MEDICAL UNIVERSITY OF VIENNA

From Birth to Adult Life: Innate Lymphoid Cells Type-2 Shape the Lung Immune Environment

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Declaration

The doctoral candidate, Simona Saluzzo, conducted her PhD studies under the supervision of Prof. Dr. Sylvia Knapp, PhD at the (i) Department of Medicine I, Laboratory of Infection Biology, Medical University of Vienna and (ii) the Research Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences. Additionally, part of her PhD studies was conducted abroad, at the Laboratory of Molecular Biology (LMB) of the Medical Research Center (MRC), University of Cambridge, UK, where the doctoral candidate spent 7 months as a research fellow under the supervision of Dr. Andrew McKenzie. The work abroad was granted by the FWF-funded PhD program Cell Communication in Health and Disease (CCHD) at the Medical University of Vienna.

For the publication "First-breath-induced type 2 pathways shape the lung immune environment" included in chapter 2 of this thesis, Simona Saluzzo performed the majority of experiments, analyzed the data and wrote the manuscript. Sylvia Knapp funded the project, supervised the work and wrote the manuscript. Andrew McKenzie provided intellectual input and supervised the experiments conducted in Cambridge. Anna-Dorothea Gorki, Batika Rana, and Philipp Starkl performed some of the in-vivo experiments. Rui Martins and Seth Scanlon provided assistance with the confocal microscopy and image processing. Omar Sharif and Joanna Warszawska provided intellectual inputs and assistance for the *in vivo* and *in vitro* experiments with *S. pneumoniae*. Karin Lakovits, Anastasiya Hladik and Ana Korosec provided technical assistance with *in vivo* and *in vitro* experiments.

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Abstract

At the moment of birth, the lung tissue is suddenly exposed to the external environment, inflated with air and subjected to dramatic vascular and anatomical changes. Little is known about the immunological environment at this delicate moment of life and its consequence on the phenotype and function of lung immune cells. Since a few players of the innate immune system already reside in the lung during embryogenesis, we hypothesized the existence of an orchestrated immune response at birth that would create a specific birth-induced immune environment in the lung and regulate the massive perinatal immune cell infiltration. We therefore investigated the role of one of the most potent epithelium-derived cytokine known in the lung, the alarmin interleukin-33 (IL-33), which is able to respond to mechanical stress. Working with newborn mice, we discovered a wave of IL-33 upregulation starting at birth, which activated tissue resident embryonic type 2 innate lymphoid cells (ILC2s), and lasted for the first three weeks of life. The beginning of ILC2 expansion coincided with the appearance of alveolar macrophages (AMs) on postnatal day 3. ILC2-derived IL-13 induced their early *in-tissue* polarization to an anti-inflammatory M2 phenotype. By postnatal day 14, newborn AMs from WT mice were less able to mount an inflammatory immune response against the pathogen *Streptococcus pneumoniae* (*S. pneumoniae*) when compared to AMs extracted from IL-13 or ILC2 deficient mice. The ILC2 and IL-13 dependent phenotype acquired perinatally was actively maintained at homeostasis later in life by the IL-33/ILC2s axis. In fact, monocyte progenitors maintained an intrinsic M1 phenotype when transferred to an ILC2 deficient or IL-13 deficient mouse recipient, proving the role of ILC2s and IL-13 in inducing a homeostatic polarization of AMs also in the adult lung at steady state. This homeostatically driven type-2 environment comes at the cost of a delayed response to *S. pneumoniae* infection. In fact, ILC2s and IL-13 deficient mice presented an increased bacterial clearance of *S. pneumoniae* and *Staphylococcus aureus*. Our work provides a new homeostatic role of ILC2s in the adult lung and its role in setting the threshold of immune activation against bacterial lung infections. Moreover, it describes for the first time the presence of an active immune response to birth, characterized by IL-33 upregulation and ILC2 activation in the newborn lung. This pathway could help explaining the susceptibility of newborns to allergy and lung infections.

Kurzfassung

Bei der Geburt wird das Lungengewebe plötzlich der äußeren Umgebung ausgesetzt, durch Lufteinstrom entfaltet und dadurch dramatischen vaskulären und anatomischen Veränderungen unterworfen. Über die immunologische Umgebung in diesem heiklen Moment des Lebens und über den Phänotyp und die Funktion lungenresidenter Immunzellen ist wenig bekannt. Da einige Spieler des angeborenen Immunsystems bereits bei der Embryogenese in der Lunge vorhanden sind, stellten wir die Hypothese auf, dass die Geburt eine orchestrierte und spezifische Immunreaktion in der Lunge auslöst. Wir untersuchten daher die Rolle eines der bekanntesten von Epithelzellen produzierten Zytokine, Interleukin-33 (IL-33), das als Antwort auf mechanischen Stress gebildet wird. Bei der Arbeit mit neugeborenen Mäusen entdeckten wir die sofortige Hochregulation von IL-33 vom ersten Lebenstag an, gefolgt von einer Welle von IL-13 produzierenden und im Gewebe proliferierenden angeborenen Typ-2-Lymphozyten (ILC2s), die die ersten drei Wochen nach der Geburt andauerte. Der Beginn der ILC2-Expansion fiel mit dem Auftreten von Alveolarmakrophagen (AMs) am postnatalen Tag 3 zusammen und induzierte ihre frühe *in situ* Polarisation zu einem IL-13-abhängigen, entzündungshemmenden M2-Phänotyp. Am postnatalen Tag 14 waren neugeborene AMs von WT-Mäusen weniger imstande, eine entzündliche Immunantwort gegen den Erreger *Streptokokkus pneumoniae* (*S. pneumoniae*) zu erzeugen, im Vergleich mit AMs, die aus IL-13- oder ILC2-defizienten Mäusen extrahiert wurden. Der perinatal erworbene ILC2- und IL-13-abhängige Phänotyp, wurde später im Leben durch die IL-33/ILC2s-Achse aktiv homöostatisch aufrechterhalten. Tatsächlich behielten Monozyten einen intrinsischen M1-Phänotyp, wenn sie in eine ILC2-defiziente oder IL-13-defiziente Empfänger-Maus übertragen wurden, was die homöostatische Rolle von ILC2s und IL-13 in der Aufrechterhaltung der Polarisierung von AMs auch in der erwachsenen Lunge untermauert. Diese homöostatisch gesteuerte Typ-2-Umgebung ging auf Kosten einer verzögerten Reaktion auf *S. pneumoniae*-Infektion. Tatsächlich zeigten ILC2- und IL-13-defiziente Mäuse eine erhöhte bakterielle Clearance von *S. pneumoniae* und *Staphylokokkus aureus*. Unsere Arbeit liefert eine neue homöostatische Rolle von ILC2s bei der Einstellung der Schwelle der Immunaktivierung gegen bakterielle Lungeninfektionen. Darüber hinaus beschreiben wir zum ersten Mal das Vorhandensein einer aktiven Immunantwort als Reaktion auf die Geburt, die durch IL-33-Hochregulation und ILC2-Aktivierung in der neugeborenen Lunge charakterisiert ist. Dieser Signalweg kann dabei helfen, die Anfälligkeit von Neugeborenen für Allergien und Lungeninfektionen zu erklären.

Publications arising from this thesis

First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment.

Simona Saluzzo, Anna-Dorothea Gorki, Batika M.J. Rana, Rui Martins, Seth Scanlon, Philipp Starkl, Karin Lakovits, Anastasiya Hladik, Ana Korosec, Omar Sharif, Joanna M. Warszawska, Helen Jolin, Ildiko Mesteri, Andrew N.J. McKenzie and Sylvia Knapp

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At the time of discussion, the publication was cited in 12 publications, in particular in the following reviews:

- "The immunology of the allergy epidemic and the hygiene hypothesis", B. Lambrecht and H. Hammad, *Nature Immunology*, 2017.
- "Development of allergic immunity in early life", Clare M. Lloyd et al., *Immunological Reviews*, 2017.
- "Innate lymphoid cells: Major players in inflammatory diseases", Ebbo M, *Nature Reviews Immunology*, 2017.
- "Interplay of innate lymphoid cells and the microbiota", L. Britanova and A. Diefenbach, *Immunological Reviews*, 2017.
- "Recent Advances in Type-2-Cell-Mediated Immunity: Insights from Helminth Infection", N.L. Harris, *Immunity*, 2017.
- "Pulmonary Susceptibility of Neonates to Respiratory Syncytial Virus Infection: A Problem of Innate Immunity?", C. Drajac, D. Laubreton, S. Riffault, and D. Descamps, *Journal of immunology research*, 2017.
- "Sentinels of the Type 2 Immune Response" J von Moltke, M Pepper, *Trends in immunology*, 2017.

Abbreviations

AD	Atopic dermatitis
AEC1	Alveolar epithelial cells type 1
AEC2	Alveolar epithelial cells type 2
AMs	Alveolar macrophages
ANOVA	One-way analysis of variance
APC	Antigen presenting cell
Arg-1	Arginase 1
BALF	Bronchoalveolar lavage fluid
BMDM	Bone marrow derived macrophages
CAP	Community acquired pneumonia
CCHD	Cell Communication in Health and Diseases
CCR	CC chemokine receptor
CeMM	Center of Molecular Medicine of the Austrian Academy of Sciences
CFU	Colony forming unit
Chil3	Chitinase 3-like protein 3
CHILP	Common helper ILC progenitor
CLR	C-type lectin receptor
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CSF-2	Colony stimulating factor type 2
CXCR	CXC chemokine receptor
DAMPS	Damage associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMPs	Erythro-myeloid precursors
E*n*	Embryonic day *n*
EOMES	Eomesodermin
FACS	fluorescence-activated cell sorting
FALC	fat associated lymphoid clusters
FIZZ1	Found in inflammatory zone 1

Foxp3	Forkhead-box P3
GATA3	GATA-binding protein 3
GITR-L	Glucocorticoid-induced tumour necrosis factor receptor-related gene
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association studies
HDM	House dust mite
HIV	Human immunodeficiency virus
HSCs	Hematopoietic stem cells
ICOS	Inducible T-cell costimulator
ICOS-L	ICOS-Ligand
Id2	Inhibitor of DNA binding 2
IFN-γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IL2Rgc	IL-2 receptor common γ-chain (cγ)
ILC	Innate lymphoid cell
ILC1	Innate lymphoid cell type 1
ILC2	Innate lymphoid cell type 2
ILC3	Innate lymphoid cell type 3
ILCP	ILC progenitor
IRAK4	Interleukin 1 receptor-associated kinase 4
IRF	Interferon refulated factor
Jak	Janus kinase
KIR	Killer-cell immunoglobulin like receptors
KLRG1	Killer cell lectin-like receptor G1
LC	Langerhans cells
LPS	Lipopolysaccaride
mAb	Monoclonal antibody
MARCO	Macrophage receptor with collagenous structure
MHC	Major histocompatibility complex
mmHg	Millimeter of mercury
MR1	Mannose receptor 1
Mrc1	Mannose receptor 1
mRNA	Messenger RNA

MyD88	Myeloid-differentiation primary-response protein 88
NCR	Natural cytotoxicity receptors
NF-HEV	Nuclear-factor for high endothelial venules
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer
NK	Natural killer cells
NO	Nitric oxide
PAMPS	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PGC1β	PPARγ coactivator 1β
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
PMs	Peritoneal macrophages
P*n*	Post-natal day *n*
PPARγ	Peroxisome proliferator–activated receptor γ
PPARδ	Peroxisome proliferator–activated receptor δ
PRR	Pathogens recognition receptor
PVR	Pulmonary vascular resistance
RA	Rheumatoid arthritis
Rag	Recombination activating gene
Retnla	Resistin like a
RNA	Ribonucleic acid
RORα	Retinoic acid receptor-related orphan receptor-α
RORγt	Retinoic acid (RA)- related orphan receptor γ isoform t
RPMI	Roswell Park Memorial Institute
RT-PCR	Real time polymerase chain reaction
S. aureus	Staphylococcus aureus
S. pneumoniae	Streptococcus pneumoniae
SEM	Standard error of the mean
SIGLEC-F	Sialic acid-binding immunoglobulin-like lectin F
SIRPa	Signal regulatory protein-a
SNPs	Single nucleotide polymorphism
SR-A	Scavenger Receptor A
sST2	Soluble ST2
ST2	Suppression of tumorigenicity 2

STAT	Signal transducers and activators of transcription
SVR	Systemic vascular resistance
TGFb	Transforming growth factor B
Th1	T Helper cells type 1
Th17	T Helper cells type 17
Th2	T helper cells type 2
TL1A	TNF-like ligand 1A
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
Tregs	Regulatory T cells
TRMs	Tissue resident macrophages
TSLP	Thymic stromal lymphopoietin
TYK	Thyrosine-protein kinase
VIP	Vasoactive intestinal peptide
WAT	White adipose tissue

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When I first arrived in Vienna, back in October 2011, I was a freshly baked medic with little lab experience and very much unaware of the potentiality of a PhD. I was determined to learn and to work with Sylvia, who decided to believe in me. I will never forget her kindness and her encouraging words. I was lucky to be part of the CCHD-PhD program, which became a bit of a family for me, and at the same time to be affiliated with the fresh and international scientific environment of CeMM, which inspired me with smart thinking and high-quality output, and where I learned how to present and communicate data at the very challenging "Friday Seminars". Back in 2012, Johanna Warszawska was the FACS-Queen of Sylvia's Lab and the one introducing me to the world of alveolar macrophages, IL-13 and IL-33. She supervised me throughout my first PhD year and that's the time I learned what it meant to work in a lab. I can't forget her precision and correctness and the care she took to explain me every single step of any experiment and how important also small things can be. I remember the first years of my PhD as some of the best of my life. I realized the value of scientific thinking and the freedom of thought that only basic research can give, bringing one in the most unexpected fields and never stops surprising. I want to thank Omar Sharif, Joanna Warszawska, Riem Gawish and Rui Martins for the hours spent talking about experiments and in general all the members of Sylvia's lab for these beautiful memories and friendship.

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IL-33, proving the importance of the mechanical stimulus for IL-33 upregulation upon the first breath. But most importantly, proving that you have to be a bit crazy in science!

When I went back to Vienna I was full of ideas and ready to perform tons of experiments that finally lead us to the preparation of the manuscript. I want to additionally thank Dorothea Gorki, Rui Martins and Philipp Starkl, Karin Lakovits, Ana Korocek and Anastasiya Hladik, for their amazing and generous support during the revision times, when we had to rush to perform the last experiments and they never let us down. From the Cambridge side, Batika Rana, Helen Jolin and Seth Scanlon were also the heroes of the revision experiments. I consider myself very lucky to have been able to work with such amazing, smart and generous people.

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1. Introduction

The sudden lung expansion due to the first breath, along with the dramatic circulatory changes in the early hours after birth, likely play a role in driving the early lung immune signature. Some of the key players of pulmonary immunity, like interstitial macrophages and alveolar epithelial cells type 2 (AEC2), are already present in the lung tissue during late embryogenesis (Hussell and Bell, 2014; Treutlein et al., 2014). However, the perinatal lung immune environment and its implication in human diseases is largely understudied. The work presented in this thesis is one of the first attempts to understand how birth could affect the immunological environment and if the perinatal changes can carry implications in the homeostasis and immune response against *Streptococcus pneumoniae* (*S. pneumoniae*), one of the deadliest pathogens in the history of humanity (van der Poll and Opal, 2009). The study of the early pulmonary immunity can also provide a better comprehension of the origin of the dysregulation of perinatal immune responses, which can lead for example to childhood asthma.

Immune responses to external pathogens are often divided in type-1 and type-2, according to the main cell type, cytokine and immunoglobulin subtype involved in pathogen elimination (Licona-Limón et al., 2013). The type-1 immune response is typically triggered in defense to viral, bacterial and fungal pathogens and is mediated by T helper cells type 1 (Th1) and Th17 cells secreting pro-inflammatory cytokines like interferon (IFN) alpha, beta, gamma or tumor necrosis factor (TNF) as well as interleukin (IL)-1, IL-6, IL-8, IL-17 - to mention a few. Type-1 immune responses are moreover characterized by the activation of CD8+ cytotoxic T cells (CD8+ T), which recognize and kill infected cells, and by the production of antibodies of the IgG class by B cells. On the other side of the coin, type-2 immune responses are mainly induced by large extracellular parasites, like helminths, and see the polarization of naive T cells to a Th2 phenotype characterized by the secretion of cytokines like IL-4, IL-5, IL-13 and the recruitment of eosinophils to the site of infection, as well as the class switch to the production of IgE by B cells. Until recently, before the discovery of Innate lymphoid cells type 2 (ILC2s) in 2010, type 2 immune responses were considered to be strictly antigen dependent and therefore a feature of adaptive immunity only (Neill et al., 2010a). ILC2s are new innate players of the tye-2 immune responses and can secrete high amounts of IL-13 and IL-5 in response to epithelial damage triggered by large parasites or airborne particles without the need of specific antigen recognition. Moreover, recent evidence suggest that ILC2s and in general innate type-2 immune responses play important roles in tissue homeostasis and metabolism (Ebbo et al., 2017).

This thesis relates to several topics in the fields of lung immunity, type 2 immune responses, immune homeostasis at tissue level and host-pathogen interaction. In the following chapter I will briefly summarize the lung embryogenesis until birth with a particular focus on the known immunological players at birth. The second chapter outlines the biology of tissue resident macrophages (TRMs) and the mechanisms of macrophage polarization, as well as the biology of one important subsets of TRMs in this thesis, i.e. the alveolar macrophages (AMs). The third chapter provides a summary of the up-to-date knowledge on innate lymphocytes, focusing on the biology of innate lymphoid cells type 2 (ILC2s) in homeostasis and disease. Finally, the fourth chapter explores the basics of the immune response to pneumococcal pneumonia. Together, the following introduction should provide the background information necessary to understand the aims of this thesis.

1.1. The lung development, first breath, alveolarization

By the vital and yet mostly unaware act of breathing we expose the airways to the potential threats of environmental air, particulate matters, oxygen, allergens and microbes. The surface available for gas exchange comprises on average 140 m^2, filtering daily around 8000-9000 liters of air (Kopf et al., 2014). At homeostasis, resident immune cells populate the lung tissue at steady state at any level of its arborization, from the mucosal layer of the trachea, deep into the finest layers of the alveolar-capillary-barrier, at the site of oxygen exchange. Their *in situ* phenotype at steady state and their threshold of activation in case of pathogen encounters is in fine balance with tissue remodeling functions and the needs to maintain the delicate alveolar architecture. However, during embryogenesis, the lung is forming in a sterile environment and it is subjected to completely different physical forces compared to life in the outside world. At the time of birth, dramatic changes in the lung elasticity, perfusion and immune cell infiltration take place, which could potentially affect the lug immunity forever.

1.1.1. Lung embryonic development

Lung development starts very early in embryogenesis, around 4-5 week of gestation in humans and at embryonic day (E) 9.5 in mice. It begins with the formation of the primary lung buds at the ventral wall of the primitive forgut, together with the primitive trachea (Figure 1) (Herriges and Morrisey, 2014; Schittny, 2017). This structure is formed by endodermal epithelium, has a simple vascular plexus and it is surrounded by mesoderm. Both

endoderm and mesoderm contribute to the morphogenesis of the lung. After the initial budding, the lungs forms through the processes of branching morphogenesis and septization. Branching morphogenesis consists in a repetitive tissue branching and growth in the surrounding mesenchyma, with the formation of conducting airways and part of the respiratory airways. This developmental strategy is also adopted for the development of glands (salivary, mammary, pancreas). The process of branching morphogenesis provides the formation of only 10 percent of the gas exchange areas. In fact, 90 percent of the surface area develops later during the fetal period and also postnatally, through septation, the main morphological mechanism of alveolar formation, also called "alveolarization" and is characterized by the formation of new alveolar septa. Septa are new inter-alveolar crests of fibrotic tissue covered by epithelial columns and sustained by a double layered capillary network. This process is paired with the maturation of the alveolar microvasculature (Woik and Kroll, 2015).

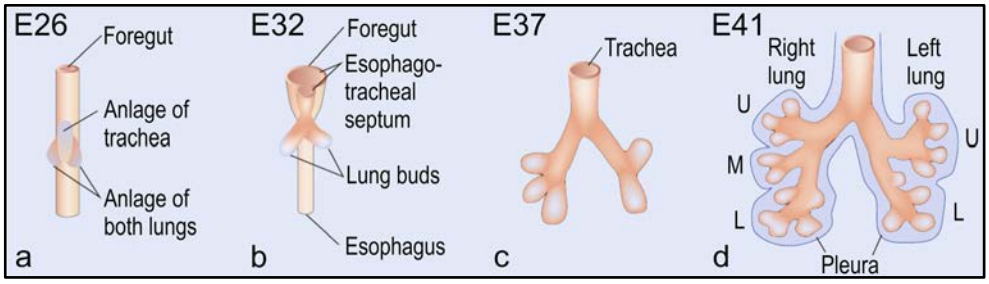


Figure 1 – The early stages of human lung development (Schittny, 2017).

Lung development can be divided into three main periods, mainly based on morphological criteria (Schittny, 2017):

- Embryonic phase: week 4-7 in humans, E9.5-E12 in mice. It is characterized by the development of the lung buds and the formation of the major airways and pleura.
- Fetal phase: week 5-38 in humans, E12-postnatal day 4 (P4) in mice. Characterized by the differentiation of alveoli and can be further divided in:
    - Pseudoglandular stage: week 5-17 in Human, E12-E16.5 in mice. This period sees the formation of the bronchial tree until the formation of a primitive respiratory acinus.
    - Canalicular stage: week 16-26 in humans, E16.5-17.5 in mice. Completion of the branching morphogenesis, with formation of the mot distal airways and development of the acinar epithelium.

- Saccular stage: week 24-38 in humans, E17.5-postnatal (P) day 4 in mice. This stage is characterized by the initial expansion of the gas exchange area through the formation of thick immature septa.

- Postnatal phase: week 36 until 3 years of age in humans, P4-P21 in mice. This is the phase of actual alveolarization, which begins by out-pocketing of the alveoli into the surrounding extracellular matrix at the level of the respiratory bronchioles. The alveoli are first lined with cuboidal epithelium and later flattened to form the alveolar epithelium and alveolar blood barrier. The actual alveolarization and microvascular maturation and can be further divided in:
  - Classical alveolarization: 36 weeks preterm-3 years in humans, P14-P36 in mice. This is a period of intense septization, however capillary network is still double layered and alveolar septa still immature.
  - Continued alveolarization: 2 years - young adulthood in humans, P14 young adulthood in mice. Reduction of the interstitial volume, final microvascular maturation, with the maturation of septa and alveoli characterized by single layered capillary network to optimize gas exchange.

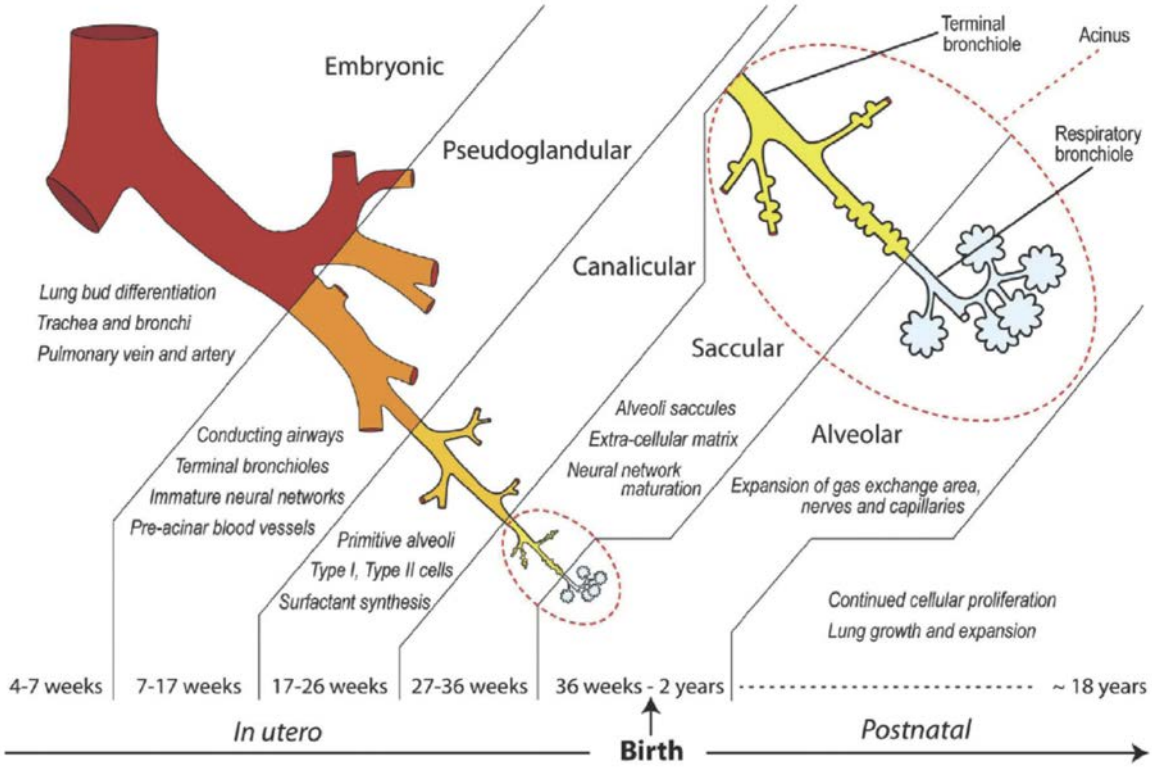


Figure 2 – Stages of lung development (Miller and Marty, 2010).

The first cells with a potential immunological role in the embryonic lung start appearing with the formation of the alveolar capillary barrier during the canalicular phase around E16.5 and E18.5 in mice. In fact, this is the time in which alveolar epithelial cells type 2 (AEC2) and type 1 (AEC1) differentiate from bipotential progenitors (Hogan et al., 2014; Schittny, 2017). AEC1 form a thin layer that covers the internal part of the alveolar ducts and of the sacculi. At this level, the endothelium of the capillary system starts forming a thick network around the primitive alveoli and enters in close contact with the AEC1, forming the basis of the future air-blood barrier. AEC2 are intercalated between AEC1 and soon after their development start producing surfactant liquid. Reaching the canalicular phase is very important for the survival of pre-term infants, as the primitive alveolar-capillary barrier is formed and surfactant is produced, avoiding alveolar collapse during respiration and promoting lung tissue elasticity (Schittny, 2017).

1.1.2. Fetal circulation and cardiovascular changes at birth

At the moment of birth, great changes occur in the circulatory system of the lungs, which are important for acquiring the primary function of oxygenation and gas exchange. Pulmonary vascular resistance (PVR) is the resistance that the blood must overcome in order to flow in the blood vessels. The more vasoconstricted the vessels are, the more difficult it will be for the blood to flow and therefore the higher the PVR will be. On the contrary, vasodilation decreases the resistance. PVR already changes during embryogenesis, because of the changes determined by the development and refinements of the fetal vascularization (Figure 3). In fact, during fetal life, the PVR is high and little blood circulates in the lung. On the contrary, the resistance is very low in the placenta, the main organ of gas exchange in the fetus (Lakshminrusimha and Saugstad, 2016). At 20 weeks, during the canalicular phase, the primitive pulmonary capillary net surrounding the premature alveolar space cannot allow high blood flow and therefore, vascular resistance in the lung is at the highest. With the development of the double capillary structure and the general increase of blood vessels during the saccular stage (30 weeks), the PVR decreases and about 25 to 30% of the cardiac blood output starts flowing to the lung (Figure 3). At this point, the heart and the lung are preparing themselves to birth.

At 38 weeks of gestation, during the alveolar phase and just in the pre-birth phase, the pulmonary vasculature develops a remarkable sensitivity to oxygen and starts sensing hypoxia. This causes a vasoconstrictory response and therefore the slow but constant increase

of the PVR along with the increased of the vasculature and alveolar surface that characterize this phase (Figure 3). Finally, at birth, ventilation causes a sudden expansion of the thorax and because of the negative pressure that is created by the pleura, the capillaries and the alvoli will be mechanically expanded and stretched to allow air flow. At this point a sudden vasodilation and sharp drop in the PVR occurs, which together with the cut of the cord blood and therefore of the placental circulation, will be cut and meaning that the cardiac output will flow almost entirely to the lungs. With the removal of the placental circulation, the systemic vascular resistance (SVR) increases, further reducing the PVR (Figure 3).

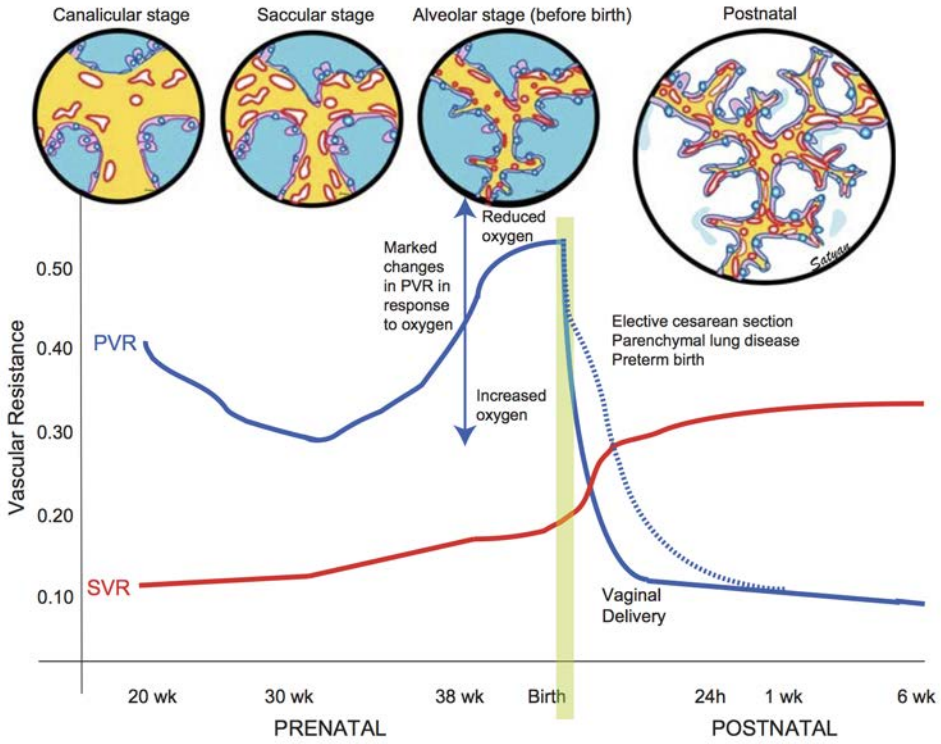


Figure 3 – Changes in systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) during gestation. (Lakshminrusimha and Saugstad, 2016).

The mode of delivery also affects the lung vascular resistance: a vaginal birth is associated with a rapid reduction of the PVR whereas birth with elective cesarean section promotes a slower drop in the PVR. Also a delayed cord clamping, as a result of the "placental transfusion", a technique recently more and more used to reduce the risk of newborn jaundice, delays the physiological drop of PVR (Lakshminrusimha and Saugstad, 2016). The immunological impact of these sudden changes in blood flow in the alveolar capillary barrier are completely unknown.

1.1.3. Postnatal refinement of the alveolar capillary barrier

In both mice and humans, the alveolarization, i.e. the final step of the development of the alveolar capillary barrier, happens postnatally with the processed of septization and thinning of the alveolar septa as well as maturation of the vasculature (Mund et al., 2008). Alveolarization continues many years after birth and reaches a plateau in young adulthood (Mund et al., 2008). In the mouse lung, new septization occurs around post-natal day 4 (Figure 4) suggesting the hypothesis of an interdependence between alveolarization and oxygenation probably thanks to the reactivation of the embryonic programs induced by hypoxia. In fact, recent evidence shows that the alveolarization program can be restarted in case of reduced oxygen intake (Herriges and Morrisey, 2014; Hogan et al., 2014). For example, the sonic Hedgehog signal, which coordinates the tissue-tissue interactions during embryogenesis and has to be actively suppressed to avoid hyper-proliferation, is re-activated in case of hypoxia or lung damage, allowing the proliferation of new alveoli (Peng et al., 2015). These results are supported by the discovery that after pneumonectomy the lung sees a re-growth and re-start of the alveolarization (Butler et al., 2012). Therefore, the healthy lung seems to have a high ability to adapt and change its structure throughout life. However, despite of the scientific progresses in understanding alveolarization, it is still not known how to reactivate a healthy and homeostatic alveolarization process in the context of human diseases, like chronic obstructive pulmonary disease (COPD) or pulmonary emphysema and

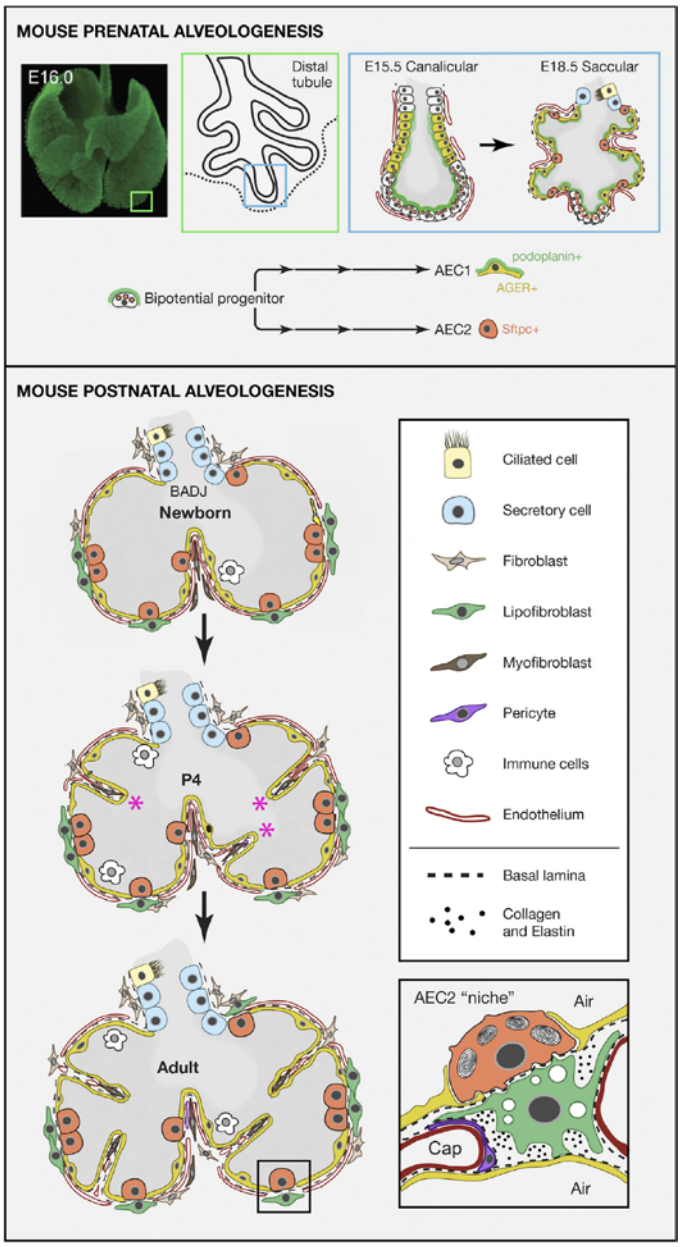


Figure 4 –Postnatal alveologenesis (Hogan et al., 2014).

fibrosis (Hogan et al., 2014). Moreover, the potential contribution of the immune system to the Hedghog signal regulation is also unknown. One of the most recent step forward in the understanding of the immunology of the delicate postnatal period, is the discovery that alveolar macrophages differentiate from fetal monocyte progenitors around postnatal day 3, therefore at the same time in which the final refinement of the alveolar-capillary barrier occurs (van de Laar et al., 2016). AMs are cells that are profoundly shaped by the tissue derived signals of their microenvironment (Okabe and Medzhitov, 2014) and they can exert different functions, like wound healing and tissue remodeling. However, mice without AMs, which develop alveolar proteinosis later in life, do not seem to have important structural malformations in the alveoli after birth (Schneider et al., 2014a). Other important cells that are part of the alveolar niche and that could potentially play immunological roles in the early life are AEC2. These cells secrete surfactant proteins, which are vital to maintain the alveolar stricture during respiration and avoid collapse, and function as long term stem cells, giving rise to AEC1, therefore contributing to the basic structure of the alveolar space. In the next chapters I will focus more in details on AEC2s and their known biological roles in embryogenesis and postnatal life. The biology of AMs will be the subject of the next introductory chapter.

