Type I Interferon Promotes Alveolar Epithelial Type II Cell Survival during Pulmonary Inflammation

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Submitted by

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Declaration

Mag. Barbara Maier performed her Ph.D. studies under the supervision of Prof. Dr. Sylvia Knapp, PhD at the University of Vienna, Department of Internal Medicine I, Laboratory of Infection Biology and the Research Center of Molecular Medicine (C-e-M-M-) of the Austrian Academy of Sciences.

Barbara Maier designed and performed all crucial experiments, analyzed data and wrote the manuscript. Sylvie Knapp provided funding, conceived the project, supervised the work and wrote the manuscript.

Riem Gawish PhD, Stefanie Sigel PhD, Rui Martins MSc and Barbara Drobits PhD assisted with experiments and data analysis. Karin Lakovits, Anastasiya Hladik, Ana Korosec and Julia Kral provided technical assistance.

Prof. Dr. Miriam Merad from the Icahn School of Medicine at Mt. Sinai, New York, provided experimental advice as well as materials and mice during the 6 months period of Barbara Maier's stay in Miriam's laboratory.

Ifnar1$^{-/-}$ mice that were generated by Prof. Dr. Michel Aguet were provided in collaboration with Prof. Dr. Mathias Müller and Birgit Strobl PhD. Ifnar$^{fl/fl}$ mice generated by Prof. Dr. Ulrich Kalinke were provided by Dr. Dagmar Stoiber. Sftpc-CreER mice were generated and provided by Prof. Dr. Brigid Hogan.

Ildiko Mesteri, MD, from the Pathology Überlingen, Germany, performed histological analyses.

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Abstract (English)

Pneumonia is a major global health risk, accounting for almost 20% of deaths among children below the age of 5 years. A frequent causative agent of pneumonia is the Gram positive bacterium *Streptococcus pneumoniae* that colonizes the upper respiratory tract, but can lead to pneumonia upon spreading to the lower airways. Upon infection of the lung severe complications such as systemic bacterial dissemination and subsequent sepsis can occur.

During pneumonia several inflammatory mediators are known to be of crucial importance to fight invading pathogens, but it is also more and more appreciated that some previously unnoticed cytokines have regulatory and tissue protective functions. We studied the contribution of type I interferons (IFN-I), a group of cytokines strongly associated with protection against viral infections, to pneumococcal pneumonia. It has been shown in several models that IFN-I exerts various immune-modulatory roles depending on the context of inflammation. Therefore we hypothesized that IFN-I could be a regulator of lung inflammation, which we studied using a mouse model of *S. pneumoniae* infection.

We found IFN-I to be protective during the course of pneumococcal pneumonia, illustrated by an increased bacterial load, more pronounced inflammation and tissue damage in lungs of mice incapable of processing IFN-I signals compared to wildtype mice. The trigger for IFN-I production in this model is bacterial DNA, which initiates IFN-I induction in alveolar macrophages, shown by transcriptional profiling of cells from infected mice. Further we found that IFN-I acts on alveolar epithelial type II cells in this model, but not on major pulmonary innate immune populations. IFN-I signaling on alveolar epithelial type II cells renders this population more resistant to inflammation-induced cell death, which counteracts the loss of epithelial cells and thereby strengthens the epithelial barrier. We furthermore found this protective mechanism to also apply in a model of sterile acute lung injury.

Concluding, we discovered that IFN-I is protecting alveolar epithelial cells in settings of bacterial and sterile lung inflammation, which stabilizes lung integrity and reduces tissue damage. This finding could be exploited in the clinics to support lung tissue regeneration of patients suffering from respiratory failure.

Abstract (Deutsch)

Lungententzündungen gelten weltweit als Gesundheitsbedrohung und sind verantwortlich für 20% der Todesfälle von Kindern im Alter bis zu 5 Jahren. Ein häufiger Erreger ist das Gram-positive Bakterium *Streptokkokus pneumoniae*, welches die oberen Atemwege oft asymptomatisch kolonisiert, jedoch durch die Ausbreitung in die unteren Atemwege Lungenentzündungen hervorruft. Im Zuge einer Pneumokokken Pneumonie können schwerwiegende Komplikationen auftreten, wie die systemische Verbreitung von Bakterien, bis hin zur Sepsis.

Eine Infektion der unteren Atmenwege führt zur Freisetzung von einer Reihe an pro- und anti-inflammatorischen Botenstoffen, Zytokine und Chemokine, die essentiell für die Abwehr von Pathogenen sind, bei gleichzeitiger Vermeidung von entzündungsinduzierten Schäden am Lungengewebe. In dieser Studie wurde die Rolle von Typ I Interferonen (IFN-I), eine Gruppe von Zytokinen, die bis jetzt vorwiegend in viralen Infektionen erforscht wurde, im Zusammenhang mit bakterieller Pneumonie untersucht. In mehreren Krankheitsmodellen wurde bereits gezeigt, dass IFN-I, abhängig vom Kontext der Entzündungsreaktion, verschiede Funktionen in der Modulation der Immunantwort übernehmen können. Basierend darauf wurde auch die Hypothese für diese Arbeit formuliert, nämlich dass IFN-I auch im Zuge einer bakteriellen Pneumonie die Entzündung in der Lunge regulieren können.

Mithilfe eines Mausmodells für *S. pneumoniae* Infektionen haben wir gezeigt, dass IFN-I eine protektive Rolle während der Pneumokokken Pneumonie spielen. Mäuse, die nicht in der Lage sind, Typ I Interferon Signale zu prozessieren, zeigen eine signifikant verstärkte Entzündungsreaktion, was z.B. durch erhöhte Freisetzung von inflammatorische Zytokinen und mehr Schäden am Lungengewebe im Vergleich zu unbeeinträchtigten Mäusen zu erkennen ist. Der Kontakt mit bakterieller DNA induziert die Produktion von IFN-I durch Alveolarmakrophagen, wobei IFN-I dann hauptsächlich auf Alveolarepithelzellen Typ II wirkt, aber nicht auf Immunzellpopulationen der Lunge. IFN-I erhöht die Widerstandskraft von Alveolarepithelzellen gegen entzündungsinduzierten Zelltod. Dadurch wird dem Verlust von Epithelzellen entgegengewirkt und die vom Epithel geformte Barriere wird verstärkt, was das Lungengewebe schützt. Außerdem konnten wir zeigen, dass dieser protektive Mechanismus auch in einem Modell des akuten Lungenversagens eine Rolle spielt.

Zusammengefasst zeigt diese Arbeit, dass Epithelzellen während bakteriellen oder sterilen Entzündungsreaktionen der Lunge durch IFN-I geschützt werden. Dadurch wird die Integrität des Lungengewebes erhalten und Gewebeschäden reduziert. Diese Erkenntnisse könnten in der Klinik genutzt werden, um die Geweberegeneration der Lunge in Patienten, die an Lungenversagen leiden, zu unterstützen.

Publications arising from this thesis

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Abbreviations

AEC	alveolar epithelial cells
AM	alveolar macrophages
AP-1	activator protein 1
BgaA	beta-galactosidase A
CbpA	choline-binding protein A
CCL20	chemokine (C-C motif) ligand 20
CD64	cluster of differentiation 64
ChoP	phosphorylcholine
Csf-1	colony stimulating factor 1
DC	dendritic cell
Fc-receptor	crystallizable fragment receptor
Fgf10	fibroblast growth factor 10
GAS	IFN-activated sites
Id2	inhibitor of DNA binding 2
IFN	interferon
IFNAR	interferonα/β receptor
IFNGR	interferonγ receptor
IgG	immunoglobulin G
IKK-I	inhibitor of nuclear factor κb kinase i
IL-22	interleukin 22
ILC	innate lymphoid cell
IM	interstitial macrophages
IRAK4	interleukin-1 receptor-associated kinase 4
Irf	interferon regulatory factor
ISG	interferon stimulated genes
ISGF3	IFN-stimulated gene factor 3

ISRE	IFN-stimulated response elements
Jak1	Janus kinase 1
LTA	lipoteichoic acid
MDA5	melanoma differentiation associated protein-5
MHC	major histocompatibility complex
Muc1	mucin 1
Mx	myxoma resistance
MyD88	myeloid differentiation primary response gene 88
NanA	neuramidase A
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK-cell	natural killer cell
NLRP3	NOD-like receptor family, pyrin domain containing 3
PI3K	phosphoinositide-3-kinase
PKR	RNA-activated protein kinase
PPAR-γ	peroxisome proliferator-activated receptor y
RIG-I	retinoic acid-inducible gene I
rPAF	receptor for platelet-activating factor
SIRPα	signal-regulatory protein alpha
Sox9	sex determining region Y box 9
Stat	signal transducer and activator of transcription 1
TBK1	tank-binding kinase 1
TGF-ß	transforming growth factor beta
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
Tyk2	tyrosine kinase 2
WHO	world health organisation
ZO	zona occludens

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1. Introduction

1.1. Lung immunity

The lung is an organ that constitutes a direct interface to the environment. As such several special requirements have to be met to ensure that on the one hand particles can be cleared from the alveolar airspace and on the other hand the immune system does not overreact to foreign material and thereby impair respiratory lung function. A layer of mucus is lining the lung epithelium which traps bacteria and other particles that enter the broncho-alveolar compartment [1,2]. Cilia that protrude from specialized lung epithelium are generating coordinated waves of movement to eliminate mucus and the foreign material it contains [3]. Nevertheless, the lung is not a sterile environment and hosts a range of commensal bacteria, termed the lung microbiota. One day to two weeks after birth the neonatal lung microbiome is established, which still changes in the course of neonatal development [4]. The adult lung is populated by a distinct set of bacterial species, among which *Firmicutes* and *Bacteroides* comprise about 80% of all commensals [5]. To keep potential pathogens, such as fungi, viruses and bacteria, in check, the lung is equipped with distinct populations of immune cells. If necessary, those cells can trigger quick and strong immune responses to fight invading pathogens. Pathogenic compounds trigger an immediate response by directly binding to innate immune receptors, such as Toll-like receptors, expressed on innate immune cells [6,7]. In the lung the most abundant innate immune cell type is alveolar macrophages, a specialized subset of macrophages that resides in the alveolar airspace. Their transcriptional and marker profile differs from other macrophage subsets and functionally alveolar macrophages are kept in an anti-inflammatory state by the epithelium [8-11]. This is crucial since the alveolar airspace is constantly exposed to the environment and therefore excessive activation of macrophages needs to be prevented. Upon pathogen recognition an immune response is mounted, which is characterized by secretion of inflammatory cytokines, recruitment of immune cell populations and disruption of the epithelial barrier. In the course of infection a more specific, adaptive immune response can be triggered and depending on the nature of the immune response B-cells and T-cells are activated.

1.2. Pulmonary innate immune cell populations

Innate immune cell populations are crucial for host defense due to their ability to respond immediately upon pathogen encounter. Distinct populations of macrophages, dendritic cells and neutrophils residing in different compartments of the lung are communicating with each other to orchestrate appropriate responses to various microbes and microbial products [12]. As illustrated in Figure 1 alveolar macrophages are residing inside the alveolar airspace. They are the first population to encounter foreign antigen. Upon recognition of pathogens, alveolar macrophages switch from their quiescent state into a highly active population phagocytosing pathogens and cell debris and secreting chemokines and cytokines to attract further immune cells and activate the epithelium and endothelium [11]. DC residing in the epithelial layer express the surface marker CD103 and are specialized in cross-presenting antigens derived from apoptotic epithelial cells via the MHC class I complex [13]. In the interstitial space, in between the epithelial and endothelial layers, cells reside, that contribute to the homeostatic balance of the alveolar microenvironment. Innate lymphoid cell populations constantly release low levels of regulatory cytokines that influence the reactivity of macrophages (IL-13) or the strength of the epithelial barrier, as in the case of IL-22 [14]. Interstitial macrophages are a population that is not very well characterized. One of their functions is the release of IL-10 to modulate DC function [15]. Interstitial DC expressing the surface marker CD11b engulf particles that have not been removed by alveolar macrophages, for example in case of bacteria that extravasate into the bloodstream. They migrate to lymph nodes and initiate adaptive immune responses mainly via the MHC class II complex [16]. Neutrophils and monocytes are circulating through the microvasculature of the lung in healthy conditions and can enter the interstitial space or the alveoli upon activation of the endothelium and sensing of chemokines.

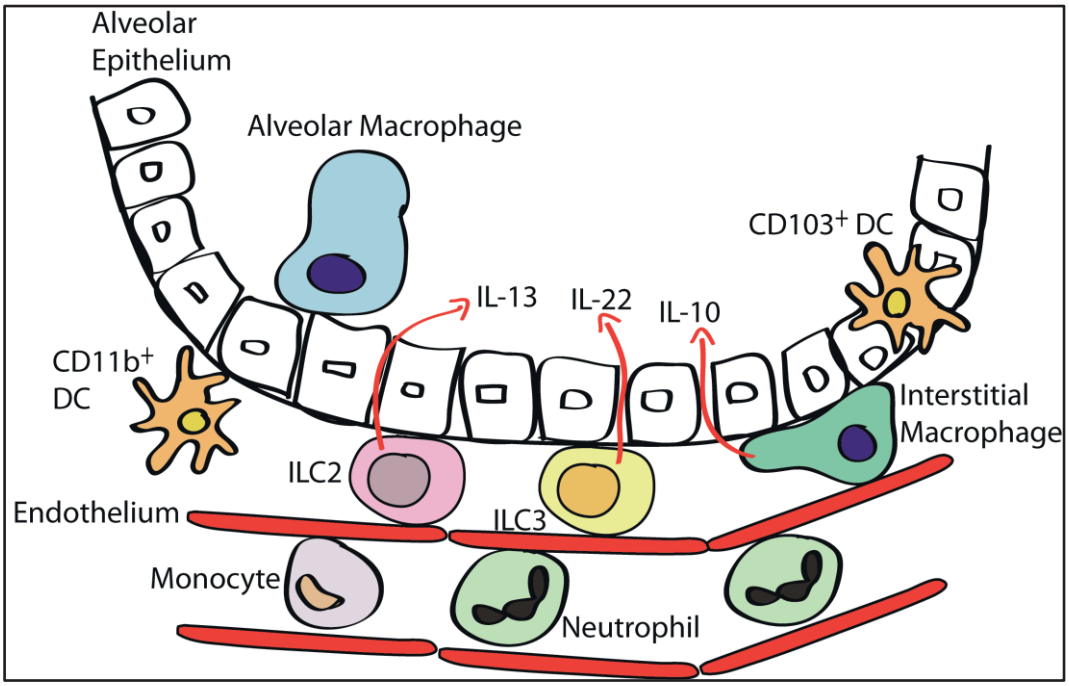

Figure 1. Innate immune populations in the healthy lung.

1.2.1. Macrophages

Macrophages are key players in innate immunity due to their superior ability to orchestrate immune responses. The main characteristics shared by most macrophages are (i) a very high phagocytic potential to clear microbes or damaged cells, (ii) the ability to present antigen to T-cells via the MHC class II complex, although with lower efficiency than dendritic cells, (iii) sensing of microbial products and (iv) subsequent secretion of large amount of cytokines thereby initiating inflammatory responses.

Due to their niche-specificity macrophages are an extremely heterogeneous population that perfectly adapt to their microenvironment to execute tissue-specific functions [17-19]. In homeostatic conditions macrophages have two main roles; firstly they are crucial for tissue maintenance by clearing cellular debris and secondly they patrol and survey their respective tissue for foreign particles. Different tissues throughout the body have very distinct requirements for their macrophage populations, largely depending on the waste products that specific tissues need to dispose, and on the probability of encounter of foreign material [20]. In the bone marrow, macrophages are crucial in engulfing erythroid nuclei, whereas spleen and liver macrophages mainly take up aged erythrocytes. In the bone, macrophages that are termed osteoclasts engulf bone material and thereby regulate the density of the

bone. Lung macrophages in steady state clear surfactant proteins to keep the alveolar airspaces clear. In fat tissue, macrophages regulate insulin sensitivity and lipolysis. Figure 2 illustrates the tissue specific functions of macrophages [20]. Factors that keep macrophages in their respective phenotype are most likely signals derived from their environment that are largely unknown. Macrophages are characterized by expression of pattern recognition receptors, which are innate receptors that bind to conserved microbial compounds in a rather unspecific manner. In different tissues, macrophages show unique expression patterns of subsets of those receptors, i.e. Toll-like receptors (TLRs), scavenger receptors, Nod-like receptors (NLRs), C-type lectins and RIG-I family proteins [21]. The diversity of different macrophage populations is also reflected on overall gene expression level. A “core signature” has been identified for all macrophage populations, which distinguishes them from other populations of the mononuclear phagocyte lineage. Further, distinct signatures have been found for macrophages from several organs [21].

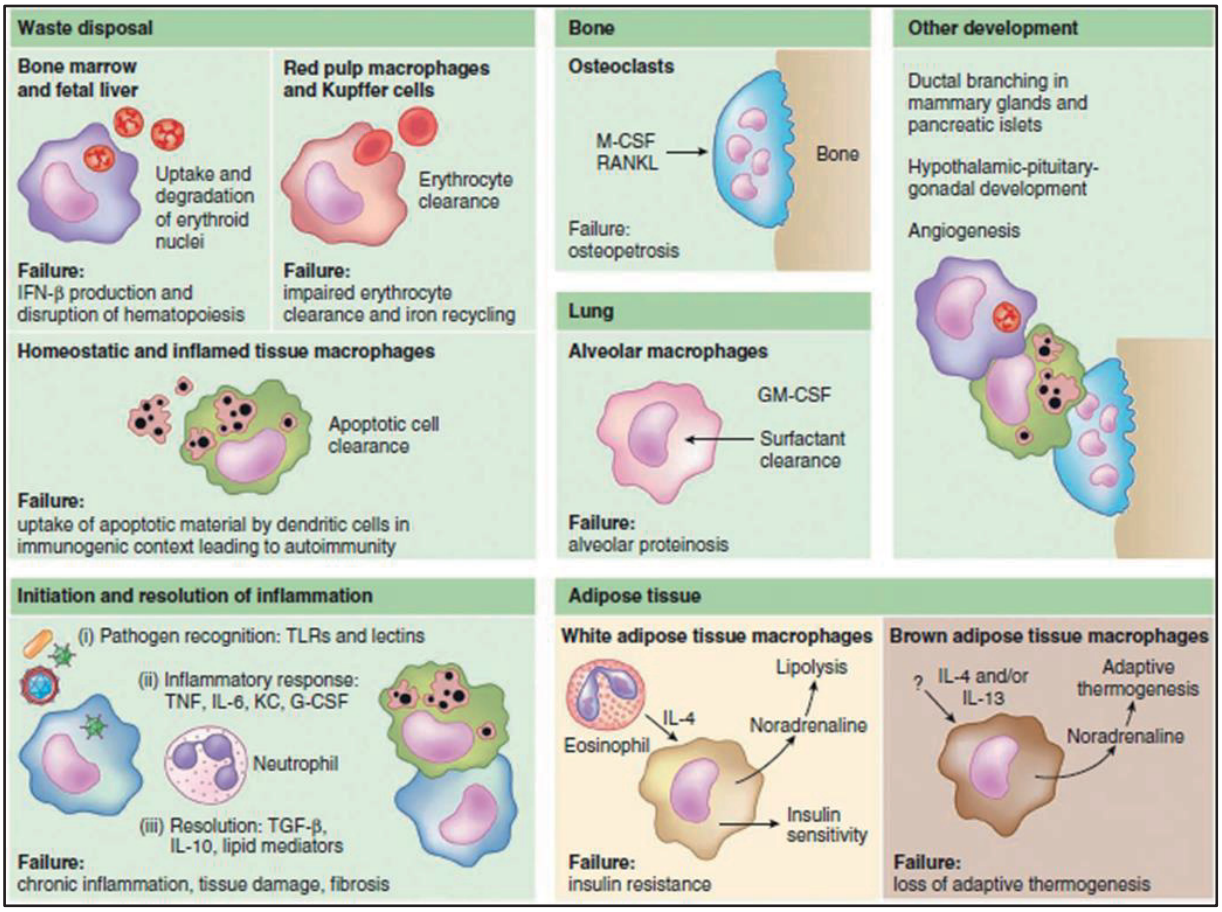

Figure 2. Tissue specific functions of different macrophage populations. Adapted from Davies *et al.*, *Nat. Immunol*. 2013

Tissue macrophages can be repopulated by bone-marrow derived blood monocytes upon major injury of the phagocyte compartment. However, in steady state conditions blood monocytes do not contribute to the renewal of tissue macrophages, but the tissue macrophage populations sufficiently maintain themselves via local proliferation [22]. One factor crucial for macrophage differentiation, proliferation and survival is Csf1. Csf1 deficient mice lack macrophages in most compartments, including bone, gut, kidney, lung and peritoneum. Their most obvious phenotype is increased bone density and lack of teeth. Notably, Langerhans cells in the skin and microglia are unaffected [23].

Macrophages can show an inflammatory phenotype (M1 classification) or an alternative anti-inflammatory phenotype (M2 classification). Importantly, those phenotypes are simplified concepts that do not represent rigid states. In reality rather a fluid passage between different macrophage phenotypes can be observed eventually with macrophages displaying features of both classifications depending on their microenvironment [24]. Generally, tissue-macrophages are classified as anti-inflammatory because of their tissue-remodeling function. This stage is maintained by mediators such as TGF-β, Csf1, glucocorticoids, IL-4 and IL-13 [25]. Upon change of microenvironments, macrophages can re-program and adapt their phenotype, which also shows that macrophage polarization is characterized by high plasticity [17]. This is of high importance in an infection setting, since macrophages often need to switch quickly between their “resting” steady-state phenotype to an inflammatory phenotype upon pathogen encounter.

1.2.2. Pulmonary macrophages

In the healthy adult lung three distinct macrophage populations have been described: alveolar macrophages (AM), interstitial macrophages (IM) and bronchial macrophages. Additionally intravascular macrophages can be found in humans, but not in rodents [26]. Alveolar macrophages constitute by far the most abundant cell type in the alveoli in steady state. Since the number of alveoli exceeds the number of macrophages about threefold, not every alveoli contains a macrophage. It has been suggested that macrophages can migrate between alveoli to be able to survey the whole surface of the lung [27]. Interstitial macrophages reside in the space between

epithelial and endothelial barrier. Their function is not very well characterized, but evidence exists suggesting they are important IL-10 producers [15] and potent stimulators of T-cell proliferation [28].

AM constantly receive deactivation signals, to prevent overshooting immune responses, such as TGF-β and C200R signaling and SIRPα binding to surfactant proteins [11]. In case several pattern recognition receptors are activated, AMs switch their phenotype and become more inflammatory. Upon infection the phagocytic capacity of AM is strongly enhanced and cytokine release is triggered.

