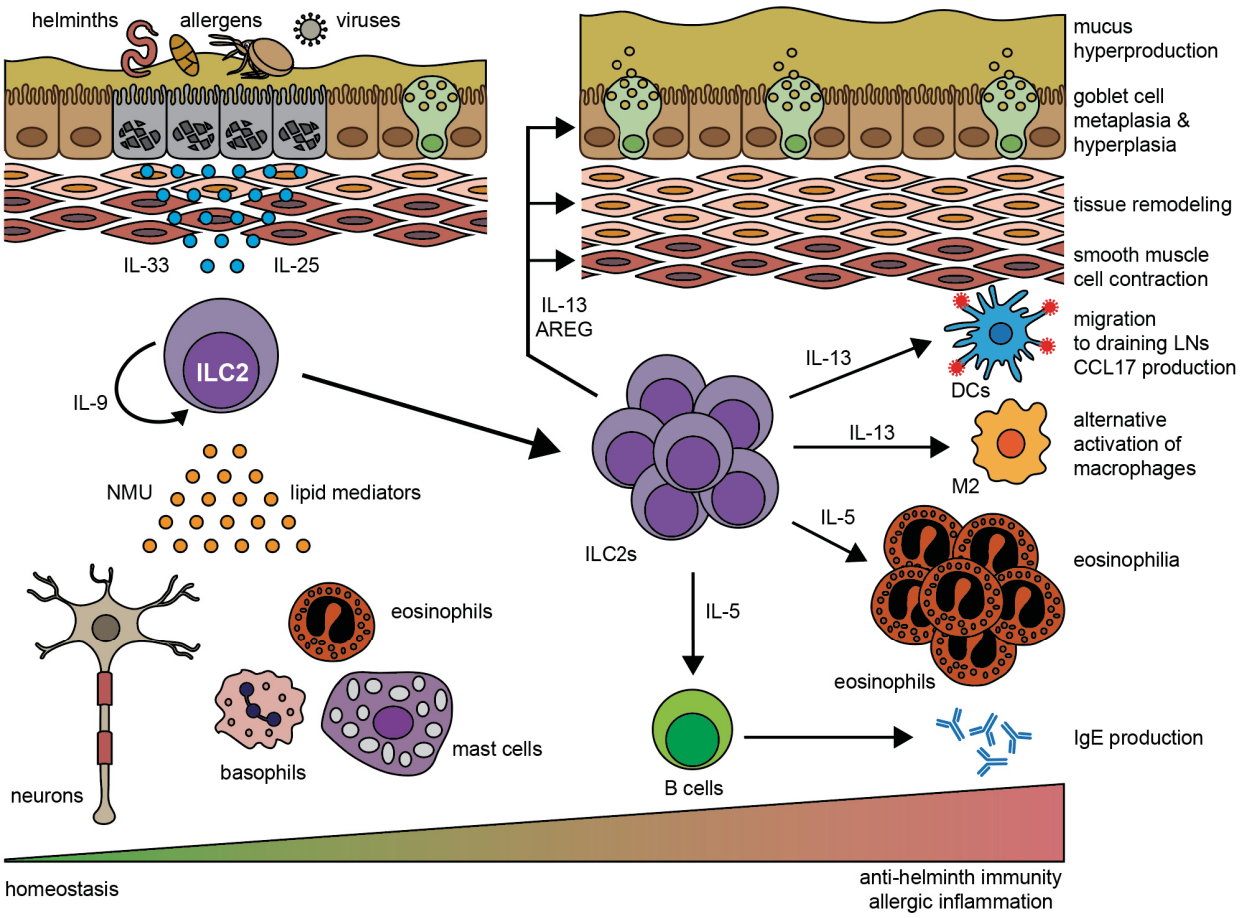

Figure 9. ILC2 effector mechanisms at barrier surfaces.

Exposure to helminths, allergens or viruses results in tissue damage and release of the alarmin(s) IL-25 and/or IL-33. Upon alarmin sensing, ILC2s rapidly start to proliferate and secrete vast amounts of type 2 signature cytokines including IL-5, IL-9, IL-13 and AREG. ILC2s can additionally become activated by the neuropeptide NMU and lipid mediators that are produced mainly by activated eosinophils, basophils or mast cells. ILC2-derived IL-9 acts in an autocrine manner to promote ILC2 survival and type 2 cytokine production. IL-5 mediates eosinophil accumulation and activation but can also facilitate IgE production by B cells. ILC2-derived AREG is important for tissue remodeling upon immune perturbation. IL-13 induces epithelial goblet cell hyperplasia and differentiation as well as mucus hyperproduction. Moreover, IL-13 acts on smooth muscle cells and other stromal cells to orchestrate smooth muscle cell contraction as well as tissue remodeling. IL-13 also promotes the migration of antigen-loaded DCs to the draining LNs and the polarization of macrophages to an M2 phenotype. Altogether, effector mechanisms of ILC2-derived type 2 cytokines cause a shift from the basal state to an environment that promotes anti-helminth immunity as well as allergic inflammation. Abbreviations: IL-, interleukin; NMU, neuromedin U; ILC2, group 2 innate lymphoid cell; AREG, amphiregulin; DCs, dendritic cells; IgE, immunoglobulin E.

Taken together, ILC2s release type 2 signature cytokines at steady state and upon activation to maintain tissue homeostasis, initiate anti-helminth immunity as well as mediate allergic inflammation. The detailed pathophysiological roles of ILC2s in allergic airway inflammation, asthma and viral lung infections will be further discussed in the following section.

1.3.7. Role of pulmonary ILC2s in inflammatory airway diseases and respiratory viral infections

Type 2 immunity is thought to have originally evolved to protect the host against parasitic infections[433]. However, type 2 responses can also be triggered by a variety of non-infectious allergens, environmental pollutants and synthetic chemicals[433]. Because these substances are generally innocuous, the resulting allergic immunopathologies were widely considered as a consequence of an accidental, misguided type 2 immune response[433]. Nevertheless, as some allergens, such as venoms are clearly noxious, it is now hypothesized that type 2 immunity to allergens may have developed to confer protection against toxins and other noxious environmental substances[434-436]. In any case, more than 3 billion people worldwide, which accounts for roughly one third of the global population, suffer from parasitic helminth infections as well as allergic and atopic diseases such as asthma, atopic dermatitis and food allergies[437,438]. Since the role of ILC2s in homeostasis, tissue repair and helminth infection are excellently reviewed elsewhere and beyond the scope of this thesis, I will focus on the pathological role of ILC2s during inflammatory allergic airway diseases as well as pulmonary infections[16,390,439,440].

1.3.7.1. ILC2s in inflammatory airway diseases

ILC2s are the predominant ILC subset in the murine lung and involved in the regulation of pulmonary immunity, inflammation, and tissue homeostasis[13]. A multitude of animal studies revealed the importance of ILC2s in allergic airway inflammation. Upon intranasal challenge with the epithelial alarmins IL-33, IL-25 and TSLP, pulmonary ILC2s rapidly produced IL-5 and IL-13 and initiated allergic airway inflammation independently of T cells[74,294,323,401,441,442]. Moreover, ILC2s were shown to play a major role in the induction of allergic airway inflammation upon exposure to model allergens such as papain, *A. alternata*, chitin and house dust-mite[382,401,443,444]. Importantly, an ILC2 population that is phenotypically and functionally similar has also been

identified in human lungs[13,22] and elevated ILC2 activity and numbers were observed in several allergic respiratory disorders including chronic rhinosinusitis and allergic rhinitis[445], suggesting that ILC2s may contribute to the pathologic type 2 response associated with these diseases. The potential importance of ILC2s in human allergic airway disease is further underlined by multiple large-scale GWAS that link variations in both IL-33 and ST2 to asthma susceptibility and pathophysiology[269-272]. Additional genetic loci associated with asthma susceptibility and pathology, include *IL5*, *IL13*, *TSLP* and *RORA*[269-271].

Allergic rhinitis

Allergic rhinitis (AR), commonly referred to as hay fever, is one of the most common allergic diseases worldwide[446]. AR is characterized by a heterogeneous IgE-mediated reaction to innocuous antigens such as pollen or animal dander which results in type 2 inflammation of the nasal mucosa. Whether there is an increase in peripheral blood ILC2s in AR patients remains controversial with no differences[284] as well as significant increases[447,448] compared to healthy controls being reported. However, ILC2 numbers in the circulation consistently increased in AR patients upon exposure to allergens[449,450]. In addition, there is limited evidence suggesting that IL-25 may be elevated in individuals suffering from AR[451], and TSLP expression was consistently shown to be significantly higher in AR patients than healthy controls[451-453]. Overall, these data indicate that ILC2 accumulation may be controlled at least in part by allergic reactions and that ILC2 activation may be mediated by TSLP and IL-25 in the nasal mucosal of AR patients.

Chronic rhinosinusitis with nasal polyps

Chronic rhinosinusitis (CRS) is a prevalent chronic inflammatory disease of the upper airway and sinuses[454]. CRS is characterized by prolonged inflammation of the nose and paranasal sinuses and can be subdivided into two main phenotypes, CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP)[454]. CRSwNP patients harboured higher frequencies of total $ST2^+$ ILC2s as well as $IL\text{-}13^+$ ILC2s in their inflamed sinonasal mucosa compared to CRSsNP patients and healthy controls [455-458]. Moreover, expression of IL-33, IL-25 and TSLP was increased in nasal polyps (NPs), implying that the diseased tissue microenvironment may promote ILC2 activation[455,459-461]. Compared to blood ILC2s or ILC2s from non-inflamed tissue, NP ILC2s expressed higher levels of ICOS and produced more IL-13 when activated with IL-33,

both indicative of a more activated phenotype[462]. Together, these findings suggest that ILC2s may contribute to the type 2 inflammation observed in individuals suffering from CRSwNP.

Asthma

According to the World Health Organization, as of 2016, an estimate of over 339 million people globally suffered from asthma resulting in over 400,000 deaths each year, rendering asthma one of the major non-communicable diseases[463,464]. Moreover, asthma represents the most common chronic disease among children and although there are cases of, mainly non-allergic, asthma onset in adulthood, most asthmatic individuals develop the disease during childhood[465]. Asthma is a heterogeneous disease characterized by chronic airway inflammation, variable airflow limitation, airway remodelling and airway hyper-reactivity. It is further defined by symptoms such as shortness of breath, chest tightness, wheezing and coughing that vary greatly over time and in intensity[465]. In asthma, the bronchial airways narrow periodically and temporarily in response to certain stimuli such as allergens. Episodes of exacerbations that can be life-threatening in very severe cases are common and can be triggered by a variety of factors, i.e. respiratory tract infections, exercise or exposure to allergens and other irritants. Characteristic pathophysiological structural changes include basement-membrane thickening through excessive collagen deposition (subepithelial fibrosis) as well as smooth muscle cell hyperplasia and hypertrophy resulting in thickening of the smooth muscle layer, which also correlates with disease severity[466,467]. Goblet cell hyperplasia and enlarged submucosal glands resulting in mucus hypersecretion are additional cardinal features of asthma. Asthma is also characterized by an elevation in VEGF levels and increased angiogenesis[468]. Several molecular asthma phenotypes have been described including allergic (eosinophilic, 'type-2 high') and non-allergic (non-eosinophilic, 'type 2 low') asthma, with the latter being further stratified into neutrophilic, mixed granulocytic or paucigranulocytic phenotypes[469]. Over 50% of severe asthma patients exhibit allergic eosinophilic airway inflammation which is driven by an excessive type 2 immune response against inhaled allergen[470,471]. Allergic asthma is characterized by eosinophilia, infiltration of activated T_H2 cells as well as activation of mast cells and ILC2s[466]. Activated ILC2s and T_H2 cells secrete type 2 signature cytokines such as IL-4, IL-5, IL-9 and IL-13 that mediate eosinophil recruitment, mast cell activation, goblet cell metaplasia and mucus hypersecretion, smooth muscle cell contraction, airway remodelling and airway hyper-reactivity as well as isotype-switching in B cells to produce

IgE antibodies[176]. Activated mast cells and other granulocytes additionally release bronchoconstrictors including histamine, leukotrienes and PGD_2 that further contribute to asthma pathogenesis[466]. Since ILC2s are major producers of type 2 cytokines and play a critical role in the pathophysiology of murine models of allergic airway inflammation, several studies investigated the role of ILC2s in the onset and progression of human asthma over the past years. It was observed that frequencies and numbers of ILC2s in peripheral blood as well as sputum and bronchoalveolar lavage fluid were elevated in asthmatic patients[211,472,473]. Moreover, ILC2s from asthmatic individuals produced more IL-5 and IL-13[284,426,474,475] . Notably, in a cohort of patients with severe asthma, ILC2s were the main producer of type 2 cytokines[475]. In addition, total numbers of circulating IL-5^+ and $IL13^+$ ILC2s in the blood of asthmatics were further increased early after allergen challenge and decreased again after 24 hours, which may indicate active recruitment to the airways[476]. Indeed, ILC2 numbers significantly increased in the BAL following segmental allergen provocation in patients with mild-to-moderate asthma accompanied by a depletion of blood ILC2s[477]. BAL-derived ILC2s isolated after allergen challenge exhibited an activated transcriptional and functional profile with an enrichment in *IL5*, *IL13*, *IL1RL1*, *IL9*, *AREG* and *VEGFA* transcript levels compared to blood ILC2s and spontaneously released IL-5 and IL-13 upon *in vitro* culture[477]. ILC2-activating cytokines IL-33 and TSLP were shown to be increased in the BAL fluid of asthmatic individuals resulting in more pronounced ILC2 activity[288,478]. It was further demonstrated that close proximity to the epithelial barrier is crucial for ILC2s to rapidly respond to allergen challenge by producing IL-13, which targets tight junctions in asthmatic patients and thereby disrupts bronchial epithelial barrier integrity[479]. As mentioned earlier, women with severe asthma exhibited a more pronounced elevation in peripheral blood ILC2s when compared to male asthmatics, most likely due to the absence of testosterone-induced suppression[247]. In addition to ILC2 activation, TSLP also induces resistance to corticosteroid therapy in ILC2s but not in T_H2 cells[288]. This implies that ILC2s may be responsible for the development of corticosteroid resistance in asthma patients[288]. Notably, some studies did not observe significantly increased ILC2 numbers in peripheral blood of asthma patients or strong correlations between ILC2 prevalence and disease severity[211,351,426]. Since asthma is a very heterogeneous disease, this discrepancy may be caused by subgroup-specific differences in pathology and the use of blood ILC2 levels as a biomarker in asthmatic disease needs to be carefully evaluated for each asthmatic endotype.

1.3.7.2. ILC2s in respiratory viral infections

With an estimate of 3 million deaths worldwide, respiratory tract infections remain the deadliest communicable disease and leading cause of death in developing countries as of 2016[480]. Influenza virus infections alone account for 290,000 – 645,000 deaths each year[481] and the ongoing coronavirus disease (COVID-19) outbreak caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has claimed more than 800,000 lives worldwide (as of August 28th, 2020)[482]. Importantly, ILC2s were shown to be induced upon respiratory virus infections in both mice and humans[13,147,257,483-487] and promote an asthma-like phenotype in mice even in the absence of adaptive immunity[257]. Indeed, asthma onset and exacerbations are predominantly triggered by infections with respiratory viruses such as rhinovirus, influenza virus or respiratory syncytial virus and impaired induction of IFN-I and IFN-II was associated with increased risk for disease exacerbations[488-490]. Moreover, high expression of type 2 inflammation genes and low expression of IFN-response genes in nasal samples has been shown to be a robust predictor of short-term asthma exacerbation risk in children[491] which is in line with previous data linking high nasal *IL13* expression to increased exacerbation likelihood[492]. Below I will briefly summarize the current knowledge of ILC2 activation in the context of respiratory viral infections.

Influenza A Virus (IAV)

Infection with influenza A virus has been shown to induce IL-33 and thereby promote murine ILC2 activation and function[13,257,304,493]. Notably, *in vitro* infection of a human lung epithelial cell line (A549) with IAV (H1N1, H3N2, H6N3) also increased IL-33 expression[493]. Using a murine model of IAV (H3N1) infection, it was demonstrated that alveolar macrophages are a main source of IL-33 and that ILC2s drive AHR independently of adaptive immunity[257]. An increase in ILC2s in combination with eosinophilia was also reported upon infection with mouse-adapted recombinant IAV (H1N1) where ILC2-derived AREG was critical for tissue repair following infection[13]. It was additionally observed that IAV (H1N1)-induced IL-33-mediated activation of ILC2s and accompanied eosinophilia and pulmonary type 2 immunopathology were significantly enhanced in *Ifnar1*$^{-/-}$ animals and thereby revealed that IFN-I is a critical negative regulator of infection-induced ILC2s[304]. Intriguingly, IFN-γ-deficient mice as well as animals treated with an IFN-γ-neutralizing antibody were protected against infection with a lethal dose of IAV (H1N1)[309]. This increased resistance to lethal challenge was dependent on the presence of ILC2-derived IL-

5[309]. These findings suggest an unexpected detrimental role of IFN-γ-mediated restriction of ILC2 activity in IAV pathogenesis. Collectively, findings from mouse models of IAV infection show that ILC2s are activated in an IL-33-dependent fashion following infection and may play a beneficial role by mediating tissue repair as well as resistance to lethal viral challenge. However, whether IAV infection mediates ILC2 activation in humans and if ILC2s play a physiologically relevant role during human IAV infection remains to be investigated.

Rhinovirus

Rhinovirus infection is a significant contributor to wheezing illnesses and has been associated with the subsequent development of asthma and asthma exacerbations[494]. Rhinovirus (strain RV-16) induces IL-25 mRNA and protein levels in asthmatic patients when compared with healthy individuals and induction of $Lin^{-}ST2^{+}ICOS^{+}$ cells was further reported, which probably correspond to ILC2s[485]. Furthermore, infection with rhinovirus (RV-16) was shown to elicit IL-33 in asthmatic subjects which was accompanied with a type 2 immune signature. Moreover, human ILC2s secreted type 2 cytokines upon co-culture with rhinovirus-infected bronchial epithelial cells, suggesting virus-induced expression and/or release of mediators activating ILC2s[486]. Infection of mice with rhinovirus (strain RV1B) also resulted in elevated protein levels of IL-25, IL-33, and TSLP, thereby triggering ILC2 expansion and activation[484,495]. Thus, ILC2 activation during rhinovirus infection may depend on parallel induction of IL-25, IL-33, and TSLP in both mouse and humans upon rhinovirus infection. Similar to above mentioned observations made during IAV infection, RSV-elicited IFN-γ suppressed type 2 cytokine production by lung ILC2s as well as goblet cell hyperplasia and mucus secretion in baby mice[496]. Together, these data show that ILC2s are induced upon rhinovirus infection and promote an asthma-like phenotype in immature mice. However, if and to what extent ILC2s contribute to rhinovirus-associated disease pathogenesis and asthma onset in humans remains elusive and will require further investigations.

Respiratory Syncytial Virus (RSV)

Infections with respiratory syncytial virus (RSV) are often associated with asthma exacerbations, especially in children[494]. Importantly, elevated numbers of ILC2s were observed in young mice after RSV (A2) infection and ILC2 induction was highly dependent on IL-33[497]. In addition, ILC2s were induced upon infection with RSV (01/2-20) in adult mice and ILC2-derived IL-13 mediated AHR, goblet cell hyperplasia, and increased mucus production[487]. Here, TSLP, most likely released by epithelial cells, was shown to be critical for ILC2 activation[487]. Importantly, elevated IL-33 levels were measured in nasal lavages of RSV-infected infants indicating that ILC2s may contribute to disease pathogenesis[497].

Despite impressive progress in understanding ILC biology, many aspects of how ILC2s are regulated and contribute to homeostasis and allergic disease pathologies remain controversial or unanswered. Therefore, future studies are needed to further elucidate the unique aspects of ILC2 identity and regulation in order to develop novel therapeutic strategies to specifically target ILC2s in human disease.

1.4. Rationale, objective and specific aims of the research

Although the precise mechanisms are still incompletely defined, dysregulated pulmonary ILC2 responses are associated with increasingly prevalent allergic airway diseases including asthma. Thus, further insights into ILC2 biology are critical to pave the way for the design and development of novel therapeutic opportunities to sustain tissue homeostasis and control allergic lung pathology. Hence, the objective of this thesis was to identify novel mechanisms of ILC2 regulation and thus potential therapeutic targets. To this end we performed transcriptomic analysis of IL-33-activated ILC2s and further validated the physiological relevance of our findings in mouse models of pulmonary disease.

Aim 1. The first aim of this thesis, presented in chapter 2, was to examine the role of the NF-κB transcription factor c-Rel during IL-33-mediated ILC2 activation *ex vivo* and during allergic airway inflammation.

Aim 2. The second aim of this thesis, presented in chapter 3, was to decipher mechanisms of IFN-I-mediated pulmonary ILC2 inhibition.

Aim 3. The third and final aim of this thesis, presented in chapter 4, was to investigate novel mechanisms of peptide-mediated lung ILC2 regulation.

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Chapter 2: The NF-κB transcription factor c-Rel modulates group 2 innate lymphoid cell effector functions and drives allergic airway inflammation.

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2.1. Abstract

Group 2 innate lymphoid cells (ILC2s) exert key roles in the initiation and orchestration of early type 2 immune responses. Upon activation by environmental cues such as the alarmin interleukin (IL)-33 they rapidly secrete large amounts of type 2 signature cytokines and mediate innate as well as adaptive immune responses. Efficient ILC2 activation is governed by a network of transcriptional regulators including nuclear factor (NF)-κB family transcription factors. While it is known that activating IL-33 receptor signaling results in downstream NF-κB activation, the underlying molecular mechanisms remain elusive. Here we found that the NF-κB subunit c-Rel is required to mount effective ILC2-driven pulmonary type 2 immune responses. IL-33-driven ILC2 activation was found to trigger c-Rel expression, nuclear translocation and subsequent binding to regulatory chromatin regions to modulate target gene expression. c-Rel-deficiency did not impact ILC2-intrinsic proliferation or type 2 cytokine production but resulted in severely reduced expression of surface 4-1BBL by IL-33-activated lung ILC2s *ex vivo* as well as *in vivo* upon IL-

33- and allergen-mediated allergic airway inflammation. In addition, we demonstrate that expression of the cognate 4-1BBL receptor, 4-1BB, is induced on $CD4^+$ conventional (Tconv) and regulatory T (Treg) cells as well as distinct airway epithelial cells following intranasal challenge with IL-33. Collectively, these observations propose a novel cellular crosstalk of ILC2s with T cells and/or epithelial cells via the 4-1BBL-4-1BB axis that may induce the release of ILC2-promoting factors which could explain the diminished numbers of ILC2s in the absence of c-Rel.

2.2. Introduction

Group 2 innate lymphoid cells (ILC2s) are a recently described innate cell population that mediate early type 2 immune responses and thereby exert key roles in the initiation and orchestration of anti-helminth immunity as well as allergic inflammation[1-5]. ILC2s exhibit striking functional similarity to adaptive type 2 T helper (Th2) cells. Like Th2 cells, they depend on the transcription factor GATA3 and produce type 2 signature cytokines such as interleukin (IL)-5 and IL-13 upon activation driving eosinophil recruitment as well as goblet cell hyperplasia and mucus production, respectively[6]. Besides initiating innate type 2 immune responses, it is becoming increasingly appreciated that ILC2s also play important roles in bridging innate and adaptive responses. ILC2s can initiate and modulate the quality and magnitude of adaptive helper T cell responses via indirect interaction mediated through bystander cells as well as directly by presenting antigens and/or providing co-stimulation[7-13]. Upon allergen-induced allergic airway inflammation, ILC2-derived IL-13 was shown to be critical for the migration of lung dendritic cells (DCs) to the mediastinal lymph node to facilitate priming of naive $CD4^+$ T cells and Th2 polarization[12]. Pulmonary ILC2-derived IL-13 also stimulates the production of the Th2-attracting chemokine CCL17 by airway DCs which is key for the recruitment of memory T cells to the airways during secondary allergen challenge[13]. Murine ILC2s can process and present antigen in complex with major histocompatibility complex class II (MHC-II) to $CD4^+$ T cells to drive type 2 immune responses *in vitro* and *in vivo*[7,8]. Besides promoting Th2 cell effector functions, this ILC2-T cell crosstalk induces IL-2 production by T cells which feeds back on ILC2s to promote proliferation and IL-13 secretion[7,8]. Moreover, it was demonstrated that IL-33-mediated activation of ILC2s induces expression of the co-stimulatory tumor necrosis factor superfamily (TNFSF) ligand OX40L *in*

vitro as well as *in vivo*[9]. Interaction of OX40L on ILC2s with OX40 on conventional T cells and regulatory T (Treg) cells, promotes Th2 proliferation and effector responses as well as Treg expansion following allergic airway inflammation and helminth infection[9]. ILC2s are further characterized by the expression of inducible T cell co-stimulator (ICOS) and interaction with ICOS ligand (ICOSL) on Treg cells facilitates Treg accumulation in response to IL-33[10,11]. ILC2s are located at barrier surfaces including the lung and, contrary to Th2 cells, lack expression of specific antigen receptors[6]. They instead become activated in an antigen-independent fashion in response to environmental cues such as alarmins IL-25, IL-33 and/or thymic stromal lymphopoietin (TSLP) that are released upon tissue perturbation[6].

Among these alarmins, IL-33 has been described as the most potent activator of lung ILC2s[14]. IL-33 signals through the heterodimeric IL-33 receptor (IL-33R) composed of the ligand-binding chain ST2 and IL-1 receptor accessory protein (IL-1RacP)[15]. While it is known that activating IL-33 receptor signalling results in downstream nuclear factor (NF)-κB activation, the underlying molecular mechanisms in ILC2s remain largely elusive[15]. NF-κB signaling is mediated by homo- or heterodimers of proteins of the Rel/NF-κB transcription factor superfamily including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel[16]. Only RelA, RelB and c-Rel harbor transcription transactivation domains and can thereby act as transcriptional activators[17]. Despite sharing a common DNA recognition motif, knockout mice lacking individual Rel/NF-κB family members exhibit non-redundant phenotypes[18,19]. Moreover, differential expression patterns in tissues and responses to receptor signals as well as target gene specificity indicate that distinct NF-κB subunits exert unique physiological roles[18,19]. While stimulation of lung ILC2s with IL-33 results in phosphorylation of RelA and treatment with a pan-NF-κB inhibitor impairs ILC2 effector functions[20-23], RelB was shown to be an intrinsic repressor of ILC2s[24]. On the contrary, the function of c-Rel in ILC2s remains elusive. c-Rel has been shown to promote type 2 immune responses upon ovalbumin (OVA)-induced allergic airway inflammation[25]. Furthermore, inhibition of c-Rel in a mouse model of house dust mite-mediated allergic inflammation resulted in reduced levels of IL-13 and airway hyper-reactivity as well as lung inflammation[26,27] and inhibited eosinophil recruitment in an OVA model of chronic asthma[28]. Since ILC2s are main drivers of allergic asthma, we aimed to investigate whether c-Rel exhibits similar effects during ILC2-driven allergic airway inflammation.

In the present study, we found that deficiency in the NF-κB transcription factor c-Rel severely diminished early pulmonary ILC2-driven type 2 responses to intranasal IL-33 administration. We further demonstrate that c-Rel is activated upon IL-33-mediated ILC2 activation and modulates ILC2 responses *ex vivo* and during allergic airway inflammation. Using RNA-seq and ChIP-seq we identified *Tnfsf9*, encoding 4-1BBL, as a novel IL-33-inducible gene that is directly regulated by c-Rel. Consistent with these findings, 4-1BBL protein was upregulated on lung ILC2s upon *ex vivo* stimulation as well as during IL-33- and allergen-induced lung inflammation in a c-Rel-dependent manner. Moreover, we showed that ILC2s represented the only cellular source of 4-1BBL after intranasal IL-33 treatment. We further demonstrated that the cognate 4-1BBL receptor, 4-1BB, is induced on conventional and regulatory T cells as well as on airway epithelial cells upon *in vivo* allergen and IL-33 challenge suggesting potential interaction with 4-1BBL-expressing ILC2s that may promote ILC2 functions.

2.3. Results

c-Rel is a critical mediator of innate type 2 immune responses.

To investigate the role of c-Rel during ILC2-driven allergic airway inflammation we challenged wild-type (WT) and c-Rel-deficient (*Rel*$^{-/-}$) mice intranasally with either PBS or IL-33 for three consecutive days and analyzed parameters of airway inflammation (**Figure 10A**). Pulmonary tissue histology showed that when exposed to IL-33, lack of c-Rel resulted in a significant reduction in perivascular and peribronchial immune cell infiltration into lung tissue compared with WT mice (**Figures 10B, C**). These findings could be recapitulated by flow cytometry, showing markedly decreased numbers of pulmonary CD45^{+} leukocytes in c-Rel-deficient animals after IL-33 treatment (**Figures 10D and 17**). Moreover, while challenge of WT mice with IL-33 resulted in a significant increase in total numbers of type 2 immunity-associated cell populations including eosinophils, type 2 dendritic cells (DC2s) as well as ILC2s, numbers were significantly diminished in *Rel*$^{-/-}$ mice (**Figures 10D and 17A-D**). Furthermore, c-Rel-deficiency led to reduced expression of lung *Il5* transcripts with a trend towards lower *Il13* levels (**Figure 10E**). In addition, the type 2-associated chemokines *Ccl17* and *Ccl22* were greatly reduced in *Rel*$^{-/-}$ compared to WT mice (**Figure 10F**). In summary, c-Rel-deficient mice failed to mount an efficient innate type 2 immune response with a significant reduction in pulmonary leukocyte

infiltration, eosinophilia, DC2s and ILC2s. Our results thus suggest that c-Rel is critical for the development of IL-33-driven allergic lung inflammation.

c-Rel-deficient ILC2s harbor no intrinsic defects in their developmental capacity, key surface molecule expression, proliferation as well as IL-5 and IL-13 production.

To assess whether lack of c-Rel results in ILC2-intrinsic alterations that may explain why *Rel*$^{-/-}$ animals fail to mount a strong innate type 2 immune response, we analyzed ILC2 progenitor numbers in the bone marrow of c-Rel sufficient (*Rel*$^{+/+}$), heterozygous (*Rel*$^{+/-}$) and c-Rel-deficient (*Rel*$^{-/-}$) littermate control animals (**Figure 11A**). We observed that mice of all three genotypes harbored comparable numbers of common lymphoid progenitors (CLP), as well as the more downstream common helper ILC progenitors (CHILP) and ILC2 progenitors (ILC2P) (**Figures 11A and 18A**). In addition, there was no difference in total numbers of mature lung ILC2s (**Figure 11B**). Together, these results indicate that c-Rel is dispensable for the development of ILC2s in the bone marrow and that c-Rel deficiency does not affect homeostatic maintenance of lung-resident ILC2s.

Besides activating signals provided by alarmins such as IL-33, ILC2s require cues from STAT5-activating cytokines such as IL-2 or IL-7 and/or costimulatory signals for optimal activation and exertion of effector functions[6,29]. Previous work demonstrated that c-Rel positively modulates expression of both IL-2 and IL-2Rα (CD25) in T cells[30-33]. We therefore assessed if observed reduction in *Rel*$^{-/-}$ ILC2 numbers during allergic airway inflammation may stem from lowered sensitivity to activating signals due to diminished expression of respective surface receptors (**Figures 11C and 19A-C**). To this end, we assessed surface expression levels of CD25 and other key activating ILC2 receptors as well as the ILC2 master transcription factor GATA3 in isolated pulmonary WT or *Rel*$^{-/-}$ ILC2s at steady state and after *ex vivo* stimulation with IL-33 (**Figure 18B**). We found that c-Rel deficiency did not alter surface expression levels of CD25 (IL-2Rα) or CD127 (IL-7R) on ILC2s at steady state or upon IL-33-mediated activation (**Figures 11C and 19B**). Moreover, the IL-33 receptor chain ST2, the costimulatory receptor ICOS as well as GATA3 expression were slightly enhanced upon ILC2 activation in c-Rel-deficient ILC2s compared to their WT counterparts (**Figures 11C and 19A, C**).

Previous studies demonstrated that c-Rel promotes cell cycle progression and expression of anti-apoptotic proteins in activated T and B cells [30,31,34-36] and we therefore analyzed the

proliferative capacity of ILC2s at steady state or upon IL-33-driven activation by Ki-67 staining (**Figure 11D**). Frequencies of Ki-67^{+} ILC2s (**Figure 11E**) as well as Ki-67 expression levels (**Figure 19D**) on ILC2s isolated from WT and *Rel*$^{-/-}$ mice were comparable, indicating that c-Rel does not impact IL-33-induced ILC2s expansion.

Genes encoding the type 2 signature cytokines IL-4 and IL-13 were proposed as putative c-Rel target genes based on differential transcript expression observed in gain-of-function and loss-of-function experiments in T cells[37]. However, intracellular protein expression levels of IL-13 as well as IL-5 were induced to a similar extent in WT and c-Rel deficient animals upon culture with IL-33 (**Figure 11F**).

Collectively, our data show that pulmonary *Rel*$^{-/-}$ do not harbor intrinsic developmental, proliferative defects at steady state or upon IL-33-dependent activation that may explain their reduced numbers upon *in vivo* IL-33 challenge. Furthermore, expression of activating surface receptors as well as production of effector cytokines IL-5 and IL-13 was comparable at steady state as well as after IL-33-induced activation.

<u>IL-33-driven *ex vivo* activation of pulmonary ILC2s induces c-Rel expression and translocation to the nucleus.</u>

It is well established that IL-33 signaling via ST2 triggers downstream activation of NF-κB pathways[15]. We therefore analyzed the direct effect of IL-33 on c-Rel expression and activation in ILC2s. *Ex vivo* culture of lung (**Figure 12A**) as well as bone marrow-derived ILC2s (**Figure 20A and 21A**) with IL-33 resulted in a rapid increase in c-Rel transcripts levels compared to unstimulated cells. Consistently, c-Rel protein expression in lung, small intestinal and bone marrow-derived ILC2s were significantly elevated over levels in unstimulated cells as early as four hours following *ex vivo* stimulation with IL-33 and further induced upon 24 hours (**Figures 12B, C and 20B**). Moreover, c-Rel protein levels increased in a dose-dependent manner and remained elevated for at least 72 hours of culture with IL-33 (**Figures 21B, C**). To determine if c-Rel is activated upon IL-33 stimulation, we assessed nuclear translocation in lung ILC2s upon *ex vivo* IL-33 stimulation by ImageStream analysis (**Figures 12D, E**) and Western blot of nuclear lysates (**Figure 21D**). Indeed, while c-Rel was confined to the cytoplasm in unstimulated ILC2s, it was predominantly localized in the nucleus upon three hours of culture with IL-33 (**Figures 12D, E and 21D**). To assess if c-Rel expression is also increased upon *in*

vivo activation of ILC2s, we induced allergic airway inflammation by treating mice intranasally with IL-33 or the fungal allergen *Alternaria alternata* for three days and stained for intracellular c-Rel protein expression. Consistent with ILC2s stimulated *ex vivo*, intranasal administration of IL-33 or *A. alternata* in WT mice resulted in a 10-fold induction of c-Rel expression in lung ILC2s over PBS-treated mice (**Figures 12F-G and 21E**). c-Rel-deficient mice that underwent the same treatment were used as negative controls. Furthermore, c-Rel transcript levels, assessed by qRT-PCR, were significantly higher in lung tissues of IL-33-challenged mice compared to mice that received PBS, while no transcripts were detected in the lungs of control *Rel*$^{-/-}$ mice (**Figure 12H**). Taken together, these findings show that c-Rel expression is induced upon *ex vivo* stimulation of ILC2s as well as during IL-33- and allergen-induced allergic airway inflammation. Furthermore, c-Rel translocates to the nucleus upon *ex vivo* culture of ILC2s with IL-33 to potentially modulate transcription of target genes.

<u>c-Rel binds to regulatory chromatin regions and promotes distinct transcriptional changes upon IL-33-driven ILC2 activation.</u>

Since we observed that c-Rel is activated and translocates to the nucleus upon stimulation with IL-33, we next investigated whether it directly modulates ILC2 gene expression. To this end, RNA-sequencing (RNA-seq) analysis on bone marrow-derived ILC2s of WT and *Rel*$^{-/-}$ mice was carried out to identify differentially regulated genes upon *ex vivo* IL-33 stimulation. Indeed, principal component analysis (PCA) of RNA-seq data revealed that, while technical replicates closely resembled each other, c-Rel-deficiency markedly affected the transcriptional profile of ILC2s at steady state as well as in response to IL-33 (**Figures 13A, B**). Using clustering analysis of transcripts that are differentially expressed based on the effect of IL-33 or c-Rel-deficiency (fold change ≤ 0.5 or ≥ 2; $p \leq 0.001$), five groups of genes were defined including genes in cluster five that are upregulated by IL-33 with lower expression in the absence of c-Rel (**Figure 13C**). Comparison of gene expression among IL-33 treated and untreated WT ILC2s revealed that more than 300 genes were differentially expressed upon IL-33 treatment ($p \leq 0.001$), the majority being upregulated (fold change ≥ 2, $p \leq 0.001$) (**Figures 13D and 22A, B**). Gene ontology (GO) enrichment analysis revealed that genes associated with inflammatory response, response to cytokines, leukocyte activation as well as regulation of signal transduction and programmed cell death are over-represented within this upregulated gene fraction (**Figure 13F**). As expected,

among the highest upregulated genes were genes encoding for type 2 signature cytokines (*Il5*, *Il6*, *Il13*, *Areg*, *Csf2*). In addition, chemokines (*Ccl1*, *Ccl3*, *Cxcl2*, *Cxcl3*), transcription factors (*Nr4a1*, *Irf4*, *Myc*, *Batf*), TNF superfamily ligands and receptors (*Tnfsf9*, *Tnfsf10*, *Tnfsf14*, *Tnfrsf25*, *Tnfrsf1b*) as well as for enzymes implicated in lipid metabolism (*Dgat2*, *Ptgs2*) were among the genes with the highest fold induction. Comparison of gene expression among WT and *Rel*$^{-/-}$ ILC2s revealed that loss of c-Rel affects the transcriptional profile of basal as well as IL-33-activated ILC2s with c-Rel-deficient ILC2s exhibiting diminished expression of more than 100 genes (**Figure 13D**). ILC2s from both genotypes showed similar expression levels of genes encoding ILC2-associated transcription factors (*Gata3, Rora, Id2, Ets1*) and cytokine receptors (*Il1rl1, Il2ra, and Il4ra, Il7r, Il9r, Crlf2*) as well as type 2 signature cytokines at steady state and upon culture with IL-33 (*Areg, Il5, Il13)* (**Figure 22C**).