1.1.4. Development and function Alveolar epithelial cells type 2

At the time of the late saccular stage (in mice between E16.5-E18.5 and in humans around Week 16-21), the cells composing the future alveolar space and blood capillary barrier are at place (Figure 4). Among those, AEC1, AEC2 and capillary endothelial cells have been shown to react to pathogen-derived stimuli in adult lungs, since they are well equipped with receptors that can recognize pathogens or damage associated molecular patterns (PAMPS and DAMPS). AEC1 and AEC2 can therefore play a role in the immune defenses as well in sensing damage signals coming from the environment, potentially being able to orchestrate the early innate immune reaction to birth (Whitsett and Alenghat, 2015). In particular, AEC2 can release cytokines, chemokines and anti-microbial peptides like lysozyme and defensins, contributing to early innate immune defenses against invading pathogens. Moreover, AEC2 help with the bacterial clearance via the production of SP-D or via direct phagocytosis of bacteria or apoptotic neutrophils, therefore also contributing to the resolution of inflammation during infection (van der Poll and Opal, 2009). Interestingly, for what concerns AEC2, it seems that their ability to respond and integrate danger signals is already established during

embryogenesis. It was recently shown that, the development of AEC2 is linked with the upregulation of genes involved in immune responses. In fact, using single cell sequencing of lung epithelial cells in distal lung regions during the late saccular phase (E18.5) Treutlein and coauthors (Treutlein et al., 2014) were able to identify five different cell populations composing the alveolar niche at this stage, in particular Clara cell, ciliated cells, AEC1, AEC2 and intermediate AEC1-AEC2. The analysis brought to the identification of a set of gene specific for AEC1 and AEC2. Interestingly, AEC2, which were enriched in the gene encoding surfactant protein C gene (*Sftpc*), also expressed increased amounts of genes encoding for the PPAR-γ signaling as well as lysosome pathways. Moreover, among the genes that best characterized AEC2 maturation were (Figure 5):

- Lcn2, Lipocalin2
- Il33, Interleukin 33
- Hc, Complement 5
- Trf, Serotransferrin
- Lyz1, Lysozyme 2
- S100g, Serum calcium binding protein G
- Lyz1, Lysozyme 1

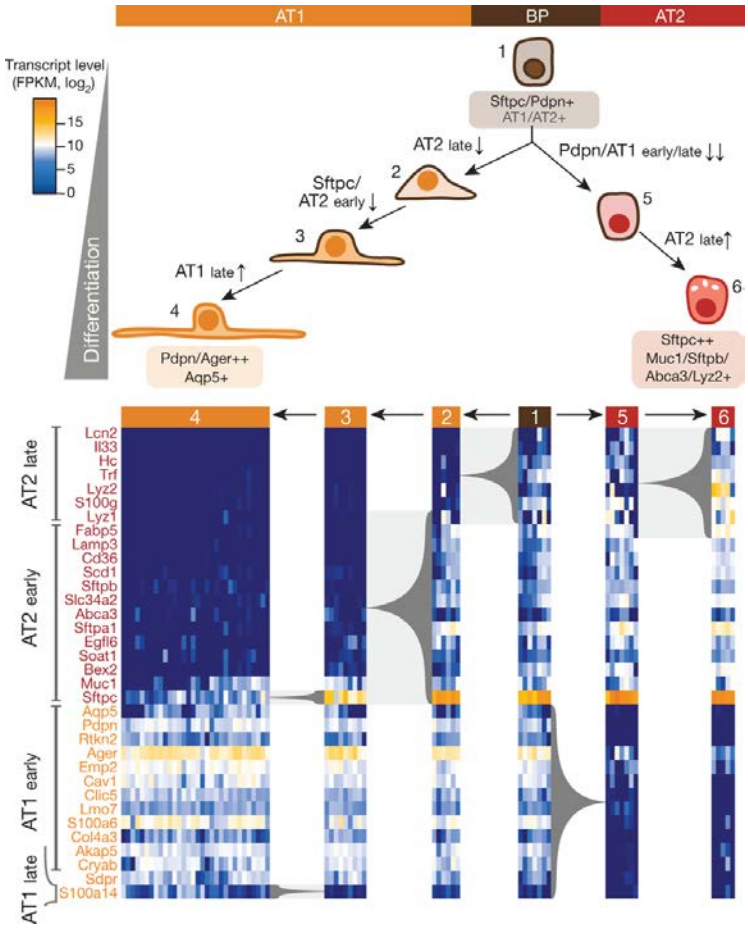


Figure 5 – Single cells transcriptome analysis of AEC1, AEC2 and Bipotential progenitors (Treutlein et al., 2014)

These results highlight the role of AEC2 in bacterial defenses and suggest that AEC2 might acquire this capacity already before birth. For example, we already described a fundamental role for Lipocalin 2 in defenses against pneumonia (Warszawska et al., 2013). Moreover, the high expression of genes like lysozyme and complement, as well as serotransferrin and S100g highlight a possible the role of AEC2 in early antimicrobial defenses also at birth. Finally, the gene encoding for the alarmin IL-33 was the only cytokine found to be already upregulated before birth and was typically expressed only in the AEC2 subset (Treutlein et al., 2014).

1.1.5. Interleukin-33

IL-33 is an epithelial derived cytokine, one of the latest identified members of the IL-1 family and now considered one of the major player of innate and adaptive immunity at tissue level, contributing to homeostasis, responses to injury or environmental stress (Liew et al., 2016). IL-33 was first identified in 2003 and described as nuclear-factor for high endothelial venules (NF-HEV), which are special endothelial cells localized at the entry site of lymphatic vessel where they allow lymphocytes extravasation from the blood circulation (Lamkanfi and Dixit, 2009). In both human and mouse, the *Il33* gene is localized on the chromosome 9 and consists of seven coding exons which produce a 31kDa protein (of 270 amino acids in humans and 266 in mice) with an N-terminal domain that is necessary for the nuclear localization and chromatin binding (Molofsky et al., 2015). In 2005 it was then discovered that IL-33 is the natural ligand for an important orphan receptor, the suppressor of tumorigenicity 2 (ST2), which was already known as a selective marker for Th2 and mast cells, but not Th1 cells (Liew et al., 2016).

One of the most recent and relevant advances in the biology of IL-33 came from the work of Lüthi et al. (Lüthi et al., 2009), which discovered how this nuclear localized cytokine in epithelial cells is released in case of cell damage and acts as a cytokine in the cell-free space constituting an important "alarm" signal, which binds and activates the ST2 on cells belonging to the classical type-2 immune response (Figure 6). Like other members of the IL-1 family, as IL1β or IL-18, IL-33 is also produced as a biologically inactive precursor, which resides in the nucleus. However, while IL-1β or IL-18 are cleaved and into their biologically active form by inflammation activated caspases, like caspase-1, and then secreted in the cell-free space, this was not the case for the un-cleaved, nuclear resident IL-33, which was rather the substrate of apoptotic caspases, like the caspase-3 and the caspase-7. Moreover, the IL-33 form cleaved by caspase-3 and caspase-7 actually failed to activate the ST2 receptor, while the non-cleaved nuclear IL-33 was perfectly able to bind to the ST2 receptor and activate cells belonging to type 2 immunity. These findings and consecutive confirmatory research revealed that IL-33 acts as an alarmin in all barrier and endothelial surfaces: it normally resides in the nucleus and it is released in case of necrotic cell death or injury, like the one seen in ischemic tissue damage or large parasite infestations (Figure 6).

IL-33 is constitutively expressed in epithelial, endothelial cells and fibroblast (Molofsky et al., 2015). It can be found in all human and mouse organs, and mainly exerts its biological activity at barrier sites, like lung, skin and gut. Importantly, it is expressed in the lung airway

epithelium in humans, whereas in mice it is mostly found in the alveolar epithelial cells type 2 (Liew et al., 2016). Signaling via ST2 on responding cells triggers the expression of the soluble ST2 decoy receptor (sST2), which has a stronger affinity to IL-33 than the cell-bound receptor, can efficiently bind to IL-33 and reduce its potentially lethal biological activity (Liew et al., 2016). In fact, mice treated with IL-33 develop a systemic inflammation characterized by airway hyper-reactivity, enlarged spleen size, mucous secretion in gut and airway, eosinophils infiltration and mast cells degranulation (Lüthi et al., 2009). Interestingly, sST2 is normally circulating in human blood and it is increased in several cardiovascular, rheumatologic and allergic diseases, potentially even serving as a marker for the disease progression in these conditions (Molofsky et al., 2015).

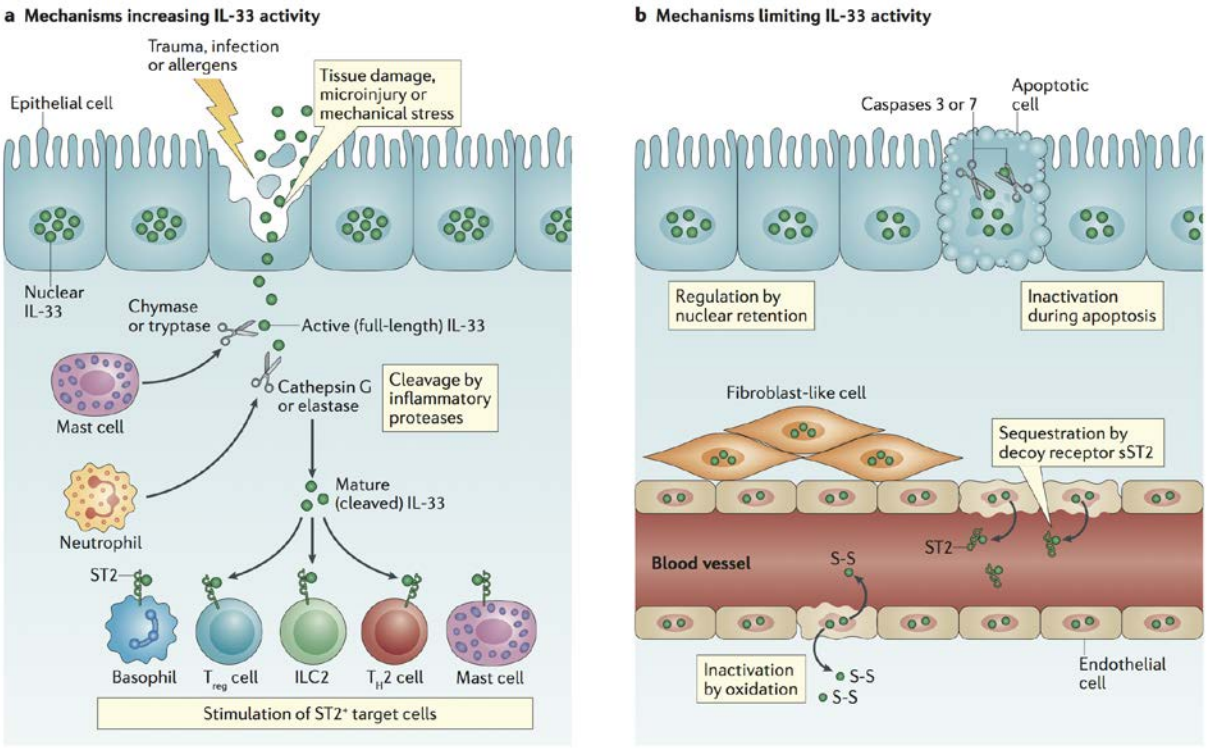


Figure 6 – Regulation of IL-33 secretion (Liew et al., 2016).

In 2010, with the discovery of Innate Lymphoid Cells type 2 (ILC2s), the cytokine IL-33 was again the subject of intense research, as it was identified as ILC2s were recognized as the main cells mediating IL-33 effects at tissue level (Neill et al., 2010b). The role of the IL-33 in the context of lung biology and innate type 2 immune responses in health and disease is a major topic of this thesis and will be further discussed in the dedicated chapters. Before that, I will focus on another important cell type of this story: the tissue resident macrophage.

1.2. Tissue resident macrophages

Tissue resident macrophages (TRMs) are key players of the innate immune system found in virtually in any tissue of the human body (Figure 7). They can assume not only the major role of local sentinels against invading pathogens, but also exert fundamental homeostatic functions at tissue levels (Lavin et al., 2015). In fact, their highly phagocytic capacity grants them the ability to take up pathogens, cellular debris or senescent erythrocytes. Moreover, TRMs are key cells in the regulation of fat metabolism, cold adaptation and tissue remodeling and exert different immunomodulatory functions (Perdiguero and Geissmann, 2015).

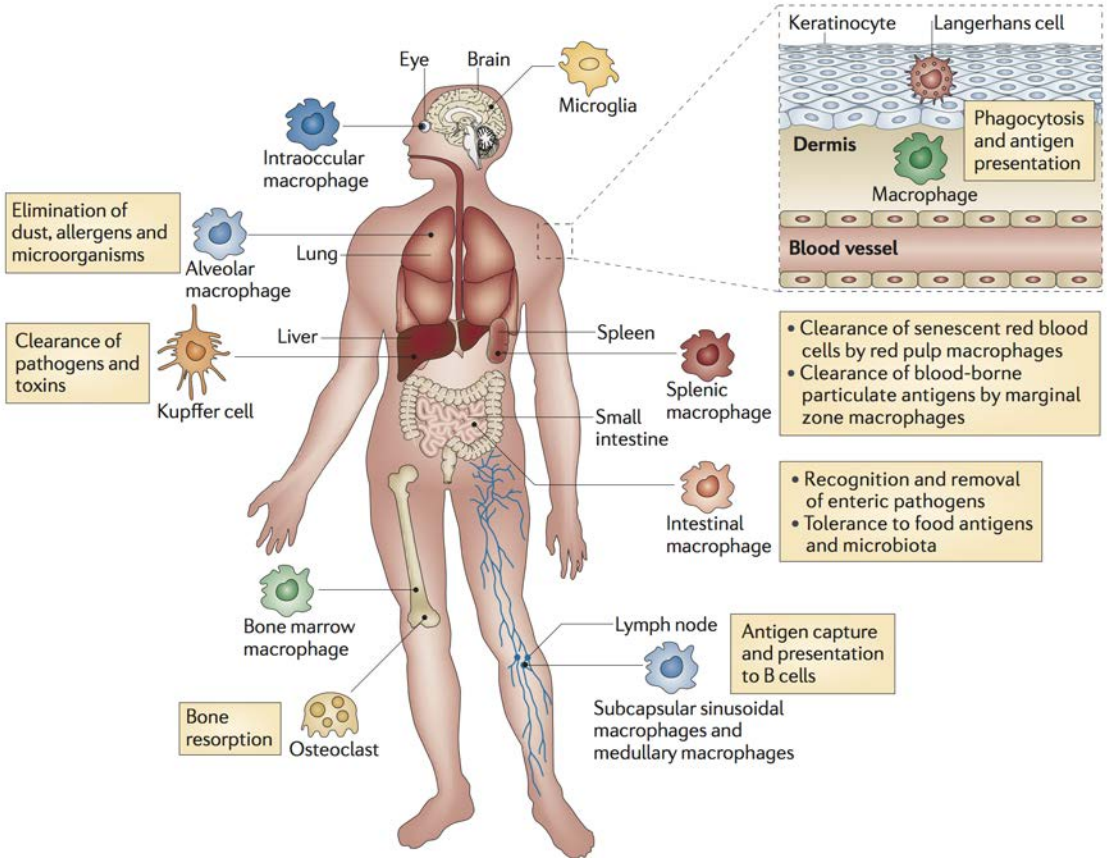


Figure 7 – Tissue-resident macrophages and associated functions (Murray and Wynn, 2011).

Macrophages have an incredible ability to adapt to the environmental niche they occupy via sensing tissue-specific signals and can also rapidly change phenotype and assume different "polarization states" based on the cytokine microenvironment at tissue level (Okabe and Medzhitov, 2014; Wynn and Vannella, 2016). However, recent exiting findings revolutionized the ontology of TRMs, as several groups showed that they can maintain themselves independently of bone marrow hematopoiesis and that they are already found

during embryogenesis (Lavin et al., 2015). They derive from yolk sac and fetal liver progenitors which colonize the embryonic tissues in several moment of development (Perdiguero and Geissmann, 2015). TRM in the adult tissues are therefore long-lived cells, derived from embryonic progenitors, which renew themselves *in-situ*. On the light of these recent finding, ant the implications for their role in human diseases and tissue homeostasis, TRM are again an intense field of investigation.

1.2.1. Origin and development

It was long believed that TRMs were derived as well as maintained by bone marrow circulating monocytes intermediates which would then constantly be recruited to the tissue and differentiate locally (Ginhoux and Guilliams, 2016). One of the first observation that confuted the prevailing monocyte-to-macrophages dogma came from Merad M. in 2002, when experiments showed that the Langerhans cells (the epidermal macrophage-like population) are only partially reduced upon lethal irradiation and that they are repopulated from cells of host origin upon bone marrow transplant (Merad et al., 2002). Further studies in parabiotic mice – i.e. a mouse model in which two mice are subjected to an operative procedure that connects their circulatory system – showed that some TRMs, like microglia, Langerhans cells and AMs do not get shared in the coupled circulation and are therefore tissue-resident, whereas macrophages in gut, dermis and hearth showed some degree of exchange with blood-derived progenitors (Ginhoux and Guilliams, 2016). Using a conditional mouse model that would allow fate-mapping studies of the murine monocytes-macrophages compartment (CX3CR1 promoter-driven *Cre* recombinase expressing mice) it was demonstrated that some the major TRMs populations, like Kupffer cells of the liver, lung AMs, microglia, epidermal Langerhans cells, spleen and peritoneal macrophages, were established prior to birth and maintained themselves *in tissue* during adulthood, independently of hematopoiesis (Yona et al., 2013).

Mammalian embryonic hematopoiesis is extremely complex and occurs in different waves arising from intra or extra embryonic sites and the precise timing of tissue colonization by embryonic macrophage progenitors remains a matter of debate. With the further use of sophisticated fate-mapping technologies several groups have attempted to investigate the precise origin and timing of tissue colonization by embryonic derived TRMs (Hoeffel et al., 2015; Perdiguero and Geissmann, 2015; Sheng et al., 2015). The current evidence suggests that TRMs originate from embryonic progenitors even before the appearance of hematopoietic

stem cells (HSCs), in two temporarily separated and functionally distinct waves of hematopoiesis originating from the yolk sac between E7 and E8.5 in mice (Ginhoux and Guilliams, 2016). The "first wave" arises as early as E7, and gives rise to primitive erythroblasts, megakaryocytes and macrophages in brain, lung, skin and liver (Figure 8). Later on in the embryonic development, the yolk sac forms a hemogenic endothelium around E8.0-E8.25, which gives rise to erythro-myeloid precursors (EMPs) and therefore to a "second wave" of hematopoiesis, distinct from the first one, which mostly enters the fetal liver (Figure 8).

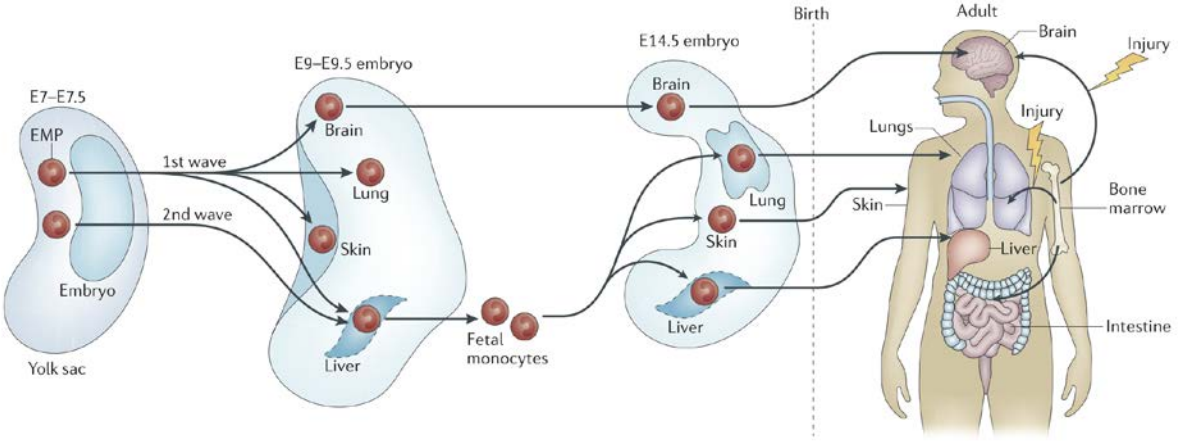


Figure 8 – Origin of tissue resident macrophages (Lavin et al., 2015).

In fact, from E8.5 onward, the fetal circulation starts to get more organized and the EMPs of the second wave are thought to first migrate to the fetal liver, where they generate progenitors with multiple myeloid potential, including the fetal liver monocyte, and then seed other organs (Lavin et al., 2015). From E12.5, the fetal liver is established as the major hematopoietic organ in the embryo and will form the hematopoietic progenitors which will eventually migrate to the bone marrow and become HSCs. The two waves of macrophage colonization that start from the yolk sac and fetal liver before E12.5 are believed to give rise to all the TRMs in the body. However, the exact contribution of early or late EMPs as well as fetal HSCs to the TRMs pool is still not clear. For example, the work of Gomez Perdiguero et. al showed that the main pool of TRMs derives directly from the early pool of EMPs originated in yolk sac, giving rise to microglia, Langerhans cells and Kupffer cells, without a monocyte intermediate coming from the fetal liver. In particular, the brain TRMs (microglia), seem to be solely of yolk sac origin, whereas in other tissue the fetal liver derived monocytes also contribute to the pool (Ginhoux et al., 2015; Gomez Perdiguero et al., 2014). Other authors (Hoeffel et al., 2015; Sheng et al., 2015) propose that the TRMs mostly derive from

fetal liver monocytes progenitors, which previously migrated from the yolk sac as late EMPs during the "second wave" of hematopoiesis. These authors maintain, however, a separate origin for the microglia, as directly derived from a first "primitive wave" of EMPs that migrated directly from the yolk sac. Subsequent studies are needed to clarify the relative contribution of the first and second "definitive wave" of hematopoiesis to the TRMs pool. It is, although, established that TRMs are of embryonic origin and it is more and more accepted that probably the EMPs-derived fetal liver monocytes pool originated during the "second wave" represents the main precursors for most TRMs (Hoeffel et al., 2015). Moreover, the observation that adoptive transfer of fetal liver monocytes can give rise to alveolar macrophages, (Guilliams et al., 2013; Kopf et al., 2014), heart macrophages and adult Langerhans cells (Ginhoux and Guilliams, 2016), reinforces this theory.

1.2.2. Homeostasis of TRMs in tissue specific context

In order to exert its physiological role, each tissue of the body needs to actively maintain a homeostatic balance. Steady conditions can vary considerably among different organ and in different stages of development and maturity. Due to their embryonic-derived strategical location and to their scavenging and wound healing capacity, as well as the ability to recognize danger and pathogens, TRMs constitute a pillar in the physiological homeostasis and immunity of mammalian tissues. It is now broadly accepted that virtually all tissues in the body are colonized by TRMs already before birth (Ginhoux and Guilliams, 2016). It remains unclear when and how they acquire and maintain different tissue specific phenotypes at homeostasis. For example, at birth, gut tissue embryonic derived macrophages are totally replenished by monocytes derived macrophages upon establishment of the gut microbiota (Bain et al., 2014), whereas central nervous system (CNS) resident microglia, probably due to its secluded environment behind the blood-brain-carrier, it's ontologically homogeneous and singularly derived from embryonic precursors (Bruttger et al., 2015).

One mechanism that can confer tissue specific characteristics to TRMs is epigenetic regulation. Modification of the histone tails of nucleosomes via methylation, acetylation, sumoylation or other chromatin regulations, can profoundly regulate the *in-tissue* phenotype of macrophages (Amit et al., 2015; Gosselin et al., 2014; Lavin et al., 2014). Enhancers are short regions of the DNA contained in "open chromatin" that can bound to promoter regions of the genome and increase the likelihood of certain gene to be transcribed. On the contrary, silencers are antagonists of enhancers and can bind to specific repressors protein inducing the

silencing of certain genes. Exciting comparative transcriptomic studies recently showed that TRMs exhibit different transcriptional and epigenetic signatures (Gautier et al., 2012; Lavin et al., 2014; Okabe and Medzhitov, 2014) specific for the tissue that they occupy. Moreover, the tissue-specific epigenetic signature is induced by the extracellular environment, as proved by experiments in which bone marrow derived monocytes (BMDMs) that were intravenously injected in lethally irradiated recipients assumed a phenotype similar to the one of TRMs (Lavin et al., 2014). Moreover, once isolated and cultured, macrophages lost their tissue specific signatures (Amit et al., 2015), proving again that the phenotype of TRMs is constantly affected by mostly unknown tssue-derived signals. The phenotype of TRMs, can vary in phagocytic capacity, secretion of tissue-specific trophic factors and of course morphological specialization. TRMs can be classified by their phenotypic characteristics according to specific epigenetically regulated transcription factors (Figure 9).

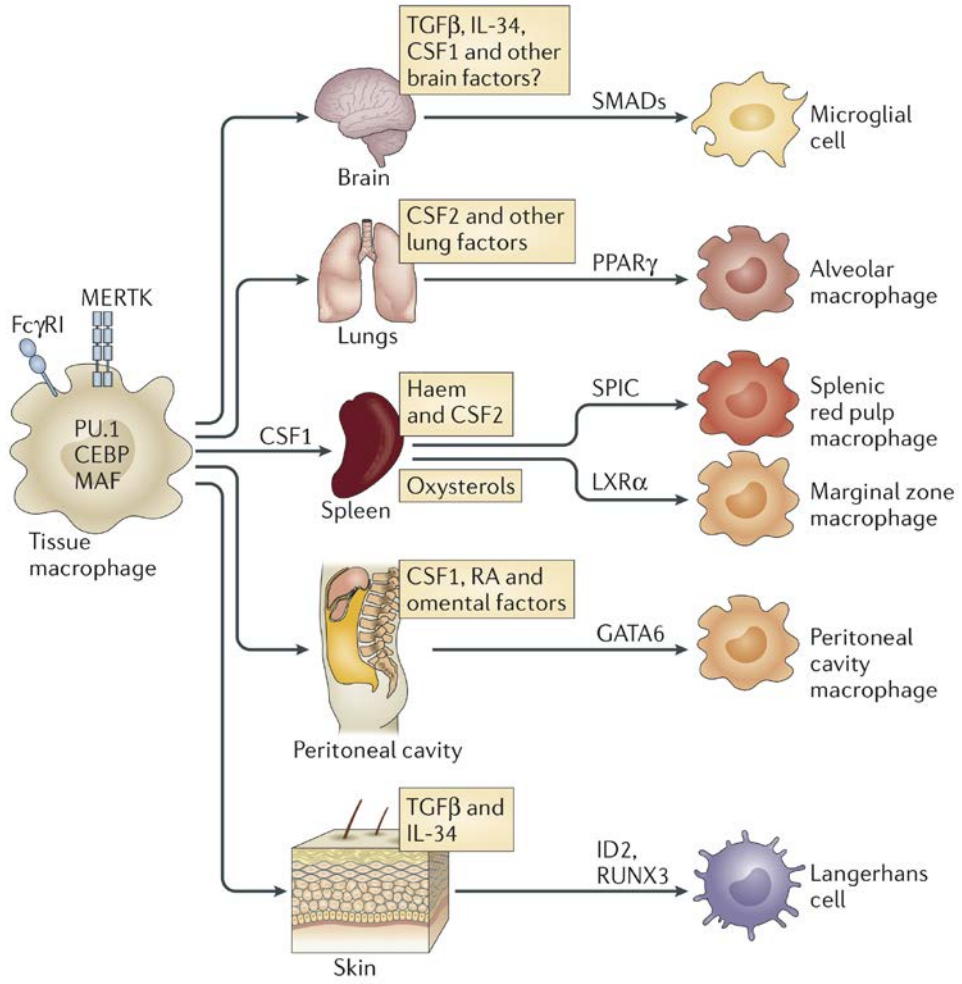


Figure 9 – A combination of epigenetically regulated tissue and lineage specific transcription factors confers *in-situ* specificity to TRMs (Lavin et al., 2015).

In the lungs, for example, AMs are characterized by the expression of genes like *Pparg*, *Car4*, *Itgax* (encoding for CD11c), *Csf2rb*, *Chi3l3* (Kopf et al., 2014; Lavin et al., 2014). Other authors concentrated also on the tissue-specific signals that affected not only the phenotype but also the function of TRMs. For example, Okabe and Medzhitov (Okabe and Medzhitov, 2014), worked on the peritoneal macrophages (PMs) and compared their whole genome sequence expression to the one of other six types of macrophages. They identified the transcription factor GATA6 as a master regulator of the *in-tissue* function of PMs only. They went on to identify retinoic acid as the fundamental signal needed to PMs for inducing GATA6 expression and characterized a role for GATA6-dependent PMs in regulating B1 cell secretion of IgA (Okabe and Medzhitov, 2014). This study aimes at characterizing the tissue-derived signals that provide specific functional role of TRMs. Before entering in the details on the function of AMs, which is one of the principal cell type involved in this work, I would like to introduce another important aspect of the general biology of TRMs, which is their ability to assume different polarization states during immune responses and which constitutes one of the main topic of the thesis.

1.2.3. Macrophage polarization

In addition to acquiring tissue-specific phenotypical characteristic at homeostasis, macrophages can also undergo a quicker functional polarization *in situ* in response to environmental stimuli, like cytokines, microbial, danger or metabolic signals (Murray et al., 2014). In fact, TRMs can rapidly mount an effective inflammatory response in case of invasion by pathogenic microorganism and, later on, assume a different phenotype, devoted to the resolution of inflammation by clearing tissue debris and keeping architectural integrity. Such plastic behavior can be reproduced *in vitro* via exposing macrophages to specific signals, which historically led to the classification of their phenotype into two different extreme polarization spectra: the M1 polarization, also called “classical” polarization, and the M2 polarization also referred to as “alternative” polarization. The M1 polarization is induced by cytokines secreted by T helper 1 cells (Th1), and promotes bacterial killing by phagocytosis, secretion of pro-inflammatory cytokines and cellular migration to the site of infection. The M2, or “alternative”, polarization is induced in the context of Th2 immune responses and is mostly devoted to tissue remodeling and wound healing. Both M1 and M2 polarization are characterized by the activation of defined signaling pathways and by the phenotypic expression of specific surface proteins that allow their identification. The M1

polarization, is mainly driven by IFN-γ, which is recognized by the heterodimeric transmembrane receptors IFNγ-R1 and IFNγ-R2 which are connected to the Janus kinases JAK1 and JAK2 an associate with the signal transducer and activators of transcription type 1 (STAT1) (Figure 10). Activated STAT1 than translocates to the nucleus and activates interferon regulatory factors (IRFs). Interestingly, the M1 polarization profile of IFN-γ activated macrophages is different than the one of IFN-γ + lipopolysaccaride (LPS) activated macrophages. In fact, the concomitant presence of LPS or other bacterial particles recognized by TLRs on macrophages, can enhance the pro-inflammatory phenotype of M1 macrophages via non-homologous signaling pathways. For example, when TLR4 binds to LPS, it activates a myeloid-differentiation primary-response protein 88 (MyD88) dependent pathways that leads to nuclear factor of kappa light polypeptide gene enhancer (NFκB) activation and translocation to the nuclei to induce pro-inflammatory genes. In macrophages, both NFκB and STAT1 can induce the expression to similar pro-inflammatory genes that characterize the M1 phenotype, like the cytokines IL-1β, TNF, IL-6, IL-12, the chemokines CCL2 and CXCL10 and the transcription of inducible nitric oxide synthase (iNOS) which exert a bactericidal function via production of the toxic molecule nitric oxide (NO). The M1 phenotype of macrophages is essential for inducing defenses against intra and extracellular bacteria, like *Mycobacterium tuberculosis* or *S. pneumoniae* respectively, as well as exerting an important anti-tumor function in solid tumors (Murray et al., 2014).

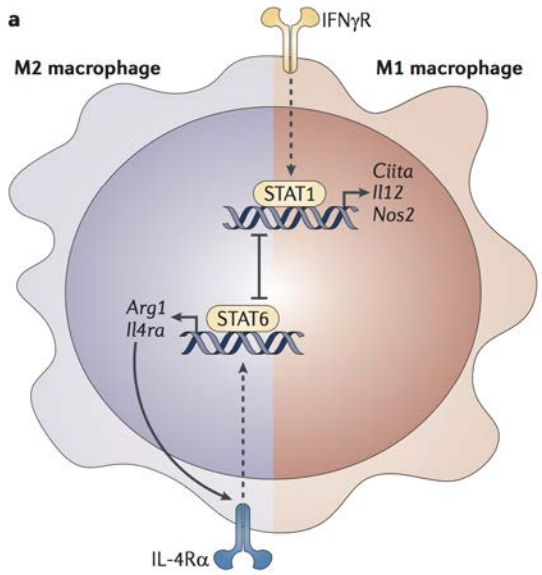


Figure 10 – The M1/M2 main inducing signals and transcription factors (Lawrence and Natoli, 2011).

On the other side of the spectra, the M2 polarization is driven by the classical Th2 cytokines IL-4 and IL-13 (Gordon and Martinez, 2010). These cytokines, like many other interleukins, also signal through heterodimeric receptors connected to JAKs. Dependently on the heterodimers complexes, two types of receptor are able to bind either to IL-4 only, or to IL-4 and IL-13 together (Figure 10) (Van Dyken and Locksley, 2013; Gordon and Martinez, 2010). The type-I receptor is composed by the IL-4Rα subunit together with the IL-4Rγc subunit and exclusively binds to IL-4. Notably, the γc subunit is shared as heterodimeric receptor by many

other interleukin receptors and in particular it is used for IL-2, IL-7, IL-9, IL-15 and IL-21 signaling. The type-II receptor is composed by the IL-4Rα and IL-13Rα1 subunits and can bind both IL-4 and IL-13. Some cell types exclusively express the type-I receptor complex and can therefore only respond to IL-4. Interestingly, the IL-13rα1 subunit is mostly expressed by non-hematopoietic cells, like Goblet cells or smooth muscle cells, with the notable exception of monocytes and macrophages (Gordon and Martinez, 2010) and in particular TRMs (Van Dyken and Locksley, 2013). Therefore, the IL-13 response can be compartmentalized accordingly to the cell type that selectively expresses the IL13α1 subunit (Bhattacharjee et al., 2013). The IL-13Rα1 subunit expression on the surface of macrophages can be modulated accordingly to the cytokine milieu, resulting in different affinity and duration of IL-13 and IL-4 signal during inflammation (Van Dyken and Locksley, 2013).

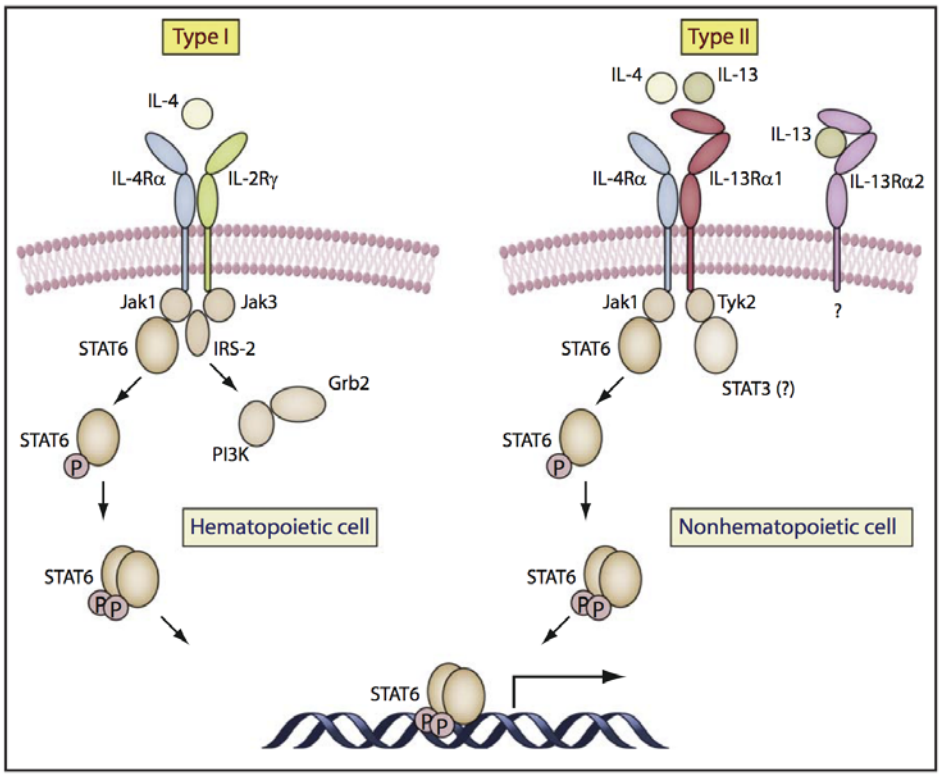


Figure 11 – IL-13 and IL-4 receptor structure and signaling (Gordon and Martinez, 2010).