AMs are derived from a fetal monocyte population that is generated in the fetal liver around embryonic day E12 and seeds the lung at E14. Around one week after birth they expand and change their phenotype and mature to alveolar macrophages, depending on the transcription factors PPAR-γ and Bach2 and expressing the surface markers F4/80, CD11c, Siglec F, CD64, but not CD11b and Ly6C (Figure 3). This pool of alveolar macrophages further self-maintains independently of bone marrow and circulating cells [11,22]. After lung injury, e.g. after influenza infection, proliferation of AM is enhanced to quickly restore a functional AM pool in the lung. AM are fully dependent on Csf1 and Csf2 as shown by mice deficient for one of the two cytokines, which are both devoid of AM. Csf2 is required to induce PPAR-γ in AM, which in turn controls cholesterol metabolism, fatty acid β-oxidation and lipid transport, storage and degradation [29]. Bach2 in contrast controls lipid and cholesterol metabolism in mature AM [30].

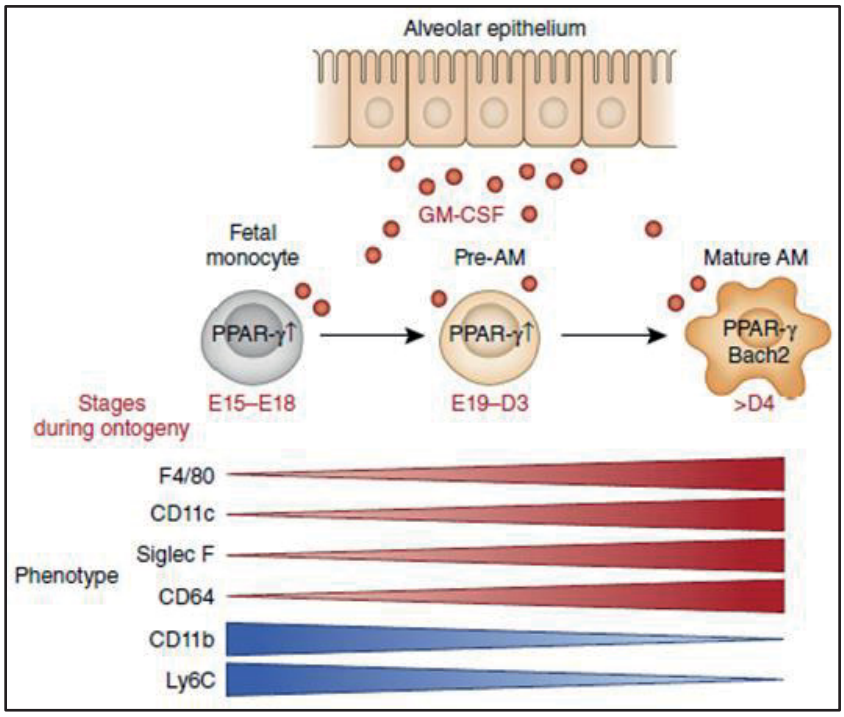

Figure 3. Development and maturation of alveolar macrophages. Adapted from Kopf *et al.*, *Nat. Immunol.* 2015.

1.3. Lung epithelium

The different structural parts of the lung impose diverse requirements to the epithelium. Therefore epithelial cell populations fulfill specialized functions in different compartments of the lung (Figure 4A). At embryonic day E9 a cluster of specific lung endoderm progenitors arises, which further differentiates into a Sox2 dependent proximal progenitor pool and a Sox9 and Id2 dependent distal progenitor pool at E12.5. Proximal progenitors give rise to epithelial cell types lining the trachea and bronchi, whereas distal progenitors differentiate into alveolar epithelial cell types [31]. To generate an organ that has a large surface area and is highly arborized and structured, a process termed branching morphogenesis drives budding of epithelial sub-structures of the lung. The initiation of branching events is mainly regulated by the local expression of Fgf10 and Fgfr2 in specific regions of the distal lung mesenchyme, which in turn is thought to be regulated by Bmp4 and Shh [32,33].

1.3.1. Epithelial cell types in the lung

Figure 4B illustrates the contribution of different epithelial cell types to the respective parts of the lung. The adult trachea is underlined by a layer of basal stem cells, which are proliferating and differentiating into other tracheal cell types. The top layer consists of ciliated cells, club cells and neuroendocrine cells. Bronchial epithelium consists of club cells, ciliated cells, neuroendocrine cells and goblet cells. Alveoli consist of alveolar epithelial cells type I (AEC I) that give structural stability to the alveolus and alveolar epithelial cells type II (AEC II) that secrete surfactant proteins [34-36]. Even though 60% of the alveolar cells are AEC II, they only account for 5% of the alveolar surface due to their size and shape [37]. AEC II also fulfill immunological functions, such as secretion of antimicrobial peptides and chemokines [38-40].

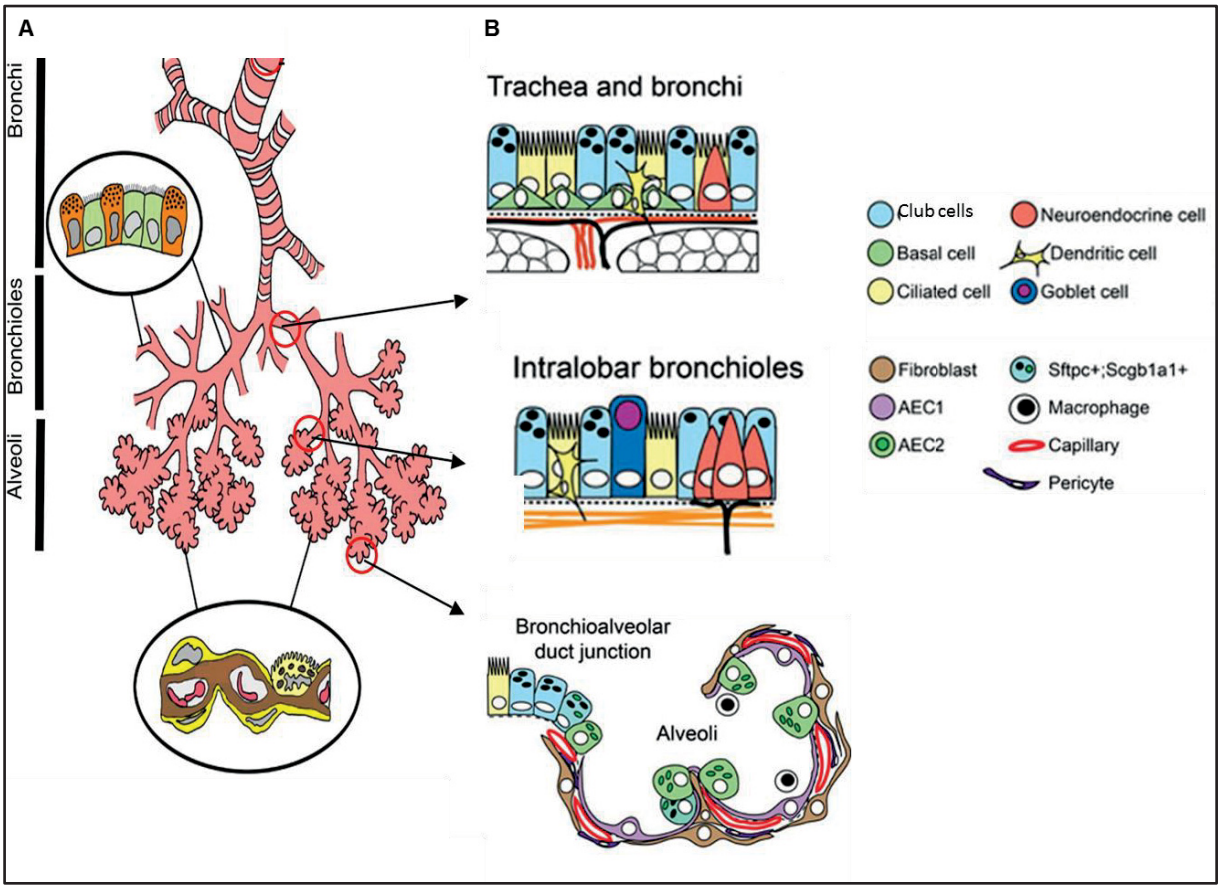

Figure 4. (A) Structural segments of the lung. (B) Pulmonary epithelial cell types. Adapted from the University of California, Cardiovascular Research Institute, *http://rocklab.ucsf.edu/research.html* and https://embryology.med.unsw.edu.au.

The lung epithelium has several mechanisms for renewal, since it is permanently subjected to the risk of damage. Although there is no constant turnover and differentiation of lung cell types, as in other organs such as the intestine, the lung can react to injury with a facultative regeneration response. The trachea can be constantly renewed by its layer of basal cells that have a high proliferative potential and can renew all tracheal cell types [41,42]. The differentiation process of basal cells is controlled by Notch signaling which promotes secretory fate and inhibits ciliary fate. In the bronchi neuroendocrine cells can self-renew upon damage [41]. Basal cells self-renew and have the potential to trans-differentiate into ciliated cells as well as bronchiolar club cells upon damage, which in turn can trans-differentiate into goblet cells. Alveolar cells are more independent from other epithelial cell types. AEC II self-renew and trans-differentiate into AEC I [41,43] (Figure 5). Upon severe damage, such as influenza infection or mechanical injury by chemicals, Notch dependent lineage negative progenitor cells migrate to the bronchioles and alveolus and give rise to club cells and AEC II [44,45].

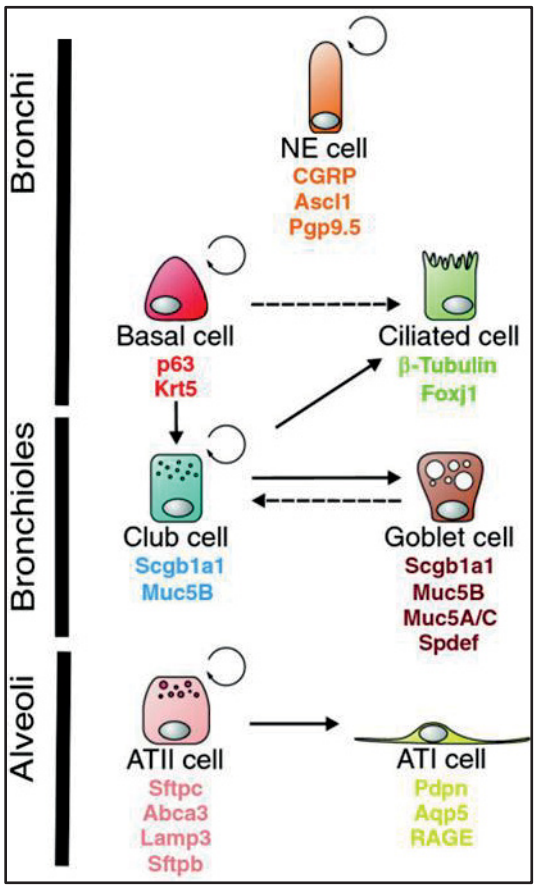

Figure 5. Regenerative potential of epithelial populations. Adapted from Volckaert *et al.*, *Fibrogenesis & Tissue Repair* 2014.

1.3.2. Epithelial barrier function

The main function of lung epithelium is to form a barrier over an area of 75 - 90m^2 in the adult lung to prevent fluid and proteins from entering the alveoli. Crucial for this barrier function are tight junctions between the epithelial cells that form a tightly controlled network of interconnected epithelial cells [46]. Tight junctions are large protein complexes that consist of transmembrane proteins, cytosolic proteins and cytoskeletal components (Figure 6). Key to the function of tight junctions are Claudin family proteins that associate with Claudins from adjacent cells and thereby form channels of varying permeability between cells [47]. Importantly, even though cell composition changes dramatically between structural parts of the lung, tight junctions form a gapless barrier throughout the whole lung surface. Therefore cells of differing phenotype have to express compatible Claudins. Other proteins crucial in epithelial barrier function are Occludin, which controls tight function formation, and intracellular *Zona Occludens* (ZO) proteins, which connect the protein complex to the cytoskeleton [48] (Figure 6). In the alveoli mainly junctions between AEC I are key to prevent barrier breakdown, although in this compartment also endothelial junctions

play an important role to tightly seal the microvessels [49]. In case of barrier failure the alveolar airspace is flooded with blood or serum and respiratory function is impaired. To maintain the fluid balance, ion transport across the epithelium is tightly regulated and controls the amount of fluid in the bronchial and alveolar airspace [50]. Regulation of ion channels can even make up for loss of Claudins, as it is the case for Claudin18, which is thought to be one of the main Claudins expressed in the lung epithelium [51].

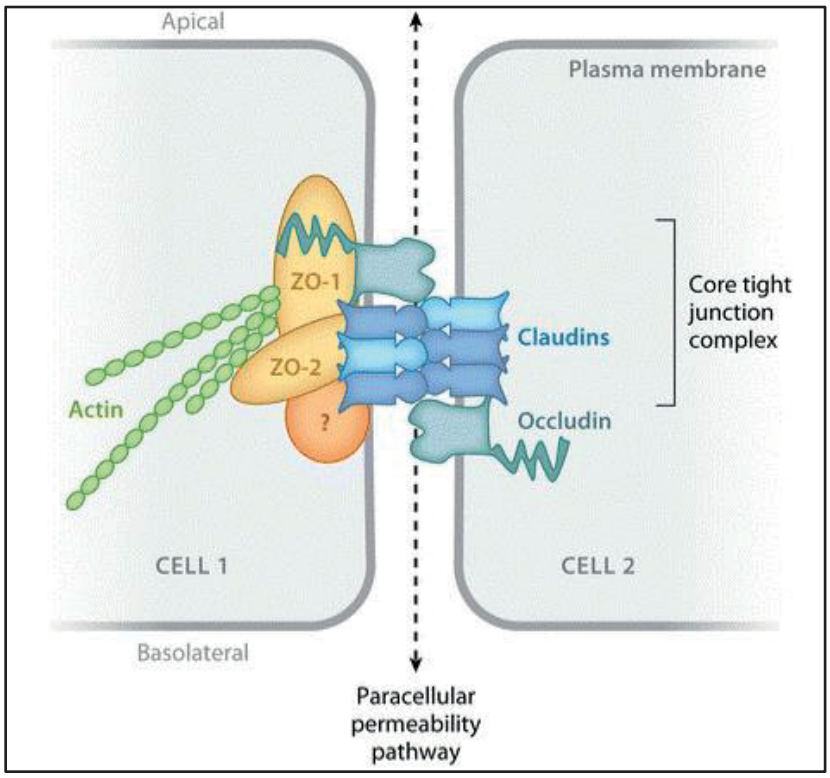

Figure 6. The core tight junction protein complex. Adapted from Koval, *Annu. Rev. Physiol.* 2013

1.3.3. Immunological functions of the pulmonary epithelium

The epithelium of the lung not only provides a structural barrier, but also fulfills immunological functions. A mechanical barrier, specific sets of receptors to react to pathogens and secreted anti-microbial compounds altogether comprise the epithelial innate immune system. Epithelial cells provide an important structural and immunological barrier in the form of the mucus layer which covers their apical surface [52]. Mucus is mainly formed by mucin proteins, which are differentially expressed by distinct epithelial cell populations along the different parts of the lung. The composition of mucins as well as their glycosylation pattern is modulated by Toll-like receptor (TLR) signaling upon pathogen encounter, which is a mechanism to

modulate the mucosal niche to the detriment of the respective pathogen (Figure 7) [40,53]. Many bacterial adhesion molecules bind to oligosaccharides present on mucins. In some cases this is a defense strategy to inhibit direct contact between bacteria and the epithelial cell surface, in other cases this mechanism might represent a way of commensal colonization of the lung [53]. Interestingly, mucins might also play a more direct role in modulating immune responses. It has been demonstrated that Muc1 in the respiratory tract inhibits flagellin-induced TLR5 activation and further NF-κB activation upon *Pseudomonas aeruginosa* infection. The exact mechanism of this interaction is still unclear, but it involves the cytoplasmic domain of Muc1 [54]. Anti-microbial substances secreted by epithelial cells are also enriched in the mucus layer which is another way of fighting invading pathogens before they directly encounter host cells (Figure 7) [38]. Those compounds cover small cationic molecules, but also larger proteins, such as enzymes and protease inhibitors. In the lung mucosa, the most prevalent small cationic molecules are ß-defensins, LL37 and CCL20. They are upregulated upon TLR signaling and interact unspecifically with negatively charged membrane components of bacteria, fungi or viruses. Due to the cationic nature of this group of anti-microbial peptides they substitute Mg^{2+} and Ca^{2+} ions, thereby destabilizing membranes. Additionally they enhance the phagocytic capacity of macrophages and the migration of several immune cells and thereby support innate and adaptive immunity [38]. The two large abundant proteins in the mucosal layer of the lung are lysozyme and lactoferrin. Lysozyme targets glycocidic bonds in peptidoglycan layers of Gram positive bacteria. Lactoferrin is an iron chelator and destabilizes membranes of Gram negative pathogens [38]. In case epithelial cells come in direct contact with pathogens they are equipped with Toll-like receptors, whose activation leads to the secretion of chemokines and cytokines such as IL-6, Cxcl8, IL-1ß, Csf1 and Csf2 to recruit and activate phagocytes. Some adapters like MyD88 and CD14 are not constitutively expressed in epithelial cells which poses a potential mechanism for controlling Toll-like receptor activation in distinct cell populations [55].

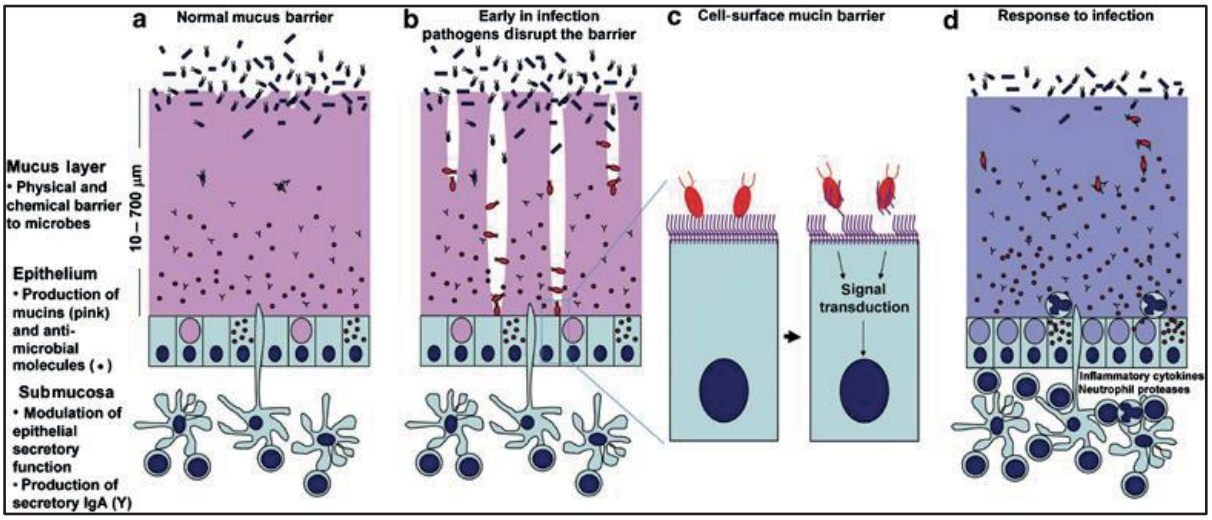

Figure 7. Schematic drawing of changes in the mucosal barrier upon infection. (A) normal barrier containing a physiological set of mucins and anti-microbial peptides. (B) Upon infection the mucosal layer is disrupted and pathogens come in contact with epithelial cells. (C) Epithelial cells sense pathogens and activate signaling pathways. (D) Differential set of mucins is expressed to adapt the mucosal layer in order to fight the invading pathogen. Adapted from Linden *et al.*, *Mucosal Immunology* 2009.

Alveolar epithelial cells (AEC) are known to be important regulators of immune cell function. AEC express integrin $\alpha_v\beta_6$ which is critical for the generation of bioactive TGF-ß. This dampens AM responses and inhibits overshooting inflammation [11]. A subset of AM forms gap junctions with AEC, enabling communication between those two cell types. In case of an inflammatory response, AM induce synchronized, periodic waves of calcium release in the epithelium. Mice that are lacking gap junctions between AM and AEC don't show synchronized calcium waves which leads to enhanced inflammation and neutrophil recruitment [56]. Another negative regulator for macrophage function is the interaction between CD200R expressed on macrophages with its ligand CD200L on epithelial cells. Both CD200R and CD200L are progressively upregulated during viral infections, highlighting its role in protection from lung injury [11]. In a model of influenza infection it has been shown that epithelial cell derived Csf2 is crucial in the recruitment of dendritic cells (DC) leading to activation of adaptive immunity and that the presence of DC in the lung parenchyma at baseline and upon influenza infection is absolutely dependent on epithelial cell derived Csf2 [57]. Altogether pulmonary epithelial cells are key players in controlling and orchestrating immune responses in the lung.

1.4. Pneumococcal pneumonia

Pneumonia is defined as an infection of the lower respiratory tract. Despite substantial efforts have been made pneumonia is still a major global health risk. According to a WHO report in 2006, pneumonia is the major cause of death for children under the age of five years with two million deaths per year and almost 20% of the overall mortality attributed to pneumonia [58]. Lower respiratory tract infections are typically caused by bacteria or viruses and are classified as either community-acquired (meaning without prior hospitalization) or hospital-acquired. 15% of community-acquired pneumonia cases have a viral cause and among those influenza virus is the most frequent pathogen. The other 85% are of bacterial cause, *Streptococcus pneumoniae* being the most prevalent causative species [59,60]. In children the distribution of pathogens is skewed towards viral infections, with 50% of community-acquired pneumonia cases caused by viruses [61]. The most common viral species among children are influenza virus, respiratory syncytical virus, parainfluenza virus and rhinovirus whereas the main bacterial species causing pneumonia in children are *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* [61,62]. Secondary pneumococcal or staphylococcal infection is a frequent complication following influenza infection, often associated with fatal outcome [63]. Further risk factors for pneumococcal pneumonia are COPD, asthma, smoking, diabetes mellitus, chronic heart disease, alcoholism, asplenia, HIV infection and defects in humoral immunity [64,65].

1.4.1. *Streptococcus pneumoniae*

Streptococcus pneumoniae is a Gram positive, aerotolerant, anaerobic, extracellular bacterium that colonizes the upper respiratory tract. If *S. pneumoniae* accesses the lower respiratory tract, it causes pneumonia which can further escalate into bacteremia, meningitis and life-threatening sepsis (Figure 8) [66]. Otitis media, which is an acute infection of the middle ear, is also frequently caused by *S. pneumoniae*. The colonization frequency of the upper airways is more than 60% in infants and decreasing with age to around 10% in adults [65]. So far, 91 serotypes of *S. pneumoniae* have been characterized that differ in the structure of their capsule and expression of virulence factors [67].

