ST2 and IL-33 differentially regulate the innate immune response to *Streptococcus pneumoniae*

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1—Introduction

1.1 The innate immune system

The immune system plays an essential role in protecting the host against infections. In addition, it monitors cellular integrity and responds to cells that have been injured or killed. The innate immune system is the first line of defense against invading pathogens and harm. It is found in all classes of plant and animal life and thus constitutes an evolutionarily ancient defense strategy (1, 2). Beside the natural anatomical epithelial barriers, the innate immune recognition relies on a limited number of germ line-encoded receptors, which detect conserved products of the microbial metabolism produced by pathogens or endogenous molecules released after tissue damage. Upon detection of these so-called pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) the innate immune system initiates an acute inflammatory response that rapidly delivers soluble and cellular defenses to the site of infection or damage. This is characterized by the production of proinflammatory cytokines and chemokines, the recruitment of leukocytes, and might lead to further activation of the adaptive immune system through antigen presenting cells. Thus, the innate immune system is important for initiating and orchestrating the immune response of the host (3, 4).

The innate immune response is initiated by tissue resident cells. In bacterial lung infections these are mainly resident alveolar macrophages (AMΦs), while other cell types including dendritic cells (DCs) and epithelial cells contribute to the response (5). These cells express pattern recognition receptors (PRRs) on the surface or within the cell in order to sense PAMPs or DAMPs. Once stimulated they produce proinflammatory cytokines and chemokines, that activate neighboring cells and attract phagocytic and antigen-presenting leukocytes such as neutrophils, exudate macrophages, and DCs. The vasodilation increases rapidly and adhesion molecules are up-regulated on endothelial cells, which allows the migration of attracted leukocytes and the leakage of proteins such as complement, lectins and antibodies into the local environment. The recruited innate immune cells attack the invading microbes by phagocytosis, secretion of antimicrobial peptides, production of reactive oxygen species (ROS) and nitrogen intermediates, and the formation of neutrophil extracellular traps (NETs). These effector mechanisms are extremely potent in killing pathogens, however, an overwhelming innate immune response can also damage and kill host cells (4, 6–8).

1.1.1 RECOGNITION OF MICROBES BY THE INNATE IMMUNE SYSTEM

The recognition of invading pathogens or injured cells by PRRs is a cornerstone of innate immunity. The evolutionarily conserved and germline-encoded PRRs belong to several protein families and functional groups including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and cytosolic DNA receptors which are able to sense various classes of molecules such as glycolipids, proteins, carbohydrates and nucleic acids (9).

1.1.1.1 Toll-like receptors

TLRs are the most widely studied PRRs. To date, ten functional TLRs have been identified in humans and twelve in mice. TLRs are either primarily expressed on the cell surface (e.g. TLR1, 2, 4, 5, 6) or exclusively within endocytic compartments (e.g. TLR3, 7, 8, 9). Extracellular TLRs recognize mainly microbial membrane components and flagellin, whereas intracellular TLRs detect nucleic acids (10). For example, TLR4 responds to bacterial lipopolysaccharide (LPS)

and TLR2 detects bacterial lipopeptides, which are major components of the outer membrane of gram-negative bacteria or the cell wall of gram-positive bacteria, respectively (11, 12). TLR5 is known to detect the flagellin of flagellated bacteria (13) and TLR9 senses unmethylated CpG motifs in double-stranded DNA (14).

TLRs belong to the TLR/interleukin-1 receptor (IL-1R) superfamily and contain an intracellular Toll/interleukin-1 receptor (TIR) domain (15). Upon ligand binding, the TLRs homo- or heterodimerize and activate downstream signaling pathways. TLR signaling is primarily meditated via the recruitment of different TIR domain-containing adaptor molecules such as MyD88 (myeloid differentiation primary response 88), TRIF (TIR-domain-containing adapter-inducing interferon-β), TIRAP (TIR-containing adaptor protein), and TRAM (TRIF-related adaptor molecule) (16). MyD88 is recruited by all TLRs, except for TLR3, and initiates the activation of nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPK) to induce proinflammatory cytokines. In addition, the endosomal TLRs 7-9 stimulate a second pathway through MyD88 leading to activation of interferon regulatory factor (IRF) 7-dependent type I IFN production. The recruitment of TRIF downstream of TLR3 and -4 induces a pathway that activates NF-κB as well as IRF3 and/or IRF7. In addition, TIRAP and TRAM function as sorting adaptors that recruit MyD88 to TLR1, 2, 4 and -6 and TRIF to TLR4, respectively (10). Since long lasting TLR stimulation can lead to overwhelming and damaging inflammation, the TLR signaling cascade is also negatively regulated by multiple mechanisms. These mechanisms include the dissociation of adapter molecules by e.g. SARM (sterile and armadillo-motif-containing protein) and perhaps ST2 (see below), the degradation of downstream signaling molecules by e.g. SHP (small heterodimer partner) and A20, and the inhibition of proinflammatory gene transcription by e.g. Bcl-3 (B-cell lymphoma 3) (17).