The activation of both type-I and type-II receptors initiates the signaling via JAK/STAT-mediated phosphorylation and activates similar intracellular signaling pathway, which are sometimes poorly defined and can change in regards to the cell type. The IL-4Rα monomer, which is shared by both type I and type II receptors, only associates with JAK1, therefore resulting in similar intracellular signaling for both IL-4 and IL-13. However, the type I and type II receptors signaling pathways also activate other poorly defined downstream signals

through their unshared heterodimers: for example, the γc subunit of the IL-4 receptor type I is associated to JAK3, and can recruit the insulin receptor substrate 2 (IRS-2), whereas IL-13Rα1 associates with TYK2 and JAK2 and might also activate additional STATs, like STAT3 (Van Dyken and Locksley, 2013). The implication of these differences in downstream signaling of IL-4 and IL-13 are largely unknown. However, the main downstream event for both pathways is STAT6 phosphorylation and binding to promoter elements on the IL-13/IL-4 responsive genes, like *Arg1*, *Retnla*, *Chil3*, trophic polyamines, genes involved in immunoregulation, like IL-10, and genes involved in matrix deposition and tissue remodeling (Martinez and Gordon, 2014).

These is a list of some genes used as markers to assess the M1/M2 polarization state of macrophages in vivo and in vitro:

- Nitric oxide synthase 2, inducible (*iNOS*): This gene promotes the formation of nitric oxide, a reactive oxygen radical that has a role as mediators of several biological processes, like neurotransmission, vasodilation, antitumor activity. The gene is also called *NOS2* and it is induced in M1 macrophages, which use NO to kill intracellular bacteria.
- Arginase 1 (Arg-1): This protein is transcribed by the *ARG1* gene in human and mice. It works as an enzyme, mostly expressed in the liver, where it catalyzes the conversion of L-arginine to L-ornithine and urea, a critical step in urea metabolism. In macrophages, Arg-1 is induced by IL-13/IL-4 and it is a classical M2 marker. Its role in the context of the M2 polarization is to counteract the generation of NO by iNOS by competing for the L-arginine metabolism.
- Mannose receptor (MR1): also known as CD206 and Mrc1. The mannose receptor 1 belongs to the family of C-type lectin receptor (CLR), which recognize mannose, fucose, and glucan structures and are widely expressed on myeloid cells, specially by TRMs and DCs. This particular gene is characteristically expressed on M2 macrophages but also found at homeostasis. It is a surface receptor with multiple carbohydrates domains and can therefore clear serum glycoproteins. At steady state it maintains normal levels of endogenous glycoproteins and clears mannosylated ligands and lysosomal hydrolases (Taylor et al., 2005). It is constitutively expressed on alveolar macrophages (Hussell and Bell, 2014) and can play a role also in the recognition of fungal antigens, like during *Cryptococcus neoformans* infection (Van Dyken and Locksley, 2013).

- Chitinase 3-like protein 3 (Chil3): also known as YM1 or Chi3l3. This protein is encoded by the gene *CHIL3* which is similar to bacterial chitinases, that can mediate the binding and degradation of the chitin molecule expressed on parasite and allergenic organisms like fungi and house dust mite (HDM). This protein, like other member of the chi-lectins, for example Ym2, are induced in the M2 macrophages by IL-4/IL13 signaling and STAT6 nuclear translocation. It is a quite useful marker of M2 polarization, as they are particularly induced in the context of allergic inflammation or parasite infection, although their role in the context of type 2 immunity is still not clearly defined (Van Dyken and Locksley, 2013).
- Resistin like alpha (Retnla): also known as resistin like molecule a (RELMa) or protein found in inflammatory zone (FIZZ1), or hypoxia-induced mitogenic factor (HIMF). *Retnla* is highly induced during allergic inflammation and parasite infection, therefore being a marker for M2 macrophage polarization. The actual function of this protein is unclear and it is mostly used as a marker for M2 macrophages. However, its overexpression can downregulate airway inflammation during acute type 2 immunity, whereas *Retnla* deficient mice display an increased inflammation, suggesting a rather regulatory role of type 2 immune responses.
- CD163: is a scavenger receptor for the haemoglobin-haptoglobin complex in humans and it is exclusively expressed on monocytes and macrophages. It works as an acute phase protein and it is involved in avoiding free hemoglobin–mediated oxidative damage. It can be induced by M2 macrophages.

The classical M2 polarization of macrophages has been studied in the context of type 2 immunity, as it can be induced by Th2 cells during asthma or helminth infection. In particular, M2 macrophages are important in providing a modulation of T cell responses during helminth infection, as well as formation of parasite-induced granulomas (Van Dyken and Locksley, 2013). The role of M2 polarized macrophages in the acute allergic lung inflammation is less clear, even if some studies suggest that they might help with induce eosinophils recruitment and tissue remodeling (Van Dyken and Locksley, 2013). However, the major role of M2 macrophages seems be in the resolution phase of type-2 inflammation, when they contribute to tissue repair processes upon parasite migration or virus induced cellular damage. Finally, an M1/M2 switch can be observed during the transition from acute infection to resolution phase and may represent a way for innate immunity to protect the body from uncontrolled and overwhelming infection (Murray et al., 2014).

In recent years the M2 polarization spectrum has been expanded to a broader range of physiological and pathological processes, including tissue integrity at homeostasis and in the tumor micro-environment or metabolic functions (Murray et al., 2014). For example, one important additional intracellular factor that regulates the M2 polarization is the proliferator–activated receptor γ (PPAR-γ) and PPAR-δ, as well as PPAR-γ coactivator 1β (PGC1β) (Schneider et al., 2014a). This transcription factor acts in dependence to the signals from IL-4, IL-13 and STAT-6 and mediates several aspects of the M2 polarization of macrophages, in particular in the context of lipid metabolism. The highest constitutive expression of PPAR-γ is found in AMs, as well as in macrophages seeded in mucosal tissues and in draining lymph nodes (Schneider et al., 2014a). The role of PPARγ, a master regulator of adipose tissue differentiation, in the differentiation of AMs might be due to the need of these cells to metabolize the lipid layer of the alveolar space, the surfactant liquid. Moreover, M2 macrophages have emerging roles in the homeostasis and metabolism of adipose tissue, where impaired M2 polarization causes insulin resistance, whereas exposure to cold temperature induces M2 polarization and mediates the browning of the white adipose tissue (Van Dyken and Locksley, 2013).

1.2.4. Alveolar macrophages

AMs are the major cell component of the alveolar space, representing it's 90-95% cellular content (Kopf et al., 2014). They are considered the sentinels of the lung environment as they are strategically positioned to be the first immune cells to sense pathogens in this delicate location. They are typical TRMs, ontologically and functionally: they descend from embryonic progenitors, self-renew in tissue, have a long half live – with a turnover of 40% in 1 year – and their phenotype is shaped by tissue-specific context, which are largely unknown (Hussell and Bell, 2014). The most accredited theory sees AMs as descending from fetal monocytes derived from the fetal liver that seeded the lung during embryogenesis (Amit et al., 2015; Hoeffel et al., 2015; Hussell and Bell, 2014; Lavin et al., 2015).

In mice, AMs can be easily identified by flow cytometry and distinguished from lung interstitial macrophages via expression of peculiar markers: AMs are $CD11c^{hi}$, sialic acid-binding immunoglobulin-like lectin F (SIGLEC-F) positive and $CD11b^{lo}$, compared to interstitial macrophages, which are $CD11b^{hi}$, CD11c negative and do not express SIGLEC-F (Fig. 12). SIGLEC-F is a typical eosinophils marker, which is expresses in high quantities on

AMs. Crosslinking of SIGLEC-F on eosinophils can reduce eosinophils mediated inflammation. However, the role of SIGLEC-F expression on AMs is unknown.

Surface marker	Peritoneal macrophage	Interstitial macrophage	Alveolar macrophage	Refs
CD11b	Intermediate expression	Intermediate expression	Not expressed	45,47
CD11c	Not expressed	Not expressed	High expression	45,47
CD14	Intermediate expression	Intermediate expression	Low expression	50,159
CD200R	Low expression*	Intermediate expression	High expression	17
DEC205	Not expressed	Expression unknown	Intermediate expression	47
F4/80	Intermediate expression	Low expression	Low expression	50,160
Mannose receptor (also known as CD206)	Low expression	Intermediate expression	High expression	50,161
MHC class II	Intermediate expression	Intermediate expression	Low expression	47,50
SIGLEC-F	Not expressed	Not expressed	High expression	50,162

CD200R, CD200 receptor; SIGLEC-F, sialic acid-binding immunoglobulin-like lectin F. *Expression shown on splenic macrophages.

Figure 12 – Phenotypic comparison and marker expression on AMs, PMs and IMs (Hussell and Bell, 2014).

The actual differentiation to AMs, as well the upregulation of SIGLEC-F and CD11c, happens perinatally in mice at day 3-4 after birth (Guilliams et al., 2013). Among the grow factors driving their perinatal differentiation, the stromal cells-derived colony stimulating factor type 2 (CSF-2) mediates the induction of the PPAR-γ activation signaling in fetal monocytes progenitors and it is therefore fundamental for their *in situ* differentiation into AMs (Ginhoux and Guilliams, 2016; Guilliams et al., 2013; Schneider et al., 2014a). These findings were obtained using a mouse model in which PPAR-γ expression was specifically missing on AMs and DCs, via crossing mice containing *Lox*P-flanked alleles encoding PPAR-γ (*Pparg*$^{fl/fl}$) with mice encoding a *Cre* recombinase under the control of *CD11c* (Schneider et al., 2014a). In their experiments, Schneider and colleagues discovered that the differentiation of AMs was abrogated at the level of the fetal monocytes precursor, translating in a profound alteration of AMs perinatal development. The GM-CSF (also called CSF-2) signal was fundamental for inducing PPAR-γ signaling in newly differentiating AMs, as mice deficient in CSF-2 receptor (*Csf2rb*$^{-/-}$) have no AMs (Hoeffel et al., 2015). The PPAR-γ pathway was responsible for imprinting the AMs specific signature to fetal liver progenitors, including genes involved in the cholesterol metabolism, a hallmark of AMs function in the high lipidic alveolar environment (Schneider et al., 2014a). Overall, these experiments suggest that AMs develop at postnatal day 3-4 in a GM-CSF-induced and PPAR-γ-mediated way and that PPAR-γ is the master regulator of the signature gene expression of AMs *in-tissue*. Interestingly, one of the most important moment in which AMs need to properly exert their tissue remodeling activity is in the perinatal period, when they have the vital role of clearing the surfactant liquid in the postnatal lung (Nakamura et al., 2013). In fact, intratracheal transfer of macrophages could

cure hereditary pulmonary alveolar proteinosis in a AMs deficient mouse model (Amit et al., 2015). This reinforce the role of AMs as keeper of the alveolar space and provide a strong argument on the role PPAR-γ expression and other genes involved in lipid metabolism. In the next paragraphs, I will address the capacity of AMs to quickly adapt to environmental changes in the airspace, both physiological and pathological, exerting vital roles in the lung homeostasis and its innate immune defenses.

AMs role in microbial defenses

AMs are strategically located to be the first cells that sense pathological microbial invasions in the deep airways and therefore represent the most important defense against bacterial pneumonia and other microbial diseases (Hussell and Bell, 2014). Like other antigen presenting cells (APC), AMs can recognize conserved microbial structures through pathogen recognition receptors (PRRs), belonging to the family of TLRs, C-type lectin receptors, NOD-Like receptors (NLRs) and scavenger receptors. Several studies proved the fundamental role of AMs in orchestrating the early defenses against bacterial, viral and fungal pathogens (Kopf et al., 2014). It is believed that the threshold of activation of AMs in case of pathogen sensing depends on the multiple binding of several PRRs, including the loss of alveolar tissue integrity and therefore of the regulatory signals coming from the intact epithelium (Kopf et al., 2014). Like other macrophages, AMs express TLR-2 (ligand of the lipoteichoic acid), TLR-4 (ligand of LPS), TLR-9, TLR-5 (ligand of flagellin) and costimulatory proteins, like CD14, CD80, CD86, CD40. Recognition of microbial by TLR-2 and TLR-4 in the context of inflammation can elicits phagocytosis and bacterial killing by inducing the surface expression of phagocytose receptors like MARCO or Scavenger Receptor A (SR-A) and the transcription of genes like *iNOS*, devoted to NO-mediated bacterial killing, hallmarks of M1 polarization (Aberdein et al., 2013; Sharif et al., 2014). AMs exert their clearance functions not only by phagocytosis and killing of bacteria, but also by secretion of pro-inflammatory cytokines and chemokines that induce the recruitment of neutrophils and monocytes on the site of infection. Once activated, AMs can serve as master regulators of inflammation in the alveolar space and can secrete IL-1β and TNF, which act also in a autocrine fashion inducing oxidative burst as well as phagocytosis of microbial pathogens, and chemokines like CXCL1, CXCL8, CCL2, which induce neutrophils and monocytes recruitment, from the circulation (Aberdein et al., 2013; Kopf et al., 2014). The early phase of antibacterial responses mediated by AMs very important for allowing proper clearance of bacterial infections (Aberdein et al., 2013). In fact, we already demonstrated how an alternative polarization of AMs can reduce bacterial

clearance in the course of bacterial pneumonia, via delaying the early neutrophils influx which is pivotal in allowing bacterial clearance (Warszawska et al., 2013).

AMs also exert a role in viral clearance, as they have been shown to be the main producers of type-I interferon (IFN) during acute influenza A virus infection (Divangahi et al., 2015) and are therefore critical to promote early viral clearance via induction of viral suppression genes. Moreover, several studies showed that AMs are critical in limiting overwhelming lung inflammation and pathology during influenza (Schneider et al., 2014b; Tate et al., 2010). In fact, efferocytosis, the process of removal of dying or dead cells by phagocytes, is substantially increased in AMs in the context of inflammation thanks to molecules like C1q, which can bind to apoptotic cells or pathogens associated molecular patterns (PAMPs) with its globular head and to AMs with the collagenous tail (Kopf et al., 2014).

AMs in lung homeostasis

At steady state, the activity of AMs seems to be tightly regulated by the availability of a few known effector molecules in the microenvironment as well as by the integrity of the alveolar epithelium (Hussell and Bell, 2014). For example, AMs express CD200R at steady state, which is a type-1 transmembrane glycoprotein receptor of the immunoglobulin superfamily, constitutively expressed on the myeloid lineages. This receptor constantly binds with the CD200 ligand on the AEC2s and this binding inhibits AMs, as shown in *CD200*$^{-/-}$ mice, whose AMs presented increased proliferation rates and a pro-inflammatory phenotype (Snelgrove et al., 2008). Another important inhibitory molecule expressed on AMs is the signal regulatory protein-a (SIRPa), a negative regulator of cellular activation. Mice deficient in SIRPa succumb of excessive lung inflammation a few weeks after birth (Hussell and Bell, 2014). Moreover, the activity of AMs is also homeostatically regulated by TGFβ in an autocrine and paracrine fashion, both in human and mice. In fact, AMs express the TGFβ-R and can sense TGFβ activated by the αvβ6 integrin on airway and alveolar epithelium (Figure 13). All these homeostatic cell-to-cell interactions in the alveolar space prevents aberrant AMs activation and expression of inflammatory cytokines (Hussell and Bell, 2014).

AMs seem to be able to exert themselves regulatory function towards naive T cells. In fact, compared to other TRMs, AMs are poor antigen presenting cells, as they express low level of MHCII as well as reduced numbers of co-stimulatory molecules, for example missing the CD40, CD80 or CD86 (Nicod et al., 2005). Since they have been shown to travel in the lymph nodes and exert antigen presentation activity, this action might result in a T cell antigen-unresponsiveness and therefore tolerance to common antigens at homeostasis. Furthermore,

AMs can directly inhibit T-cell activation by producing TGFβ and eventually even directly induce forkhead-box P3 (Foxp3) expression in naive T cells and therefore driving the development of regulatory T cells (Tregs). However, these interactions have been only studied *in vitro* and it is not clear if they really happen *in situ*, even if extremely probable, considering that naive T cells have been observed in the lumen of healthy lungs (Hussell and Bell, 2014).

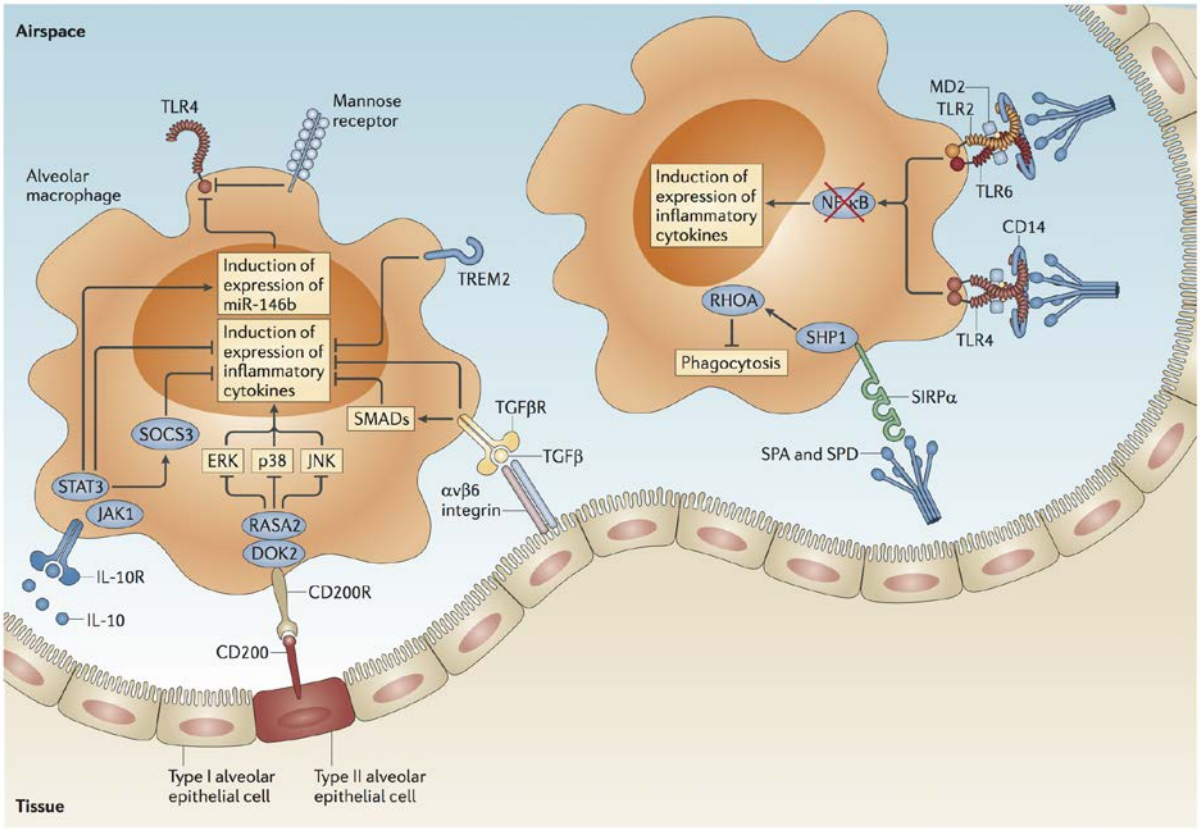


Figure 13 – Alveolar Macrophages activation in healthy lungs is actively downregulated. Taken from Hussel T. and Bell J.T. 2014.

These anti-inflammatory and regulatory functions seem to be important in the context of infection. For example, efferocytosis is a very important homeostatic function of AMs, which constantly clear dying or dead cells in the epithelium, maintaining the alveolar space intact and healthy, also with the help of some scavenger receptors, like CD36 (Divangahi et al., 2015; Sharif et al., 2013; Taylor et al., 2005). AMs-mediated efferocytosis is also vital in in the context of bacterial pneumonia and tissue damage where it maintains tissue integrity and avoids excessive inflammation (Sharif et al., 2013).

Besides presenting a phenotype particularly devoted to tissue remodeling, AMs also seem to have a tendency to an M2 phenotype at homeostasis. Interestingly, CD200R is can be induced in blood monocytes derived macrophages upon *in vitro* treatment with IL-4 and IL-13, which

are classical M2 polarizing cytokines suggesting that AMs could be actively maintained in an M2 polarization state (Hussell and Bell, 2014). Moreover, AMs constitutively express a classical M2 marker: the mannose receptor-1 (MR1), a carbohydrate-binding C-type lectin, expressed also on other myeloid cells, like some DCs and macrophages. MR1 can recognize un-opsonized bacteria and prevent the activation of AMs, inducing tolerance against non-pathogenic microbes in the airways. Compared to other TRMs, AMs present reduced phagocytic capacity and a reduced respiratory burst (Hussell and Bell, 2014). Studies in human suggest that 50% of the AMs in healthy individuals express CD206 and can therefore phenotypically fall into the M2 category. Moreover, AMs seem to be particularly sensitive to M2 polarization which it has been observed in many pathological contexts. A dysregulated M2 polarization of AMs has been mostly associated with asthma or chronic lung fibrosis, suggesting that the M2 program is rather induced in pathological settings. AMs can relatively rapidly induce T cells costimulatory molecules during infection or upon lung transplantation and therefore eventually lose the capacity to induce tolerance at steady state (Nicod et al., 2005), potentially promote the pathogenesis of chronic interstitial fibrosis. For example, AM can adapt to hypoxia by inducing the hypoxia inducible factor (HIF), at the consequence of acquiring a more pro-inflammatory state via activation of NFκB, potentially contributing to chronic inflammation (Hussell and Bell, 2014). AMs with an M2 phenotype, characterized by expression of *Retnla* and *Chil3*, have been identified in patients with lung fibrosis (Nicod et al., 2005). AMs from asthmatic patients are also found in an M2 state and are believed to contribute to the chronic tissue remodeling and airway pathology in these patients. Moreover, patients with emphysema also present a particular phenotype of AMs, characterized by induction of MMP1 and MMP12, metallo-proteinases which contribute to lung structural changes (Hussell and Bell, 2014; Kopf et al., 2014). We previously showed

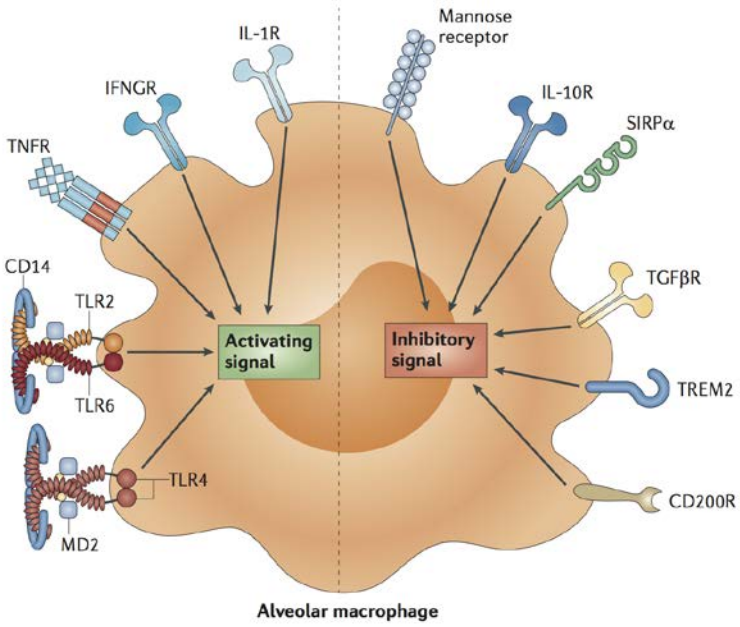


Figure 14 – Alveolar Macrophages activation signals. Taken from Hussel T. and Bell J.T. 2014.

that a major consequence of the phenotypic alteration of AMs towards an M2 phenotype is the increased susceptibility to bacterial lung infections, like *S. pneumoniae* (Warszawska et al., 2013). The origins of the M2 polarization state of AMs and its consequences in bacterial infections are the main focus of this study.

The major drive of the M2 polarization of AMs in pathological context is not well known. It was shown that upon intranasal chitin treatment, which is a wide-spread polymer present on parasite, fungi and arthropods and responsible for the allergic sensitization in many asthmatic patients, AMs assume an IL-13 dependent M2 polarization state, driven selectively by innate lymphoid cells type 2 (ILC2s) (Van Dyken et al., 2014). Interestingly, in their studies, IL-4 did not contribute at all to the polarization of AMs. Another report on the effective role of IL-13 in the polarization of AMs, comes from the studies of Kurowska-Storlaska, who showed how AMs can become much more sensitive to IL-13 if they also sense the concomitant presence of IL-33, an important tissue derived alarmin (Kurowska-Stolarska et al., 2009). The role of ILC2s and IL-13 in polarizing TRMs will be discussed in the next chapters.

1.3. Innate lymphoid cells, new player in innate immunity

Innate lymphoid cells (ILCs) are recently identified members of the innate immune response which, like TRMs, are also strategically located at natural barriers in the body, like mucosal tissues, gut, lung and skin and can secrete high amounts of classical Th cells associated cytokines in response to environmental stimuli, without need of antigen recognition (Walker et al., 2013). ILCs share also another important characteristic with TRMs, which is the fact that they seed the various tissues during embryogenesis and after birth are mostly renewed by local proliferation instead of replenishment from peripheral blood precursor (Gasteiger et al., 2015a).

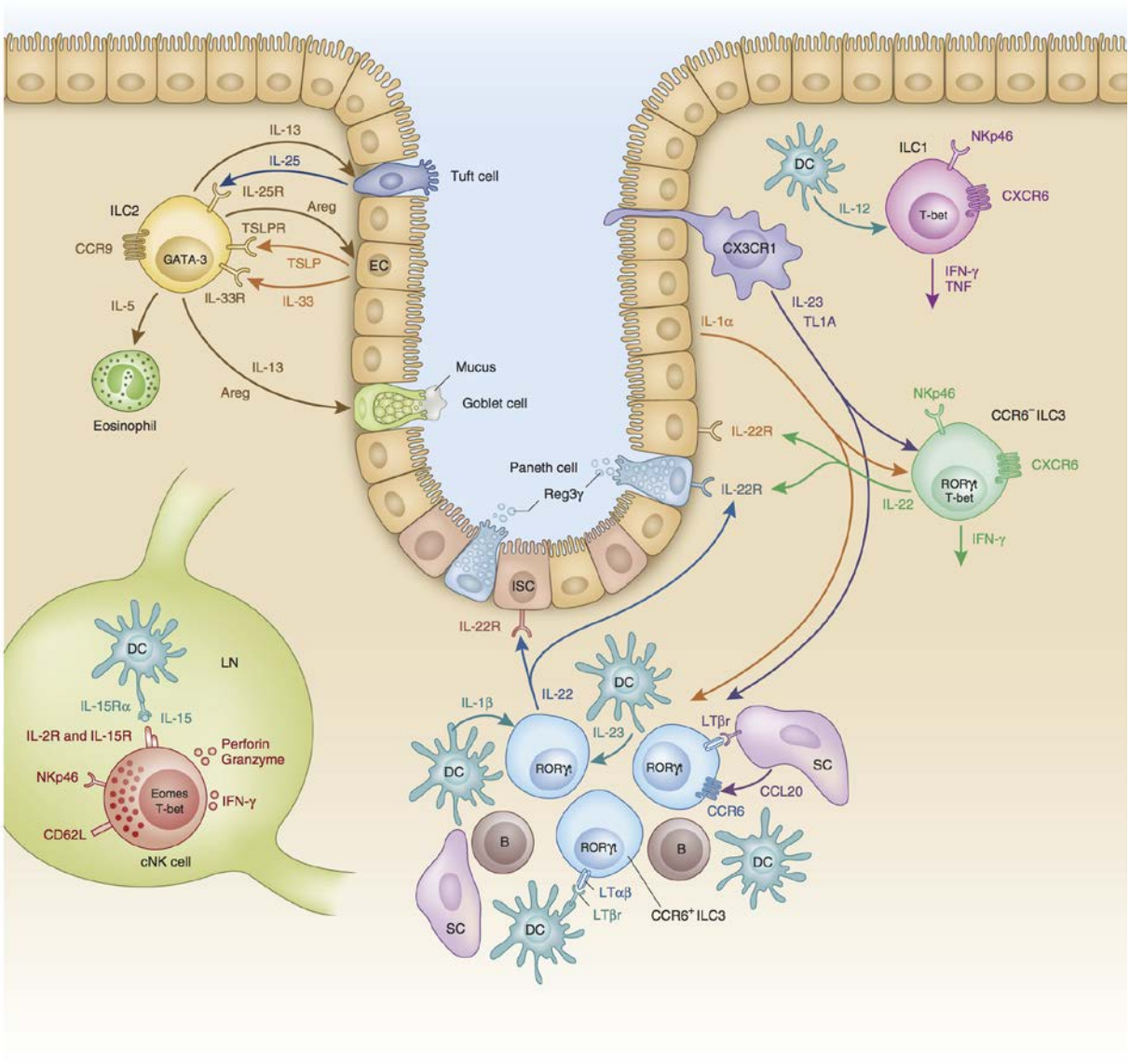


Figure 15 – ILCs at tissue barrier (Klose and Artis, 2016).

Together with their strategic location at tissue level, one of the remarkable characteristic of ILCs is that their biological activity is strictly dependent on their microenvironment, making them a very important player in the initiation and regulation of the inflammatory response *in situ*. In response to environmental cytokines, tissue damage, extra or intracellular pathogens, ILCs can trigger a potent immune response through the release of inflammatory as well as regulatory cytokines at the site of infection. Recent research revealed an immunomodulatory role for ILCs in adaptive immune responses as well as important roles in the resolution of inflammation and homeostasis (Ebbo et al., 2017; Klose and Artis, 2016; Roediger and Weninger, 2015, 2017), making ILCs an attractive target of immunotherapies in chronic and acute infection.

In the next pages, I will briefly present the family of ILCs, with a particular focus on ILC2s, the main member involved in this study. I will then expand on the recent findings on ILCs and their role at homeostasis and disease.

1.3.1. The family

The family of ILCs include NK cells as well as three groups of the non-cytotoxic innate lymphocytes, which have been created divided according to their cytokine secretion profile and in the transcription factors driving their differentiation: the ILC1, ILC2s and ILC3s. The nomenclature process was problematic, as these cells were discovered from different groups and called with different names, like nuocytes, natural helper cells or non-B-non-T cells. In 2013 a consensus meeting took place among the main investigators involved in the discovery of ILCs and the nomenclature was unified (Spits et al., 2013).

The development of ILCs has been a subject of intense research, as many groups predicted the existence of a progenitor for all ILCs (Constantinides et al., 2014). It is now clear that all ILCs arise from a common committed precursor in the bone marrow, which first gives rise to a progenitor of NK cells, and then forms a common helper ILCs progenitor (CHILP) characterized by the expression of the IL-2 receptor common γ-chain (cγ) and the inhibitor of DNA binding 2 (Id2) for development (Constantinides et al., 2014; Withers and Hepworth, 2017). In further differentiations, all ILCs are formed (Figure 16): ILC1s acquire T-bet, ILC2s the GATA-binding protein 3 (GATA-3) as well as the retinoic acid receptor-related orphan receptor-α (RORα), and ILC3s express retinoic acid related orphan receptor γ isoform t (RORγt) (Klose and Artis, 2016).

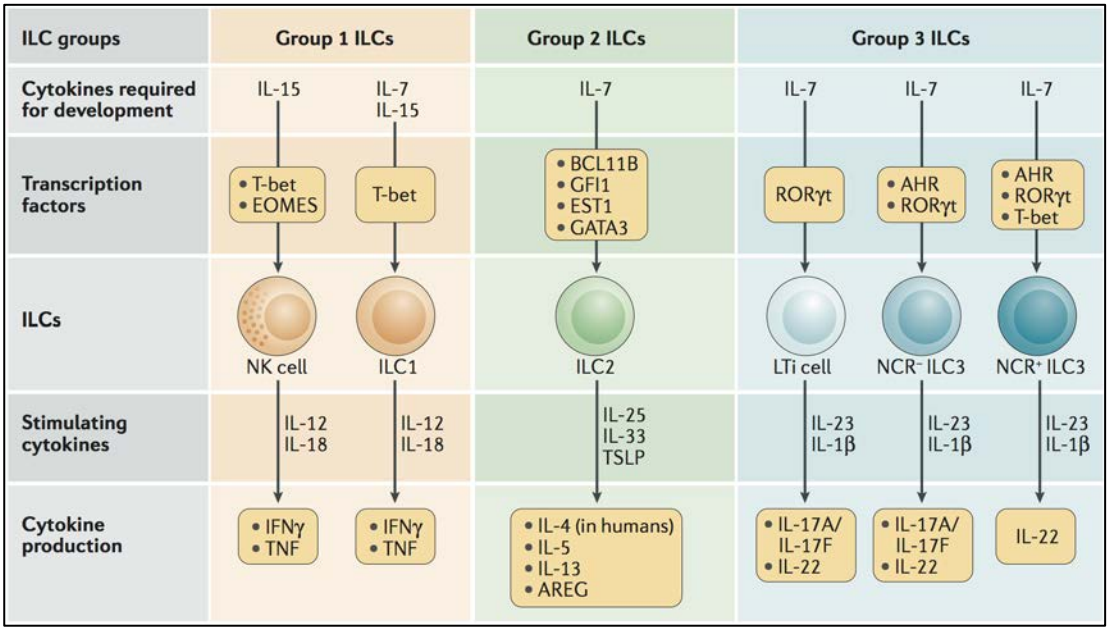


Figure 16 – ILCs subsets (Ebbo et al., 2017).

The best way to identify non-cytotoxic ILCs is by flow cytometry in tissue or peripheral blood, both in mice and humans. The most accredited gating strategy is to select the population of total lineage negative (Lin–) leukocytes, which also has to include an NK cells lineage marker, like CD49.1/NK1.1 in mice, or CD94/NKG2A in humans. After gating for CD45+ Lin- cells, then one should further gate for cells expressing the IL-7 receptor (CD127) (Björklund et al., 2016). For human samples, it is also useful to add the marker CD161, as all human ILCs subsets will express CD127 and CD161. This gate will contain all the ILCs subpopulations which can then be further identified by specific surface markers, which we will address in the next chapters. I will now briefly introduce the three subtypes of ILCs and then focus on ILC2s only and in particular on their role at homeostasis and disease.

ILC1

Group 1 ILCs are characterized by the expression of the transcription factor T-bet and produce IFN gamma and TNF in response to IL-12 (Klose and Artis, 2016). Some authors include in this group also NK cells, suggesting a further differentiation between "cytotoxic" ILC1s, which are the *bona fida* NK cells, and the "non-cytotoxic" ILC1s (Ebbo et al., 2017; Jiao et al., 2016). Classical NK cells have been identified as Lin– NK1.1+ cells and have an a historic role in tumor surveillance and intracellular pathogen recognition, in particular concerning antiviral defenses (Klose and Artis, 2016), for their ability to recognize and kill cells that downregulated MHCI expression. In particular, they can recognize target cells via

expression of specific natural cytotoxicity receptors (NCRs), like NKp46 (also known as NCR1) and can induce granule mediated cytotoxic cell killing via perforine and granzymes or via engagement of death receptors (Jiao et al., 2016). The capacity of NK cells to induce cell killing is dependent on the balance between activating and inhibiting signals on the target cell surface. For example, they famously express inhibitory receptors, like the Killer-cell immunoglobulin like receptors (KIRs) in humans or the Ly49 in mice, which are the main receptors for MHC class I molecules and the binding of these receptors inhibits NK cell killing (Jiao et al., 2016).