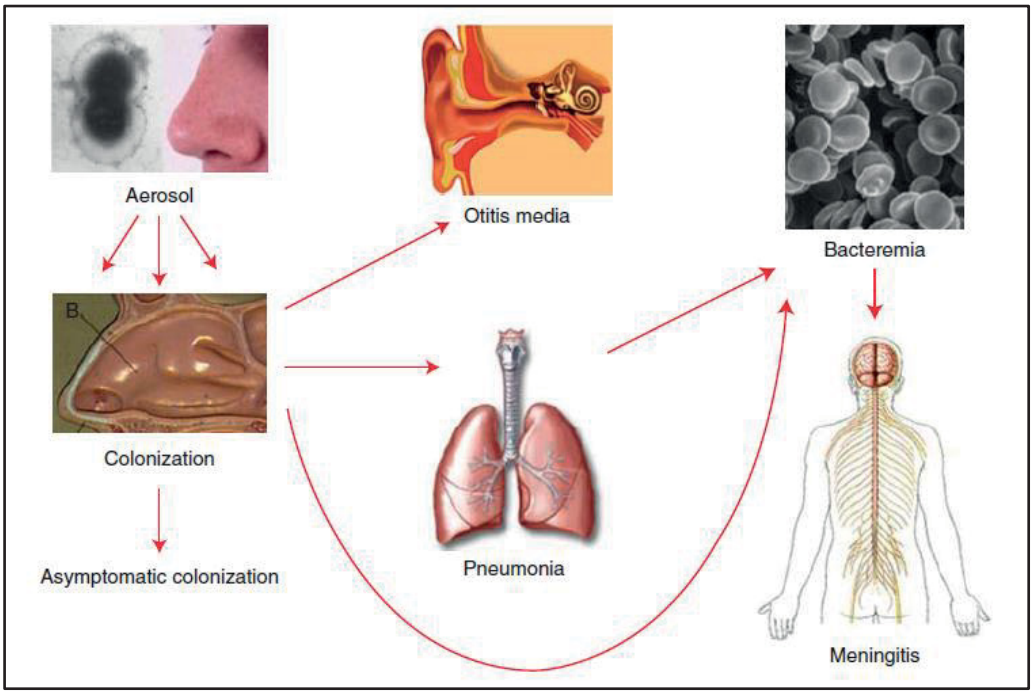

Figure 8. Progression of pneumococcal disease. Adapted from Henriques-Normark *et al.*, *Cold Spring Harb Perspect Med* 2013.

To successfully invade the lower respiratory tract, *S. pneumoniae* has an array of virulence factors that need to be expressed in a coordinated fashion, depending on tissue niche and population density. Upon establishment of infection, a distinct subset of virulence factors is differentially expressed in the lung as compared with bacteria isolated from the blood or secondary infected organs [68]. The major virulence factor determining successful infection in humans is the pneumococcal capsule, which inhibits mucosal defense mechanisms of the host, IgG deposition, binding of complement factors and accessibility of the bacteria to several antibiotics [65]. Generally, two forms of the capsule can be distinguished: (i) a thick capsule that masks pneumococcal structures and thereby inhibits recognition by the immune system and (ii) a thin capsule that allows adherence to host tissues. Many strains can switch between those two capsular stages to exploit the advantages of both. The bacterial cell membrane that is covalently linked to the capsule in most strains consists of three major groups of proteins which are choline-binding proteins, lipoproteins and proteins bound to the bacterial capsule [67]. PspA, one of the 10 – 15 choline binding protiens, inhibits C3 deposition on the bacterial cell wall, which is an important mechanism of pneumococcal immune evasion. In addition Factor H is bound by PspC, further inhibiting complement activation. Up to 45 lipoproteins have been described on the pneumococcal surface, including metal-binding proteins and

iron-acquisition proteins, two mechanisms crucial for bacterial virulence [69]. PsaA for example has specificity for manganese, which is needed to resist oxidative stress and reactive oxygen species generated by the host. Three proteins are able to bind iron, PiaA, PiuA and PiT [67].

Several components of the pneumococcal capsule mediate adherence to different host structures. Phosphorylcholine (ChoP), component of the integral membrane glycoprotein lipoteichoic acid (LTA), binds to the receptor for platelet-activating factor (rPAF) and thereby mediates bacterial transmigration through the epithelial barrier [70]. Another bacterial cell wall component CbpA binds to the human immunoglobulin receptor and secretory immunoglobulin [71]. Other pilus-like structures bind to yet unidentified host compounds. To unmask glycoconjugates for their adherence at the epithelial surface, pneumococci express the exoglycolases NanA, BgaA and StrH. Pneumococcal PavA and enolase bind to host extracellular matrix protiens fibronectin and plasminogen, respectively [72,73]. All of those factors promote pneumococcal dissemination and systemic spread of disease.

Invasive strains of *S. pneumoniae* express protein virulence factors next to their capsular virulence factors. The most prominent one is pneumolysin, a pore forming toxin that damages host cell membranes, inhibits ciliary movement of epithelial cells and impairs respiratory burst of phagocytes [74]. Pneumolysin is a member of the family of bacterial cytolysins. It is secreted and forms multimers which integrate into host membranes and act as pores. Approximately 40 monomers are sufficient to form one pore. Its other effects are also evident at a sub-lytic dose. Studies have shown that certain pneumolysin mutants activate TLR4 or stimulate IFNγ secretion [75,76]. Deficiency of pneumolysin significantly reduces the ability of most *S. pneumoniae* strains to induce fatal disease [67]. Pneumolysin is released from the bacterial cytoplasm after autolysis, a process that occurs after expression of LytA which is induced by a high density of bacteria and cleaves the peptidoglycans of *S. pneumoniae*. Table 1 summarizes virulence factors of *S. pneumoniae* and their range of action.

Pneumococcal virulence factors and disease	Main role in colonization
Upper-airway colonization	
Capsule	Prevents entrapment in the nasal mucus, thereby allowing access to epithelial surfaces. Also inhibits effective opsonophagocytosis.
ChoP	Binds to rPAF on the epithelial surface of the human nasopharynx.
CbpA (also known as PspC)	Binds to human secretory component on a polymeric Ig receptor during the first stage of translocation across the epithelium.
NanA, BgaA and StrH	Act sequentially to cleave terminal sugars from human glycoconjugates, which might reveal receptors for adherence.
Hyl	Breaks down hyaluronan-containing extracellular matrix components.
PavA	Binds to fibronectin.
Eno	Binds to plasminogen.
Competition in upper airway	
Bacteriocin (pneumocin)	Small antimicrobial peptide that targets members of the same species.
Respiratory-tract infection and pneumonia	
Ply	Cytolytic toxin that also activates complement. An important determinant of virulence in *in vivo* models of disease. Wide range of effects on host immune components at sub-lytic concentrations.
PspA	Prevents binding of C3 onto pneumococcal surface. Also binds lactoferrin.
LytA	Digests the cell wall, which results in the release of Ply.
PsaA	Component of the ABC transport system, which is involved in resistance to oxidative stress.
PiaA and PiuA	Component of the ABC transport system.
NanA and NanB	Aid colonization by revealing receptors for adherence, modifying the surfaces of competing bacteria that are within the same niche and/or modifying the function of host clearance glycoproteins.
IgA	Cleaves human IgA1.

Table 1. Virulence factors expressed by *S. pneumoniae*. Modified from Kadioglu *et al.*, *Nat Rev Microbiol* 2008.

1.4.2. Immune response to *S. pneumoniae*

The pneumococcus is recognized by the immune system by an array of different receptors to guarantee an efficient immune response. Pattern recognition receptors, including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), are innate receptors that are constitutively expressed by a range of innate immune cells. They bind rather unspecifically to conserved microbial structures to immediately recognize a big range of invading pathogens. TLR2 is activated by pneumococcal lipoteichoic acid (LTA) and lipoproteins, both integral components of the Gram positive cell wall [77]. TLR9 binds to unmethylated CpG motifs present in *S. pneumoniae* DNA and TLR4 can be activated by pneumolysin or endogenous danger-associated ligands during the course of *S. pneumoniae* infection [75,78]. Mice deficient for the TLR adapter protein MyD88, which is necessary for TLR2, TLR4 and TLR9 signaling, show impaired immune responses and enhanced mortality upon pneumococcal

pneumonia [79]. Nod-like receptors NLRP3 and Nod2 are also involved in the immune recognition of *S. pneumoniae*. Nod2 binds intracellular peptidoglycan fragments and in turn activates NF-κB, whereas NLRP3, which is also intracellular, induces inflammasome activation in a pneumolysin dependent manner [80]. After activation of TLRs and NLRs an inflammatory response is triggered that is characterized by strong activation of NF-κB dependent cytokines such as IL-6, Cxcl1 and TNFα and inflammasome dependent production of IL-1. The first cells to encounter *S. pneumoniae* are alveolar macrophages (Figure 9) [81]. They phagocyte bacteria and initiate an inflammatory response, in case bacterial number is high. Epithelial cells sense *S. pneumoniae* after the bacteria have successfully invaded the mucosal layer. Upon chemokine and cytokine production neutrophils are attracted and migrate into the alveolus. They are the predominant cell type in lavage fluids of pneumococcal pneumonia patients. The main function of neutrophils is the phagocytosis of large numbers of bacteria [81,82]. At this stage alveolar macrophages that are loaded with bacteria undergo apoptosis [83]. The remaining macrophage population clears the alveolar airspace by taking up epithelial and neutrophilic cell debris. Inflammatory monocytes infiltrate into the alveolus and secrete inflammatory mediators. Other cell types such as innate lymphoid cells type 2 (ILC2) and interstitial macrophages release regulatory cytokines into the alveolar airspace. Cell populations contributing to the alveolar response against *S. pneumoniae* are summarized in Figure 9. This major inflammatory response in the lung is accompanied by impaired respiratory function and tissue injury [84]. In case bacteria cannot be cleared in this phase of infection they enter the bloodstream, where C-reactive protein recognizes the pneumococcal cell wall and induces complement activation [85]. If bacteria spread to distinct organs, systemic infection and sepsis that can end in fatal outcome is the consequence [65,81,86].

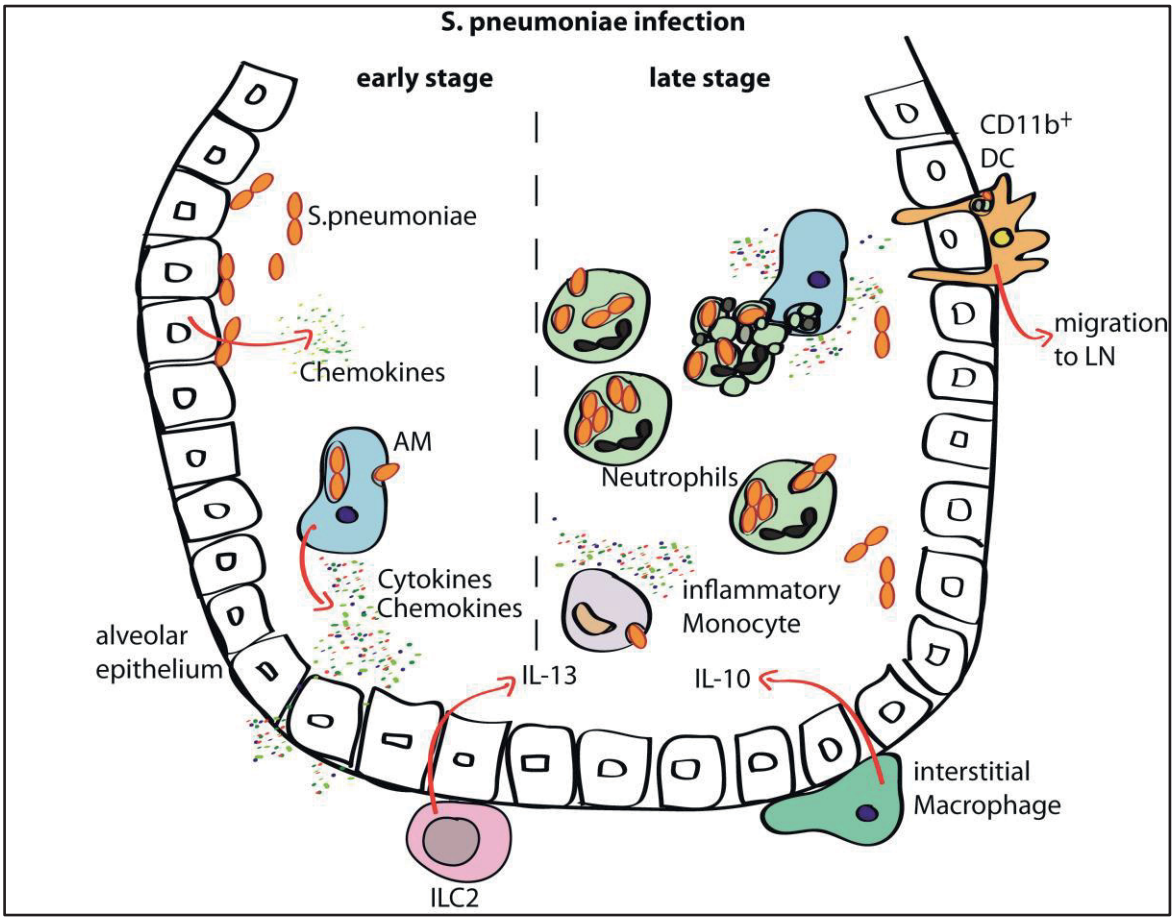

Figure 9. Immune populations contributing to an immune response against *S. pneumoniae* in the alveolus.

1.5. Type I interferon

Interferons have been discovered in 1957 when a system was described, that renders cells resistant to viral infection after treatment with supernatant from virus infected cells [87]. A classification into type I interferon and type II interferon has been made after the finding that two different receptors are used by the two groups of interferons respectively. All type I interferons, which are IFN-α, IFN-β, IFN-ω, IFN-κ and IFN-ε, signal via the interferon alpha/beta receptor (IFNAR), consisting of two subunits IFNAR1 and IFNAR2. Type II interferon, which is only IFN-γ, binds to the interferon gamma receptor (IFNGR), also consisting of two subunits IFNGR1 and IFNGR2 (Figure 10) [88]. All type I interferons share regions of structural homology, whereas IFN-y is structurally unrelated. Both type I and type II interferon receptors share the signal transducer Janus kinase 1 (JAK1), which is permanently associated with the IFNAR2 subunit and the IFNGR1 subunit, respectively. Functionally, type I and type II interferons activate distinct sets of target genes after signaling through

their respective receptors [89]. Type I interferons stimulate the upregulation of genes that render cells intrinsically resistant to viral infection, whereas type II interferons upregulate antimicrobial genes and genes involved in cellular metabolism and generation of radicals. Some target genes can be shared between the two interferon groups depending on the context and cross-talk between the two signaling pathways also exists, mainly mediated by abundance of the shared signal transducing factors [90].

A third group of interferons, called type I-like interferons or type III interferons, is the IFN-λ family. Signaling through their receptor, consisting of IL-28R and IL-10R2, leads to the formation of a complex of Stat1 (signal transducer and activator of transcription 1), Stat2 and Irf9 (interferon regulatory factor 9), which is also induced upon IFNAR signaling. However, in contrast to type I interferons, type III interferons are only expressed at epithelial surfaces [91].

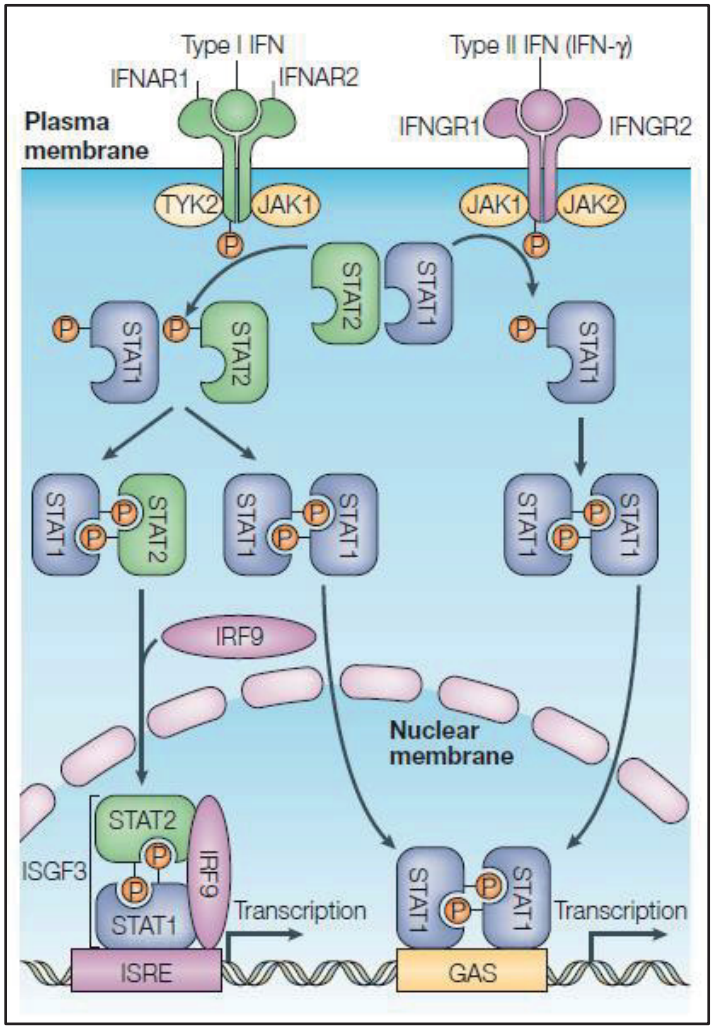

Figure 10. Signaling by interferon receptors. Adapted from Decker *et al.*, *Nat. Rev*. 2005.

Type I interferon (IFN-I) is secreted upon infection or inflammation in very high quantities. However, also in steady state IFN-I is constantly produced at very low levels to regulate organ homeostasis [92,93]. Notably, in contrast to inflammatory situations, IFN-I expression is dependent on activator protein 1 (AP-1) and NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) in resting conditions. Whereas upon infection IFN-I induction requires IRF-3 and IRF-9, deficiency in those two factors does not reduce tonic IFN-I expression. Loss of constitutive IFN-I signaling leads to reduced expression levels of IRF and STAT proteins and biologically to a deregulation of the hematopoietic stem cell niche and defects in several immune populations [92]. IRF-2 is a negative regulator of constitutive IFN-I secretion and loss of IRF-2 leads to increased IFN-I levels and exhaustion of the hematopoietic stem cell niche [94].
The homeostasis of several immune cell populations is dependent on constitutive low-level secretion of IFN-ß. IFNAR deficient mice, that are not able to process IFN-I signals, show decreased numbers of NK-cells and B-cells in the spleen and enhanced responsiveness of myeloid populations to Csf-1 [95,96]. Macrophage function is modulated by constitutive IFN-I signaling, indicated by the decreased phagocytic capacity of macrophages incapable of mounting IFN-I responses in response to LPS [97]. Also the bone resorption function of osteoclasts is impaired in the absence of IFNAR in mice [98]. Another important aspect of baseline IFN-I signaling is the modulation of expression levels of STAT proteins. In case the relative abundance between STAT proteins is shifted, hetero- or homodimer formation using specific forms of STAT is favored which in turn leads to a bias in signal transduction [99]. In steady state thymic epithelial cells show the highest detectable IFN-ß expression shown with a luciferase reporter mouse. Other IFN-ß expressing tissues are spleen, lymph nodes, liver, kidney and intestine [100].

Upon infection or inflammatory conditions vast amounts of IFN-I are secreted. Nearly all cells can induce IFN-I upon contact with intracellular nucleic acids or TLR stimulation. Via activation of IFNAR in an autocrine or paracrine manner the IFN-I signal amplifies itself and leads to the establishment of cell-intrinsic effects that allow the cell to clear intracellular infections, as well as systemic effects such as modulation of the inflammatory response [88,101]. Cell-intrinsic effects can be quickly established by an autocrine feedback loop of IFN-I signaling mainly affecting the local

environment, whereas systemic effects are only achieved if IFN-I is secreted in large quantities. This is executed by so-called professional interferon-producing cells, which is a subpopulation of dendritic cells, termed plasmacytoid dendritic cells (pDC) [88].

1.5.1. Type I interferon induction and signaling

Signal transduction upon IFN-I release occurs, when free IFN-I binds to the extracellular domain of the IFNAR complex, consisting of IFNAR1 and IFNAR2. The intracellular domains of IFNAR1 and IFNAR2 are permanently associated with the kinases tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) respectively, which are activated upon ligand binding. The receptor complex gets phosphorylated which enables binding of the signal transducer STAT that in turn also get phosphorylated (Figure 10) [102]. Phospho-STATs can either form heterodimers consisting of one STAT1 and one STAT2 molecule or homodimers consisting of two STAT1 molecules. The phospho-STAT1/phospho-STAT2 dimer further associates with IRF9 and thereby the IFN-stimulated gene factor 3 (ISGF3) complex is formed, that translocates into the nucleus and binds to distinct DNA elements called IFN-stimulated response elements (ISRE) [102,103]. Those elements are regulating the transcription of many interferon stimulated genes (ISG), highlighting the importance of ISGF3 in the induction of transcriptional interferon responses. Phospho-STAT1 homodimers directly associate with DNA elements called IFN-activated sites (GAS) that induce transcription of a distinct set of ISGs (Figure 10). Importantly, IFN-γ signaling also induces STAT1 homo-dimerization, but not the formation of STAT heterodimers, which is an important aspect regarding cross-talk between the two signaling cascades. Another level of regulation is the relative abundance of STAT proteins that can shift the ratio between homo- and hetero-dimer formation [88,101]. In addition, two STAT3 molecules can form a homodimer, which activates a set of genes which represses inflammatory pathways. In certain cell types also the less common forms STAT4, STAT5 and STAT6 can be involved in IFNAR signaling [90]. To modulate and control the IFN-I signature upon a certain stimuli, several mechanisms exist apart from STAT expression levels. STAT proteins are post-translationally modified, they interact with other transcription factors, repressors, enhancer elements etc. and epigenetic factors determine the accessibility of target DNA sequences [90].

Alternatively, a soluble form of IFNAR2 (sIFNAR2) exists, which is a splice variant of the same gene. sIFNAR2 enhances IFN-I signaling by capturing free IFN-I and associating with membrane bound IFNAR1 to trigger the downstream signaling cascade [104].
Some evidence suggests that phosphoinositide-3-kinase (PI3K) and p38 miogen-activated protein kinase (p38) signaling pathways are activated by IFNAR, however their contribution to IFN-I immune responses is less well studied [105].