1.1.1.2 NOD-like receptors

NLRs are cytosolic PRRs that can be functionally divided into different subgroups of which two are of particular interest. The first group includes NOD1 and NOD2 and mainly activates NF-κB, MAPKs, and possibly IRFs to induce the production of proinflammatory cytokines, antimicrobial peptides, and type I IFNs (18–20). Members of the second group are involved in inflammasome formation and consist of NLRs such as NLRP3 (NLR family, pyrin domain containing 3), NLRP6, and NLRC4 (NLR family caspase recruitment domain (CARD) domain-containing protein). Inflammasomes are multi-protein complexes that mediate the

processing of caspase-1, which in turn promotes the subsequent cleavage and the release of mature IL-1β and IL-18 in response to microbes and danger signals (21, 22). More recently a novel mechanism of non-canonical inflammasome activation was identified. This pathway is dependent on caspase-11 and triggers both caspase-1-dependent and -independent production of IL-1β and IL-18 (23). The stimulation of inflammasomes also protects against infections by the induction of pyroptosis, a form of proinflammatory and lytic cell death. The activation of all NLR-signaling complexes is mediated by the characteristic central nucleotide binding and oligomerization domain (NACHT). The N-terminal domain mediates downstream signaling through homotypic protein-protein interactions (22).

1.1.1.3 C-type lectin receptors

The C-type lectin superfamily comprises more than 1000 proteins classified into 17 subgroups based on domain organization and phylogeny. They contain a conserved C-type lectin-like domain, which is able to bind to carbohydrates in a calcium-dependent manner (24). A large number of C-type lectins are involved in the recognition of PAMPs and DAMPs. Membrane-bound CLRs such as Dectin-1, Mincle (Macrophage-inducible C-type lectin), and Clec9A are expressed on innate myeloid cells including macrophages, neutrophils and DCs. Most CLRs signal via the spleen tyrosine kinase (Syk), which in turn activates NF-κB. This can be either indirectly, through the adaptors Fc receptor γ chain (FcRγ) or DAP12, which bear classical Syk-recruiting ITAM motifs, or directly via a hemITAM motif in the cytoplasmic tail of the receptor. A distinct group of CLRs contains ITIM motifs that negatively regulate signaling through kinase-associated receptors. Moreover, there are CLRs without ITAM or ITIM domains (25).

1.1.1.4 RIG-I-like receptors

RLRs are DExD/H box RNA helicases that primarily sense viral infection. The family consists of the three members RIG-I, MDA5 (melanoma differentiation associated gene 5), and LGP2 (laboratory of genetics and physiology 2) (26). RIG-I and MDA5 play non-redundant roles in cytosolic RNA sensing by recognizing different RNA molecules and groups of viruses (27). RIG-I respond to short ssRNA and dsRNA with a 5’ triphosphate end and panhandle-like secondary structures, whereas MDA5 detects longer dsRNA substrates. The RIG-I pathway can additionally be activated by 5’ phosphate-RNA that is transcribed from AT-rich

DNA by RNA polymerase III. RIG-I and MDA5 signaling is mediated through homotypic interactions of the CARD domain with the essential signaling adaptor protein MAVS (mitochondrial antiviral signaling). This results in the activation of IRF3, IRF7, and NF-κB and the production of type I IFN and inflammatory cytokines (26).

1.1.1.5 Cytosolic DNA receptors

Cytoplasmic DNA from microbial or self-origin is sensed by the recently identified cyclicGMP-AMPsynthase (cGAS), which catalyzes the production of cyclic GMP-AMP (cGAMP) from cytosolic ATP and GTP. The generated cGAMP, as well as other cyclic dinucleotides such as the bacterial second messenger's cyclic diguanylate and cyclic diadenylate, directly activate the adaptor protein stimulator of IFN genes (STING), which is localized in the endoplasmic reticulum. This leads to the activation of IRF3 and the production of type I IFN (28–30). Before the discovery of cGAS as a non-redundant and general cytosolic DNA sensor that activates STING, several other proteins, including DAI (DNA-dependent activator of IRFs) and IFI16 (interferon gamma-inducible protein 16), were suggested as candidates for cytosolic DNA sensors (26). In addition to inducing type I IFNs, cytosolic DNA also activates the absent in melanoma 2 (AIM2) inflammasome, leading to caspase-1 activation and IL-1β and IL-18 maturation. Similar to some NLR family members such as NLRP3, AIM2 is able to form an inflammasome complex through homotypic protein interactions (31, 32).

1.1.2 RECOGNITION OF CELL AND TISSUE INJURY BY THE INNATE IMMUNE SYSTEM

In 1989, Charles Janeway proposed for the first time that the innate immune system responses to infection after sensing of PAMPs (33). However, this model could not explain all immunological responses, like for example the adjuvant effect of non-bacterial alum or the inflammation after tissue trauma. Considering these expectations, Polly Matzinger postulated in 1994 that the immune system does not respond to infection per se but to non-physiological cell death and damage (34). Recent studies have shed light on the molecular mechanisms behind those immune responses, and have introduced the concept of DAMPs as important mediators of sterile inflammation.