We further focused our analysis on genes induced by IL-33 treatment with significantly lower expression in c-Rel-deficient ILC2s (cluster 5) that may explain the observed *in vivo* differences. To evaluate which of these genes are potential direct targets of c-Rel, we performed ChIP-seq analysis to determine c-Rel binding sites within associated promoter regions. While there was very little constitutive binding of c-Rel to target regions in ILC2s at the basal level, activation with IL-33 resulted in a marked increase of c-Rel recruitment (**Figure 13F**) with a significant increase in binding peak heights (**Figure 13G**). In parallel, transcriptional activity at the respective binding sites was increased upon ILC2 activation, as assessed by H3K27 acetylation (**Figure 13F**). Importantly, around 70% of identified binding sites were located within a one kb distance to the nearest transcription start site (TSS) indicating that c-Rel may act as a transcriptional modulator of respective genes (**Figure 13H**). Collectively, our data strongly suggests that IL-33 elicits downstream binding of c-Rel to regulatory chromatin regions in ILC2s to promote expression of target genes.

<u>c-Rel regulates 4-1BBL expression on ILC2s *ex vivo* and during allergic airway inflammation.</u>

To identify differentially expressed genes that are directly regulated by c-Rel in activated ILC2s, we analyzed the promoter regions of respective genes for c-Rel binding sites by combining our RNA-seq and ChIP-seq data (**Figure 14A**). Overlay of both datasets revealed that c-Rel binds within the promoter region of *Tnfsf9*, encoding for the costimulatory ligand 4-1BBL (**Figure 14A**). Furthermore, expression of *Tnfsf9* was strongly induced in WT lung ILC2s

cultured with IL-33 and transcript levels were significantly lower in the absence of c-Rel (**Figure 14A**). c-Rel binding to the *Tnfsf9* promoter was further validated by ChIP-qPCR, showing 40-fold enrichment over control IgG in ILC2s stimulated with IL-33 while no difference was observed in untreated cells (**Figure 14B**). To confirm the RNA-seq findings, we assessed *Tnfsf9* expression in naive and *ex vivo* stimulated lung ILC2s by qRT-PCR analysis. Again, *Tnfsf9* expression was significantly reduced in c-Rel-deficient ILC2s upon IL-33 stimulation, further validating the RNA-seq results (**Figure 14C**). We next evaluated if the observed induction of *Tnfsf9* expression in *ex vivo* IL-33-activated ILC2s translates to an increase in surface 4-1BBL protein on lung ILC2s. Indeed, while 4-1BBL was not detectable on unstimulated ILC2s, expression gradually increased over time of culture with IL-33, plateaued at 24 hours (**Figure 14D**). Furthermore, induction of 4-1BBL protein expression on lung ILC2s was dose-dependent and stable over the course of 48 hours (**Figures 23A, B**). Importantly, c-Rel-deficient ILC2s exhibited greatly diminished surface levels of 4-1BBL following *ex vivo* stimulation with IL-33 for two days (**Figures 14E, F**). To determine if 4-1BBL surface levels were also upregulated upon *in vivo* activation of pulmonary ILC2s we challenged mice with PBS, IL-33 or the fungal allergen *Alternaria alternata* and assessed 4-1BBL levels on lung ILC2s (**Figure 14G**). Indeed, while little basal expression was observed in mice treated with PBS, challenge with both IL-33 and *A. alternata* resulted in 8- and 5-fold increases in surface 4-1BBL expression, respectively (**Figures 14H, I**). 4-1BBL-deficient mice served as negative controls to properly adjust 4-1BBL gating (**Figure 23C**). 4-1BBL expression is typically induced in professional antigen-presenting cells such as dendritic cells, B cells and macrophages upon activation but was also reported in $CD4^+$ and $CD8^+$ T cells[38-41] . To determine whether ILC2s represent the only source of 4-1BBL we screened 4-1BBL expression on other lung immune cell subsets. While surface 4-1BBL protein could not be detected on any of the analyzed pulmonary myeloid and lymphoid cell populations (ILC2s, NK cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, conventional DC1s and DC2s, alveolar macrophages (AVM), interstitial M1 and M2 macrophages, eosinophils and neutrophils) in PBS-challenged mice, induction of 4-1BBL was only observed on ILC2s upon intranasal IL-33 challenge (**Figures 14J, K, 17A-D and 24A, B**). Moreover, myeloid cDCs which can directly react to IL-33[42,43], did not show elevated expression of 4-1BBL upon IL-33 administration (**Figures 14J, K)**. We next evaluated whether we can recapitulate our *ex vivo* findings and determined whether c-Rel

affects 4-1BBL expression on ILC2s *in vivo* following intranasal instillation of WT and *Rel*$^{-/-}$ mice with PBS or IL-33. Indeed, while total numbers of 4-1BBL^{+} ILC2s were markedly increased in IL-33-treated WT mice, we observed a significant reduction in the absence of c-Rel (**Figure 14L**). Moreover, while *Tnfsf9* transcript levels were elevated in lungs of WT mice, no induction was observed in *Rel*$^{-/-}$ animals (**Figure 14M**). Together, these data show that c-Rel positively regulates 4-1BBL expression in ILC2s following *ex vivo* as well as *in vivo* activation with IL-33.

Expression of 4-1BB is upregulated on distinct lung T cell and airway epithelial cell subsets upon IL-33 or allergen-mediated allergic airway inflammation.

The cognate 4-1BBL receptor, 4-1BB, is an inducible costimulatory receptor mainly expressed on activated CD4^{+} and CD8^{+} T cells but expression has also been reported on DCs, macrophages and other hematopoietic as well as non-hematopoietic cells[44]. To determine potential cellular interaction partners of activated 4-1BBL-expressing ILC2s during allergic airway inflammation, we analyzed distinct lymphoid and myeloid immune cells as well as airway epithelial cell subsets for 4-1BB expression after intranasal PBS or IL-33 challenge (**Figure 15A**). qRT-PCR analysis of whole lung tissue revealed strong upregulation of 4-1BB (*Tnfrsf9*) transcript expression in both WT and *Rel*$^{-/-}$ mice upon IL-33 challenge, indicating that receptor expression may indeed be induced upon IL-33 challenge (**Figure 15B**). While we did not observe basal 4-1BB protein expression by any of the analyzed pulmonary cell populations (ILC2s, CD4^{+} conventional T cells and regulatory T cells, CD8^{+} T cells, B cells, alveolar macrophages, interstitial M2 and M2 macrophages as well as cDC1s, cDC2s and airway epithelial cell subsets, **Figures 25A-C**), surface levels of 4-1BB were increased upon IL-33 challenge on CD4^{+} T cell and distinct airway epithelial cell subsets (**Figures 15C and 26A, B**). Specifically, frequencies (**Figure 15D**) and total numbers (**Figure 15 E**) of CD4^{+} GATA3^{-} Tconv, GATA3^{+} Th2, GATA3^{-} Treg, GATA3^{+} Treg as well as CD74^{+}MHC-II^{-} airway epithelial cells were markedly increased following intranasal IL-33 challenge. The highest increases in 4-1BB frequencies were found in CD74^{+}MHC-II^{-} epithelial cells and GATA3^{+} Treg cells, with around 50% and 20% or cells expressing 4-1BB after IL-33 challenge, respectively (**Figure 15D**). CD74^{+}MHC-II^{-} epithelial cells also represented the highest number of 4-1BB-expressing cells in the lung followed by Tconv cells (**Figure 15E**). Moreover, intranasal challenge with *A. alternata* (**Figure 15F**)

resulted in a similar increase in frequencies (top) and total numbers (bottom) of 4-1BB$^+$ FoxP3$^-$ Tconv (**Figure 15G**) and FoxP3$^+$CD25$^+$ Treg cells (**Figure 15H**) when compared to IL-33-challenged animals. Together, these findings show that 4-1BB receptor expression is robustly induced on distinct T cells and airway epithelial subsets following IL-33 or allergen challenge and these cells may thereby constitute potential interaction partners for 4-1BBL-expressing ILC2s.

Antigen-specific CD4$^+$ T cell subsets show no enrichment in 4-1BB expression and 4-1BB$^+$ CD4$^+$ T cells exhibit increased expression of Treg signature and type 2-associated markers.

The most prominent function of 4-1BB is its role in T cell co-stimulation in response to antigen where ligation of 4-1BB on T cells by 4-1BBL on antigen-presenting cells provides critical signals to promote survival, expansion and acquisition of effector phenotypes[44,45]. Recent work demonstrated that the co-stimulatory ligand OX40L is induced on IL-33-activated ILC2s and is critical for allergen-induced adaptive type 2 responses[9]. Therefore, we investigated whether 4-1BB$^+$ lung T cells that arise upon IL-33 challenge exhibit specificity towards exogenous antigen and 4-1BBL-expressing ILC2s may thus be potentially implicated in the initiation of antigen-specific T cell responses. To this end, we intranasally challenged WT mice with IL-33 in combination with the immunogenic peptide 2W1S or peptide only as a control, as described previously (**Figure 16A**)[9,46]. Antigen-specific T cell populations (tetramer$^+$) were tracked by staining with 2W1S:I-A^b MHC-II tetramer (**Figure 27A**). As expected, we observed few tetramer$^+$ T cells when peptide was administered by itself and a potent increase in frequencies and total numbers of antigen-specific CD4$^+$ T cells when IL-33 was given in parallel (**Figure 16B**). Moreover, when CD4$^+$ T cells were stratified by FoxP3 and GATA3 expression we showed an increase in all subsets GATA3$^-$ Tconv, GATA3$^+$ Th2, GATA3$^-$ Treg, GATA3$^+$ Treg (**Figure 16C**). Comparison of frequencies of 4-1BB$^+$ antigen-specific and non-specific Tconv and Th2 cells revealed that only around 5% of tetramer$^+$ cells co-expressed 4-1BB protein as opposed to around 15% of tetramer$^-$ T cells (**Figure 16D**), suggesting that 4-1BBL-expressing ILC2s are not implicated in promoting Ag-specific T cell responses and may rather interact with non-specific bystander T cells.

To further characterize the nature of 4-1BB$^+$ T cells and thereby draw conclusions about their function, we compared expression of T cell activation markers (ICOS), Th2 markers

(GATA3, ST2) as well as Treg markers (FoxP3, CD25, Helios) among lung 4-1BB$^+$ and 4-1BB$^-$ Tconv and Treg cell subsets following challenge with IL-33 (**Figure 16F,G**). 4-1BB-expressing FoxP3$^-$ Tconv cells (**Figure 16F**) and FoxP3$^+$CD25$^+$ Treg cells (**Figure 16G**) exhibited significantly increased expression of the Treg associated markers CD25 and FoxP3 as well as GATA3 and ICOS, while Tconv cells also showed elevated levels of ST2 and Treg cells harbored increased expression of Helios. These findings indicate that 4-1BBL-expressing ILC2 may influence the functions of select CD4$^+$ T cell subsets during IL-33-driven responses.

2.4. Discussion

Rel/NF-κB transcription factors play critical roles in the modulation of immune responses by regulating the expression of numerous genes involved in lymphoid cell development, proliferation, survival as well as immune cell effector functions[17]. Despite sharing a common DNA recognition motif, knockout mice lacking individual Rel/NF-κB family members exhibit non-redundant phenotypes[18,19]. Moreover, differential expression patterns in tissues and responses to receptor signals as well as target gene specificity indicate that distinct NF-κB subunits exert unique physiological roles[18,19]. It has recently been demonstrated that activating IL-33 receptor signalling results in downstream activation of the Rel/NF-κB family members RelA and RelB, promoting or restraining ILC2 function, respectively[20-24]. On the contrary, the function of the NF-κB subunit c-Rel in ILC2s remains elusive. c-Rel has been shown to promote airway hyper-reactivity and allergic airway inflammation in murine models of antigen- and allergen-induced allergic lung inflammation[25,26,28]. ILC2s are main drivers of asthma-associated type 2 immune responses and we therefore hypothesized that c-Rel positively regulates ILC2 activation and function.

Upon intranasal IL-33 challenge, we observed a marked reduction in ILC2 numbers, lung type 2 signature cytokine transcripts and associated parameters of lung inflammation in c-Rel-deficient mice which could not be explained by ILC2-intrinsic proliferative defects or differential expression of activating receptors or key transcription factors. Using RNA-seq and ChIP-seq approaches we identified *Tnfsf9*, encoding the co-stimulatory ligand 4-1BBL, as a direct target of c-Rel in activated ILC2s. We moreover demonstrated that lung ILC2s upregulate 4-1BBL upon intranasal IL-33 or allergen challenge and represent the sole source of lung 4-1BBL. Furthermore, we show that 4-1BB receptor is induced on MHC-II$^-$CD74$^+$ airway epithelial cells as well as

GATA3^{+} and GATA3^{-} Tconv and Tregs during allergic airway inflammation rendering them potential cellular interaction partners of 4-1BBL^{+} ILC2s. Taken together, we identified c-Rel as a positive modulator of IL-33-mediated ILC2 function and ILC2-driven allergic lung inflammation.

Previous studies demonstrated that expansion of lung Th2 and Treg cells upon intranasal IL-33 or allergen challenge is dependent on ILC2s[9,12]. Furthermore, analysis of IL-33-challenged mice showed co-localization of Treg cells and ILC2s in the lung[9,11] and ILC2s have been described to directly regulate the activation and function of CD4^{+} T helper cells as well as Treg cells via antigen presentation[7,8] as well as expression of the co-stimulatory ligands ICOSL[11] and OX40L[9]. In this study we now reveal that lung ILC2s additionally express the co-stimulatory ligand 4-1BBL upon IL-33-driven activation *ex vivo* as well as *in vivo* following intranasal IL-33 or allergen administration. 4-1BBL is typically expressed on professional antigen-presenting cells such as DCs, B cells and macrophages and is elicited upon ligation of innate pattern recognition receptors as well as adaptive antigen receptors[38,39]. We now describe for the first time that 4-1BBL is induced on activated ILC2s and that IL-33R-mediated signaling as well as c-Rel are implicated in upregulating 4-1BBL expression. Similar to IL-33, other IL-1 family members activate NF-κB signaling upon binding to their cognate receptors on target cells[47,48] and both IL-1β[49,50] and IL-18[51,52] have been described to directly promote ILC2 functions. Furthermore, ILC2s can be stratified into two distinct subsets based on their responsiveness to alarmins, namely IL-33-responsive natural ILC2s (nILC2s) and IL-25-responsive inflammatory ILC2s (iILC2s). Binding of IL-25 to its cognate receptor has been reported to induce NF-κB activation[29,53] and while c-Rel-deficiency clearly impairs nILC2s, the impact on pulmonary iILC2s frequency and function remains to be investigated. Besides activating downstream STAT5 phosphorylation, IL-2 has been shown to induce activation and translocation or RelA[54] and it would be of interest if c-Rel can be activated by IL-2 itself and/or if synergistic effects are observed in combination with IL-33.

The cognate receptor of 4-1BBL, 4-1BB, is inducible on a variety of immune cells but functions mainly as a costimulatory molecule for T cells[44,45]. Interaction of 4-1BB with 4-1BBL or agonistic antibodies typically promotes proliferation and survival of T cells thereby augmenting immunity against viruses[55,56] and tumors[57,58]. However, 4-1BB signaling has also been shown to suppress T cell responsiveness and inflammation in the context of autoimmune and inflammatory

diseases[59,60]. Our data show that 4-1BB transcript levels are markedly upregulated upon intranasal IL-33 administration and that MHC-II$^-$CD74$^+$ airway epithelial cells as well as GATA3$^+$ and GATA3$^-$ Tconv and Tregs upregulate surface 4-1BB upon IL-33 and allergen challenge. 4-1BB is widely considered as a marker for antigen-experienced Tconv and Treg cells[61], however, our data indicate that 4-1BB-expressing T cells rather represent a non-antigen-specific bystander population with increased expression of Treg cell associated markers such as FoxP3 and CD25. Importantly, IL-33-activated lung ILC2s are found in close proximity to airway epithelial cells[62] as well as CD4$^+$ T cells subsets[9,11], thereby enabling potential physical interactions. While previous work reported 4-1BB expression on human intestinal epithelial cell lines in response to TNF/lymphotoxin signaling[63], this is the first time 4-1BB induction was described on airway epithelial cells. The precise nature of these 4-1BB-expressing airway epithelial cells still needs to be investigated in more detail, since CD74 has been described as a specific type II pneumocyte marker but lack of MHC-II expression is indicative of ciliated epithelial cells[64]. Therefore, inclusion of additional type II pneumocyte markers such as pro-surfactant protein C and/or syndecan-1 (CD138) as well as sort-purification and transcriptomic analysis of 4-1BB$^+$ epithelial cells will be carried out in the future to further define the 4-1BB$^+$ epithelial cell population[64,65].

In addition, the underlying molecular mechanisms of 4-1BB induction on ECs and CD4$^+$ T cells during ILC2-driven allergic airway inflammation remain elusive. Airway epithelial cells constitutively express ST2[65,66] and ST2 expression was further reported on subsets of pulmonary Th2[67] and Treg cells[68-70]. It is therefore tempting to speculate that expression of 4-1BB-induction upon intranasal allergen or IL-33 challenge may be a direct consequence of IL-33 signaling in the respective cellular subsets.

The presented study shows several limitations which will be discussed in detail below. First, since c-Rel is expressed in a variety of cells within the hematopoietic compartment[18,71] it cannot be excluded that c-Rel-deficiency impacts effector functions of other innate pulmonary immune cell populations such as IL-33-reactive macrophages or dendritic cells. These cells could potentially promote ILC2 activation by providing co-stimulation or activating cytokines. The ILC2-intrinsic effects in the absence of c-Rel will be addressed in the future by adoptive transfer of WT or *Rel*$^{-/-}$ ILC2s in ILC-deficient recipient mice followed by intranasal allergen or IL-33 challenge.

Second, the molecular consequences of 4-1BB ligation within epithelial cells and T cells and whether they directly promote ILC2 functions remain elusive. Previous work demonstrated that IL-2 enhances ILC2 survival as well as activation-mediated expansion and type 2 cytokine production[7,8,54]. Moreover, 4-1BB ligation has been shown to stimulate production of IL-2 in T cells[72]. We thereby put forward that ligation of 4-1BB by 4-1BBL-expressing ILC2s may induce the production of ILC2-promoting cytokines such as IL-2 which further elicit ILC2 functions. This issue can be addressed by single-cell RNA sequencing upon IL-33 or allergen challenge and validated *ex vivo* by co-culture experiments of activated ILC2s with their respective cellular interaction partners.

Another limitation which needs to be addressed in the future is whether c-Rel affects human ILC2 function and expression of 4-1BBL upon IL-33-mediated activation as well as the physiological role of the 4-1BBL-4-1BB axis during allergic lung disease in humans.

The specific function of the 4-1BBL-4-1BB axis during allergic airway inflammation remains controversial with 4-1BB-agonism displaying either activating or inhibitory effects depending on the experimental model system. Here, treatment of mice with agonistic anti-4-1BB antibody inhibited Tconv cell-driven allergic lung inflammation upon OVA administration[73,74] while anti-4-1BB administration exacerbated NKT cell-mediated AHR and inflammatory cell accumulation[75].

In this work we show that c-Rel positively regulates IL-33-mediated ILC2 functions *ex vivo* as well as *in vivo* and we thereby show for the first time that c-Rel plays an essential role in ILC2-driven allergic airway inflammation. We further identified 4-1BBL as an IL-33-inducible c-Rel target in ILC2s, revealing a novel cellular 4-1BBL source as well as pathway of 4-1BBL induction. Moreover, we describe upregulation of 4-1BB on T cell and epithelial cell subsets during IL-33-mediated allergic airway inflammation suggesting cellular crosstalk of these subsets with 4-1BBL-expressing ILC2s that may further promote disease progression. Elucidating mechanisms of ILC2 activation as well as their cellular interaction partners during allergic type 2 immune responses will not only provide a greater understanding of ILC2 biology but may aid in the development of novel therapeutics that target ILC2 function. While RelA-deficiency results in embryonic lethality and *RelA*$^{-/-}$ cells exhibit severe defects regarding survival, proliferation, and effector functions[76], mice lacking c-Rel are viable and only show limited immunological defects[30]. Thus, targeting c-Rel specifically may avoid the adverse side effects that

have halted advancement of pan-NF-κB-inhibitors for therapeutic applications[27,77]. IL-33-dependent ILC2 activation has been reported during cancer[78,79], viral infections[80-82] as well as murine models of autoimmune diseases[83-86] and given the clinical importance of 4-1BB in these conditions it would be important to assess whether 4-1BBL expression on ILC2s may impact disease outcome in these contexts. Finally, different approaches relying on 4-1BB agonism are currently in clinical trials as cancer immunotherapeutics[57,58,87-92] and assessing the consequences of 4-1BB ligation in different disease settings is therefore of strong biological and therapeutic importance.

2.5. Materials and Methods

Mice

C57BL/6J wildtype mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house. $Rel^{-/-}$ as well as $4\text{-}1BB^{-/-}$ and $4\text{-}1BBL^{-/-}$ mice have been previously described and were kindly provided by Dr. Steve Gerondakis (Monash University) and Dr. Tania Watts (University of Toronto), respectively[30,93,94]. All animals were maintained on a C57BL/6J background and were bred and housed under specific pathogen-free conditions with *ad libitum* access to food and water. Unless otherwise stated, experiments were conducted with adult female age-matched mice (8 – 16 weeks) in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University.

Primary cells

Primary murine lung or bone marrow ILC2s were cultured in complete ILC2 medium (RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml gentamicin and 55 µM β-mercaptoethanol) at 37°C and 5% CO_2.

In vivo stimulation

Mice were anaesthetized with isoflurane followed by intranasal administration of 500 ng carrier-free IL-33 (R&D Systems) or 50 µg *Alternaria alternata* extract (Greer Laboratories) per mouse in 40 µL PBS for three consecutive days. Lungs were isolated and analyzed 24 h after the last treatment. For detection of antigen-specific T cells, mice were administered with 500 ng carrier-

free IL-33 (R&D Systems) in combination with 25 µg 2W1S peptide (EAWGALANWAVDSA; GenScript) in 40 µL PBS for two consecutive days (day 0, 1) and lungs were harvested and analyzed four days after the last administration (day 5).

Preparation of single cell suspensions from tissue

Lung were isolated, finely minced and digested in RPMI-1640 containing 5% FBS, 0.2 mg/ml Liberase™ TM (Roche) and 0.1 mg/ml DNase I (Roche) at 37°C. Digested lungs were homogenized with a 5 ml syringe attached to an 18G 1½ needle, filtered through a 70 µm cell strainer and washed with PBS supplemented with 2% FBS. Red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich).

Flow cytometry

Pelleted cells were resuspended in 2.4G2 hybridoma supernatant and incubated for 15 min on ice to block Fc receptors. Cells were subsequently stained with antibody dilutions prepared in PBS supplemented with 2% FBS for 30 min on ice. Dead cells were excluded by staining with Fixable Viability Dye eFluor™ 780 (eBioscience) following the manufacturer's instructions. Intracellular staining was performed using the FoxP3 / Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Stained cell suspensions were acquired on a BD FACSCanto™ II System (BD Biosciences), a BD LSRFortessa™ Cell Analyzer (BD Biosciences) or sorted on a BD FACSAria™ III Cell Sorter (BD Biosciences). Flow cytometry data were analyzed using FlowJo X (BD Biosciences). All antibodies used for flow cytometry analyses are listed in Table 1.

Lung histopathology

Lungs were inflated with 10% buffered formalin and incubated overnight. After formalin fixation they were transferred to 70% ethanol, embedded in paraffin, sectioned and stained with Hematoxylin and Eosin following standard procedures. A histologic disease score from 0 to 4 was attributed based on peribronchial, perivascular and parenchymal immune cell infiltration.

Isolation and *ex vivo* expansion of bone marrow ILC2

Bone marrow and lung ILC2 were isolated and expanded as described previously with minor modifications[80]. Briefly, bone marrow from tibias and femurs were pooled, subjected to red blood cell lysis using Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma-Aldrich) and sorted as lineage (CD3ε, CD5, CD11b, CD11c, Gr1 (Ly6G), CD45R (B220), NK1.1, TCRβ, TCRγδ, Ter-119)-negative, Sca-1$^+$c-kit$^-$CD25$^+$ cells. Isolated cells were expanded in complete ILC2 medium supplemented with IL-2, IL-7, IL-25, IL-33 (all 50 ng/ml; R&D Systems) and TSLP (20 ng/ml; R&D Systems). After 2 – 3 weeks of expansion, cells were rested for 72 h in IL-2 and IL-7 (both 10 ng/ml), incubated in medium without cytokines for 4 h and used in experiments.

Ex vivo stimulation

Isolated, expanded and rested ILC2 were pooled, washed and incubated in complete medium without cytokines for 4 hours before treatment with indicated stimuli.

Protein quantification

IL-5 and IL-13 protein levels in tissue culture supernatants were determined using the respective DuoSet mouse ELISA kits (R&D Systems) according to the manufacturer's instructions. Absorbance was measured using an Enspire™ 2300 Multilabel Reader (PerkinElmer).

Western Blot

Sub-cellular fractionation of ILC2 was performed as previously described[95]. Briefly, pelleted cells were resuspended in 900 µL PBS/0.1% NP-40 containing protease inhibitor and triturated with a micropipette to lyse the cell membranes. 300 µL of lysate was collected, (= whole-cell lysate, WC). The remaining 600 µL were centrifuged at 13,000 g for 10 seconds, and supernatant was collected (= cytoplasmic fraction, C). Finally, the remaining pellet, containing intact nuclei, was resuspended in 300 uL PBS/0.1% NP-40 with protease inhibitor. All fractionated samples were probe-sonicated for 15 seconds at 60% amplitude. Protein was quantified using the Bio-Rad Protein Assay (#500-0006), as per manufacturer's instructions. Prior to loading samples denatured in 3X Laemmli buffer containing SDS and 15% β-mercaptoethanol at 95°C for 5 minutes. 15 µg of protein for whole-cell lysate and cytoplasmic fraction samples, or 30 µL/ one tenth of the total nuclear fractionated samples, were separated on 8% polyacrylamide gels, with a 3%

polyacrylamide stacking gel. Proteins were wet-transferred onto nitrocellulose membranes. For immunoblotting, membranes were probed with anti-c-Rel; anti-GAPDH or anti-H2A (all Cell Signaling Technology) in 0.1% T-TBS + 5% milk powder followed by incubation with anti-rabbit IgG-HRP (Cell Signaling Technology). Details for antibodies are listed in **Table 1**.

Imagestream

Expanded and rested lung ILC2 were left for 4 h in medium without cytokines and left untreated or stimulated with IL-33 (10 ng/ml) for the indicated time points. Cells were fixed and permeabilized using the FoxP3/transcription factor staining kit (eBioscience) according to the manufacturer's instructions and stained with anti-c-Rel, anti-CD25 and DAPI (NucBlue™ Fixed Cell ReadyProbes™ Reagent, Life Technologies). Samples were run on an ImageStreamX Mark II imaging flow cytometer (Amnis) and nuclear translocation of c-Rel in $CD25^{+}$c-$Rel^{+}DAPI^{+}$ cells was analyzed using the similarity feature of the IDEAS software (Amnis). Antibodies are listed in Table 1.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from cultured ILC2 was extracted using the Quick-RNA MicroPrep Kit (ZymoResearch) according to the manufacturer's instructions. For preparation of total lung RNA, tissue was mechanically disrupted in a MagNA Lyser (Roche) followed by RNA extraction with TRIzol™ Reagent (Life Technologies) and clean-up using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. cDNA was prepared using $Oligo(dT)_{12\text{-}18}$ Primer (Life Technologies) and SuperScript™ III Reverse Transcriptase (Life Technologies). qRT-PCRs were performed with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Transcript expression was normalized to *Hprt* expression levels and quantified using the comparative $2^{-\Delta\Delta CT}$ method. Data are depicted either as relative expression or relative fold change compared to the mean of the control group. Primers used in this study were designed using the PrimerQuest Tool (Integrated DNA Technologies) and purchased from Integrated DNA Technologies. Sequence details are provided in **Table 2**.

RNA sequencing (RNA-seq)

Bone marrow (BM) and lung WT and Rel$^{-/-}$ ILC2 were isolated as described above. BM ILC2s were expanded for 5 d, rested for 72 h in IL-2 and IL-7 (both 10 ng/ml) and incubated for 4 h in medium without cytokines before stimulation. Lung ILC2s were incubated for 18 h in IL-2 and IL-7 (both 10 ng/ml) after sorting and left for 4 h in medium without cytokines. Cells were either left untreated or stimulated with IL-33 (10 ng/ml) for the indicated time points. Total RNA was extracted from BM ILC2s using the Quick-RNA MicroPrep Kit (ZymoResearch) and from lung ILC2s using the MagMAX™ mirVana™ Total RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. RNA-seq was performed as previously described[96]. Briefly, RNA integrity was evaluated using a Bioanalyzer RNA Pico kit (Agilent). Then, total RNA was depleted of rRNA and libraries were prepared using the KAPA Stranded RNA-Seq kit (Roche). The libraries were sequenced on an Illumina HiSeq 2500 sequencer in paired-end 50bp configuration. The quality of sequence reads was assessed with the FastQC tool (Babraham Bioinformatics), and low-quality bases were trimmed and adapter sequences were removed using Trimmomatic v.0.33[97] using the following arguments: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 HEADCROP:4 LEADING:3 TRAILING:3 SLIDINGWINDOW:6:25 MINLEN:30. Sequence reads were mapped to the mouse UCSC mm9 reference assembly using TopHat v2.0.9 with Bowtie v1.0.0 algorithms[98,99]. The number of reads mapping onto gene exons was quantified by counting the number of strand-specific reads using featureCounts[100]. The differential gene expression analysis was performed by comparing IL-33-treated with non-stimulated ILC2s using the edgeR Bioconductor package[101]. First, residual rRNA reads were excluded and genes with an expression level > 10 counts per million reads (CPM) in at least 3 of the samples were retained for TMM normalization and differential gene expression. Genes with changes in expression ≥ |2| fold and Benjamini-Hochberg adjusted *p* values ≤ 0.001 were considered significant. The heatmap presenting the log2 fold change between IL-33-treated vs non-stimulated ILC2 for the selected genes was prepared using MeV software[102].

Chromatin immunoprecipitation (ChIP) assay

Bone marrow ILC2 were sorted, expanded and rested ILC2 as described above. 20 x 10^6 cells were stimulated with IL-33 (100 ng/ml) or left unstimulated for 3 h. ChIP was performed as described previously with slight modifications[103]. Briefly, cells were fixed in culture medium containing 1%

formaldehyde for 10 min at room temperature followed by addition of 0.125 M glycine to quench crosslinking and extraction of nuclei. Nuclear pellets were resuspended in sonication buffer and chromatin was sheared for twelve cycles of 30 seconds with a digital sonifier (Branson Ultrasonics) at 80%, with 30 seconds rest between cycles in cooled circulating water to obtain fragments of 200-500 bp in size. Bead-antibody complexes were prepared by 3 h incubation of 40 µL Dynabeads Protein G (Life Technologies) with anti-cRel (Thermo Fisher Scientific), anti-H3K27Ac (Abcam) or antibodies or goat IgG (Thermo Fisher Scientific) as a control. Immunoprecipitation was performed by overnight incubation of antibody-bead matrices with sheared chromatin from the equivalent of $10x10^6$ cells, followed by successive medium-stringency washes[96]. Samples were de-crosslinked by overnight incubation at 65°C in 1% SDS buffer, followed by RNase A (Thermo Fisher Scientific) and Proteinase K (New England Biolabs) digestions and ChIP DNA purification using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol. ChIP relative enrichment was assessed by qRT-PCR analysis with PowerUp™ SYBR™ Green Master Mix in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Enrichment to the *Csf2* promoter was evaluated using the ΔΔCt method using input DNA as negative control and the pro-opiomelanocortin (*Pomc*) gene as a negative binding region. Enrichment was further calculated as fold change over control IgG. Primer sequences are provided in **Table 2**.

ChIP-sequencing (ChIP-seq)

ChIP-seq libraries were prepared using the Kapa Library Preparation kit (Roche) and sequenced on an Illumina HiSeq 2500 sequencer with a v4 flowcell using a paired 50 bp configuration. Input DNA was sequenced as negative control. The resulting sequence reads were mapped to the UCSC mouse mm9 reference genome with Bowtie 1.0.0[99], and c-Rel peak detection was performed using MACS1.4.1[104] with a p-value threshold $<10^{-5}$; peak heights were retrieved using Homer annotatePeaks tool[105] and peaks with fold enrichment >5 and peaks heights >6 reads per million were retained. Sequence read density profiles (bigwig) normalized to counts per 10^7 reads were generated with Homer tool and visualized with the Integrative Genomics Viewer (IGV)[106]. *De novo* motif enrichment analysis was performed using findMotifsGenome Homer tool and visualized with WebLogo[107]. ChIP-seq heatmap was generated by extracting normalized read counts with ± 1kb for the input control and c-Rel and ± 2kb for the H3K27Ac ChIPs using the

Homer toolkit and visualized with Java TreeView[108].

Statistical analyses

All data were analyzed with GraphPad Prism software (GraphPad Software). P values below 0.05 were defined as statistically significant (*p<0.05, **p<0.01, ***p<0.001). Unless otherwise indicated, figures display means ± standard deviation (SD). Experiment sample sizes (n), experiment replicate numbers and statistical tests used are included in the respective figure legends.

2.6. Acknowledgements

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2.7. Figures and tables

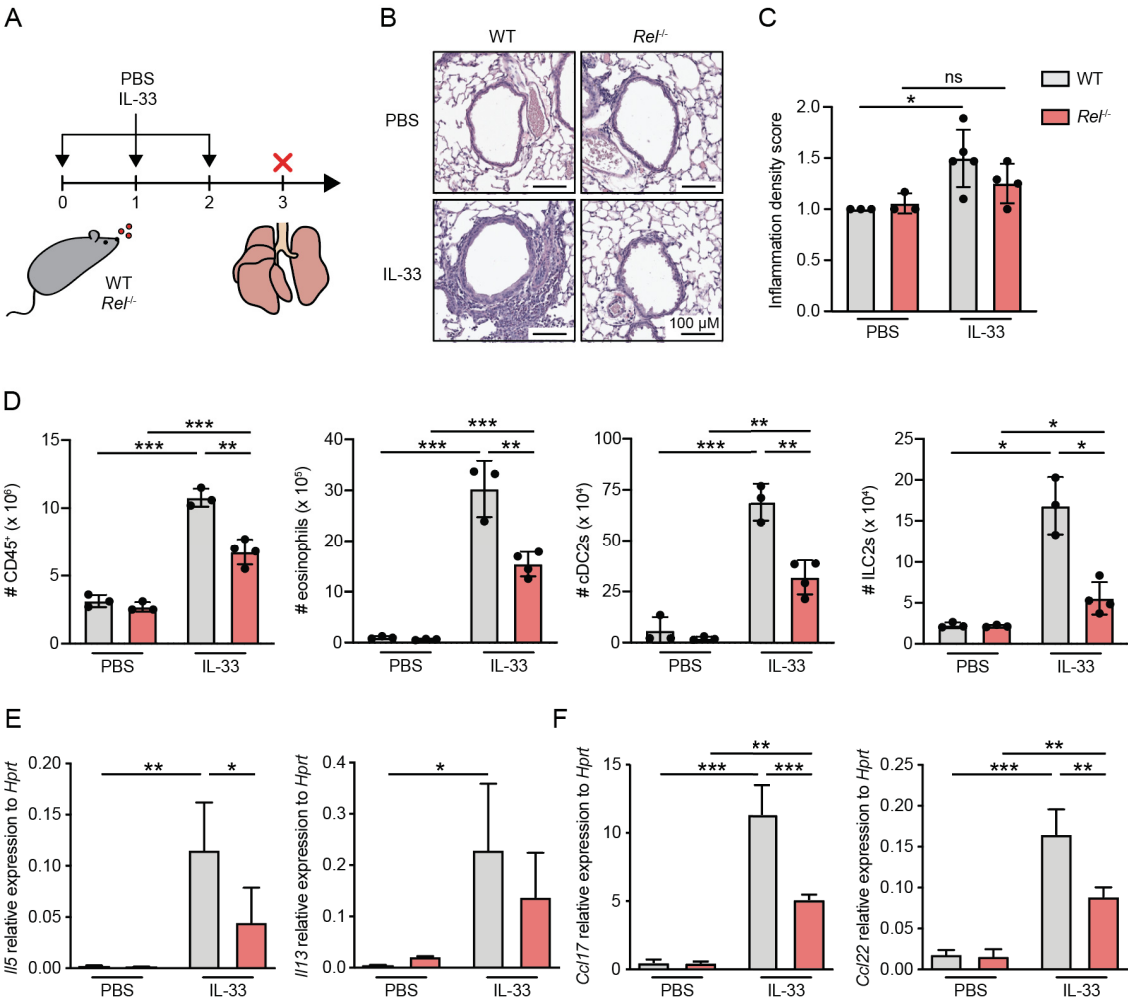


Figure 10. c-Rel is a critical mediator of innate type 2 immune responses.