IFN-I are induced upon pathogen encounter and activation of a distinct subset of pathogen recognition receptors. Depending on the nature of the pathogen, those receptors are extracellular, intracellular or endosomal. Membrane bound receptors that initiate IFN-I responses are TLR3 and TLR4. Endosomal TLR3 binds double-stranded viral RNA, whereas TLR4 that resides in the plasma membrane, binds extracellular bacterial patterns such as lipopolysaccharide and others [106]. Both TLR3 and TLR4 are associated with the cytosolic adaptor molecule TIR-domain containing adaptor protein inducing IFN-β (TRIF) and TLR4 additionally requires TRIF-related adaptor molecule (TRAM) for IFN-I induction (Figure 11) [88]. In pDCs endosomal TLR7 and TLR9 bind single stranded RNA and nonmethylated CpG DNA respectively, and initiate a pathway via the adapter proteins MyD88 (myeloid differentiation primary response gene 88) and IRAK4 (interleukin-1 receptor-associated kinase 4) to induce IRF7 [106]. In addition cytosolic sensors retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated protein-5 (MDA-5) recognize single-stranded viral RNA and lead to the activation of IRF3 (Figure 11).
The key factors for IFN-I induction are IRF3 and IRF7. IRF3 is phosphorylated and thereby activated by one of the two kinases tank-binding kinase 1 (TBK1) or inhibitor of nuclear factor κb kinase i (IKK-i) (Figure 11) [102]. Which kinase is transducing the signal depends largely on the source of the activating factor. IRF3 dimerizes upon phosphorylation and associates with NF-κB, AP-1 and high mobility group (HMG) proteins to form enhancer complexes that bind and activate the *Ifnβ* promotor and, in some situations, also the *Ifnα4* promoter. IRF7 is activated upon IFNAR signaling after the first wave of IFN-I production has occurred, amplifies the IFN-I signal and activates another subset of ISGs, mainly containing genes important in the later response against pathogens [88,90].

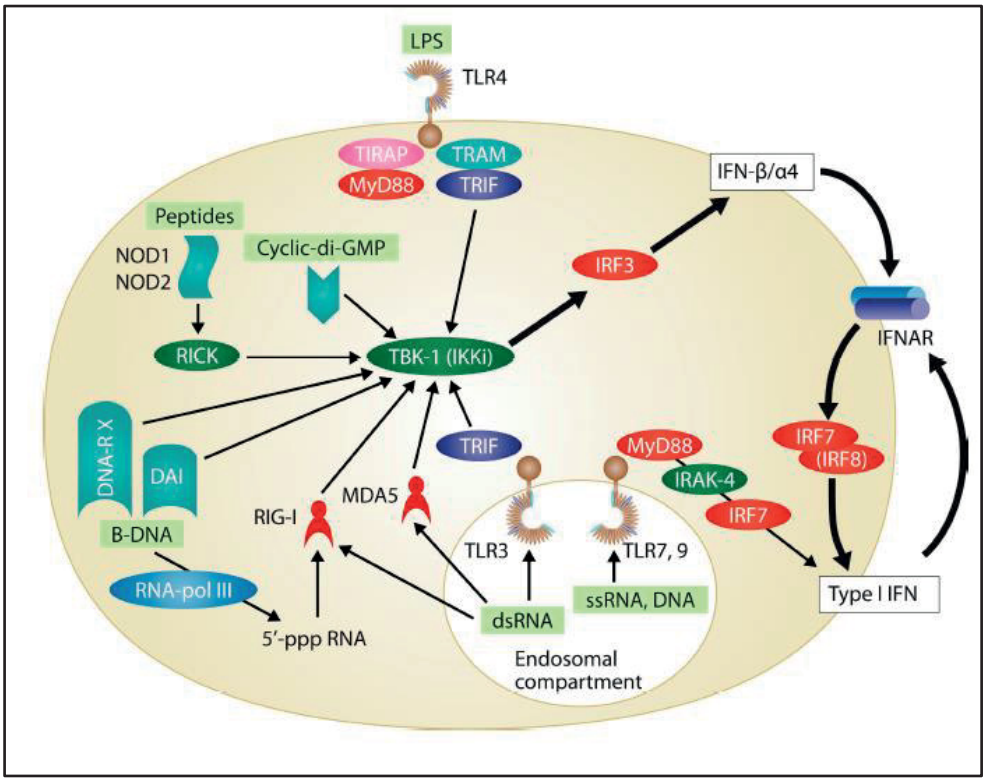

Figure 11. Mechanisms of type I interferon induction. Adapted from Trinchieri *2010* JEM.

1.5.2. Range of action of type I interferon: anti-viral properties

IFN-I have very diverse functions depending on the tissue environment, the nature of the stimulus and the cell types involved. Generally three main functions are attributed to IFN-I signaling, which are (i) generation of a cell-intrinsic, anti-viral state in virus infected cells, (ii) enhancing antigen presentation and adaptive immune responses and (iii) modulation of inflammatory responses. This diversity in possible reactions to one stimulus is enabled by the differential regulation of hundreds of interferon stimulated genes [90,91].

Viruses are a major threat for the host organism, therefore IFN-I have evolved to block the viral life-cycle at many stages, to be able to target a big variety of virus species in different host cell types [103]. Upon virus infection a cell detects viral nucleic acids and IFN-I secretion is induced. This activates IFNAR signaling not only on the infected cell itself, but also on bystander cells, which is an important aspect in limiting viral spreading. Figure 12 summarizes the so-called anti-viral state that is generated in cells to successfully fight viral infection and propagation of virus particles, of which a few mechanisms will be discussed here [107]. To render a cell

resistant to virus infection a complex machinery with diverse functions is upregulated to inhibit several steps in the viral life-cycle. A well-studied family of anti-viral proteins is the IFITM protein family (interferon-induced transmembrane proteins). Next to non-immunological functions in development they have been shown to restrict replication of Influenza A virus and other enveloped viruses by inhibiting early entry and membrane fusion steps [108]. Reverse transcription of viral RNA and nuclear import is inhibited my myxoma resistance proteins (Mx) that have a ring-like structure to trap uncoated viral particles [109]. Viral uncapped RNA that has been transcribed in the host cell nucleus is recognized by RNA-activated protein kinase (PKR) and translation is thereby blocked [110]. Other important anti-viral activities are blocking uncoating of virus particles, inhibiting viral RNA transcription in the host nucleus and blocking viral particle assembly and release.

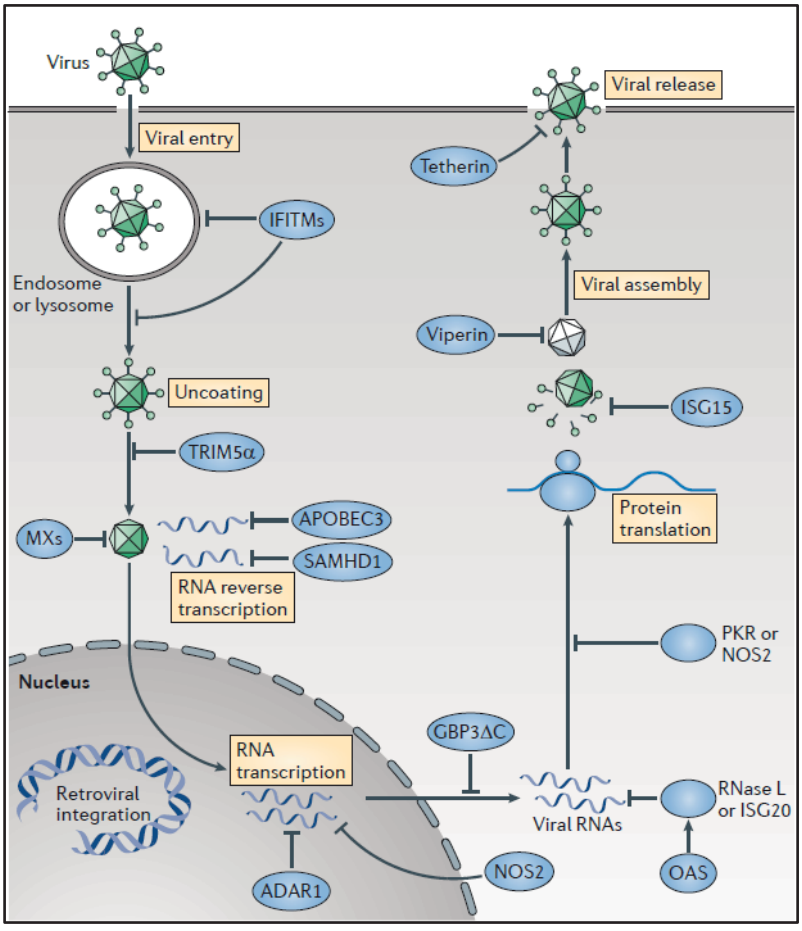

Figure 12. Cell-autonomous mechanisms induced by IFN-I against virus infections. Adapted from MacMicking *2012* Nat. Rev. Immunol.

1.5.3. Range of action of type I interferon: immune cell homeostasis

IFN-I has been demonstrated to modulate T-cell differentiation, maturation and survival. In several models it has been shown that IFN-I promotes T_{H1}-cell differentiation and inhibits T_{H2}-cell and T_{H17}-cell differentiation. IFN-I signaling together with IL-12, IL-18 and other cytokines activates Stat4 signaling in naïve T-cells, which induces T-bet, a key transcription factor for T_{H1}-cell commitment. T-bet drives IFN-γ expression, which in turn positively feeds back to amplify T-bet activation [111]. After antigen presentation IFN-I signaling on T-cells blocks apoptosis and thereby prolongs their active phase and further drives memory T-cell generation by driving IL-2 expression [112,113]. On the contrary, T_{H2}-cell and T_{H17}-cell differentiation is actively blocked by IFN-I signaling. Important signals for T_{H2}-cell commitment are IL-4, IL-5 and IL-13, of which IL-5 in mouse and IL-4 in human cells is blocked by IFN-I and thereby expression of the transcription factor GATA3 is inhibited [114].The TH_{17}-cell program is repressed by IFN-I signaling, which is of clinical significance for IFN-α treatment of multiple sclerosis patients [115].

IFN-I has been shown to have enhancing effects on the effector function of $CD8^+$ T-cells. $CD8^+$ T-cells can differentiate into effector cells independent of STAT4 and IL-12, however both IL-12 and IFN-I are additional signals that fully activate effector function, as opposed to tolerance, if present [111,116]. When IFN-I directly acts on innate $CD8^+$ T-cells, IL-15 secretion is induced and memory $CD8^+$ T-cells are generated.

Additionally to the effects on T-cell maturation, IFN-I also indirectly boosts adaptive immunity by promoting dendritic cell (DC) function. The antigen-presenting ability of DCs is significantly increased and their survival is promoted upon IFN-I stimulation [117]. IFN-I together with Csf2 promotes the maturation of precursors to mature DC. In the serum of systemic lupus erythematosus IFN-α/β concentrations reach levels high enough to stimulate DC differentiation [118]. Immature DCs upregulate co-stimulatory molecules such as MHC II, MHC I, CD40, CD80 and CD86 upon IFN-I signaling [119]. Thereby also the ability to stimulate T-cell proliferation increases and the cytokine signature released by DCs is modulated.

1.5.4. Range of action of type I interferon: modulation of inflammatory responses against bacteria

In addition to its cell-intrinsic effects, IFN-I also modulates the cytokine response in several bacterial infection models (Figure 13). A strong connection is established between IFN-I signaling and inflammasome activation. IFNβ signaling has been shown to directly inhibit the NLRP1 and NLRP3 inflammasomes [120]. Additionally, IL-10 is induced by IFN-I, which also negatively regulates IL-1 production, next to other anti-inflammatory effects. During bacterial infection with *Francisella tulnarensis* or *Listeria monocytogenes* IFN-I has been shown to enhance the AIM2 inflammasome activation leading to increased IL-1 production which is an important factor in the immune response against intracellular pathogens [121]. To the contrary, IFN-I produced upon *Mycobacterium tuberculosis* infection represses the production of cytokines, such as IL-1, which is crucial for the host defense against this pathogen [122]. In the case of *Listeria monocytogenes* infection IFN-I causes apoptotic cell death of lymphocytes and macrophages and thereby skews cytokine production towards a pro-inflammatory response, altogether representing a detrimental effect of IFN-I [123-125]. Upon lung infection with *Chlamydia muridarum* IFN-I signaling also induces macrophage apoptosis, which results in increased susceptibility to infection [126].

On the other hand, IFN-I exerts protective roles in infections with certain bacterial species, such as *Chlamydia pneumoniae*, where it modulates type II interferon responses, a mechanism which is also important in the defense against *Mycobacterium leprae* and the fungal pathogen *Pneumocystis murina* [127-129]. During infection with *Legionella pneumophilia* IFN-I orchestrates an innate immune response by polarizing macrophages towards an inflammatory phenotype, which is protective for the host [130].

IFN-I has been shown to modulate TNF signaling, an important pathway regulating inflammatory responses and apoptosis [131]. Vice versa the pattern of ISG expression upon infection can be modulated by TNF signaling [132].

In the chronic phase of infections, such as lymphocytic choriomeningitis virus, *mycobacterium tuberculosis* and *mycobacterium leprae* infection, IFN-I is an important immune-suppressing factor by inducing IL-10 and programmed cell death ligand 1 (PDL1), which are dominating over pro-inflammatory mediators [133,134].

1.5.5. Type I interferon in non-infectious disease

IFN-I is associated with various diseases of which some examples will be discussed here (Figure 13). Several autoimmune-diseases are characterized by high levels of circulating and tissue pDCs and high levels of IFN-I subsequently. Under auto-inflammatory conditions IFN-I is detrimental for the host and driving pathology of disease by activating and enhancing antigen uptake and DC function and autoantibody production (Figure 13) [135]. In psoriasis and other auto-inflammatory skin diseases self-DNA and self-RNA in lesions are bound by the peptide LL-37 and further transported into pDCs where an IFN-I response is induced via activation of TLR7 and TLR9 [91,136]. Systemic lupus erythematosus (SLE) patients display high levels of IFN-I in the serum and blood cells show expression of ISG. In this case immune complexes consisting of auto-antibodies and self-DNA and self-RNA stimulate pDCs via Fc-receptor (crystallizable fragment receptor) activation to secrete IFN-I [135]. The importance of IFN-I in the pathogenesis of SLE is also demonstrated by the fact that many genes that have been associated with SLE are in fact regulating or modulating the IFN-I response or Fc receptor binding [137].

IFN-I also plays a major role in various cancer types. Generally, IFN-I is protective in many cancer models due to effects such as enhancing adaptive immunity and importantly NK cell mediated immunity, inhibiting angiogenesis and promoting apoptosis (Figure 13). As a consequence, IFN-I has been tested extensively in anti-cancer therapy, in many cases with some success. Unfortunately severe side effects of IFN-I treatment make it only applicable in cases where more targeted therapies fail [138,139].

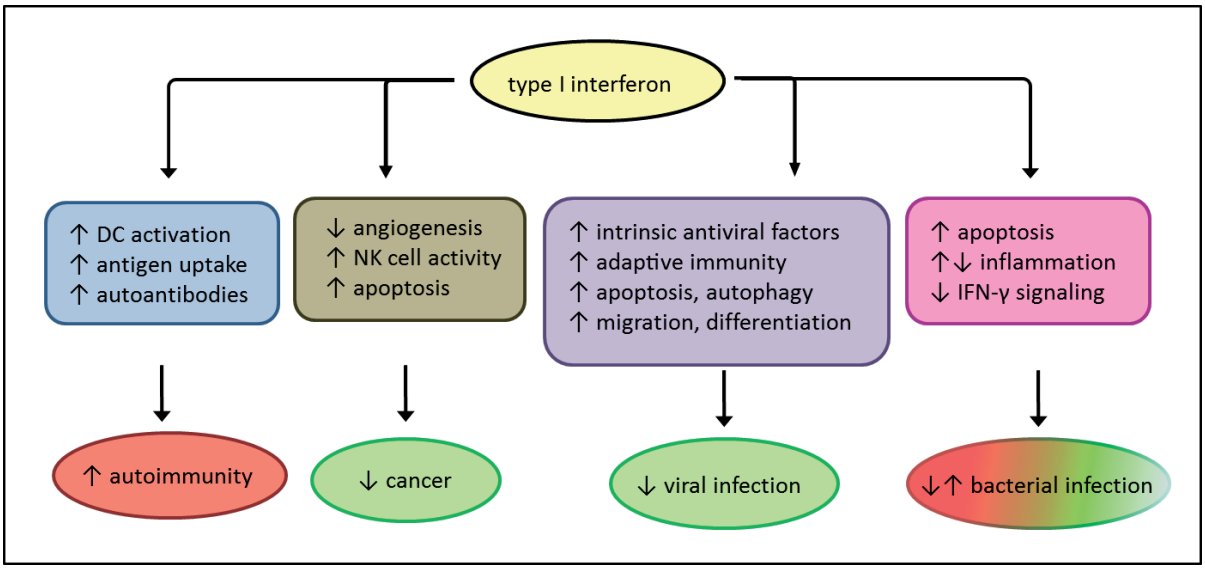

Fig13. Effects of type I interferon in different disease conditions.

2. Results

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Type I Interferon Promotes Alveolar Epithelial Type II Cell Survival during Pulmonary Inflammation

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Short title: **IFN-I Prevents AECII Death in Pneumonia and ALI**

At a Glance Commentary:
Scientific Knowledge on the Subject
Bacterial and sterile lung inflammations are accompanied by collateral damage to the epithelial compartment, resulting in the breakdown of epithelial barriers with the consequences of respiratory failure and facilitated systemic bacterial spread. Preserving the integrity of respiratory epithelial barriers is of great clinical importance and an unmet medical need in critical care medicine.

What This Study Adds to the Field
Our study reveals a barrier-protective role for type I interferon as it prevents alveolar epithelial type II cells (AECII) from inflammation-associated cell death. This protective function was evident not only during pneumococcal pneumonia but also in sterile acute lung injury, suggesting type I interferon might be employed therapeutically in these important conditions to prevent respiratory failure.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

Abstract

Rationale: Protecting the integrity of the lung epithelial barrier is essential to ensure respiration and proper oxygenation in patients suffering from any type of lung inflammation.

Objectives: We aimed to investigate the importance of type I interferon (IFN-I) in pneumococcal pneumonia as well as in acute lung injury to better understand immune-modulating effects in the lung with potential medical implications.

Methods: We used conventional and conditional type I interferon receptor deficient mice and applied mouse models of *S. pneumoniae* infection and acid-induced acute lung injury. We employed cell culture based experiments to complement *in vivo* data.

Measurements and Main Results: Here we reveal a new and protective function of IFN-I by dissecting the biological contribution of IFN-I to the host response during pneumococcal pneumonia. We found alveolar macrophage-derived IFN-I to selectively protect alveolar epithelial type II cells (AECII) from inflammation-induced cell death upon pneumonia. Mechanistically we could show that direct signaling via the IFN-I receptor on AECII is sufficient to promote AECII survival, whereas IFNAR signaling on immune populations does not contribute. This IFN-I triggered mechanism importantly restricts tissue damage in *S. pneumoniae* infected lungs and thereby prevents bacteria from spreading systemically. Significantly, we find that this protective role of IFN-I is not restricted to bacterial infections but that it is also pertinent to acute lung injury.

Conclusions: Our data suggest IFN-I as a potential therapeutic mediator that might be employed to antagonize inflammation associated damage and to protect the respiratory epithelial barrier in infectious and sterile lung injuries.

Number of Words: 249; Key Words: Pneumococcal Pneumonia, ALI, Epithelial Barrier

Introduction

Type I interferons (IFN-I) are strongly upregulated upon viral infections to fulfill three major functions, namely i) inducing a cell-intrinsic anti-viral state in infected cells, ii) promoting antigen presentation and adaptive immune responses and iii) modulating innate inflammatory responses [1]. In many diseases the cell biology of IFN-I responses is poorly understood, since initial expression levels can be low and IFN-I secretion rapidly results in propagation of the IFN-I signal via binding to the interferon-α/β receptor (IFNAR) that is expressed on nearly all cell types [2]. Many bacteria induce IFN-I, thereby modulating innate immune responses by different, often ill-defined mechanisms [3]. Detrimental as well as beneficial effects of IFN-I have been reported in the context of bacterial infections. The mechanisms underlying these heterogeneous effects range from the promotion of cell death by IFN-I [4,5], to interference with type II interferon responses [6,7], and shifting of macrophage phenotypes [8].

In this study we investigated the cell biology of the IFN-I response to *Streptococcus pneumoniae*, a Gram-positive bacterium that is the most frequent cause of community-acquired pneumonia. Upon infection, a substantial inflammatory response in the lung is triggered, which rapidly escalates into systemic inflammation and life-threatening sepsis [9]. Several studies have provided evidence for IFN-I expression in the lung after infection with *S. pneumoniae* and connected IFN-I induced in the respiratory tract with progression of invasive disease [10-12]. *In vitro,* multiple cell types have the capacity to upregulate IFN-I upon *S. pneumoniae* challenge, such as alveolar and bone marrow derived macrophages, epithelial cells or conventional dendritic cells and some cell-intrinsic defects have been attributed to loss of IFN-I after *S. pneumoniae* stimulation, e.g. reduced production of RANTES and decreased IL-18 secretion by macrophages [13,14]. However, most of the cited studies rely on *in vitro* experiments and it is not well understood which cells contribute *in vivo* to IFN-I responses against *S. pneumoniae*.

During respiratory viral infections alveolar macrophages are known as potent IFN-I producers, and IFN-I responsive cells are both infected cells and their bystander cells [15,16]. In contrast, during bacterial respiratory infections or sterile acute lung injury (ALI) the IFN-I producing and responding cell types *in vivo* are completely uncharacterized. Using RNA profiling we here present alveolar macrophages as the

source of IFN-I upon pneumococcal pneumonia *in vivo* and we provide data generated with conditional *Ifnar1*$^{-/-}$ mice that for the first time identify the precise target cells of IFN-I suggesting that IFN-I acts on alveolar epithelial type II cells (AECII) in this setting. Further, we found that IFN-I protects AECII from cell death; a mechanism which we found not only upon *S. pneumoniae* infection but also in a model of acid-induced ALI.

Results

Type I interferon prevents lung damage and bacterial dissemination during *S. pneumoniae* infection

To assess the role of IFN-I during pneumococcal pneumonia we infected WT and *Ifnar1*$^{-/-}$ mice with *S. pneumoniae* and analyzed bacterial numbers, cytokine levels and lung histology. Forty hours post-infection (p.i.) *Ifnar1*$^{-/-}$ mice exhibited elevated blood bacterial counts and aggravated signs of inflammation, indicated by increased levels of pulmonary and systemic IL-6, Cxcl1 and IL-1ß together with more pronounced lung infiltrates seen by histology (Figure 1A, B, C). This was accompanied by a higher proportion of TUNEL-positive cells in *Ifnar1*$^{-/-}$ lungs (Figure 1D) and increased lung weights together with elevated protein concentrations and cell influx in the broncho-alveolar lavage fluids (BALF) of *Ifnar1*$^{-/-}$ mice 16h p.i. (Figure 1E, F, G).