1.1.2.1 Cell death and DAMP release

DAMPs can be defined as intracellular or extracellular matrix molecules that are released upon necrotic cell death and tissue damage and which are recognized by receptors of the innate immune system. Necrotic cell death is characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent release of the intracellular content into the extracellular milieu (35). This is in contrast to apoptosis, which is accompanied by reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation, plasma membrane blebbing and engulfment by resident phagocytes. Apoptosis and autophagy are known as forms of programmed cell death, whereas necrosis has been considered merely as accidental and uncontrolled for a long time (36). However, recent genetic evidence, as well as the discovery of chemical inhibitors of necrosis, have greatly changed this view, and revealed the existence of multiple pathways of regulated necrosis. The most understood form of regulated necrosis, termed necroptosis, is negatively regulated by caspase-8 and dependent on the kinase activity of RIPK (receptor-interacting protein kinase)-1 and/or RIPK3-MLKL (mixed lineage kinase domain-like). Further examples of regulated necrosis that are independent of RIPK1 or RIPK3 are emerging. These include cell death mechanisms known as parthanatos, oxytosis, ferroptosis, NETosis, pyronecrosis and pyroptosis (37). The latter is dependent on the inflammasome and mediated by activated caspase-1 or -11 to restrict the replication of some intracellular pathogens (22). Furthermore, it is known that in the absence of phagocytic capacity, apoptotic cells proceed to secondary necrosis characterized by the same features of necrotic cell death (38).

In general DAMPs can be divided into two groups: (i) molecules that perform non-inflammatory functions in living cells (e.g. ATP) and acquire immunomodulatory properties when released, secreted, modified, or exposed on the cell surface during cellular stress, damage, or injury or (ii) alarmins which are stored inside cells, released upon cell lysis, and then possess cytokine-like functions (e.g. IL-33). DAMPs belonging to the first group can be further classified according to their location. Intracellular DAMPs are normally located inside cells and are released upon necrotic death. Extracellularly located DAMPs, are released by extracellular matrix degradation during tissue injury. Prototypical DAMPs are, for example, high-mobility-group-protein B1 (HMGB1), S100 proteins, heat shock proteins, purine metabolites, such as ATP and uric acid, SAP130, genomic and mitochondrial DNA, actin, hyaluronan, and others (35, 39, 40).

DAMPs are recognized by the innate immune system though different receptors. Some of these receptors also detect PAMPs such as TLR2, TLR4, NLRP3, RIG-I, and Mincle. In addition, there are several other receptors that do not recognize specific PAMPs including RAGE (receptor for advanced glycation endproducts), purinergic P2X/Y receptors, and ST2. The binding of many DAMPs is not limited to one receptor, which underlines the redundancy of the innate immune system (39, 41).

1.1.2.2 Di- and trinucleotides

Cellular ATP serves as an energy carrier that drives virtually all cell functions. However, ATP and other purine nucleotides such as ADP, UTP and UDP can be released upon cell death or stress to alert the immune system (42). Extracellular nucleotides activate a large group of cell surface receptors including P2X, P2Y or adenosin P1 receptors. P2X receptors are ligand gated ion channels that open in response to the binding of extracellular ATP. On the contrary, members of the G protein-coupled P2Y6 receptor family can be activated by different nucleotides (ATP, UTP, ADP, UDP, UDP-glucose). The P2X or P2Y family consists of seven or eight members, respectively (P2X1-7, P2Y1, 2, 4, 6, 11, 12, 13, 14) (43). Extracellular ATP is crucial for the activation of inflammasomes and the subsequent release of IL-1β and IL-18 or the initiation of pyroptosis (22). Notably, ATP is not only released in response to tissue damage but can be actively secreted from intact cells in a tightly regulated manner. Transient increase in extracellular ATP is ubiquitously used for cell-to-cell communication in the nervous, vascular, and immune system. Moreover, ATP is released from apoptotic cells and serves as a find-me signal (44).

1.1.2.3 Uric acid

Uric acid is generated as part of the normal turnover of nucleic acids, when purine nucleotides are oxidized by xanthine oxidase. The molecule is released upon necrotic cell death (45), which leads to the crystallization of uric acid to biological active monosodium urate (MSU). Notably, uric acid overproduction leads to nucleation of MSU crystals in the joints and thereby causes gout. Apart from MSU, many other irritant particles and crystals (e.g. asbestos, silica, calcium pyrophosphate, and alum) are highly proinflammatory and can cause disease. MSU and other crystals activate the NLRP3 inflammasome. Moreover, it has been shown

that MSU can also stimulate inflammasome-independent pathways and activates TLR2, CD14 and the purinergic P2Y6 receptor (46, 47). Uric acid is oxidized by uricase to allantoin, which is highly soluble in water. The enzyme is present in most mammals, however, it is absent in humans (48).