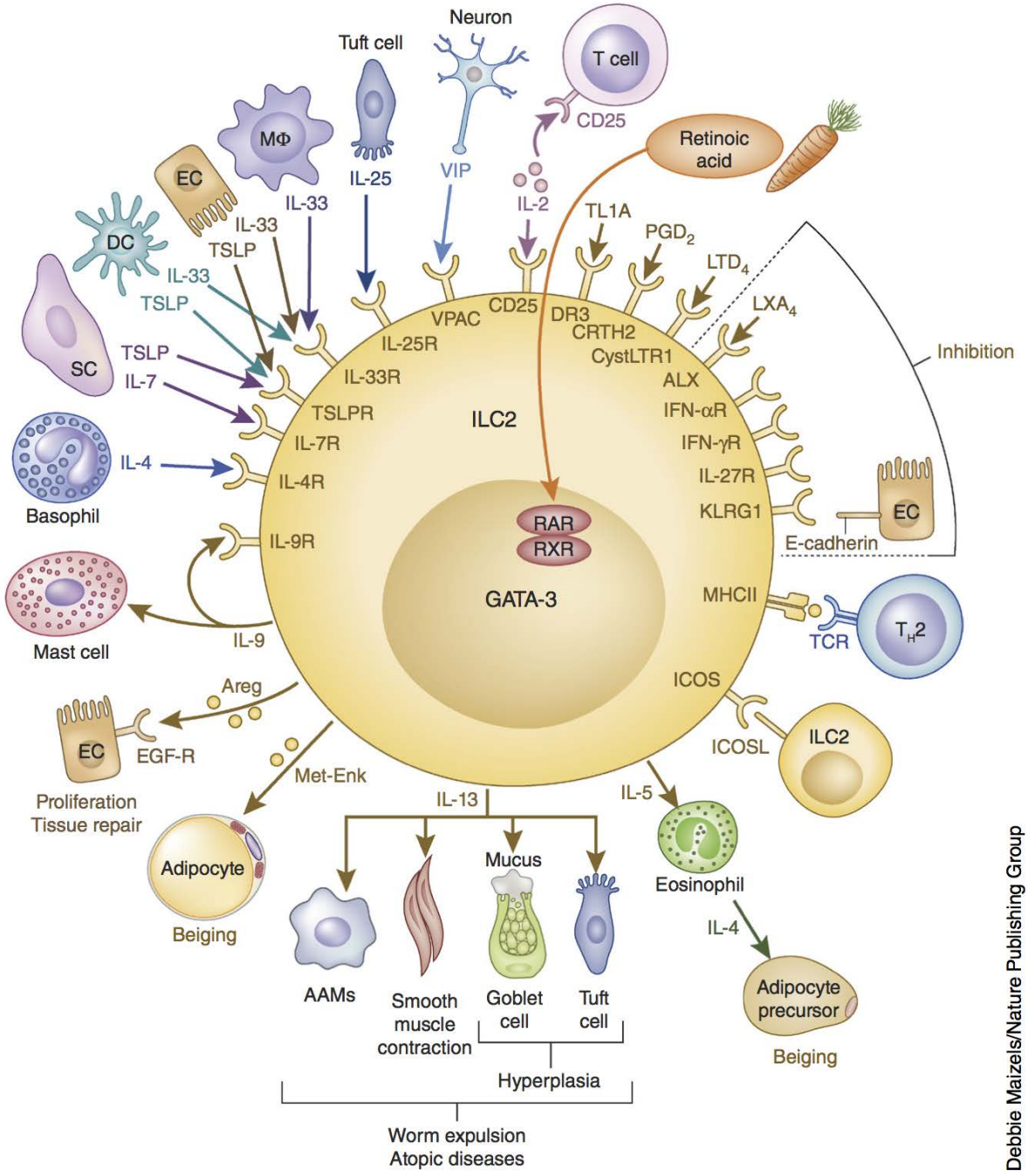
ILC1s are usually identified by flow cytometry by exclusion of NK, ILC2s and ILC3s markers. They have at first been discovered as tissue associated subsets of NK cells, with no cytotoxic activity and expressing the tumor necrosis factor related apoptosis inducing ligand (TRAIL) (Takeda et al., 2005). Later on, further ILC1s subsets were identified in the thymus, gut, lymphoid organs and salivary glands. ILC1s preferentially reside in tissues, while NK cells abundantly circulate in the peripheral blood and secondary lymphoid organs at homeostasis. Like all ILCs, long term maintenance of ILC1 is accomplished by self-renewal in tissue, not only at mucosal level, but also in secondary lymphoid organs, such as spleen and lymph nodes (Gasteiger et al., 2015a). This is not the case for NK cells, which undergo bone marrow replenishment at steady state and both in peripheral tissues and secondary lymphoid organs. In 2014, after the discovery of a common lymphoid progenitor for all ILCs subsets (ILCP), it was shown that NK cells directly differentiate from the ILCP via the transcription factor PLZF, whereas all other ILCs differentiate later from an Id2+ precursor (Artis and Spits, 2015; Constantinides et al., 2014). Moreover, recent evidence suggest that conventional NK cells depend from the transcription factor EOMES for their development, while ILC1 are strictly T-bet dependent (Roediger and Weninger, 2015). Finally, non-cytotoxic ILC1s where shown to have non redundant role compared to NK cells through their capacity to be an early source of TNF and IFNγ at tissue level (Abt et al., 2015). In summary, NK cells and ILC1s might be considered separately, due to their quite diverse role in innate immunity, as well as their different tissue localization and embryonic development (Jiao et al., 2016): ILC1s could be classified as the innate counterpart of Th1 cells due their localization in tissue and their ability to produce TNF and IFNγ in response to intracellular pathogens, whereas the classical NK cells, or "cytotoxic" ILC1s, can be seen as an innate counterpart of antigen specific CD8+T cells (Klose and Artis, 2016).

ILC2

ILC2s can be identified in mouse tissues as Lin^{-} $CD127^{+}ST2^{+}$ cells, which is the main gating strategy used in this study. In humans, they are identified from the $Lin^{-}CD161^{+}CD127^{+}$ gate as being the subgroup expressing CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) and, depending on the tissue, CD117, also known as c-Kit, a classical mast cell marker. They were first characterized in 2010, when three different groups simultaneously discovered an innate cellular source of IL-13 and IL-5 in mice deficient in B and T cells (Moro et al., 2010; Neill et al., 2010a; Price et al., 2010). Like Th2 cells, ILC2s express the transcription factor GATA-binding protein 3 (GATA3). However, they are the only cell in the immune system that specifically the retinoic acid receptor-related orphan receptor-α (RORα) for differentiation, as shown in RORα staggerer mice ($Ror\alpha^{sg/sg}$) which are selectively devoid of ILC2s (Halim et al., 2012a). Interestingly, RORα knock out mice are non-viable, whereas the staggerer mice, which have a strong deficiency of RORα, besides presenting a selective deficiency of ILC2s, are viable but show important neurological deficits and do not survive more than 3-6 weeks of age. Healthy mice selectively deficient in ILC2s could be created by floxing the gene *RORα* only in the IL7 expressing cells (i.e. all lymphocytes) by using $IL7R^{Cre}$ mice (Oliphant et al., 2014a). The $Il7r^{Cre}Ror\alpha^{sg/fl}$ mice are therefore devoid of ILC2s, whereas all other lineages and other ILCs are perfectly conserved and functional.

Although they form only a small quiescent population in the resting tissues – for example in the mouse lung they represent around 0,25-1% of lung (Drake and Kita, 2014) – once activated, ILC2s can quickly proliferate in tissue and secrete high quantity of type 2 cytokines, like IL-5, IL-13, IL-9. ILC2s have been described in mucosal tissues, lung, gut, skin, fat associated lymphoid clusters (FALC), spleen, liver, visceral adipose tissue, bone marrow and virtually in any tissue in the body (Klose and Artis, 2016). To understand the biology of ILC2s it is important to know which are the main stimuli that determine their activation, which happens to be for the most part epithelial-derived. Interestingly, the strongest recognized stimulus so far is IL-33, the epithelial derived alarmin that we introduced in the chapter related to AEC2. IL-33 is mostly expressed in epithelial cells and fibroblasts, where it localizes in the nucleus and it is released as a cytokine upon cellular damage or destruction, therefore acting as an alarmin (Liew et al., 2016). Mice treated intranasally or intravenously with IL-33, display a generalized state of inflammation, with airway hyperactivity, spleen enlargement, gastro-intestinal inflammation with hypertrophy and hyperplasia of goblet cells (Lüthi et al., 2009) and a systemic proliferation of ILC2s (Barlow

et al., 2013). ILC2s are the first cells sensing IL-33 in the lung and the cells mostly responsible for mediating its biological activity at tissue level (Halim et al., 2014; Kearley et al., 2015). Some other of the most studied triggers of ILC2s activation are IL-25 and the thymic stromal lymphopoietin (TSLP). IL-25, also known as IL-17E because of its similarity with IL-17A, is secreted by epithelial cells, basophils, eosinophils and mast cells (Oliphant et al., 2011). IL-25 is not an alarmin, as it is actively secreted, instead of being released upon cellular damage, and it seems to be less potent in inducing ILC2s (Barlow et al., 2013).



Figure–17 ILC2s signaling integration and effector cytokines (Klose and Artis, 2016).

It binds to the IL-25R, also known as IL-17RB, on the surface of ILC2s. The direct association of IL-25 with the initiation of type 2 immune responses was first reported in 2007, when it was revealed that intraperitoneal IL-25 injection could induce airway hyper-reactivity, a hallmark of type 2 inflammation (Angkasekwinai et al., 2007). Another important epithelial derived stimulus for ILC2s proliferation is TSLP, which was first identified in the late 1990's and takes its name from its ability to promote the growth of a thymic stromal cell line in vitro. Subsequently, it was noted that TSLP can stimulate dendritic cells (DCs) to induce Th2 polarization of naive Th cells, initiating type 2 immune responses (Zhou et al., 2005). Compared to IL-33 and IL-25, TSLP seem to have little role in the activation of lung ILC2s and to be instead more involved in the activation of skin ILC2s, suggesting an organ-dependent sub-specialization of ILC2s (Roediger and Weninger, 2015). ILC2s were also reported to sense IL-15, IL2, IL-4, IL7, Prostaglandin D2, leukotriene D4, TL1A and other stimuli (Figure 17) (Klose and Artis, 2016). For example, upon sensing the circadian synchronizer vasoactive intestinal peptide (VIP), ILC2s can secrete IL-5 depending on the circadian rhythm fluctuations and therefore regulate eosinophils homeostasis at tissue level (Nussbaum et al., 2013). They express ICOS and ICOSL and this interaction can promote their self-activation and self-amplification (Maazi et al., 2015). ILC2s can also receive inhibitory signals, and are for example inhibited by type I and II interferons, as well as by IL-27 (Duerr et al., 2015). Importantly, regulatory T cells (Tregs) can exert immune-regulatory functions on activated and proliferating ILC2s, mainly via competing for IL-2 or directly inhibiting ILC2s via IL-10 and TGFβ, or inhibiting the ICOS-ICOSL interaction. In fact, the depletion of Tregs can result in the exaggerated proliferation of ILC2 (Doherty and Broide, 2017). Another characteristic of ILC2s worth mentioning, is that in special conditions, like in *in vivo* infections with *Nippostrongilus brasiliensis* of *Candida albicans*, ILC2s can additionally assume an inflammatory phenotype, characterized by the expression of KLRG1 and IL-17, as well as upregulation of RORγt. These "inflammatory ILC2s" are only developing in presence of IL-25 and seem to be an intermediate progenitor of ILC2s or ILC2s that appear in fungal and helminth infections (Huang et al., 2014).

ILC3

ILC3s encompass a more heterogeneous group of innate lymphocytes, all characterized by the expression of the transcription factor RORγt and the chemokine receptor CCR6. ILC3s are commonly considered the innate counterpart of Th17 cells (Klose and Artis, 2016). In

response to signals from tissue resident myeloid or epithelial cells, such as IL-1β, IL-1α and IL-23, ILC3s can produce pro-inflammatory cytokines like IL-17A, IL-17F, IL-22 and GM-CSF. Both in mice and humans, ILC3s can be further subdivided into two groups, based on the expression of the NK associated receptor NKp46 (also called natural-cytotoxicity-receptor or NCR) or the chemokine receptor CCR6: $CCR6^{-}NCR^{+}$ILC3s and $CCR6^{+}NCR^{-}$ ILC3s. The $CCR6^{+}NCR^{-}$ ILC3s comprises other ILCs, like the Lymphoid tissue inducers cells (LTi) and the LTi-like ILC3s (Figure 16). Both subgroups have a similar cytokine profile, in particular both $CCR6^{+}NCR^{-}$ and $CCR6^{-}NCR^{+}$ ILC3s can secrete IL-22 in response to IL-23 and IL-1β. The two subgroups differ for what concerns the location, as $CCR6^{-}NCR^{+}$ ILC3s are mostly found in the small intestine mucosa, while $CCR6^{+}NCR^{-}$ ILC3s are mostly dominant in the colon and in the lymphoid tissues (Withers and Hepworth, 2017). These differences mirrors their functions, as for example during development, CCR6+ ILC3s, have an essential role in the actual formation of lymphoid organs and in adult mice they mostly cluster in cryptopatches and lymphoid follicles (Klose and Artis, 2016).

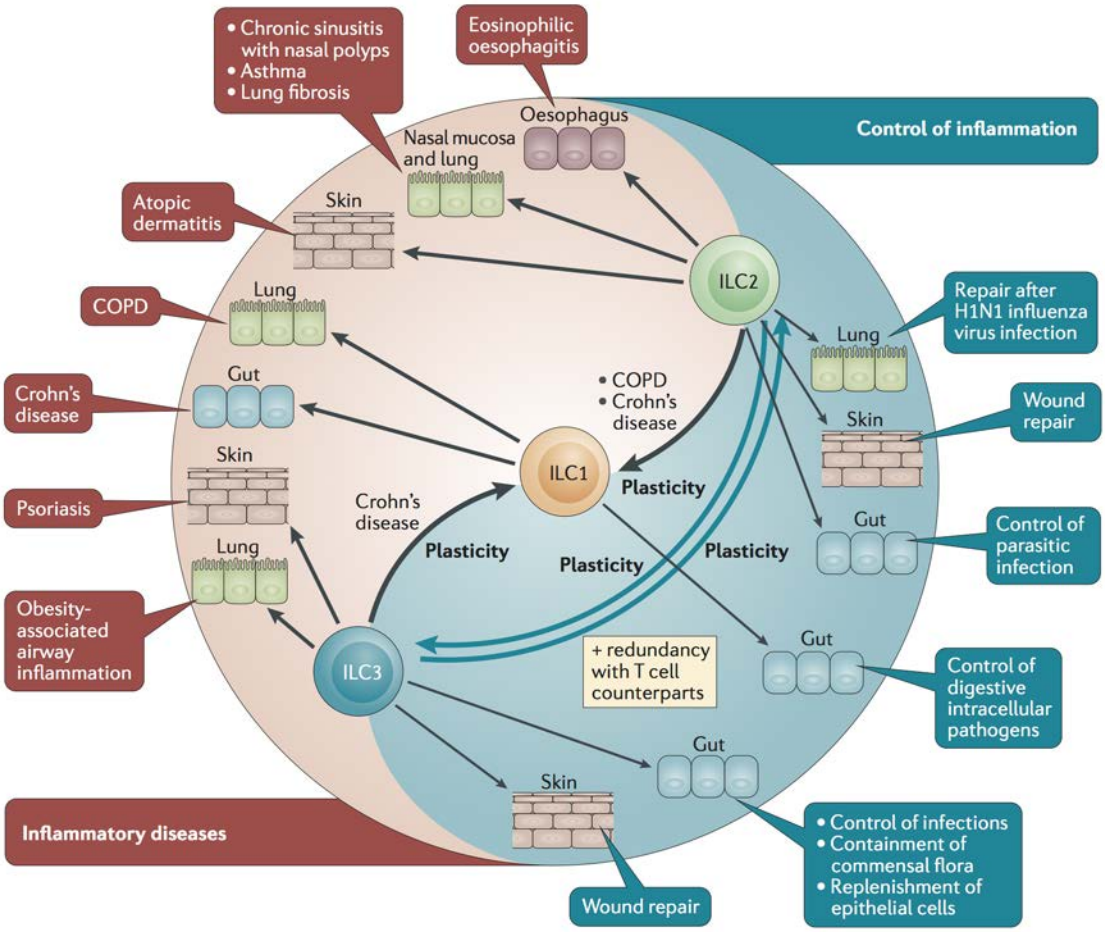
ILC3s have a prominent role in the defenses against bacterial and fungal infections as well as a recognized role in the establishment and maintenance of the gut microbiome and it is mostly their role in the intestinal mucosa at homeostasis and defenses that has caught the attention of researches worldwide, especially for the possible implication of these cells in the pathogenesis of intestinal bowel disease (Withers and Hepworth, 2017). In fact, ILC3s are among the first cells to seed gut mucosal barrier during embryogenesis and therefore to sense the microbiome colonization as well as the diet derived changes upon weaning (Hepworth et al., 2015). Moreover, also in adult life ILC3s constantly sense the signals deriving from diet and microbiota and dynamically controls the microbiome homeostasis via directly inducing cell death of activated commensal bacterial-specific T cells, therefore providing some sort of commensal selection (Hepworth et al., 2015).

At homeostasis, intestinal ILC3s also integrate signals from myeloid or epithelial derived mediators and are therefore very sensitive to dietary changes, vitamin intake or fluctuations in the microbiota, which influences their homeostatic proliferation (Withers and Hepworth, 2017). One of the most important effector cytokine of ILC3s is IL-22, which is fundamental in regulating host-pathogen interaction at homeostasis, as well as in mediating the innate immune response to intestinal pathogens. For example, vitamin A derived metabolites sensed by the retinoic acid receptors can favor ILC3 responses, which are fundamental for controlling the intestinal bacterium *Citrobacter rodentium* and mice deficient in vitamin A cannot control the infection (Klose and Artis, 2016). Another example of dietary-sensing by

the ILC3s is their ability to sense metabolites from cruciferous vegetables, like cabbage and broccoli, via the aryl hydrocarbon receptor (AHR), whose activation is fundamental not only for ILC3s function, but also for their development itself (Klose and Artis, 2016). ILC3s-derived IL-22 acts on the IL-22R on epithelial cells and Panneth cells and promotes barrier function and the segregation of bacteria via the induction of antimicrobial peptides and tight junction formation (Sonnenberg et al., 2012). Altogether, evidences support the hypothesis of ILC3s playing an important role in the pathogenesis of microbiome dysbiosis and therefore intestinal bowel disease, as well as maintaining homeostasis of the gut tissue.

1.3.2. Innate lymphoid cells type-2 in health and disease

Type-2 immune responses probably evolved to protect against intestinal nematodes, as these were ubiquitous infections before modern medicine (Hammad and Lambrecht, 2015). They are characterized by polarization of naive Th cells into a Th2 phenotype, able to secrete IL4, IL-5 and IL13, which drives eosinophils recruitment and IgE class-switch of B cells and finally mast cells degranulation in response to even minimal amounts of antigen (Licona-Limón et al., 2013). These responses potently kill, paralyze and eliminate parasites via the gastrointestinal tract and at the same time provide tissue remodeling stimuli to reconstruct the architectural damage (Allen and Maizels, 2011). However, aberrant type 2 responses are also responsible for allergic reactions and implicated in the pathogenesis of childhood asthma, allergic rhinitis or atopic dermatitis (Klose and Artis, 2016). Th2 cells have long been considered the master regulators of type-2 allergic immune responses, which typically affects barrier tissues, like skin, lungs and intestine. However it was always a bit unclear how these responses exactly initiate in tissue, since antigen presentation and IgE class switching usually are events that occur in the lymph nodes (Roediger and Weninger, 2015). The discovery of ILC2s shed some lights on this immunological mystery. In fact, quiescent populations of ILC2s are strategically located at mucosal tissues and at the interphase with the outside environment and can be directly activated by epithelium-derived cytokines induced by barrier breach, like IL-25, IL-33 and TSLP, without the need of antigen recognition. ILC2s serve as a fundamental early innate source of IL-13 and IL-5 at tissue level and might even be important in initiating type 2 immune responses itself, via skewing tissue resident dendritic cells to a phenotype that might preferentially drive the polarization of naive T cells into Th2 instead of Th1, once they reach the lymph nodes (Halim et al., 2015; Oliphant et al., 2011).



Figure–18 Dual role of ILC2s in inflammatory diseases and repair (Ebbo et al., 2017).

Role of ILC2s in the pathogenesis of Asthma

According to the pathogenesis, the asthmatic disease can be subdivided in two main groups: the allergic asthma and the non-allergic (also called intrinsic) asthma. Allergic asthma is typically associated with allergic sensitization to airborne particles already during childhood and with presence of elevated serum immunoglobulins IgE and positive skin prick-test to defined airborne antigens. It usually progresses also in allergic rhinitis and atopic dermatitis in a stepwise fashion that has been called "atopic march". Non-allergic asthma, instead, is not correlated with the sensitization to a specific antigen, and often develops later in life and in association with obesity and nasal polyps and it is more difficult to treat, often being corticosteroid resistant (Lambrecht and Hammad, 2014) . The importance of ILC2s in allergic immune responses has been predicted in genome wide association studies (GWAS) which strongly linked the allergic phenotype with the presence of single nucleotide polymorphism (SNPs) in genes involved in ILC2s biology to be strongly linked with the allergic phenotype: among these *IL-33*, *ST2*, *TSLP*, *Rorα*, *IL-4*, *IL-5* and *IL-13* (Li et al., 2015). While antigen

sensitization and type-2 adaptive immunity plays well-defined roles in the pathogenesis of allergic asthma, ILC2s could be responsible for symptomatology and pathogenesis of both forms. In fact, ILC2s-derived IL-5, IL-9 and IL-13 induce eosinophils recruitment, goblet cells hyperplasia, smooth cell muscle contraction and airway remodeling, which are the hallmarks of airway hyper-responsiveness (AHR) seen in asthma, without the need of antigen presentation. Moreover, some evidence suggests that ILC2s are involved in the early events at tissue level that lead to antigen sensitization (Halim et al., 2012b, 2015). In the quiescent lung, ILC2s are quite abundant and rpsent cell type with the highest expression of the IL-33 receptor (ST2), therefore being probably the first cells to react to IL-33 release in case of barrier damage (Halim et al., 2014; Nussbaum et al., 2013). Molecules like papain or the major house dust mite (HDM) allergen Derp-1 and Derp-2 have protease and enzymatic activity that directly cause damage of epithelial cells by cleaving tight junction proteins. This leads to the release of IL-33, one of the major molecule used by ILC2s to sense the external environment (Hammad and Lambrecht, 2015). Other allergens with protease activity are for example chitin, *Alternaria alternata* and some cockroach antigens and they have all shown to activate ILC2s and induce airway hyper-reactivity independently of adaptive immunity (Roediger and Weninger, 2015). Another way in which proteolytic antigens can activate ILC2s following antigen exposure is via inducing the expression of the leukotriene LTD4, the main ligand of the cysteine leukotriene receptor CysLT1R present on ILC2s (Lambrecht and Hammad, 2014). Therefore, an intrinsic hyper-reactivity of mucosal barriers to airborne particles could determine exaggerated ILC2s activation and airway hyper responsiveness in some individuals, without the need of antigen presentation (Lambrecht and Hammad, 2014).

ILC2s might also play an important role in exacerbating allergic asthma. In fact, even in ovalbumin or HDM sensitized mice, ILC2s still represent the major type-2 producing cytokines at tissue level, even more than activated Th2 cells (Wolterink et al., 2012). Mice lacking ILC2s, like the *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice used in this study, develop a less severe lung inflammation upon allergic sensitization and re-challenge with papain or HDM (Halim et al., 2014). Therefore, also in the context of Th2 activation upon allergen re-encounter, ILC2s seem to contribute to the lung inflammation. Moreover, as already mentioned, this innate source of type-2 cytokine can to play a role in the allergic sensitization itself, as it was shown in the case of the sensitization to papain, for which an ILC2-dependent source of IL-13 can skew migratory DCs to induce naive T cells polarization towards Th2 (Halim et al., 2015). Moreover, ILC2s have been shown to express MHCII and other co-stimulatory molecules, as

well as being able to travel to the lymph node and even act asan antigen presenting cells during the sensitization phase, driving the proliferation of Th2 cells (Oliphant et al., 2014b).

New therapeutic approaches to asthma involve the blocking of cytokines downstream of Th2 or ILC2s activation. For example Dupilumab, an antibody against the alpha-chain of the IL-4 receptor which is also shared by the IL-13 receptor (Figure 11) therefore blocking both IL-4 and IL-13, two of the major cytokine produced by Th2 and ILC2s (Lambrecht and Hammad, 2014). Antibodies against IL-13ra1 (Lebrikizumab) and the cytokine IL-5 (Mepolizumab) or the IL-5 receptor (Benralizumab) are also under investigation in asthma. However, many clinical trials with IL-4 or IL-13 blocking strategies, either with systemically delivered or inhaled blocking antibodies, for now failed to show benefit in severe asthma, even if providing significant improvements in a subset of patients with eosinophilic asthma (Parulekar et al., 2017). Biologicals that that could possibly target cytokines lying upstream to ILC2s activation, like IL-33, TSLP and IL-25, are under investigation (Parulekar et al., 2017).

Role of ILC2s in the pathogenesis of atopic dermatitis

Like for the lung, also skin allergens can induce the transient destabilization of tight junction and adhesion molecule degradation, which promotes type-2 immune responses via secretion of keratinocytes derived TSLP (Hammad and Lambrecht, 2015). These phenomena are relevant in the pathogenesis of atopic dermatitis (AD), a classical allergic skin disease. In fact, patients affected by AD show signs of reduced barrier controls. Moreover, the increased frequency of mutations of filaggrin, a filament-associated protein involved in the correct differentiation of epidermal cells, strongly correlates with the development of allergic skin disease (Rerknimitr et al., 2017).

At steady state, ILC2s reside in the dermis and have been observed in increased numbers in atopic dermatitis (AD) (Roediger and Weninger, 2015). They can be recruited in the skin upon allergen sensitization and re-challenge both in mice and humans. Dermal ILC2s are constantly inhibited by E-cadherins, which suppress ILC2s activation via binding to KLRG, expressed in abundance on skin resident ILC2s (Salimi et al., 2013). Dermal ILC2s preferentially reside close to the vessel and can produce small quantities of IL-13 also at steady state and localize in close proximity with mast cells, implicating a concerted response during inflammation (Roediger and Weninger, 2015). In fact, both mast cells and ILC2s can be activated by IL-33 and TSLP derived from fibroblasts and keratinocytes and in response to stimulation ILC2 can produce IL-9, which is an important survival factor for mast cells during

acute inflammation (Roediger et al., 2014). Like it was observed for the mouse lung, also in the mouse skin ILC2-derived IL-13 can feed dermal dendritic cells which then travel to the lymph node and polarize naive T cells to a Th2 phenotype, thereby contributing to the pathogenesis of AD (Halim et al., 2014).

The skin of AD patients often becomes colonized with *Staphylococcus aureus* (*S. aureus*), which can further degrade skin barrier and exacerbate the disease (Hammad and Lambrecht, 2015). One possible explanation to *S. aureus* pathogenic replication could be the fact that IL-13 was shown to reduce the production of antimicrobial peptides in the keratinocytes and might therefore contribute to staphylococcal colonization or replication (Roediger and Weninger, 2015). Dupilumab, the IL-4Ra antagonist which acts downstream of Th2 and ILC2 activation, is currently in study for the treatment of severe AD (ClinicalTrials.gov Identifier: NCT02612454). Other potential therapies that act upstream or downstream of ILC2s activation, like TSLP inhibitors or CRTH2 antagonist respectively, are under investigation (Rerknimitr et al., 2017).

Role of ILC2s in the maintenance of tissue integrity and homeostasis.

Upon activation, ILC2s have an established role in the maintenance of lung and gut tissue integrity via secretion of amphiregulin, which regulates proliferation and differentiation of epithelial cells via binding to the epidermal growth factor receptor (EGFR) (Monticelli and Artis, 2012). Moreover, the M2 polarization of TRMs induced by activated ILC2s promotes wound healing and tissue remodeling (Klose and Artis, 2016). The role of tissue resident ILC2s, however, seems to go beyond the one played in case of barrier damage. Since resident ILC2s are the main target of IL-33 at tissue level, they might also be implicated in mediating its homeostatic effects (Molofsky et al., 2015). For example, the white adipose tissue (WAT) of healthy mice and humans is populated with M2 macrophages, Tregs and $ST2^{+}$ILC2s (Brestoff and Artis, 2015). Mice lacking IL-33 expression or signaling, show reduced numbers of ILC2s and M2 macrophages and are more susceptible to obesity and insulin resistance upon high-fat-diet (Molofsky et al., 2013). Moreover, recent intriguing results demonstrate that the IL-33/ILC2s axis promote the commitment of adipocytes precursors to beige adipocytes via epinephrine and catecholamine secretion and subsequent uncoupling protein activation, thereby controlling energy expenditure and adipose tissue homeostasis (Brestoff et al., 2014). Another mechanism by which ILC2s seem to control metabolism is via inducing IL-4 production from eosinophils, which in turn can act on the IL-4R expressed on pre-adipocytes and drive their differentiation into metabolically active beige adipose tissue

(Lee et al., 2015). Finally, eosinophils homeostasis in the lung, gut and adipose tissue has also been shown to depend on ILC2s-derived IL-5 secretion in the quiescent lung, which in turn depends on the circadian fluctuations and on the post-nutritive secretion of the vasoactive intestinal peptide (VIP), linking homeostatic eosinophils number oscillations with nutrient intake and circadian rhythm (Nussbaum et al., 2013).

Role of ILC2s in the resolution of inflammation

Because of the production of amphiregulin, which can mediate tissue repair via induction of epithelial cells replication, ILC2s have an established role in the resolution of the lung epithelial damage created by cytopathic viruses, like influenza (Monticelli et al., 2012). Moreover, IL-13 has also been reported to induce epithelial proliferation upon airway injury or inflammation (Monticelli and Artis, 2012). Recently, a new role for ILC2s in mediating the resolution of chronic inflammation has been proposed, which could have important clinical implications in all chronic inflammatory diseases, like rheumatoid arthritis (RA), diabetes, asthma and atherosclerosis (Roediger and Weninger, 2017). In a model of antigen mediated RA, Rauber et al. showed that ILC2s can stimulate the suppressive capacity of Tregs via IL-9 (Rauber et al., 2017). In fact, mice deficient in IL-9 could not resolve the chronic inflammation in an antigen-mediated arthritis mouse model, because of impaired Tregs suppression capacity. With the use of IL-9 reporter mice, the authors demonstrated that the main source of this cytokine during the post inflammatory phase of arthritis were in fact ILC2s. Mechanistically, the secretion of IL-9 provided a positive feed-back on ILC2s, determining their *in situ* proliferation and upregulation of the ligand for glucocorticoid-induced tumour necrosis factor receptor-related gene (GITR-L), highly expressed on Tregs. ILC2s promoted Tregs activity via direct contact through GITR-L and ICOS-L. Adoptive transfer of *IL-9*$^{-/-}$ Tregs that were pre-activated with GITR-L and ICOS-L *ex vivo* via co-culture with ILC2s promoted the resolution of the chronic inflammation in *IL-9*$^{-/-}$ mice (Rauber et al., 2017). These data are not only valid for mice, as the author showed that also in humans the major source of IL-9 during the remission phase of RA was coming from ILC2s. ILC2s number in blood were also particularly higher in patients in clinical remission compared to patients during the acute phase of arthritis (Rauber et al., 2017). These data demonstrate a pivotal role of ILC2s in the resolution of inflammation in humans and have potential implication in other chronic inflammatory diseases.

1.4. Pneumococcal pneumonia

1.4.1. Epidemiology and risk factors

Streptococcus pneumoniae (*S. pneumoniae*) is the most common cause of community acquired pneumonia (CAP) and the most frequent cause of death due to an infectious disease worldwide (World Health Organization, 2008). Human lungs, particularly at very early age, but also in elderly patients, seem to be very susceptible to this disease, which before the advent of antimicrobial therapy has been the number one killer of humanity (van der Poll and Opal, 2009). *S. pneumoniae* is responsible for 11% (8-12%) of all deaths in children aged from 1-59 months, excluding deaths in HIV+ children (Falleiros-Arlant et al., 2015). Besides age, two other main risk factors for the development of *S. pneumonia* induced CAP have been identified and these are asthma and influenza (Chien et al., 2009; Talbot et al., 2005a). Interestingly, both diseases are characterized by exacerbation of type 2 immunity in the lung, with an M2 polarization of alveolar macrophages, tissue remodeling and activation of ILC2s to produce IL-13 (Chang et al., 2011; Lambrecht and Hammad, 2014; Monticelli and Artis, 2012). Moreover, epidemiological evidence suggest that exacerbation of type 2 immunity, for example parasitic infections, increase the risk for bacterial diseases worldwide, probably via inducing an alternative polarization of macrophages (Salgame et al., 2013). We also previously showed, that an M2 polarization of alveolar macrophages is detrimental in case of pneumococcal pneumonia (Warszawska et al., 2013). Other groups also suggested that Arginase 1 expression in AMs could be detrimental (Knippenberg et al., 2015). In summary, besides the known risk factors for severe CAP, like age and immunosuppression, also patients with conditions characterized by an exacerbation of type 2 immune responses in the lung are to be considered at risk of developing CAP. However, the reason for the increased susceptibility in young children is not known and empirically attributed to the “immaturity” of the immune system. Moreover, the contribution of ILC2s in the susceptibility to *S. pneumoniae* has not been addressed so far.

1.4.2. Pathogenesis

S. pneumoniae is a highly pathogenic capsulated gram-positive bacterium, classified in 91 different serotypes on the base of the capsular antigens. It is quite commonly found in the nasopharyngeal tract of the general population and it is acquired via direct contact with contaminated respiratory secretions among children and family members, as a prolonged contact is required for the infection (van der Poll and Opal, 2009). *S. pneumoniae* presents

several virulence factors that are critical for invasiveness, the most effective being the polysaccharide capsule: this thin layer of highly negatively charged polysaccharides sterically inhibits the interaction of with phagocytic receptors on macrophages, therefore avoiding early recognition. Moreover, it also prevents mechanical clearance by mucous secretion and provides adherence to the epithelial surface, while even being able to reduce the exposure to several antibiotics (van der Poll and Opal, 2009). Pneumococci also produce pore-forming exotoxins, like pneumolysin, which upon its release during autolysis, is highly toxic to host cells: it can for example reduce the motility of the ciliary epithelium and impair the respiratory burst of macrophages and neutrophils (van der Poll and Opal, 2009). Other known virulence factors include the presence of pili, which can also inhibit the phagocytosis and the production of a biofilm and IgA protease, which inhibit bacterial clearance.

When the pneumococci deposits in the hier airways, they are usually efficiently expelled by the mucociliary system, by couching or by antimicrobial peptides produced by local innate epithelial defenses. The nasopharyngeal colonization usually precedes the development of a disseminated disease if not readily controlled by the immune system, a process that can take few weeks in adults, but up to a few months in children. Failing in controlling the nasopharyngeal colonization results in progression of the bacteria to the lower respiratory tract and eventually in invasive disease, which usually manifests itself in form of pneumonia, but can also cause meningitis, otitis media or septicemia (Weinberger et al., 2008). In the case of pneumonia, if the bacteria reach the lower respiratory tract and manages to proliferate, it will the cause a full-blown pneumonia, with abrupt severe illness, fever, cough and dyspnea. The cough is usually productive with a purulent sputum. If left untreated, the disease can progress to acute respiratory failure and death in days upon onset (van der Poll and Opal, 2009). At this point of the infection, some immunological mechanism in the lower airways are vital to assure survival.

1.4.3. Key mechanism of resistance to S. pneumoniae infection

When the pneumococci reach the alveolar space, a few key cells are still able to contain bacterial and avoid exaggerated proliferation which then together with the help of antimicrobial therapy will increase the chances of survival of the infected individual. The very early immune responses to *S. pneumonia* infection in the alveolar space are orchestrated by alveolar macrophages, epithelial cells and neutrophils (van der Poll and Opal, 2009).