(**A**) WT and c-Rel-deficient (*Rel*$^{-/-}$) mice were intranasally challenged for three consecutive days (day 0, 1 and 2) with PBS as a control or IL-33 (500 ng/mouse). Lungs were isolated and analyzed 24 hours after the last challenge (day 3). (**B**) Microscopy of lung sections stained with hematoxylin and eosin (H&E) from WT (left panel) and *Rel*$^{-/-}$ mice (right panel) treated with PBS or IL-33. Scale bars, 100 µM. (**C**) Pathology score of inflammatory infiltration density assessed microscopically from H&E-stained lung sections. **(D)** Total numbers of CD45^{+} leukocytes, eosinophils, cDC2s and ILC2s in lungs of WT (grey) or *Rel*$^{-/-}$ (red) mice were determined by flow cytometric analysis. Expression levels of (**E**) *Il5* and *Il13* as well as (**F**) *Ccl17* and *Ccl22* in whole lung tissue of WT (grey) and *Rel*$^{-/-}$ (red) animals were assessed by qRT-PCR. Data are representative of two independent experiments with n = 3 – 4 mice per group. Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

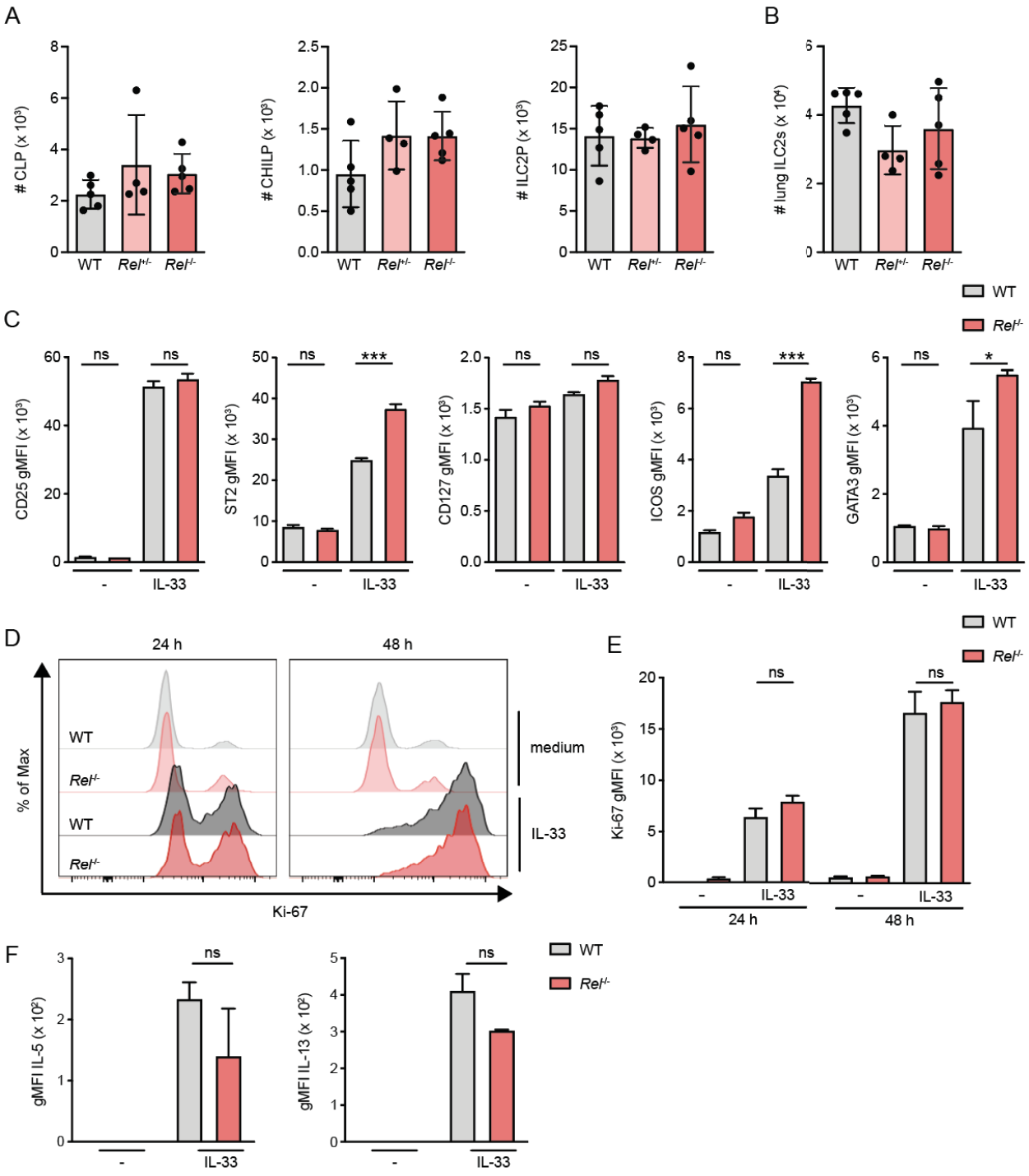

A
CLP (x 10³)
CHILP (x 10³)
ILC2P (x 10³)
WT
Rel+/-
Rel-/-
B
lung ILC2s (x 10⁴)
C
WT
Rel-/-
CD25 gMFI (x 10³)
ST2 gMFI (x 10³)
CD127 gMFI (x 10³)
ICOS gMFI (x 10³)
GATA3 gMFI (x 10³)
ns
***
*
-
IL-33
D
24 h
48 h
% of Max
Ki-67
medium
IL-33
E
Ki-67 gMFI (x 10³)
24 h
48 h
F
gMFI IL-5 (x 10²)
gMFI IL-13 (x 10²)

Figure 11. c-Rel-deficient ILC2s harbor no intrinsic defects in their developmental capacity, key surface molecule expression, proliferation as well as IL-5 and IL-13 production.

Flow cytometric analysis of total numbers of **(A)** ILC2 precursor populations CLP (left), CHILP, (middle) and ILC2P (right) and **(B)** pulmonary ILC2s in WT, heterozygous *Rel*$^{+/-}$ and homozygous *Rel*$^{-/-}$ littermate control mice. (**C-F**) Lung ILC2s from WT (grey) and *Rel*$^{-/-}$ (red) mice were isolated by flow cytometry and cultured *ex vivo* in the absence (-) or presence of IL-33 (10 ng/ml). **(C)** Surface expression levels of CD25, ST2, CD127, ICOS and intracellular expression of GATA3 in isolated WT (grey) and *Rel*$^{-/-}$ (red) lung ILC2s after 24 h stimulation with medium only (-) or IL-33. **(D)** Representative flow cytometry histogram plots of intracellular Ki-67 expression by WT (grey) and *Rel*$^{-/-}$ (red) lung ILC2s left untreated or cultured with IL-33 for 24 hours (left panel) or 48 hours (right panel). **(E)** Ki-67 expression levels by lung ILC2s shown as gMFI. **(F)** Flow cytometric assessment of intracellular IL-5 (left) and IL-13 (right) expression in untreated lung ILC2s (-) or following stimulation with IL-33 for 24 hours. Data points are representative of two independent experiments with n = 4 – 5 mice per treatment group (A - B) or at least two independent experiments with two biological replicates for each stimulation condition (C-F). Data are shown as mean ± SD with *p < 0.05, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test or by two-tailed t test (unpaired). CLP, common lymphoid progenitor; CHILP, common helper ILC2 progenitor; ILC2P, ILC2 progenitor; gMFI, geometric mean fluorescence intensity; ns, not significant.

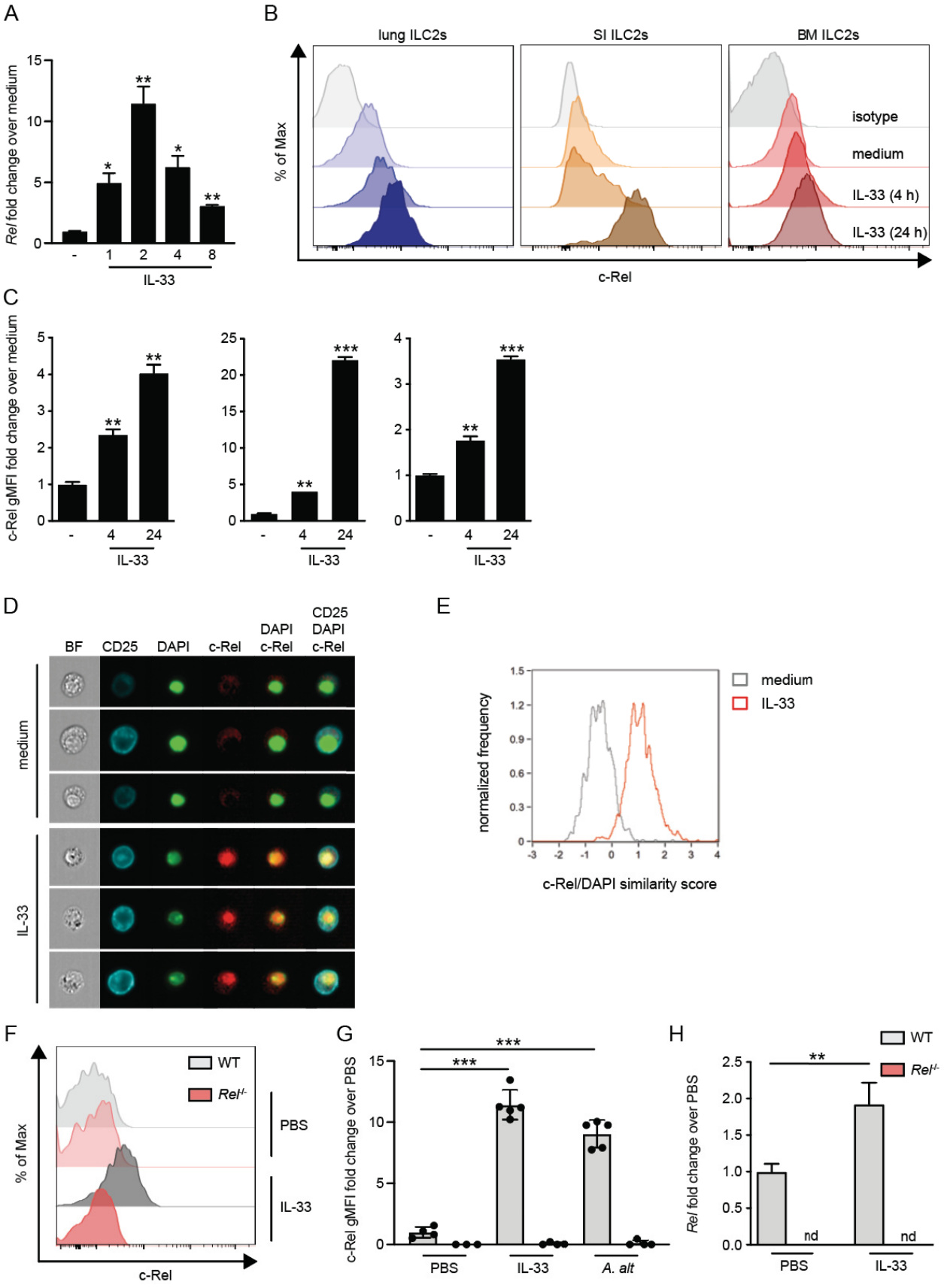

A
Rel fold change over medium
IL-33
B
lung ILC2s
SI ILC2s
BM ILC2s
% of Max
isotype
medium
IL-33 (4 h)
IL-33 (24 h)
c-Rel
C
c-Rel gMFI fold change over medium
IL-33
D
BF
CD25
DAPI
c-Rel
DAPI c-Rel
CD25 DAPI c-Rel
medium
IL-33
E
normalized frequency
medium
IL-33
c-Rel/DAPI similarity score
F
WT
Rel-/-
PBS
IL-33
% of Max
c-Rel
G
c-Rel gMFI fold change over PBS
PBS
IL-33
A. alt
H
WT
Rel-/-
Rel fold change over PBS
nd
PBS
IL-33

Figure 12. IL-33-driven *ex vivo* activation of pulmonary ILC2s induces c-Rel expression and translocation to the nucleus.

(**A-C**) Murine lung, small intestinal or bone marrow-derived ILC2s were isolated by multicolor fluorescence activated cell sorting (FACS) and left unstimulated (-) or cultured in the presence of recombinant murine IL-33 (10 ng/ml) for the indicated time points. **(A)** Kinetics of c-Rel (*Rel*) transcript expression in isolated murine lung ILC2s assessed by qRT-PCR. Asterisks indicate significance over untreated control. **(B)** Representative histogram plots of intracellular flow cytometric staining of c-Rel in lung (left), small intestinal (middle) or bone marrow-derived (right) ILC2s left untreated or cultured with IL-33 for either 4 or 24 hours. c-Rel staining is shown in comparison to staining with an isotype control antibody (grey). **(C)** c-Rel staining intensity in lung (left), small intestinal (middle) and bone-marrow-derived (right) ILC2s quantified as gMFI fold change over unstimulated (medium) cells. Asterisks indicate significance over untreated control. **(D-E)** ImageStream analysis of isolated lung ILC2s left unstimulated (medium) or cultured with IL-33 (10 ng/ml) for 3 hours. (**D**) Panels show representative images (from left to right) of brightfield (BF), CD25, DAPI and c-Rel as well as merged images of DAPI + c-Rel and CD25 + DAPI + c-Rel. **(E)** Quantification of nuclear translocation of c-Rel by overlay of c-Rel/DAPI similarity scores of untreated (grey histogram) and IL-33-activated ILC2s (red histogram). **(F-H)** WT (grey) and $Rel^{-/-}$ (red) mice were intranasally challenged for three consecutive days (day 0, 1 and 2) with either PBS, IL-33 (500 ng/mouse) or *Alternaria alternata* extract (50 µg/mouse) and lungs were analyzed 24 hours after the last administration. **(F)** Representative histogram plots of intracellular c-Rel staining in pulmonary WT (grey) and $Rel^{-/-}$ (red) ILC2s following intranasal challenge with PBS or IL-33 determined by flow cytometry. **(G)** Quantification of c-Rel expression levels as gMFI in lung ILC2s following PBS, IL-33 or *A. alternata* administration. **(H)** qRT-PCR analysis of *Rel* expression in whole lungs of WT (grey) and $Rel^{-/-}$ (red) mice intranasally challenged with PBS or IL-33. Data points are representative of at least two independent experiments with either two biological replicates (A–E) or n = 3 – 5 mice per treatment group (F–H). Data are shown as mean ± SD with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test or by two-tailed t test (unpaired). gMFI, geometric mean fluorescence intensity; nd, not detectable; *A. alt*, *Alternaria alternata*.

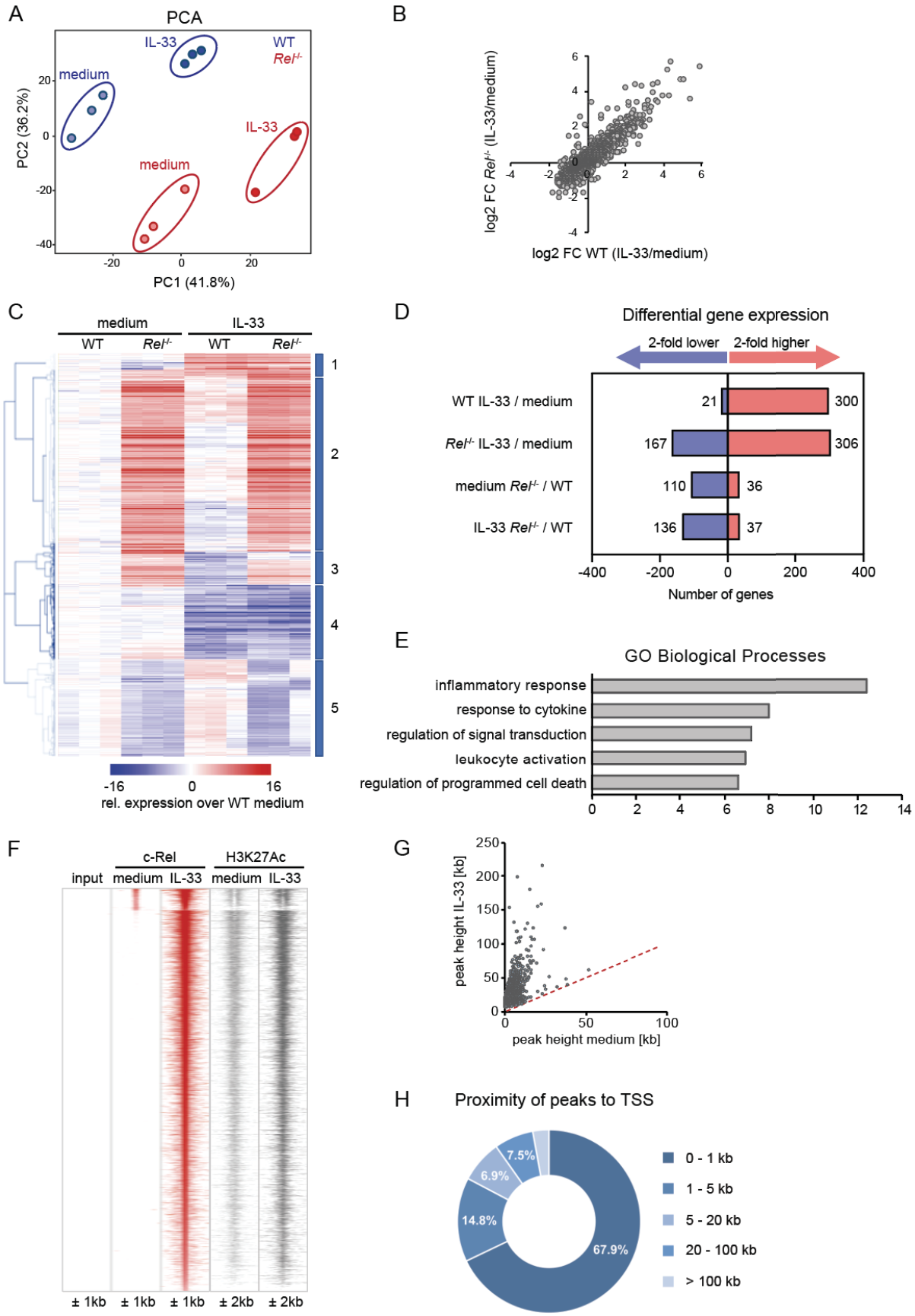

A
PCA
IL-33
WT
Rel-/-
medium
IL-33
medium
PC2 (36.2%)
PC1 (41.8%)
B
log2 FC Rel-/- (IL-33/medium)
log2 FC WT (IL-33/medium)
C
medium
IL-33
WT
Rel-/-
WT
Rel-/-
1
2
3
4
5
-16
0
16
rel. expression over WT medium
D
Differential gene expression
2-fold lower
2-fold higher
WT IL-33 / medium
21
300
Rel-/- IL-33 / medium
167
306
medium Rel-/- / WT
110
36
IL-33 Rel-/- / WT
136
37
Number of genes
E
GO Biological Processes
inflammatory response
response to cytokine
regulation of signal transduction
leukocyte activation
regulation of programmed cell death
F
c-Rel
H3K27Ac
input
medium
IL-33
medium
IL-33
± 1kb
± 1kb
± 1kb
± 2kb
± 2kb
G
peak height IL-33 [kb]
peak height medium [kb]
H
Proximity of peaks to TSS
7.5%
6.9%
14.8%
67.9%
0 - 1 kb
1 - 5 kb
5 - 20 kb
20 - 100 kb
> 100 kb

Figure 13. c-Rel binds to regulatory chromatin regions and mediates distinct transcriptional changes upon IL-33-driven ILC2 activation.

RNA-seq (**A-E**) and ChIP-seq (**F-H**) analysis of murine bone-marrow-derived ILC2s isolated by flow cytometry and expanded *ex vivo* for either 5 days (A-E) or 21 days (F-H). Cells were rested after expansion and left unstimulated (medium) or were cultured in the presence of IL-33. **(A)** Principal component analysis (PCA) of RNA-seq gene expression data of WT (blue) and *Rel*$^{-/-}$ (red) ILC2s cultured with medium or IL-33 (10 ng/ml) for 4 hours. **(B)** Comparison of gene expression in WT and *Rel*$^{-/-}$ ILC2s in response to IL-33. **(C)** Clustered heat map of differentially expressed genes (fold change ≤ 0.5 or ≥ 2; $p \leq 0.001$) in *Rel*$^{-/-}$ and WT ILC2s. Five groups of genes (1-5) were defined based on the effect of IL-33 and c-Rel-deficiency using hierarchical clustering. **(D)** Pairwise comparison of gene epxression among IL-33-treated vs unstimulated WT and *Rel*$^{-/-}$ ILC2s as well as unstimulated *Rel*$^{-/-}$ vs WT or IL-33-treated *Rel*$^{-/-}$ vs WT ILC2s (fold change ≤ 0.5 or ≥ 2; $p \leq 0.001$). **(E)** Gene ontology (GO) term enrichment analysis for biological processes of significantly upregulated genes in WT ILC2s upon culture with IL-33 for 4 hours. **(F)** Determination of chromatin binding sites of c-Rel in ILC2s that were unstimulated or treated with IL-33 (100 ng/ml) for 3 hours by ChIP-seq analysis. Each horizontal line represents the read density in a ± 1 kb region around a unique peak. H3K27Ac epigenetic marks are shown for a ± 2 kb region surrounding associated c-Rel binding peaks. **(G)** Peak heights for c-Rel binding sites determined by ChIP-seq in untreated and IL-33-treated ILC2s. **(H)** Distance of c-Rel binding sites (in kb) to transcription start sites (TSS) given in percentage of total binding peaks. Data are representative of one experiment with three technical replicates (A–E) or two independent experiments (E–G). PCA, principal component analysis; GO, gene ontology; TSS, transcription start sites.

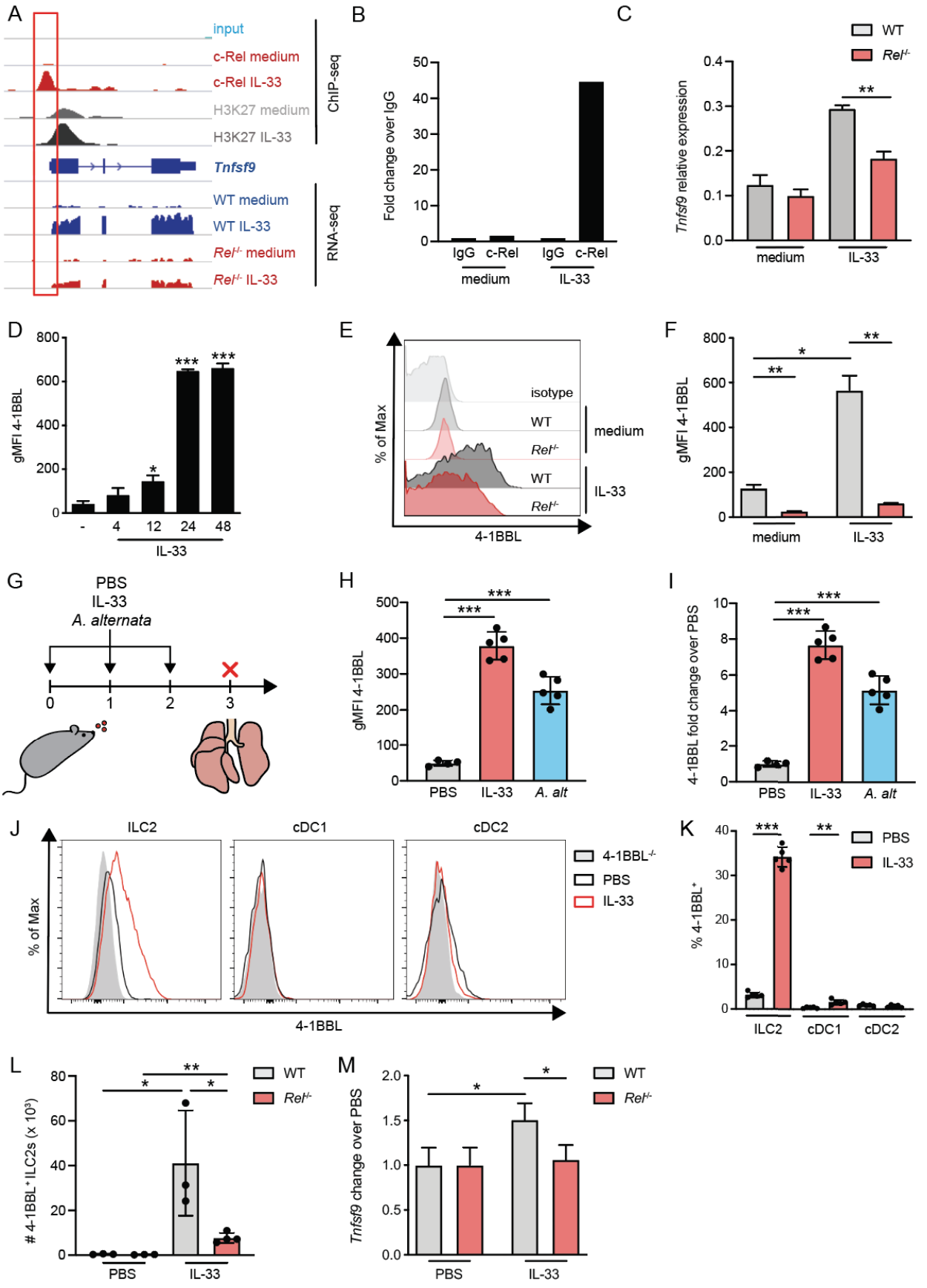
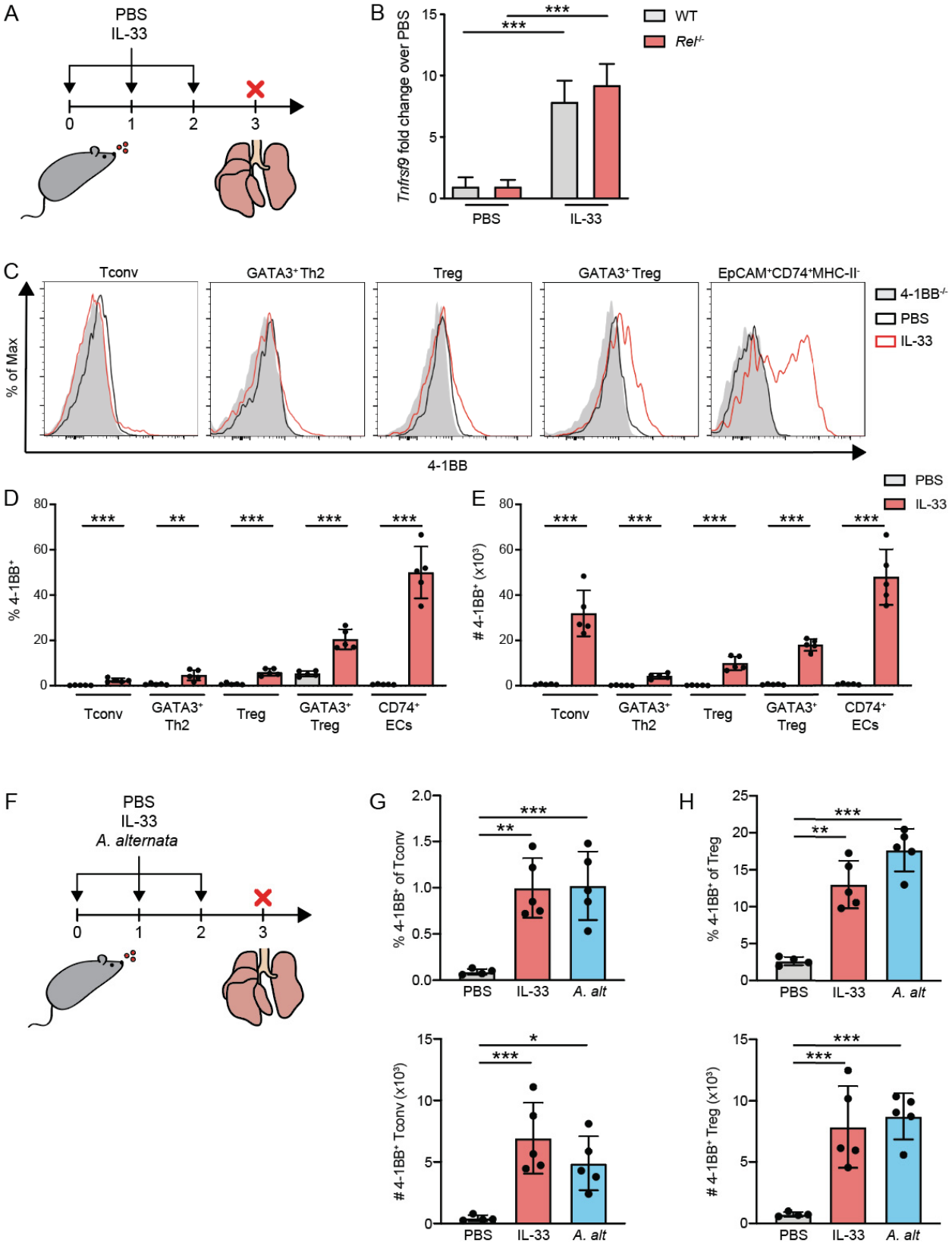

A
input
c-Rel medium
c-Rel IL-33
H3K27 medium
H3K27 IL-33
Tnfsf9
WT medium
WT IL-33
Rel-/- medium
Rel-/- IL-33
ChIP-seq
RNA-seq
B
Fold change over IgG
IgG c-Rel IgG c-Rel
medium
IL-33
C
WT
Rel-/-
Tnfsf9 relative expression
medium
IL-33
D
gMFI 4-1BBL
IL-33
E
% of Max
isotype
WT
Rel-/-
WT
Rel-/-
medium
IL-33
4-1BBL
F
gMFI 4-1BBL
medium
IL-33
G
PBS
IL-33
A. alternata
H
gMFI 4-1BBL
PBS
IL-33
A. alt
I
4-1BBL fold change over PBS
PBS
IL-33
A. alt
J
ILC2
cDC1
cDC2
% of Max
4-1BBL-/-
PBS
IL-33
4-1BBL
K
% 4-1BBL+
PBS
IL-33
ILC2
cDC1
cDC2
L
4-1BBL+ ILC2s (x 10³)
WT
Rel-/-
PBS
IL-33
M
Tnfsf9 change over PBS
WT
Rel-/-
PBS
IL-33

Figure 14. c-Rel regulates 4-1BBL expression on ILC2s ex vivo and during allergic airway inflammation.
(**A**) Genomic snapshot of the *Tnfsf9* (4-1BBL) locus showing density of ChIP-seq (for c-Rel and H3K27Ac) and RNA-seq reads (WT and *Rel*$^{-/-}$) in bone marrow-derived expanded ILC2s. (**B**) ChIP-qPCR validation of c-Rel levels (relative to control IgG) within the *Tnfsf9* promoter region. (**C**) Validation of *Tnfsf9* expression by qRT-PCR in lung ILC2s isolated from WT (grey) or *Rel*$^{-/-}$ (red) mice and stimulated with either medium or IL-33 (10 ng/ml) for 4 hours. (**D**) Kinetics of surface 4-1BBL expression on WT lung ILC2s left unstimulated (-) or cultured in the presence of IL-33 (10 ng/ml) for the indicated time points (hours). Expression levels were assessed by flow cytometry and are shown as gMFI. Asterisks indicate significance over untreated control. (**E-F**) Flow cytometric analysis of surface 4-1BBL levels on isolated WT (grey) and *Rel*$^{-/-}$ (red) lung ILC2s stimulated with medium or IL-33 (10 ng/ml) for 48 hours. 4-1BBL expression is shown as **(E)** representative histogram plots in comparison to staining with an isotype control antibody (light grey) or as (**F**) gMFI levels. (**G-M**) Mice were intranasally administered with either PBS as a control, IL-33 (500 ng/mouse) or *Alternaria alternata* extract (50 μg/mouse) for three consecutive days (day 0, 1 and 2) and lungs were analyzed 24 hours after the last challenge (**G**). Flow cytometric analysis of surface 4-1BBL levels on lung ILC2s following intranasal challenge of WT mice with PBS (grey bars), IL-33 (red bars) or *A. alternata* (blue bars). 4-1BBL expression is shown as (**H**) gMFI or (**I**) fold change over PBS-treated control mice. (**J**) Representative flow cytometry histogram plots of surface 4-1BBL staining on pulmonary WT ILC2s, cDC1s and cDC2s after *in vivo* challenge with PBS (black histogram) or IL-33 (red histogram). Staining is shown in comparison to 4-1BBL-deficient animals (filled histogram) **(K)** Frequencies of 4-1BBL-expressing ILC2s, cDC1s and cDC2s following intranasal PBS (grey bars) or IL-33 challenge (red bars). **(L)** Total numbers of 4-1BBL-expressing lung ILC2s in WT (grey) and *Rel*$^{-/-}$ (red) mice following *in vivo* administration of PBS or IL-33. **(M)** qRT-PCR analysis of *Tnfsf9* expression in whole lungs of WT (grey) and *Rel*$^{-/-}$ (red) mice intranasally challenged with PBS or IL-33. Data are representative of at least two independent experiments with either two biological replicates per stimulation condition (A–F) or n = 3 – 5 mice per treatment group (G–M). Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test or by two-tailed t test (unpaired). gMFI, geometric mean fluorescence intensity; *A. alt*, *Alternaria alternata*.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PBS
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1
2
3
B
Tnfrsf9 fold change over PBS
WT
Rel-/-
PBS
IL-33
C
Tconv
GATA3+ Th2
Treg
GATA3+ Treg
EpCAM+CD74+MHC-II-
% of Max
4-1BB
4-1BB-/-
PBS
IL-33
D
% 4-1BB+
Tconv
GATA3+ Th2
Treg
GATA3+ Treg
CD74+ ECs
E
4-1BB+ (x10³)
F
PBS
IL-33
A. alternata
G
% 4-1BB+ of Tconv
# 4-1BB+ Tconv (x10³)
H
% 4-1BB+ of Treg
# 4-1BB+ Treg (x10³)
A. alt

Figure 15. Expression of 4-1BB is upregulated on distinct lung T cell and airway epithelial cell subsets upon IL-33 or allergen-mediated allergic airway inflammation.

(**A-H**) WT and *Rel*$^{-/-}$ or 4-1BB-deficient (4-1BB$^{-/-}$) mice were intranasally challenged with PBS as a control, IL-33 (500 ng/mouse) or *Alternaria alternata* extract (50 μg/mouse) for three consecutive days (day 0, 1 and 2) and lungs were analyzed 24 hours after the last administration (day 3). (**B**) Assessment of *Tnfrsf9* (4-1BB) expression levels in whole lungs of WT (grey) and *Rel*$^{-/-}$ (red) mice intranasally challenged with PBS or IL-33 by qRT-PCR. (**C**) Representative flow cytometry histogram plots of surface 4-1BB staining by indicated pulmonary T cell and epithelial cell subsets following intranasal *in vivo* challenge with PBS (black histogram) or IL-33 (red histogram). Staining is shown in comparison to 4-1BB-deficient animals (filled histogram). (**D**) Frequencies and (**E**) total numbers of indicated 4-1BB-expressing CD4^{+} T cell subsets and CD74^{+}MHC-II^{-} epithelial cells following intranasal PBS (grey bars) or IL-33 (red bars) challenge. Frequencies (top panel) and total numbers (bottom panel) of 4-1BB^{+} lung (**J**) Tconv and (**K**) Treg cells following intranasal challenge of WT and 4-1BB$^{-/-}$ mice with PBS (grey bars), IL-33 (red bars) or *A. alternata* (blue bars). Data points are representative of at least two independent experiments with n = 3 – 5 mice per treatment group. Data are shown as mean ± SD with **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. *A. alt*, *Alternaria alternata*.

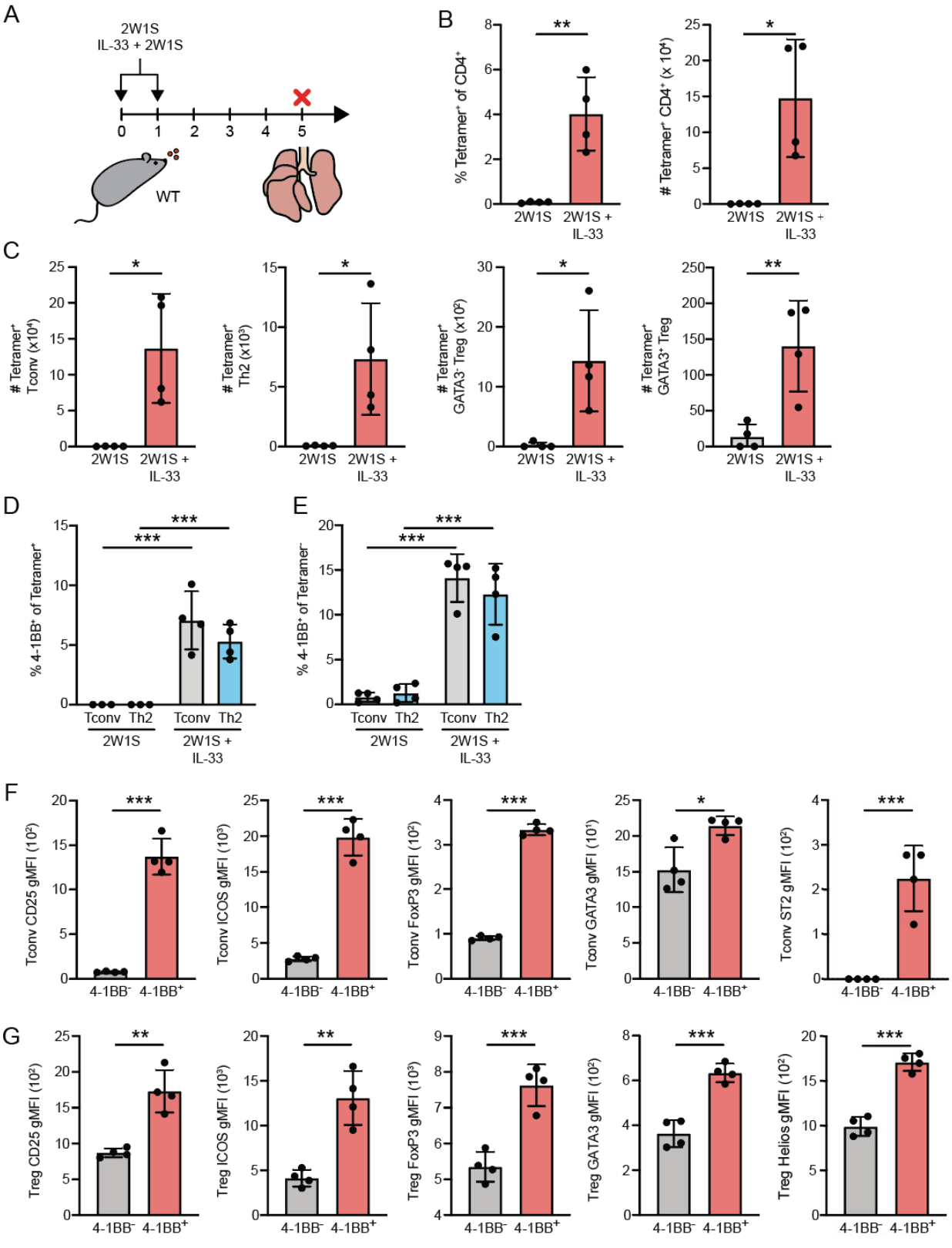

A
2W1S
IL-33 + 2W1S
0 1 2 3 4 5
WT
B
% Tetramer+ of CD4+
# Tetramer+ CD4+ (x 10⁴)
2W1S
2W1S + IL-33
C
Tetramer+ Tconv (x10⁴)
Tetramer+ Th2 (x10³)
Tetramer+ GATA3- Treg (x10²)
Tetramer+ GATA3+ Treg
D
% 4-1BB+ of Tetramer+
Tconv Th2 Tconv Th2
2W1S
2W1S + IL-33
E
% 4-1BB+ of Tetramer-
F
Tconv CD25 gMFI (10²)
Tconv ICOS gMFI (10³)
Tconv FoxP3 gMFI (10²)
Tconv GATA3 gMFI (10¹)
Tconv ST2 gMFI (10²)
4-1BB- 4-1BB+
G
Treg CD25 gMFI (10²)
Treg ICOS gMFI (10³)
Treg FoxP3 gMFI (10³)
Treg GATA3 gMFI (10²)
Treg Helios gMFI (10²)

Figure 16. Antigen-specific $CD4^+$ T cell subsets show no enrichment in 4-1BB expression and $4\text{-}1BB^+$ $CD4^+$ T cells exhibit increased expression of Treg signature and type 2-associated markers.