1.1.2.4 SAP130

The SAP130 protein is a component of a small nuclear ribonucloprotein (snRNPs), which is associated with the spliceosome. In 2008, SAP130 has been identified as a DAMP that is recognized by the C-type lectin Mincle (49). The transmembrane receptor Mincle is associated with the ITAM-bearing adaptor FcRγ and activates Syk-kinase signaling on myeloid cells (25). In addition to SAP130, Mincle has been shown to recognize the mycobacterial glycolipid trehalose-6,6'-dimycolate (50, 51), glycolipid ligands in *Malassezia* (52) and *Candida* strains (53), and is protective during *Klebsiella pneumoniae* infections (54).

1.2 The ST2/IL-33 axis

1.2.1 THE DUAL FUNCTION CYTOKINE AND ALARMIN IL-33

IL-33 is a member of the IL-1 family which additionally consist of IL-1α, IL-1β, IL-18 as well as other molecules (55). The protein was initially discovered as a nuclear factor present in endothelial cells and first named NF-HEV (nuclear factor from high endothelial venules) (56). In 2005, Schmitz *et al*. identified IL-33 as the extracellular ligand for the orphan IL-1 receptor family member ST2 (57). Like other IL-1 family members, IL-33 is synthesized as precursor that lacks a signal peptide. However, different from e.g. IL-1 and IL-18, the full-length IL-33 is biologically active and does not require processing into a mature form (58). IL-33 is released as an alarmin following necrotic cell damage (59). Moreover, recent evidences suggest that IL-33 can be actively released through an unconventional secretory mechanism following stimulation with the DAMP ATP (60) and after mechanical stress (61). In addition to IL-33, the IL-1 family member IL-1α was identified as an alarmin (62–64). In contrast to other cytokines, IL-33 and IL-1α

are constitutively expressed in the nucleus of the cell and can be immediately released after cell death. Both proteins are associated with the chromatin suggesting a function as transcriptional regulators (64–69). Therefore, they are considered as "dual-function cytokines".

1.2.1.1 IL-33: expression, processing and secretion

IL-33 is mainly expressed by non-hematopoietic cells. In humans, IL-33 is found in endothelial cells, epithelial cells, fibroblasts, lymphoid organs, the brain and in the lung (59). Using an IL-33 LacZ reporter mouse strain, Pichery *et al*. demonstrated that the expression is mainly similar in mouse tissues (70). The analysis of *Il33Citrin* reporter mice further revealed that type 2 alveolar epithelial cells represent the predominant source of IL-33 in the lungs of naive mice (71). IL-33 is strongly expressed in inflamed tissues e.g. in the lungs of patients and mice with allergic airway inflammation (70–72) or during influenza virus infections (73). Furthermore, several reports have shown that IL-33 is up-regulated *in vitro* after stimulation with IFN-γ (74) or various Toll-like receptor ligands such as LPS (TLR4), Pam3Cys (TLR2), CpG oligonucleotides (TLR9) and others (75–78).

The N-terminal region of full-length IL-33 is necessary and sufficient for the protein to be expressed in the nucleus (65, 66) whereas binding to its receptor occurs through the C-terminal IL-1-like domain (57). Surprisingly, it has been shown that activated caspase-1 and apoptotic capases-3 or -7 cleave IL-33 inside the IL-1 domain which results in the inactivation of the protein (79–82). Thus, unlike IL-1β and IL-18, IL-33 is biologically active in its full-length form. Moreover, recent findings indicated that the activity of IL-33 can be amplified by proteases. Elastase, cathepsin G and proteinase 3 are released from recruited neutrophils during inflammation and can cleave full-length IL-33 into forms that possess a ten times greater potency to activate its receptor (83) (Fig. 1).

1.2.1.2 Intracellular IL-33: A nuclear factor

Inside the living cell, full-length IL-33 is acting as a nuclear factor independent of its extracellular alarmin-like function. In 2007, Carriere *et al*. described that the N-terminal part of IL-33 possesses a chromatin binding helix-turn-helix motive and is associated to the heterochromatin in primary human endothelial cells as well as in human and murine cell lines. Although they did not identify specific target genes, their data suggest that nuclear IL-33 has transcriptional repressor