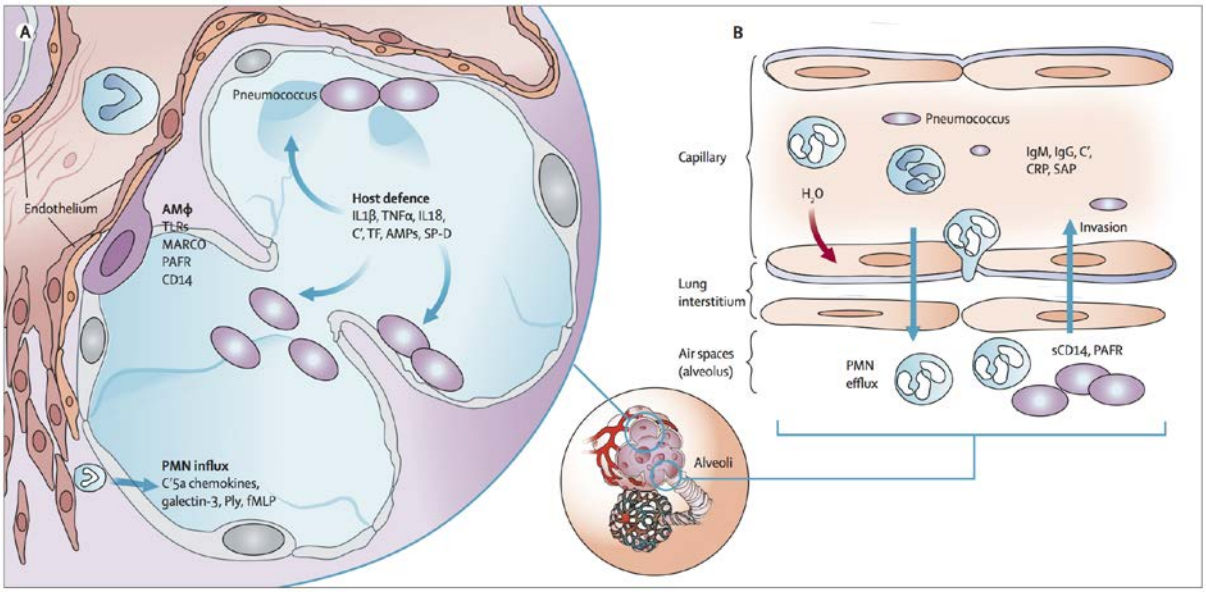


Figure 19 – Major pathogenic events initiated by *S. pneumoniae* in case of airway invasion (van der Poll and Opal, 2009)

Early recognition from the innate immune system is vital to induce an first wave of neutrophils and allow the begin of effective bacterial clearance, reducing the pathogenic burden. Alveolar macrophages are among the first cells to see the bacteria and to start an early innate response. For example, AMs recognize and internalize *S. pneumoniae* via the surface receptor MARCO (macrophage receptor with collagenous structure). Moreover, some PAMPs of *S. pneumoniae* are recognized by TLRs present on AMs, like the lipoteichoic acid (LTA), a component of the capsular wall, which can be recognized by TLR-2. Additionally, the bacterial toxin pneumolysin is detected by TLR4, whereas the intracellular TLR9 recognizes the bacterial DNA and is essential for the effective phagocytosis and killing in lung macrophages (Lee et al., 2007). Upon TLR ligation, the common TLR-adaptor protein MyD88 is recruited at the C-terminal domain of TLRs and activates intracellular kinases, above all the and interleukin 1 receptor-associated kinase 4 (IRAK4). Both MyD88 and IRAK4 are essential for nuclear translocation of NFκB and for initiating the pro-inflammatory cascade that leads to recruitment of neutrophils at the site of infection. In fact, children with genetic deficiency in MyD88 or IRAK4 are at increased risk of death due to invasive pneumococcal pneumonia (van der Poll and Opal, 2009).

Activated macrophages – and to a certain extent also AEC2 – start secreting inflammatory cytokines like IL-1β, IL-18, IL-6, TNF and alpha-chemokines like CXCL1, CXCL5 and CXCL8, that that can attract neutrophils at the site of infection (Soehnlein and Lindbom, 2010). Neutrophils are the dominant component of the cellular infiltrate in the early phases of

pneumonia and the primary phagocytes devoted to bacterial killing. Neutrophils are short-lived innate effector cells in acute inflammation and represent the most abundant polymorphonuclear leukocytes (PMN) in the human blood. CXCL1, CXCL5 and CXCL8 induce the activation, rolling and adhesion of neutrophils to the endothelium, and ultimately, their extravasation into the site of infection (Soehnlein and Lindbom, 2010). Once in tissue, neutrophils bind to antibody- or opsonin-coated pathogens, phagocytose them and direct them to intracellular ROS-mediated killing. An early neutrophil infiltration is determinant for the clearance of S. pneumoniae, as it can significantly reduce the bacterial burden during the early proliferation phase and therefore give time to the adaptive immune system to develop CD4+ and CD8+ antigen specific T cells (Schliehe et al., 2015; Warszawska et al., 2013). Any cause of delay in neutrophils influx during the early phase of infection can dangerously allow bacterial proliferation to reach a point of non-return, in which the body can only benefit from an early begin of an antimicrobial therapy.

1.5. Thesis Aim

The perinatal lung immune environment is largely "understudied", both in humans and in mice. The alarmin IL-33 has been shown to play fundamental immunological roles in the homeostasis of the lung and to be upregulated in AEC2 already during late embryogenesis. However, it's potential role at birth is unknown. Some cells that populate the lung tissue, like alveolar macrophages, appear in the lung right after birth and could potentially be affected by the alarmin IL-33 or its downstream effectors, like ILC2s and IL-13. By understanding the phenotype and function of the lung immune cells in newborn mice, we could obtain new insights in the pathogenesis of common lung diseases in newborns, like asthma and pneumonia.

With this work we therefore aimed at:

- understanding the possible role of the alarmin IL-33 in lung tissue at birth and in the perinatal period.
- characterizing the tissue-derived signals that provide specific functional cues to AMs at birth and in post-natal homeostasis.
- studying the phenotype of alveolar macrophages in the newborn lung as well as in adults, with special focus at their capacity to react to microbial pathogens.
- shedding new light on the homeostatic role of IL-33 and ILC2s in the lung defenses against bacterial pneumonia at birth and during adult life.

2. Results

First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment

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Running title

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2.1. Abstract

From birth onward, the lungs are exposed to the external environment and therefore harbor a complex immunological milieu to protect this organ from damage and infection. We investigated the homeostatic role of the epithelium-derived alarmin interleukin-33 (IL-33) in newborn mice and discovered the immediate upregulation of IL-33 from the first day of life, closely followed by a wave of IL-13- producing type 2 innate lymphoid cells (ILC2s), which coincided with the appearance of alveolar macrophages (AMs) and their early polarization to an IL-13-dependent anti-inflammatory M2 phenotype. ILC2s contributed to lung quiescence in homeostasis by polarizing tissue resident AMs and induced an M2 phenotype in transplanted macrophage progenitors. ILC2s continued to maintain the M2 AM phenotype during adult life at the cost of a delayed response to Streptococcus pneumoniae infection in mice. These data highlight the homeostatic role of ILC2s in setting the activation threshold in the lung and underline their implications in anti-bacterial defenses.

2.2. Introduction

The integrity of the alveolar-capillary barrier is essential to ensure sufficient blood oxygen levels, and the mechanisms driving its maintenance, renewal and protection are flourishing fields of research (Beers and Morrisey, 2011; Chiu and Openshaw, 2015; Hogan et al., 2014; Hussell and Bell, 2014; Kopf et al., 2014; Peng et al., 2015). Lung development begins at embryonic day 9 (E9) in mice and proceeds through stages of branching morphogenesis, giving rise to pre-alveolar spaces at the saccular stage, and the differentiation of type-1 and type-2 airway epithelial cells (AEC1 and AEC2) by E18.5 (Mund et al., 2008; Woik and Kroll, 2015). At birth, alveolar sacs are suddenly exposed to the external environment and subjected to the mechanical forces of spontaneous ventilation (Orr et al., 2006; Wirtz and Dobbs, 2000). It is after the previously sterile lung tissue has been exposed to the outside environment around postnatal day 4 (P4), when the process of alveologenesis continues with the formation of primary septa (Hogan et al., 2014). These postnatal adaptations are paralleled by the development of the early innate immune environment. Alveolar macrophages (AMs) differentiate on P3 from CD11b^{hi} F4/80^{int} Ly6C^{hi} fetal monocyte progenitors into long lived, self-renewing cells (Guilliams et al., 2013; Murphy et al., 2008). Since tissue-derived signals were found to govern the gene expression signature of macrophages (Lavin et al., 2014; Okabe and Medzhitov, 2014), the lung cytokine milieu in newborns likely determines the

phenotype of AMs during this delicate developmental period. However, the postnatal immunological environment in lungs is largely unexplored, as are the innate immune signals that influence the function of AMs early in development and during homeostasis.

Under different pathological conditions, AMs have the ability to assume either an IFNγ and TLR ligand-induced inflammatory phenotype (M1) or an IL-4, IL-13 or IL-10-induced wound healing and tissue remodeling phenotype (M2) (Gordon and Martinez, 2010). M1 macrophages are potent producers of inflammatory cytokines such as TNF and CXCL1 (Guery et al., 2011; Mantovani et al., 2004). CXCL1 is a chemokine that critically determines the early recruitment of neutrophils (De Filippo et al., 2013), thereby exerting a protective role in bacterial lung infections (Schliehe et al., 2015; Warszawska et al., 2013). M2 macrophages, phenotypically defined by the expression of *Retnla* (referred to here as *Fizz1*), *Mrc1*, *Chil3* (referred to here as *Ym1*) and *Arg1*, are less efficient in triggering inflammatory responses to bacterial pathogens than M1 macrophages (Warszawska et al., 2013).

IL-33 is an alarmin belonging to the IL-1 family of cytokines best known for its capacity to drive type-2 immune responses (Liew et al., 2010; Schmitz et al., 2005) and increasingly recognized as an important mediator of homeostasis and tissue tolerance (Molofsky et al., 2015). Upon mechanical strain or cell necrosis (Kakkar et al., 2012; Lamkanfi and Dixit, 2009; Sanada et al., 2007), IL-33 is released from cells, activating the ST2 receptor expressed on several lung cell types, including Tregs, dendritic cells (DCs), mast cells, group-2 innate lymphoid cells (ILC2s), natural killer (NK) cells and AMs (Lu et al., 2015). At steady state, lung resident ILC2s are the most abundant ST2-expressing cells and are found in close proximity to bronchovascular structures (Halim et al., 2014; Nussbaum et al., 2013), where they can be rapidly activated by IL-33 to secrete IL-13, IL-5, IL-6, IL-9, GM-CSF and amphiregulin (Roediger and Weninger, 2015). ILC2s are involved in host protection against parasitic helminths, promotion of airway hyperreactivity in asthma or upon influenza infection and are important for adipose tissue homeostasis (Barlow et al., 2012a; Brestoff et al., 2014; Chang et al., 2011; Molofsky et al., 2013; Monticelli et al., 2011; Neill et al., 2010a).

Human lungs are highly susceptible to bacterial infections. Pneumonia caused by *Streptococcus pneumoniae* (*S. pneumoniae*), is the primary cause of death by an infectious disease in Western countries (van der Poll and Opal, 2009). Notably, risk factors for developing community acquired pneumonia are asthma and influenza (Chien et al., 2009; Talbot et al., 2005b), which are both characterized by IL-13 induced airway hyperreactivity

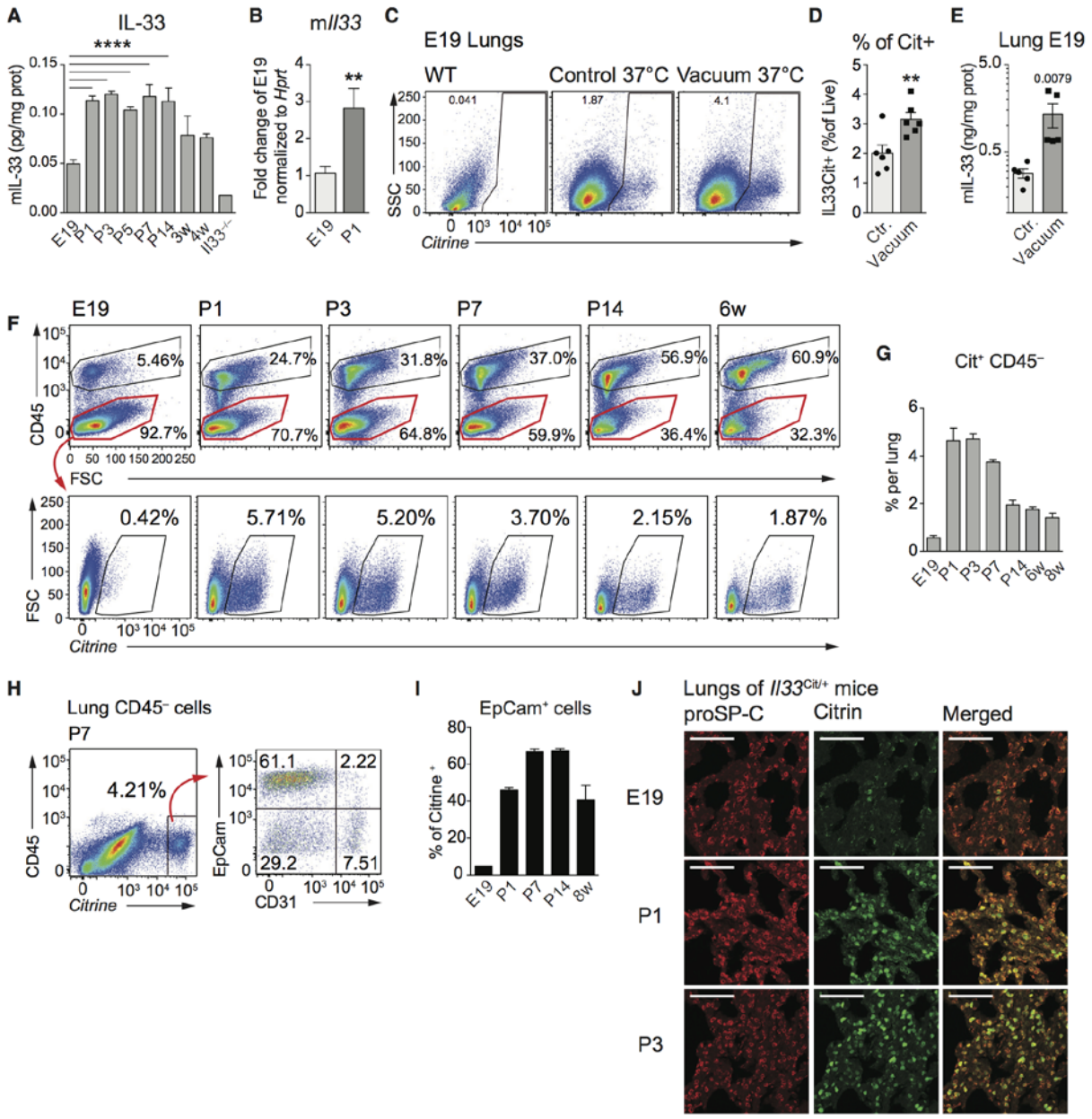
(Kim et al., 2012; Lambrecht and Hammad, 2014) and the presence of M2 polarized AMs (Chen et al., 2012). Here, we investigated the physiological role of the IL-33/ILC2/IL-13 axis in shaping the pulmonary immune environment from birth to adult life, and the consequences of these pathways on the innate defense against *S. pneumoniae*.

2.3. Results

2.3.1. Postnatal lung inflation is associated with the upregulation of IL-33 by AEC2

With the first breath a number of profound changes occur in the newborn's lung. We hypothesized that the sudden inflation of the previously liquid-filled lungs may cause considerable mechanical stress and potential tissue injury, which could result in IL-33 induction (Kakkar et al., 2012). We discovered a substantial increment in pulmonary IL-33 on P1 compared to E19 both at protein (Figure 1A) and mRNA levels (Figure 1B). To investigate if an abrupt exposure to negative pressure, as occurring upon spontaneous breathing in the alveolar space, might cause the induction of *Il33*, we placed the lungs of E19 *Il33*$^{Cit/+}$ reporter (Hardman et al., 2013) and WT mice in a vacuum chamber (Figure S1A) and discovered a significant induction of Citrine^{+} viable cells (Figure 1C, D and S1B-C) and IL-33 protein (Figure 1E) in lungs 6h post exposure to negative pressure as compared to ambient atmospheric pressure.

To study the cellular origin of pulmonary IL-33 over time, we analyzed lungs of *Il33*$^{Cit/+}$ reporter mice by flow cytometry. We observed a strong upregulation of *Il33* among the CD45^{-} cell fraction starting on P1 (Figures 1F-G and S1D-E). About 60% of CD45^{-} Citrine^{+} cells were further classified as EpCam^{+}CD31^{-} cells (Figure 1H and 1I). Immunohistochemistry revealed AEC2 (surfactant protein C^{+}) being the most abundant cell population upregulating *Il33* in the first days after birth (Figure 1J). Postnatally infiltrating CD45^{+} cells (Figure 1F upper panel and S1F) did not show substantial *Il33* expression (Figure S1G), except for few Citrine^{+} cells in the fetal macrophage fraction (CD45^{+} F4/80^{+} CD11b^{+} CD11c^{-} SiglecF^{-}) (Figure S1H-I). In summary, we determined that postnatal lung inflation or exposure to abrupt changes of pressure was associated with the immediate induction of IL-33.



Publication figure 1 - Alveolar epithelial cells type 2 (AEC2) induce IL-33 at birth. **(A)** Whole lung IL-33 quantification by ELISA at E19 and postnatal days 1, 3, 5, 7, 14 (P1-14), 3 and 4 weeks (3-4w) after birth. **(B)** Quantitative RT-PCR of pulmonary Il33 expression in WT mice at E19 and P1. **(C)** FACS analysis of viable Citrine+ cells from Il33Cit/+ mice at E19 exposed to vacuum or atmospheric pressure (control) for 6h. **(D)** Quantification of (C). **(E)** Whole lung IL-33 quantification by ELISA of WT lungs at E19 exposed to vacuum or atmospheric pressure (control) for 6h. **(F)** FACS analysis of lung CD45 and Citrine expression in Il33Cit/+ reporter mice at indicated time points (gates are set using WT as controls). **(G)** Percentage of Cit+CD45–cells among lung cells, gated as in (F). **(H)** Flow cytometry of viable CD45– lung cells from Il33Cit/+ mice at P7, stained for EpCam and CD31. **(I)** Quantification of the Cit+ proportion of EpCAM+ cells between E19 and 8 weeks of age. **(J)** Micrographs of lung sections at E19, P1 and P3 from Il33Cit/+ reporter mice. Red = Surfactant protein-C (SP-C). Green = IL-33-driven citrine. Scale bars represent 75 µm. Data are representative of two independent experiments with 3-5 mice per time point and graph bars represent mean ± SEM. **p < 0.01 and ****p < 0.0001.

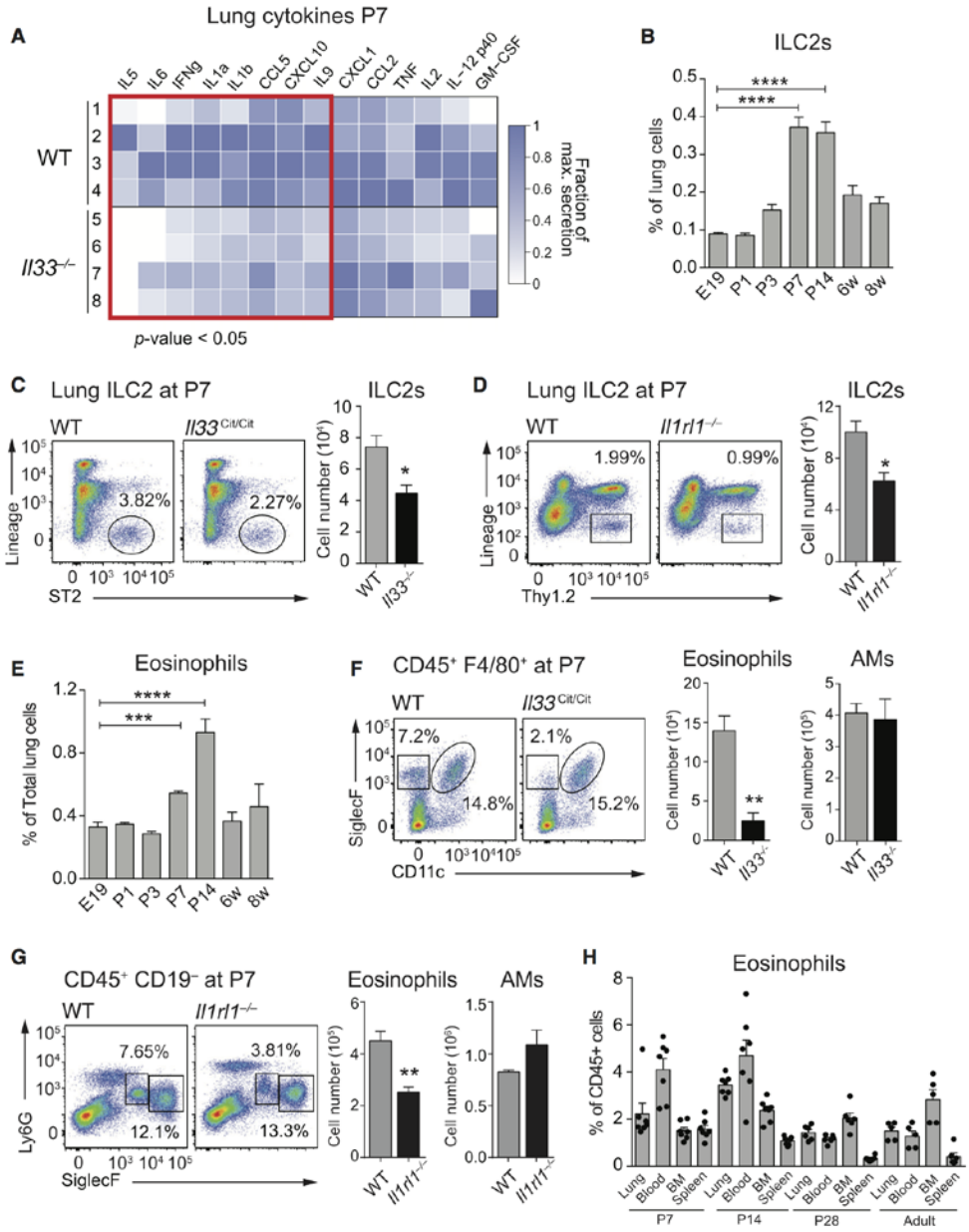
2.3.2. IL-33 shapes the lung environment

To understand IL-33 dependent effects on the immune environment in neonatal lungs, we first analyzed a panel of pulmonary cytokines and chemokines at P7 in wild type (WT) and IL-33-deficient (*Il33*$^{Cit/Cit}$) mice. *Il33*$^{Cit/Cit}$ mice showed reduced expression of the type-2 cytokines IL-5 and IL-9, and of inflammatory mediators like IL-6, IFNγ, IL-1α, IL-1β, CCL5, and CXCL10 (Figure 2A and S2A). Since ST2^{+} ILC2s are major producers of IL-5 and Il-9 and considered the primary targets of IL-33 in the lung (Halim et al., 2014; Kearley et al., 2015), we analyzed newborn lungs for the presence of ILC2s (Lin^{-} CD127^{+} ST2^{+} ICOS^{+}). We detected few ILC2s at E19, with markedly increased numbers by P7 that stabilized by week 6 (Figure 2B and S2B). We found IL-33 to be contributory in populating lungs with ILC2s, as illustrated by reduced ILC2 numbers in *Il33*$^{Cit/Cit}$ (Figure 2C) and ST2-deficient (*Il1rl1*$^{-/-}$) mice at P7 (Figure 2D). In accordance with the ability of ILC2s to regulate eosinophil homeostasis via IL-5 secretion (Nussbaum et al., 2013), eosinophils populated the lungs a few days after ILC2s (Figure 2E and S2C), with clear reductions in the absence of IL-33 (Figure 2F) or ST2 (Figure 2G). IL-5+ ILC2s expanded locally in the lungs (Figure S2E-G), whereas eosinophil numbers increased systemically (Figure 2H). The numbers of AMs, polymorphonuclear cells (PMN), B cell and T cells were not changed in *Il33*$^{Cit/Cit}$ mice (Figure 2F, 2G and S2D). Importantly, the postnatal alveolarization process (Hogan et al., 2014) was not affected by the absence of IL-33 (Figure S2H).

Collectively, these data indicate a critical role for IL33 in shaping the immune cell infiltrate in the neonatal lung, by promoting the appearance of ILC2s and eosinophils. This early period, in which the lung immunological environment is being established, may have subsequent effects on adult lung homeostasis and host defense.

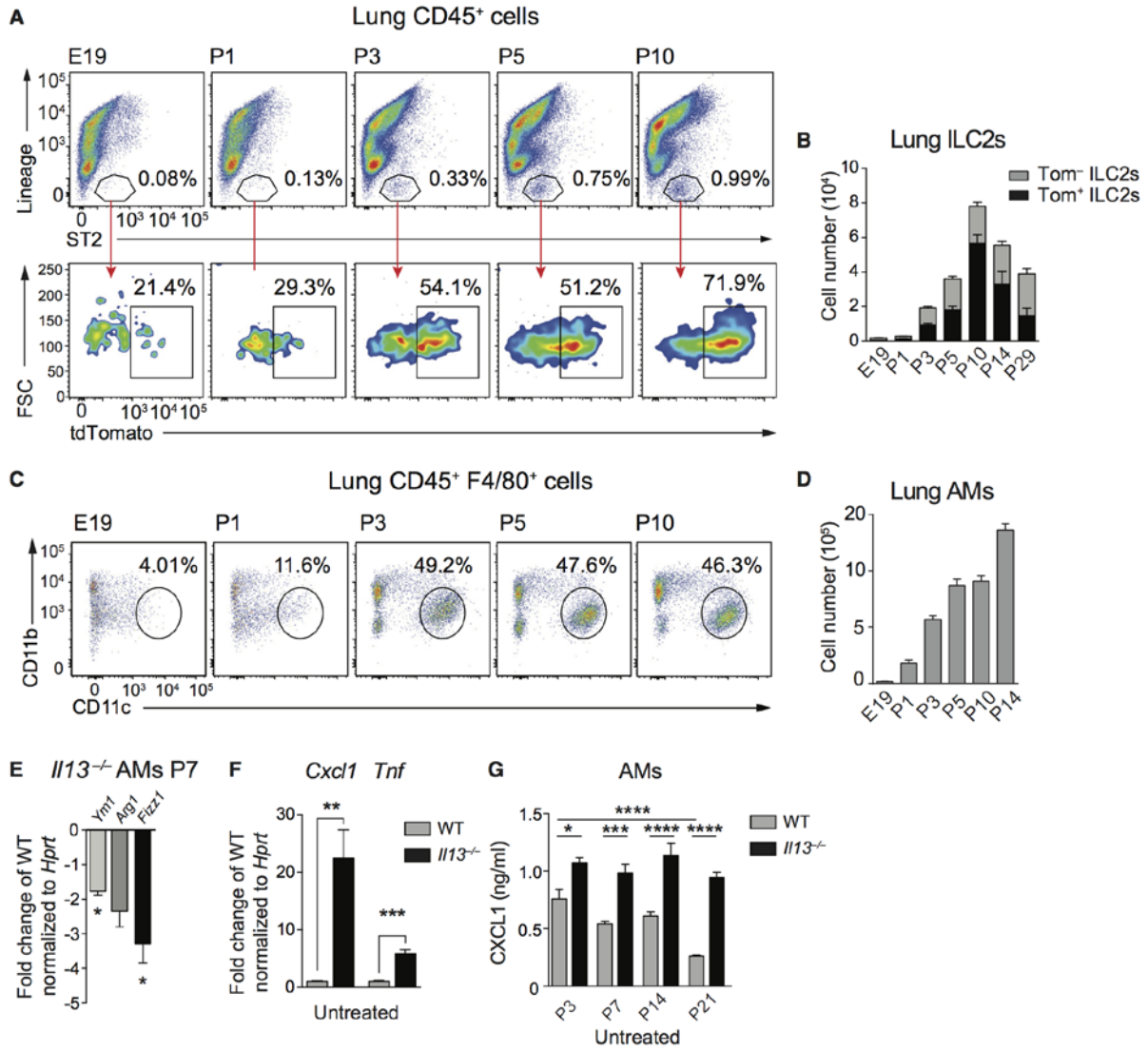
2.3.3. AM development in the neonatal lungs coincides with ILC2 activation

We next examined the activation state of postnatally expanded ILC2s in lungs using *Il13* tdTomato (*Il13*$^{Tom/+}$) reporter mice (Barlow et al., 2012b). IL-13-expressing ILC2s began to expand at P3, peaked at 70% by P10 and started to decline by P14 (Figure 3A and 3B, and S3A-C). Perinatal IL-13 expression was restricted to Lin^{-} cells (Figure S3D), and depended on the presence of IL-33 (Figure S3E). The expansion of activated ILC2s coincided with the emergence of AMs (Figure 3C-D and S3F).



Publication figure 2 - IL-33 drives a type-2 immune environment in lungs of newborns.
(A) Heat-map representation of cytokine levels in whole lung homogenates comparing WT and *Il33*$^{Cit/Cit}$ mice at P7. Original values (see Figure S2A) were rescaled between zero and the maximum value detected for each cytokine, and presented as the fraction of maximum secretion. **(B)** Percentage of lung ILC2s (Lin$^-$ ST2$^+$ Thy1.2$^+$ CD25$^+$ ICOS$^+$) analyzed by FACS at indicated time points. **(C)** FACS analysis of lung ILC2s (Lin$^-$ ST2$^+$) in WT and *Il33*$^{Cit/Cit}$ mice at P7; further gated for CD25$^+$ and ICOS$^+$ and quantified (right panel). **(D)** FACS analysis of lung ILC2s (Lin$^-$ Thy1.2$^+$) in WT and *Il1rl1*$^{-/-}$ mice at P7, further gated for CD25$^+$ and ICOS$^+$ and quantified (right panel). **(E)** Percentage of lung eosinophils (F4/80$^+$ CD11b$^+$ SiglecF$^+$ CD11c$^-$) analyzed by FACS at indicated time points. **(F-G)** FACS analysis of lung eosinophils (CD11b$^+$ SiglecF$^+$ CD11c$^-$) and AMs (CD11b$^-$ SiglecF$^+$ CD11c$^+$) at P7 in WT and *Il33*$^{Cit/Cit}$ mice (F) and WT and *Il1rl1*$^{-/-}$ mice (G). **(H)** Lung, blood, bone marrow and spleen cells from *Il5*$^{Cer/+}$ mice were analyzed for eosinophils (CD11b$^+$ SiglecF$^+$ F480$^+$ CD11c$^-$) at P7, P14, P28 and adult (6 – 8 weeks) by FACS. Data are representative of one (A, H), or two (B-G) independent experiments with 4 mice per group. Graph bars represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. For flow cytometry, all cells were pre-gated on viable, single, CD45$^+$. E=embryonic, P=postnatal, w=week.

Considering the critical role of IL-13 in driving the alternative activation of macrophages, we tested to what degree this postnatal wave of ILC2-derived IL-13 might contribute to the immediate polarization of newly differentiated AMs (Gordon and Martinez, 2010). We discovered reduced expression levels of the M2 markers *Ym1*, *Arg1*, and *Fizz1* in *Il13*$^{-/-}$ and *Il1rl1*$^{-/-}$ AMs (Figure 3E, S3G). Further, we found elevated spontaneous expression levels of *Cxcl1* and *Tnf* in *Il13*$^{-/-}$ as compared to WT AMs on P7 (Figure 3E, F). Remarkably, the amount of CXCL1 released by WT AMs declined with age, whereas AMs from *Il13*$^{-/-}$ mice continued to produce high levels of CXCL1 until P21 (Figure 3G). These data demonstrate that postnatal AMs exhibit an M1 phenotype and that IL-13 promotes the deactivation and M2 polarization of AMs over time.



Publication figure 3 - Lung ILC2s expansion and activation coincides with AM differentiation and M2 polarization. **(A)** Representative FACS profiles of expanding lung ILC2s (Lin– ST2+) (upper panel) and proportion of Tom+ cells (lower panel) in Il13Tom/+ mice between E19 and P10. **(B)** Quantification of absolute numbers of lung Lin– ST2+ Thy1.2+ Tom+/– cells at indicated time points. **(C)** FACS plots illustrating

percentages of AMs (F4/80+CD11b-CD11c+) at indicated time points. **(D)** Absolute numbers of AMs gated as in (C) between E19 and P14. **(E)** AMs (F4/80+CD11b–CD11c+SiglecF+) were sorted on P7 from WT and Il13Tom/Tom mice and M2 markers were assessed by RT-PCR. **(F)** AMs from WT and Il13Tom/Tom (IL-13 deficient) mice on P7 were isolated as in (E), cultured for 6h and Cxcl1 and Tnf gene induction assessed by RT-PCR. Values were normalized to HPRT and are expressed as fold-change versus WT. **(G)** AMs from WT and Il13Tom/Tom (IL-13 deficient) mice on P3, P7, P14 and P21 were isolated as in (E), cultured for 6h and spontaneous CXCL1 secretion was quantified by ELISA. Data are representative of three (A-D) or two (E-G) independent experiments with 3-4 mice per group. Values were normalized to HPRT and are expressed as fold-change versus indicated control. Bars represent mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

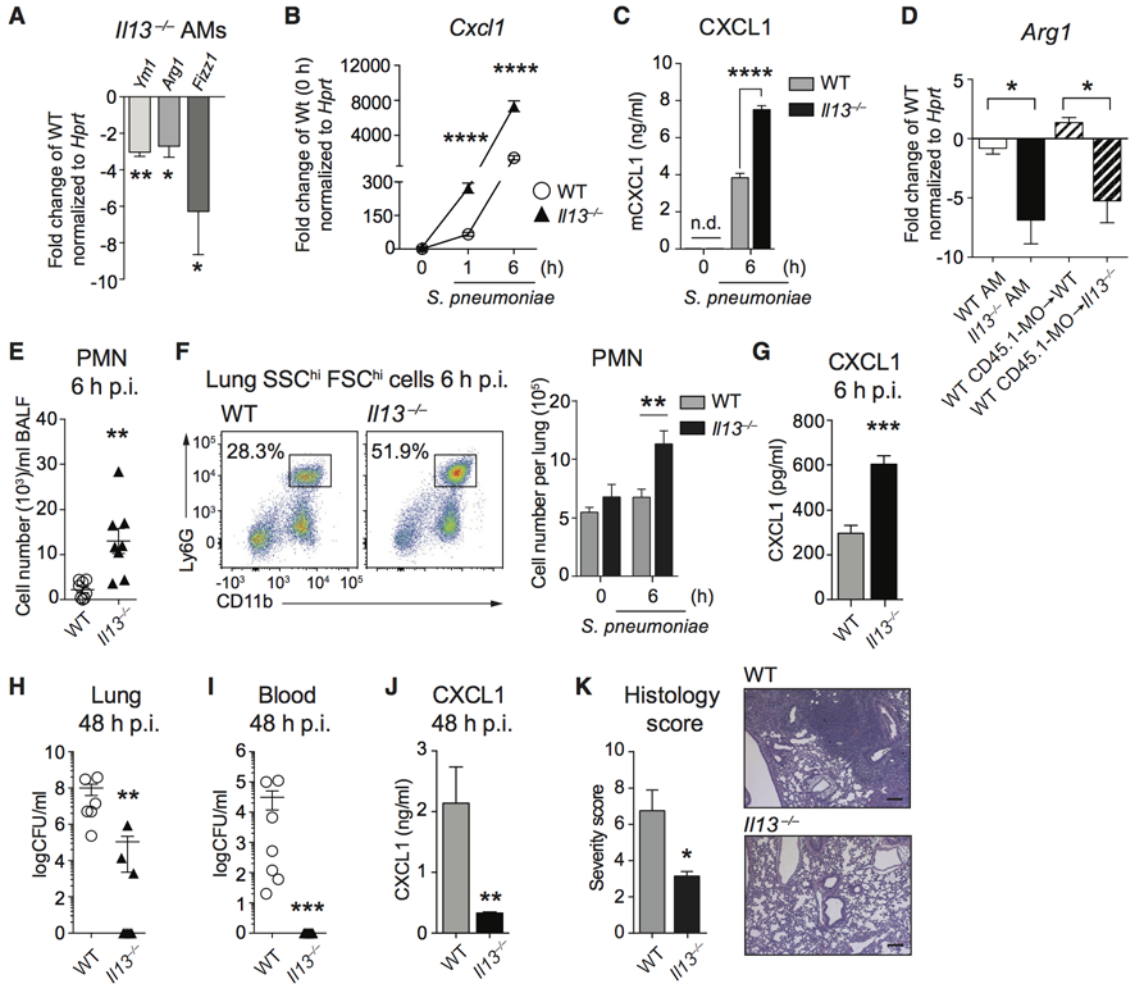
2.3.4. IL-13 maintains adult resident AMs in an M2 state

AMs are long-lived cells with local self-renewal capacity, which are - like other macrophages - strongly influenced by the environment they inhabit (Guilliams et al., 2013; Lavin et al., 2014; Murphy et al., 2008). We reasoned that the need for an unremittingly quiescent lung environment throughout life would favor an M2 AM phenotype and discovered that pulmonary IL-13 continued to affect the M2 polarization of AMs in adult mice (Figure 4A). Adult *Il13*$^{-/-}$ AMs stimulated with the respiratory pathogen *S. pneumoniae*, or the TLR2 ligand lipoteichoic acid (LTA), consistently induced higher levels of CXCL1 as compared to WT AMs (Figure 4B-C, S4A). WT monocytes adoptively transferred to the lungs of WT or *Il13*$^{-/-}$ mice differentiated towards a SiglecF^{+} AM phenotype within two weeks (Figure S4B), and upregulated M2 markers in WT but not *Il13*$^{-/-}$ recipient animals (Figure 4D and S4C). These results confirm that a tissue-derived source of IL-13 is required to polarize and maintain AMs in an M2 state in adult mice. Of note, IL-33 itself was not sufficient to shape the polarization and activity of AMs from adult mice, as *Il1rl*$^{-/-}$ AMs did not differ from WT AMs in their expression of M2 markers nor response to *S. pneumoniae* (Figure S4D-F). In summary, endogenous IL-13 contributes to the M2 phenotype of resident and monocyte-derived AMs in healthy adult mice and is required to suppress potentially excessive inflammation.