(**A-D**) WT mice were intranasally challenged with 2W1S peptide (25 μg /mouse) or 2W1S (25 μg /mouse) + IL-33 (500 ng/mouse) for two consecutive days (day 0 and 1) and lungs were isolated and analyzed on day 5 followed by quantification of 2W1S-$Tetramer^{+/-}$ T cell subsets (**A**). (**B**) Frequencies (left) and total numbers (right) of $CD4^+Tetramer^+$ T cells following administration of 2W1S or 2W1S + IL-33. (**C**) total numbers of 2W1S-$Tetramer^+$ $GATA3^-FoxP3^-$ Tconv, $GATA3^+FoxP3^-$ Th2, $GATA3^-FoxP3^+$ Treg, $GATA3^+FoxP3^+$ after 2W1S or 2W1S + IL-33 challenge. Frequencies of $4\text{-}1BB^+$ (**D**) $Tetramer^+$ and (**E**) Tetramer- lung $CD4^+FoxP3^-$ Tconv as well as $CD4^+CD25^+FoxP3^+$ Treg cells. Flow cytometric analysis of CD25, ICOS, FoxP3, GATA3 and Helios or ST2 expression by $4\text{-}1BB^-$ and $4\text{-}1BB^+$ lung (**F**) $CD4^+FoxP3^-$ Tconv and (**G**) $CD4^+CD25^+FoxP3^+$ Treg cells after intranasal administration of IL-33 (500 ng/mouse on day 0, 1 and 2). Lungs were collected and analyzed on day 3. Data points are representative of at least two independent experiments with n = 3 – 4 mice per treatment group. Data are shown as mean ± SD with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as determined by two-tailed t test (unpaired). gMFI, geometric mean fluorescence intensity.

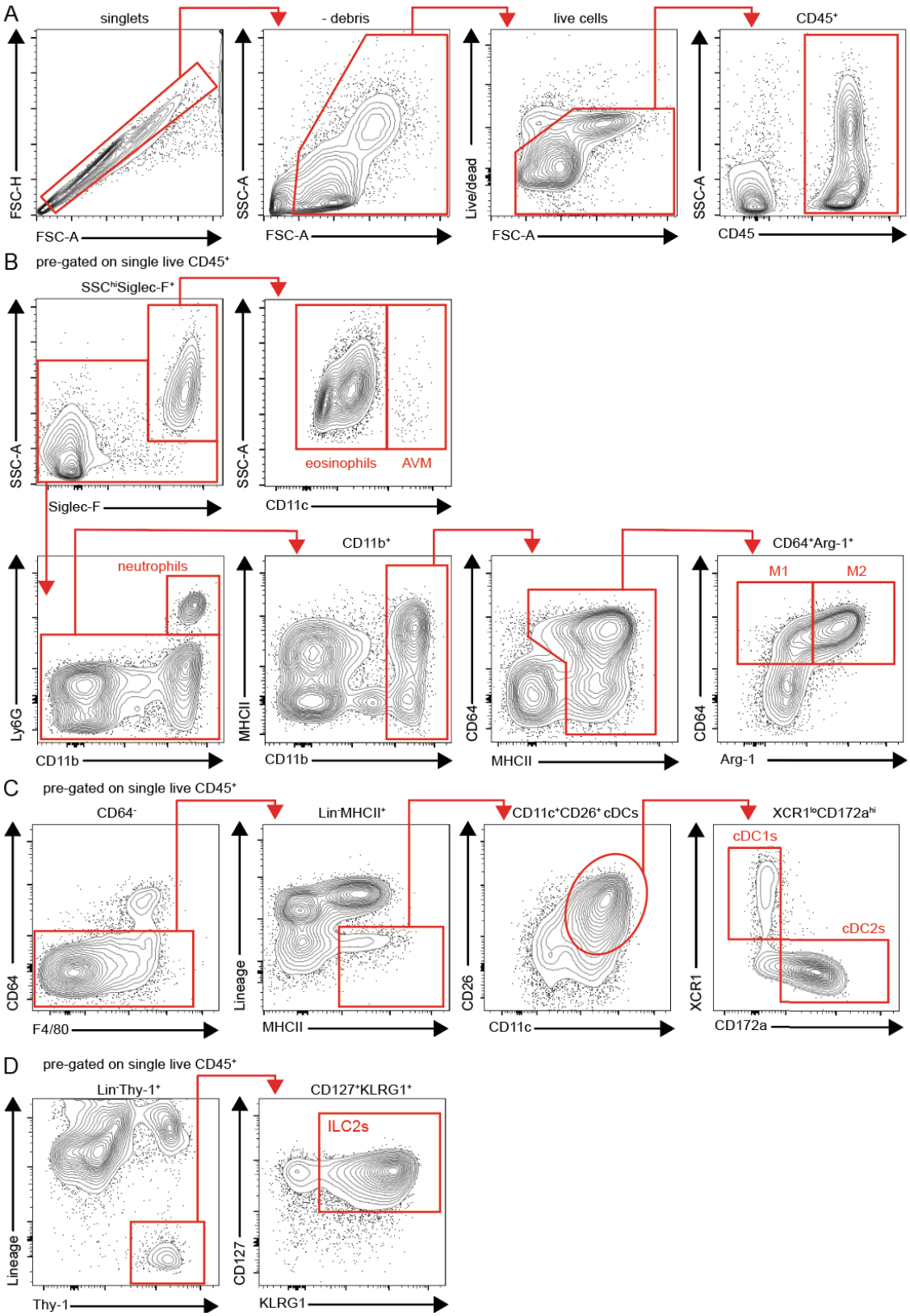

A
singlets
- debris
live cells
CD45+
FSC-H
FSC-A
SSC-A
FSC-A
Live/dead
FSC-A
SSC-A
CD45
B
pre-gated on single live CD45+
SSChiSiglec-F+
SSC-A
Siglec-F
SSC-A
CD11c
eosinophils
AVM
neutrophils
CD11b+
CD64+Arg-1+
M1
M2
Ly6G
CD11b
MHCII
CD11b
CD64
MHCII
CD64
Arg-1
C
pre-gated on single live CD45+
CD64-
Lin-MHCII+
CD11c+CD26+ cDCs
XCR1loCD172ahi
cDC1s
cDC2s
CD64
F4/80
Lineage
MHCII
CD26
CD11c
XCR1
CD172a
D
pre-gated on single live CD45+
Lin-Thy-1+
CD127+KLRG1+
ILC2s
Lineage
Thy-1
CD127
KLRG1

Figure 17 (Suppl. Fig. 1). Gating strategies for the identification of pulmonary myeloid cell populations and ILC2s.

(A) Multicolor flow cytometry gating strategies to identify murine pulmonary **(B)** eosinophils (single, live $CD45^+SSC^{hi}Siglec\text{-}F^+CD11c^-$), AVMs (single, live $CD45^+SSC^{hi}Siglec\text{-}F^+CD11c^+$), neutrophils (single, live $CD45^+Ly6G^+CD11b^+$), M1 (single, live $CD45^+Ly6G^-CD11b^+CD64^+Arg\text{-}1^-$) and M2 macrophages (single, live $CD45^+Ly6G^-CD11b^+CD64^+Arg\text{-}1^+$) as well as **(C)** cDC1s (single, live $CD45^+CD64^-Lin^-MHCII^+CD26^+CD11c^+XCR1^+CD172a^-$), cDC2s (single, live $CD45^+CD64^-Lin^-MHCII^+CD26^+CD11c^+XCR1^-CD172a^+$) and **(D)** ILC2s (single, live $CD45^+$ $Lin^-Thy\text{-}1^+CD127^+KLRG1^+$). AVM, alveolar macrophage; cDC1, conventional type 1 dendritic cell; cDC2, conventional type 2 dendritic cell.

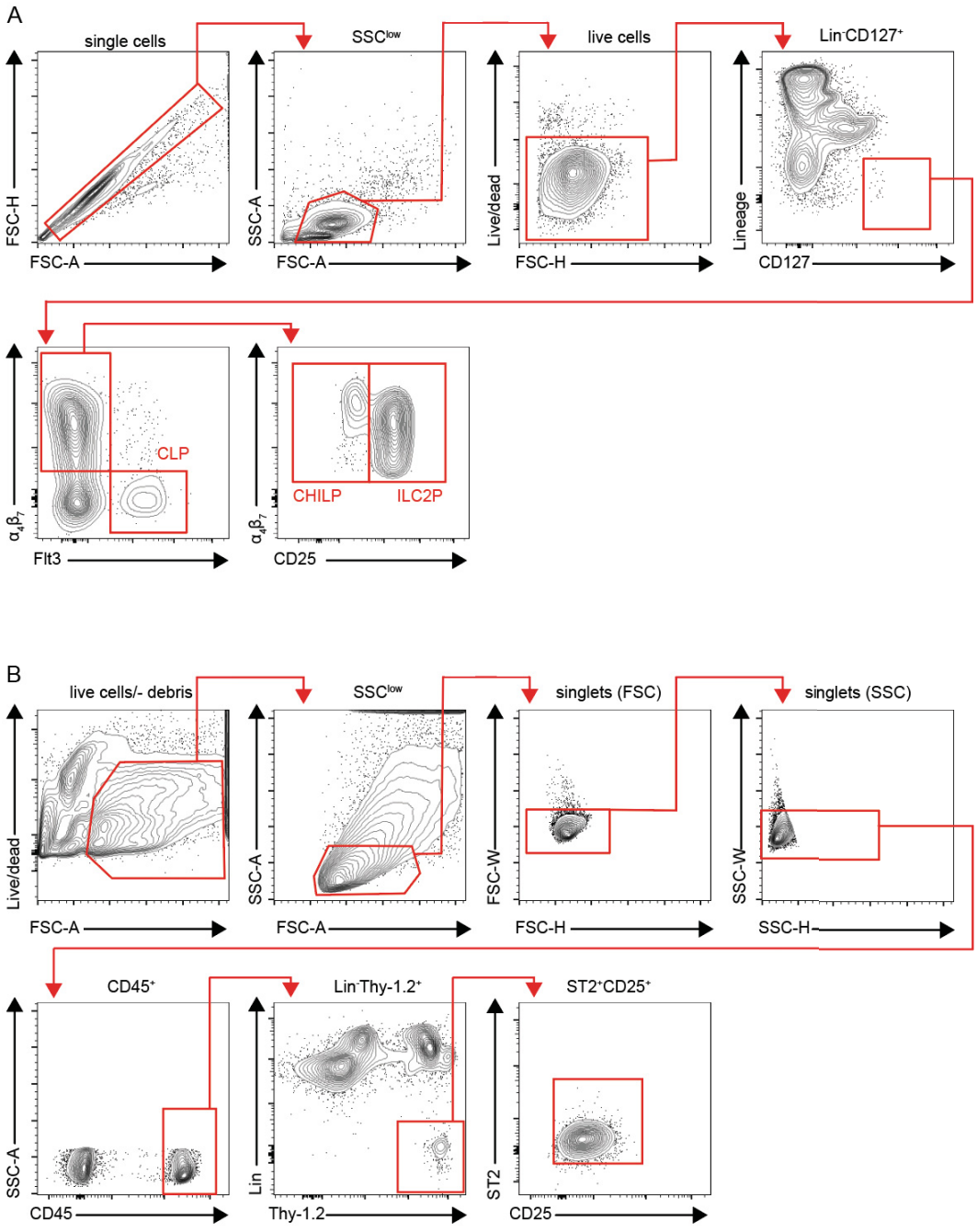


Figure 18 (Suppl. Fig. 2). Gating strategies for the identification of bone marrow ILC2 progenitor populations and isolation of murine lung ILC2s.

Flow cytometry gating strategies to **(A)** identify bone marrow CLP (single live Lin$^-$CD127$^+$Flt3$^+$$\alpha_4\beta_7^-$), CHILP (single live Lin$^-$CD127$^+$Flt3$^+$$\alpha_4\beta_7^+$ CD25$^-$) and ILC2P (single live Lin$^-$CD127$^+$Flt3$^+$$\alpha_4\beta_7^+$ CD25$^+$) populations and **(B)** isolate murine lung ILC2s (single live Lin$^-$CD45$^+$Thy-1$^+$ST2$^+$CD25$^+$). CLP, common lymphoid progenitor; CHILP, common helper ILC2 progenitor; ILC2P, ILC2 progenitor.

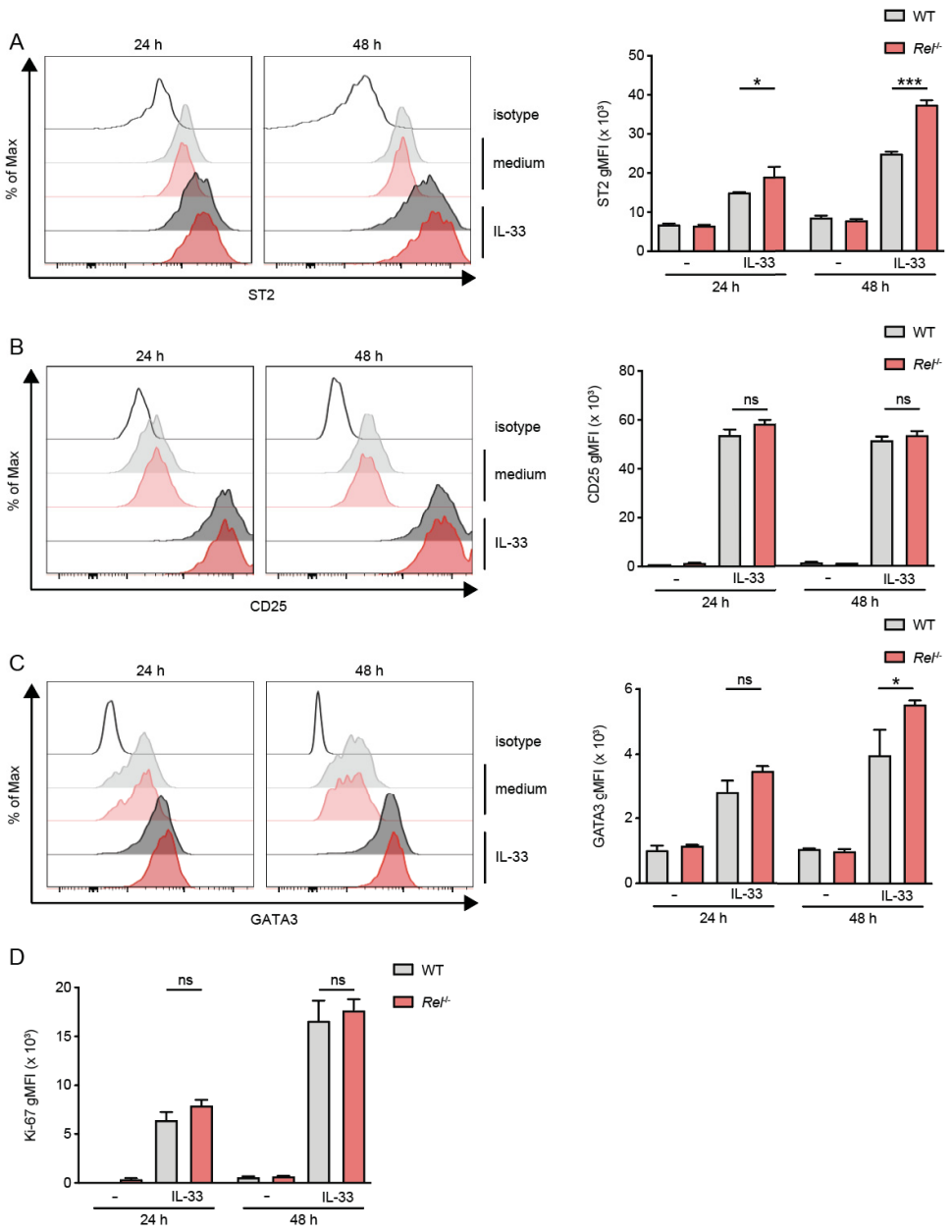


Figure 19 (Suppl. Fig. 3). c-Rel deficiency does not impair expression of key surface receptors, GATA3 and proliferation.

(**A-D**) ILC2s from lungs of WT (grey) and *Rel*$^{-/-}$ (red) mice were isolated by flow cytometry and cultured *ex vivo* in the absence (-) or presence of IL-33 (10 ng/ml) for 24 and 48 hours and analyzed by flow cytometry. Analysis of surface expression of **(A)** ST2 and (**B**) CD25 by isolated WT (grey) and *Rel*$^{-/-}$ (red) lung ILC2s shown as representative histogram plots (left panel) or as gMFI (right panel). **(C)** Intracellular expression of GATA3 in pulmonary WT (grey) and *Rel*$^{-/-}$ (red) ILC2s depicted as histogram plots (left panel) or gMFI (right panel). **(D)** Frequency of Ki-67^{+} of total ILC2s. Data are representative of two independent experiments with two biological replicates for each stimulation condition. Data are shown as mean ± SD with *p < 0.05, ***p < 0.001 as determined by two-tailed t test (unpaired). gMFI, geometric mean fluorescence intensity; ns, not significant.

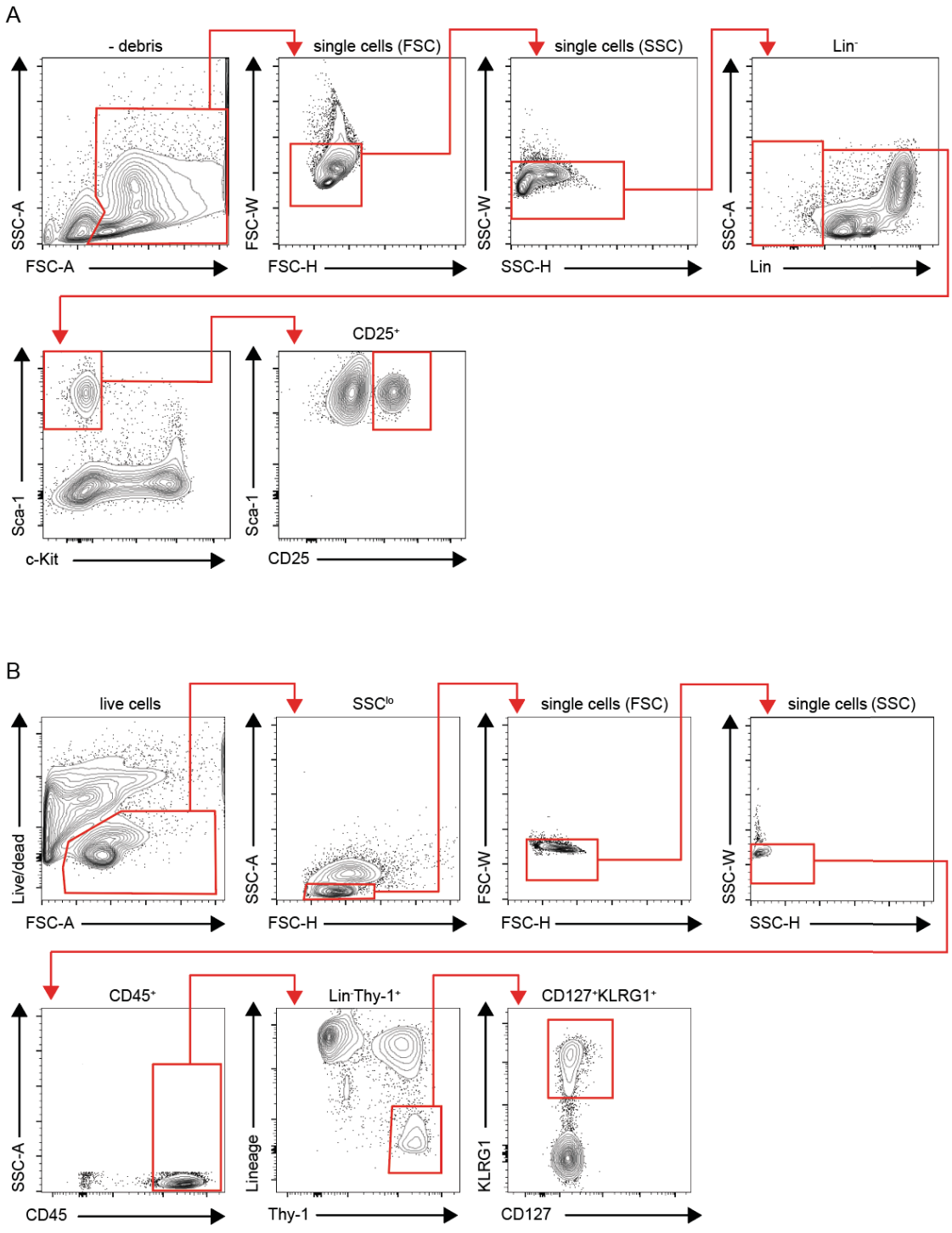
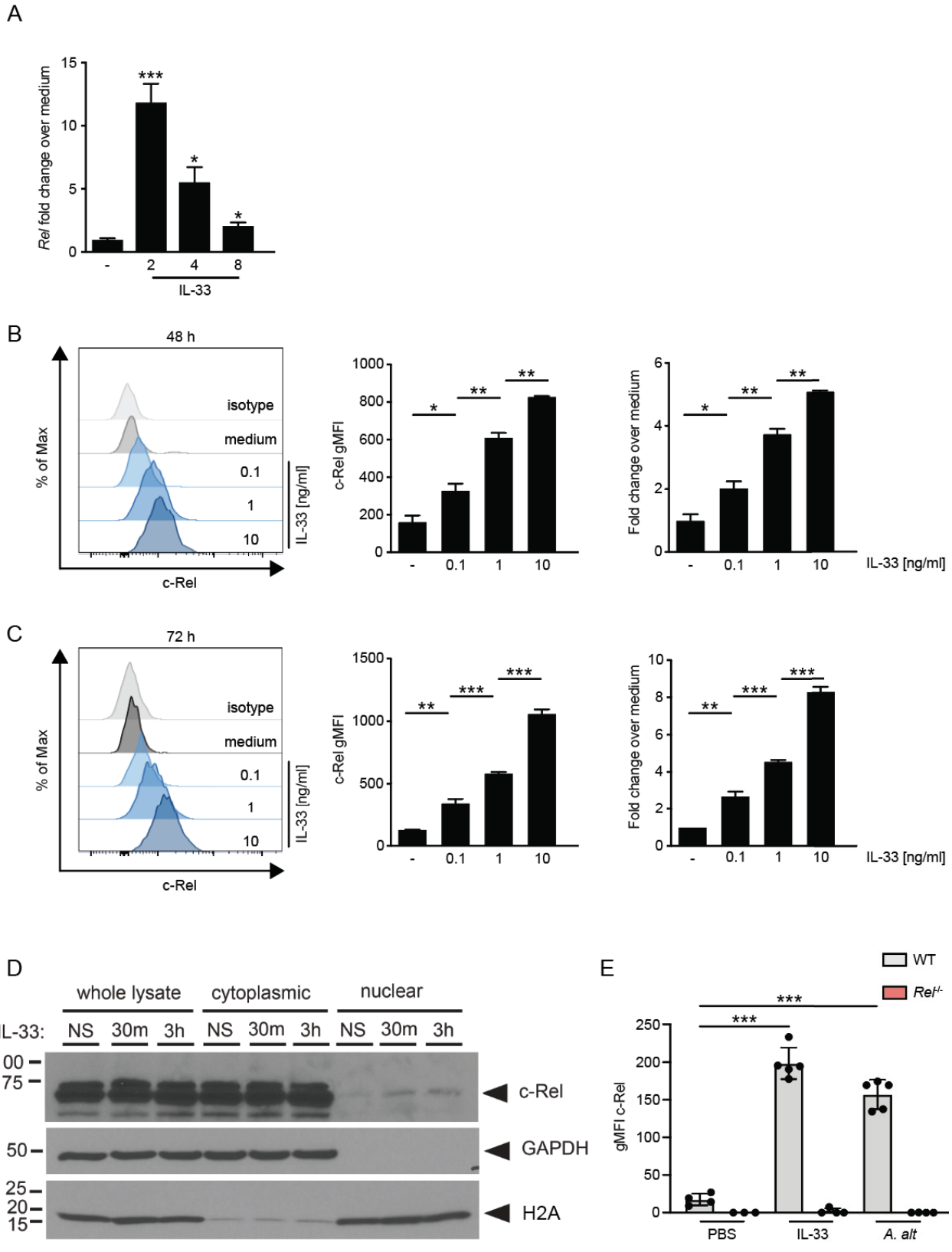


Figure 20. (Suppl. Fig. 4). Gating strategies for the isolation of murine bone marrow-derived ILC2 progenitors and small intestinal ILC2s.

Flow cytometry gating strategies to isolate **(A)** bone marrow-derived ILC2 progenitors (single $Lin^-Sca\text{-}1^+c\text{-}Kit^-CD25^+$) and **(B)** murine small intestinal ILC2s (single live $CD45^+Lin^-KLRG1^+CD127^+$).


A
Rel fold change over medium
15
10
5
0
***
*
*
-
2
4
8
IL-33
B
48 h
% of Max
isotype
medium
0.1
1
10
IL-33 [ng/ml]
c-Rel
c-Rel gMFI
1000
800
600
400
200
0
*
**
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-
0.1
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10
Fold change over medium
6
4
2
0
*
**
**
IL-33 [ng/ml]
C
72 h
% of Max
isotype
medium
0.1
1
10
IL-33 [ng/ml]
c-Rel
c-Rel gMFI
1500
1000
500
0
**
***
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-
0.1
1
10
Fold change over medium
10
8
6
4
2
0
**
***
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IL-33 [ng/ml]
D
whole lysate
cytoplasmic
nuclear
IL-33:
NS
30m
3h
NS
30m
3h
NS
30m
3h
100
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15
c-Rel
GAPDH
H2A
E
WT
Rel-/-
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250
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0
PBS
IL-33
A. alt

Figure 21 (Suppl. Fig. 5). IL-33-driven *ex vivo* activation of pulmonary ILC2s induces c-Rel expression and translocation to the nucleus.

(**A-D**) Murine lung or bone marrow-derived ILC2s were isolated by flow cytometry and left unstimulated (-) or cultured in the presence of recombinant murine IL-33 (10 ng/ml) for the indicated time points. **(A)** Kinetics of c-Rel (*Rel*) transcript expression in *ex vivo* expanded bone marrow-derived ILC2s stimulated for 2, 4 or 8 hours with IL-33 (10 ng/ml). Transcript levels were assessed by qRT-PCR and are depicted as fold change over unstimulated ILC2s (-). Asterisks indicate significance over untreated control. (**B, C**) Flow cytometric analysis of dose-dependent intracellular expression of c-Rel by isolated lung ILC2s after (**B**) 48 hours or (**C**) 72 hours of *ex vivo* stimulation with medium (-) or IL-33 (0.1, 1, 10 ng). c-Rel staining is shown as histogram plots (left panel) in comparison to an isotype control antibody (grey histogram), gMFI (middle panel) or fold change over unstimulated ILC2s. **(D)** *Ex vivo* expanded bone-marrow derived ILC2s were left unstimulated (NS) or cultured with IL-33 (100 ng/ml) for 30 min or 3 hours and whole-cell, cytoplasmic and nuclear lysates were probed for c-Rel by Western blot. Expression of GAPDH and H2A served as fractionation and loading controls. **(E)** WT (grey) and $Rel^{-/-}$ (red) mice were intranasally challenged for three consecutive days with either PBS, IL-33 (500 ng/mouse) or *Alternaria alternata* extract (50 μg/mouse) and lungs were analyzed 24 hours after the last administration. c-Rel expression levels in lung ILC2s following intranasal challenge was assessed by flow cytometry and are shown as gMFI fold change over the PBS-treated control group. Data are representative of at least two independent experiments with two biological replicates (A–D) or n = 3 – 5 mice per treatment group (E). Data are shown as mean ± SD with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test or by two-tailed t test (unpaired). gMFI, geometric mean fluorescence intensity; nd, not detectable; *A. alt*, *Alternaria alternata*.

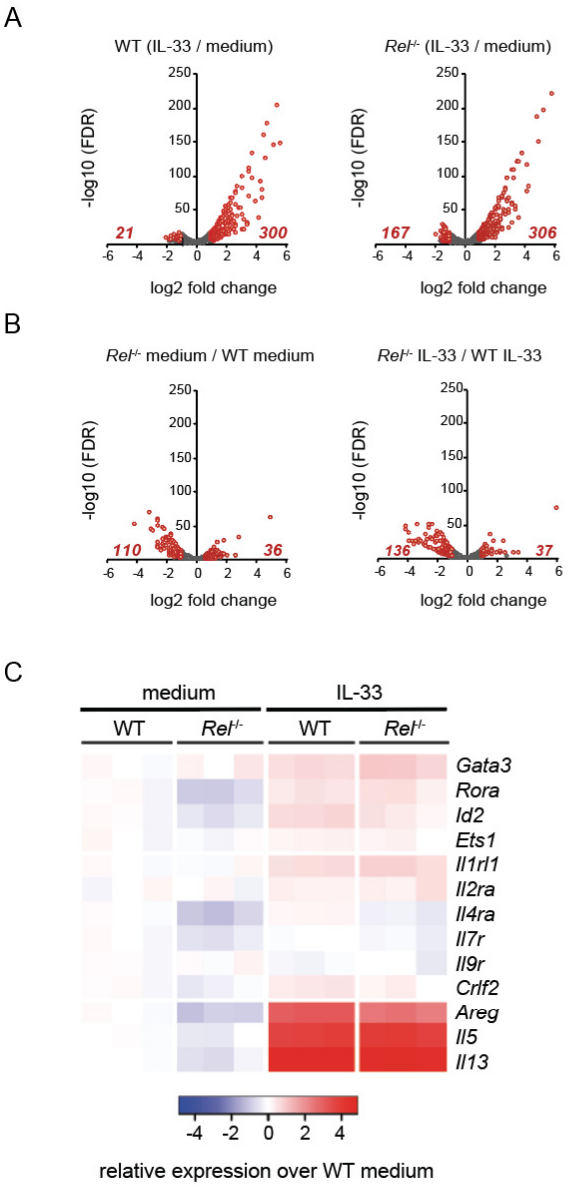

Figure 22 (Suppl. Fig. 6). Expression of key ILC2 transcription factors, surface receptors and signature cytokines are comparable among WT and c-Rel-deficient ILC2s.

RNA-seq analysis of murine bone-marrow-derived WT and *Rel*$^{-/-}$ ILC2s isolated by flow cytometry and expanded *ex vivo* for 5 days. Cells were rested after expansion and left unstimulated (medium) or were cultured in the presence of IL-33 for 4 hours. **(A-B)** Volcano plots showing pairwise comparison of differential gene expression among **(A)** IL-33-treated vs unstimulated WT (left) or *Rel*$^{-/-}$ ILC2s (right) as well as (**B**) unstimulated *Rel*$^{-/-}$ and WT ILC2s (left) or IL-33-treated *Rel*$^{-/-}$ and WT ILC2s (right); fold change ≤ 0.5 or ≥ 2; $p \leq 0.001$. **(C)** Heatmap depicting gene expression fold changes (Log2 cpm; relative to unstimulated WT ILC2s) of selected key ILC2 transcription factors, surface receptors and cytokines under indicated conditions. Data are representative of one experiment with three technical replicates.

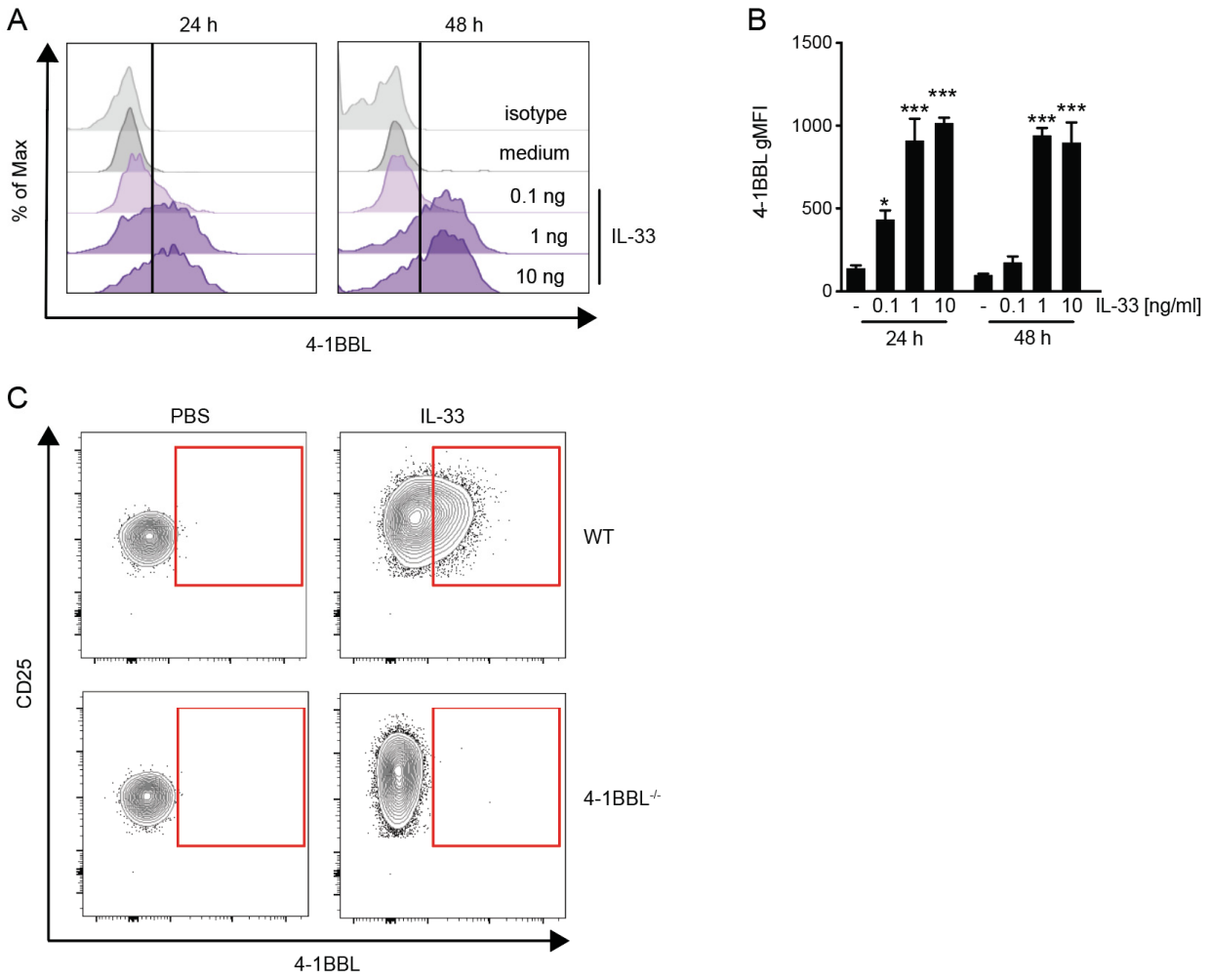

Figure 23 (Suppl. Fig. 7). c-Rel regulates 4-1BBL expression on ILC2s *ex vivo* and during allergic airway inflammation.

(**A, B**) Murine lung ILC2s were isolated by flow cytometry and left unstimulated (-) or cultured in the presence of recombinant murine IL-33 for the indicated time points. Flow cytometric analysis of dose-dependent 4-1BBL surface expression by isolated lung ILC2s after 24 hours or 48 hours of *ex vivo* stimulation with medium (-) or IL-33 (0.1, 1, 10 ng). 4-1BBL expression is shown as (**A**) histogram plots in comparison to an isotype control antibody (grey histogram) and (**B**) gMFI. Asterisks indicate significance over untreated control (-). (**C**) WT and 4-1BBL-deficient mice (4-1BBL$^{-/-}$) mice were intranasally treated with PBS or IL-33 (500 ng/mouse on day 0, 1 and 2) and lungs were analyzed 24 hours after the last administration (day 3). 4-1BBL expression levels by lung ILC2s were subsequently assessed by flow cytometry and contour plots of respective stainings are shown. Data are representative of at least two independent experiments with two biological replicates (A–C) or n = 3 – 5 mice per treatment group (D). Data are shown as mean ± SD with *p < 0.05, ***p < 0.001 as determined by two-tailed t test (unpaired). gMFI, geometric mean fluorescence intensity.