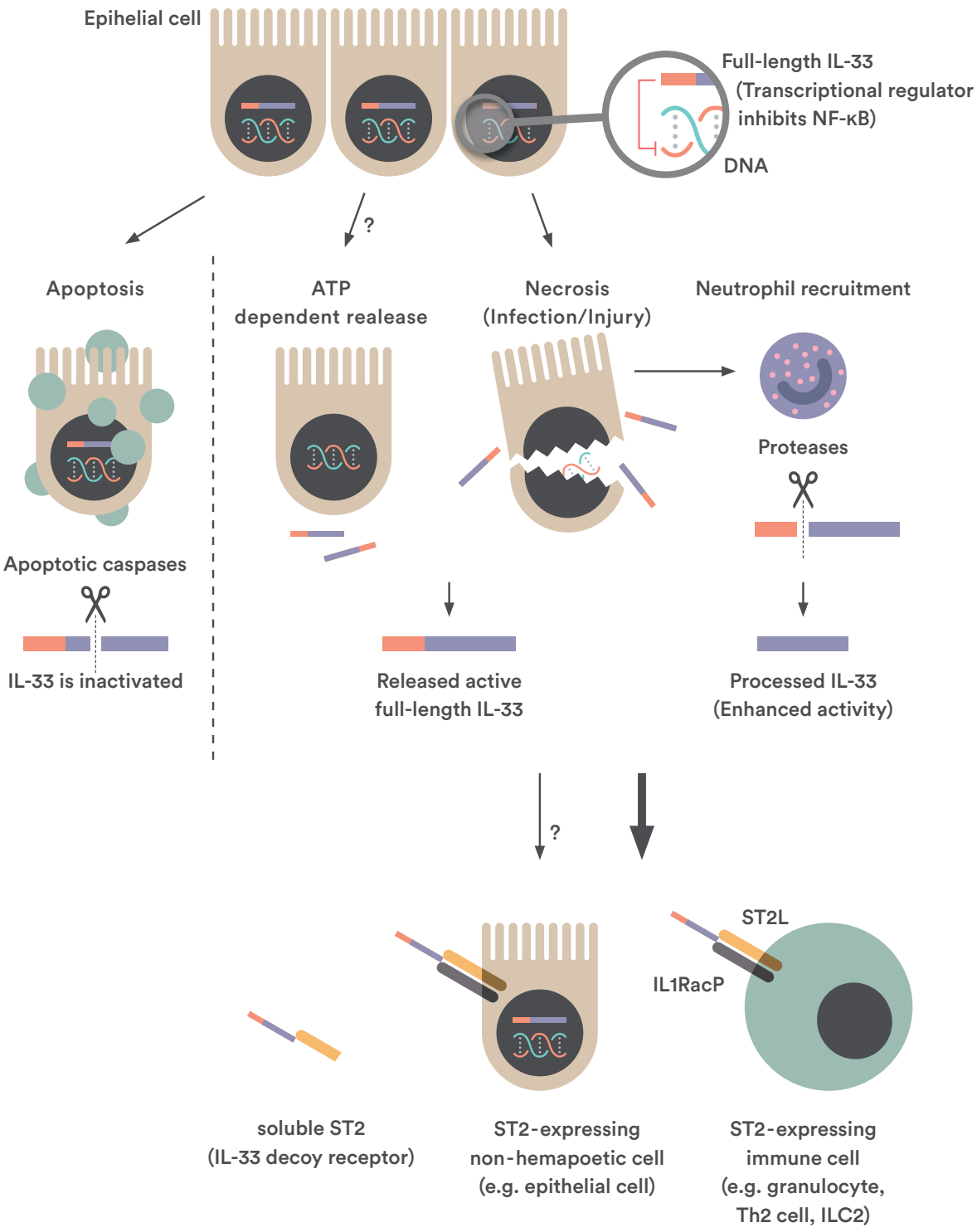

FIGURE 1: Schematic representation of the ST2/IL-33 axis. IL-33 is constitutively expressed and stored in the nucleus of e. g. epithelial cells, where it can act as a transcriptional regulator. During apoptosis, IL-33 is cleaved and inactivated by caspases, whereas it is released as an active full-length protein after necrotic cell death and possibly also after ATP stimulation. Neutrophils, recruited to the site of injury, secrete proteases that are able to cleave and amplify IL-33 bioactivity. Extracellular IL-33 activates ST2-expressing immune cells and probably non-hematopoietic cells. ST2L/IL-33 signaling can be inhibited by the decoy receptor sST2.

functions (65). Another study confirmed this finding and showed that IL-33 binds to the histone dimer H2A-H2B and hence regulates chromatin condensation (66). A recent study demonstrated that IL-33 interacts the with the NF-κB p65 subunit and thereby dampens NF-κB-dependent gene transcription (67). In addition, two independent studies showed that the knock-down of IL-33 results in the transcriptional up-regulation of inflammatory cytokines in human fibroblast-like synoviocytes (68) or endothelial cells (69).

1.2.2 THE ST2 RECEPTOR AND THE ROLE OF ST2L/IL-33 SIGNALING

1.2.2.1 The ST2 receptor

ST2 was discovered over 20 years ago as an orphan member of the Toll-like/IL-1 receptor (TIR) family (84–86). Members of this family consist of an extracellular domain, a transmembrane segment and the characteristic cytoplasmic TIR domain, which interacts with TIR-domain containing adaptor molecules (15). The ST2 gene generates at least four different gene products by alternative splicing; the soluble secreted form (sST2), the transmembrane form (ST2L), and two variant forms (ST2V and ST2LV) (87–89). In 1998, ST2L was shown to be preferentially expressed on type 2 but not on type 1 helper T cells and was therefore suggested as a stable marker for Th2 cells (90, 91). It took another seven years until Schmitz *et. al* identified IL-33 as a specific extracellular ligand for ST2L, which forms a heterodimer with its co-receptor IL-1RAcP. They demonstrated that binding of IL-33 to ST2L/IL-1RAcP triggers the recruitment of the adapter molecule MyD88 to ST2, which activates NF-κB and MAPK pathways and leads to the induction of a proinflammatory response (57). Interestingly, another report demonstrated that ST2 can exert antiinflammatory effects by negatively regulating TLR4 and IL-1 receptor (IL-1R) signaling through sequestrating the adaptors proteins MyD88 and TIRAP (92). Six years later, it was further demonstrated that ST2 also negatively regulates TLR2 signaling after bacterial lipoprotein (BPL) stimulation (93). These studies, however, did not examine whether the antiinflammatory functions of ST2 were dependent on IL-33 or not.