2.3.5. Pulmonary IL-13 is detrimental upon pneumococcal infection.

We hypothesized that the IL-13 driven M2 polarization of AMs might impact on innate defenses against *S. pneumoniae*. Upon infection of *Il13*$^{-/-}$ and WT mice with *S. pneumoniae*, we observed a more pronounced early (6h) influx of neutrophils in BALF and lung (Figure 4E-F) and enhanced amounts of lung CXCL1 (Figure 4G) in *Il13*$^{-/-}$ mice. This augmented early inflammatory response in the absence of *Il13* translated into an improved bacterial

clearance from lungs 48h post-infection and completely prevented the systemic spread of bacteria (Figure 4H, I). In accordance with the reduced bacterial burden we found decreased CXCL1 levels (Figure 4J), lower numbers of infiltrating monocytes (Figure S5A) and less pronounced lung infiltrates at 48h post-infection in *Il13*$^{-/-}$ as compared to WT animals (Figure 4K).



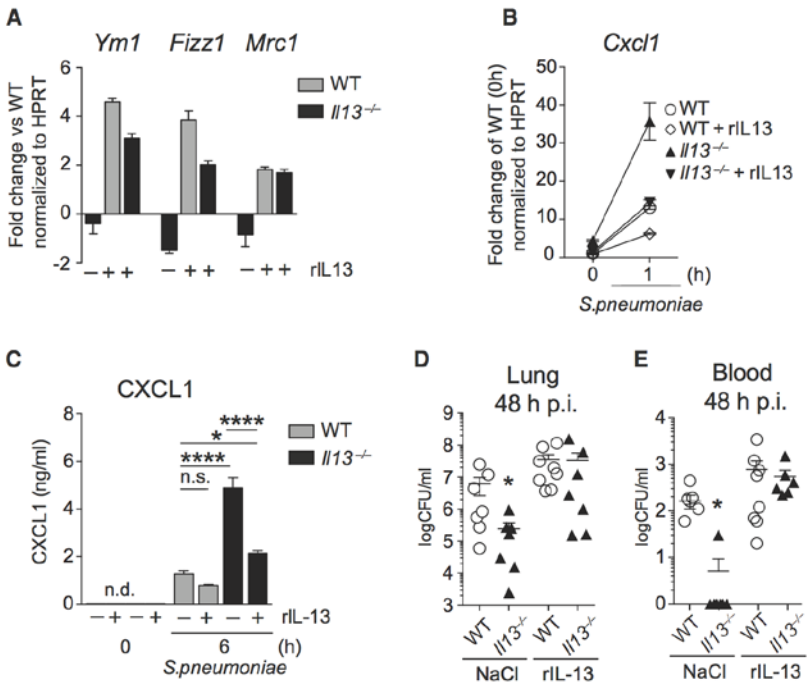
Publication figure 4 - AMs from IL-13 deficient mice present a pro-inflammatory phenotype and improved defenses against S. pneumoniae. **(A)** AMs from adult WT and *Il13*$^{-/-}$ mice were isolated by BAL and analyzed for expression of M2 polarization markers by RT-PCR. **(B-C)** AMs isolated as in (A) were in vitro stimulated with S. pneumoniae (MOI 100) and the induction of Cxcl1 was quantified by RT-PCR (B) and supernatant protein levels by ELISA (C). **(D)** CD45.1 WT monocytes were intra-tracheally transferred to WT and *Il13*$^{-/-}$ CD45.2 recipients and bronchoalveolar cells were harvested by lavage 2 weeks later. FACS-sorted recipient AMs and monocyte-derived AMs were analyzed for expression of M2 polarization markers by RT-PCR. **(E-G)** WT and *Il13*$^{-/-}$ mice were i.n. infected with S. pneumoniae and sacrificed after 6h. PMN numbers in BALF were assessed on cytospins (E) and in lungs by FACS analysis (CD45+ SSChi FSChi CD11b+ Ly6G+) (F). Lung CXCL1 was quantified by ELISA (G). **(H-K)** WT and Il13–/– mice were i.n. infected with S. pneumoniae and sacrificed after 48h. CFU counts in lung homogenates (H) and blood (I). Lung CXCL1 was quantified by ELISA (J). Hematoxylin-eosin stained lung sections were scored by a pathologist (see Methods) (K, left). Representative H-E lung sections (K, right). Scale bars represent 180 μm. Data are representative of at least three independent

experiments with four (A-C) and 7-8 (E-K) mice per group. Data in (D) are from a single experiment with 6 mice per group. PCR values were normalized to HPRT and expressed as fold-change versus indicated control. Mean ± SEM are depicted; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. BAL = bronchoalveolar lavage; CFU = colony forming units; MOI = multiplicity of infection; PMN = polymorphonuclear cells.

To assess the broader relevance of these findings, we investigated the contribution of pulmonary IL-13 to host defense against *Staphylococcus aureus* (*S. aureus*) as well as upon induction of LPS-induced acute lung injury. Similar to our observations in pneumococcal pneumonia, we discovered an augmented early inflammatory response to LPS (Figure S5B and S5C), and an improved clearance of *S. aureus,* associated with a reduced disease-associated temperature drop in *Il13*$^{-/-}$ animals (Figure S5D and, S5E). Together, these results support the notion that pulmonary IL-13 shapes the immune environment in the lung, which upon infection delays the induction of innate defenses against pathogens.

We could not detect any baseline differences in immune cells involved in the defense against bacteria (NK, T or B cells, PMN and monocytes – Figure S5F, and data not shown) or in ILC2 levels (Figure S5G), except for an increased number of eosinophils in *Il13*$^{-/-}$ mice (Figure S5H). To exclude the possibility that eosinophils contributed to the phenotype, we repeated the infection studies in *Il5*$^{-/-}$ mice, which have severely reduced pulmonary eosinophilia (Figure S5I), and could not identify any differences in bacterial counts (Figure S5J), nor in the inflammatory response elicited by AMs *in vitro* (Figure S5K).

A short-term *in vivo* exposure to rmIL-13 was sufficient to "re-polarize" resident AMs from IL-13-deficient mice towards an M2 phenotype (Figure 5A) and to reduce *S. pneumoniae* induced CXCL1 releases (Figure 5B, 5C). Finally, the i.n. administration of rmIL-13 to *Il13*$^{-/-}$ mice impaired bacterial clearance in lungs and blood, with bacterial counts being comparable to WT controls (Figure 5D, 5E). In summary, these data demonstrate that the lung immune environment at homeostasis is profoundly shaped by IL-13, at the expense of impaired anti-bacterial defenses.



Publication figure 5 - Intranasal rmIL-13 treatment reversed the inflammatory phenotype of AMs in Il13$^{-/-}$ mice and the responses to *S. pneumoniae*.
(A-C) WT and *Il13*$^{-/-}$ mice were treated daily with rmIL-13 (6ng in 50µl NaCl) i.n. and AMs were isolated by BAL on day 3. M2 markers were assessed by RT-PCR (A). Cultured AMs were stimulated with *S. pneumoniae* (MOI 100) and fold-induction of *Cxcl1* was measured by RT-PCR (B). AMs were treated as in (B) and CXCL1 protein was quantified by ELISA (C). **(D-E)** WT and *Il13*$^{-/-}$ mice were treated with mrIL-13 as in (A-C), infected i.n. with *S. pneumoniae* on day 3 and sacrificed after 48h. CFU counts in lung homogenates (D) and blood (E). Data in (A-C) are representative of two independent experiments with four mice per group. Data in (D-E) are from a single experiment with eight mice per group. Mean ± SEM are depicted; *p < 0.05, and ****p < 0.0001. BALF = bronchoalveolar lavage fluid; CFU = colony forming units; MOI = multiplicity of infection; i.n. = intranasal.

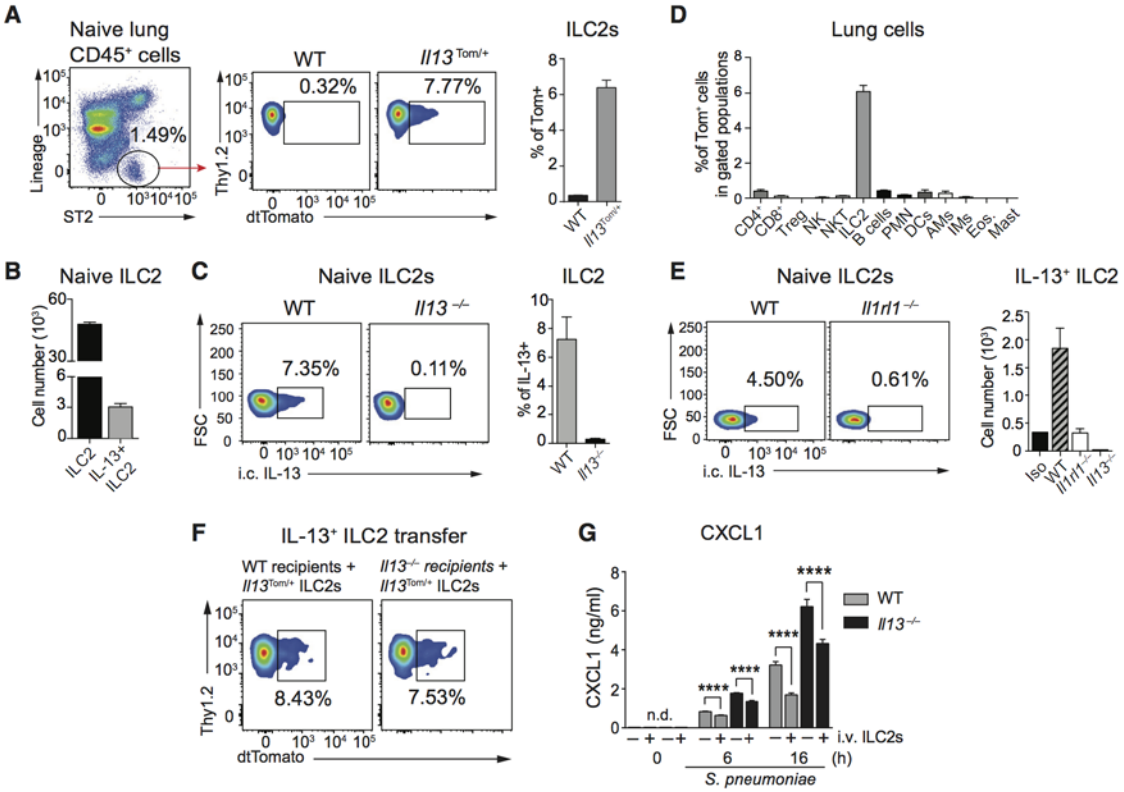
2.3.6. Resident ILC2s are the sole source of IL-13 in heathy adult lungs.

To determine the potential contribution of ILC2s to the AM phenotype, we evaluated the activity of lung ILC2s in adult mice using *Il13*$^{Tom/+}$ reporter mice and intracellular cytokine staining. We found that ~7-8% of ILC2s in the lungs of naïve adult mice (~3×10^3 cells) produced IL-13 (Figure 6A-C). Notably, we excluded that Th2 cells, eosinophils, mast cells, macrophages, natural killer (NK) cells, natural killer T (NKT) cells and invariant natural killer T (iNKT) cells, which have all been shown to produce IL-13 in different lung pathological conditions (Kim et al., 2008; Price et al., 2010; Rijavec et al., 2011), were the source of IL13 at steady state in healthy adult lungs (Figure 6D and S6A). In fact, ILC2s were the only cells expressing *Il13* in healthy adult lungs at homeostasis (Figure 6D), a finding we confirmed by intracellular staining for IL-13 (Figure S6B).

Constitutive IL-13 production by lung resident ILC2s did not depend on T or B-cells in adult mice (Figure S6C-G). However, homeostatic IL-13 production depended on ST2 (Figure 6E) and less so on IL-25, another cytokine capable of inducing IL-13 production by ILC2s via IL17rb (Roediger and Weninger, 2015) (Figure S6H). The absolute number of lung ILC2s did

not change in the absence of ST2 or IL17rb (Figure S6I). In summary, lung resident ILC2s are a constant and unique source of pulmonary IL-13 in healthy adult lungs at steady state.

To test if IL-13-producing ILC2s alone were sufficient to determine the responsiveness of AMs to *S. pneumoniae*, we adoptively transferred IL-33-expanded lung Tom^{+}ILC2s to WT and *Il13*$^{-/-}$ mice (Figure 6F). We observed a significant reduction of *S. pneumoniae* induced CXCL1 secretion by AMs isolated from *Il13*$^{-/-}$ recipients that received Tom^{+}ILC2s (Figure 6G). Of note, adoptively transferred ILC2s were also able to reduce the responsiveness of WT AM to *S. pneumoniae in vitro.* Collectively, these data show that pulmonary ILC2-derived IL-13 maintains lung resident AMs in an M2 state in healthy adult mice.



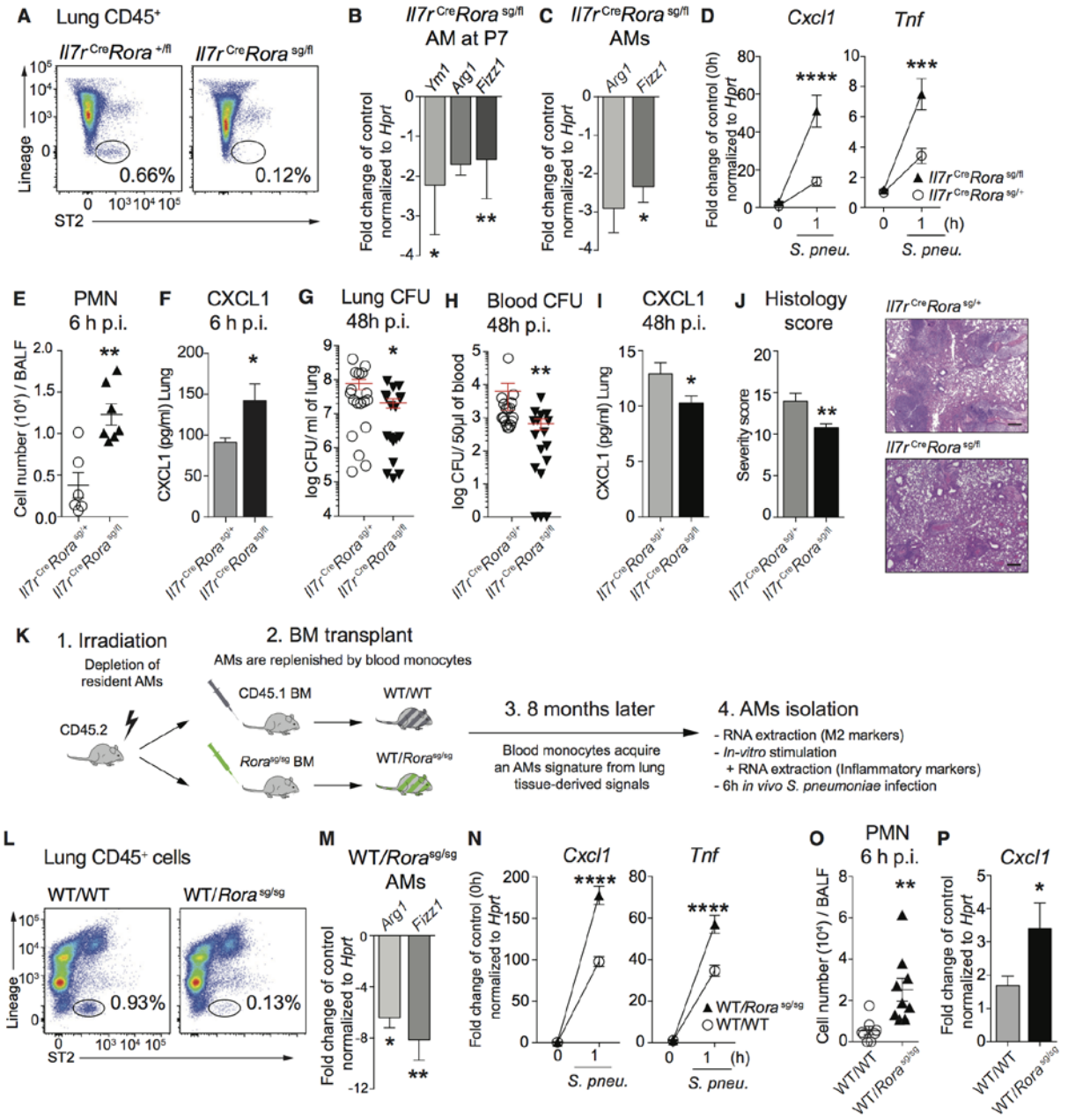
Publication figure 6 - ILC2 are the only cells producing IL-13 in the lung at homeostasis. **(A)** IL-13 expression in ILC2s (Lin– ST2+ ICOS+ Thy1.2+ CD25+) assessed by flow cytometry in adult, naive Il13Tom/+ mice. Representative plots and percentage of tdTomato+ ILC2s. **(B-C)** ILC2s and IL-13+ ILC2s were quantified by flow cytometry and i.c. staining for IL-13 in naive WT lungs. (B) Absolute numbers of total lung ILC2s (Lin– ST2+ ICOS+ Thy1.2+ CD25+) and IL-13+ ILC2s. (C) Representative plots of IL-13+ ILC2s gated as in (A) and percentage of IL-13 producing ILC2s (right panel). **(D)** Lung cell populations were tested by FACS for IL-13 expression in healthy adult Il13Tom/+ mice. Gating strategies are shown in Supplemental Table 2. **(E)** IL-13 production by lung ILC2s in WT, *Il1rl1*$^{-/-}$ and *Il13*$^{-/-}$ assessed by i.c. staining using flow cytometry (Iso = isotype control); representative plots and absolute numbers are depicted. **(F-G)** ILC2s were first expanded in lungs of Il13Tom/+ mice via i.n. administration of rmIL-33 (0.5 µg/50µl for 5 days), then sorted Tom+ILC2s were transferred i.v. to WT and *Il13*$^{-/-}$ mice. (F) Representative FACS plots showing the homing of Il13Tom/+ ILC2s in lungs 5 days after adoptive transfer. (G) AMs were isolated by BAL from WT and *Il13*$^{-/-}$ recipients 5 days after adoptive transfer, in-vitro stimulated with S. pneumoniae (MOI 100) and CXCL1 release was assessed

by ELISA in supernatants. Data are representative of three (A-C), two (E-G) and one (D) independent experiments with four mice per group. Mean ± SEM are depicted; ****p < 0.0001. BAL = bronchoalveolar lavage; MOI = multiplicity of infection; i.v. = intravenous; i.c. = intracellular; i.n. = intranasal.

2.3.7. ILC2 maintain the M2 polarization of AMs early in development and at homeostasis

We then asked if the congenital absence of ILC2s would mirror the phenotype observed in *Il13*$^{-/-}$ mice. AMs extracted from newborn (P7) and adult *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice, congenitally deficient in lung resident ILC2s (Oliphant et al., 2014a) (Figure 7A), showed a reduced expression of M2 markers (Figure 7B, C and S7A), and increased *Cxcl1* and *Tnf* expression when stimulated with *S. pneumoniae* (Figure 7D). Infection of *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice with *S. pneumoniae* resulted in increased neutrophil influx and higher lung CXCL1 levels 6h post-infection (Figure 7E-F, and S7B). This translated into an improved bacterial clearance with reduced systemic dissemination of pneumococci (Figure 7G-H), lower pulmonary CXCL1 levels (Figure 7I) and less severe lung infiltrates in *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice 48h after infection (Figure 7J). We concluded that the congenital absence of ILC2s impacted on the M1 versus M2 polarization of AMs in neonatal and adult mice, with implications on the ability to fight bacterial lung infections.

We then asked to which degree ILC2s might contribute environmental signals to shape the functionality of bone-marrow-derived AMs (Lavin et al., 2014) and generated bone marrow chimeras using ILC2-deficient *Rora*$^{sg/sg}$ mice as donors (Wong et al., 2012) (Figure 7K, 7L). AMs isolated from WT/*Rora*$^{sg/sg}$ chimeras expressed lower levels of the M2 markers *Arg1* and *Fizz1* (Figure 7M) and higher levels of *Cxcl1* and *Tnf* in response to *S. pneumoniae* (Figure 7N). *In vivo*, WT/*Rora*$^{sg/sg}$ chimeras exhibited an augmented early inflammatory response upon pneumococcal infection (Figure 7O-P, and S7C). Collectively, lung ILC2s convey important cues that maintain quiescence by shaping the functional state of lung macrophages at homeostasis.



Publication figure 7 - Lung resident ILC2s polarize tissue resident AMs towards an M2 phenotype and dampen early inflammatory responses against bacteria. **(A)** Flow cytometry plots of ILC2s in naive *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice and *Il7r*^{Cre}*Rora*$^{+/fl}$ controls. **(B)** AMs isolated by flow cytometry from healthy *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice and controls at P7 and M2 markers evaluated by RT-PCR. **(C-D)** AMs isolated by BAL from healthy adult *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice and controls. (C) M2 markers evaluated by RT-PCR. (D) Primary AMs stimulated for 1h with S. pneumoniae (MOI 100) to assess the induction of Cxcl1 and Tnf. **(E-J)** *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice and controls were i.n. infected with S. pneumoniae (105 CFUs) and sacrificed after 6h (E,F) or 48h (G-J). (E) PMN influx on BALF cytospins. (F) CXCL1 induction in whole lung homogenate. (G) CFUs in lung and (H) in blood. (I) CXCL1 induction in whole lung homogenate. (J) Hematoxylin-eosin stained lung sections were scored by a pathologist (see Methods) (J, left). Representative H-E lung sections (J, right). Scale bars represent 180 µm. **(K-N)** CD45.2 recipients were lethally irradiated and transplanted with WT or *Rora*$^{sg/sg}$ bone marrow and sacrificed 8 months later. (K) Experimental set-up. (L) Representative FACS plots of ILC2s in healthy WT/WT and WT/*Rora*$^{sg/sg}$ bone marrow chimeras. (M) AMs isolated via BAL and assessed for M2 markers by RT-PCR or (N) stimulated with S. pneumoniae (MOI 100) to evaluate Cxcl1 and Tnf induction. **(O-P)** WT/WT and WT/*Rora*$^{sg/sg}$ chimeras were infected with 105 CFUs S. pneumoniae and sacrificed after 6h to assess (O) PMN influx, and (P) Cxcl1 induction in lung tissue. Data are representative of at least 2 independent experiments with 4 (A-D; M,N) and 7-

8 (E-J; O,P) mice per group. Data in G, H are pooled from two independent experiments. Mean ± SEM are depicted; *p ≤ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. BALF = bronchoalveolar lavage fluid; CFU = colony forming units; i.n. = intranasal. MOI = multiplicity of infection; PMN = polymorphonuclear cells.

2.4. Discussion

With the first breath, lungs are suddenly exposed to the external environment, therefore requiring regulatory forces in place to avoid continuous inflammatory reactions to environmental stimuli. Here, we show a perinatal wave of IL-33-mediated expansion and activation of ILC2s, resulting in an IL-13-driven polarization of newly differentiating AMs to an M2 phenotype. This exerts important homeostatic functions that contribute to a quiescent lung environment shortly after birth and throughout adult life.

AEC2 are the main source of IL-33, as shown earlier (Hardman et al., 2013; Pichery et al., 2012), further confirmed by a recent study of developing AEC2 in embryonic lungs (Treutlein et al., 2014). Even though the mode of homeostatic IL-33 release remains to be elucidated, the mechanical stress induced by physiological ventilation possibly contributes to pulmonary IL-33 (Martin and Martin, 2016), and we discovered that exposing E19 lungs to negative pressure, was sufficient to induce IL-33. Moreover, the release of bioactive IL-33 from living cells upon encounter of environmental allergens, extracellular ATP or mechanical stress has been reported (Chen et al., 2015; Kakkar et al., 2012; Kouzaki et al., 2011; Sanada et al., 2007).

IL-33g and ILC2s are increasingly recognized as fundamental regulators of tissue homeostasis (Molofsky et al., 2015; Moltke and Locksley, 2014). A such, recent reports described an IL-33 driven, ILC2 dependent mechanism for adipose tissue homeostasis, which involves the presence of eosinophils and M2 macrophages (Lee et al., 2015; Qiu et al., 2014). Excitingly, perinatal IL-33 induction was recently found to license adipocytes for uncoupled respiration and thermoregulation after birth (Odegaard et al., 2016).

The IL-33 and ILC2 dependent physiological type-2 milieu that we describe, might play a role in the reportedly exaggerated airway hyperreactivity upon house dust mite exposure in newborns and strengthens the concept of a “window of immune development” (Gollwitzer et al., 2014). In fact, while this manuscript was under revision, a report demonstrated a casual link between perinatal IL-33 induction and asthma (de Kleer et al., 2016). Here we propose a unique and homeostatic role for ILC2s in shaping the lung immune environment in early life, as the appearance of activated ILC2s around P3 gradually de-activated AMs.

While type-2 responses, as seen upon helminth infections, are known to impair defenses against mycobacteria (Monin et al., 2015; Salgame et al., 2013), we now report that even homeostatic type-2 conditions impact on lung immunity, illustrated by reduced lung inflammation upon LPS challenge and a delayed clearance of medically important lung pathogens such as *S. pneumoniae*.

Our experiments in mice congenitally deficient in ILC2s corroborated the concept that ILC2s affected the AM phenotype from birth until adult life. Analysis of bone marrow chimeras using *Rora*$^{sg/sg}$ mice further demonstrated that pulmonary ILC2s provided essential, tissue-specific signals to even polarize bone marrow precursors that arrive in lungs. Supporting our notion that ILC2s contribute to the *in vivo* phenotype of AMs, a recent publication identified tissue-specific transcriptional signatures of resident macrophages, and found AMs to be characterized by two IL-13 and IL-5 inducible genes, namely *Ym1* (*Chi3l3*) and *Car4*, respectively (Lavin et al., 2014).

In conclusion, we show that IL-33-driven ILC2 activation dominates the lung milieu early after birth by inducing a type-2 immune environment. Lung resident ILC2s are major contributors to the phenotype and function of tissue resident AMs at homeostasis, favoring a quiescent immune environment. While this effect might prove beneficial at steady state and upon sterile lung injury, it comes at the expense of a delayed response to the common lung pathogen *S. pneumoniae.*

2.5. Experimenta procedures

Mice

Il13$^{-/-}$ (McKenzie et al., 1998), *Il13* $^{tdTomato/+}$ (Barlow et al., 2012a) (referred as *Il13*$^{Tom/+}$), *Il1rl1*$^{-/-}$(Townsend et al., 2000), *Il5*$^{-/-}$ (Kopf et al., 1996), *Rag2*$^{-/-}$ (Shinkai et al., 1992), *Il7r*^{Cre} (Schlenner et al., 2010) and *Rora*$^{+/fl}$ (Oliphant et al., 2014a), Staggerer *Rora*$^{sg/+}$ (JAX Laboratories) mice were on a C57Bl/6 background. We obtained *Il7r*^{Cre}*Rora*$^{sg/fl}$ mice (experimental) or *Il7r*^{Cre}*Rora*$^{+/fl}$ littermate controls by crossing *Il7r*^{Cre} with *Rora*$^{+/fl}$ and *Rora*$^{sg/+}$. *Il33*$^{Cit/+}$ (Hardman et al., 2013) mice and *Il5*$^{Cer/+}$ mice (Saunders et al., 2016) were on a Balb/c background. Mice were bred in a specific pathogen-free (SPF) facility and all mice were matched for age, gender and background in individual experiments. All animal experiments were approved by the Austrian Federal Ministry of Sciences and Research (BMWFW-66.009/0122-II/3b/2013) and the UK Home Office.

Isolation, culture and stimulation of AMs.

AMs from newborn mice were isolated by cell sorting using a FACSAria II (BD Bioscience) by gating on viable $CD45^+$ $F4/80^+$ $CD11b^{low}$ $CD11c^+$ $SiglecF^+$ $Ly6C^-$ cells. In adult mice, AMs were isolated by bronchoalveolar lavage followed by cell adhesion. Purity of isolated AM with both methods was consistently above >95%. AMs were stimulated in RPMI containing 3% FCS with heat inactivated *S. pneumoniae* at a MOI 100, or *S. aureus* LTA (10µg/ml). In Figure 3E-G, 4A-D, 5A-C, 7B-D and 7 M, N, cells were pooled from 3-4 mice per group and analyzed in technical quadruplicates.

Cytokine administration

Recombinant mouse IL-13 and IL-33 were purchased from Biolegend. Anesthetized mice were treated daily with rmIL-13 (6ng/50µl NaCl for 3 consecutive days) or rmIL-33 (0.5µg/50µl NaCl for 5 consecutive days). Mice were sacrificed one hour after the last administration.

Adoptive transfer of ILC2s

Lung ILC2s were FACS purified as defined by $lineage^-$ (CD3a, CD4, CD8a, CD19, CD11c, CD11b, Gr1, FcεR1, CD49b), $Thy1.2^+$ $ST2^+$ $ICOS^+$ and Tom^+ from *Il13*$^{Tom/+}$ mice that had been treated intranasally (i.n.) with rmIL-33 for five days. Cells were transferred intravenously to *Il13*$^{-/-}$ or WT mice recipients ($1x10^5$ cells per mouse) and assessed for localization in lungs five days later.

Generation of bone marrow chimeras.

Six-weeks old CD45.2 *Rora*$^{sg/sg}$ or WT littermates served as bone marrow donors. CD45.1 recipients were irradiated (9Gy) and reconstituted on the same day with $2x10^6$ bone marrow cells/recipient by intravenous injection. Mice were analyzed for reconstitution and absence of lung resident ILC2s after eight months.

Murine pneumonia model.

Mice were infected i.n. with 10^5 CFUs *S. pneumoniae* serotype 3 (ATCC 6303) as described (Sharif et al., 2014; Warszawska et al., 2013), or with $5x10^7$ CFUs *S. aureus* (USA300). Acute lung injury was induced by i.n. administration of 100ng LPS (*E. coli* O55:B5). Bronchoalveolar lavage fluid (BALF) was collected, cells were counted with an automated cell counter (Z2 Coulter Counter, Beckman) and Giemsa stained cytospin preparations were used for differential cell counts. Lung tissues were homogenized in sterile saline using a Precellys 24TM (Peqlab) and lung CFUs were determined by 10-fold serial dilutions of

homogenates on blood agar plates. An aliquot of lung homogenates was incubated in RA1 buffer (Macherey-Nagel) containing 10% of beta-mercaptoethanol (Calbiochem) and stored at -80° for RNA extraction. The remaining lung homogenates were incubated in Greenberger lysis buffer as described (Sharif et al., 2014) and supernatants were stored at -20°C until cytokines were assayed.

Pneumonia severity score.

Paraffin embedded lung sections were stained with H&E and scored by a trained pathologist, blinded to experimental groups. The final pneumonia score was the sum of the following parameters: severity of pleuritis, interstitial inflammation, edema and thrombi formation were scored as 0 = absent, 1 = mild, 2 = moderately severe, 3 = severe; bronchitis was scored as 1 if present; endothelitis was scored as 0 = absent, 2 = present, 3 = present with endothelial wall necrosis; the existence of a lobar confluent infiltrate was scored as 1 and a score of 0.5 was added for every infiltrate covering 10% of the lung area.

Statistical analysis.

Data are expressed as mean ± SEM. Statistical significance in 2-group comparisons was assessed with unpaired Student's *t*-test. When indicated, a Mann-Whitney U-test was used for analysis of nonparametric data. For multivariable comparisons we performed one-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. Results were analyzed with Graph Pad Prism software version 6 and a $p < 0.05$ was regarded as statistically significant.

Vacuum-induced lung stress.

Lungs were harvested, stored on ice and transferred to either a pre-heated 37oC humidified control chamber or a pre-heated humidified vacuum chamber (Vacuubrand model MZ2C) (see Fig S1A). The vacuum was initiated and both groups were incubated for 6hs. Lungs were then dissociated using scissors and digested in collagenase 1 (750U/ml Invitrogen) and DNase1 (0.31mg/ml Roche) in RPMI (Life technologies) for 45 mins at 37ºC. Tissues were passed through a 70μM filter using PBS with 2% FCS and processed for flow cytometry.

Lung flow cytometry and cell sorting.

Lung single cell suspensions were prepared by incubating finely minced lung tissue for 1h at 37°C in RPMI containing 5% FCS, collagenase I (ThermoFischer), and DNase I (Sigma-Aldrich), homogenized with a glass homogenizer and then passed through a 70μm strainer. Cells were incubated for 5 min on ice in red blood cell lysis buffer (Sigma-Aldrich), washed

and finally passed through a 40µm cell strainer. Single cell suspensions were counted with a hemocytometer and 2x106 cells/stain were incubated with anti-mouse Fc receptor blocking antibody CD16/CD32 (eBioscience) and stained with a mix of fluorochrome labeled antibodies (**see Table 1**). The alpha-GalCer/CD1d loaded dimer and unloaded control were provided by the NIH tetramer core facility. DAPI or fixable viability dye (eBioscience 65-0865) was added to the surface antibody mix to allow dead cells exclusion by flow cytometry. For gating strategies see **Table 2**. For intra-cellular (i.c.) staining, lung cell suspensions were further purified using Percoll (Sigma), incubated with PMA (60ng/ml), ionomycin (500ng/ml) and 1x protein transport inhibitor (eBioscience), or with brefeldin A (GolgiPlug™, BD Biosciences) for 4h at 37°C. Cells were then stained with surface antibodies, fixed and permeabilized before addition of anti mouse IL-13 mAb or anti-IL-5 mAbs, respectively, or isotype control Abs (Fixation and Permeabilization Solution Kit, BD Biosciences). For nuclear staining, cells were permeabilized and processed using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience). Acquisition was performed with a LSRFortessa (BD Biosciences) and data were analyzed using the FlowJo software version vX.0.7 (TreeStar). Cell sorting was performed with BD FACSAria™ III.

Reagents for isolation, culture and stimulation of alveolar macrophages (AMs).

AMs from newborn mice were isolated by cell sorting from lung single cell suspensions as described above. In adult mice, AMs were isolated by bronchoalveolar lavage (BAL) followed by cell adhesion. In brief, mice were lethally anesthetized using 100 mg/kg body weight ketamine (Ketaset) and 10 mg/kg body weight xylazine (Rompun, Bayer), administered in sterile PBS i.p. AMs were isolated by inserting a tracheal cannula (Venflon, BD Bioscience) and flushing the lungs 10 times with 1ml NaCl. Isolated cells were counted and allowed to adhere at 37°C for 2h in RPMI containing 10% FCS and 1% penicillin/streptomycin at a concentration of 5x104 cells/well in a 96 well plate. Wells were washed twice with PBS and adherent cells were used for further experiments.

Real time PCR.

Total mRNA was isolated using the NucleoSpin RNA XS kit (Macherey-Nagel) or the RNeasy Micro kit (Qiagen) according to the manufacturers' instructions. Real-time PCR was performed using either the SYBR Green Master Mix (Applied Biosystems), or the TaqMan universal PCR mix (Applied Biosystems) and the StepOnePlusTM Real-Time PCR System (Applied Biosystems) or using a ViiA 7 (Thermofisher). Commercially available Taqman probes were used for the expression of mCCL17 (Mm01244826_g1), mYm1

(Mm00657889_mH), mFizz1 (Mm00445109_m1), mArg1 (Mm00475988_m1) and GAPDH (Cat. 4352932E). Designed primers were purchased from Sigma-Aldrich and are listed in the **Table 3**. Gene expression was normalized to GAPDH or HPRT and expressed as fold change versus indicated controls.

Lung tissue homogenates and ELISA.

Lungs were homogenized in the presence of protease inhibitors and total protein content was quantified by Pierce BCA Protein Assay Kit (Thermo Scientific). The Multiplex bead array (MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel - Premixed 25 Plex) was performed according to the manufacturer's instructions (Millipore), using MAGPIX multiplexing instrument and MILLIPLEX analysis 5.1 software (Millipore). Mouse IL-33 was measured using the eBioscience ELISA kit, according to the manufacturers' instructions. For protein quantification in S. pneumoniae infected lungs, CXCL1 and TNF were measured in lung homogenates using specific ELISA kits from RnD Systems, according to the manufacturers' instructions.