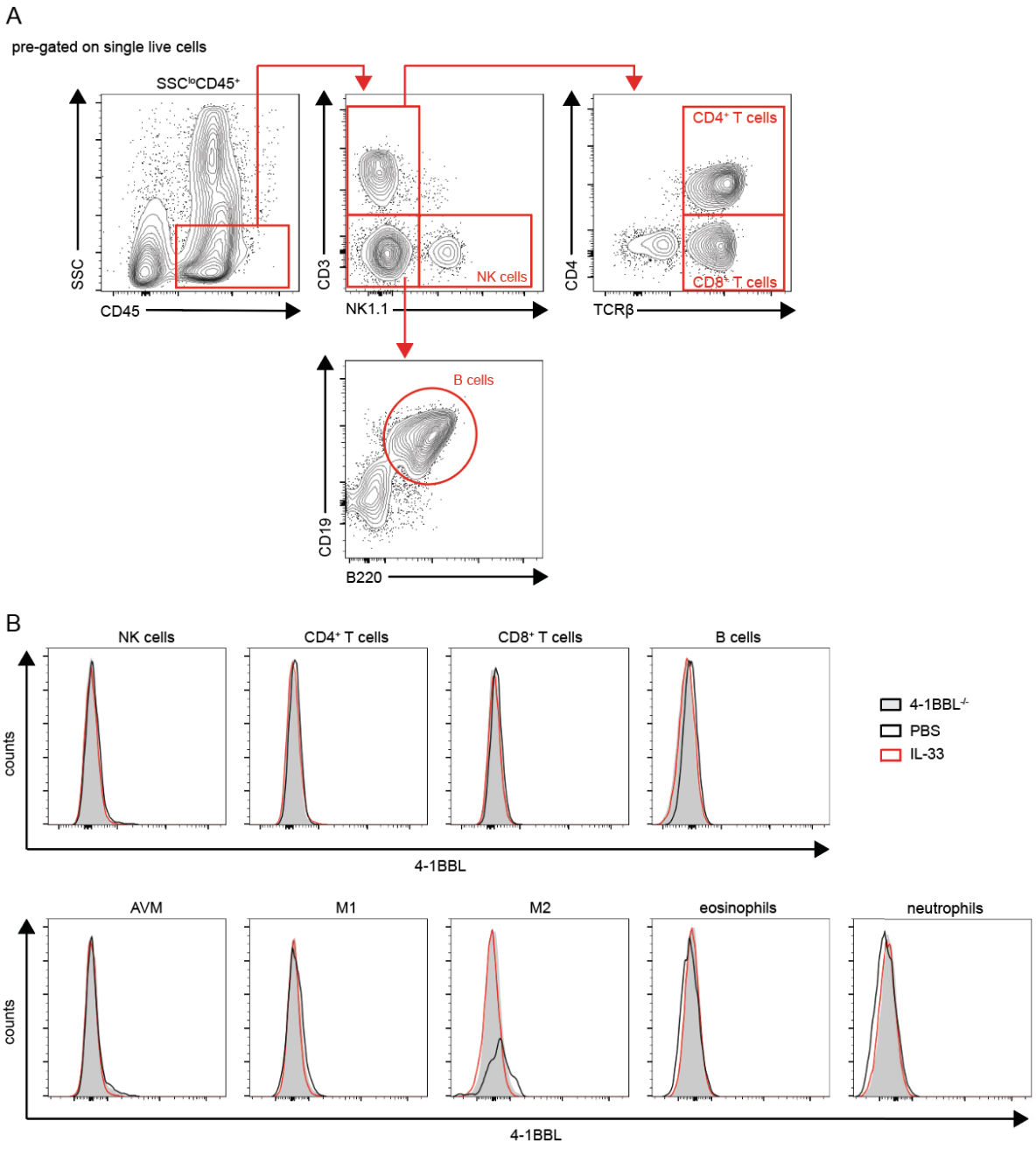

Figure 24 (Suppl. Fig. 8). Expression of 4-1BBL on immune cell populations.

(**A**) Multicolor flow cytometry gating strategies to identify murine lung NK cells (single, live $SSC^{lo}CD3^{-}NK1.1^{+}$), B cells (single, live $SSC^{lo}CD3^{-}NK1.1^{-}CD19^{+}B220^{+}$) $CD4^{+}$ T cells (single, live $CD45^{+}SSC^{lo}CD4^{+}TCR\beta^{+}$) and $CD8^{+}$ T cells (single, live $CD45^{+}SSC^{lo}CD4^{-}TCR\beta^{+}$). **(B)** Representative histogram plots of 4-1BBL expression on indicated lung lymphoid (top panel) and myeloid (bottom panel) immune cell subsets after intranasal challenge with IL-33 (red histogram; 500 ng/mouse) or PBS (black histogram) for 3 days (day, 0, 1 and 2). Lungs were analyzed 24 hours after the last challenge. Data are representative of three independent experiments. Staining is shown in comparison to respective populations in 4-1BBL-deficient animals (filled histogram). AVM, alveolar macrophages.

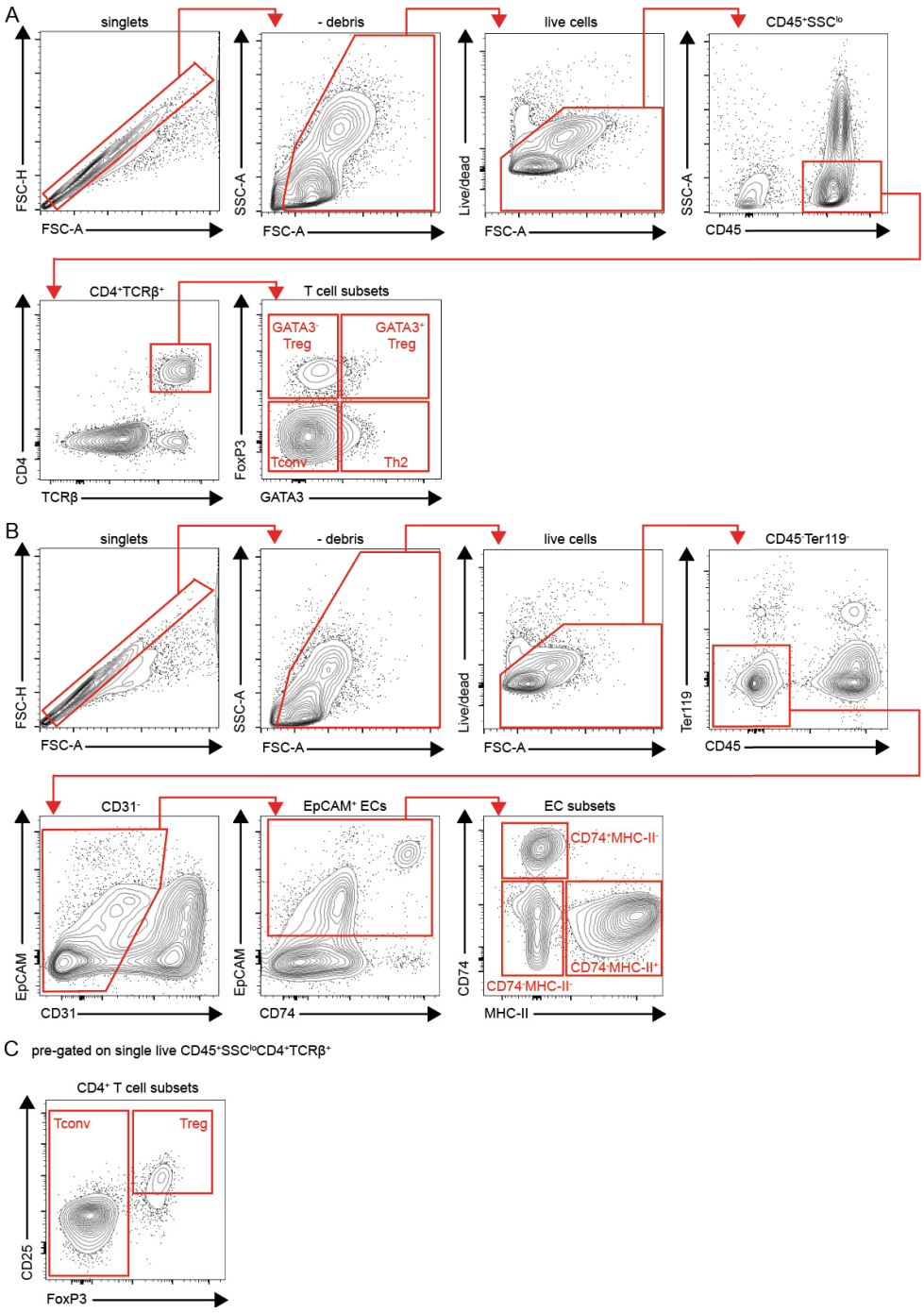

Figure 25 (Suppl. Fig. 9). Gating strategies for the identification of lung T cell and epithelial cell subsets.

Multicolor flow cytometry gating strategies to identify murine lung **(A)** $CD4^+TCR\beta^+$ T cell subsets (gated as single, live cells that are $CD45^+SSC^{lo}CD4^+TCR\beta^+$ and further defined as $GATA3^-FoxP3^-$ Tconv, $GATA3^+FoxP3^-$ Th2, $GATA3^-FoxP3^+$ Treg and $GATA3^+FoxP3^+$ Treg) and **(B)** epithelial cell subsets (gated as single, live cells that are $CD45^-Ter119^-CD31^-$ and further defined as $CD74^+MHC\text{-}II^-$, $CD74^-MHC\text{-}II^-$ or $CD74^-MHC\text{-}II^+$). **(C)** Gating strategy for $CD4^+TCR\beta^+$ T cell subsets (gated as single, live cells that are $CD45^+SSC^{lo}CD4^+$ $TCR\beta^+$ and further defined as $FoxP3^-$ Tconv.and $CD25^+FoxP3^+$ Treg).

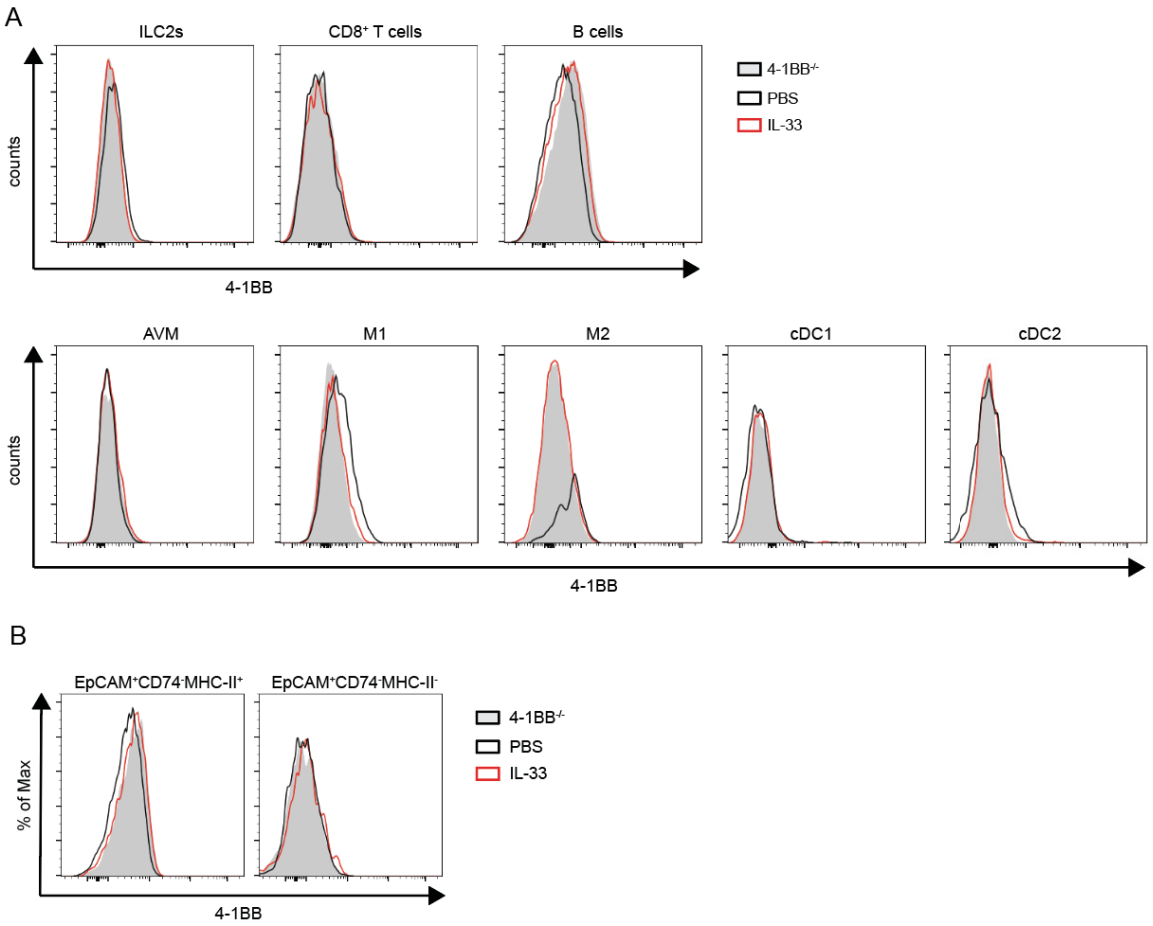

Figure 26 (Suppl. Fig. 10). Expression of 4-1BB on immune and epithelial cell populations.

Representative histogram plots of surface 4-1BB expression by indicated (**A**) lymphoid (top panel) and myeloid (bottom panel) immune cell population as well as (**B**) CD74$^-$MHC-II$^+$ and CD74$^-$MHC-II$^-$ airway epithelial cells after intranasal challenge of WT mice with PBS (black histogram) or IL-33 (red histogram; 500 ng, day 0, 1 and 2, analysis on day 3). Staining is shown in comparison to respective populations in 4-1BB-deficient animals (filled histogram). Data are representative of three independent experiments. AVM, alveolar macrophages, cDC1, type 1 conventional dendritic cells; cDC2, type 2 conventional dendritic cells.

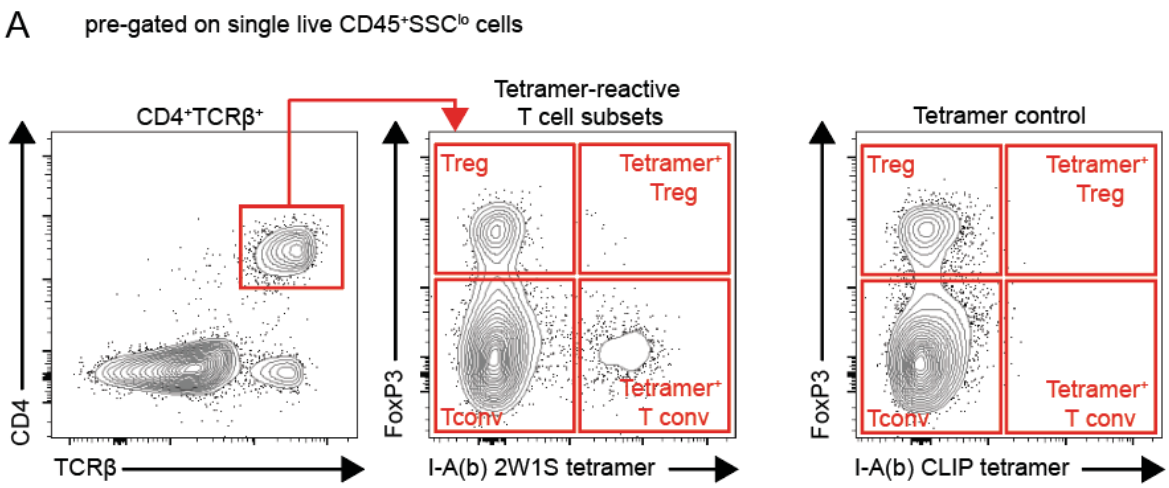

Figure 27 (Suppl. Fig. 11). Gating strategy for the identification of Tetramer-specific lung T cell subsets.

(**A**) Multicolor flow cytometry gating strategy to identify murine lung $CD4^{+}TCR\beta^{+}$ T cell subsets reactive with $I\text{-}A^{b}$ 2W1S:Tetramer. Cells were gated as single, live cells that are $CD45^{+}SSC^{lo}CD4^{+}TCR\beta^{+}$ and further stratified by reactivity with 2W1S tetramer into $Tetramer^{-}FoxP3^{-}$ Tconv, $Tetramer^{+}FoxP3^{-}$ Tconv, $Tetramer^{-}FoxP3^{+}$ Treg as well as $Tetramer^{+}FoxP3^{+}$ Treg cells.

Table 1. Genes upregulated in Rel$^{-/-}$ ILC2s.

medium		IL-33	
Gene	**Fold change**	**Gene**	**Fold change**
Chi3l3	29.04	*Chi3l3*	37.45
Klra7	6.48	*Ccl6*	11.27
Mmp12	5.25	*Mmp12*	8.33
Mmp13	4.09	*Klra7*	5.05
Sidt1	3.36	*Cd36*	4.90
Lck	3.26	*Lck*	3.59
Mrc1	3.05	*Gpnmb*	3.27
Nkg7	2.96	*Fa2h*	3.17
Perp	2.81	*Fn1*	3.16
Rab11fip4	2.64	*Mrc1*	3.15
Zfp503	2.55	*Klrg1*	3.02
Stab2	2.53	*Sidt1*	2.98
Abcb9	2.52	*Nkg7*	2.90
Lhfpl1	2.51	*Mmp13*	2.87
Klrg1	2.49	*Rab11fip4*	2.87
Gpnmb	2.47	*Ccr8*	2.61
Shmt1	2.42	*Peg10*	2.57
Cd27	2.31	*Reln*	2.52
St3gal6	2.27	*Tle4*	2.45
Gzmc	2.26	*Ikzf2*	2.37
Peg10	2.25	*Stab2*	2.34
Arg1	2.19	*Shmt1*	2.31
Cd3g	2.15	*Arg1*	2.24
Mthfd1	2.08	*Mreg*	2.19
Ly9	2.00	*Perp*	2.16
		Zfp503	2.12
		Cald1	2.11
		Abcb9	2.08
		Hc	2.07
		Cd101	2.05
		Pcsk1	2.00

Table 2. Genes downregulated in Rel$^{-/-}$ ILC2s.

medium		IL-33	
Gene	**Fold change**	**Gene**	**Fold change**
Gzmg	0.06	*Mcpt2*	0.06
Gzmn	0.08	*Gzmg*	0.06
Igfbp4	0.09	*Apol7c*	0.07
Apol7c	0.11	*Mcpt1*	0.07
Mcpt2	0.11	*Ccl22*	0.08
Tmem176b	0.12	*Il22*	0.08
Tmem176a	0.13	*Iltifb*	0.08
Mcpt1	0.14	*Igfbp4*	0.10
Angptl2	0.14	*Il17rb*	0.11
Serpine2	0.14	*Gzmf*	0.12
Gzmd	0.16	*Ido1*	0.12
Gpr114	0.16	*Klra5*	0.12
Gzmf	0.17	*Gzmn*	0.14
Tnfsf11	0.17	*Gpr114*	0.15
Upp1	0.17	*Scin*	0.15
Il22	0.18	*Aldh1a3*	0.16
Olah	0.20	*Olah*	0.16
Crispld2	0.20	*Tmem176b*	0.16
Tmem45a	0.21	*Gzmd*	0.17
Gzme	0.21	*Crispld2*	0.18
Ido1	0.22	*Dsc2*	0.18
Il17rb	0.22	*Tmem45a*	0.18
Fam5c	0.22	*5430421N21Rik*	0.19
Csf2	0.22	*Tmem176a*	0.19
Cxxc5	0.22	*Tnfsf11*	0.19
Gja1	0.24	*B3galt5*	0.20
Ccdc80	0.24	*Mcam*	0.22
Penk	0.25	*Abcb1a*	0.22
Serpinb1a	0.25	*Gja1*	0.22
Abcb1a	0.25	*Dhrs3*	0.23
Xdh	0.25	*Cyp1b1*	0.23
Irf4	0.26	*Hapln4*	0.24
Ctla4	0.27	*Klrd1*	0.24

Ccl22	0.27	*Gzme*	0.25
Dsc2	0.29	*Fam5c*	0.25
Ifitm1	0.29	*Angptl2*	0.25
Klra5	0.29	*Csf2*	0.25
Acpp	0.29	*Ccl1*	0.25
Gpr34	0.30	*Car2*	0.26
Scin	0.30	*Grm6*	0.26
Akr1c18	0.30	*Serpinb6b*	0.26
Palld	0.30	*Acpp*	0.26
Nt5e	0.30	*Serpine2*	0.27
Dhrs3	0.30	*Csn2*	0.28
Mcam	0.30	*Xdh*	0.29
Scn1b	0.31	*Nt5e*	0.30
Car2	0.31	*Slpi*	0.30
4632428N05Rik	0.32	*Serpinb1a*	0.32
Csn2	0.32	*Akr1c18*	0.32
Klrd1	0.32	*Plat*	0.32
9930013L23Rik	0.32	*Fam49a*	0.32
Cd74	0.33	*Abi3*	0.32
Pecam1	0.33	*Cables1*	0.32
Gadd45a	0.34	*Ifitm1*	0.32
Apol7b	0.35	*9930013L23Rik*	0.33
Edil3	0.35	*Bmf*	0.33
B3galt5	0.36	*Apol7b*	0.33
Il1r2	0.36	*Upp1*	0.34
Cd96	0.37	*Plscr4*	0.34
Cables1	0.38	*Rundc3b*	0.34
Cyp1b1	0.38	*Havcr2*	0.34
Serpinb6b	0.38	*Amica1*	0.35
Spp1	0.38	*Arhgef40*	0.36
5430421N21Rik	0.38	*Gcnt2*	0.36
Gpr128	0.39	*Abhd15*	0.37
AA467197	0.40	*Ctla4*	0.37
Rgs18	0.40	*Ier5l*	0.37
Abi3	0.40	*Eps8*	0.37
Rnls	0.41	*Ttyh2*	0.37

Gstt1	0.41	*Scn7a*	0.37
Hic1	0.42	*Tmem71*	0.38
Pla2g7	0.42	*Ccdc80*	0.38
Cdkn2b	0.42	*D10Bwg1379e*	0.38
Cxcr4	0.42	*Hs6st2*	0.38
Ece1	0.42	*Lama5*	0.39
Trf	0.43	*Tnfrsf23*	0.39
Tnfrsf23	0.44	*Pla2g7*	0.39
Mcpt8	0.44	*Dnajc6*	0.39
AW112010	0.44	*Cdkn2b*	0.39
Mt2	0.44	*Il6*	0.39
Scpep1	0.45	*Cilp2*	0.39
Scn7a	0.45	*Cd55*	0.39
Gcnt2	0.45	*Tnfsf9*	0.40
Bmf	0.46	*Trf*	0.40
Ltb	0.46	*Gpr34*	0.40
St6galnac6	0.46	*Cd96*	0.40
Gngt2	0.46	*Ccr7*	0.40
Ier5l	0.46	*Emp1*	0.40
Tubb2a	0.47	*Cntn1*	0.40
Prelid2	0.47	*Appl2*	0.41
Dtx4	0.47	*Gstt1*	0.41
Dapk2	0.48	*Cd160*	0.42
Igf2r	0.48	*Hic1*	0.42
Irak3	0.48	*Add2*	0.42
Plscr4	0.48	*Nov*	0.42
Slc20a1	0.48	*Frmd6*	0.42
Cdip1	0.48	*Dtx4*	0.42
Sestd1	0.48	*Acsf2*	0.43
Chst12	0.48	*Mcpt8*	0.43
Emp1	0.49	*Rasa4*	0.43
Procr	0.49	*Il1r2*	0.43
Cers6	0.49	*Ndrg1*	0.43
Pde7a	0.49	*Penk*	0.44
Dkk3	0.49	*Palld*	0.44
Mt1	0.49	*Slco2b1*	0.44

Socs3	0.49	*Rnls*	0.44
Slc24a3	0.49	*Ncald*	0.44
Sdc1	0.50	*Cxxc5*	0.44
Akr1c12	0.50	*Ramp3*	0.45
Rel	0.50	*Adora2a*	0.45
Anxa1	0.50	*Gadd45a*	0.45
		Slc39a8	0.45
		Rgs18	0.45
		Akr1c12	0.46
		Plekhf1	0.46
		Anxa1	0.46
		Sestd1	0.46
		Rnf157	0.46
		Ppp3cc	0.46
		Prss35	0.46
		Dapk2	0.46
		Tubb2a	0.47
		Mt2	0.47
		Scpep1	0.47
		Slc15a3	0.47
		Prkd3	0.47
		Mt1	0.47
		Tiam2	0.47
		Nlrp3	0.48
		Mical1	0.48
		Cd93	0.48
		B4galnt4	0.48
		Ece1	0.48
		Sema3e	0.48
		Edil3	0.48
		Bcl2a1a	0.48
		Ntng2	0.48
		Gadd45g	0.48
		Optn	0.48
		Tubb2b	0.49
		AA467197	0.49

Igf2r	0.49
Bcl2a1d	0.49
Spp1	0.49
Gpr183	0.49
Evi2a	0.49
Fcer1g	0.49
Sgsm2	0.49
Tnfrsf26	0.50
Cd28	0.50
Bcl2	0.50
Ank	0.50

Table 3. Antibodies and fluorescent reagents.

TARGET	CLONE	SOURCE
Flow cytometry antibodies		
Arg-1	A1exF5	eBioscience
CD3ε	145-2C11	eBioscience
CD4	GK1.5	BioLegend
CD5	53-7.3	eBioscience
CD8α	53-6.7	BioLegend
CD11b	M1/70	eBioscience
CD11c	N418	eBioscience
CD19	eBio1D3	eBioscience
CD25	PC61.5	eBioscience
CD26	H194-112	eBioscience
CD45	30-F11	BioLegend
CD45.2	104	eBioscience
CD45R (B220)	RA3-6B2	eBioscience
CD64	X54-5/7.1	BD Biosciences
CD90.2 (Thy-1.2)	53-2.1	BioLegend
CD117 (c-Kit)	2B8	eBioscience
CD127	A7R34	BioLegend
CD135 (Flt3)	A2F10	BioLegend

CD137 (4-1BB)	17B5	BioLegend
CD137L (4-1BBL)	19H3	custom made by Bio X Cell
CD172a (SIRPa)	P84	eBioscience
CD278 (ICOS)	7E.17G9	BD Biosciences
c-Rel	1RELAH5	eBioscience
F4/80	BM8	eBioscience
FcεR1α	MAR-1	eBioscience
FOXP3	FJK-16s	eBioscience
GATA3	TWAJ	eBioscience
IL-5	TRFK5	BioLegend
IL-13	eBio13A	eBioscience
Ki-67	SolA15	eBioscience
KLRG1	2F1	eBioscience
Integrin α4β7	DATK32	BioLegend
Ly-6A/E (Sca-1)	E13-161.7	BioLegend
Ly6C	AL-21	BD Biosciences
Ly6G/Ly6C (Gr-1)	RB6-8C5	eBioscience
Ly6G	1A8	BD Biosciences
MHC Class II (I-A/I-E)	M5/114	BD Biosciences
NK1.1	PK136	eBioscience
Siglec-F	E50-2440	BD Biosciences
IL-33R	RMST2-2	eBioscience
TCRβ	H57-597	eBioscience
TCR γ/δ	eBioGL3	eBioscience
TER-119	TER-119	eBioscience
XCR-1	ZET	eBioscience
Flourescent reagents		
I-A(b) mouse 2W1S Tetramer Brilliant Violet 421		NIH Tetramer Core
I-A(b) human CLIP Tetramer Brilliant Violet 421		NIH Tetramer Core
Streptavidin eFluor 660		eBioscience
Streptavidin Brilliant Violet 711		eBioscience
Streptavidin PE-Cyanine7		eBioscience
Western Blot antibodies		

c-Rel	D4Y6M	Cell Signaling Technology
GAPDH	14C10	Cell Signaling Technology
Histone H2A	D6O3A	Cell Signaling Technology
Anti-rabbit IgG, HRP-linked	polyclonal	Cell Signaling Technology
ChIP antibodies		
c-Rel	polyclonal	Thermo Fisher Scientific
Histone H3K27Ac	polyclonal	Abcam
Goat IgG	polyclonal	Thermo Fisher Scientific

Table 4. qRT-PCR primers.

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Reference
Ccl17	GGAAGTTGGTGAGCTGGTATAA	GATGGCCTTCTTCACATGTTTG	Duerr et al., 2016[80]
Ccl22	CTTCTTGCTGTGGCAATTCAG	TCACTAAACGTGATGGCAGAG	Duerr et al., 2016[80]
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	Hernandez *et al*., 2015[109]
Il5	CTCTGTTGACAAGCAATGAGACG	TCTTCAGTATGTCTAGCCCCTG	Mohapatra *et al*., 2016[110]
Il13	GCAGCATGGTATGGAGTGT	TATCCTCTGGGTCCTGTAGATG	this study
Rel	GGATCAACTGGAGAAGGAAGATT	ATGGACCCGCATGAAGAATAG	this study
Tnfsf9	CCGAGAGAATAATGCAGACCAG	CAGTAGCTTGGCGAACACA	this study
Tnfrsf9	CCAAGTACCTTCTCCAGCATAG	GTTGTGGGTAGAGGAGCAAA	this study
ChIP primers			
Tnfsf9	GGAGGAGGAGAGAAAGTTCC	CGTGCTTTATAGGCTACCGA	this study

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Preface to Chapter 3

In chapter 2 we investigated transcriptional regulation of ILC2 activation by the NF-kB transcription factor c-Rel. In chapter 3 we focused on inhibitory mechanisms of ILC2 regulation. Previous work from our lab and others demonstrated that type I interferons restrain ILC2 proliferation, survival and type 2 cytokine production. However, the precise molecular mechanisms initiated in ILC2s upon IFN-I-stimulation remain elusive. We therefore performed RNA-seq analysis on IFN-I-treated ILC2s early upon stimulation with IL-33 to decipher immediate transcriptomic changes and further validated our findings during allergic airway inflammation and influenza virus infection.

Chapter 3: Type I interferon limits ILC2 effector functions via suppression of the CCR8-CCL1/CCL8 axis.

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3.1. Abstract

It is well established that type 1 interferons (IFN-I) restrain ILC2 effector functions and associated type 2 immunopathologies. Long-term exposure to IFN-I inhibits ILC2 proliferation, survival as well as type 2 cytokine production, however, the early effects of IFN-I on ILC2s remain largely elusive. Using RNA-sequencing analysis, we demonstrate that IFN-β treatment inhibits the production of the chemokine CCL1 by ILC2s upon *ex vivo* activation. Moreover,

ex vivo stimulation with IFN-β resulted in downregulation of CCR8, the cognate chemokine receptor for CCL1 which is critical for tissue migration of activated ILC2s during allergic airway inflammation as well as for autocrine CCL1-mediated ILC2 effector functions. We additionally show that IFN-I restrains CCR8 expression on ILC2s as well as transcript levels of CCL8, the chemotactic ligand of CCR8, *in vivo* in a mouse model of influenza A virus infection. Furthermore, IFN-I inhibits CCL1 expression in an IL-33-mediated model of allergic airway inflammation. By targeting the CCR8-CCL1/CCL8 axis IFN-I thus impairs ILC2 effector functions.

3.2. Introduction

Group 2 innate lymphoid cells (ILC2s) reside at barrier surfaces, including the lungs. where they become activated following tissue perturbation[1-3]. ILC2s exert critical roles in anti-helminth immunity and the pathogenesis of allergic diseases by rapidly secreting vast amounts of the type 2 signature cytokines IL-5 and IL-13 upon activation, thereby mediating eosinophil recruitment, goblet cell hyperplasia and mucus production[4,5]. Besides initiating innate type 2 immune responses, it is becoming increasingly appreciated that ILC2s also play important roles in instructing adaptive immunity[6-12]. Unlike type 2 T helper (Th2) cells which exert similar effector functions, ILC2s are activated in an antigen-independent manner by environmental cues such as the alarmins interleukin (IL)-25, IL-33 and/or thymic stromal lymphopoietin (TSLP)[4]. Regulation of ILC2 activation at steady state and upon allergic inflammation have been the subject of extensive research and it was revealed that in addition to alarmins, ILC2s require signals from STAT5 activators such as IL-2, IL-7 and IL-9 and/or costimulatory molecules for efficient activation[13].

A recent study demonstrated that the chemokine CCL1 is produced by murine as well as human ILC2s[14]. CCL1 expression in ILC2s was further augmented upon IL-33-mediated *ex vivo* activation[14] and elevated CCL1 protein levels were observed in murine lungs upon allergic airway inflammation[15]. Moreover, the majority of murine lung ILC2s highly expressed the cognate CCL1 receptor, CCR8, at steady state as well as after intranasal IL-25, IL-33 or *Alternaria alternata* challenge and *Nippostrongylus brasiliensis* infection[14,15]. It was further demonstrated that CCL1 acts as an autocrine ILC2-stimulating factor and potentiates

proliferation, survival and IL-9 production *in vitro* in a CCR8-dependent manner[14]. ILC2-intrinsic CCR8-deficiency resulted in impaired anti-helminth immunity upon *N. brasiliensis* infection, suggesting a critical role of CCR8 in ILC2-mediated type 2 immune responses[14]. CCL8, another CCR8 ligand was also induced during allergic airway inflammation and ILC2s co-localized with CCL8 deposits in the peribronchial region[15]. Strikingly, blocking of the CCR8-CCL1/CCL8 axis with a CCR8 blocking antibody significantly impaired ILC2 migration and accumulation within the peribronchial space following pulmonary IL-33 challenge[15]. Importantly, CCL8 also promoted migration of human peripheral blood ILC2s *in vitro* suggesting that similar migratory mechanisms may be employed[15].

ILC2 are negatively regulated by cytokines such as IL-10[16], IL-27[17,18] and IFN-γ[11,18] as well as androgen receptor signaling[19,20], α7 nicotinic acetylcholine receptor (α7nAChR) ligation[21], stimulation of β2-adrenergic receptor (β2-AR)[22] as well as cAMP-accumulation mediated by calcitonin gene-related peptide (CGRP) signaling[23-25]. In addition, recent work by our laboratory and others revealed that the type I interferons (IFN-I) IFN-α and IFN-β restrain ILC2 effector function, inhibiting proliferation, survival as well as type 2 cytokine production[17,18,26]. However, the underlying mechanisms that regulate these processes as well as immediate effects of IFN-I-stimulation on ILC2s remain elusive.

In the current study we investigated the early mechanisms of IFN-I-mediated ILC2 inhibition. Using RNA-sequencing (RNA-seq) analysis, we found that IFN-β restrains production of the chemokine CCL1, a known autocrine activation and survival factor for ILC2s. We further show that expression of the cognate CCL1 chemokine receptor CCR8 is downregulated upon IFN-I-stimulation *ex vivo* as well as upon sublethal influenza A virus infection (IAV). In addition, expression of CCL8, the chemotactic ligand of CCR8, was suppressed in an IFN-I dependent manner in the lungs of IAV-infected mice. Moreover, IFN-β potently restrained pulmonary CCL1 expression in a model of ILC2-driven allergic airway inflammation. Thus, this study sheds new light on the underlying mechanisms of ILC2 regulation by identifying the CCR8-CCL1/CCL8 axis as a target for IFN-I-driven ILC2 inhibition.

3.3. Results

Type I interferon inhibits CCL1 production from murine ILC2s *ex vivo*.

To investigate the early molecular mechanisms of IFN-I mediated ILC2 suppression, we used RNA-sequencing (RNA-seq) analysis to determine how IFN-I alters gene expression in IL-33 activated ILC2s isolated from murine lungs (**Figure 34**). Pulmonary ILC2s were left either untreated or cultured *ex vivo* in the presence of IL-33 alone or in combination with IFN-β for 4 hours. Previous RNA-seq analyses on ILC2s stimulated with IFNs were performed at later time points[26] and we therefore additionally analyzed differential gene expression at 24 hours to compare early to late transcriptomic changes. Indeed, partial least squares (PLS) projection of the RNA-seq dataset revealed that, while biological replicates closely resembled each other, IFN-β treatment markedly altered the transcriptional response of ILC2s to IL-33 and induced distinct transcriptional states at 4 as well as 24 hours (**Figure 28A**). Differential gene expression analysis of IL-33 treated ILC2s compared to IL-33 + IFN-treated cells at 4 h revealed that 503 genes were significantly modified by IFN-treatment ($p \leq 0.01$). Of these genes, 107 were significantly downregulated (fold change ≤ 0.5, $p \leq 0.01$) and 396 were upregulated (fold change ≥ 2, $p \leq 0.01$). The top 20 down- (**Figure 28B)** and upregulated (**Figure 35**) genes are depicted as clustered heatmaps. As expected, IFN-β potently induced prototypical IFN stimulated genes (ISGs) such as *Oasl2* and *Isg15* (**Figures 28E, 35**) while *Ccl1* encoding for the chemokine CCL1 was the gene with the most significant decrease in expression following IFN-stimulation (**Figures 28D, 36**). Notably, expression of ILC2 signature genes encoding type 2 cytokines (*Areg*, *Csf2*, *Il5*, *Il13*), key surface receptors (*Il1rl1*, *Il2ra*, *Il7r*) and transcription factors (*Gata3*, *Rora*) remained unaffected after 4 hours of IFN-I stimulation compared to IL-33 treated cells (**Figure 28C**). To confirm RNA-seq findings and verify the direct effects of IFN-β, we validated Oasl2, Isg15 and CCL1 (**Figure 28G**) transcript expression by quantitative real-time PCR (qRT-PCR) in lung ILC2s isolated from wild-type (WT) and IFN-I-receptor deficient mice (*Ifnar1*$^{-/-}$) that were left unstimulated or treated with IL-33 or IL-33 + IFN-β for 4 hours. Consistent, with the RNA-seq data, expression of *Oasl2* and *Isg15* was not or only barely detectable in untreated and IL-33 stimulated ILC2s while IFN-treatment resulted in a marked induction in WT mice which was completely abrogated in ILC2s from *Ifnar1*$^{-/-}$ animals (**Figure 28E**). On the other hand, *Ccl1* expression was low in unstimulated cells and increased upon IL-33 stimulation in WT as well as *Ifnar1*$^{-/-}$ mice (**Figure 28G**).

Importantly, addition of IFN-β suppressed IL-33-driven *Ccl1* expression only in WT but not in IFN-I-receptor deficient animals (**Figure 28G**) recapitulating the RNA-seq results (**Figure 28B, D**). To determine if the suppressive effects of IFN-β on *Ccl1* transcription are observed at the protein level as well, we measured secreted CCL1 levels in WT and *Ifnar1*$^{-/-}$ lung ILC2 culture supernatants after 24 hours of stimulation with either medium, IL-33 or IL-33 in combination with IFN-β. In accordance with the effects observed at the RNA level and with recent work[14], CCL1 was not detectable in unstimulated ILC2s and induced with IL-33 in both WT and *Ifnar1*$^{-/-}$ animals, with *Ifnar1*$^{-/-}$ ILC2s exhibiting slightly increased CCL1 production (**Figure 28H**). While cell viability was not affected (**Figure 28F**), CCL1 protein levels were significantly repressed in the presence of IFN-β while no effect was observed with ILC2s isolated from *Ifnar1*$^{-/-}$ animals (**Figure 28H**). Taken together, our data show that IFN-I rapidly induces distinct transcriptional changes in IL-33-activated pulmonary ILC2s and potently suppresses IL-33-induced CCL1 production *ex vivo*.