To more precisely understand the impact of IFN-I triggered effects we examined mice eight hours p.i. and found elevated bacterial counts in *Ifnar1*$^{-/-}$ lungs and BALF and higher lung weight and alveolar protein concentrations, but no differences in cytokine levels and cell influx (Figure 2A, B). More pronounced alveolar protein levels in *Ifnar1*$^{-/-}$ mice (Figure 2B) can be explained by the previously reported barrier-protective role of IFN-I [10], which we could verify in our model (Figure E1A, online data supplement). By analyzing lung cell populations by flow cytometry we excluded baseline differences in the pulmonary immune cell composition between WT and *Ifnar1*$^{-/-}$ mice (Figure E1B, C, D, online data supplement). Collectively, loss of IFN-I signaling exerted detrimental effects during pneumococcal pneumonia, illustrated by augmented bacterial dissemination and inflammation together with more pronounced signs of epithelial barrier disruption.

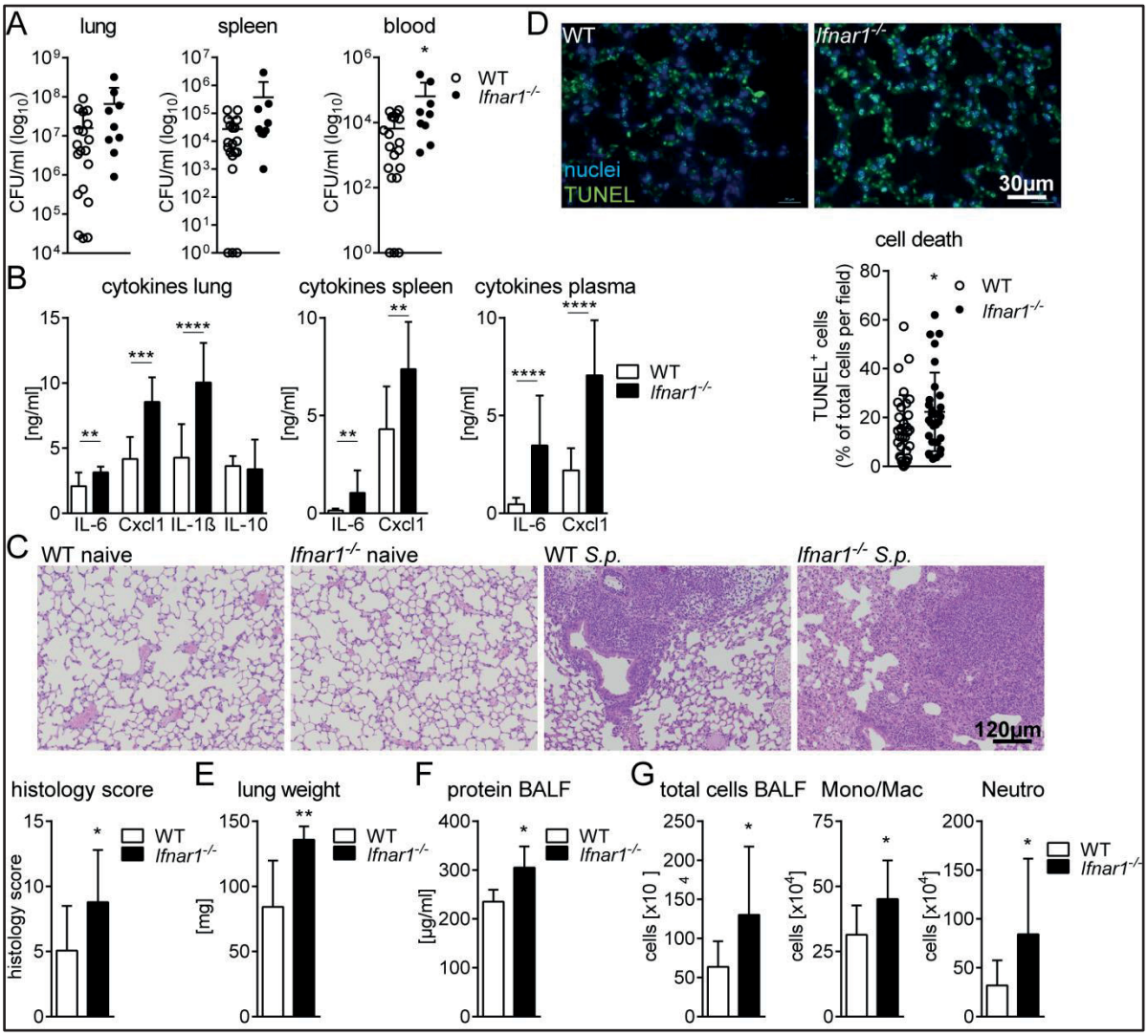

Figure 1. Loss of IFN-I signaling aggravates the pathology of pneumococcal pneumonia. Wild-type (WT) and *Ifnar1*$^{-/-}$ mice were infected i.n. with 10^5 CFU *S. pneumoniae* (ATCC 6303). 40h p.i. bacterial load was assessed (A), cytokines of homogenized organs or plasma were quantified by ELISA (B), lung tissue was stained with H&E (C) and TUNEL (D) and lung weight was determined (E). 16h p.i. protein levels in the BALF were quantified (F) and differential cell counts of BALF cells were done on cytospin preparations (G). Representative images of n≥8 mice per group are depicted (magnification 100x (C) and 40x (D)), next to a quantification of all samples. Values are mean +/- SD and results are representative of at least 2 independent experiments with n≥8 for each group. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

Type I interferon is produced in lungs of *S. pneumoniae* infected mice in a DNA dependent manner

Next we investigated the timing and triggers of IFN-I induction upon pneumococcal infection. We could detect IFN-β in lungs eight hours p.i. (Figure 2C), which is in line with previous studies [10,13]. Infection with a pneumolysin-deficient mutant of *S. pneumoniae* [Δply] or heat-inactivated (HI) *S. pneumoniae* also led to the induction of IFN-β (Figure 2D). This finding suggests that neither pneumolysin, the bacteria's major virulence factor [17], nor RNA or other factors which are not preserved during

heat-inactivation were required for the induction of IFN-I. *Ifnar1*$^{-/-}$ mice infected with either *S. pneumoniae* [Δply] or HI *S. pneumoniae* showed the same phenotype as upon infection with WT bacteria (Figure 2E, F, G).

Because it has been reported that pneumococcal DNA induces IFN-I in macrophages *in vitro* [13] we tested the contribution of bacterial DNA *in vivo* and infected mice with WT and DNAse treated HI *S. pneumoniae* [ΔDNA]. Doing so, IFN-β was no longer detectable and induction of the interferon-stimulated genes *Ifit1* and *PKR* was significantly reduced (Figure 2H). Accordingly, differences in lung weight between *Ifnar1*$^{-/-}$ and WT mice were abolished when we infected animals with HI *S. pneumoniae* [ΔDNA], indicating a lung barrier strengthening role for IFN-I produced upon *S. pneumoniae* infection (Figure 2I). Taken together, we identified pneumococcal DNA as the inducer of IFN-I upon *S. pneumoniae* infection *in vivo.*

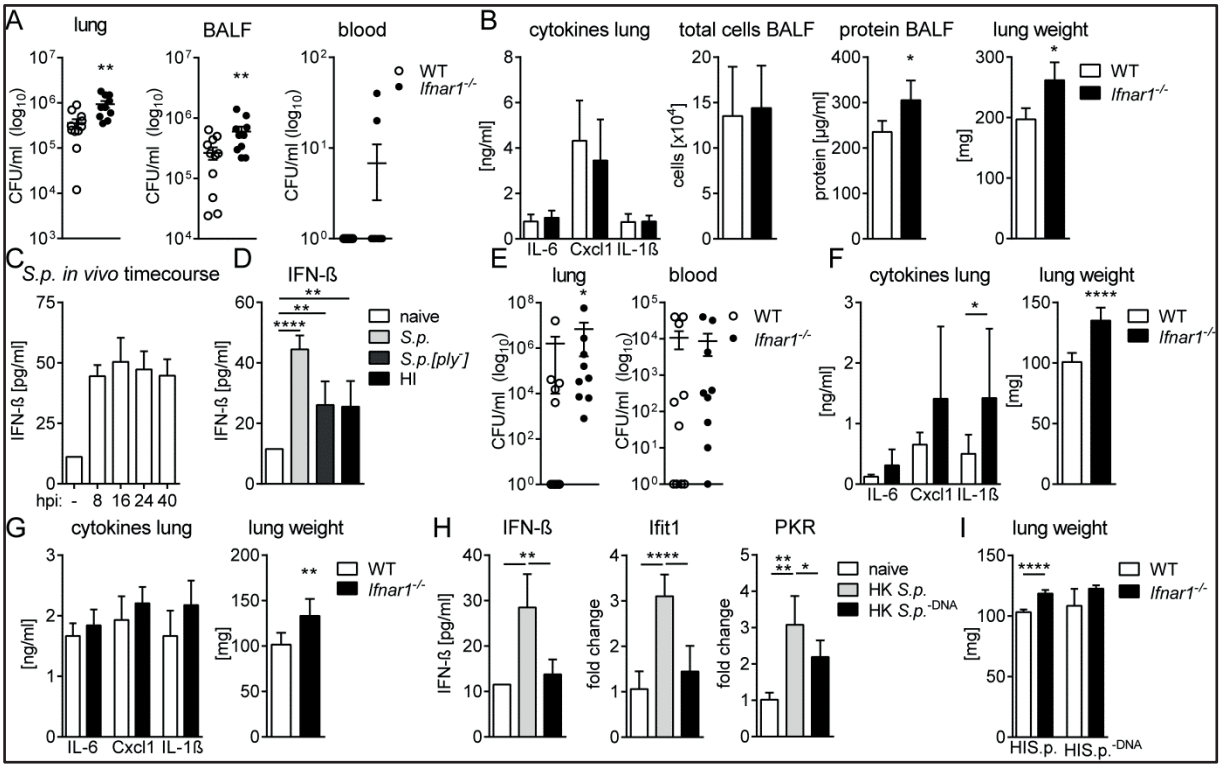

Figure 2. IFN-β is induced in a DNA dependent manner during *S. pneumoniae* infection. WT and *Ifnar1*$^{-/-}$ mice were infected i.n. with 10^5 CFU *S.* pneumoniae (ATCC 6303) and bacterial load (A), cytokine levels, lung weight as well as cell numbers and protein concentrations in the BALF (B) were analyzed 8h p.i. (C) WT mice were infected i.n. with 10^5 CFU *S. pneumoniae* (ATCC 6303) and IFN-β was quantified in lung homogenates by ELISA at the indicated time points. (D-G) Mice were infected with 10^7 CFU of either WT *S. pneumoniae* (D39), pneumolysin deficient *S. pneumoniae* [Ply$^-$] (D39) or heat-inactivated (HI) *S. pneumoniae* (D39). 40h p.i. lung homogenates of WT mice were analyzed for IFN-β by ELISA (D). Bacterial load (E), cytokine levels and lung weight of *S. pneumoniae* [Ply$^-$] infected WT and *Ifnar1*$^{-/-}$ mice (F) as well as cytokine levels and lung weight of HI *S. pneumoniae* infected WT and *Ifnar1*$^{-/-}$ mice (G) were assessed 40h p.i. (H, I) Mice were infected with 10^7 CFU HI *S. pneumoniae* or DNAse treated HI *S. pneumoniae* (both D39). 40h p.i. lung homogenates of WT mice

were analyzed for IFN-β by ELISA, and for *Ifit1* and *PKR* by qRT-PCR (H) and lung weight of WT and *Ifnar1*$^{-/-}$ mice is given in (I). Values are mean +/- SD and results are representative of 2 independent experiments with n≥8 for each group. * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001.

Alveolar macrophages upregulate type I interferon during the onset of *S. pneumoniae* infection *in vivo*

We hypothesized that macrophages are the source of IFN-I upon bacterial challenge, and we quantified IFN-β released by an alveolar macrophage (AM)-derived cell-line (MHS) stimulated with *S. pneumoniae* (Figure 3A). The induction of IFN-β *in vitro* strongly depended on the uptake of bacteria and the acidification of lysosomes, as *S. pneumoniae* triggered IFN-I releases were abolished in the presence of cytochalasin D or bafilomycin A (Figure 3B). In these settings, there was a strong induction of IL-6, although its levels were also reduced upon cytochalasin D or bafilomycin A treatment (Figure 3C).

As bacterial uptake seemed to be a prerequisite for macrophages to produce IFN-β, we tested the *in vivo* contribution of individual phagocytes to bacterial ingestion by infecting mice with a GFP expressing strain of *S. pneumoniae[GFP+]*, followed by flow cytometric analysis. We identified AM as the main cell population phagocytosing *S. pneumoniae[GFP+]* early upon infection (Figure 3D). While monocytes partly contributed to phagocytosis, neutrophils strongly participated later after infection (Figure E2A, online data supplement). The GFP signal was verified by microscopy of sorted GFP^{-} or GFP^{+} populations (Figure E2B, online data supplement). To test if IFN-I was induced in AM *in vivo*, we FACS-sorted CD45^{+} CD11c^{hi} Siglec F^{+} AMs from the BALF of *S. pneumoniae* infected mice three hours p.i. (Figure E2C, online data supplement) and performed a qPCR array covering IFN-I response genes using mRNA isolated from these cells. We found *Ifnb1* and *Ifna4* upregulated, as well as several genes required for the augmentation of the primary IFN-I signal e.g. *Irf9*, *Irf3*, *Stat1* and *Ifnar1* (Figure 3E). In contrast, genes that are involved in driving mass production of ISG, such as *Irf7* and *Stat2*, remained uninduced [2] (Figure 3E). Collectively, these data show that AM are important early phagocytes that can internalize *S. pneumoniae* and induce IFN-I upon infection *in vivo*.

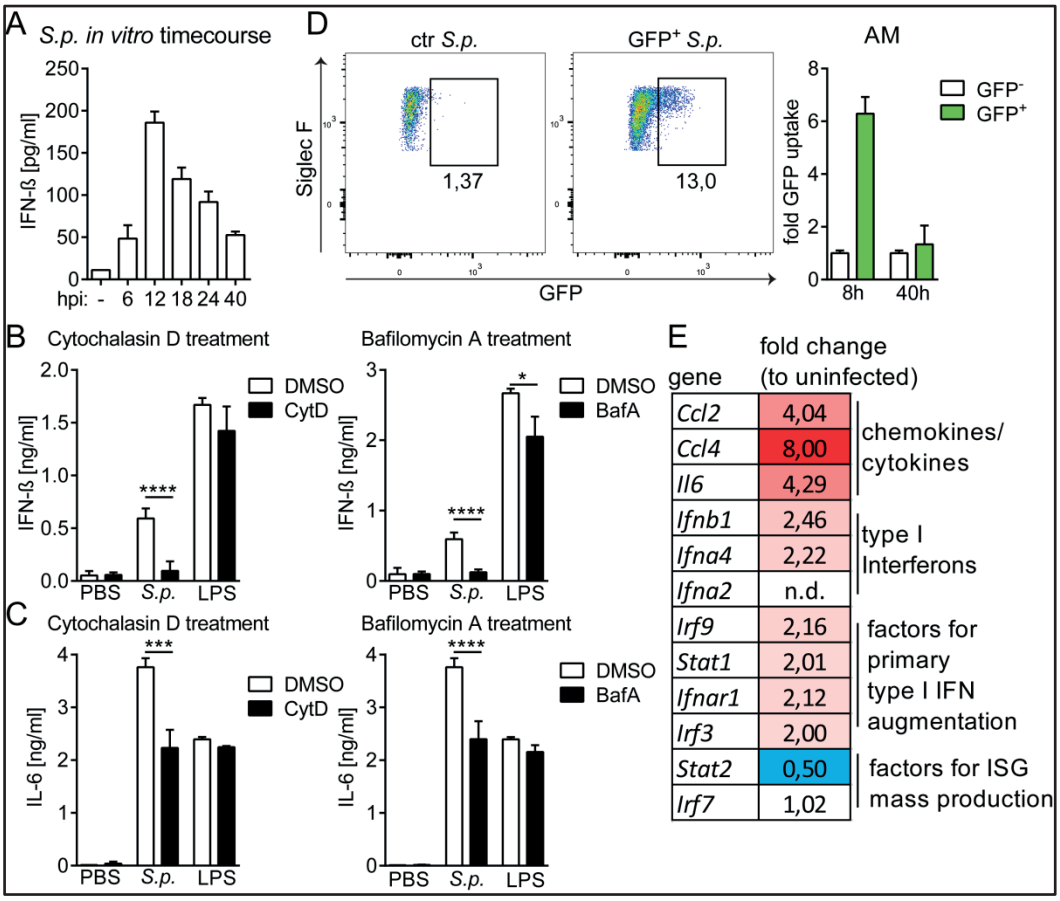

Figure 3. Alveolar macrophages upregulate IFN-I and factors necessary for IFN-I expression upon *S. pneumoniae* infection. (A) MHS cells were stimulated with 10^7 CFU/ml *S. pneumoniae* (ATCC 6303). IFN-β was quantified by ELISA at the indicated timepoints. MHS cells were pretreated for 40min with either cytochalasin D or bafilomycin A and stimulated with 10^7 CFU/ml *S. pneumoniae* (ATCC 6303) or 1µg/ml LPS for 12h. IFN-β (B) and IL-6 (C) was quantified by ELISA. (D) WT mice were infected with 10^7 CFU GFP$^+$ *S. pneumoniae* (D39). Lungs were digested at the indicated timepoints and GFP$^+$ cells were quantified by flow cytometry (n=4 per group). (E) WT mice were infected with 10^5 CFU *S. pneumoniae* (ATCC 6303). BALF was taken 3h p.i. and AM were sorted by flow cytometry. A qRT-PCR array was performed on the extracted RNA (AM were pooled from 3 mice per group). Values are mean +/- SD and results are representative of at least 2 independent experiments, the qRT-PCR array was performed once and all depicted genes were validated by qRT-PCR in an independent experiment. In vitro stimulations were performed in quadruplicates. Representative plots of n=4 mice per group are depicted next to a quantification of all samples. * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Autocrine IFN-I signaling does not alter alveolar macrophage functions

To assess if autocrine IFN-I signaling in AM is relevant during pneumococcal pneumonia, we analyzed WT and *Ifnar1*$^{-/-}$ AM for functional parameters that have been associated with IFN-I signaling. Upon stimulation with *S. pneumoniae*, *Ifnar1*$^{-/-}$ AM showed no defects in inflammatory cytokine production (Figure 4A). Since macrophage polarization is an important parameter that determines the AM response to *S. pneumoniae* and considering that IFN-I was found associated with M1 polarization of macrophages infected with *Legionella sp.*, we investigated markers

that signify polarization of macrophages [8,18]. We discovered comparable levels of *Ym1* and *Mrc*, and although baseline expression of *Cd74* and *Fizz1* were higher in *Ifnar1*$^{-/-}$ AM, these differences disappeared after stimulation, thus suggesting an unaltered polarization state of AM (Figure 4B). Finally, we studied the propensity of AM to undergo apoptosis, which was found to contribute to pneumococcal clearance and has been shown earlier to be regulated by IFN-I [19,20]. We isolated AM of *S. pneumoniae* infected WT and *Ifnar1*$^{-/-}$ mice eight hours p.i. and analyzed cell death by TUNEL assay and Annexin V staining, neither of which indicate any effect of IFN-I signaling on AM apoptosis (Figure 4C, D). Altogether our data suggest that AM from *Ifnar1*$^{-/-}$ mice were not impaired or altered in their response to *S. pneumoniae*.

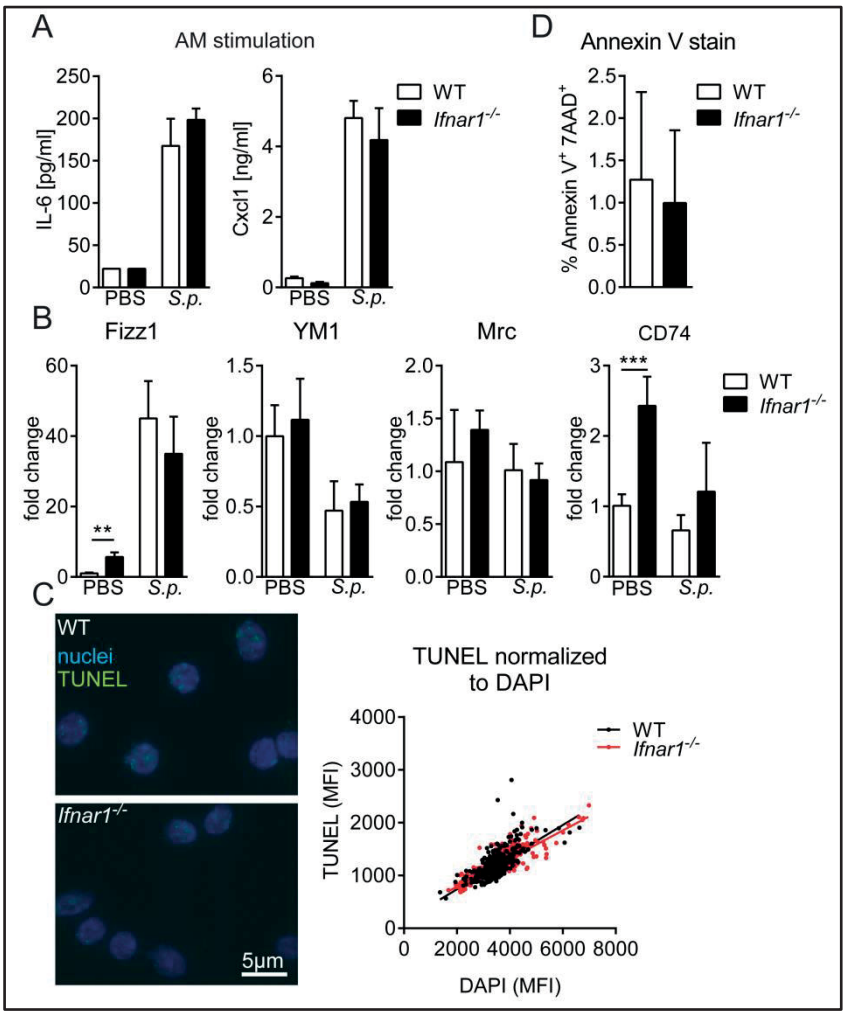

Figure 4. Alveolar macrophages deficient for IFN-I signaling are functionally unimpaired. AM from WT and *Ifnar1*$^{-/-}$ mice were isolated from BALF, plated and stimulated with 10^7 CFU/ml *S. pneumoniae* (ATCC 6303). AM were pooled from 4 mice per group and plated in quadruplicates. After 12h cytokines were quantified by ELISA (A) and polarization markers were assessed by qRT-PCR (B). WT and *Ifnar1*$^{-/-}$ mice were infected i.n. with 10^5 CFU *S. pneumoniae* (ATCC 6303) (n=6 per group). AM were isolated from BALF 8h p.i and stained for TUNEL (C) or for Annexin V and 7-AAD and analyzed by flow cytometry (D). Representative images of n=6 mice per group are depicted

(magnification 20x), next to a quantification of all samples. Values are mean +/- SD and results are representative of 2 independent experiments. ** $p \leq 0.01$.