ST2 is expressed by a broad range of innate and adaptive immune cells including granulocytes (neutrophils, eosinophils, basophils), macrophages, mast cells, DCs, natural killer (NK) cells, invariant NKT cells, Th2 cells, cytotoxic T cells (CTLs), regulatory T cells, B cells and the recently identified type 2 innate

lymphoid cells (ILC2s) (94, 95). Moreover, ST2 expression was detected in fibroblasts and lung endothelial and epithelial cells (84, 96).

1.2.2.2 The role of extracellular IL-33 and signaling via ST2L

Once released into the extracellular space, IL-33 acts as an alarmin and signals via its receptor ST2L. Extracellular IL-33 induces mostly type 2 immune responses and plays an important role in allergy, rheumatic diseases, and the immune defense against parasites (94, 97). For example, ST2L/IL-33 signaling is protective during infections with *Trichuris muris* (98), *Toxoplasma gondi* (99), and *Nippostrongylus (N.) brasiliensis* (100). In 2010, Oboki *et al*. described that IL-33 is a crucial amplifier of mucosal and systemic innate, rather than acquired, immune responses (101), which is plausible when considering that ST2L is expressed on a broad range of innate immune cells but only on selective populations of adaptive immune cells (94).

Type 2 immune responses are characterized by the release of classical type 2 cytokines such as IL-4, IL-5 and IL-13, tissue eosinophilia, and goblet-cell hyperplasia, however, dysregulation of these responses can promote allergic diseases (102). One of the major functions of type 2 immunity is the initiation and regulation of tissue repair and a large body of evidence confirmed that ST2L/IL-33 signaling is crucial for this (103). IL-33 binding induces the production of type 2 cytokines and other type 2 mediators from Th2 cells (57, 91), mast cells (104, 105), basophils (106), eosinophils (107, 108), DCs (109), and ILC2s (100, 110–113). Furthermore, several studies demonstrated that IL-33 can shift macrophage polarization towards an alternatively activated phenotype, which also initiates type 2 responses (114–117).

Nevertheless, the IL-33/ST2 axis can promote both Th1 and Th2 immune responses depending on the type of activated cell and surrounding microenvironment. It has been shown that IL-33 directly interacts with NK cells, invariant NKT cells (118, 119), and CTLs (120) to stimulate IFN-γ production. Additionally, IL-33 treatment can trigger the secretion of typical proinflammatory cytokines such as IL-6, TNF-α or IL-1β e.g. from LPS-treated macrophages (101, 121), mast cells (122) and endothelial cells (123). Alves *et al*. reported that IL-33 treatment leads to enhanced neutrophil recruitment during experimental sepsis, which mediates increased bacterial clearance and survival in these mice. They proposed that IL-33 prevents the TLR-induced down-regulation of the neutrophil chemokine receptor CXCR2, which is crucial for the recruitment of neutrophils

from the circulation to the site of infection (124). This was confirmed by two other groups, who showed that IL-33 treatment increases the expression of CXCR2 in response to *Candida albicans* (125) or *Staphylococcus aureus* infections (126). In line with these findings are observations of Buckley *et al*. who reported that ST2-deficient mice exhibit an increased susceptibility to polymicrobial sepsis induced by cecal ligation puncture (CLP) with impaired bacterial clearance (127). On the other hand, Oboki *et al*. demonstrated that IL-33 deficiency increases the survival rate of mice during LPS-induced sepsis (101), which is in contrast to the findings of Alves *et al*. (124). Moreover, it has been described that St2-deficient mice are more resistant to secondary *Pseudomonas aeruginosa* pneumonia after CLP-induced sepsis (128).

1.2.2.3 Soluble ST2

The secreted soluble form of ST2 (sST2) represents the extracellular part of ST2 and arises from alternative splicing (87). sST2 binds and sequestrates extracellular IL-33 and acts consequently as a decoy receptor for the proinflammatory cytokine. Thus, sST2 is considered as an antiinflammatory factor in conditions such as asthma (129). However, recent reports suggested that sST2 can also act independently of IL-33 by a so far undefined non-canonical mechanism (130, 131). It is interesting to note that increased serum levels of sST2 have been observed in various diseases, including asthma, sepsis, chronic obstructive pulmonary disease, lung fibrosis, cardiovascular diseases and autoimmune diseases (132–138). For example, sST2 serves as a novel biomarker of cardiac stress, which correlates with the severity of acute and chronic heart failure and is able to predict the risk of mortality (136, 139). Additionally, enhanced plasma concentrations of sST2 were associated with a higher mortality rate during severe sepsis in humans and mice (124, 140).