IFN-I suppresses CCR8 expression by lung ILC2s *ex vivo*.

CCL1 induction upon IL-33 stimulation was observed in a previous report which further states that CCL1 acts as an autocrine ILC2-stimulating factor that that drives ILC2 functions by binding to its cognate receptor CCR8[14]. We therefore investigated whether IFN-I can modulate surface CCR8 expression on WT lung ILC2s in comparison to ILC2s isolated from *Ifnar1*$^{-/-}$ mice to potentially further interfere with the CCL1/CCR8-axis. We first assessed viability of WT versus *Ifnar1*$^{-/-}$ ILC2s left unstimulated or cultured with IFN-β, IL-33 or a combination thereof for 24 hours. No major differences in cell viability were observed after 24 hours except for a slight decrease in WT ILC2s that were treated with IFN-β only (**Figure 29A**). Consistent with previous reports[14,15] we observed that the majority of lung ILC2s highly expressed CCR8 (**Figures 29B-E**) and no difference in percentage of CCR8^{+} ILC2s (**Figure 29D**) as well as CCR8 expression levels (quantified as gMFI; **Figure 29E**) were observed in WT or *Ifnar1*$^{-/-}$ cells that were left untreated or stimulated with IL-33 alone for 24 hours. However, in the presence of IFN-β, CCR8 expression was markedly reduced in unstimulated as well as IL-33-activated WT ILC2s while surface expression levels (**Figure 29E**) and CCR8^{+} ILC2 percentages (**Figure 29D**) remained unchanged in ILC2s that were isolated from *Ifnar1*$^{-/-}$ animals. In summary, we confirmed that pulmonary ILC2s highly express the cognate CCL1 receptor CCR8 on their surface and that *ex vivo* CCR8

expression is downregulated upon IFN-I treatment. These findings indicate that IFN-β may suppress the CCL1/CCR8-axis by negatively regulating both ligand production and receptor expression.

IFN-I negatively regulates type 2 immune responses upon influenza A virus (IAV) infection.

We and others recently demonstrated that ILC2s are activated upon influenza A virus (IAV) infection in an IL-33-dependent manner[17,27,28] and that infection-mediated ILC2 activation as well as associated type 2 immunopathologies are restrained by IFN-I[17]. Mechanistically, IFN-I promoted ILC2 apoptosis and inhibited proliferation as well as cytokine production[17]. To confirm our *ex vivo* findings *in vivo,* we utilized an established model of murine IAV infection where WT and *Ifnar1*$^{-/-}$ mice were infected with a sublethal dose of IAV (strain A/Puerto Rico/8/1934 H1N1) and total numbers of lung immune cell populations were analyzed five days post infection (**Figures 30A, 37**). We observed that total numbers of pulmonary cells (**Figure 30B**) as well CD45^{+} immune cells (**Figure 30C**) were markedly increased upon infection, albeit to a similar extent in WT and *Ifnar1*$^{-/-}$ animals. Ly6C^{+} monocytes (**Figure 30D**) were induced upon infection but their abundance was significantly reduced in the absence of IFN-I signaling. Consistent with our previous findings[17], numbers of pulmonary neutrophils (**Figure 30E**) and eosinophils (**Figure 30F**) remained unchanged in infected WT animals but were induced and significantly higher in IFNAR-deficient mice following infection. In addition, lungs of *Ifnar1*$^{-/-}$ animals harbored less interstitial M1 macrophages (**Figure 30G**) while numbers of alternatively activated M2 macrophages (**Figure 30H**) were significantly elevated. Moreover, while cDC1 numbers (**Figure 30I**) were slightly enhanced in WT mice upon infection, numbers of cDC2s (**Figure 30J**) were induced in mice of both genotypes and were higher in the absence of functional IFN-I signaling. Recent work showed that macrophage-derived CCL8, another CCR8 ligand, is critical for ILC2 migration and positioning during allergic airway inflammation[15]. CCL8 expression was described to be initiated upon Th2 immunization in mice[29] and expression is also induced upon IAV infection[30]. To determine whether CCL8 expression is affected by IFN-I during IAV infection, we assessed whole lung CCL8 transcript levels by qRT-PCR. While we did not observe induction at the chosen time point in WT mice, CCL8 levels were significantly elevated in the lungs of *Ifnar1*$^{-/-}$ mice (**Figure 30K**). Taken together, we recapitulated and expanded on our earlier findings, confirming that the absence of IFN-I signaling during sublethal IAV infection results in elevated type 2 immunity. We

furthermore demonstrated that IFN-I negatively regulates expression of *Ccl8*, a critical mediator of ILC2 functions.

IFN-I suppresses CCR8 expression on ILC2s and T cells following IAV infection.

To determine whether IFN-I modulates CCR8 expression *in vivo* we again infected WT and *Ifnar1*$^{-/-}$ mice with a sublethal dose of IAV and analyzed CCR8 expression on ILC2s as well as CD4^{+}FoxP3^{-} conventional T helper cells (Tconv) and CD4^{+}CD25^{+}FoxP3^{+} regulatory T cells (Treg) (**Figures 31A, 38**). Contrary to previous reports[17,27,28], we did not observe an increase in ILC2 numbers in either WT or IFNAR-deficient mice upon infection (**Figure 31B**). Importantly, CCR8 expression on ILC2s was significantly elevated in mice lacking IFN-I-signaling (**Figure 31C**). In addition, an increase in total numbers of Tconv (**Figure 31D**) but not Treg cells (**Figure 31E**) was observed in infected WT as well as *Ifnar1*$^{-/-}$ animals when compared to mock infected animals. CCR8 is preferentially expressed by Th2[31] as well as Treg[32] cells and in accordance with elevated type 2 immunity in the absence of IFNAR-signaling upon IAV infection[17], numbers of CCR8^{+} Tconv (**Figures 31F, H**) and CCR8^{+} Treg cells (**Figures 31G, I**) were induced upon infection in *Ifnar1*$^{-/-}$ but not WT mice. Consistently, expression levels of CCR8 on Tconv (**Figure 31J**) and Treg cells (**Figure 31K**), quantified by gMFI, were increased upon infection in the absence of IFNAR signaling. Collectively, these findings indicate that IFN-I negatively regulates expression of CCR8 on ILC2s as well as on CD4^{+} conventional T cells and regulatory T cells.

IFN-I inhibits pulmonary CCL1 expression upon IL-33-induced allergic airway inflammation.

Induction of CCL1 expression in murine lungs was demonstrated recently following *Nippostrongylus brasiliensis* infection[14] as well as upon administration of recombinant IL-33[14]. To assess whether IFN-I can suppress CCL1 expression *in vivo* during ILC2-mediated allergic airway inflammation we intranasally administered PBS, IL-33 or IL-33 + IFN-β to WT or *Ifnar1*$^{-/-}$ animals for three consecutive days (**Figure 32A**). Lungs were isolated 24 hours after the last administration and transcript levels of CCL1 and the type 2 signature cytokines IL-5 and IL-13 were analyzed by qRT-PCR. Consistent with previous work, lung *Ccl1* expression was increased by more than 10-fold following IL-33 administration while combined administration with IFN-β significantly reduced IL-33-mediated *Ccl1* induction (**Figure 32B**). Similar effects were observed when assessing gene expression levels of the type 2 signature cytokines IL-5 (**Figure 32C**) and

IL-13 (**Figure 32D**) which is consistent with previous observations[17]. Taken together, these data confirm that IFN-β is a potent negative regulator of not IL-5 and IL-13 but also suppresses CCL1 production in an ILC2-mediated model of allergic airway inflammation.

Pulmonary ILC2s exhibit an amoeboid pattern of movement upon in vitro IL-33 treatment and do not migrate towards CCL1 or CCL22.

Since CCR8 has been described as a critical mediator of ILC2s migration, we aimed to ascertain if lung ILC2s were able to migrate towards CCR8 ligands CCL1 and CCL8 and whether IFN-I may interfere with directed CCR8-dependent ILC2 motility. Besides CCR8, CCR4 expression was reported on murine ILC2s[14,15] and recent work showed migration of ILC2s towards the CCR4 ligand CCL22[14]. Using LifeAct-GFP mice we aimed to examine the migratory behavior of IL-33-activated pulmonary ILC2s in an under-agarose migration assay (**Figure 33A**). Consistent with previous findings[15], activated ILC2s adapted an amoeboid shape with prominent actin polymerization at the leading edge (**Figure 33B**). While ILC2s exhibited amoeboid non-directed movement, no directed migration towards either CCL1 or CCL22 alone was observed (**Figures 33C, D**).

3.4. Discussion

Chemokines guide trafficking and positioning of immune cells during development, homeostasis, as well as inflammation. Several of these chemokines are known to recruit innate immune cells such as eosinophils, neutrophils and monocytes and guide migration of dendritic cells (DCs) from the perturbed tissue to the draining lymph nodes[33]. During allergic inflammation, chemokines such as CCL1 have been implicated in the activation of ILC2s[14] and Treg cells[32] and CCL8 has been shown to guide homing of Th2 cells[34], as well as migration of type 2 DCs (DC2s)[29] and ILC2s[15]. CCL1 and CCL8 are both ligands of the chemokine receptor CCR8. Several studies demonstrated that CCR8 plays a critical role in the development of inflammation in murine models of allergic asthma [35-38] and using CCR8 antagonists for the treatment of allergic asthma has been debated[39,40]. CCL1 and CCL8 have been described to induce chemotaxis of Th2 cells[31] and CCR8-deficient mice displayed impaired lung eosinophilia as well as type 2 cytokine levels in the lung following allergen challenge[41]. The importance of CCR8 in allergic asthma remains controversial, since additional studies have shown that CCR8 may be dispensable in the recruitment of Th2 cells into

lung tissue during allergic airway inflammation[42-44] and conflicting results were obtained regarding the role of CCR8 regarding the migration of activated ILC2s.

Our findings indicate a critical role for IFN-I in restraining CCL1 as well as CCR8 expression by ILC2s and thereby potentially inhibiting the CCL1-CCR8 feed forward mechanism. We moreover demonstrate that IFN-I negatively regulates lung CCL8 expression and may therefore interfere with ILC2 trafficking within the tissue. Furthermore, IFN-I downmodulated expression of CCR8 on $CD4^+$ T cells and may thereby also target adaptive T cell responses.

It was recently recognized that migration of dermal $CD301^+$ DC2s into the parenchymal regions of the lymph node (LN) is mediated through CCR8 and guided by LN macrophage-derived CCL8[29]. Here, activated $CCR8^+$ DC2s facilitated priming and differentiation of $CD4^+$ T cells toward a Th2 cell fate to promote allergic skin inflammation[29]. It would thus be important to determine whether CCR8-expressing lung DCs are detectable upon IAV infection or allergic airway inflammation and whether IFN-I additionally downregulates CCR8 on these DCs and thereby alters adaptive effector responses.

In accordance with previous work[14,15] we demonstrate that pulmonary ILC2s do not migrate towards CCL1 *ex vivo*. We also observed no directed movement towards the CCR4 ligand CCL22, which has been described recently to promote ILC2 chemotaxis[14]. However, ILC2s used in this study[14] were activated with IL-25 *in vivo*, subsequently isolated from spleens and lymph nodes and expanded *ex vivo* before experimental use. In the present study we used freshly sorted naive lung ILC2s and it remains to be determined whether *ex vivo* manipulation may impact expression of chemokine surface receptors and if tissue-specific expression patterns could explain these controversial observations. Future studies will involve investigating whether IFN-I modulates CCL8-induced lung ILC2 migration *ex vivo* based on its ability to downmodulate CCR8 expression. To further determine the influence of IFN-I on *in vivo* migration and positioning of ILC2s following allergic airway inflammation, intravital lung microscopy will be utilized.

Importantly, findings with murine ILC2s were directly translatable to human cells in previous studies. Human ILC2s were shown to express surface CCR8 and produced CCL1 upon activation which promoted their expansion[14]. Moreover, CCL8 has been described to induce human ILC2 chemotaxis[15]. Therefore, it will need to be addressed whether IFN-I inhibits CCL1 production and CCR8 expression in human ILC2s. The chemokine CCL18 has been described as an additional CCR8 ligand in humans and has been shown to promote migration of polarized

human Th2 cells[45]. CCL18 is constitutively expressed in lung tissue and elevated levels of CCL18 were found in sputum, bronchoalveolar lavage and sera of allergic asthmatics compared to healthy control group[46,47]. CCL18 is primarily expressed by antigen-presenting cells and expression is inducible by IL-4 and IL-13 in polarized M2 macrophages[48]. Importantly, more recent work showed that IL-33 as well can directly elicit CCL18 production in macrophages[49]. Hence, it would be of interest to determine whether CCL18 exhibits similar functions to CCL1 and/or CCL8 regarding ILC2 maintenance and migration during ILC2-driven allergic responses in the human airways.

Our study highlights that modulating the IFN-I signaling pathway may represent a promising therapeutic avenue in the treatment of allergic diseases by not only restraining the CCL1-CCR8 feed forward mechanism in ILC2s but also by potentially inhibiting migration of ILC2s as well as Th2 cells.

3.5. Materials and Methods

Mice

C57BL/6J wild-type mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. *Ifnar1*$^{-/-}$[50] and LifeAct-GFP[51] animals were described previously and kindly provided by Dr. Jennifer Gommerman (University of Toronto) and Dr. Janis Burkhardt (University of Pennsylvania), respectively. All animals were maintained on a C57BL/6J background and were bred and housed under specific pathogen-free conditions with *ad libitum* access to food and water. Unless otherwise stated, experiments were conducted using adult female age-matched mice (8 – 16 weeks) in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University.

Allergic airway inflammation model

Mice were anaesthetized by isoflurane inhalation, followed by intranasal administration of either 40 µl carrier-free recombinant murine IL-33 (500 ng/mouse in PBS; R&D Systems) in the presence of absence of murine IFN-β (5,000 U/mouse; PBL Assay Science). Mice were challenged for three consecutive days and lungs were analyzed 24 hours after the last treatment.

Influenza A virus infection

Mice were anaesthetized by intraperitoneal injection of ketamine/xylazine, followed by intranasal administration of a sublethal dose of 10 PFU IAV H1N1 (strain A/Puerto Rico/8/34) per 20 g body weight. Lungs were analyzed on day five post infection.

Preparation of single cell suspensions from murine lung tissue

To obtain single cell suspensions, mouse lungs were processed as described previously with slight modifications[17]. Briefly, lungs were perfused with cold PBS, isolated and finely minced. Lung pieces were subsequently digested in RPMI-1640 supplemented with 5% FBS, 0.2 mg/ml Liberase™ TM (Roche) and 0.1 mg/ml DNase I (Roche) for 50 min. Digested lungs were homogenized using a syringe and passed through a 70 µm cell strainer to obtain single cell suspensions. Cells were washed with PBS and red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma).

Flow cytometry

Single cells were incubated for 15 min on ice with 2.4G2 hybridoma supernatant to block Fc receptors and stained with respective fluorochrome-labeled antibodies for 30 min on ice. Mouse lineage cocktail was composed of antibodies against B220, CD3ε, CD5, CD11b, CD11c, CD19, FcεRIα, NK1.1, Ly-6C/G, TCRαβ, TCRγδ and TER-119. Dead cells were excluded by staining with Fixable Viability Dye™ eFluor™ 780 (eBioscience) following the manufacturer's instructions. Intracellular transcription factor and cytokine staining was performed using the FoxP3 / Transcription factor staining kit (eBioscience) according to the manufacturer's protocol. Stained cell suspensions were acquired on a BD LSRFortessa™ Cell Analyzer (BD Biosciences) and data were analyzed using FlowJo™ software (version 10, BD Biosciences). All antibodies used for flow cytometry analyses are listed in **Table 3**.

Isolation and culture of murine lung ILC2

Lung single cell suspensions were obtained as described above, blocked with 2.4G2 hybridoma supernatant followed by staining with fluorochrome-labeled antibodies and Fixable Viability Dye™ (eBioscience). ILC2s were isolated with a BD FACSAria™ Fusion cell sorter based on surface marker expression as viable lineage negative $CD45^+CD90^+CD25^+ST2^+$ cells. All

antibodies used are listed in detail in **Table 3**. Isolated ILC2 were incubated for 18 – 24 h in complete medium (RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin and 55 µM 2-mercaptoethanol) containing murine IL-7 (10 ng/ml), washed and rested for 4 h in complete medium without cytokines before use in experiments. Cells were stimulated as indicated with IL-7, IL-33 (both at 10 ng/ml, R&D Systems), IFN-β (500 U/ml; PBL Assay Science) or combinations thereof.

Protein quantification

CCL1, IL-5, IL-9 and IL-13 in tissue culture supernatants and lung grinds were determined using the respective mouse or human DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. Absorbance was measured using an Enspire™ 2300 Multilabel Reader (PerkinElmer).

RNA extraction and quantitative real time-PCR (qRT-PCR)

Total RNA from cultured cells was extracted using the Quick-RNA™ MicroPrep kit (ZymoResearch) according to the manufacturer's instructions. For extraction of tissue RNA, tissue was mechanically disrupted in TRIzol™ Reagent (Life Technologies) using a MagNA Lyser (Roche). RNA was extracted as per the manufacturer's instructions and cleaned up with the Quick-RNA™ MicroPrep kit (ZymoResearch). cDNA was synthesized with Oligo(dT)$_{12-18}$ Primer and SuperScript™ III Reverse Transcriptase (both Life Technologies) and qRT-PCR was carried out using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative gene expression was calculated using the comparative ΔCt method after normalization to expression levels of the housekeeping gene *Hprt*. All primers used in this study were designed with PrimerQuest Tool (Integrated DNA Technologies) and purchased from Integrated DNA Technologies (IDT, Coralville, IA). Primer sequences are provided in **Table 4**.

RNA sequencing (RNA-seq)

Murine lung ILC2s were isolated as described above, incubated for 18 h in complete medium containing murine IL-7 (10 ng/ml), washed and rested for 4 h in complete medium without cytokines. Cells were left untreated or stimulated for 4 and 24 h with rm-IL-33 (10 ng/ml) in the

presence or absence of IFN-β (500 U/ml) and total RNA was extracted using the MagMAX™ mirVana™ Total RNA Isolation Kit (Applied Biosystems) as per the manufacturer's protocol. RNA-seq was performed as previously described [52]. Briefly, RNA integrity was assessed using a Bioanalyzer RNA Pico kit (Agilent). Then, total RNA was depleted of rRNA and libraries were prepared using the KAPA Stranded RNA-Seq kit (Roche). Libraries were sequenced on an Illumina HiSeq 2500 sequencer in paired-end 50 bp configuration. The quality of sequence reads was assessed with the FastQC tool (Babraham Bioinformatics), and low-quality bases were trimmed and adapter sequences were removed using Trimmomatic v.0.33 [53] using the following arguments: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 HEADCROP:4 LEADING:3 TRAILING:3 SLIDINGWINDOW:6:25 MINLEN:30. Sequence reads were mapped to the mouse UCSC mm9 reference assembly using TopHat v2.0.9 with Bowtie v1.0.0 algorithms [54,55]. The number of reads mapping onto gene exons was quantified by counting the number of strand-specific reads using featureCounts[56]. The differential gene expression analysis was performed by comparing IL-33-treated with non-stimulated ILC2s using the edgeR Bioconductor package[57]. First, residual rRNA reads were excluded and genes with an expression level > 10 counts per million reads (CPM) in at least 3 of the samples were retained for TMM normalization and differential gene expression. Genes with changes in expression ≥ |2| fold and Benjamini-Hochberg adjusted *p* values ≤ 0.01 were considered significant. The heatmap presenting the log2 fold change between IL-33-treated vs non-stimulated ILC2 for the selected genes was prepared using MeV software[58].

Under-agarose cell migration assay

ILC2s from LifeAct-GFP mice were sort-purified as described above, cultured in complete medium containing IL-7 (10 ng/ml) for 18 h and incubated with the indicated stimuli for 24 h. ILC2s were stained with 2 μg/mL Hoechst 33342 (Thermo Fisher Scientific) diluted in RPMI-1640 without phenol red for 20 min at 37°C, 5% CO_2. To prepare migration chambers 4.8% UltraPure agarose (Life Technologies) was dissolved in nuclease-free water and further diluted at 1:3 ratio in a warm mixture of complete RPMI without phenol red and HBSS as previously described[59]. The gel was cast into glass-bottom dishes (WPI) coated with 10 μg/mL fibronectin (Sigma-Aldrich) to create migration chambers. After complete polymerization, two 2-mm well were punched 2 mm apart in the agarose pad. One well was filled with the indicated chemokine to generate a soluble chemokine gradient, and 50,000 activated ILCs in 10 μL imaging medium were

added to the second well. Before imaging, loaded devices were incubated 2 hours at 37°C, 5% CO_2. Cell migration was recorded by time-lapse widefield microscopy on an Axiovert 200M Fully Automated Inverted Microscope (Zeiss) equipped with a 20X/0.8 NA Plan-Apochromat objective (Zeiss), a top-stage incubation system set at 37°C and delivering 5% CO_2 in humidified air (Live Cell Instrument), and a monochrome camera. Imaging medium consisted in RPMI without phenol red (Wisent) supplemented as described beforehand. Brightfield and fluorescence emitted from Hoechst 33342 and GFP were collected from a single *z*-stack using an X-Cite 120 LED (Excelitas Technologies) as a light source and commercial filter cubes to excite and collect signals from Hoechst 33342 (Zeiss) and GFP (Zeiss). Images were acquired every 30 s for a total of 1 h, with an exposure time of 50 (Hoechst) or 200 ms (GFP). Movies were acquired using Zen pro (Zeiss) and processed using Imaris (Bitplane).

Statistical analysis

Differences between datasets were analyzed with GraphPad Prism software (GraphPad Software, La Jolla, CA) using either a two-tailed *t* test (unpaired) for the comparison of two groups or a one-way ANOVA followed by Tukey's multiple comparisons test for three or more groups. P values below 0.05 were defined as statistically significant with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Unless otherwise indicated, figures display means ± standard deviations (SD). Experiment sample sizes (n) and experiment replicate numbers are included in the respective figure legends.

3.6. Acknowledgements

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3.7. Figures and tables

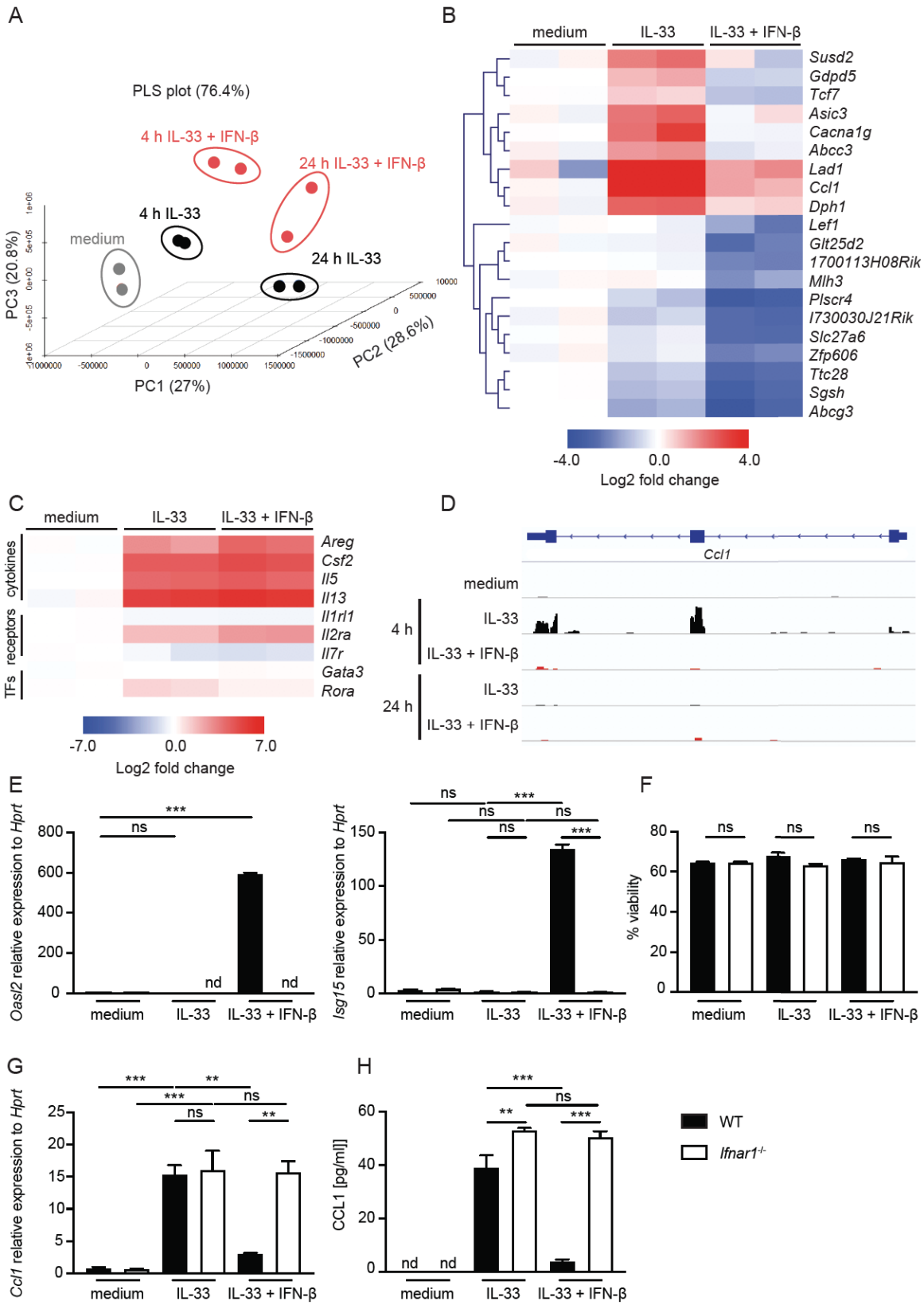

Figure 28. Type I Interferon inhibits CCL1 production from murine lung ILC2s *ex vivo*.

Murine lung ILC2s were isolated by multicolor fluorescence activated cell sorting (FACS), left unstimulated (medium) or cultured in the presence of IL-33 (10 ng/ml) alone or in combination with IFN-β (500 U/ml) for either 4 or 24 hours. **(A)** Partial least square (PLS) projection of RNA-seq data from lung ILC2s left untreated (medium) or stimulated with IL-33 or IL-33 + IFN-β for the indicated time points. **(B)** Clustered heat map of 20 most significantly downregulated genes (fold change ≤ 0.5; p ≤ 0.01) after 4 hours in IL-33 + IFN-β-stimulated ILC2s as compared to IL-33 treatment alone. **(C)** Heatmap depicting gene expression fold changes of selected key ILC2 signature cytokines, surface receptors and transcription factors after 4 hours of stimulation under indicated conditions. **(D)** RNA-seq read coverage of *Ccl1* locus in unstimulated ILC2s as well as after 4 or 24 hours of stimulation under indicated conditions. **(E)** qRT-PCR analysis of interferon-stimulated genes *Oasl2* and *Isg15* in ILC2s obtained from WT (black) or *Ifnar1*$^{-/-}$ (white) mice left untreated or cultured in the presence of IL-33 or IL-33 + IFN-β for 4 hours. **(F)** Viability of WT (black) and *Ifnar1*$^{-/-}$ (white) ILC2s assessed by flow cytometry after 24 hours of culture at indicated conditions. **(G)** qRT-PCR analysis of *Ccl1* expression in WT (black) and *Ifnar1*$^{-/-}$ (white) ILC2s cultured for 4 hours with medium only, IL-33 or IL-33 + IFN-β. **(H)** WT (black) and *Ifnar1*$^{-/-}$ (white) ILC2 were stimulated for 24 hours with medium only, IL-33 or IL-33 + IFN-β and CCL1 concentrations in supernatants were determined by ELISA. Data points are representative of one experiment with two biological replicates (A – D) or technical replicates from at least two independent experiments (E – G). Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. ns, not significant; nd, not detectable; TFs, transcription factors.

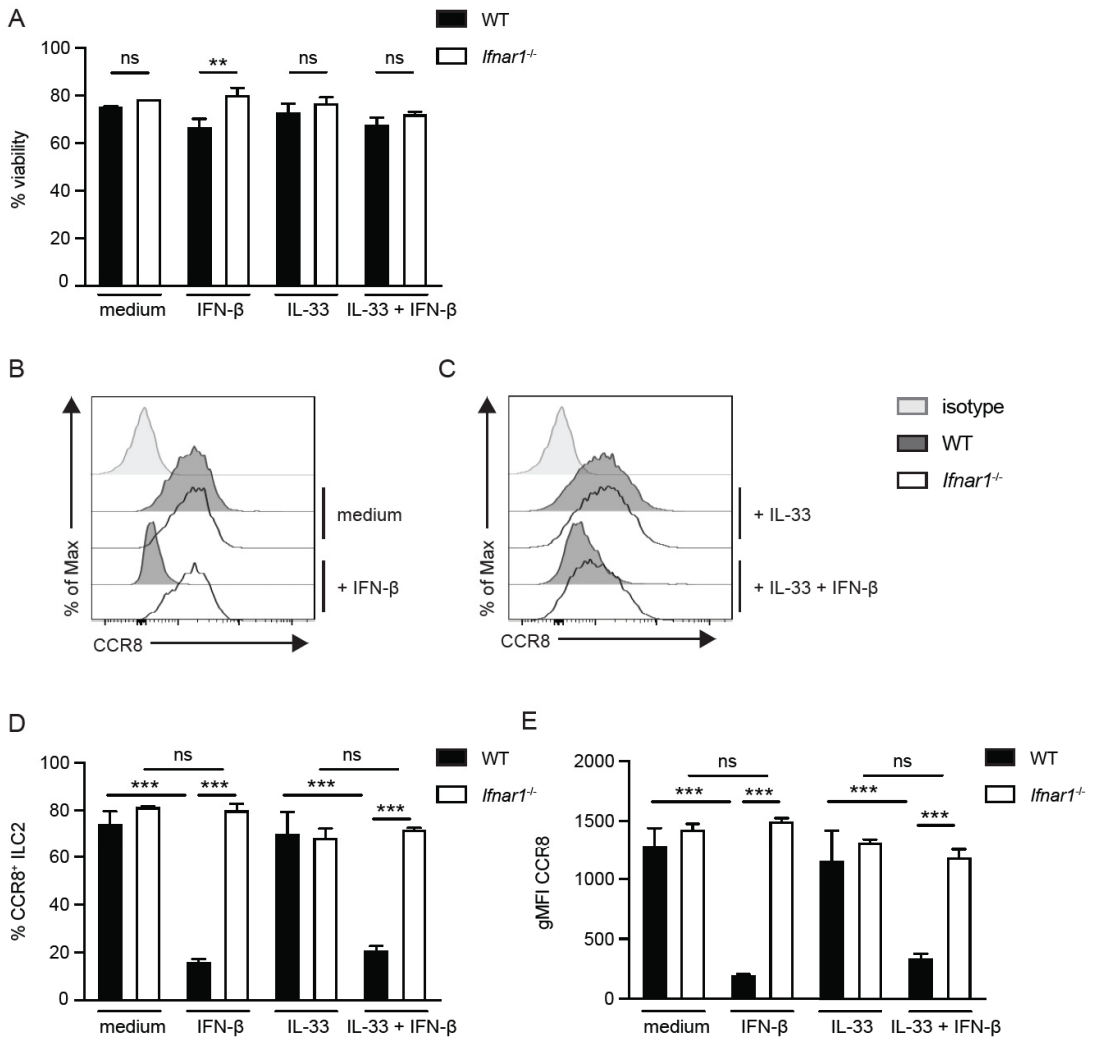

Figure 29. IFN-I suppresses CCR8 expression by lung ILC2s *ex vivo*.

Murine lung ILC2s were sort-purified from WT and *Ifnar1*$^{-/-}$ mice and left unstimulated (medium) or cultured in the presence of IL-33 (10 ng/ml) or IFN-β (500 U/ml) alone or in combination for 24 hours. **(A)** ILC2 viability was determined by flow cytometry after 24 hours of culture. **(B, C)** WT (filled histograms) and *Ifnar1*$^{-/-}$ (empty histograms) ILC2s were cultured with (B) medium only or IFN-β, (C) IL-33 or IL-33 + IFN-β for 24 hours and CCR8 expression levels were analyzed by flow cytometry. Representative flow cytometry histogram plots of CCR8 expression as compared to isotype control stainings (grey histograms) are shown. **(D)** Frequencies of CCR8^{+} WT (black) and *Ifnar1*$^{-/-}$ (white) ILC2s cultured under the indicated conditions were determined by flow cytometry. **(E)** gMFI of CCR8 expression by ILC2s isolated from WT (black) and *Ifnar1*$^{-/-}$ (white) mice and cultured under the indicated conditions for 24 hours were determined by flow cytometry. Data are representative of technical replicates from three independent experiments. Data are depicted as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. ns, not significant; gMFI, geometric mean fluorescence intensity.

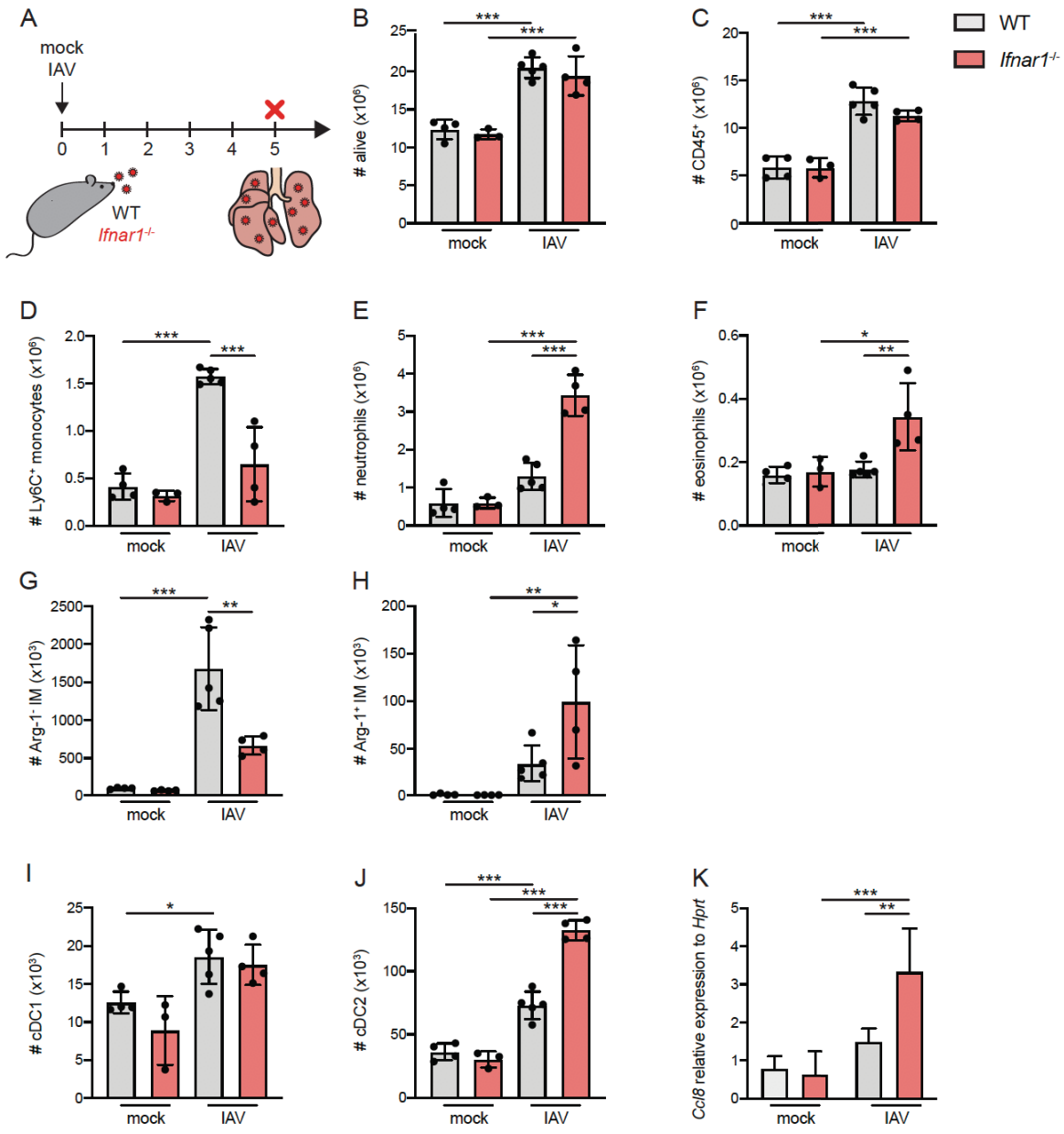

Figure 30. IFN-I negatively regulates type 2 immune responses upon influenza A virus (IAV) infection.

(A) WT and *Ifnar1*$^{-/-}$ mice were intranasally infected with a sublethal dose of 10 PFU influenza A virus or PBS (mock) as a control and lungs were isolated on day 5 post infection for analysis. Total numbers of **(B)** viable cells, **(C)** CD45^{+} leukocytes, **(D)** Ly6C^{+} monocytes, **(E)** neutrophils, **(F)** eosinophils, **(G)** Arg-1^{-} IMs, **(H)** Arg-1^{+} IMs, **(I)** cDC1s and **(J)** cDC2s in lungs of uninfected (mock), IAV-infected WT (grey) or *Ifnar1*$^{-/-}$ (red) mice were determined by flow cytometric analysis. **(K)** qRT-PCR analysis of *Ccl8* expression in whole lung tissue of mock- and IAV-infected WT (grey) or *Ifnar1*$^{-/-}$ (red) mice day 5 post infection. Data are representative of two independent experiments with n = 3 – 5 mice per group. Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. IAV, influenza A virus; IM, interstitial macrophages; cDC1, conventional type 1 dendritic cell; cDC2, conventional type 2 dendritic cell.