IFN-I mediates protective effects upon *S. pneumoniae* infection via alveolar epithelial type II cells *in vivo*

Since we found AM of *Ifnar1*$^{-/-}$ mice to be unaffected, we generated conditional *Ifnar1*-deficient mice to discriminate between cell types responsive to IFN-I during pneumococcal pneumonia *in vivo*. To obtain *Ifnar1* deficiency in lung cell types relevant in the defense against *S. pneumoniae* we crossed *Ifnar1*$^{fl/fl}$ mice with mice expressing Cre recombinase under the control of the Lysozyme M promoter, the CD11c promoter or the SP-C promoter deleting in AECII, one of the two epithelial cell types that line alveoli. Forty hours after *S. pneumoniae* challenge *Ifnar1*$^{fl/fl}$ *Lysm-Cre*$^{+}$ mice and *Ifnar1*$^{fl/fl}$ *Cd11c-Cre*$^{+}$ mice showed no difference in bacterial burden, inflammatory cytokine levels nor lung weight compared to *Ifnar1*$^{fl/fl}$ *Cre*$^{-}$ control mice (Figure 5A, B, C, D). In Tamoxifen (Tx) induced *Ifnar1*$^{fl/fl}$ *Sftpc-CreER*$^{+}$ mice we found elevated bacterial counts, higher levels of inflammatory cytokines and higher lung weights compared to Tx treated *Ifnar1*$^{fl/fl}$ *Cre*$^{-}$ control animals (Figure 5E, F). Lung histological analyses revealed that tissue damage was more pronounced in *Ifnar1*$^{fl/fl}$ *Sftpc-CreER*$^{+}$ lungs, whereas no difference was found in *Ifnar1*$^{fl/fl}$ *Lysm-Cre*$^{+}$ or *Ifnar1*$^{fl/fl}$ *Cd11c-Cre*$^{+}$ mice when compared to controls (Figure 5G). Protein concentrations in BALF of *Ifnar1*$^{fl/fl}$ *Sftpc-CreER*$^{+}$ mice were elevated 16 hours p.i (Figure 5H). Overall, *Ifnar1*$^{fl/fl}$ *Sftpc-CreER*$^{+}$ mice pheno-copied conventional *Ifnar1*$^{-/-}$ mice thus suggesting that IFN-I directly acts on AECII to exert its barrier protective effects.

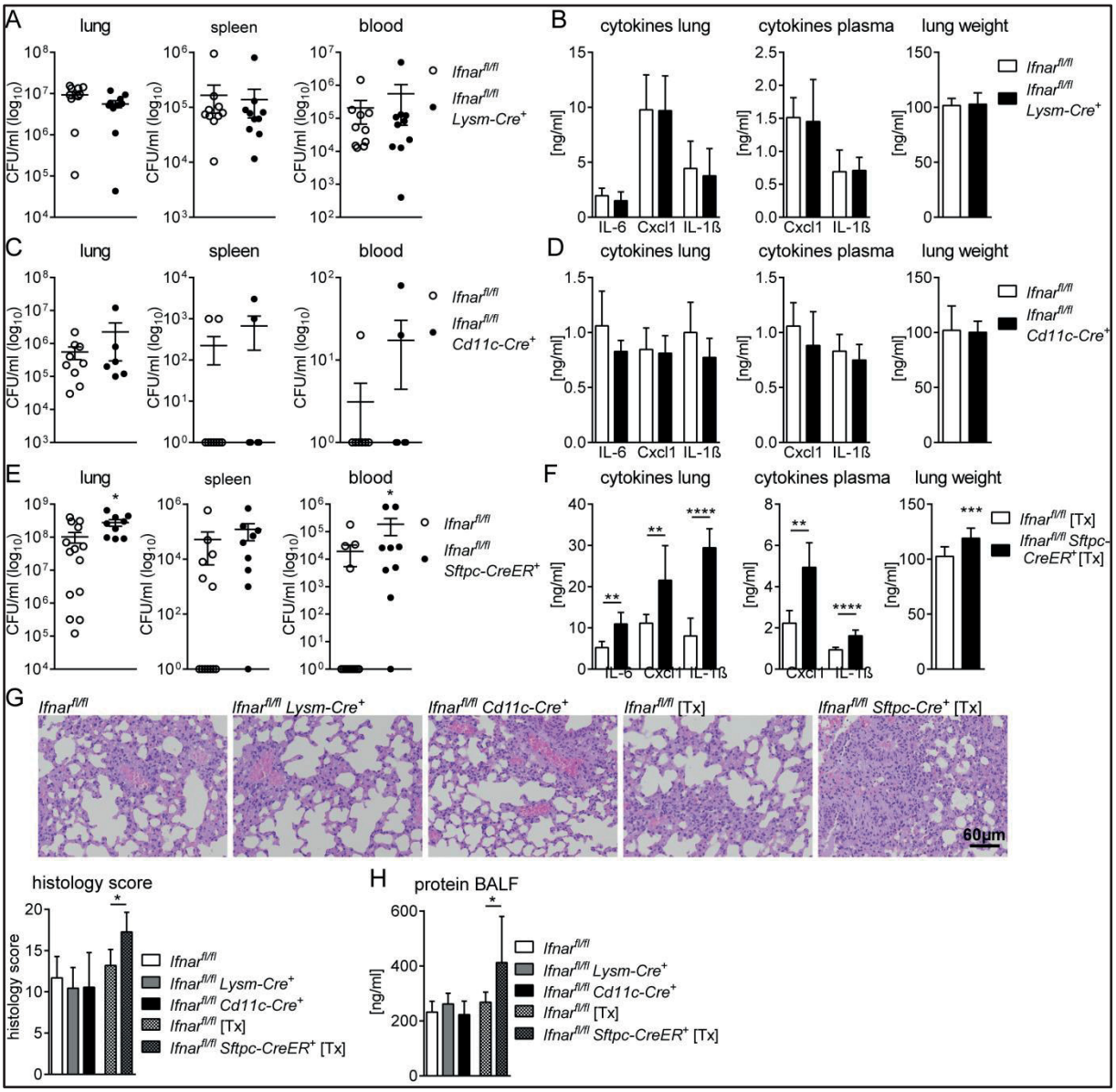

Figure 5. IFN-I acting on alveolar epithelial type II cells prevents bacterial dissemination and damage during pneumococcal pneumonia. All mice were infected i.n. with 10^5 CFU *S. pneumoniae* (ATCC 6303). Bacterial loads, cytokine levels and lung weight were analyzed 40h p.i. from (A, B) $Ifnar^{fl/fl}$ vs. $Ifnar^{fl/fl}$ $Lysm\text{-}Cre^+$ mice and (C,D) $Ifnar^{fl/fl}$ vs. $Ifnar^{fl/fl}$ $Cd11c\text{-}Cre^+$ mice. $Ifnar^{fl/fl}$ and $Ifnar^{fl/fl}$ $Sftpc\text{-}CreER^+$ mice were injected with 5mg tamoxifen on 4 consecutive days and infected 10 days after the last Tx injection. 40h p.i bacterial loads, cytokine levels and lung weight were analyzed (E, F). Lung tissue taken 40h p.i. was stained with H&E for all groups (G) and protein concentrations were quantified in the BALF taken 16h p.i. (H). Representative images of n≥6 mice per group are depicted (magnification 40x), next to a quantification of all samples. Values are mean +/- SD and results are representative of 2 independent experiments with n≥6 mice per group and genotype. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Type I interferon signaling protects form alveolar epithelial type II cell death upon *S. pneumoniae* infection

As IFN-I directly acts on AECII, we investigated changes in this population upon pneumococcal pneumonia. We found the expression of the AECII-specific markers *Sftpc* and *Lpcat1* reduced in *S. pneumoniae* infected *Ifnar1*$^{-/-}$ lungs compared to infected WT lungs (Figure 6A). We performed IHC and stained for the presence of AECII in lung samples from infected mice and detected decreased numbers of proSP-C positive cells in *Ifnar1*$^{-/-}$ mice compared to WT animals. Notably, AECII also decreased upon infection in WT lungs, indicating that epithelial damage upon *S. pneumoniae* infection affects the AECII population (Figure 6B). When evaluating proSP-C staining of infected lungs from conditional mice, only *Ifnar1*$^{fl/fl}$ *Sftpc-CreER*$^{+}$ mice displayed decreased numbers of AECII upon infection compared to *Ifnar1*$^{fl/f}$ *Cre*$^{-}$ animals after Tx treatment, whereas AECII numbers were unaffected in *Ifnar1*$^{fl/fl}$ *Lysm-Cre*$^{+}$ and *Ifnar1*$^{fl/fl}$ *Cd11c-Cre*$^{+}$ mice (Figure E3, online data supplement). Our data indicate that the AECII compartment is damaged in *S. pneumoniae* infected lungs and that this damage is more pronounced in the absence of IFN-I signaling on AECII.

We hypothesized that IFN-I either protects AECII from cell death or promotes their regeneration. We analyzed cell death of AECII by TUNEL assay and co-stained with *proSP-C*. In infected *Ifnar1*$^{-/-}$ lungs a significantly higher proportion of AECII was TUNEL positive showing that functional IFN-I signaling indeed promoted AECII survival upon *S. pneumoniae* infection (Figure 6C). To corroborate the direct effect of IFN-I on AECII survival we assessed the death rate of MLE cells, an AECII derived cell line, in the presence or absence of IFN-β. MLE cells were protected from TNF-induced cell death in the presence of IFN-β, whereas cell death triggered by live bacteria or pneumolysin was unaffected, most likely due to the irreversibility of pore-formation (Figure 6D). Altogether our data suggest that IFN-I directly protects AECII from inflammation-induced cell death *in vivo*.

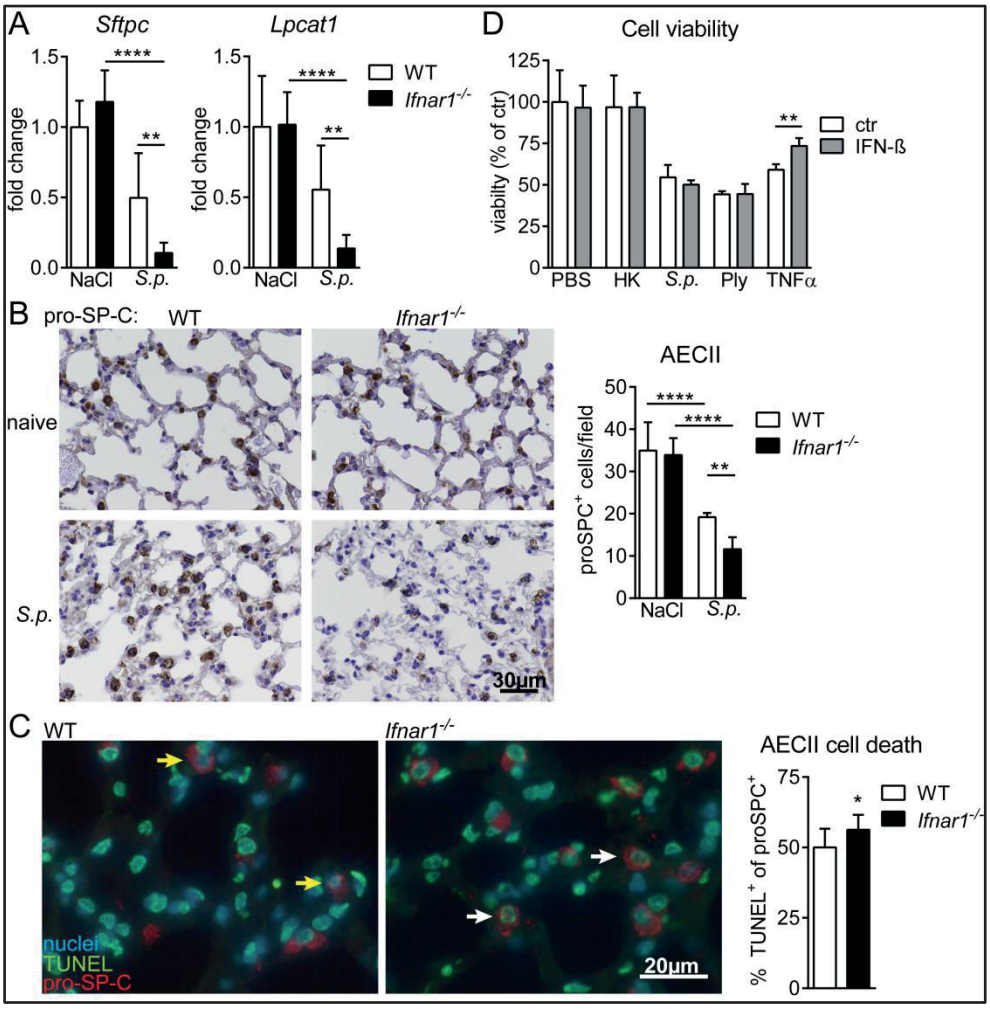

Figure 6. IFN-I signaling maintains AECII numbers and prevents AECII cell death upon pneumococcal pneumonia. WT and *Ifnar1*$^{-/-}$ mice were infected i.n. with 10^5 CFU *S. pneumoniae* (ATCC 6303) or mock infected (n≥8 for each group). 40h p.i. *Sftpc* and *Lpcat1* levels were quantified by qRT-PCR from lung homogenates (A) and paraffin embedded lung tissue was stained for *proSP-C* (B) and co-stained for *proSP-C* and TUNEL (C). In (C) yellow arrows indicate *proSP-C*$^+$ TUNEL$^-$ cells and white arrows indicate *pro-SP-C*$^+$ TUNEL$^+$ cells. Serum-starved MLE cells were pretreated with 1ng/ml IFN-β for 30min and subsequently stimulated with 10ng/ml pneumolysin, 10^7 CFU/ml *S. pneumoniae* (ATCC 6303) or 5µg/ml TNFα. After 4h cell survival was measured using a commercial kit (Glomax) (D). Representative images of n≥8 mice per group are depicted (magnification 40x), next to a quantification of all samples. In vitro stimulations were performed in quadruplicates. Values are mean +/- SD and results are representative of at least 2 independent experiments. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

Protective effects of type I interferon signaling are also evident during sterile lung injury

To assess if the protective activity of IFN-I on AECII *in vivo* is specific for *S. pneumoniae* infection, we investigated lung permeability as well as AECII numbers in acid induced ALI, a sterile model of lung epithelial damage. Eight hours after intratracheal (i.t.) application of hydrochloric acid, we found increased lung weights and protein concentrations in BALF of *Ifnar1*$^{-/-}$ mice (Figure 7A). Also, the leakage of

i.v. injected Evans Blue dye into lungs was augmented in the absence of IFN-I signaling, indicating a lung barrier defect in *Ifnar1*$^{-/-}$ mice (Figure 7B). By studying *Sftpc* and *Lpcat1* expression levels and *proSP-C* staining of histological samples we discovered a reduction of AECII numbers in *Ifnar1*$^{-/-}$ mice upon ALI as well as an increased proportion of dead AECII cells as indicated by higher numbers of TUNEL^{+} AECII co-stained for *proSP-C* (Figure 7C, D, E). These data suggest a broader, epithelial-protective role for IFN-I by promoting AECII survival upon inflammatory or chemical injury.

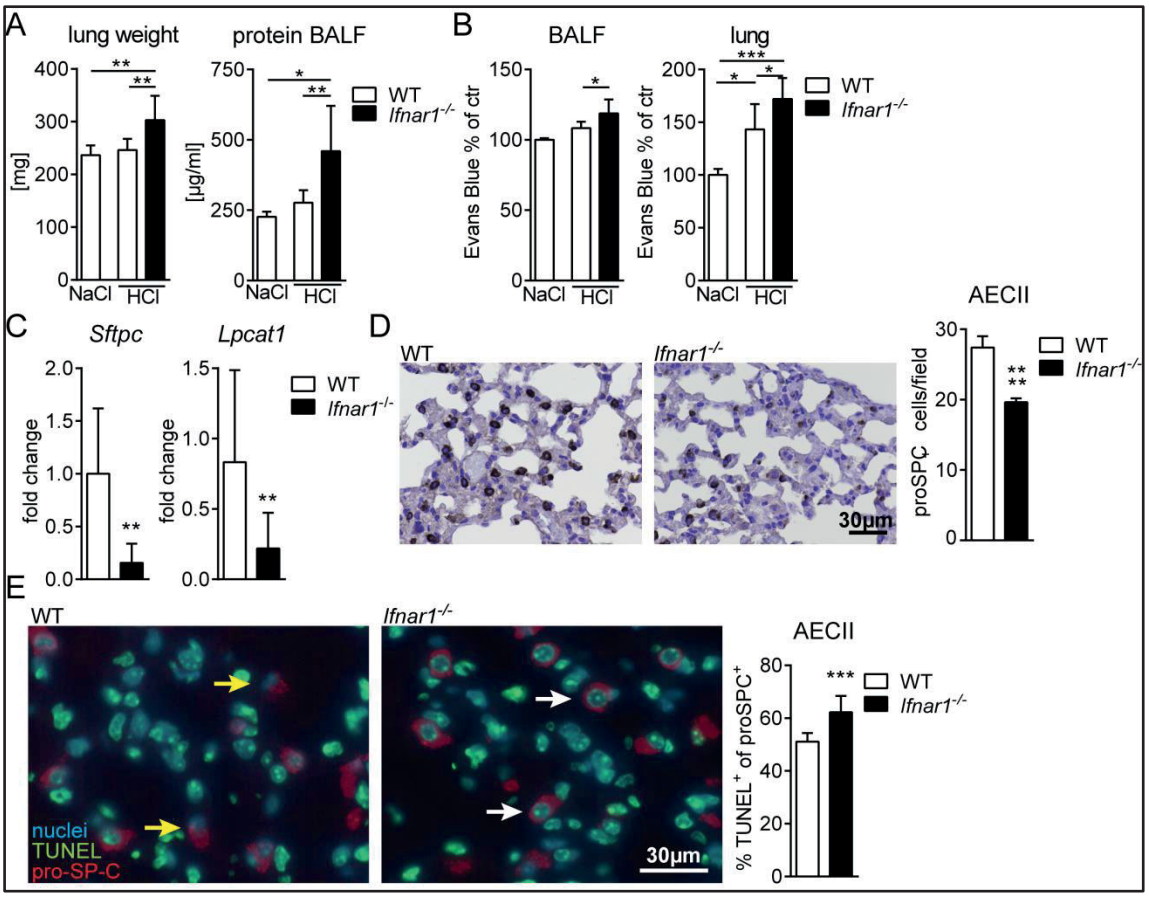

Figure 7. Absence of IFN-I signaling is linked to reduced AECII numbers upon acid induced lung injury. 60µl 0.1N HCl was instilled i.t. into WT and *Ifnar1*$^{-/-}$ mice and all mice were sacrificed 8h later. (A) Lung weight and protein concentrations in BALF were assessed. (B) Some mice received 100µl Evans Blue i.v. 60 min before BALF and lungs were collected. Evans Blue was quantified in BALF and formamide-pretreated lungs. (C) *Sftpc* and *Lpcat1* were quantified by qRT-PCR from lung tissues; (D) paraffin embedded lung tissue was stained for *proSP-C* and (E) co-stained for *proSP-C* and TUNEL. In (E) yellow arrows indicate *proSP-C*$^{+}$ TUNEL^{-} cells and white arrows indicate *pro-SP-C*$^{+}$ TUNEL^{+} cells. Representative images of n≥8 mice per group are depicted (magnification 40x), next to a quantification of all samples. Values are mean +/- SD and results are representative of at least 2 independent experiments with n≥8 per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

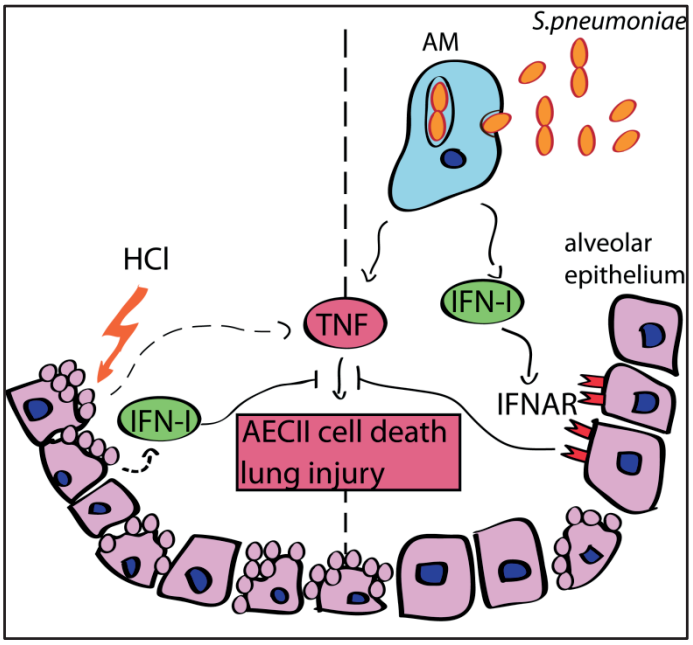

Figure 8. Proposed model of IFN-I mediated protective effects on AECII. Upon infection, AM encounter and ingest *S. pneumoniae* to then induce an inflammatory response that involves the secretion of IFN-I. In a similar manner, sterile acute lung injury is associated with tissue damage and resulting inflammation. Respiratory epithelial cells are damaged in the course of pulmonary inflammation, which is diminished in the presence of IFN-I that directly protects AECII from death.

Discussion

The data we present here highlight the importance of IFN-I in controlling AECII survival upon bacterial or acid-triggered lung injury, as summarized in a graphical synopsis in Figure 8. By focusing on pneumococcal pneumonia, we discovered that bacterial DNA is the main trigger of IFN-I *in vivo* and that macrophages are a major source. Using a number of conditional IFNAR-knockout mice, we revealed that the presence of IFN-I protected AECII from *S. pneumoniae* or sterile lung injury induced cell death *in vivo* and from TNFα induced cell death *in vitro*. The net effect of this barrier-protective activity of IFN-I was the local containment of bacteria and prevention of systemic inflammation upon pneumococcal infection. Importantly so, we extend these findings of IFN-I's barrier protective properties to sterile ALI, suggesting a beneficial role for IFN-I in settings of acute lung damage.