1.2.3 THE ROLE OF ILC2s IN LUNG INFLAMMATION

The recently identified ILC2s belong to the heterogeneous family of innate lymphoid cells which appear to functionally correspond to the Th cells of the adaptive immune system. ILCs can be categorized into three groups on the basis of their ability to produce cytokines associated with Th1, Th2 or Th17 cells. The type I ILCs are dependent on the transcription factor T-bet, are characterized by the production of IFN-γ and include NK cells as well as ILC1s. The type 2 ILCs are

dependent on GATA3, consist of the ILC2s and produce type 2 cytokines (IL-5, IL-9, IL-13) in response to IL-25, IL-33 and TSLP. The type 3 group of ILCs is dependent on the transcription factor RORγt and includes lymphoid tissue-inducer cells as well as IL-17A and IL-22 producing ILCs (102, 141, 142).

ILC2s were discovered in 2010 by three independent groups, who named them either nuocytes (100), natural helper cells (143) or innate helper 2 cells (113). Although some differences were noted in phenotypes and tissue distributions, it has been agreed that these cells should all be referred to as ILC2s (144). In parallel with these studies, a fourth report identified a population of IL-25-elicited multi-potent progenitor type 2 cells (MPP^{type2} cells), which also promotes type 2 immunity, but are functionally distinct from other ILC2s (145). ILC2 have the characteristics of lymphoid cells but lack the expression of classical lineage markers associated with DCs, macrophages, granulocytes, T cells and B cells and are positively identified by the expression of c-Kit, CD90 (Thy1), CD25 (IL-2Rα) and CD127 (IL-7Rα) and ST2 (141). Similar to other ILCs, ILC2s develop from a common bone marrow-derived lymphoid precursor in dependency of the transcription factors Id2 (inhibitor of DNA binding 2) (143, 144). ILC2s are independent of RORγt, but they specifically require the transcription factors GATA3 (146, 147) and RORα (148, 149).

ILC2s serve as crucial regulators of inflammation and tissue repair at barrier surfaces including the respiratory tract (150). In 2011, Chang *et al*. discovered an ILC2 population in the murine lung that mediates airway hyper-reactivity (AHR) in response to influenza virus infection. They showed that IL-33 derived from macrophages stimulates ILC2s to induce AHR through an IL-13-dependent mechanism (110). A few month later, Monticelli and colleagues identified lung-resident ILC2s in mice and humans. They reported that IL-33-dependent ILC2s regulate epithelial cell repair following influenza virus infection through production of amphiregulin (112).

In the last years, various studies showed that lung ILC2s contribute to the development of allergic airway inflammation and the initiation of adaptive Th2 responses (151, 152). Moreover, they play a crucial role in helminth infections, which can cause substantial tissue damage via the migration of the worms through the lung (102). For example, it has been reported that ILC2s are recruited due to elevated IL-33 levels during *N. brasiliensis* infections and further promote tissue repair in the recovery phase following inflammation (111, 153). Nevertheless, the biology and function of ILC2s is an emerging field of research and a large number of questions still need to be answered. Since their discovery in 2010, several

studies highlighted a role of lung ILC2s in allergic inflammation, host defense and airway epithelial repair, however, their function during bacterial infections remains largely unknown.

1.3 *Streptococcus pneumoniae*

1.3.1 EPIDEMIOLOGY AND DISEASE

Streptococcus (S.) pneumoniae is a gram-positive bacterium that frequently colonizes the upper respiratory tract of humans. However, depending on the immune status of the host and the pneumococcal serotype, this asymptomatic colonization can progress to invasive and life-threatening diseases. Moreover, viral infections of the respiratory tract are frequently associated with an increased incidence of invasive pneumococcal disease (IPD). Influenza virus, in particular, predisposes to secondary pneumococcal pneumonia, which results in increased morbidity and mortality during seasonal and pandemic influenza (154, 155). *S.pneumoniae* is the most common cause of community-acquired pneumonia, and a major cause of meningitis, and bacteremia in children and adults (156). The significance of the bacterium as a human pathogen is highlighted by the fact that pneumonia is the leading cause of death in children under five years worldwide (157, 158). In 2011, an estimated 1.3 million children died from pneumonia, which is more than AIDS, malaria and tuberculosis combined. Around 82% of deaths occurred in the first 2 years of life (157). But not only the smallest children are at highest risk, also the elderly (>65 years) and immunocompromised individuals are highly susceptible towards pneumococcal pneumonia and other IPD. Since the introduction of routine childhood immunization, elderly people suffer the greatest burden of IPD in developed countries (159).

S.pneumoniae is transmitted via droplets and aerosols from person to person. The colonization of the nasopharyngeal mucosa is a prerequisite for pneumococcal disease. The carriage rate peaks around two and three years of age with around 60% and decreases thereafter to 10% in the adult population (160). The bacterium usually persists as a colonizer for weeks, but it can also spread from

the nasopharynx to the lower respiratory tract or migrate through the epithelial barrier into sterile compartments, such as lung, brain, middle ear, and blood, where it causes disease (161). The pneumococcus is a highly diverse pathogen and at least 90 different capsular serotypes are known (162). Some serotypes, such as 1 and 7F, are more prominent in invasive disease, whereas other types are mainly involved in carriage. However, the highest mortality rates were found in patients infected with serotypes of a lower invasive disease potential, such as serotypes 3, 6B, and 19F (163, 164).