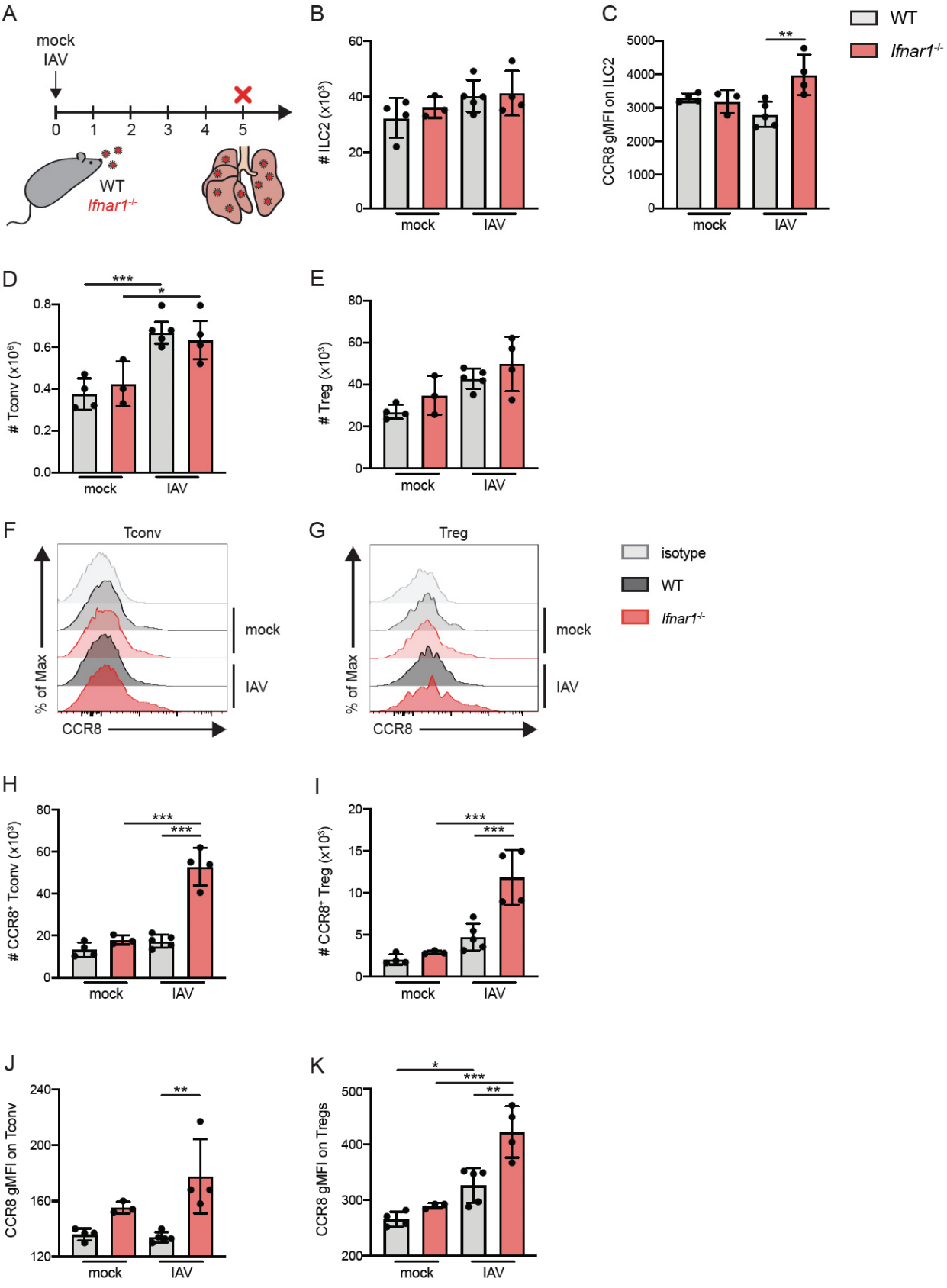
A
mock
IAV
0 1 2 3 4 5
WT
Ifnar1-/-
B
ILC2 (x10³)
mock
IAV
C
CCR8 gMFI on ILC2
WT
Ifnar1-/-
D
Tconv (x10⁶)
E
Treg (x10³)
F
Tconv
% of Max
CCR8
G
Treg
isotype
H
CCR8⁺ Tconv (x10³)
I
CCR8⁺ Treg (x10³)
J
CCR8 gMFI on Tconv
K
CCR8 gMFI on Tregs

Figure 31. IFN-I suppresses CCR8 expression on ILC2s and T cells following IAV infection.

(A) WT (grey) and *Ifnar1*$^{-/-}$ (red) mice were subjected to intranasal infection with a sublethal dose of 10 PFU IAV or PBS (mock) as a control. Lungs were isolated on day 5 post treatment for analysis. Immune cell populations were analyzed by flow cytometry. **(B)** Total numbers of pulmonary ILC2s. **(C)** gMFI of CCR8 expression on ILC2s. Total numbers of **(D)** CD4^{+} Tconv cells and **(E)** Treg cells. Representative histograms of CCR8 expression by **(F)** pulmonary Tconv cells and **(G)** Treg cells from WT (black histograms) and *Ifnar1*$^{-/-}$ (red histograms) mice infected with IAV or mock-treated compared to an isotype controls (grey histograms) are shown. Total numbers of **(H)** CCR8^{+} Tconv cells and **(I)** Treg cells from lungs of infected and control WT (grey) and *Ifnar1*$^{-/-}$ (red) animals. Expression levels of CCR8 by pulmonary **(J)** Tconv and **(K)** Treg cells in WT (grey) and *Ifnar1*$^{-/-}$ (red) mice post infection. Data are representative of two independent experiments with n = 3 – 5 mice per group. Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. IAV, influenza A virus; Tconv, conventional CD4^{+} T cells; Treg, regulatory T cells; gMFI, geometric mean fluorescence intensity.

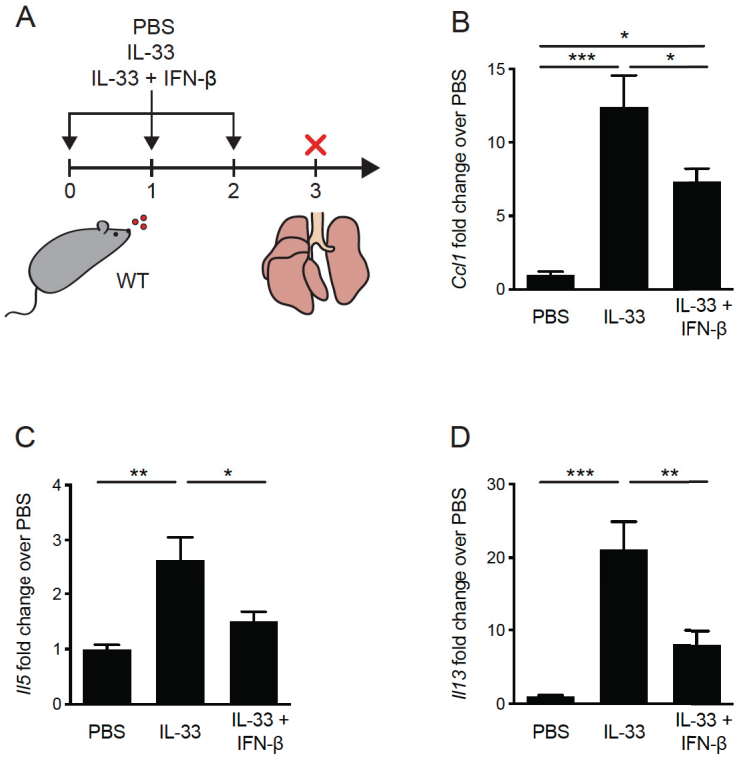

Figure 32. IFN-I inhibits pulmonary CCL1 expression upon IL-33-induced allergic airway inflammation.

(A) WT mice were intranasally challenged on three consecutive days (days 0, 1, 2) with either PBS as a control, recombinant murine IL-33 (500 ng/mouse) or IL-33 (500 ng/mouse) in combination with IFN-β (500 U/mouse). Lungs were isolated 24 hours after the last treatment (day 3) and expression of **(B)** *Ccl1*, **(C)** *Il5* and **(D)** *Il13* in whole lung tissue were assessed by qRT-PCR. Data are representative of two independent experiments with n = 3 – 5 mice per group. Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

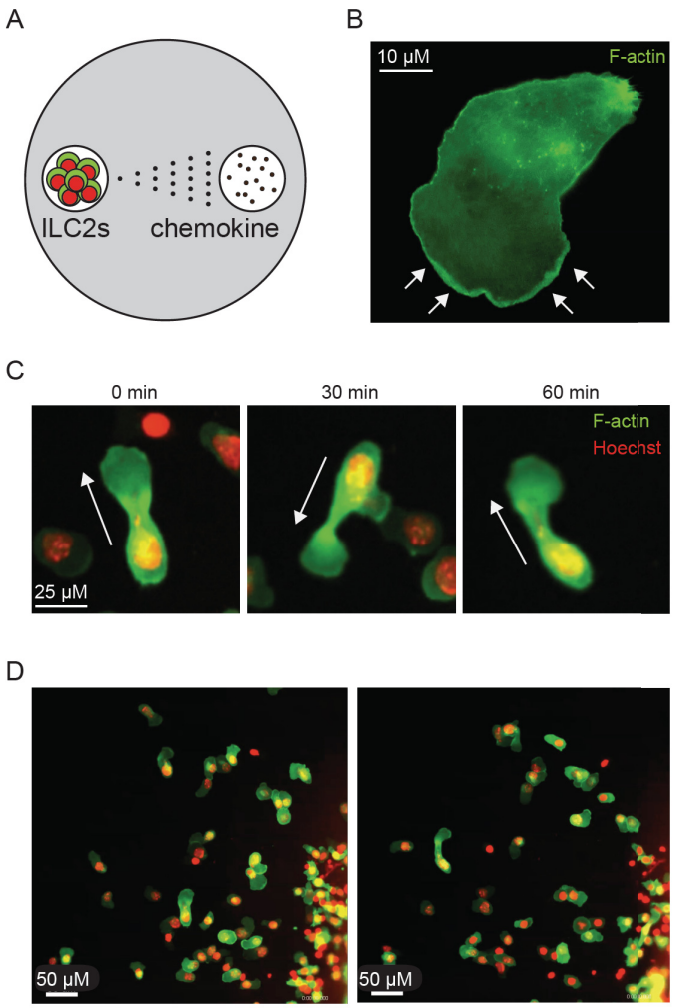

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Figure 33. Pulmonary ILC2s exhibit an amoeboid pattern of movement upon *in vitro* IL-33 treatment and do not migrate towards CCL1 or CCL22.

(A-D) Murine lung ILC2s from LifeAct-GFP mice were isolated and rested for 18 h in medium containing IL-7 (10 ng/ml) followed by stimulation with IL-33 (10 ng/ml) for 24 h. ILC2s were stained with Hoechst nuclear dye and seeded into respective well of fibronectin-coated migration chamber. Chemokines (100 nM) were added to the chemokine reservoir and incubated for 2 h. Cells were imaged, and migration was recorded by time-lapse widefield microscopy. **(B)** Actin remodeling in ILC2s. F-actin (green) polymerization at leading edge is highlighted by white arrows. Scale bar, 10 µM **(C)** Change in shape during migration over time. F-actin is depicted in green and nuclear dye Hoechst in red. White arrows indicate direction of movement. Scale bar, 25 µM **(D)** ILC2 migration within agarose matrix over the course of 1 hour. Scale bar, 50 µM. Data are representative of one experiment.

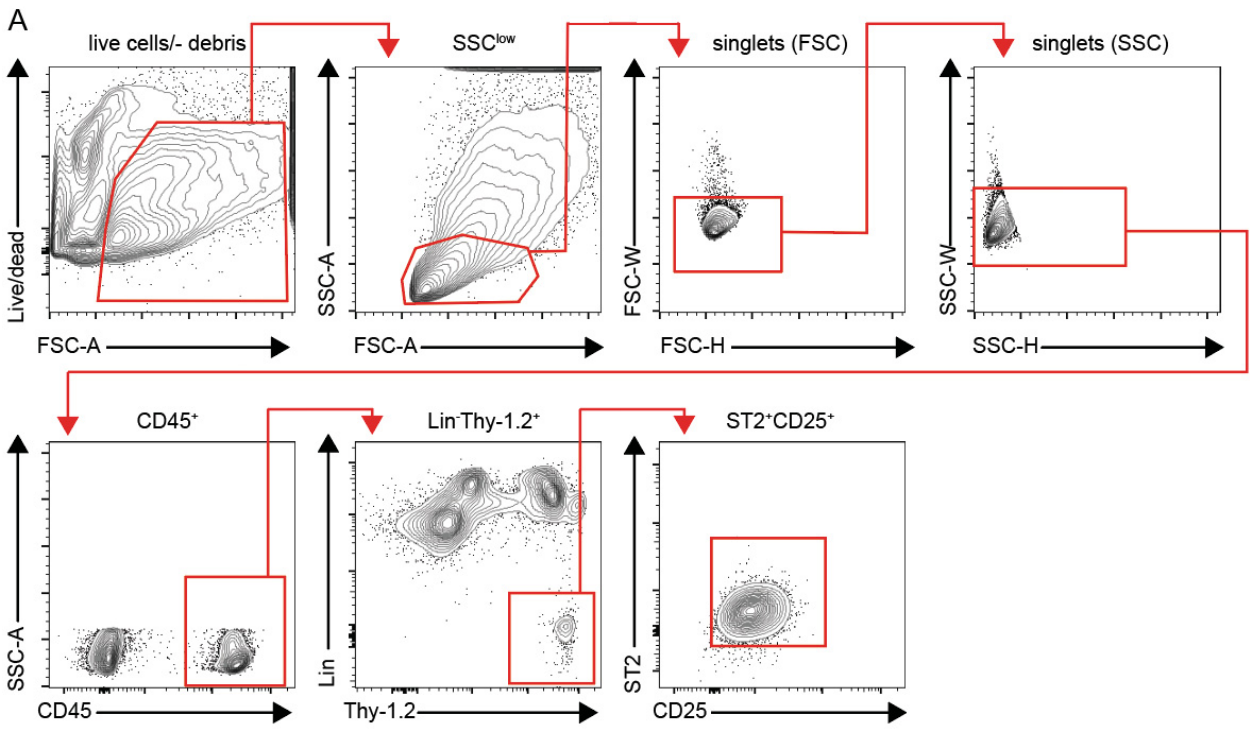

Figure 34 (Suppl. Fig. 1). Gating strategy for the isolation of murine lung ILC2s.

(A) Gating strategy to isolate murine lung ILC2s by multicolor fluorescence activated cell sorting (FACS). Dead cells and doublets were excluded and ILC2s were further defined as lineage-negative (Lin^-) $CD45^+Thy\text{-}1^+ST2^+CD25^+$ cells.

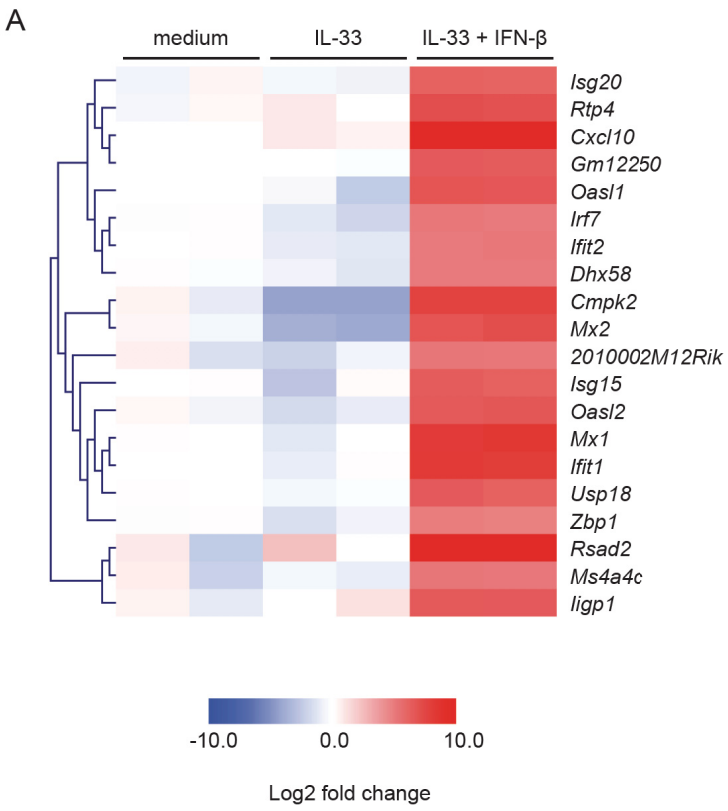

Figure 35 (Suppl. Fig. 2). Interferon-stimulated genes (ISGs) are upregulated in pulmonary murine ILC2s upon culture with IFN-I.

RNA-seq analysis of isolated lung ILC2s that were either left unstimulated (medium), cultured with IL-33 (10 ng/ml) or IL-33 in combination with IFN-β (500 U/ml) for 4 hours. **(A)** Clustered heat map depicting fold change of 20 most upregulated genes (fold change ≥ 2; $p \leq 0.01$) in IL-33 + IFN-β-stimulated ILC2s over IL-33 treatment alone.

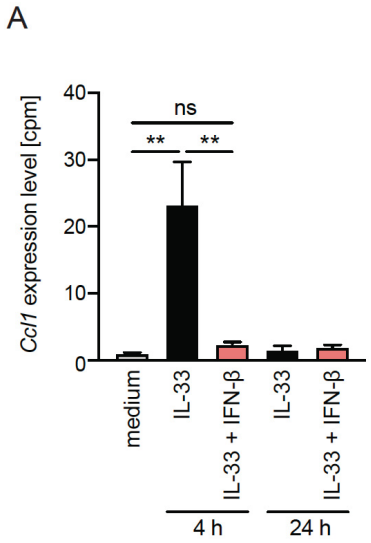

Figure 36 (Suppl. Fig. 3). Expression of *Ccl1* transcripts is induced early upon IL-33-mediated activation in murine lung ILC2s.

(A) Kinetics of *Ccl1* gene expression in sort-purified pulmonary murine ILC2s left unstimulated (medium), cultured with IL-33 (10 ng/ml, black bars) or IL-33 in combination with IFN-β (500 U/ml, red bars) for 4 or 24 hours. Transcript expression was determined by RNA-seq analysis and is shown in counts per million reads. Data are shown as mean ± SD with **$p < 0.01$, as determined by one-way ANOVA followed by Tukey's multiple comparisons test. cpm, counts per million; ns, not significant.

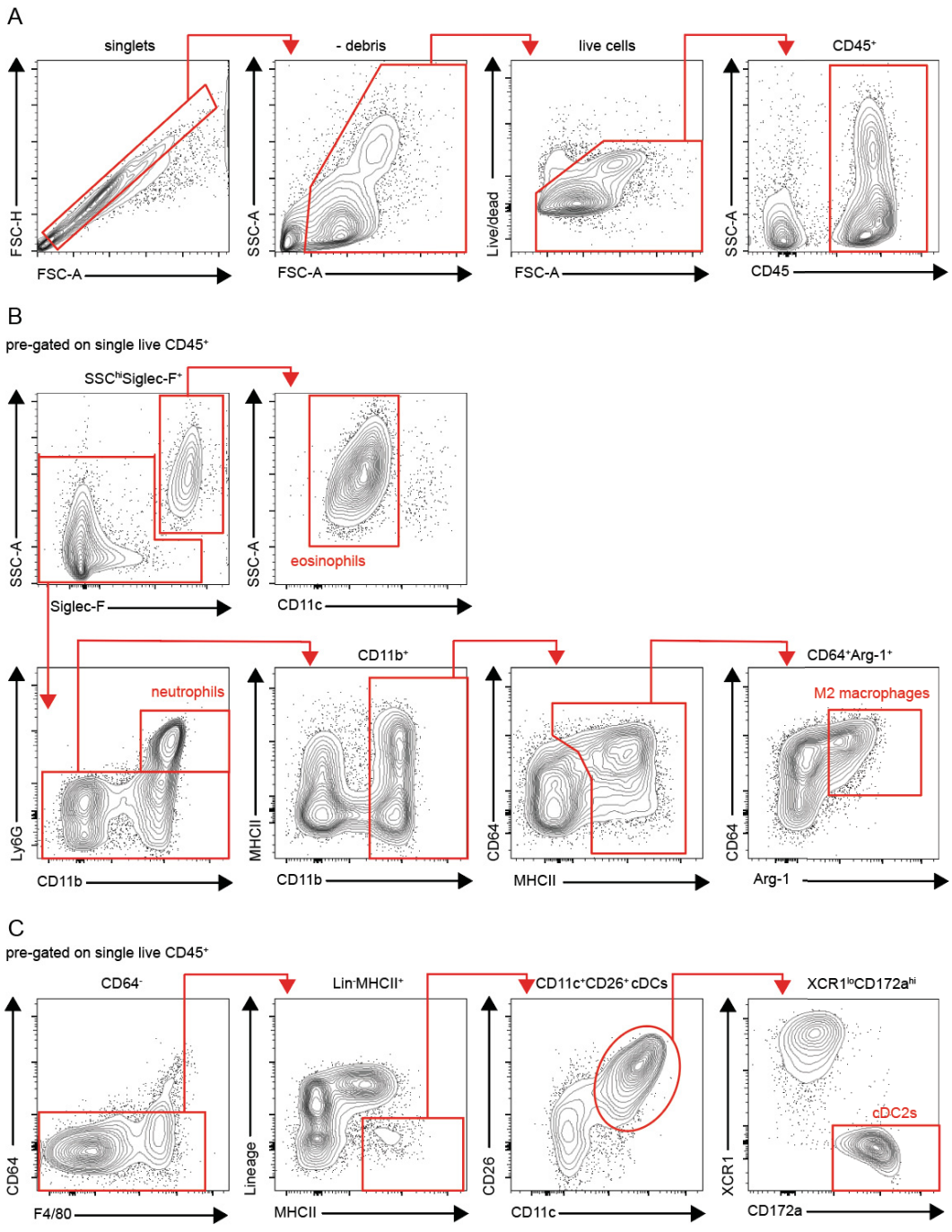

Figure 37 (Suppl. Fig. 4). Gating strategies for the identification of pulmonary myeloid cell populations.

(A) Multicolor flow cytometry gating strategies to identify murine pulmonary **(B)** eosinophils (defined as single, live cells that are $CD45^+SSC^{hi}Siglec\text{-}F^+CD11c^-$), neutrophils (defined as single, live cells that are $CD45^+Ly6G^+CD11b^+$) and M2 macrophages (defined as single, live cells that are $CD45^+Ly6G^-CD11b^+CD64^+Arg\text{-}1^+$) as well as **(C)** cDC1s (defined as single, live cells that are $CD45^+CD64^-Lin^-MHCII^+CD26^+CD11c^+XCR1^+CD172a^-$) and cDC2s (defined as single, live cells that are $CD45^+CD64^-Lin^-MHCII^+CD26^+CD11c^+XCR1^-CD172a^+$).

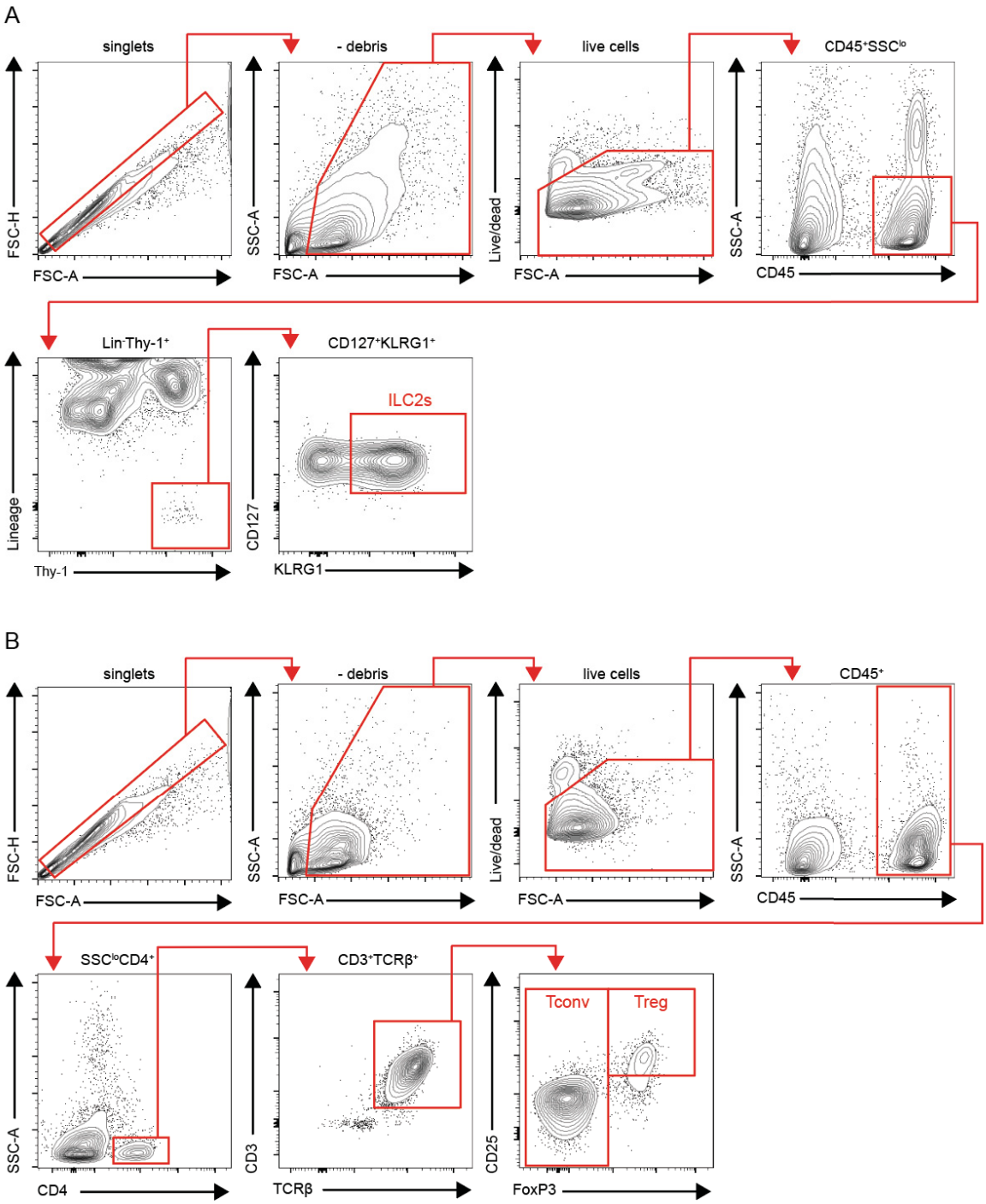

Figure 38 (Suppl. Fig. 5). Gating strategies for ILC2s and CD4$^+$ T cell subsets in murine lungs.

Multicolor flow cytometry gating strategies to identify murine pulmonary **(A)** lung ILC2s (defined as single, live cells that are CD45$^+$SSC^{lo}Lin$^-$Thy-1$^+$CD127$^+$KLRG1$^+$) as well as **(B)** Tconv cells (defined as single, live cells that are CD45$^+$SSC^{lo}CD4$^+$CD3$^+$TCRβ$^+$FoxP3$^-$) and Treg cells (defined as single, live cells that are CD45$^+$SSC^{lo}CD4$^+$CD3$^+$TCRβ$^+$FoxP3$^+$CD25$^+$).

Table 5. Flow cytometry antibodies.

Target	Clone	Source
Arg-1	A1exF5	eBioscience
CD3ε	145-2C11	eBioscience
CD4	GK1.5	BD Biosciences
CD5	53-7.3	eBioscience
CD11b	M1/70	eBioscience
CD11c	N418	eBioscience
CD19	1D3	eBioscience
CD25	PC61.5	eBioscience
CD26	H194-112	eBioscience
CD45.2	104	BioLegend
CD45	30-F11	BioLegend
CD45R (B220)	RA3-6B2	eBioscience
CD64	X54-5/7.1	BD Biosciences
CD90.2 (Thy-1.2)	53-2.1	BioLegend
CD127	A7R34	BioLegend
CD172a (SIRPa)	P84	eBioscience
CD198 (CCR8)	SA214G2	BioLegend
F4/80	BM8	BioLegend
FoxP3	FJK-16s	eBioscience
FcεR1	MAR-1	eBioscience
GATA3	TWAJ	eBioscience
Ki-67	SolA15	eBioscience
Ly6C	AL-21	BD Biosciences
Ly6G	1A8	BD Biosciences
Ly6G/Ly6C (Gr-1)	RB6-8C5	eBioscience
MHC Class II (I-A/I-E)	M5/114	BD Biosciences
KLRG1	2F1	eBioscience
NK1.1	PK136	eBioscience
Siglec-F	E50-2440	BD Biosciences
ST2	RMST2-2	eBioscience
TCRβ	H57-597	BioLegend
TCRγ/δ	eBioGL3	eBioscience
TER-119	TER-119	eBioscience
XCR-1	ZET	BioLegend

Table 6. qRT-PCR primers.

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Source
Ccl1	GGCTGCCGTGTGGATACAG	AGGTGATTTTGAACCCACGTTT	Liu *et al.*, 2013[60]
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	Hernandez *et al.*, 2015[61]
Il5	CTCTGTTGACAAGCAATGAGACG	TCTTCAGTATGTCTAGCCCCTG	Mohapatra *et al.*, 2016[62]
Il13	GCAGCATGGTATGGAGTGT	TATCCTCTGGGTCCTGTAGATG	this study
Isg15	GAGCTAGAGCCTGCAGCAAT	TTCTGGGCAATCTGCTTCTT	Pott *et al.*, 2011[63]
Oasl2	GGATGCCTGGGAGAGAATCG	TCGCCTGCTCTTCGAAACTG	Pott *et al.*, 2011[63]

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Preface to Chapter 4

In chapter 3, we investigated mechanisms of IFN-I-mediated negative regulation of ILC2s. In particular, we focused on how IFN-I affect the CCR8-CCL1/CCL8 axis using *ex vivo* stimulation and *in vivo* allergic airway inflammation and influenza A virus infection models. We showed that IFN-I suppresses both CCR8 expression and CCL1 production by ILC2s which, based on recent work, may impact their migratory behaviour.

Besides interferons, the neuropeptide CGRP was recently described to be a potent negative regulator of alarmin-driven pulmonary ILC2 responses. Based on these findings, the focus of chapter 4 was to investigate if lung ILC2s are able to integrate inhibitory signals from other peptides that are structurally related to CGRP. We thereby analyzed expression of peptide receptors on pulmonary ILC2s by RNA-seq analysis and validated our findings in functional *ex vivo* studies.

Chapter 4: Adrenomedullin negatively regulates IL-33-mediated group 2 innate lymphoid cell responses.

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4.1. Abstract

Neuropeptides have emerged as critical modulators of ILC2 functions at steady state as well as upon allergen challenge and helminth infection. To decipher additional mechanisms of peptide-mediated pulmonary ILC2 regulation, we analyzed transcript expression of peptide receptors on ILC2s. Here we observed that in addition to known neuropeptide receptor chains, ILC2s highly expressed *Calcrl* and *Ramp3* transcripts, components of the adrenomedullin (ADM) peptide receptor. Furthermore, relative to other known ILC2-regulating peptides which are preferentially expressed in the gastrointestinal tract, endogenous ADM was highly abundant in the lung under homeostatic conditions. *In vitro* stimulation of pulmonary ILC2s with ADM inhibited IL-33-driven ILC2 responses in a dose- and cAMP-dependent manner. Collectively, these data identify ADM as a novel negative regulator of pulmonary ILC2s responses.

4.2. Introduction

Inflammatory allergic airway diseases such as allergic asthma are characterized by an excessive type 2 immune response against inhaled allergens[1]. The initiation of associated type 2 immunopathologies was long thought to be solely orchestrated by type 2 T helper (Th2) cells producing interleukin (IL)-4, IL-5 and IL-13 which mediate classical hallmarks of type 2 inflammation, class switch recombination to immunoglobulin E (IgE), eosinophilia as well as goblet cell hyperplasia and mucus hyperproduction[2]. However, recent reports highlighted that

group 2 innate lymphoid cells (ILC2s) exert critical roles in the initiation and orchestration of allergic airway inflammation and can act in concert with Th2 cells to drive development and progression of disease pathogenesis[3-5]. Unlike Th2 cells, ILC2s are enriched at barrier surfaces and are activated in an antigen-independent manner by integrating cues from their tissue microenvironment, including alarmins such as IL-25 and IL-33, that are released upon tissue perturbation[5]. ILC2s are potent sources of early type 2 cytokines and must therefore be tightly regulated. It was shown that their function can be positively or negatively modulated by cytokines[6-10], hormones[11-13], lipid mediators[14-23], as well as co-stimulatory receptors[24-31]. Recent reports also indicated that lung as well as small intestinal ILC2 functions can be regulated by peptides such as neuromedin U (NMU), vasoactive intestinal peptide (VIP) and calcitonin-gene related peptide (CGRP) that mediated their effects by binding to G protein-coupled receptors (GPCRs) on ILC2s and differential activation of downstream signaling proteins results in either inhibitory or activating effects [32-39]. In the lung, NMU can induce ILC2-mediated inflammation[32] and further amplifies ILC2 responses during allergic airway inflammation[33]. Short-term *in vitro* culture of IL-33-activated pulmonary ILC2s in the presence of CGRP enhanced IL-5[36-38] while repressing IL-13 production[37,38]. In addition, CGRP suppressed lung ILC2 proliferation in response to IL-33 *in vitro* and restrained ILC2 effector responses and associated immunopathology during IL-33-elicited allergic airway inflammation[37,38]. Pulmonary ILC2s are able to integrate a diversity of environmental cues and are strategically located in close proximity to epithelial cells[40], endothelial cells[41] as well as neurons[33,34], which are potent sources of immunomodulatory peptides.

To delineate additional mechanisms of peptide-mediated ILC2 regulation, we analyzed the spatial distribution of regulatory peptide expression as well as their receptor subunits by ILC2s, followed by functional *in vitro* studies using primary pulmonary ILC2s. In accordance with previous reports we observed that transcript levels of the peptide adrenomedullin (ADM) are enriched in murine lungs at steady state and additionally demonstrated that pulmonary ILC2s express *Calcrl* and *Ramp3* genes, encoding ADM receptor components. We further showed that *in vitro* stimulation with ADM restrained IL-33-driven ILC2 effector functions in a cAMP-dependent manner indicating that ADM functions as a negative regulator of lung ILC2 responses.

4.3. Results

ADM is enriched in murine lungs and pulmonary ILC2s express ADM receptor subunits Calcrl and Ramp3.

To identify potential regulatory peptides that influence murine ILC2 responses we screened expression of peptide receptors by RNA-sequencing (RNA-seq) in isolated primary bone marrow-derived ILC2s (**Figure 41A).** We subsequently validated our findings with quantitative real-time PCR (qRT-PCR) in sort- purified lung ILC2s (**Figure 41B**). Consistent with previous studies[32-35], naive lung ILC2s expressed *Nmur1* and *Vipr2* transcripts (**Figure 42A**), encoding for NMU and VIP receptors, respectively, as well as the CGRP receptor components, *Calcrl* and *Ramp1* [37-39] ((**Figures 39A, C**). In addition to these described neuropeptide receptor chains, lung-resident ILC2s were found to highly express *Ramp3* but not *Ramp2* transcripts, which together with the gene product of *Calcrl* constitute receptors for the peptide adrenomedullin (ADM)[42] (**Figures 39B, C**). To analyze how IL-33-driven ILC2 activation affects expression of ADM receptor chains, we cultured isolated lung ILC2s with IL-33 over the course of 24 hours. While *Ramp1* levels remained stable 8 hours post stimulation, *Ramp3* expression gradually declined and was significantly lower after 4 h of culture with IL-33 (**Figure 42B**). *Calcrl* levels were induced after 4 hours of activation and declined again at 8 hours (**Figure 42B**). We next investigated if ADM was present in the lungs of mice at steady state to exert a potential regulatory role for pulmonary ILC2s. In addition, we measured endogenous transcript expression of the known ILC2-modulating peptides NMU (*Nmu*), VIP (*Vip*), and CGRP (*Calca*). Relative to other known ILC2-modulating peptides and consistent with previous studies[43,44], *Adm* was found to be highly abundant in the murine lung under homeostatic conditions (**Figure 39D**). In contrast, NMU, VIP and CGRP transcripts were preferentially expressed in the gastrointestinal tract (**Figure 39E**). Contrary to CGRP, which is produced by ILC2s upon IL-33-induced activation[37-39], ILC2-intrinsic *Adm* expression was not observed (data not shown). Taken together these observations demonstrate that lung ILC2s express transcripts for both ADM receptor chains which are differentially modulated by IL-33-mediated activation and that ADM is highly abundant in the lungs of naive mice.

ADM inhibits IL-33-mediated activation of pulmonary ILC2s in a cAMP-dependent manner.

To examine the effects of ADM on ILC2 effector functions, we stimulated sort-purified murine lung ILC2s with IL-33 and increasing concentrations of ADM. We observed that addition of ADM impaired IL-33-driven ILC2 proliferation in a dose-dependent manner (**Figure 40A**). In addition, levels of the type 2 cytokines IL-5 and IL-13 in culture supernatants were also significantly diminished with increasing concentrations of ADM (**Figure 40B**). Binding of ADM to either of its cognate receptors, induces production of the secondary messenger molecule cyclic AMP (cAMP)[45-48]. To determine if ADM also induces cAMP production in lung ILC2s, we measured intracellular cAMP levels in unstimulated cells, or after stimulation with either IL-33 or ADM (**Figure 40C**). Indeed, culture of ILC2s with ADM resulted in rapid increase of intracellular cAMP levels (**Figure 40C**). In contrast, no cAMP was detected in unstimulated cells or ILC2s treated with IL-33 (**Figure 40C**). To investigate if the observed inhibitory effects of ADM on ILC2s are mediated by cAMP, we cultured ILC2s in the presence of IL-33 and increasing concentrations of the cell permeable cAMP analog di-butyryl-cAMP (db-cAMP) for five days. Similar to what was observed with ADM, treatment with db-cAMP lead to restrained ILC2 proliferation and cytokine production in a dose-dependent manner (**Figures 40D, E**). On the contrary, inhibition of cAMP generation with an adenylate cyclase inhibitor (SQ22,536) alleviated the suppressive effect of ADM on IL-33-induced ILC2 proliferation (**Figure 40F**). Taken together, ADM restrains effector functions of IL-33-activated ILC2s *in vitro* in a cAMP-dependent manner indicating that ADM is a potent negative regulator of ILC2s.