Our findings, in which IFN-I restrains inflammation-induced epithelial damage, extend and corroborate earlier reports that described epithelial barrier-protective properties elicited by IFN-I [10]. Interestingly, in observations made with viral pneumonia models IFN-I has been demonstrated to promote apoptosis of alveolar epithelium via sTRAIL induction [21-23]. These studies in conjunction with the data we report here support

the attractive possibility that IFN-I acts as a context-specific regulator of epithelial cell viability in the lungs. As such, IFN-I differentiates between situations in which the host benefits from tissue protection to keep alveolar damage under control and conditions in which epithelial cells are infected by respiratory viruses and need to be eliminated [24-26].

IFN-Is are known to contribute to hematopoietic stem cell renewal [27] and it is important to reconsider that also the AECII compartment exhibits stem cell characteristics important for the regeneration of alveolar epithelial type I cells [28,29]. It is therefore of great significance for the host to protect this cell population in order to ensure the integrity and renewal capacity of alveoli that are damaged during inflammatory responses. This aspect is of particular relevance in patients suffering from ALI-associated respiratory failure, as is our observation that IFN-I executes barrier-strengthening effects in a model of ALI. It is tempting to speculate that potential treatments that preserve AEC-II viability will not only protect the epithelial barrier, but subsequently support the regeneration of the alveolar lining.

Another clinically very significant condition, where patients might benefit from treatments that support the epithelial barrier, is post-influenza bacterial pneumonia. In this setting IFN-I has been shown to play a major, albeit detrimental role for the host [30,31]. Mechanisms attempting to explain the role of IFN-I in the course of secondary infection include a reduction in IL-17 [32,33], apoptosis of bone marrow granulocytes [34] and impairment of neutrophil recruitment [30]. We speculate that IFN-I-mediated enhancement of epithelial cell death upon viral infection likely contributes to the worsened pneumonia outcome observed in super-infected mice, since in the setting of bacterial pneumonia the epithelial barrier needs to be strengthened rather than disrupted.

Cell homeostasis in the intestinal epithelium has been shown to be regulated by IFN-I. *Ifnar1*$^{fl/fl}$ *Villin-Cre* mice, depleted specifically for *Ifnar1* in the intestinal epithelium, exhibit a decreased number of Paneth and goblet cells and an increased proliferation of intestinal epithelial cells at baseline [35]. Those changes are dependent on the intestinal microflora and can be reversed when *Ifnar1*$^{fl/fl}$ *Villin-Cre* mice are co-housed with WT animals. Antibiotic treated or germ-free mice show substantial degrees of epithelial degeneration, lower pulmonary *Ifnb* expression and impaired adaptive immune responses upon influenza infection, which can be partially restored by

administering pI:C, a potent trigger of IFN-I [36]. The exact causative relationship between IFN-I, pulmonary epithelium and lung microflora still remains to be investigated. Taking into account that IFN-I plays an important role in the regulation of the pulmonary epithelium ([21], our data), impacts on microbiota [35] and the host's microbiota being crucial for mounting IFN-I responses [36], we hypothesize that IFN-I and the microbiota could represent two important interdependent factors not only involved in the priming of immune cell populations in the lung but also in controlling the functionality of the alveolar epithelium.

Although the importance of IFN-I in various diseases is widely acknowledged, research has almost exclusively focused on the molecular biology of IFN-I induction and subsequent transcription of ISGs. A large proportion of publications is based on *in vitro* studies performed with cell lines, while less is known about the significance of those findings *in vivo*. Recently a number of novel tools have been developed that facilitate the dissection of IFN-I responses in mice. We demonstrate in this study that AMs induce IFN-β and IFN-α4 upon pneumococcal pneumonia. This suggests that AMs are a potent source of IFN-I upon various stimuli, which is not surprising given their prime position that enables them to sample antigens in the alveolar space. Taken together, these data highlight the importance of AMs, as the prime cells orchestrating an immune response against extracellular bacteria, not only as initiators of inflammation, but also as regulators of factors that ultimately promote and protect lung integrity.

Materials & Methods

Ethics statement

All mouse experiments were performed in accordance with Austrian law and after approval by the Institutional Review Board of the Medical University of Vienna and the Austrian Ministry of Sciences (BMWF-66.009/0284-WF/V/3b/2014). All experiments were performed under anesthesia with a mixture of ketamine (100mg/kg) and xylazine (20mg/kg) and all efforts were made to reduce, refine and replace animal experiments.

Mice

Eight weeks old female C57BL/6 wild-type, *Ifnar1*$^{-/-}$ mice [37], Ifnar$^{fl/fl}$ mice [38], Lysm-Cre$^{+/-}$ mice [39], Cd11c-Cre$^{+/-}$ mice [40] and Sftpc-CreER$^{+/-}$ mice [41] (kindly provided by Prof. Brigid Hogan) were used. Littermate Cre^{+} mice and Cre^{-} control mice were used for experiments.

Bacterial strains and infection models

Mice were infected with the indicated doses of *S. pneumoniae* as previously described [18,42-44]. *S. pneumoniae* strain ATCC 6303 was used for all experiments involving only wildtype bacteria. Manipulated bacteria, such as pneumolysin deficient *S. pneumoniae* and GFP^{+} *S. pneumoniae* were on the D39 background and respective wild-type D39 control strains were used. Infection of mice and processing of samples is described in the online data supplement.

Histology, Immunohistochemistry and Immunofluorescence

Hematoxylin and eosin staining, proSP-C immunostaining, TUNEL staining and proSP-C and TUNEL co-staining are described in the online data supplement.

qRT-PCR and RNA profiling

RNA was isolated as previously described [18]. Data is presented as fold change over the appropriate control group, i.e. mock infected mice in the case of *in vivo* experiments and vehicle treated cells for *in vitro* studies. A list of used primers is included in the online data supplement. For RNA profiling the RT^{2} Profiler Array "Type I Interferon Response" from Quiagen (Cat.Nr. 330231 PAMM-016ZA) was used. Data is presented as fold change over control AM sorted from mock infected mice.

Flow cytometry

The preparation of lung cell suspension was performed as previously described [18]. Antibodies used are described in the online data supplement.

Cell culture and stimulations

Cell lines, culture conditions and reagents used for cell culture stimulations are described in the online data supplement.

Sterile acute lung injury

ALI was performed as previously described [45] with minor modifications. 60µl of 0.1N HCL (Sigma) was instilled into the trachea of anesthetized mice with the help of an otoscope. After 8h mice were sacrificed with a mixture of ketamine and xylazine. After collecting BALF for cell-counts and protein measurements, lungs were removed and homogenized as described for pneumonia models. In some experiments 750µg Evans Blue (Sigma) was injected i.v. 7.5h after acid aspiration. After 30min, mice were sacrificed. The measurement of Evans Blue extravasation is described in the online data supplement.

Statistics

Comparisons between groups were performed using unpaired, two-sided *t*-test or one-way ANOVA followed by Tukey's multiple comparisons analysis, where appropriate. Sample sizes for *in vivo* experiments were calculated based on previous experience. Data is presented as mean ± S.D.

Acknowledgement

We thank Brigid L.M. Hogan and Dagmar Stoiber for generously providing us with mouse strains.

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Online Data Supplement:

Type I Interferon Promotes Alveolar Epithelial Type II Cell Survival during Pulmonary Inflammation

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Online Data Supplement Figure Legends.

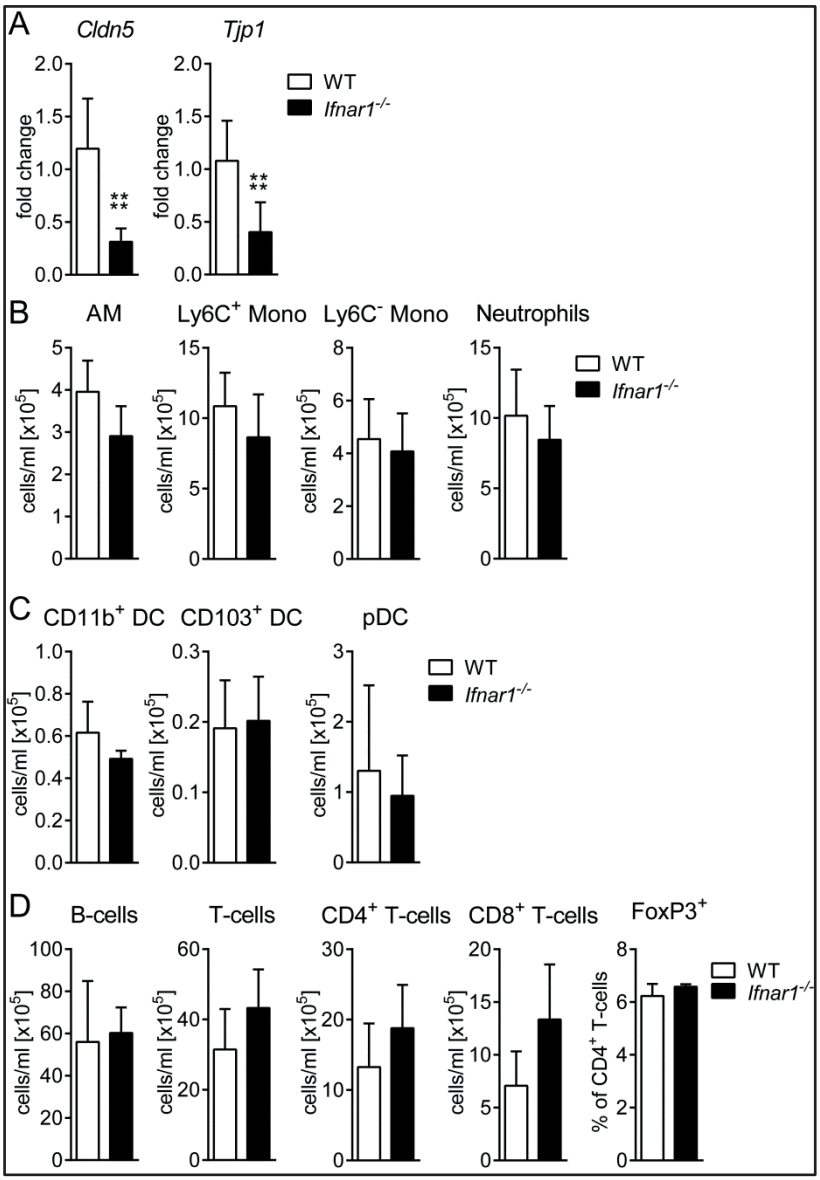

Figure E1. *Ifnar1*$^{-/-}$ mice exhibit reduced epithelial barrier protective genes, whereas immune populations are unaltered. (A) WT and *Ifnar1*$^{-/-}$ mice were infected i.n. with 10^5 CFU *S. pneumoniae* (ATCC 6303) (n≥8 for each group). 40h p.i. *Cldn5* and *Tjp1* levels were quantified by qRT-PCR from lung homogenates. (B,C,D) Lungs of naïve WT and *Ifnar1*$^{-/-}$ mice were digested and analyzed by flow cytometry using the following marker panels: AM: CD45$^+$ CD11c^{hi} Siglec F$^+$; Ly6C$^+$ Mono: CD45$^+$ CD11c$^-$ CD11b$^+$ Ly6C$^+$; Ly6C$^-$ Mono: CD45$^+$ CD11c^{int} CD11b$^+$ Ly6C$^-$; Neutrophils: CD45$^+$ CD11c$^-$ Ly6G$^+$; CD11b$^+$ DC: CD45$^+$ CD11c$^-$ MHCII^{hi} CD11b$^+$; CD103$^+$ DC: CD45$^+$ CD11c$^-$ MHCII^{hi} CD103$^+$; B-cells: CD45$^+$ CD19$^+$; T-cells: CD45$^+$ CD3$^+$

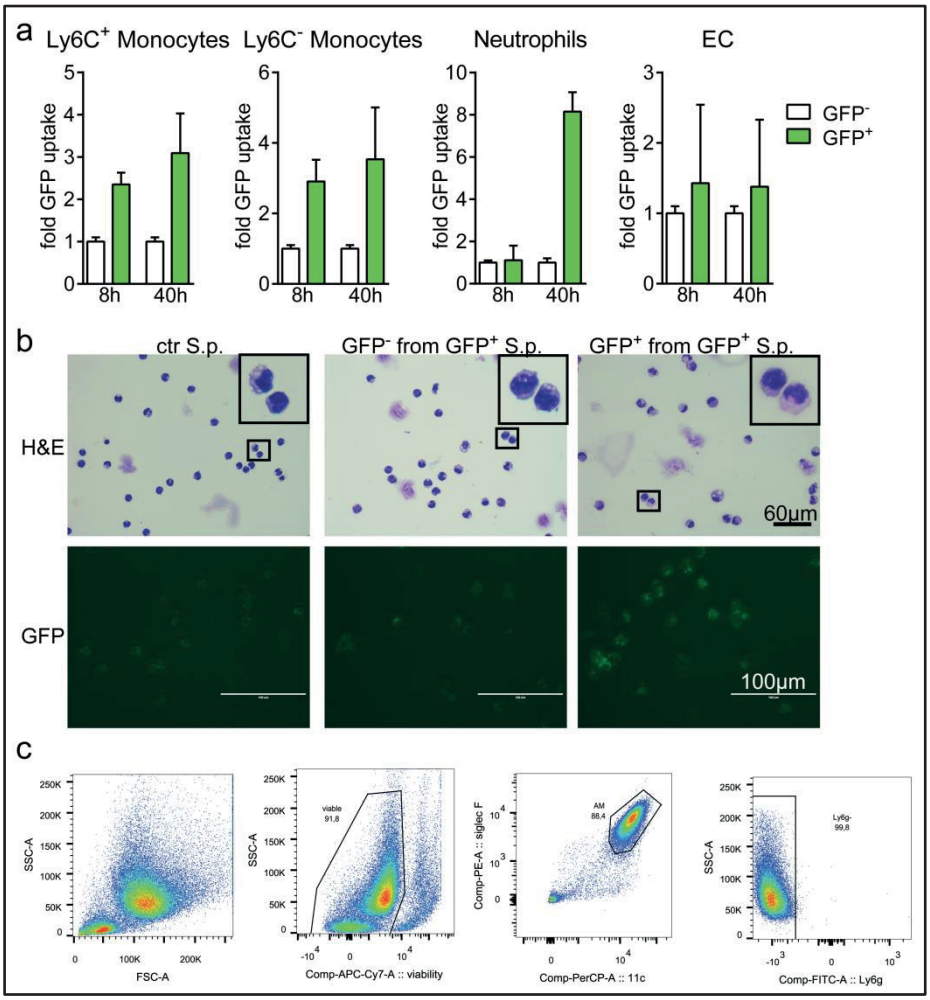

Figure E2. Uptake of GFP+ bacteria by lung immune cells. (A) WT mice were infected with 10^7 CFU GFP$^+$ *S. pneumoniae* strain D39 or GFP$^-$ control D39. Lungs were prepared for flow cytometry and immune cell subsets were analyzed for GFP positivity at indicated time points. (B) 40h p.i. CD45$^+$ CD11c$^-$ F4/80$^+$ Ly6C$^+$ CD11b$^+$ Ly6G$^-$ monocytes were sorted by FACS from D39 GFP$^-$ ctr infected mice. From D39 GFP+ infected mice GFP$^+$ and GFP$^-$ monocyte populations (same markers as above) were sorted separately. Cytospin preparations of sorted cells were immediately analyzed for GFP. (C) Gating strategy to obtain pure AM populations for RNA extraction. Representative images of n=4 mice per group are depicted (magnification 40x (H&E) and 20x (GFP)), next to a quantification of all samples. Data are mean +/- SD and results are representative of 2 independent experiments.

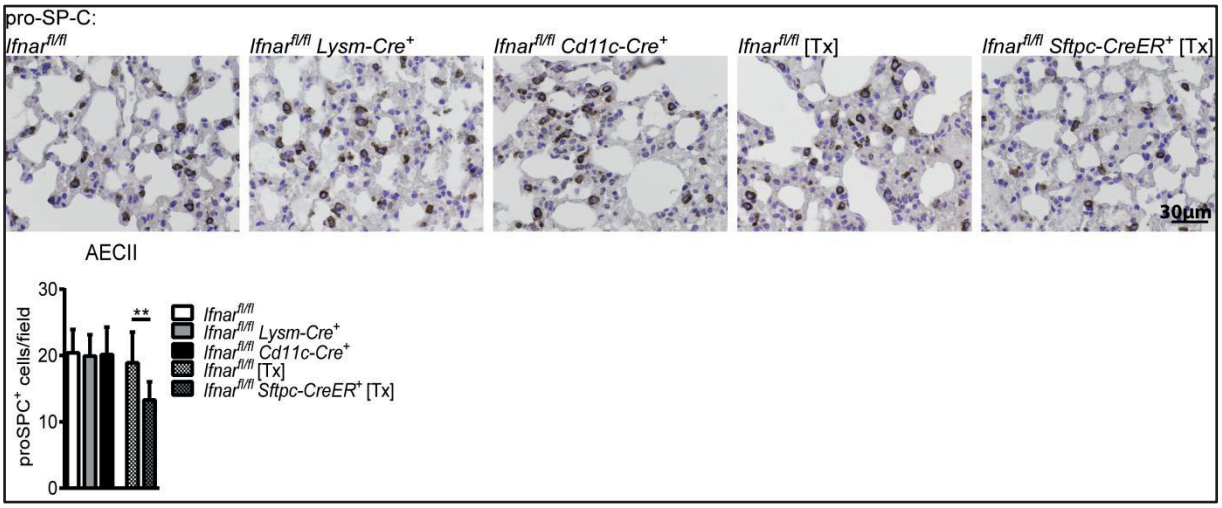

Figure E3. Ifnarfl/fl Sftpc-CreER+ mice show decreased AECII numbers upon pneumococcal pneumonia. *Ifnar*$^{fl/fl}$, *Ifnar*$^{fl/fl}$ *Lysm-Cre*$^{+}$, *Ifnar*$^{fl/fl}$ *Cd11c-Cre*$^{+}$ and tamoxifen treated *Ifnar*$^{fl/fl}$ and *Ifnar*$^{fl/fl}$ *Sftpc-CreER*$^{+}$ mice were infected with *S. pneumoniae* (ATCC 6303) and lungs removed 40h p.i. were stained for ProSP-C. Representative images of n≥6 mice per group are depicted (magnification 40x), next to a quantification of all samples. Data are mean +/- SD and results are representative of 2 independent experiments. ** $p \leq 0.01$.

Online Data Supplement Materials and Methods.

Bacterial strains and infection models

Mice were infected with the indicated doses of *S. pneumoniae* as previously described [1-3]. *S. pneumoniae* strain ATCC 6303 was used for all experiments, unless indicated otherwise. Pneumolysin deficient *S. pneumoniae* and GFP^{+} *S. pneumoniae* were on the D39 background and respective wild-type D39 control strains were used. Bacteria were cultured in Todd-Hewitt broth to mid-logarithmic phase, washed and inoculated into anesthetized mice intra-nasally. Bacteria were incubated at 65°C for 30min for heat-inactivation. DNAse treatment of HI bacteria was performed with DNAse I (Sigma) for 1h at 37°C followed by DNAse inactivation by incubation at 75°C for 10min. 8, 16 or 40 hours post infection mice were sacrificed with a mixture of ketamine and xylazine and blood samples were collected in heparin coated tubes. BALF was taken in some experiments by flushing the lung with 1ml of sterile NaCl through a needle inserted into the trachea via a midline incision as described earlier [4]. BALF cell numbers were determined using a hemocytometer and Giemsa stained cytospin preparations were used for differential cell counts. BALF supernatants were stored at -80°C until cytokine measurements and protein quantifications were performed. Total protein in BALF was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Lung and spleen were harvested and

homogenized using Precellys 24TM (Peqlab) and bacterial counts were quantified by plating 10-fold serial dilutions of blood and tissue homogenates on blood agar plates. Remaining lung and spleen homogenates were incubated for 30min in lysis buffer (containing 300mM NaCl, 30mM Tris, 2mM $MgCl_2$, 2mM $CaCl_2$, 1%Triton X-100, and pepstatin A, leupeptin, and aprotinin (all 20ng/ml; pH 7.4; Sigma-Aldrich)) at 4°C, centrifuged at 1500*g* at 4°C, and supernatants were stored at −80°C until cytokine measurements were performed [3]. ELISA kits for mouse IL-6, IL-1ß, Cxcl1 and IL-10 were purchased from R & D Systems, the ELISA kit for mouse IFN-β was purchased from PBL interferon source.

10 days prior to infection, tamoxifen-inducible Cre was activated by i.p. injecting 5mg tamoxifen (Sigma) dissolved in corn oil on 4 consecutive days.