Analysis of alveolarization.

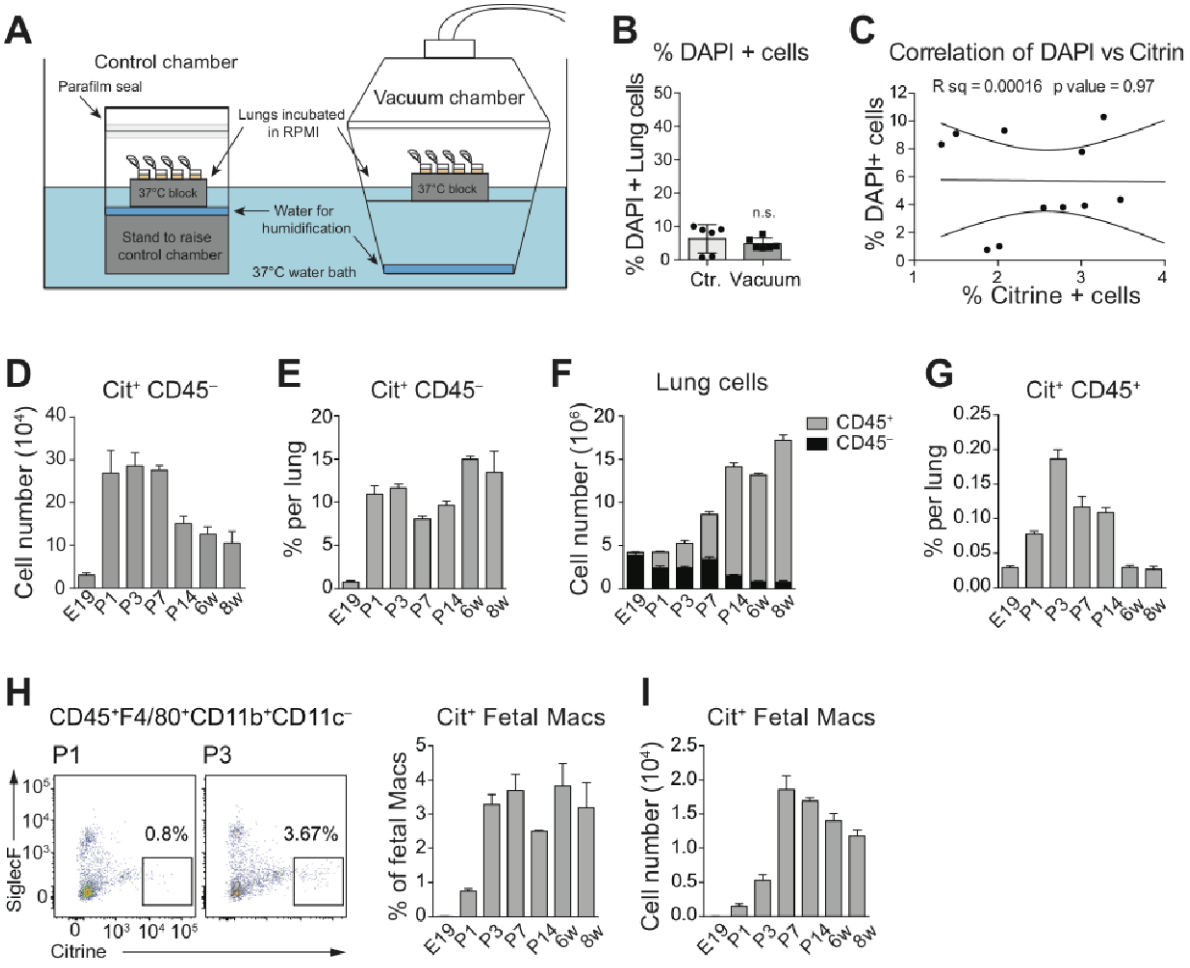
H&E stained lung sections of newborn mice at postnatal day 7 were imaged using an Olympus FSX100 automated microscope and a 20X magnification (10 fields per lung). Lung alveolarization was quantified using the automatic image analysis software CellProfiler (Lamprecht et al., 2007) (http://www.cellprofiler.org). Briefly, image masks for each field were generated by converting the images to grayscale and applying a binary threshold using the threshold function in ImageJ (version 1.49v; http://imagej.nih.gov/ij/). The original images were loaded into CellProfiler and alveoli were identified using the binary images generated in ImageJ (as described above), as the thresholding method using the *IdentifyPrimaryObjects* module. The shape and size features of the identified alveoli were then calculated using the *MeasureObjectSizeShape* module and exported to a spreadsheet format.

Immunofluorescence and analysis

Lungs from *Il33*Cit/+ reporter mice were isolated at embryonic day 19 (E19) and postnatal days 1 (P1) and 3 and fixed overnight in 1% formaldehyde–PBS at 4 °C. After extensive washing with PBS, lungs were then incubated overnight in 30% sucrose solution at 4 °C. On the third day, lungs were embedded in 15% sucrose + 7.5% porcine skin gelatin (Sigma) in

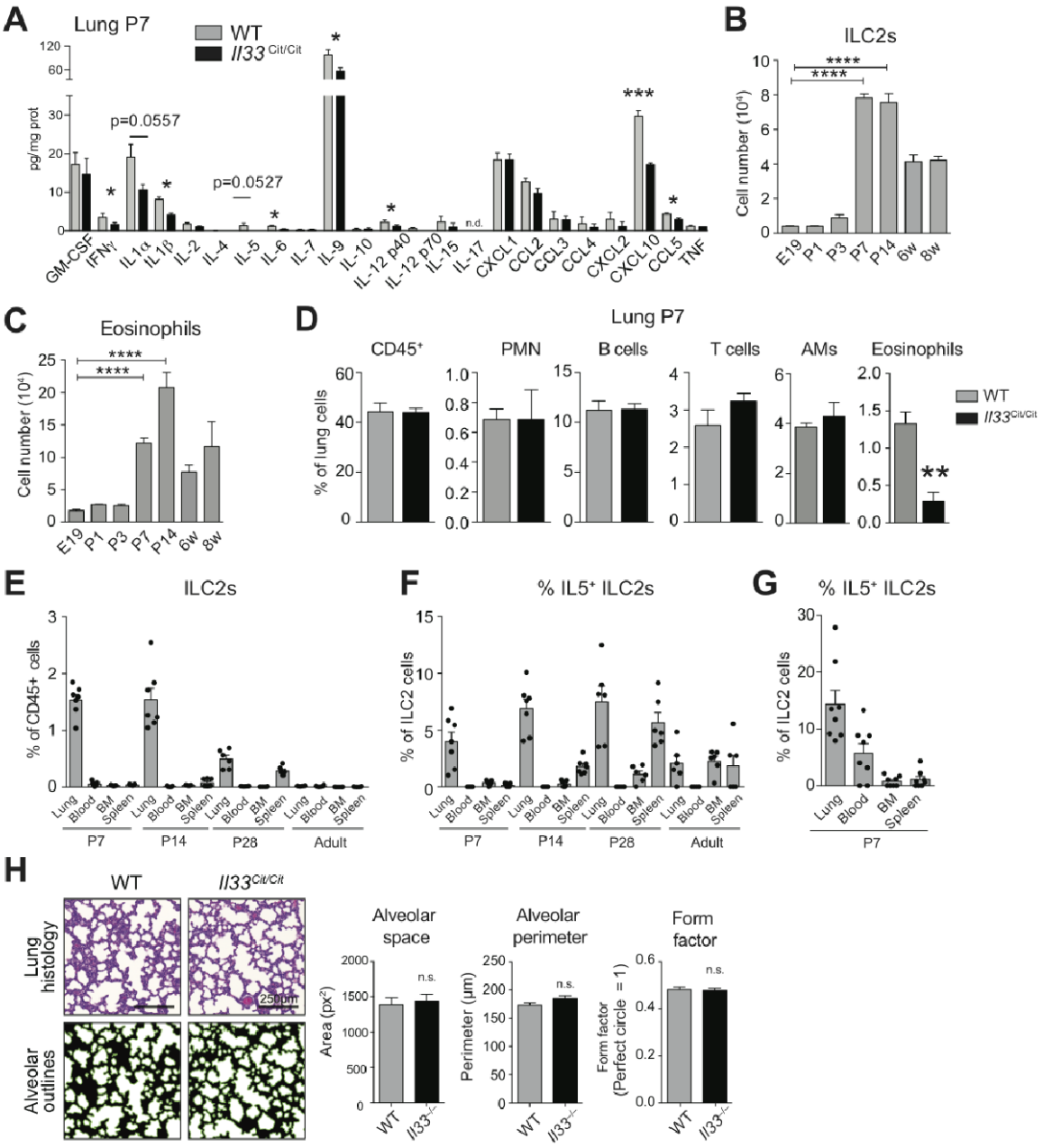
PBS and flash-frozen in isopentane chilled to −80 °C with liquid nitrogen. Lobes were sectioned to a 20-µm thickness onto Superfrost Plus slides (Thermo Scientific) and stored at −20 °C. Sections were air-dried for 1 h, rehydrated with PBS and then incubated in blocking buffer (3% goat serum (Jackson Immunoresearch) + 0.05% Triton-X in PBS) for 30 min. Sections were then incubated with a polyclonal rabbit anti-pro-surfactant protein-C antibody (Merck Millipore, AB3786) (1:500) in blocking buffer for 1 h. After washing, sections were then incubated with an Alexa Fluor 546-labeled polyclonal goat anti-rabbit antibody (4 µg/ml) (Thermo Fisher Scientific) and DAPI (300 nM) for 30 min in blocking buffer. After additional washes, Prolong Gold (Invitrogen) was added to slides plus a coverslip. Sections were imaged on a laser-scanning microscope (TCS SP8, Leica) with a 20×/ 20x/0.75NA HC PL APO CS2 air objective. Data were processed and analyzed using the open-source software ImageJ (Fiji package) (Schindelin et al., 2012).

2.6. Supplementary figures



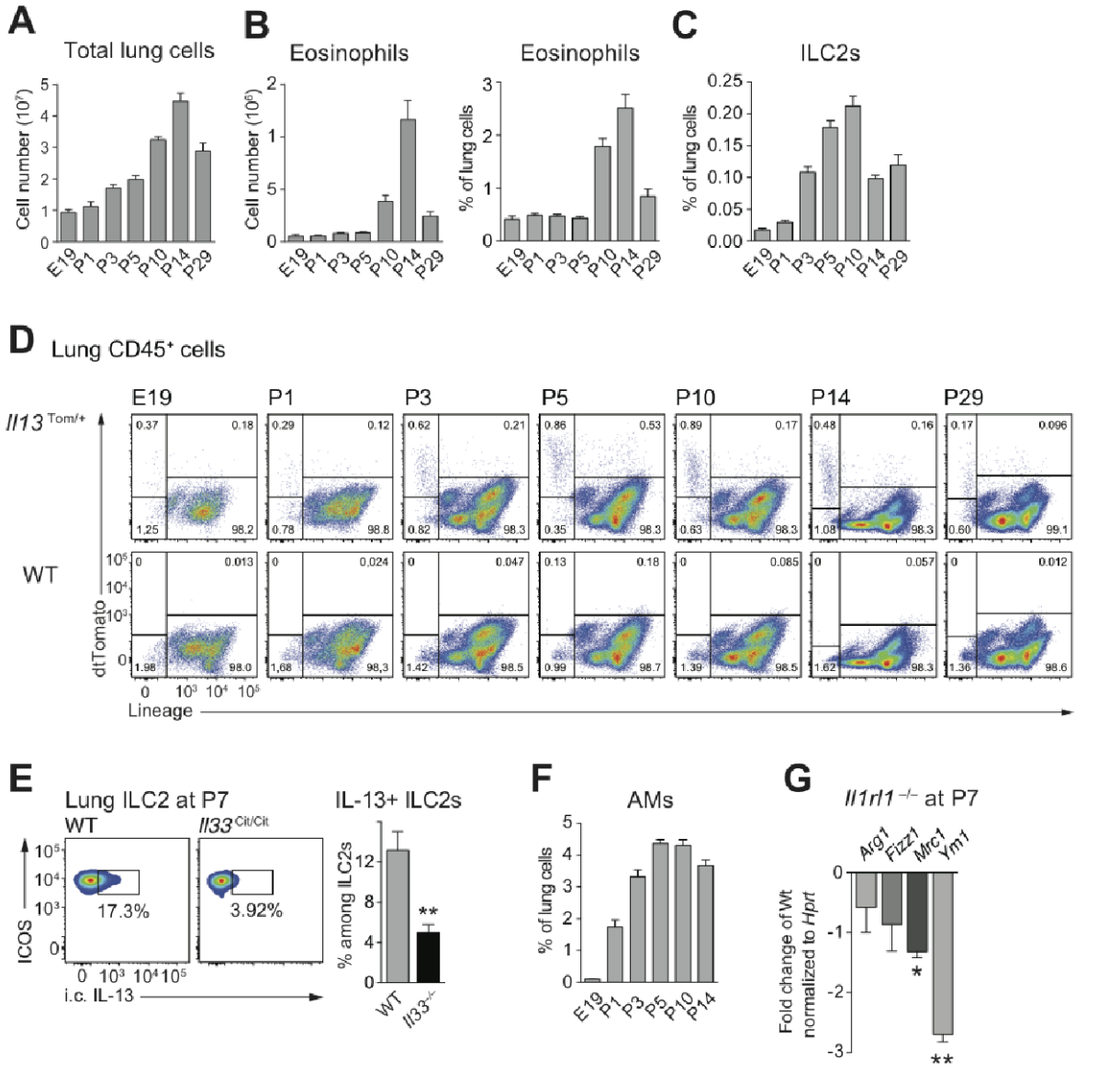
Supplementary figure 1 - Integrated analysis of Citrine expression in lung cells of newborns and upon negative pressure.

Related to Figure 1. **(A)** Experimental set-up. Embryonic lungs (P19) were placed at 37°C and subjected to a negative pressure for 6h (see experimental procedures) (referring to Figure 1C, D, E). **(B)** Viability of lung cells assessed by DAPI staining (referring to Figure 1C, D, E). **(C)** Linear regression analysis of viability and IL-33 expression (Citrin) of embryonic lung cells subjected to negative pressure (vacuum) for 6h (referring to Figure 1C, D, E). **(D)** Absolute numbers of Cit+ CD45– lung cells (referring to Figure 1F lower panel and 1G). **(E)** Proportion of CD45– cells that up-regulate Citrine at indicated time points (referring to Figure 1F lower panel) **(F)** Relative contribution of CD45+ and CD45– cells to total lung cell numbers from E19 to 8 weeks of age (referring to Figure 1F upper panel). **(G)** Quantification of Citrine expression among CD45+ cells in newborn mice. **(H)** Left: blots showing expression of Citrine among fetal macrophages gated as CD45+ F4/80+ CD11b+ CD11c– SiglecF–. Right: quantification of Citrine expression among fetal macrophages. **(I)** Absolute numbers of lung fetal macrophages gated as in (H) at indicated time points. Graph bars represent mean ± SEM. Data are representative of two independent experiments with 3-4 mice per time point.



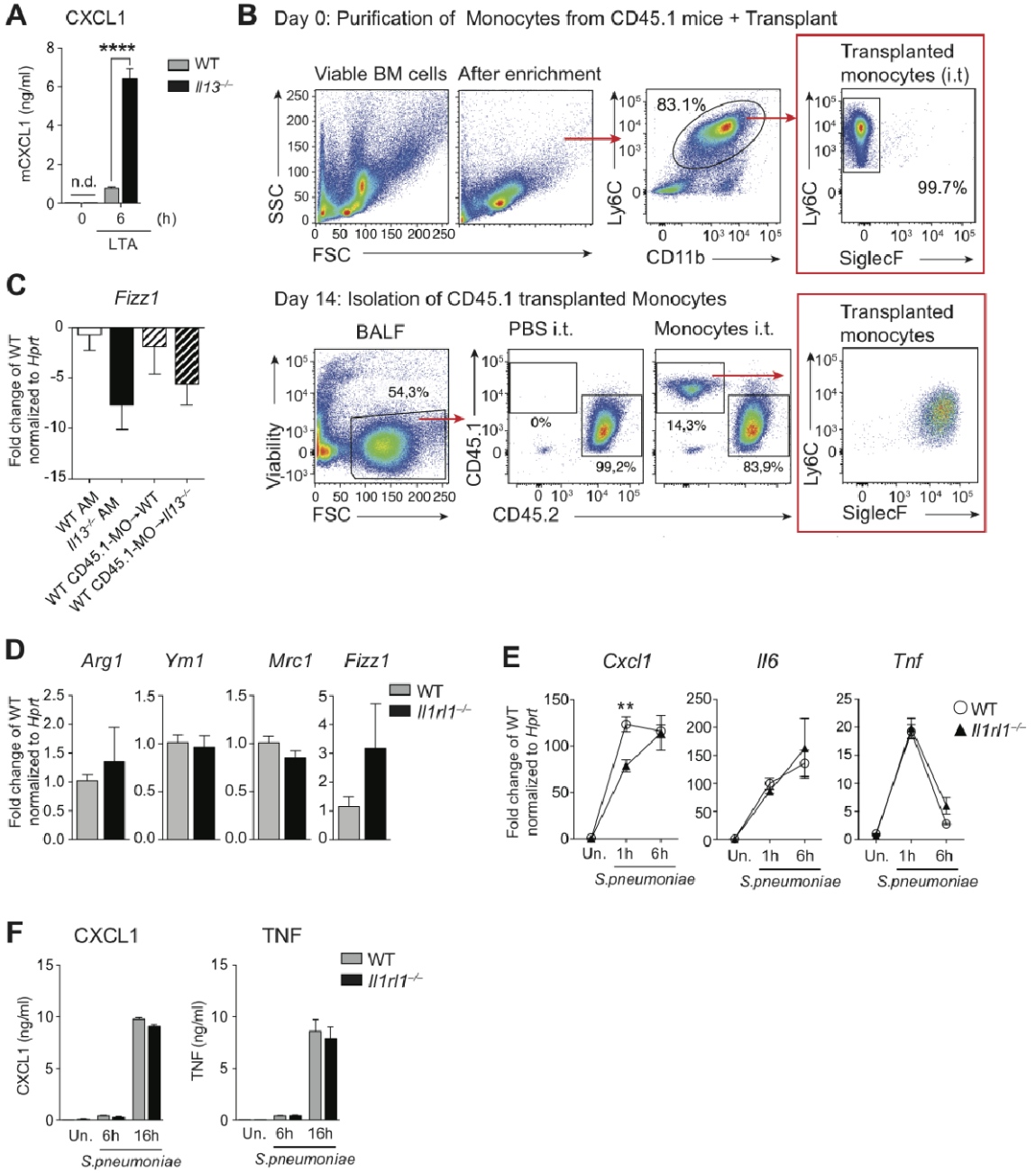
Supplementary figure 2 - Integrated analysis of Il33Cit/Cit mice on P7, postnatal eosinophil expansion and perinatal alveolarization. Related to Figure 2. (A) Absolute values of multiplex ELISA (referring to Figure 2A). (B) Absolute numbers of lung ILC2s between E19 and week 8, (referring to Figure 2B).

(C) Absolute numbers of lung eosinophils between E19 and week 8, (referring to Figure 2E). (D) Analysis of lung immune cell populations in WT and *Il33*$^{Cit/Cit}$ mice, depicted is the percentage of total lung cells, (referring to Figure 2F). PMN = polymorphonuclear cells gated as CD45+ Ly6G+; B cells = CD45+ CD19+; T cells = CD45+ CD3+; AMs = CD11b– SiglecF+ CD11c+; eosinophils = CD11b+ SiglecF+ CD11c–. (E, F) Proportion of ILC2s (among CD45+cells) and IL5-expressing ILC2s (*Il5Cer/+* mice) in indicated body compartments between P7 and adulthood. (G) Quantification of IL5 production by ILC2s assessed by i.c. cytokine stain, (referring to Figure 2H). (H) Lung alveolarization was quantified on H&E stained lung sections from WT and *Il33*Cit/Cit mice at P7 using the automatic image analysis software CellProfiler. For details see Supplemental experimental procedures. Graph bars represent mean ± SEM. *p < 0.05, ***p<0.001, ****p < 0.0001. Data are representative of two independent experiments with 4 mice per group and/or time point.

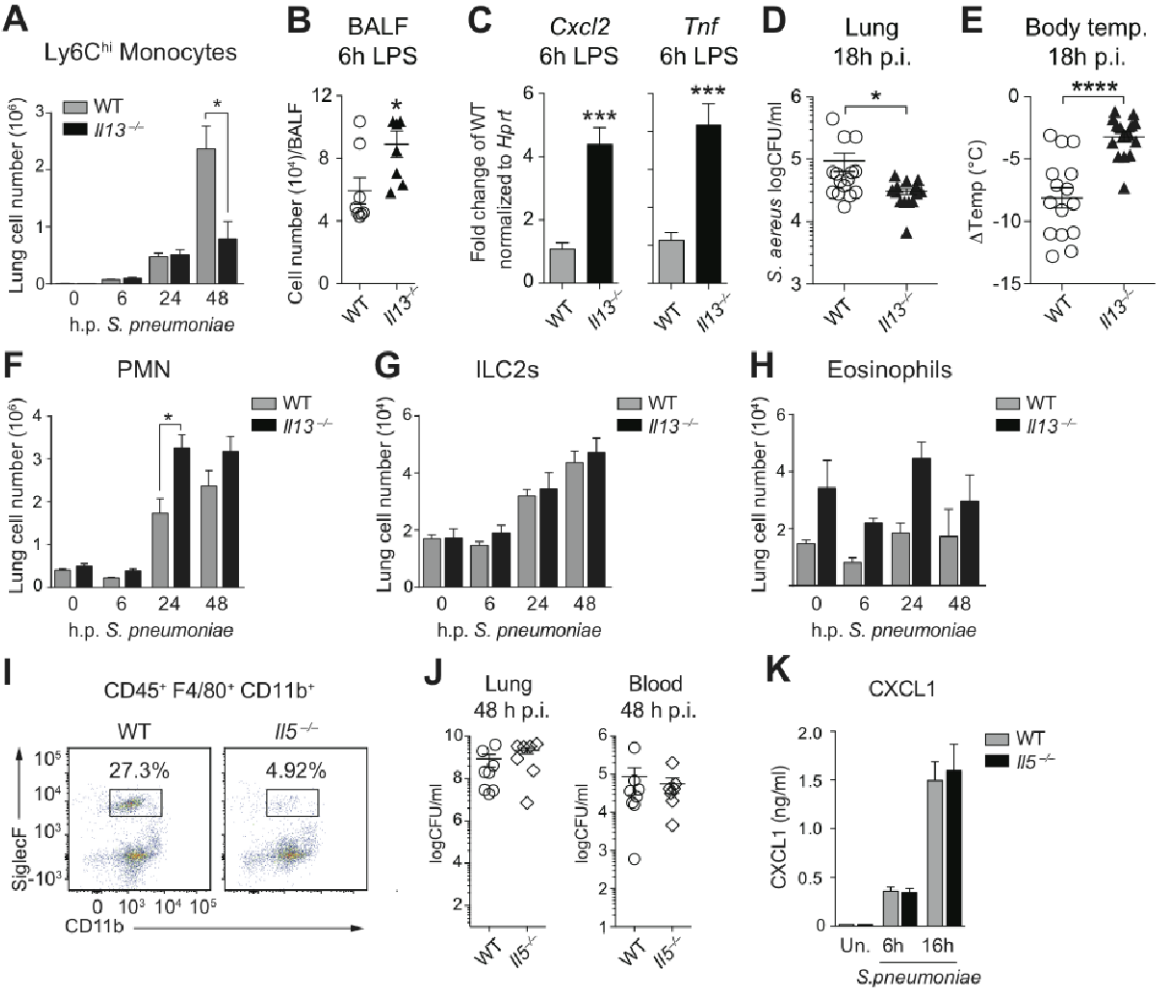


Supplementary figure 3 - Lin⁻cells are the only IL-13 expressing cells in the first two weeks after birth. Related to Figure 3. (A-C) Quantification of lung ILC2s and eosinophils at the indicated time points in *Il13*$^{Tom/+}$ mice. Of note, *Il13*$^{Tom/+}$ mice, which are on a C57BL/6 background, show the same cell influx dynamic as *Il33*$^{Cit/+}$ mice, which are on a Balb/c background, (referring to Figure 3A-D). (A) Absolute number of total lung cells. (B) Absolute numbers and percentages of eosinophils gated as in Figure 2E. (C) Percentage of ILC2s, gated as in Figure 2B. (D) FACS of lung cells gated for Lineage (CD3, CD4, CD8, CD19, F4/80, CD11b, CD11c, FceRI, NK1.1, Ly6C/G) and *Il13*^{dtTomato} expression at indicated time points.

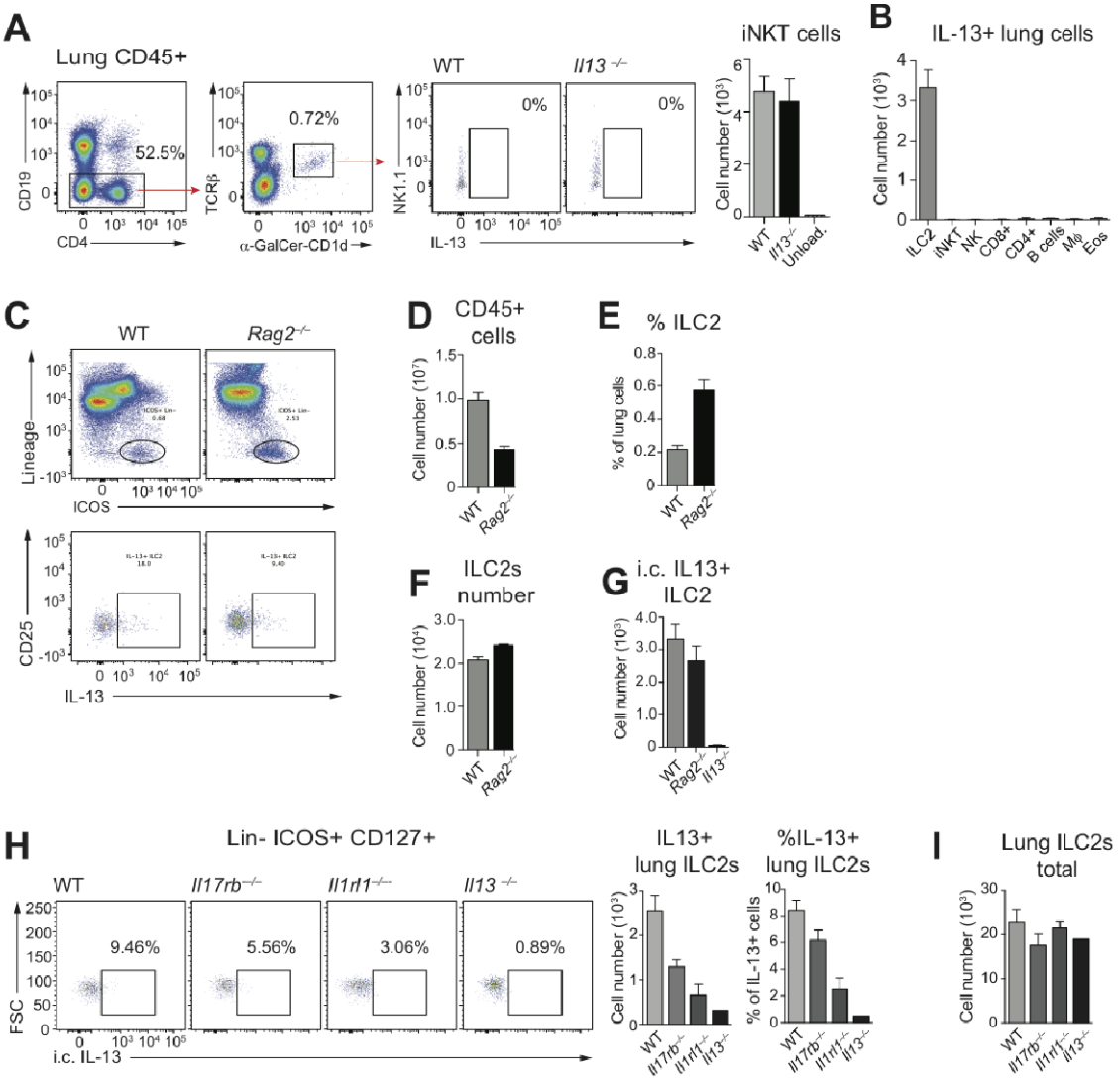
(E) IL-13 expression in lung ILC2s at P7 quantified by i.c. cytokine stain. (F) Percentage of AMs gated as in Figure 3C, (referring to Figure 3C-D). (G) AMs (F4/80+CD11b–CD11c+SiglecF+) were sorted on P7 from WT and *Il1rl1*$^{-/-}$ mice and M2 markers were assessed by RT-PCR. Data are representative of two (A-F) and one (G) independent experiments with 3-4 mice per group. Bars represent mean ± SEM; *p < 0.05; **p < 0.01.



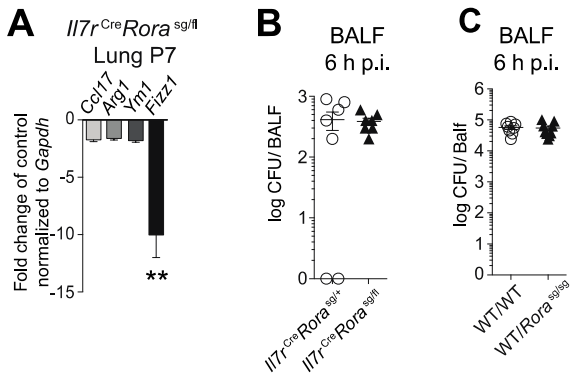
Supplementary figure 4 - BM derived monocytes assume an AMs phenotype 2 weeks upon intratracheal transplant. *Il1rl1* alone plays no significant role in AM polarization in adult mice. Related to Fig 4. (A) AMs from WT and *Il13*–/– mice were isolated by BAL and *in vitro* stimulated with LTA (10µg/ml). CXCL1 was quantified in supernatants, (refers to Figure 4B-C). (B) FACS plots representing bone marrow derived monocytes isolated from WT CD45.1 mice and transferred i.t. into WT or *Il13*–/– mice. Two weeks later transferred monocytes showed upregulation of SiglecF, (refers to Fig 4D). (C) *Fizz1* expression was quantified in FACS-sorted AMs upon monocytes transplant, (refers to Fig 4D. (D, E) AMs isolated via BAL from WT and *Il1rl1*–/–mice analyzed for M2 markers by RT-PCR (D), or *in vitro* stimulated with *S. pneumoniae* (MOI 100) and analyzed for cytokine expression by RT-PCR (E). Values were normalized to HPRT and are expressed as fold-change versus WT at t = 0h. (F) AMs were isolated as in (D), and stimulated *in vitro* with *S. pneumoniae* (MOI 100) and CXCL1 and TNF protein levels were quantified in supernatants by ELISA. Data are representative of at least two independent experiments with four (A and D-F) and 6-7 (B, C) mice per group and/or time point. Mean ± SEM are depicted; **p < 0.01, ****p < 0.0001. I.t. = intra-tracheally; MOI = multiplicity of infection; BAL = bronchoalveolar lavage.



Supplementary figure 5 - Broader role of IL-13 in the inflammatory response to LPS and S. aureus. Eosinophils do not impact on AM polarization, or responses to S. pneumoniae. Related to Fig 4. **(A)** Numbers of lung Ly6Chi monocytes (F4/80+ CD11b+ Ly6Chi) during *S. pneumonia* infection, (refers to Figure 4E-K).
(B-C) Mice were treated i.n. with LPS (100ng/50µl) and harvested 6h later to assess cell influx in BALF (B) and cytokine expression in BALF (C). **(D-E)** Mice were infected i.n. with *S. aureus* and assessed 18h later for body temperature (E), and harvested to quantify lung CFUs (D). **(F-H)** Absolute numbers of (F) PMN (SSChi FSChi CD11b+ Ly6G+), (G) ILC2s (Lin– ST2+ ICOS+ CD25+ Thy1.2+), (H) eosinophils (CD11b+ F4/80+ SSChi CD11c– SiglecF+) during the course of *S. pneumoniae* infection; (refers to Figure 4E-K). **(I)** FACS plots illustrating lung eosinophil numbers in healthy WT and *Il5*–/– mice. **(J)** WT and *Il5*–/– mice were i.n. infected with 105 CFU *S. pneumoniae* and sacrificed after 48h. CFU counts were assessed in lung homogenates (left) and blood (right). **(K)** BAL isolated AMs from WT and *Il5*–/– mice were *in vitro* stimulated with *S. pneumoniae* (MOI 100). CXCL1 was quantified in supernatants. Graph bars represent mean ± SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001. Data are representative of two independent experiments with 4 (A, I and K) and at least 8 (B-H, and J) mice per group and/or time point.



Supplementary figure 6 - iNKT cells are not a source of IL-13 at homeostasis. Baseline ILC2-derived IL-13 expression does not depend or on B and T cells, but partially on Il17rb. Related to Figure 6. **(A)** FACS gating strategy for iNKT cells (CD19– CD4+/– TCRβ+ α-GalCer-CD1d dimer+ NK1.1+). Unloaded CD1d dimer was used as negative control. Right panel: absolute number of iNKT cells in naive adult lungs. IL- 13 production was assessed by i.c. staining. **(B)** Absolute number of IL-13 secreting lung cells at homeostasis (cells gated as in Figure 6D). IL-13 production was assessed by i.c. staining. iNKT cells were gated as in Figure S6A. **(C-G)** ILC2s were assessed for intracellular IL-13 production in naive WT and *Rag2*–/– mice at homeostasis. (C) Representative plots of ILC2s gating strategy. (D) Absolute lung cell number. (E) Percentage of ILC2s out of total lung cells. (F) Absolute number of ILC2s. (G) Absolute number of IL-13 expressing ILC2s. **(H)** Representative FACS plots of ILC2s (Lin– ST2+ ICOS+ Thy1.2+ CD25+) assessed for IL-13 expression via i.c. staining in indicated mouse strains at homeostasis. Right: Absolute number and percentage of IL-13 secreting ILC2s. **(I)** Absolute number of lung ILC2s at steady state in indicated mouse strains. Single cells were pre-gated for viability and CD45+ expression. Graph bars represent mean ± SEM. Data are representative of at least two independent experiments with 4 mice per group. i.c. = intracellular.



Supplementary figure 7 - Reduced M2 marker expression in lungs of neonatal ILC2-deficient mice. Related to Fig 7. (A) M2 markers were assessed by RT-PCR in whole lung homogenates of naive *Il7r*Cre*Rora*sg/fl mice as compared to *Il7r*Cre*Rora*+/fl controls on postnatal day 7 (P7), (refers to Figure 7B). (B, C) BALF CFU in indicated mouse strains 6h post i.n. infection with *S. pneumoniae*, (refers to Figure 7E-J, and Figure 7O-P, respectively). Graph bars represent mean ± SEM. **p < 0.01. Data are representative of at least two independent experiments with 4 mice per group (A) or 7-8 mice per group (B-C).

2.7. Supplementary tables

Supplementary table 1. Antibodies.