4.4. Discussion

It has recently been demonstrated that neuropeptides such as NMU or CGRP can modulate ILC2 responses at steady state as well as upon helminth infections and allergen challenge[32-36,38,39]. To decipher novel pathways of peptide-mediated ILC2 regulation we analyzed peptide receptor expression by murine ILC2s and observed that bone marrow-derived as well as pulmonary ILC2s expressed transcripts for ADM receptor subunits. Culture with ADM suppressed IL-33-driven ILC2 effector functions *in vitro*, including proliferation and type 2 cytokine release of pulmonary ILC2s, thereby indicating that ADM negatively regulates ILC2s.

ADM is a member of the CGRP superfamily of peptides and exerts its effector functions by binding to ADM receptors composed of calcitonin receptor-like receptor (CLR) and either

receptor activity-modifying protein (RAMP)2 or RAMP3[42,49,50]. ADM receptors are widely distributed on non-hematopoietic cells including endothelial cells, epithelial cells and smooth muscle cells but expression has also been reported on immune cells such as macrophages[50]. Initially identified as a vasoactive peptide exhibiting vasodilatory and hypotensive effects, it is becoming increasingly evident that ADM also exerts potent immunomodulatory functions[50-52]. While we observed expression of the ADM receptor chains CLR (*Calcrl*) and RAMP3 (*Ramp3*) by ILC2s, no RAMP2 transcripts could be detected. *Calcrl* as well as *Ramp3* expression was gradually lost over the course of stimulation which may indicate that ILC2s downmodulate their expression to allow for efficient effector activation. Hence, it would be of interest to analyze if these findings could be recapitulated upon allergic airway inflammation *in vivo*.

ADM is highly expressed in human lungs under homeostatic conditions[53,54]. Pulmonary ADM sources include, bronchial epithelial cells[55,56], type II pneumocytes[55-57], neuroendocrine cells[55,56], vascular endothelial cells[54-57], smooth muscle cells[58,59] and alveolar macrophages[57]. Consistently, we observed high expression of ADM transcripts in lung tissue of naive mice, while known ILC2-regulatory peptides VIP, NMU and CGRP were barely detectable and predominantly confined to the gastrointestinal tract. ILC2s are located in close proximity to both airway epithelial[40] and endothelial cells[41] as well as neurons[33,34] allowing for direct regulation by factors derived from these non-hematopoietic cell lineages. As opposed to CGRP expression, which has been described to be upregulated upon IL-33-mediated ILC2 activation and may therefore act in an autocrine manner, we did not observe induction of *Adm* expression upon culture with IL-33 (data not shown).

Previous reports stated that ADM is rapidly downregulated in the airway epithelium and pulmonary vascular endothelium upon allergic airway inflammation and might therefore allow for efficient ILC2 induction[57]. Moreover, it was shown that ADM exhibits anti-inflammatory properties in rodent models of lung injury[43,44,60-64], attenuates pulmonary hypertension in animal models as well as humans[65-68] and negatively regulates plasma extravasation and airway hyperresponsiveness in mouse models of allergic airway inflammation[57,69]. In line with these findings, atopic asthmatic children showed significantly reduced expression of ADM when compared to a nonatopic healthy control group[57] and nasal ADM levels were reduced in allergic rhinitis patients[70]. However, these studies neither detailed the cell type(s) regulated by ADM nor investigate the molecular mechanisms of ADM actions. Since lung-resident ILC2s are key drivers

of allergic airway inflammation it is tempting to speculate that the previously reported effects may be at least partly due to ADM-mediated ILC2 suppression.

ADM elicits its cellular functions by activating adenylate cyclase and elevating cAMP levels[45-48]. In accordance, we observed rapid intracellular accumulation of cAMP in ILC2s upon ADM stimulation and additionally showed that the inhibitory effects of ADM on ILC2 functions were dependent on cAMP and reversible by inhibiting adenylate cyclase function. It is well established that cAMP is a potent inhibitor of NF-κB signaling[71]. Indeed, ADM treatment was described to dampen proinflammatory cytokine production by inhibiting lung NF-κB activation and translocation[44]. Since NF-κB signaling is critical for alarmin-driven ILC2 effector functions and treatment with pan-NF-κB inhibitor completely abolishes ILC2 activation following stimulation with IL-33 or neuropeptides[37], it is intriguing to speculate that ADM may exert its inhibitory effects via cAMP-mediated NF-κB inhibition.

Furthermore, CGRP has been shown to counteract not only alarmin-elicited ILC2 responses but also the ILC2-stimulating effects of NMU[37,38], it would hence be of interest if ADM exerts similar effects.

The present study revealed that lung ILC2s possess a functional ADM receptor and that ADM potently suppresses alarmin-driven pulmonary ILC2 functions *in vitro* by inducing cAMP generation. Thus, ADM receptor agonists may be a potential therapeutic target in battling allergic lung disease. However, the physiological role of ADM under homeostatic conditions and during allergic airway inflammation as well as the precise downstream effector mechanisms remain elusive and will be addressed in future studies.

4.5. Materials and Methods

Mice

C57BL/6J wild-type mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house for experimental use. Animals were bred and maintained under specific pathogen-free conditions with *ad libitum* access to food and water. Unless otherwise stated, experiments were conducted with adult female age-matched mice (8 – 16 weeks) in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University.

Preparation of single cell suspensions from lung tissue

To obtain single cell suspensions, lungs were processed as described previously[7] with minor modifications. Briefly, lungs were finely minced and digested in RPMI-1640 containing 5% FBS, 0.2 mg/ml Liberase™ TM (Roche) and 0.1 mg/ml DNase I (Roche). Digested lungs were homogenized with a 5 ml syringe attached to a 18G 1½ needle and passed through a 70 μm cell strainer to obtain single cell suspensions. Cells were washed with PBS and red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma).

Flow cytometry

Single cells were incubated with 2.4G2 hybridoma supernatant to block Fc receptors and stained with antibodies as indicated. Mouse lineage cocktail was composed of antibodies against B220, CD3ε, CD5, CD11b, CD11c, CD19, FcεRIα, NK1.1, Ly-6C/G, TCRαβ, TCRγδ and TER-119. Dead cells were excluded by staining with Fixable Viability Dye™ eFluor™ 780 (eBioscience) following the manufacturer's instructions. Intracellular transcription factor and cytokine staining was performed using the FoxP3 / Transcription factor staining kit (eBioscience) according to the manufacturer's instructions. Stained cell suspensions were acquired on a BD LSRFortessa™ Cell Analyzer (BD Biosciences) and data was analyzed using FlowJo™ 10 software (BD Biosciences). All antibodies used for flow cytometry analyses are listed in **Table 5**.

Isolation and culture of murine bone marrow ILC2 precursors

Bone marrow from tibias and femurs were pooled, subjected to red blood cell lysis using Hybri-Max Red Blood Cell Lysis Buffer (Sigma) and sorted as lineage (CD3ε, CD5, CD11b, CD11c, Gr1 (Ly6G), CD45R (B220), NK1.1, TCRβ, TCRγδ, Ter-119)-negative, Sca-1$^+$c-kit$^-$CD25$^+$ cells. Isolated ILC2 precursors were expanded in complete ILC2 medium (RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin and 55 µM 2-mercaptoethanol) supplemented with IL-2, IL-7, IL-25, IL-33 (all 50 ng/ml) and TSLP (20 ng/ml). After 5 days of expansion, cells were rested for 72 hours in IL-2 and IL-7 (both 10 ng/ml), incubated in medium without cytokines for 4 hours and used in experiments.

Isolation and culture of murine lung ILC2

Lung single cell suspensions were obtained as described above, blocked with 2.4G2 hybridoma supernatant followed by antibody and Fixable Viability Dye^{TM} (eBioscience) staining. ILC2 were sorted into on a BD FACSAria™ III Cell Sorter (BD Biosciences) based on surface marker expression as viable lineage-negative CD45$^+$CD90$^+$CD25$^+$ST2$^+$ cells. All antibodies used are listed in detail in **Table 6**. For RNA extraction, freshly isolated ILC2 were incubated for 18 hours in complete ILC2 medium containing IL-7 (10 ng/ml), washed and rested for 4 hours in complete medium without cytokines before use in experiments. For 5 day assays, cells were sorted and either stimulated directly as indicated with IL-7, IL-33 (all 10 ng/ml, R&D), adrenomedullin (1 – 100 nM; Phoenix Pharmaceuticals) and/or di-butyryl-cAMP (10 – 500 µM; Sigma) or pre-treated with SQ22,536 (10 µM; Cayman Chemical) for 30 minutes before stimulation.

Quantification of cytokines and cAMP

IL-5 and IL-13 in tissue culture supernatants were analyzed using IL-5 and IL-13 DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. cAMP concentration was determined with the Cyclic AMP XP® Chemiluminescent Assay Kit (Cell Signaling). Absorbance and luminescence were measured on an Enspire^{TM} 2300 Multilabel Reader (PerkinElmer).

RNA extraction and quantitative real time-PCR (qRT-PCR)

Total RNA from cultured cells was extracted using the Quick-RNA^{TM} MicroPrep kit (ZymoResearch) according to the manufacturer's instructions. For extraction of tissue RNA, tissue

was mechanically disrupted in TRIzol™ Reagent (Life Technologies) in Lysing Matrix D tubes (MP Biomedicals) using a MagNA Lyser (Roche). RNA was extracted according to the manufacturer's instructions and cleaned up with the Quick-RNA™ MicroPrep kit (ZymoResearch). cDNA was synthesized with Oligo(dT)$_{12-18}$ Primer and SuperScript™ III Reverse Transcriptase (both Life Technologies) and qRT-PCR was carried out using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). All primers and respective sequences are listed in **Table 6**. Gene expression was calculated using the comparative ΔCt method after normalization to expression levels of the housekeeping gene *Hprt*.

Statistical analysis

Differences between datasets were analyzed with GraphPad Prism software (GraphPad Software) using one-way ANOVA followed by Tukey's multiple comparisons test. P values below 0.05 were defined as statistically significant with *$p<0.05$, **$p<0.01$ and ***$p<0.001$. Unless otherwise indicated, figures display means ± standard deviations (SD). Experiment replicate numbers are included in the respective figure legends.

4.6. Acknowledgements

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4.7. Figures and Tables

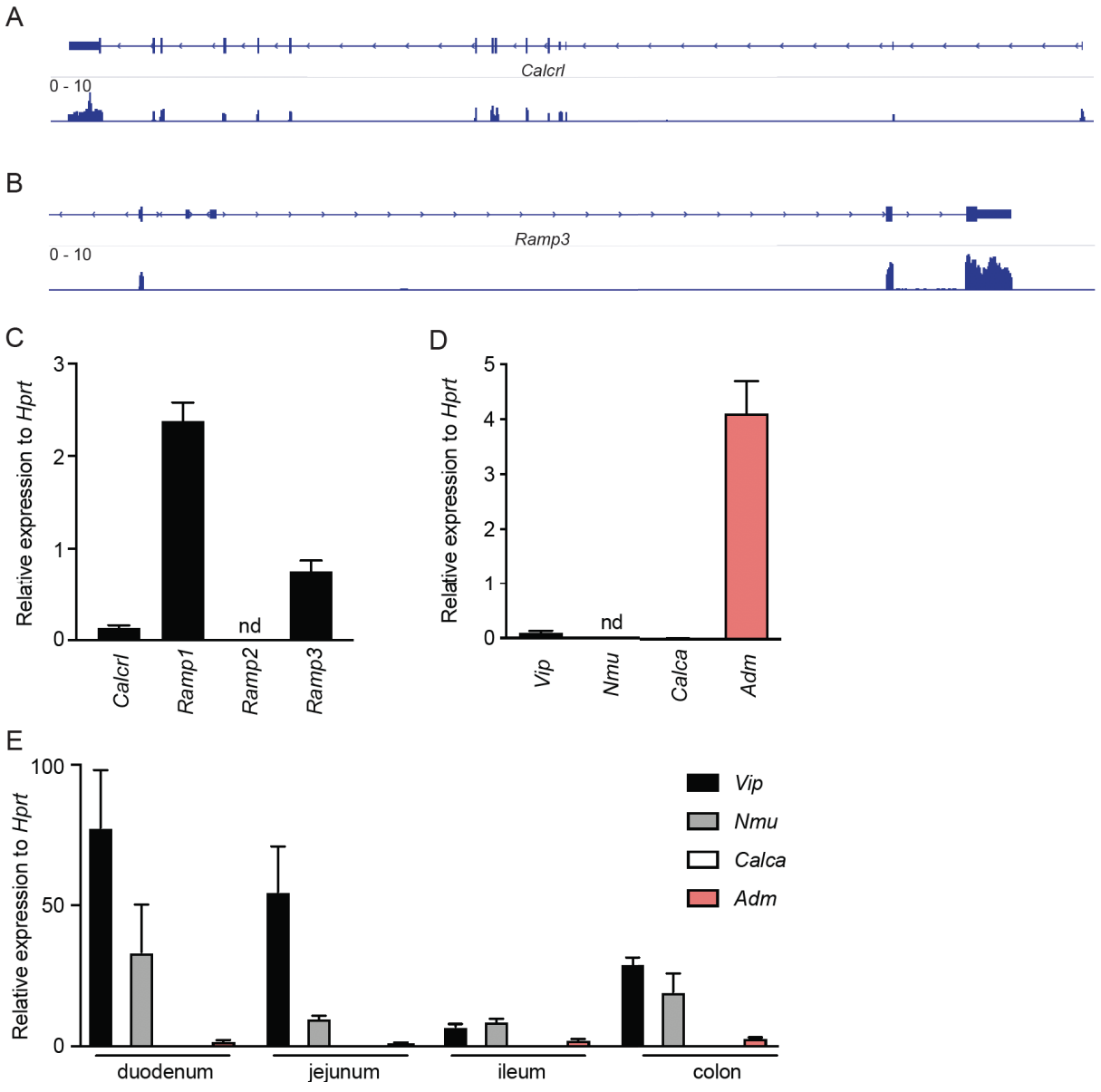

Figure 39. ILC2s express ADM receptor subunits and *Adm* is enriched in lung tissue.

Genomic track view of ADM receptor chains (**A**) *Calcrl* and (**B**) *Ramp3* loci from bone marrow-derived ILC2 RNA-seq analysis. (**C**) Normalized expression of ADM and CGRP receptor subunits by qRT-PCR in sort-purified lung ILC2s. (**D**) Relative expression of *Adm* and ILC2-regulatory neuropeptides in whole lung tissues as measured by qRT-PCR. (**E**) Transcript expression levels of *Adm* and neuropeptides in indicated tissues of the gastrointestinal tract, quantified by qRT-PCR. Data are representative of averages from technical replicates of at least two independent experiments and are shown as mean ± SD; nd, not detectable.

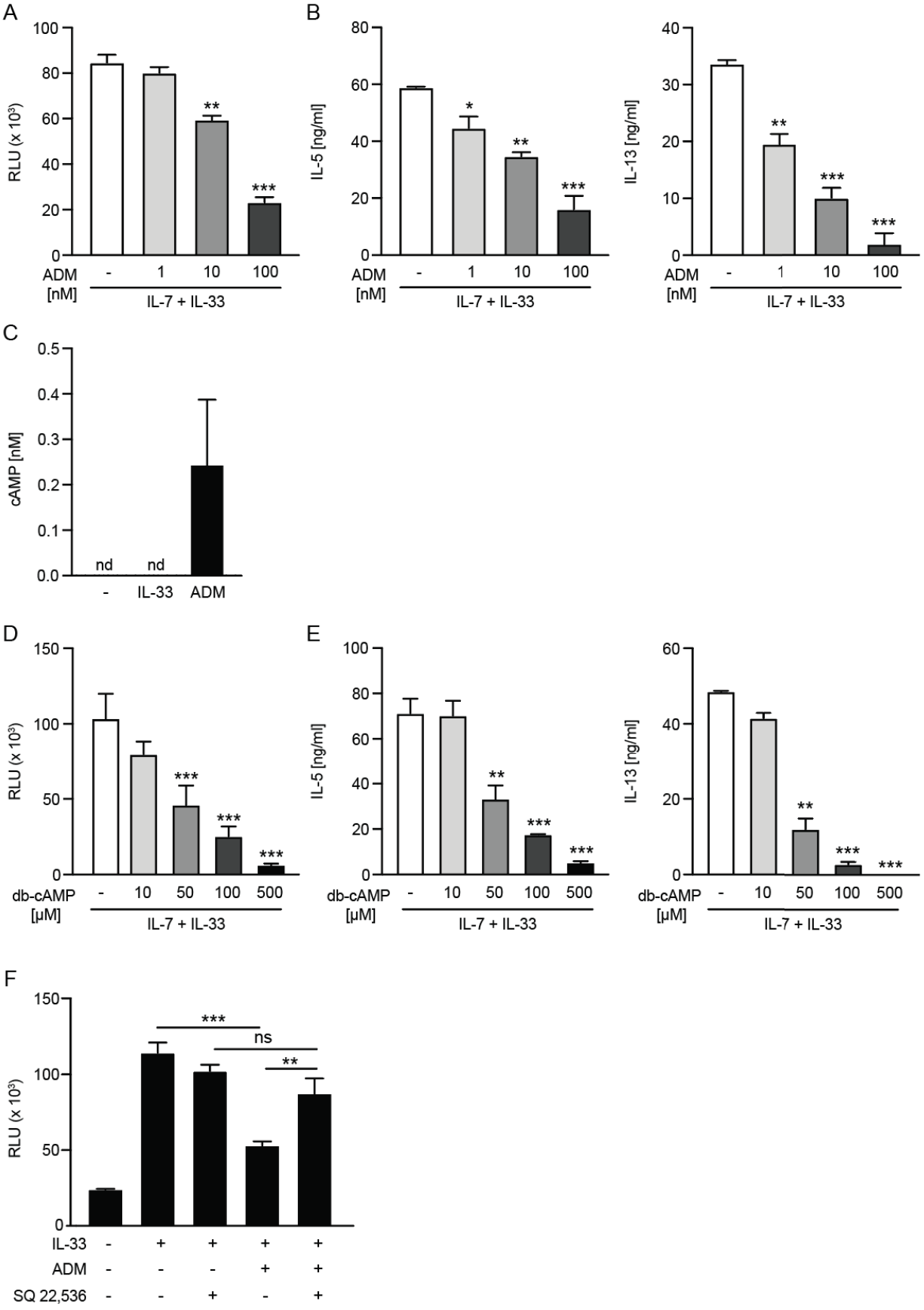
A
RLU (x 10³)
ADM [nM]
IL-7 + IL-33
B
IL-5 [ng/ml]
IL-13 [ng/ml]
C
cAMP [nM]
nd
IL-33
ADM
D
db-cAMP [µM]
E
F
ns
IL-33
ADM
SQ 22,536

Figure 40. ADM inhibits IL-33-mediated ILC2 activation in a cAMP-dependent manner.

Lung ILC2s were isolated by multicolor fluorescence activated cell sorting und cultured in the presence of IL-7 and IL-33 (both 10 ng/ml) as well as indicated stimuli. (**A**) Cell viability and (**B**) IL-5 and IL-13 concentrations in ILC2 supernatants after 5 days of culture in the presence of indicated concentrations of ADM. Viability was determined by AlamarBlue cell viability reagent and cytokine levels were assessed by ELISA. (**C**) cAMP concentrations in lung ILC2 lysates left unstimulated (-) or stimulated with either IL-33 or Adm for 20 min as measured by ELISA. (**D**) ILC2 viability and (**E**) IL-5 and IL-13 concentrations in culture supernatants after 5 days stimulated with indicated concentrations of dibutyryl-cAMP. (**F**) ILC2 viability quantified by AlamarBlue cell viability reagent after 5 days of culture with IL-33 (10 ng/ml) in the presence or absence of ADM (100 nM) and SQ22,536 (10 μM). All data are averages from technical replicates representative of three independent experiments (A, B, D, E) or representative of two independent experiments with similar results (C, F). Data are shown as mean ± SD with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test; ns, not significant; nd, not detected.

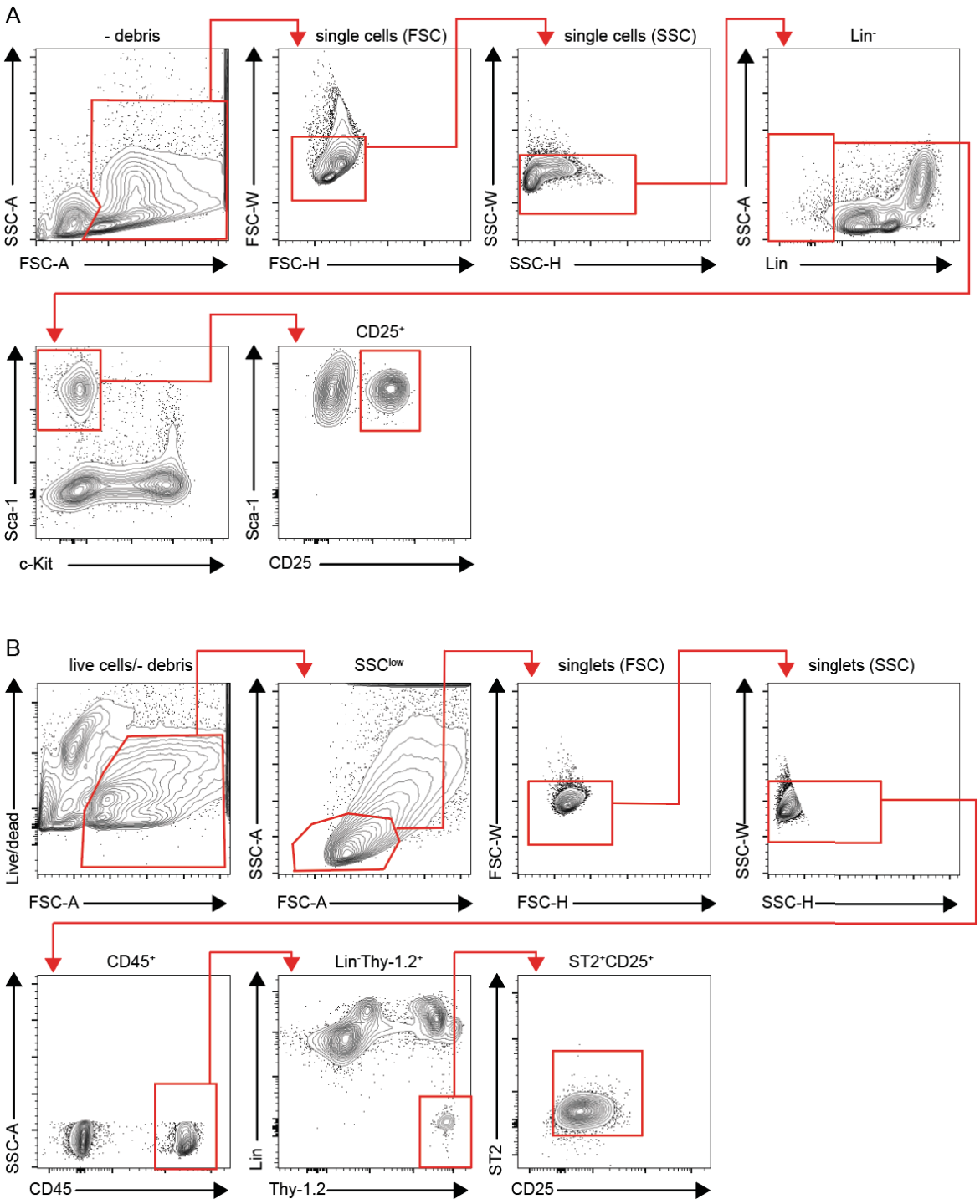

Figure 41 (Suppl. Fig. 1). Gating strategies for the isolation of murine lung ILC2s and bone marrow ILC2 progenitors.

(**A**) Murine bone marrow-derived ILC2 progenitors were isolated by multicolor fluorescence activated cell sorting (FACS). After exclusion of debris and doublets, ILC2 progenitors were defined as lineage-negative (Lin^-) c-$kit^-Sca\text{-}1^+CD25^+$ cells. (**B**) Murine lung ILC2s were defined and isolated by flow cytometric sorting as live single $Lin^-CD45^+Thy\text{-}1.2^+ST2^+CD25^+$ cells.

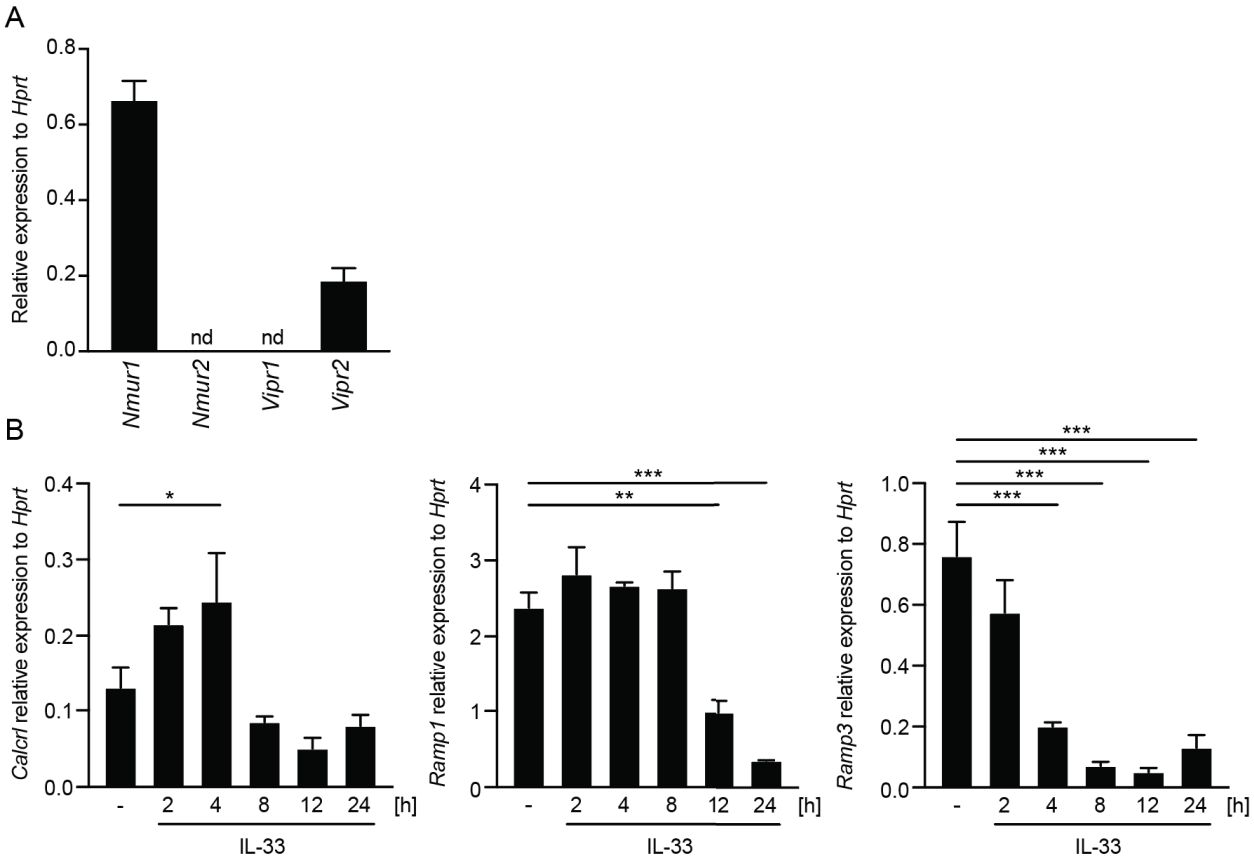

Figure 42 (Suppl. Fig. 2). IL-33 mediated activation differentially modulates expression of Adm and CGRP receptor components by ILC2s.

Gene expression analysis by qRT-PCR of (**A**) neuropeptide receptors and (**B**) CGRP and ADM receptor components *Calcrl*, *Ramp1* and *Ramp3* in isolated murine lung ILC2s cultured in the absence (-) or presence of IL-33 (10 ng/ml) for the indicated time points. Data are averages from technical replicates representative of two independent experiments. Data are shown as mean ± SD with *p < 0.05, **p < 0.01, ***p < 0.001 as determined by one-way ANOVA followed by Tukey's multiple comparisons test. nd, not detectable

Table 7. Flow cytometry antibodies.

Target	Clone	Source
CD3ε	145-2C11	eBioscience
CD5	53-7.3	eBioscience
CD11b	M1/70	eBioscience
CD11c	N418	eBioscience
CD19	eBio1D3 (1D3)	eBioscience
CD25	PC61.5	eBioscience
CD45.2	30-F11	BioLegend
CD45R (B220)	RA3-6B2	eBioscience
CD90.2 (Thy-1.2)	53-2.1	BioLegend
CD117 (c-Kit)	2B8	eBioscience
FcεR1	MAR-1	eBioscience
Ly6A/E (Sca-1)	E13-161.7	BioLegend
Ly6G/Ly6C (Gr-1)	RB6-8C5	eBioscience
NK1.1	PK136	eBioscience
ST2	RMST2-2	eBioscience
TCRβ	H57-597	eBioscience
TCRγ/δ	eBioGL3	eBioscience
TER-119	TER-119	eBioscience

Table 8. qRT-PCR primers.

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')	Reference
Adm	GCCACAGAATGAAGCTGGTT	TTAGCGCCCACTTATTCCAC	Fernandez *et al*., 2008[72]
Calca	ATGGGCTTCCTGAAGTTCTC	TGGGCTGCTTTCCAAGAT	this study
Calcrl	GCAGAGGAGGTGTATGACTATG	GAGAGCATCAGAGTGGGAAA	this study
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	Hernandez *et al*., 2015[73]
Nmu	GCAGAATACCAGAGTCCTTCC	CTTGTTGACCTCTTCCCGTT	this study
Ramp1	CTCACCATCTCTTCATGGTCAC	CACCATAGCGTCTTCCCAATAG	this study
Ramp2	TCTCCGGAGTCCCTGAAC	TTTGACACAAGGCTGTCCTC	this study
Ramp3	TCGCTGACATGATGCAGAAG	CCATGATGTTGGTCTCCATCTC	this study
Vip	TGCAGAATCCCTTAGCAGAAA	GCATCAGAGTGTCGTTTGATTG	this study

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Chapter 5: General Discussion

According to the World Health Organization, as of 2016, an estimate of over 339 million people globally suffered from asthma resulting in over 400,000 deaths each year[1,2]. Asthma thereby represents one of the major non-communicable diseases worldwide. Over 50% of severe asthma patients exhibit allergic eosinophilic airway inflammation which is driven by an excessive type 2 immune response against inhaled allergen[3,4]. ILC2s were shown to play a major role in the induction of allergic lung inflammation in mice upon exposure to model allergens such as papain, *A. alternata*, chitin and house dust mite[5-8]. In addition, frequencies and numbers of ILC2s in peripheral blood as well as sputum and bronchoalveolar lavage fluid were elevated in asthmatic patients and produced more type 2 signature cyotkines[9-15]. The potential importance of ILC2s in human allergic airway disease is further highlighted by multiple large-scale GWAS that link variations in both IL-33 and ST2 to asthma susceptibility and pathophysiology[16-19].

Despite impressive progress during the past years in understanding ILC2 biology, many aspects of how ILC2s are regulated during allergic disease remain controversial or unanswered. Thus, further elucidating the unique aspects of pulmonary ILC2 regulation will be critical to pave the way for the design and development of novel therapeutic intervention strategies to counteract ILC2-driven type 2 immunopathologies such as asthma. Hence, the objective of this thesis was to investigate mechanisms of ILC2 regulation and thereby reveal novel potential therapeutic targets and treatment options.

5.1. Summary of main results and therapeutic implications

The first aim of this thesis was to examine the role of the NF-κB transcription factor c-Rel during IL-33-mediated ILC2 activation *ex vivo* and upon allergic airway inflammation. Here, we demonstrated that c-Rel promotes lung inflammation in an ILC2-driven murine model of allergic airway inflammation. Furthermore, we showed that c-Rel is activated following IL-33-induced ILC2 activation and directly modulates ILC2 gene expression. Specifically, c-Rel directly regulated expression 4-1BBL on ILC2s *ex vivo* and *in vivo* following intranasal IL-33 or allergen challenge. Moreover, we described that expression of the cognate 4-1BBL receptor, 4-1BB, is induced upon allergic airway inflammation on a distinct airway epithelial cell subset as well as

Tconv and Treg cells suggesting a novel cellular ILC2 crosstalk. Taken together, we identified c-Rel as a novel modulator of transcriptional ILC2 regulation and showed for the first time that ILC2s express the co-stimulatory molecule 4-1BBL upon activation. Our data using *Rel*$^{-/-}$ mice suggests that c-Rel promotes acute ILC2-driven allergic airway inflammation and thereby represents a promising target for the treatment of allergic airway disease. Therefore, evaluating the effect of established c-Rel inhibitors on ILC2-driven allergic airway inflammation would be of great interest and targeting of the 4-1BB-4-1BBL axis with respective blocking antibodies may represent a novel approach regarding the treatment of allergic lung inflammation. Elucidating the role of 4-1BBL in asthma is of particular importance in the light of agonizing anti-4-1BB antibodies currently being evaluated in clinical trials as cancer immunotherapeutics[20]. Here, the possibility that these agents could promote ILC2 responses and associated type 2 inflammation should therefore be considered and carefully investigated.

The second aim of this thesis was to decipher mechanisms of IFN-I-mediated pulmonary ILC2 inhibition. We revealed that CCL1 expression and production in lung ILC2s is inhibited by IFN-I and moreover demonstrated that the cognate CCL1 receptor CCR8 is downregulated on pulmonary ILC2s upon IFN-I stimulation *ex vivo* as well as upon infection with influenza A virus infection. CCL1 and CCR8 are important for maintenance of ILC2s and CCR8 was further implicated in lung ILC2 migration during allergic lung inflammation[21,22]. We thereby revealed a new IFN-I-mediated inhibitory mechanism of ILC2s that may be of biological significance and could therefore be exploited as a therapeutic target in the treatment of allergic airway disease.

Finally, we investigated mechanisms of peptide-mediated lung ILC2 regulation and identified the calcitonin-gene related peptide superfamily member adrenomedullin as a novel negative regulator of ILC2 functions. Our research thus highlights that activation of the ADM receptor by agonizing antibodies or direct administration of ADM may present a novel therapeutic intervention strategy for counteracting ILC2-mediated lung pathologies in allergic human disease. Further elucidating the role of ADM in lung inflammation will certainly present an important area of future research.

5.2. Future studies

To further delineate the roles of c-Rel, 4-1BBL as well as ADM- and IFN-I-mediated ILC2 inhibition, several key issues need to be addressed.

To investigate the ILC2-intrinsic effect of c-Rel, in vivo allergic airway inflammation models should be repeated in ILC-deficient mice (*Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$) following adoptive transfer of WT or *Rel*$^{-/-}$ ILC2s rather than using full body c-Rel knock-out mice. Furthermore, knockout animals are available lacking either 4-1BBL or 4-1BB and hereby the ILC2-intrinsic role of 4-1BBL may be determined after adoptive transfer of WT or 4-1BBL-deficient ILC2s into animals lacking ILC2s. To further determine the functional relationship between 4-1BBL^{+} ILC2s and 4-1BB^{+} T cell subsets and epithelial cells, sc-RNA-seq analysis following IL-33 and/or *A. alternata* administration is key. Differentially expressed genes in the respective cellular subsets compared to their 4-1BB/4-1BBL-negative counterparts will provide critical insights into this potential cross-talk and can be validated in co-culture experiments using 4-1BBL$^{-/-}$ ILC2s or T cells and epithelial cells lacking 4-1BB. Moreover, it is of critical interest to determine whether c-Rel promotes 4-1BBL expression in human ILC2s and whether ILC2s isolated from asthmatic patients display altered expression levels of both c-Rel and 4-1BBL.

Whether IFN-I inhibits migration towards CCL8 in both mouse and human ILC2s will need to be addressed in the future. To this end, migration of IL-33 +/- IFN-treated LifeAct^{+} ILC2s towards a CCL8 gradient through microchannels can be imaged. The effect of IFN-I on T cell migration towards CCL8 would be of additional interest. Moreover, intranasal co-administration of IFN-I and IL-33 and subsequent determination of lung CCL1 and CCL8 expression levels as well as CCR8 expression on ILC2s will reveal the direct effect of IFN-I on ILC2-driven allergic airway inflammation. Ultimately, intravital imaging of the lung should be performed following IL-33- or IL-33 + IFN-I administration to track positioning and migration of pulmonary ILC2s *in vivo*.

Future experiments for chapter 4 should address whether ADM inhibits IL-33-induced ILC2 proliferation (Ki-67 or proliferation dye staining) and/or ILC2 survival (viability + Annexin V staining). Depletion of endogenous ADM with an anti-ADM antibody would give further insights into the homeostatic role of ADM on ILC2s. In addition, ADM could be co-administered intranasally alone or in combination with IL-33 or *A. alternata* to determine its effect on steady state versus activated lung ILC2s. Finally, to obtain detailed mechanistic insights into ADM-

mediated ILC2 inhibition, RNA-seq analysis of ILC2s stimulated with IL-33 in the presence or absence of ADM should be carried out.

5.3. Conclusions

ILC2s were appreciated as key players in allergic disease shortly after their discovery a decade ago. Since then, as our knowledge of the biological scope and complexity of ILC2s and their functions and regulatory pathways increases so too has our understanding on their importance in allergic airway inflammation. The thesis presented here reveals several novel strategies to target pulmonary ILC2s and thereby alleviate allergic lung inflammation. We additionally describe a novel potential cross-talk between ILC2s and T cells and/or epithelial cells with may serve as an additional target in the future. Whether inhibition of c-Rel, modulation of the ADM pathway and type IFN signaling may be of clinical significance will need to be determined in thorough investigations involving the use of *in vitro* cultures of human ILC2s as well as allergic airway inflammation models in humanized mice before eventually moving into clinical studies to determine safety and efficacy.

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