Histology, Immunohistochemistry and Immunofluorescence

For histologic examination lungs were fixed for two days in 7.5% formaldehyde and embedded in paraffin. 4µm thick lung sections were stained with hematoxylin and eosin or processed further for immunohistochemistry or immunofluorescence. Hematoxylin and eosin stained sections were analyzed by a pathologist who was blinded for groups. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, edema, endotheliitis, bronchitis, pleuritis, perivascular inflammation and thrombi formation. Each parameter was graded on a scale of 0 to 4 as follows: 0, absent; 1, mild; 2, moderate; 3, moderately severe; and 4, severe. The “inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 28. The presence of confluent infiltrates was termed “pneumonia” and scored for its presence (1, pneumonia present; 0, pneumonia absent) and quantified in relation to the total lung surface (0.5 points per 10% infiltrate). The “histology score” was expressed as the sum of the scores for the latter parameters. For proSP-C immunostaining, endogenous peroxidase activity was quenched and antigen was retrieved with Antigen Unmasking Solution (Vector, H-3300) for 5min. Blocking was done in normal goat serum for 10min and the slides were stained with anti-proSP-C (Abcam, ab40879) for 90min. A secondary goat-anti-rabbit antibody (Vector Lab, BA-1000) was used for 30min and the signal was amplified with Vectastain ABC kit (Vector, PK-6100) and Peroxidase Substrate Kit (Vector, SK-4100) was applied. Cell structures were stained with hematoxylin and pictures were taken on an Olympus

FSX100 Microscope. For analysis, ten pictures per lung were taken and manually screened for proSP-C$^+$ cells per field. For TUNEL staining, slides were stained with TUNEL reaction mix (Roche, In situ cell death detection kit, AP, 11684809910). Nuclei were stained with DAPI and slides were kept in Prolong Antifade Reagent (Life Technologies P36930). Automatic image analysis with CellProfiler software was performed to quantify TUNEL stain [5] (http://www.cellprofiler.org/). CellProfiler was programed to: a) split images into DAPI (blue), and TUNEL (green) channels; b) load the split channels into the pipeline and identify primary objects (nuclei, DAPI) using the Otsu adaptive method with 2-class thresholding (threshold range 0.14-1), minimized weighted variance and intensity method to distinguish clumped objects; c) retrieve the respective DAPI and TUNEL integrated intensities of each identified nucleus. The integrated DAPI and TUNEL intensities were then normalized to the average DAPI integrated intensity. Primary objects touching the image borders are excluded from the analysis. For TUNEL and proSP-C co-staining slides were first stained with TUNEL reaction mix (Roche, In situ cell death detection kit, TMR red 12156792910), then blocked with normal goat serum for 10min and stained for proSP-C for 60min. As a secondary antibody an A647 conjugated goat-anti-rabbit antibody (Life Technologies, A-21244) was used for 30min. Then nuclei were stained with DAPI and slides were kept in Prolong Antifade Reagent. Pictures were taken on a Zeiss Axioimager microscope. For analysis, ten pictures per lung were taken and manually screened for TUNEL$^+$ proSP-C$^+$ cells per field.

qRT-PCR and RNA profiling

Sequences of primers used for RT-PCR

Gene	**5'**	**3'**
Ifit1	CAGCAACCATGGGAGAGAATGCTGA	GGCACAGTTGCCCCAGGTCG
PKR	AAGTACAAGCGCTGGCAGAA	GCACCGGGTTTTGTATCGAC
Cldn5	GTGCCGGTGTCACAGAAGTA	TTGACCGGGAAGCTGAACTC
Tjp1	CGTACGACAGTAGAACACGCT	AGATGCTACTTCCGGAGGCTTA
Fizz1	TCCAGCTGATGGTCCCAGTG	AAAGCCACAAGCACACCCAGT
YM1	TCTGGGTACAAGATCCCTGAACTG	GCTGCTCCATGGTCCTTCCA
Mrc	TCTGGGCCATGAGGCTTCTC	CACGCAGCGCTTGTGATCTT
CD74	TCCTTGGGCCTGTGAAGAAC	GTGGGCTTCTTCTCCTCCAG
Sftpc	TAGCATCCACAGGGTCGGTA	TTGTTTTCCAATCAGGCTGC
Lpcat1	GCACGAGCTGCGACTGAG	AGTCCACGACCTTCCTCCATA

Flow cytometry

To analyze alveolar cell populations, 5ml BALF was retrieved as described above, washed twice with PBS and cells were directly stained with respective Abs. For Annexin V staining cells were washed in 1.25mM $CaCl_2$ in PBS and stained for 20min with Annexin V-FITC Apoptosis Detection Kit (eBioscience) and 7-AAD Viability Dye (eBioscience).To prepare whole lung cell suspensions, lungs were perfused in situ via injection of PBS through the right ventricle, cut into small pieces and digested in RPMI containing 5%FCS, 150U/ml Collagenase Type I (Gibco, Cat.Nr. 17100) and 50U/ml DNAse I (Sigma) for 40min at 37°. Single cell suspensions were obtained by repeatedly flushing the samples through an 18G needle followed by a 70µm filter. Cells were counted and $2x10^6$ cells were stained in PBS containing 2%FCS for 30min at 4°C. Viability of cells was assessed using either DAPI or Fixable Viability Dye eFluor 780 (eBioscience). Antibodies used were: BV510 coupled anti-CD45 (Biolegend, 30-F11), PE or APC coupled anti-Siglec F (BD, E50-2440), PerCP-Cy5.5 coupled CD11c (Biolegend, N418), FITC coupled Ly6G (Biolegend, 1A8), Pacific Blue coupled MHCII (Biolegend, M5/114.15.2), PE-Cy7 coupled F4/80 (Biolegend, BM8), BV605 coupled Ly6C (Biolegend, HK1.4), A700 coupled CD11b (eBioscence, M1/70). Flow cytometry and sorting was performed using a BD LSRFortessa and data were analyzed using the FlowJo software Version 10. Pictures of cells sorted

from GFP^+ *S. pneumoniae* infected cells were taken on a Zeiss Axioimager microscope.

Cell culture and stimulations

AM were isolated as described above from BALF [1] and cultured in RPMI containing 10%FCS and 1% penicillin and streptomycin. MHS cells were cultured in the same medium with the addition of 50nM 2-mercaptoethanol. AM and MHS cells were plated at a density of $5x10^5$ cells/ ml 16h prior to stimulation. All stimulations were carried out in medium containing 3%FCS. Bafilomycin A and cytochalasin D (both Calbiochem) pretreatments were performed at 1µM concentrations, respectively, 40min before addition of stimuli or bacteria. Heat-inactivated *S. pneumoniae* were used for stimulations at a concentration of 10^7CFU/ml. LPS (Sigma) was used at a concentration of 1µg/ml. Supernatants of stimulated cells were directly used for cytokine quantification and cells were taken up in 100µl Trizol for RNA extraction. MLE-12 cells were cultured in RPMI containing 2%FCS, 1% penicillin and streptomycin, 0.5% insulin-transferrin-sodium-selenite, 5mg/l transferrin, 10nM hydrocortisone and 10nM ß-estradiol. All stimulations were carried out in this medium without FCS. MLE cells were plated at a density of 10^6cells/ml in serum-free medium 16 hours prior to stimulation. Cells were pretreated with recombinant IFN-β (Biolegend) at a concentration of 1ng/ml for 30min. Purified pneumolysin purchased from a commercial source was used at a concentration of 10ng/ml. Recombinant TNFα (Biolegend) was used at a concentration of 5µg/ml. Viable *S. pneumoniae* was used for stimulations at a concentration of $5x10^5$ cells/ml and heat-inactivated S. pneumoniae were used at 10^7/ml. After stimulation supernatants were stored at -20°C until cytokine measurements were performed. Cells were lysed in CellTiter-Glo reagent (Promega, CellTiter-Glo Luminescent Cell Viability Assay) and viability was assessed with a Glomax 96 Microplate Luminometer (Promega).

Sterile lung injury

60µl of 0.1N HCL (Sigma) was instilled into the trachea of anesthetized mice with the help of an otoscope. After 8h mice were sacrificed with a mixture of ketamine and xylazine. After performing a BAL, and collecting BALF for cell-counts and protein measurements, lungs were removed and homogenized as described for pneumonia models. In some experiments 750µg Evans Blue (Sigma) was injected i.v. 7.5h after

acid aspiration. After 30min, mice were sacrificed. Evans Blue extravasation was directly measured from BALF at OD_{620}. Lung tissue was placed into 2ml formamide (Sigma) for two days at RT to extract Evans Blue dye, which was quantified using a photospectrometer at OD_{620}. A standard curve for Evans Blue dissolved in formamide at known concentrations was used to determine total amount of extracted dye from tissues. Lungs and BALF from mice not injected with Evans Blue were used to correct for hemoglobin present in the tissue. Data was normalized to mock treated, Evans Blue injected mice.

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3. Discussion

In this study we provide evidence for (i) the beneficial role of IFN-I during pneumococcal pneumonia, (ii) pneumococcal DNA to be the triggering molecule for IFN-I induction in this model, (iii) IFN-I induction in alveolar macrophages in vivo upon *S. pneumoniae* infection (iv) IFN-I to act on alveolar epithelial type 2 cells, but not on major immune populations, and (v) the survival promoting function of IFN-I on alveolar epithelial type 2 cells.

Although IFN-I has been associated with many different disease conditions and its role therein has been studied for decades, most research has focused on the signaling pathways induced by IFN-I and on the subsequent induction of ISGs. The majority of studies rely on *in vitro* data and only little is known about the relevance of those findings in *in vivo* models or patients. Some novel tools have been developed recently to allow for studying IFN-I responses and its cell biology *in vivo*. The generation of luciferase reporter mice led to the appreciation that IFN-β is expressed constitutively at low levels, with thymic epithelial cells showing the highest baseline expression in mice mice [100]. In naive lymph nodes, spleen, liver, and small intestine IFN-β has been detected and to a lesser extent also in kidney, lung and large intestine, although the precise cellular source of IFN-I remains unclear. Upon infection, DCs are important IFN-β producers in the spleen, and TNF/iNOS-producing DCs (tipDCs) were revealed as the source of IFN-β in murine listeriosis and plasmacytoid DCs upon cytomegalovirus infection [93,140]. In the respiratory tract alveolar macrophages are a source for IFN-I in many viral infections, such as Influenza, Vesicular Stomatitis Virus, Sendai Virus and Newcastle Disease Virus [141-143]. In this study we provide evidence, that also in the context of bacterial infection alveolar macrophages induce a IFN-I response. Even though the IFN-I signal on alveolar macrophages upon *S. pneumoniae* infection is not potentiated extensively, since some genes for the massive production of secondary ISGs are not upregulated, and autocrine IFN-I signaling on macrophages seems to be less important for the course of pneumococcal pneumonia, its levels are sufficient to modulate epithelial integrity. Unfortunately many lung cell populations are not easily accessible and it is therefore unclear how much other cells as for example epithelial cells contribute to the secreted IFN- β found in the whole lung. The generation of

more sensitive reporter mice that would also indicate low level IFN-I expressing cells *in vivo* would significantly increase our understanding of the cell biology of IFN-I responses.

In a study using *Ifnar1*$^{fl/fl}$ *villin-Cre* mice, which are deleted for IFN-I specifically in intestinal epithelial cells, it was observed that epithelial cell proliferation and total cell numbers of epithelial cells are controlled by IFN-I [144]. In the intestine, IFN-I is contributing to Paneth cell and goblet cell proliferation and to maintaining their numbers, as seen by reduced Paneth and goblet cells in *Ifnar1*$^{fl/fl}$ *Villin-Cre* mice. These changes lead to an accelerated tumorigenesis upon DSS induced colitis, although inflammatory parameters are unchanged [144]. When co-housing intestinal epithelium-specific IFN-I deleted mice with wildtype controls their defects disappear, indicating that the intestinal microbiota is changed in those animals, which is ultimately the underlying cause for the observed phenotype. Although we didn't detect any changes in AECII numbers in IFN-I signaling deficient mice at steady state, it would be interesting to quantify other specialized lung epithelial cell types at baseline and upon *S. pneumoniae* challenge or other stimuli and determine the effect of intestinal and/or pulmonary microbiota on lung epithelial cells, since our data together with other published reports suggest a role for IFN-I as an important regulator of lung epithelial cell types [141]. Mice deficient for microbiota, either by housing them under germ-free conditions (gnotobiotic mice) or by administering antibiotics for a prolonged period of time, display heavy degeneration of the lung epithelium, and their *Ifnb* expression in the respiratory tract upon influenza virus infection is significantly reduced which leads to defects in mounting an adaptive immune response. These effects can be partially reversed by treatment with pI:C, which induces strong IFN-I secretion [145]. This data could indicate that baseline expression of IFN-I is one factor that helps maintaining the niche for lung microbiota which is in turn important for a functional epithelium. We hypothesize, that IFN-I and the microbiota are two important interdependent factors regulating the epithelial barrier, given that on the one hand IFN-I is involved in regulating the pulmonary epithelium ([141], our data) but also the microbiota [144] and on the other hand the host microbiota is needed in order to establish IFN-I responses [145].

Here we demonstrate a protective role for IFN-I on epithelial cells in an inflammatory setting, which is likely to be mediated by interference with TNF signaling. This is a remarkable finding since it is well established that IFN-I executes pro-apoptotic or pro-pyroptotic functions in many cell types. In infectious settings it has been shown that inflammasome activation as well as IL-1 expression and processing are inhibited by IFN-I in the lung [120,122]. In tumor cells and virus infected cells IFN-I directly drives apoptosis via induction of different cellular mediators like TRAIL receptors, CD95, PKR or caspases [146]. IFN-I driven apoptosis can be an important factor in clearing virus infected cells, if only infected cells are targeted and if apoptosis is executed before the virus could spread to other cells. In the case of encephalomyocarditis virus (EMCV) infection IFN-I induced PKR regulates apoptosis which is an important mechanism to prevent the virus infection from fully establishing infection of different host sites [147]. The importance of IFN-I driven apoptosis in protecting from viral infections is also illustrated by the fact that many viruses have evolved strategies that counter-act apoptosis of host cells, for example the direct expression of viral proteins that block host PKR. In some malignancies IFN-I has been shown to promote survival of immune cells, as for example B-cells survival in chronic lymphocytic leukemia [148].

For bacterial infections it has been shown in various models that bacteria tend to exploit IFN-I signaling to induce immune cell death and thereby weaken host immune responses [149-152]. *In vivo* studies indicate that splenic apoptosis is reduced in IFN-I signaling deficient mice upon *L. monocytogenes* infection and more specifically it has been demonstrated that splenic macrophage apoptosis is driven by IFN-I upon *S. typhimurium* infection [123,153]. In the lungs, IFN-I was shown to promote macrophage apoptosis upon *C. muridarium* infection whereas no difference was reported in lymphocytes and epithelial cells [126]. Interestingly we didn't find enhanced macrophage apoptosis in the absence of *Ifnar1* in alveolar macrophages, which can be explained by the fact that although IFN-I is induced, genes especially involved in the production of ISGs in large quantities such as *Stat2* or *Irf7* are not upregulated in AM and we therefore hypothesize that pro-apoptotic or pro-pyroptotic IFN-I dependent genes are not induced in AM upon infection with *S. pneumoniae*. On the contrary, we see that AECII are protected from cell death by IFN-I in our model. This finding is a rare indication for a pro-survival signal induced by IFN-I signaling. We speculate that IFN-I might play a dual role in controlling cell death of different

populations in the context of bacterial infections by on the one hand promoting cell death of immune cells in many bacterial infections but on the other hand protecting non-immune cells from cell death. This could be an important mechanism for resolution of inflammation and restriction of tissue damage and represents IFN-I as an important regulator of cell death upon bacterial infection. Further, IFN-I contributes to actively limiting tissue damage, which is an important process to ensure the basic functions of a tissue can be carried out.

Our results indicate that IFN-I directly strengthens the respiratory epithelial barrier under inflammatory conditions, which could be exploited in the clinics for treatment of pneumonia patients. We hypothesize, that IFN-I could support respiratory function by limiting edema formation in the lung. Further, IFN-I treatment could result in the reduction of systemic spread of pathogens and inflammation-triggering compounds. IFN-I therapy has been widely used in the past for treatment of various conditions, such as multiple sclerosis, hepatitis B and C, melanoma and other solid tumors and hematologic disorders [154-158]. Unfortunately, the effects of IFN-I are very unspecific and therefore patients undergoing IFN-I treatment suffer from system wide side-effects. Shortly after IFN-I treatment a cytokine response is triggered in patients, characterized by the upregulation of IL-6, IL-1 and TNF, which leads to flu-like symptoms like fever, fatigue, chills, etc. Blood leukocyte counts also significantly decline after IFN-I administration, caused by a differentiation and growth arrest of bone marrow precursors. This is accompanied by anemia and thrombocytopenia [154]. Liver and gastro-intestinal cytotoxicity is also a frequent side effect of IFN-I treatment, indicated by elevated transaminase levels in the blood and symptoms like nausea, vomiting and diarrhea. In addition also side effects on the renal, pulmonary, endocrine and cardiovascular systems have been described. Chronic effects include the occurrence of various autoimmune conditions, such as most frequently autoimmune thyroiditis, but also hepatitis, arthritis, psoriasis, diabetes, systemic lupus erythematosus, multiple-sclerosis-like symptoms and other neurological disorders. The mechanisms for IFN-I driven induction of autoimmunity are not entirely clear and probably multifactorial. Exacerbation of already pre-existing subclinical autoimmunity and driving expression of antigens that are known to be involved in autoimmune symptoms are two possibilities that have been suggested [154]. Due to

those diverse side-effects IFN-I therapy is being substituted by more targeted agents that don't show systemic effects.

However, many of the more severe side-effects only occur after long-term treatment with IFN-I, meaning therapy for several weeks and months up to years. The course of pneumonia is rather quick and treatment is needed only for a period of few weeks, which would make IFN-I therapy possible. One of the difficulties would be to counter-act the systemic pro-inflammatory effects of IFN-I that could potentially exacerbate fever and sepsis in patients. Another important aspect is the context and the underlying cause of lung inflammation. In pneumonia models other than *S. pneumoniae* infection, IFN-I have been described as detrimental to the host, including acute tuberculosis infection and infection with *Chlamydia muridarum* [126,159]. Those observed differences between dominant effects of IFN-I depending on the pathogenic cause of lung inflammation most likely is caused by different inflammatory microenvironments that differentially affect the involved cell types. This highlights the immune-modulatory role of IFN-I acting in a context dependent manner. Thus, a more detailed understanding of the precise conditions that promote the protective functions of IFN-I has to be achieved to further speculate about potential therapeutic implications. To compare the inflammatory niche and the profile of all contributing and involved cell types in conditions under which IFN-I is protective during lung inflammation versus conditions under which IFN-I is detrimental during lung inflammation could help to find distinguishing factors between the two scenarios to find potential co-factors influencing IFN-I responses.

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Curriculum Vitae

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Personal Information

Date of birth: 07/31/1987
Place of birth: Vienna, Austria
Nationality: Austrian

Academic Education

01-06 2014: Research in external laboratory within PhD studies: Prof. Miriam Merad's laboratory, Tisch Cancer Institute, Icahn School of Medicine at Mt. Sinai, New York, USA; In vivo uptake of fluorescently labelled bacteria
10 2011: start of PhD studies: Laboratory of Infection Biology, Medical University of Vienna, Vienna. Supervisor: Prof. Dr. Sylvia Knapp
2010 – 2011: Master of Science: Division of Microbiology and Immunobiology, Max F. Perutz Laboratories, University of Vienna, Vienna. Supervisor: Prof. Dr. Manuela Baccarini
2005 – 2011: Studies of Molecular Biology: University of Vienna, Vienna.

Teaching Experience

Supervision of an internship student (Judith Konrat)

Professional Experience

2011 – present: PhD studies on Innate Immune Recognition of *Streptococcus pneumoniae*. Topics: 1) Haploid genetic screen for host factors in bacterial infection. 2) Role of type I interferon in *Streptococcus pneumoniae* infection. 3) Effect of endotoxin tolerance on infection *in vivo*.
2010 – 2011: Master thesis: "B-Raf in tumor-driven angiogenesis"

04/2011 Internship: "Effect of B-Raf/C-Raf 3 Alleles Knock-out on Embryonic Angiogenesis". Department of Microbiology and Immunobiology, Max F. Perutz Laboratories, University of Vienna, Vienna. Supervisor: Prof. Dr. Manuela Baccarini

01-02/2011 Internship: "Role of MicroRNA-193a in Kidney Development". Institute of Molecular Biotechnology, Vienna. Supervisor: Dr. Xavier Martinez

11-12/2010 Internship: "Involvement of SUN-1 in meiotic chromosome homology search and movement". Department of Chromosome Biology, Max F. Perutz Laboratories, University of Vienna, Vienna. Supervisor: Prof. Dr. Verena Jantsch

05-06/2010 Internship: "Genetic Generation of Chordin and Smad1 Knock-in *Nematostella vectensis* strains by Intranuclear Microinjection" Department of Molecular Evolution and Development, University of Vienna, Vienna. Supervisor: Prof. Dr. Ulrich Technau

Career related activities

2012 & 2013 Member of Organizing Committee of the annual Workshop "Bridging the Gap"

Attended conferences

1. **8th PhD-Symposium of the Medical University of Vienna, 2012**, (Poster presentation)
2. **Symposium of the Austrian Society for Allergology and Immunology, Klosterneuburg, 2012** (Poster Presentation)
3. **9th PhD-Symposium of the Medical University of Vienna 2013** (Short Talk)
4. **5th – 7th International Bridging the Gap Symposium, Vienna 2012 – 2014** (part of the organizing committee)
5. **28th annual conference of the European Macrophage and Dendritic Cell Society (EMDS), Vienna, 2014** (Poster presentation)
6. **8th International Bridging the Gap Symposium, Vienna 2015** (Short Talk)
7. **Systems Biology of Infection Symposium, 2nd Edition, Ascona, Switherland 2015** (Poster Presentation)

Abstracts

1. Maier B, Sigel S, Knapp S (2012) **Intracellular recognition machinery for Streptococcus pneumonia**. (PhD-Symposium of the Medical University of Vienna)
2. Maier B, Sigel S, Strobl B, Müller M, Knapp S (2012) **Role of type I interferon in Streptococcus pneumoniae infection in vivo.** (Symposium of the Austrian Society for Allergology and Immunology)
3. Maier B, Knapp S (2013) **Identifying novel genes involved in the recognition of S.pneumoniae by haploid genetic screening.** (Short Talk at the PhD-Symposium of the University of Vienna)
4. Maier B, Sigel S, Strobl B, Müller M, Knapp S (2014) **Role of type I interferon in Streptococcus pneumoniae infection in vivo.** (Conference of the European Macrophage and Dendritic Cell Society)
5. Maier B, Gawish R, Knapp S (2015) **LPS induced disease tolerance in Gram-negative sepsis.** (Short Talk at Bridging the Gap Symposium)
6. Maier B, Hladik A, Martins R, Lakovits K, Korosec A, Kral J, Mesteri I, Strobl B, Merad M, Knapp S (2015) **Alveolar macrophage derived type I interferon directly protects alveolar epithelial type II cells from cell death in a murine pneumonia model.** (Systems Biology of Infection Symposium, 2nd Edition, Sept 2015, Ascona, Switzerland)

Awards

06/2013 **Award for best oral presentation** at the YSA PhD Symposium in Vienna

Publications

2013 A reversible gene trap collection empowers haploid genetics in human Cells.

Bürckstümmer, T, Banning, C, Hainzl, P, Schobesberger, R, Kerzendorfer, C, Pauler, FM, Chen, D, Them, N, Rebsamen, M, Smida, M, Fece de la Cruz, F, Lapao, A, Liszt, M, Eizinger, B, Guenzl, P, Konopka, T, Gapp, B, Paparatics, K, Maier, B, Stöckl, J, Fischl, W, Salic, S, Taba, R, Knapp, S, Bennett, KL, Bock, C, Colinge, J, Kralovics, R, Ammerer, G, Casari, G, Brummelkamp, TR, Superti-Furga, G and Nijman, SMB. Nat Methods, 2013 Oct;10(10):965-71. doi: 10.1038/nmeth.2609.

2012 Angiogenic sprouting requires the fine tuning of endothelial cell cohesion by the Raf-1/Rok alpha complex.

Wimmer, R, Cseh, B, Maier, B, Scherrer, K, Baccarini, M. Dev Cell, 2012 Jan 17;22(1):158-71. doi: 10.1016/j.devcel.2011.11.012.

Memberships in professional organizations

Austrian Association of Allergy and Immunology (ÖGAI)