Ab	Fluorochrome	Clone ID	Manufacturer	Isotype
B220	PerCP/Cy5.5	RA3-6B2	eBioscience	Rat IgG2a
CD45	V500	30-F11	BD Biosciences	Rat IgG2b
CD45	BV510	30-F11	Biolegend	Rat IgG2b
CD45.1	Pacific Blue	A20	Biolegend	Mouse IgG2a
CD45.2	PE	104	Biolegend	Mouse IgG2a
CD3	FITC, eFluor450	17A2	Biolegend	Rat IgG2b
CD3	PE/Cy7	145-2C11	Biolegend	Hamster IgG
CD4	FITC, PE/Cy7	GK1.5	eBioscience	Rat IgG2a
CD4	AF700, PE/Cy7, eFluor450	RM4-5	eBioscience	Rat IgG2a
CD8a	FITC, eFluor450, PE/Cy7	53-6.7	Biolegend or eBioscience	Rat IgG2a
CD11b	APC, FITC, AF700, PercP/Cy5.5, PE/Cy7, Pacific Blue	M1/70	Biolegend or eBioscience	Rat IgG2b
CD11c	FITC	HL3	BD Biosciences	Hamster IgG1
CD11c	AF700, PE/Cy7, BV421, AF647	N418	Biolegend or eBioscience	Hamster IgG
CD19	FITC, PE/Cy7	eBio1D3	eBioscience	Rat IgG2a
CD19	PE/Cy7, BV421, AF700	6D5	Biolegend	Rat IgG2a
CD31	PE/Cy7	390	eBioscience	Rat IgG2a
CD49b	FITC, Pacific Blue, PE/Cy7	DX5	Biolegend or eBioscience	Rat IgM
CD127	APC, PE/Cy7	A7R34	Biolegend	Rat IgG2a
CD138	APC	281-2	Biolegend	Rat IgG2a
c-Kit	AF700	ACK2	eBioscience	Rat IgG2b
EpCam	PE/Cy7	G8.8	Biolegend	Rat IgG2a
F4/80	BV421, BV785, FITC, PE/Cy7, APC, PerCp/Cy5.5	BM8	Biolegend or eBioscience	Rat IgG2a
FcεRIa	FITC, Pacific Blue,	MAR-1	Biolegend or	Hamster IgG

	PE/Cy7, PE		eBioscience	
Foxp3	APC	FJK-16s	eBioscience	Rat IgG2a
Gr-1	FITC, BV421, PE/Cy7	RB6-8C5	Biolegend or eBioscience	Rat IgG2b
ICOS	APC, PE, PE/Cy7, BV421	C398.4A	Biolegend or eBioscience	Hamster IgG
IL5	APC	TRFK5	Biolegend	Rat IgG1
IL13	PE	eBio13A	eBioscience	Rat IgG1
Ly6C	BV605	HK 1.4	Biolegend	Rat IgG2c
Ly6G	PE, PE/Cy7	1A8	Biolegend	Rat IgG2a
MHCII	FITC	2G9	BD Biosciences	Rat IgG2a
MHCII	Pacific Blue, BV510	M5/114.15.2	Biolegend	Rat IgG2b
NK1.1	eFluor450	PK136	eBioscience	Rat IgG2a
RORγt	PE	AFKJS-9	eBioscience	Rat IgG2a
Siglec-F	APC, AF647	E50-2440	BD Biosciences	Rat IgG2a
Sca-1	APC	D7	Biolegend	Rat IgG2a
ST2	Biotin, PerCp/Cy5.5, FITC	DJ8	MD Bioproducts	Rat IgG1
ST2	eFluor710	RMST2-2	eBioscience	Rat IgG2a
Ter-119	BV421, PE/Cy7	Ter-119	Biolegend	Rat IgG2b
Thy1.2	eFluor450	53-2.1	eBioscience	Rat IgG2a

Supplementary table 2. Gating strategy for lung cells in the study.

Cell population	**Gating strategy**
B cells	CD19+ MHCII+
CD4+ T cells	CD3+/CD4+ CD8-
CD8+ T cells	CD3+/CD4- CD8+
T regs	CD3+ CD4+ /CD25+ Foxp3+
ILC2	Lin-(CD3-CD4-CD5-CD8-CD19-CD49b-Fc□RIa-CD11b-Cd11c-F4/80-Gr1-) Thy1.2+ICOS+ST2+CD25+
NK cells	CD3- /NK1.1+ CD49b+

NKT cells	CD3+ /NK1.1+CD49b+
DCs	CD8-/F4/80-CD11c^{hi} /CD11b^{lo} MHCII^{hi}
AMs	F4/80+ CD11c+ /CD11b^{lo} SiglecF+
PMN	CD19-F4/80- Ly6G+
Eosinophils	CD19-F4/80+ CD11b+ CD11c- SiglecF+ SSC^{hi}
Mast cells	CD19- CD11b+ cKit+ FceRIa+

Supplementary table 3. List of primers.

Gene target	**NM name**	**Fragment size**	**Sequence**
mArg1	NM_007482	158bp	F: CAGTGTGGTGCTGGGTGGAG R: ACACAGGTTGCCCATGCAGA
mYm1	NM_009892.1	191bp	F: TCTGGGTACAAGATCCCTGAACTG R: GCTGCTCCATGGTCCTTCCA
mMrc1	NM_008625	116bp	F: TCTGGGCCATGAGGCTTCTC R: CACGCAGCGCTTGTGATCTT
mFizz1	NM_020509	197bp	F: TCCAGCTGATGGTCCCAGTG R: AAAGCCACAAGCACACCCAGT
mCXCL1	NM_008176	235bp	F: GACCATGGCTGGGATTCACC R: TCAGAAGCCAGCGTTCACCA
mTNF	NM_013693	200bp	F: GAACTGGCAGAAGAGGCACT R: GGTCTGGGCCATAGAACTGA
mHPRT	NM_013556	96bp	F: GTTAAGCAGTACAGCCCCAAAATG R: AAATCCAACAAAGTCTGGCCTGTA

3. Discussion

One of the major conceptual advances in the field of type 2 immunity is the recent discovery that barrier epithelial cells are crucial upstream regulators of the type-2 immune response, determining the outcome of a pathogen encounter via sensing cellular damage or PAMPS and releasing alarmins and cytokines, like IL-33, IL-25, GM-CSF, IL-1β (Hammad and Lambrecht, 2015). Such cytokines can license ILC2s and Th2 cells to produce cytokines independently of antigen specificity and promote a polarization of antigen presenting cells like DCs or TRMs towards an anti-inflammatory phenotype (Lambrecht and Hammad, 2017).

In this work, we explored the impact of birth and tissue expansion on the immunology of the newborn lung. We have been among the first researchers to describe that perinatal lung inflation is associated with a wave of *Il33* upregulation in AEC2 and consequent peak in IL-33 protein level in the lung. Once released, the nuclear cytokine IL-33 acted as an alarmin, triggering a potent activation of embryonic ILC2s and therefore an unexpected type 2 innate immune response to birth. This physiological "immune awakening" of the lung was characterized by IL-13 and IL-5 release, eosinophils infiltration and M2 polarization of newly developing alveolar macrophages (AMs). We believe that these perinatal immune activation is responsible for a "type-2 imprint" of the lung tissue, since AMs maintained an ILC2s and IL-13 driven M2 polarization also in adult life, which was proven detrimental in case of infection with *S. pneumoniae* and *S. aureus*. Our results have important implication in the understanding of the susceptibility of newborns to allergen sensitization and bacterial infections like pneumococcal pneumonia, which is still nowadays, despite of antibiotic therapy, the first cause of death by an infectious disease worldwide (van der Poll and Opal, 2009).

In our experiments with newborn $Il33^{Cit/+}$ reporter mice, *Il33* was highly upregulated on the day of birth and then slowly lost strength in the course of the first two weeks of life and it reached a low constant activity around the third week, that was maintained throughout the mouse life. The upregulation of *Il33* on P1 was linked with protein production and release, which we attributed to cellular death, damage or mechanical stress (Kakkar et al., 2012), very likely to happen at birth upon the first breaths of air and alveolar expansion. However, while the IL-33 release by AEC2 is easily explained by cellular damage or mechanical stress, the genetic upregulation of *Il33* in AEC after birth and to some sort also during late embryogenesis is quite puzzling. In mice, *Il33* transcription is usually constitutive but it can be induced in the context of inflammation or TLR ligation, which can also happen in the

perinatal lung environment (Molofsky et al., 2015). Interestingly, IL33 is constitutively expressed at barrier tissue levels during embryogenesis (Pichery et al., 2012) and highly expressed in AEC2 already before birth (Treutlein et al., 2014), confirming the importance of this protein in early life. However, we cannot explain what could induce the previously unsuspected strong genetic upregulation of *Il33* in mice AEC2 on the first day of life. We could surprisingly reproduce this happening in-vitro by inducing mechanic expansion in late stage embryonic lungs (i.e. short time before birth). We can at least say that the cells that upregulated Il33 were perfectly viable, hinting at mechanical stress as the actual contributor for Il33 gene upregulation. Indeed, some promoters can be mechanically induced by so called "mechanosensors" or "mechanotransducers" (Low et al., 2014), but the roles of such promoters in the upregulation of *Il33* has not be investigated so far. Interestingly, fetal breathing movements in humans already start in uterus, around week 10 post conception, causing stretching and mechanical changes that could potentially activate mechanoreceptor-dependent upregulation of immune mediators. For example, it has been shown that specific mechanosensitive channels can induce the upregulation of serotonin already during the fetal phase, promoting the differentiation of epithelial cells (Pan et al., 2006). Moreover, evidence that AEC2 present mechanoreceptors are also coming from a study from Scott at al. in which they showed that mechanical stretch of AEC2 can induce surfactant lipid production (Scott et al., 1993). Intriguingly, surfactant related phospholipid synthesis in AEC2 begins at the same time as constitutive *Il33* expression (Treutlein et al., 2014), suggesting that episodic fetal breathing movement could also be responsible for the small constitutive expression of *Il33* in AEC2. Such a theory could explain for example the expression of *Il33* in AEC2 during late stage embryogenesis and maybe even explain the limited but consistent presence of ILC2s in the embryonic mouse lung already before birth (Ebbo et al., 2017). On a general note, epithelial cells throughout the body at other barrier levels, for instance skin keratinocytes or gut epithelial cells, are strongly subjected to mechanical stress at birth and could therefore undergo perinatal *Il33* upregulation. It is reasonable to think that this wave of birth associated IL-33 secretion characterizes the whole newborn body at epithelial barrier level and could confer a type 2-imprint also to other tissues, like the skin, establishing an anti-inflammatory phenotype to skin resident long-lived immune cells, like Langerhans cells.

Once released to the extracellular space, IL-33 acts as an alarmin of tissue damage in the newborn lung, by activating cells that express the ST2 receptor. In the adult lung, ILC2s have been confirmed as the major IL-33 responsive cell population (Halim et al., 2012b). However, if the small population of embryonic ILC2s that already populate the lung before birth (Artis

and Spits, 2015) are also the most effective IL-33 responsive cells was not clear so far. Our data show that the murine lung embryonic ILC2 population rapidly expands under IL-33 stimulation in the course of the first three days of life and that they starts producing high amounts of IL-13 and IL-5, thereby recruiting eosinophils. Our experiments in ST2 KO mice confirmed the importance of IL-33 in determining the eosinophil influx and ILC2 expansion in early life. ILC2 numbers peaked in the lung around P7, when they reached a plateau that lasted until P14 and then slowly went down to reach the number commonly found in adult mice, in parallel with a decline in *Il33* gene expression. Accordingly, eosinophils peaked at P14, recruited by ILC2s-derived IL-13 and IL-5. Moreover, we showed that ILC2s was the most abundant IL-13 expressing cell population during the first two weeks after birth. We therefore think that ILC2s represent the most effective IL-33 responsive and IL-13 secreting population in the newborn lung. Of course, perinatal ILC2 expansion is probably due to local expansion rather than recruitment from the circulation, as it was recently shown that ILC2s are rather tissue resident cells that expand locally (Gasteiger et al., 2015b).

In humans, pulmonary surfactant starts being produced by week 23-24 and reaches sufficient levels around week 35 of gestation. Antenatal corticosteroid administration to mothers in preterm labor stimulates surfactant production, and the endotracheal administration of exogenous surfactant significantly improves respiratory physiology in preterm babies with acute respiratory distress syndrome (ARDS) (Neumann and Von Ungern-Sternberg, 2014). Besides supportive care, no other pharmacological therapies are nowadays available to treat this disease. It is unknown if immunological factors play an important role in the pathogenesis of ARDS, but our data suggest that the mouse lung could be in an IL-33-mediated inflammation and that therapies that target IL-33 might be useful to reduce the inflammation and allow lung regeneration.

Another important finding described in this thesis is that right at the time of their differentiation in the lung, AMs are subjected to an M2 polarization driven by IL-13 producing ILC2s. We show that during *in tissue* development and differentiation at P3, AM are exposed to type-2 polarizing factors, like IL-33 derived from AEC2 and IL-13 derived from ILC2s. This combination of IL-13 and IL-33 is highly polarizing in AMs, as IL-33 was shown to amplify the IL-13 signal (Kurowska-Stolarska et al., 2009). Mice deficient in ILC2s or IL-13 had normal numbers of AMs, which meant that ILC2s did not affect their numeric development. However, AMs extracted from ILC2 or IL-13 deficient mice presented a clear loss of the physiological M2 phenotype not only at P14, but also later in adult life. Considering that AMs are long lived cells that replicate in tissue and acquire their phenotype

by tissue derived signals, we think that our findings strongly contribute to the understanding of the origins of AMs polarization at homeostasis. In fact, it was previously noted that lung AMs are in an intrinsic M2 state, for example expressing PPARγ, a regulator of M2 macrophage polarization (Schneider et al., 2014a), and Chil3l3, an established M2 marker (Lavin et al., 2014). However the mechanism for the constitutive M2 phenotype of AMs in in tissue was so far unknown (Hussell and Bell, 2014). We therefore believe that our findings provide a new pathway that explains the constitutive M2 phenotype of AMs, which is of high importance for our understanding of the immunological state of lung tissue.

The ILC2 mediated polarization of lung macrophages at birth might constitute an important immune-regulatory system that can provide tolerance to the external environment and also induce tissue remodeling and wound healing in the expanding lung. As we further show in our experiments, the M2 polarization of AMs, which might be important in achieving tolerance and maintaining a healthy epithelium, comes at the expenses of a reduced immune response to bacterial pathogens. Even if IL-33 was important in inducing ILC2 activation and IL-13 expression in the newborn lung, the absence of IL-33 alone was not sufficient to abrogate the physiological M2 phenotype in AMs, which was definitely induced by ILC2s or IL-13, hinting at other possible sources of ILC2 activating cytokines in the absence of IL-33. This is certainly a pitfall of our findings. Even if IL-33 is the strongest ILC2 activating cytokine in the lung, it is by far not the only one and probably alternative pathways induce ILC2 activation in the absence of IL-33, like TSLP, LT4, or IL-25, which we did not investigate.

Interestingly, we observed that the wave of IL-33 expression in the newborn lung coincided with the infiltration of many other immune cells. In fact, in late fetal life, the lung is populated by very few leukocytes, among those, mostly fetal macrophages and monocytes (Hussell and Bell, 2014). The lung expansion and probably the vascular changes that occur at birth determine the sudden tissue infiltration with immune cells, like B cells, neutrophils, T cells and eosinophils. Infiltrating immune cells reach homeostatic numbers two weeks after birth in the mouse lung. It is tempting to speculate that the type-2 immune environment that we described in our work and that characterizes the newborn lung in the first weeks of life, might also affect the function of other cells that infiltrate the lungs at this early stage, exactly like it happens with AMs. In fact, other lung resident cell types can express IL-33 or IL-13 receptors, like B cells, T cells ad Tregs. Therefore, the IL-33/ST2/ILC2s mediated immune-regulatory mechanism that we discovered for newborn AMs, might influence also other lung resident cell types, avoiding over-reaction to new antigens coming from the environment.

It was long believed, that immunity in newborns was defined as an intrinsic "tolerant" state, in order not to over-react to the outside environment. It was already noted, for instance, that upon challenge with immune stimuli, children younger than 2 months develop an innate Th2/Th17 naive cell polarization, and a much weaker Th1 polarization (Goenka and Kollmann, 2015). The reduced Th17 pathway, could explain the increased risk for bacterial infection with *Listeria monocytogenes*, candidiasis, *Mycobacterium tuberculosis* or herpes simplex virus (Dowling and Levy, 2014). The reason for the impaired Th1 responses in early life is also not well understood and historically attributed to a rather poorly defined "immaturity" of the immune system. Indeed, some authors already suggested that the newborn lung immune system undergoes "maturation" in the first two weeks of life. In fact, it was noted that newborn mice present an increased susceptibility to allergen sensitization which is then regulated by p14 by newly developing PD-L1 responsive Tregs (Gollwitzer et al., 2014). Moreover, Tregs generated during the perinatal period have been shown to express ST2 and be highly suppressive and proliferative, thereby indicating the need for a strong control of the immune system at birth (Yang et al., 2015). Considering the recent findings on the role of ILC2s in enhancing the function of Tregs via IL-9 secretion, it is not excluded that in the newborn lung, ILC2s might contribute to the Treg-mediated suppression of perinatal inflammation (Rauber et al., 2017). These observations support the hypothesis of a "window of immune development" of newborns adaptive immunity. However, while the adaptive immune system needs a few days to mature, the innate immune system of mice and humans seems perfectly able to mount pro-inflammatory immune responses already at birth. In fact, recent evidence suggests that human and murine PBMCs extracted during the post-natal periods are perfectly able to mount the same immune responses as adult PBMCs when stimulated *in vitro*, pointing at poorly perinatal circulating soluble factors as the cause for the anti-inflammatory phenotype. We show in our results that early AMs are still quite responsive to TLR stimuli, but then, few days after birth, they start undergoing an IL-13 dependent polarization process that renders them M2-like and reduces their capacity to respond to bacterial molecules. Moreover, the AMs of newborn mice deficient in IL-13 or ILC2s presented an M1 phenotype and increased responses to bacterial products, confirming the role of ILC2s in polarizing AMs during the perinatal period. These findings highlight the possibility that ILC2s provide an important immune regulatory mechanism in newborns, giving time to adaptive immunity to mature and therefore bridging the gap between the two systems to prevent excessive inflammation.

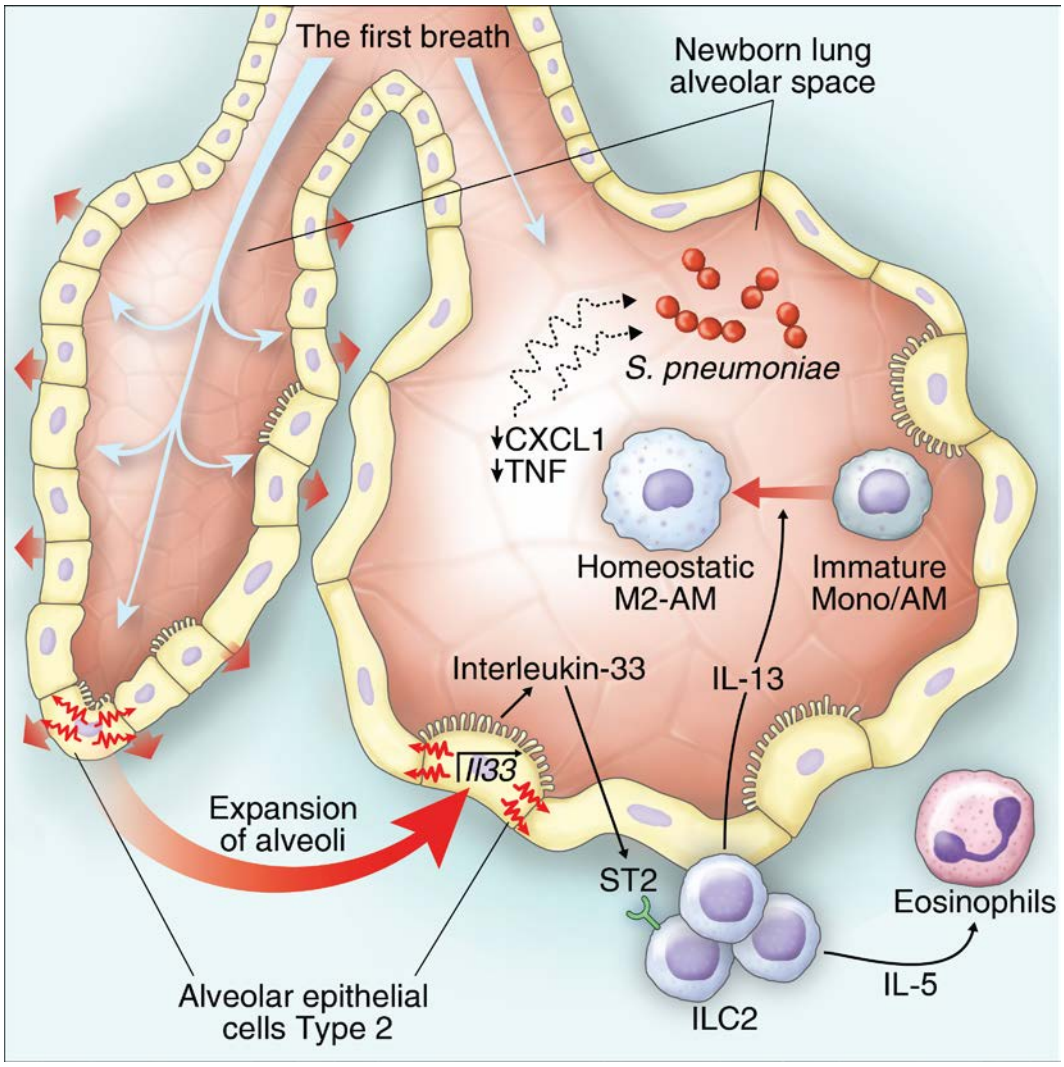


Figure 20 – Working model of how first breath induces alveolar expansion resulting in IL-33 release by AEC2 is able to induce the expansion of ILC2s and their activation to produce IL-13 consequently skewing the phenotype of newborn developing AMs to an M2 response, leading to reduced intrinsic responses of tissue resident AMs to lung bacterial pathogens such as *S. pneumoniae*. AEC2 = alveolar epithelial cells type 2, ILC2 = Innate lymphoid cells type 2, AMs = alveolar macrophages. Image from Rui Martins.

What concerns the translation of our findings to human health, it is important to point out that all the players of the immune mechanism we describe are also present in human lungs, meaning that it is highly likely that the same IL-33/ILC2 mediated immune activation also takes place in human tissues. For example, ILC2s have been observed in human fetal lungs (Mjösberg et al., 2011) and evidence suggests that also in humans AMs develop postnatally and present an intrinsic M2 polarization phenotype characterized by CD206 expression (Bharat et al., 2016). Even if it is yet not clear if human AEC2 also express and release IL-33, it is known that this alarmin is abundantly expressed in the nuclei of human lung bronchial epithelial and endothelial cells, which might represent the major source of lung IL-33 in

humans. The biology of IL-33 is practically identical to the one in mice, in particular IL-33 was also observed to be secreted upon mechanical stress in human fibroblasts (Kakkar et al., 2012). Moreover, additional evidence supports the hypothesis that the IL-33-derived type-2 immune reaction in murine newborns might also happen in human neonates. As I mentioned in the introduction, the sensing of IL-33 triggers the upregulation and expression of soluble ST2 (sST2), which serves as a decoy receptor. Soluble ST2 is also released in humans and it is considered a marker for cellular damage and IL-33 release throughout the body (Chen et al., 2015). Strikingly, human newborns also have high levels of plasma circulating immune-suppressors, in particular sST2 as well as IL-13, strongly suggesting that our findings could probably be applied also to human newborns (Belderbos et al., 2013). Mover, other studies show highly increased ILC2 numbers in cord blood from human newborns compared to young children and adults. Finally, recent evidence on the expansion of uterine ILC subtypes during pregnancy strongly support the hypothesis that ILC play a role in tissue homeostasis during reproduction and birth (Boulenouar et al., 2016). The study of early immune responses in humans is of course limited to the availability of samples and the conclusions are mostly drawn from *in vitro* experiments using human placenta derived cord blood cells (Dowling and Levy, 2014). However, since a wave of soluble immune-mediators belonging to the ILC2 and Treg biology, like IL-10, IL-4, IL-13 and CD25 and since sST2 was detected at high levels in the circulation of human newborns, we can conclude that there is strong supporting evidence that our findings can be equally translated to humans.

Sensitization to allergens classically occurs in neonatal life and early childhood (Gollwitzer et al., 2014; Lambrecht and Hammad, 2017). GWAS studies confirmed the association of genes important in ILC2 biology as well as of *Il33* and *Il1rl1* in the susceptibility to allergic asthma (Moffatt et al., 2010). Moreover, a loss of function mutation in the *Il33* gene was shown to protect from asthma (Smith et al., 2017). Even if the increased type 2 immunity in early life might play a role in allergen sensitization, our findings describe a physiological type-2 immune response to birth, which does not necessarily translate in an allergic phenotype, but rather tells us that the lung tissue is preferentially primed towards wound healing and tissue remodeling and type 2 immunity instead of fighting microbial pathogens. It is probably rather a dysregulation of the physiological immune pathway that we describe, that could be responsible for antigen sensitization in early life. As alveolarization and lung tissue remodeling last longer in humans (up to three years after birth), it is possible that similar mechanism that ease the sensitization to allergens in mice are still active in humans for longer periods of time, until early childhood. Newborns are highly susceptible to viral infections, for

example to Respiratory Syncytial Virus (RSV), which has been suggested as one of the possible risk factors for immune dysregulation and asthma development in early life (Lynch et al., 2016). A dysregulation of the perinatal immune response, maybe also due to dysbiosis of the lung microbiome, might be responsible for allergen sensitization. In fact, the lung microbiome is also formed in the first two weeks of life, when changes in the lung microbiota are associated with maturation of Tregs and DCs and in absence of microbiota colonization, mice are hyper responsive to aeroallergens (Gollwitzer et al., 2014). However, despite few important reports showing how certain airborne particles, like farm dust, can regulate the response of epithelial cells towards a less allergic response (Schuijs et al., 2015), more research is needed to understand how microbial particles can regulate epithelial cell barrier in early life, avoiding allergic sensitization. Interestingly, many authors attributed the susceptibility to RSV infection in neonates to an exaggerated type 2 immunity, whose origins were also not clear (Lambert et al., 2014). However, our findings, which were at the same time confirmed by another independent group (de Kleer et al., 2016), point towards epithelial derived alarmins as the real players of the first innate immune response to birth and consequently to a physiological type-2 immune response that is later regulated by the adaptive immune system, eventually involving also a cross talk between ILC2s and Tregs (Rauber et al., 2017).

Last but not least, an important finding of this work is that the type-2 pathways activated perinatally are responsible for a long-lasting polarization of tissue resident AMs, with the consequence of a constitutive reduced defense capacity against pneumococcal pneumonia in adult life. Lung bacterial diseases have been and still are nowadays, in the antibiotic and vaccine era, among the major killers of humanity (World Health Organization, 2008). Our findings show that the lung tissue is physiologically less able to fight bacteria because of homeostatically active IL-33 and ILC2-derived type-2 pathways. In fact, ILC2s are constantly active in the lung, secrete low amounts of IL-13 and maintain an M2 polarization of lung AMs, which is detrimental in *S. pneumoniae* and *S. aureus* lung infections. Even if we do not support our findings with in vivo models of pneumococcal pneumonia in newborn mice, our experiment in adult mice deficient in IL-13 and ILC2s confirm the role of ILC2s in reducing the responses to pneumonia.

On a general note, one of the main open questions of ILC research is to understand if ILCs can have non-redundant role in humans, in comparison to Th cells and to the newly identified resident memory T cells. In principle, ILC2s are an innate and immediate source of effector cytokines and are activated by environmental signals. On the contrary, Th cells act against

specific antigens only and also need few days to develop upon first antigen encounter. Memory Th cells can go back to the tissue in which they first encountered the antigen and assume a quiescent state, becoming tissue resident. These cells can mount an effective immune response in case of antigen re-encounter, without causing the potential excessive damage of ILCs, due to their antigen specificity (Park and Kupper, 2015). For what concerns the capacity to sense tissue damage also Th cells and Tregs express ST2 and can therefore respond to IL-33 even so to a significantly lesser extent compared to ILC2 (Griesenauer and Paczesny, 2017; Schiering et al., 2014). Therefore, considering the immunity at tissue level, is the biological role of ILC2s non-redundant to the one of tissue resident memory T cells?

One first step in understanding the biology of ILC2s compared to the one of T cells comes from Vely F. et al (2016). The authors investigated ILC numbers in patients that underwent myeloablative or non-myeloablative hematopoietic stem cell transplantation (HSCT) in children affected by severe combined immunodeficiency (SCID), which presented a IL2Rγc mutation. These children, besides not having B and T cells, also miss all ILC lineages (Vély et al., 2016). Patients that underwent non-myeloablative regimens continued to display very low numbers of ILC1, ILC2 and ILC3 in circulating blood, gut and skin samples. Upon HSC transplantation with non myeloablative regimens, the authors noted that the selective deficiency in ILCs also at tissue level that was not associated with major clinical consequences in 7-39 years follow up (Vély et al., 2016). These data therefore suggest that ILCs could be dispensable in humans with a functional adaptive immune system and point towards a rather supportive role for ILCs in regards to Th cells, at least in the context of modern medicine and hygiene, which might in principle mask some of their immunological functions. However, the follow up time in this study (7-39 years) was probably too short to address the hypothesis of a role for ILCs in tumor immunosurveillance. Finally, since these patients are often treated with immunosuppressive regimens, it is actually impossible to rule out if the reduced number of ILC2s might be beneficial or detrimental in protecting them against lung infections. Further investigations on the role of ILCs in embryonic life, early childhood and in the context of hereditary or acquired adaptive immune deficiencies (like in HIV or in patients upon immunosuppressive therapy) is essential to further characterize the biology of ILCs.

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Curriculum vitae

Qualification: Physician, 2nd year Resident in Dermatology, PhD

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Academic education:

15th March 2016 – present	Resident at the Dep. of Dermatology, Medical University of Vienna
15th March 2016 – present	Research fellow at the Stary Lab, Translational Immunology in Mucosa and Skin (TIMS), Department of Dermatology
Oct. 2011 – Feb. 2017	PhD fellow, CCHD Program at the MUW with focus in Immunology Supervisor: Prof. Dr. Sylvia Knapp
March 15th 2011	License for Medical Practice – *Jus practicandi*
Oct. 22th 2010	MD Degree at the Medical University of Turin, grade 110/110 and honors. Thesis title: "Pharmacognomic of Ribavirin in Chronic Hepatitis C Infection" Supervisor: Prof. Dr. Giovanni Di Perri.
2004 – 2010	Human Medicine studies at the Medical University of Turin, IT
2003 – 2004	Pharmacology Studies, University of Turin, IT.

Notable research experiences abroad

May – July 2014 and Jan. – May 2015	Research fellow in the lab of Prof. Dr. Andrew McKenzie – Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK. Total of 7 months.
May – June 2008	Research fellow at the Laboratory of Anatomical Pathology, Faculty of Medicine, Madrid. Supervisor: Prof. Dr. Emilio Alvarez Fernandez.
September – October 2006	Research fellow at the IRCC (Institute for Cancer Research and Treatment), Division of Angiogenesis, University of Torino, Faculty of Medicine. Supervisor: Prof. Dr. Federico Bussolino

Notable clinical experiences abroad

Feb. – April 2011	Tropical Medicine Course at the Institute of Tropical Medicine "Alexander von Humboldt", Universidad Peruana Cayetano Heredia, Lima, Perù. Department: Prof Eduardo Gotuzzo.
2008 – 2010	Clinical internship at the University Clinics of Infectious Diseases and STDs, Hospital "Amedeo di Savoia", Prof. Dr. Giovanni Di Perri. Turin, IT. Experience in the HIV in-patient and out-patient clinic.

Notable educational experience abroad

Sept. 20th 2007 – June 20th 2008	Fellow of the Erasmus Program *(European Region Action Scheme for the Mobility of University Students)*. "Universidad Complutense" of Madrid Universitary Hospital "Gregorio Marañon", Madrid, Spain.
2002, June 6th	A-level Diploma Certificate at Lebanon High School, 38300 St Luois Street, Lebanon,OR, USA

Scholarships, awards, grants

2018	MUW Researcher of the Month – January 2018
2017	Clemens von Pirquet Prize of the OEGAI
2016	Best Oral Presentation Award at the OEGAI Symposium, Innsbruck 2016
2015	Best Poster Presentation Award at the YSA 11th PhD Symposium of the MUW
2012	Best Oral Presentation Award at the YSA 7th PhD Symposium of the MUW
2014	Boeringer Inghelheim Travel Grant
2007	ERASMUS Scholarship to study in Madrid, Complutense University of Medicine

Activity within the Medical University

- 5th, 6th 7th CCHD Symposium organization as member of the CCHD PhD Program. (http://www.meduniwien.ac.at/phd-cchd/workshop/)
- 9th YSA Symposium organization as member of the Young Scientist Organization of the Medical University of Vienna (Board Member: Communication Manager 2012/2013).

Research Interests:; Innate lymphoid cells (ILC1, ILC2, ILC3); tissue specific innate and adaptive immune responses at mucosal and skin level.

Memberships

- ÖGAI: Österreichische Gesellschaft für Allergologie und Immunologie
- ÖGDV: Österreichischen Gesellschaft für Dermatologie und Venerologie

Publications:

1. **Saluzzo S**, Layer F, Stingl G, Stary G, „Staphylococcal Scalded Skin Syndrome Caused by a Rare Variant of Exfoliative-toxin-A + S. aureus in an Adult Immunocompromised Woman". Saluzzo S, Layer F, Stingl G, Stary G. *Acta Derm Venereol*. 2017 Aug 30

2. **Saluzzo S**, Gorki AD, Sharif O, Rana BMJ, Martins R, Scanlon ST, Starkl P, Lakovits K, Hladik A, Jolin H, Korosec A, Sharif O, Warszwaska JW, Jolin H, Mesteri I, McKenzie ANJ & Knapp S, "First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment", *Cell Reports* 2017.

3. Martins R, Maier J, Gorki AD, Huber KV, Sharif O, Starkl P, **Saluzzo S**, Quattrone F, Gawish R, Lakovits K, Aichinger MC, Radic-Sarikas B, Lardeau CH, Hladik A, Korosec A, Brown M, Vaahtomeri K, Duggan M, Kerjaschki D, Esterbauer H, Colinge J, Eisenbarth SC, Decker T, Bennett KL, Kubicek S, Sixt M, Superti-Furga G, Knapp S, Heme drives hemolysis-induced susceptibility to infection via disruption of phagocyte functions", *Nat Immunol.* 2016.

4. Jais A, Einwallner E, Sharif O, Gossens K, Tsai-Hsiu Lu T, Soyal SM, Medgyesi D, Neureiter D, Paier-Pourani J, Dalgaard K, Duvigneau JS, Lindroos Christensen J, Zapf T, Amann S, **Saluzzo S**, Jantscher F, Stiedl P, Todoric J, Martins R, Oberkofler H, Müller S, Hauser-Kronberger C, Kenner L, Casanova E, Sutterlüty-Fall H, Bilban M, Miller K, Kozlov AV, Krempler F, Knapp S, Lumeng CN, Patsch W, Wagner O, Pospisilik JP, Esterbauer H, "Heme Oxygenase-1 Drives Metaflammation and Insulin Resistance in Mouse and Man", *Cell* 2014.

5. Sharif O, Matt U, **Saluzzo S**, Lakovits K, Haslinger I, Furtner T, Doninger B, Knapp S. „The scavenger receptor CD36 downmodulates the early inflammatory response while enhancing bacterial phagocytosis during pneumococcal pneumonia". *J Immunol*. 2013

6. **Saluzzo S,** "Pharmacogenomics of Ribavirin in Patients Treated for HCV Chronic Infection". San Luigi University Press 2010. Pubblitesi 2011.

Posters and oral presentations (short list)

Jan. 2011	"Pharmacology of Ribavirin in Chronic HCV infection" 6th Residential Course on Clinical Pharmacology of ARVs. Turin, Italy (**poster presentation**)
June 2012	"The role of IL-13 in Pneumococcal Pneumonia" 8th YSA PhD Symposium, Vienna (**oral presentation**)
Nov. 2012	"Homeostatic type 2 pathways in lung infection" OEGAI 2012, Vienna (**poster presentation**)
Dec. 2012	"ILC2-derived IL-13 plays a detrimental role in pneumococcal pneumonia" Viennese Symposium on Immunology, Infection biology and Inflammation (VI3) (**poster presentation**)
Sept. 2015	"Innate lymphoid cells type-2 contribute to macrophage polarization and reduce defenses against pneumococci", ECI 2017 – Vienna (**poster presentation**)
Nov. 2016	"Homeostatically driven type 2 pathways shape the lung immune environment" OEGAI – Annual Meeting 2016 – Innsbruck (**oral presentation**)
Dec. 2016	"Homeostatically driven type 2 pathways prime the lung immune environment" EMBO Conference on Innate Lymphoid Cells 2016 – Berlin (**poster presentation**)
Nov. 2016	"Staphylococcal Scalded Skin Syndrome Caused by a Rare Variant of Exfoliative-toxin-A + S. aureus in an Adult Immunocompromised Woman" - OEGDV Annual Meeting 2017 – Vienna (**oral presentation**)
Jan. 2017	"First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment" OEGDV –, Science Days 2017 – Salzburg (**oral presentation**)
Nov. 2017	OEGAI – Austrian Society of Immunology and Allergology. Symposium Vienna (**chair**)
Jan. 2018	"Understanding HIV Latency in the skin" OEGDV – Science Days 2018 – Kaprun (**poster presentation**)