The prevention and treatment of IPD remains a challenge despite the usage of vaccines and antibiotics. In the last decades, an increasing number of antibiotic-resistant pneumococci have been isolated. The drug resistance and the prevalence of specific clones are variable among different regions and countries and evolve over time. Since antibiotic-resistant clones are introduced into a region, they spread rapidly and the dissemination is further enhanced by the transmission in hospitals and other crowed places (156). To date, two different types of vaccines are available. The pneumococcal polysaccharide vaccines consist of purified polysaccharides from 7 (PPV7) or 23 serotypes (PPV23). However, these vaccines are not immunogenic in the smallest children due to their immature immune system. The conjugated vaccines (PCVs) consist of capsular polysaccharides covalently bound to the diphtheria toxoid, which is highly immunogenic. But these vaccines only provide protection against a limited number of serotypes (7, 10, and recently 13). Vaccination has tremendously decreased the incidence of IPD caused by the serotypes covered by the vaccines. However, an increase of IPD caused by non-vaccine serotypes and clonal expansion of non-vaccine clones have now been observed (165, 166).

1.3.2 BIOLOGY

The pneumococcal genome is a covalently closed, circular DNA structure and consists of 2 to 2.16 million base pairs, depending on the individual strain (167). It contains a core set of 1553 genes that are essential for viability and 154 genes that contribute to virulence. The genome of *S. pneumoniae* is highly variable and about 10% of it can differ between strains. Pneumococcal virulence and also antibiotic resistance have been acquired by horizontal gene transfer during the evolutionary past (168).

Pneumococci are surrounded by a polysaccharide capsule that determines their serotype. So far, more than 90 different serotypes of *S. pneumoniae* have

been described (162). The capsule is probably the most important virulence factor of the bacterium. It protects the bacterium against host immunity by impairing pneumococcal opsonization with complement factors and antibodies and thereby inhibits phagocytosis (169). The production of capsular material can be regulated by the pathogen. Transparent capsules are favored in early colonization, which aids the attachment to nasopharyngeal epithelial cells, whereas opaque capsules are favored during invasion to evade opsonophagocytosis (160).

Another important virulence factor is the pore-forming toxin pneumolysin (PLY), which is released during autolysis as a soluble monomer. PLY binds to membrane cholesterol and forms large pores by oligomerization of up to 50 monomers (170). High amounts of PLY lead to host cell lysis and contribute to lung injury and neuronal damage (171). However, PLY has also biological effects at sublytic concentrations, including the activation of the classical complement pathway (172). PLY-deficient mutants are less virulent than wild-type strains and usually less likely to mediate lethal pulmonary infections (173, 174). However, some serotypes expressing non-cytolytic PLY are also associated with invasive diseases (175). Apart from the capsule and PLY, *S. pneumoniae* expresses further virulence factors including, hyaluronidase, neuraminidase, pneumococcal surface antigen A and B (PsaA, PsaB), pneumococcal surface protein A and C (PspA, PspC), pneumococcal adherence and virulence factor A (PavA), and others (176).

1.3.3 INNATE IMMUNE RECOGNITION OF *S. PNEUMONIAE*

S. pneumoniae is sensed by various membrane-bound and cytosolic PRRs of the innate immune system. In particular, TLR2, -4 and -9, NOD2 and the NLRP3 inflammasome have been identified as crucial detectors of the bacterium (177). TLR2 is known to detect pneumococcal cell wall components such as lipopeptides and lipoteichoic acid (LTA), which also involves the co-receptors CD14 and the lipopolysaccharide-binding protein (12, 178). The role of TLR2 has been studied in different mouse models. TLR2 deficiency resulted in impaired pneumococcal clearance in a model of nasopharyngeal colonization (179). Moreover, *Tlr2*$^{-/-}$ mice showed an enhanced susceptibility to pneumococcal meningitis (180) but only a moderately increase in susceptibility during pneumococcal pneumonia (181). However, the infection with PLY-deficient bacteria led to increased mortality in *Tlr2*$^{-/-}$ mice compared to wild-type mice suggesting that PLY-induced signaling can compensate for TLR2 deficiency (182).

Some studies demonstrated that PLY is sensed by TLR4 (183, 184), however, the role of TLR4 signaling during pneumococcal infections remains controversial. Malley *et al*. showed that TLR4-deficient mice have increased colonization levels and enhanced risk of invasive disease (183). In contrast, others observed that TLR4 was not necessary for efficient clearance of colonization (179). In pneumococcal pneumonia, *Tlr4*$^{-/-}$ mice showed a reduced survival only after infection with low-level bacterial doses (185). Moreover, TLR4 plays a limited role in sepsis and meningitis (186, 187). Finally, Mc Neela *et al*. reported that PLY induces cytokine production independently of TLR4 (188). In addition to membrane-bound TLR2 and -4, *S.pneumoniae* is recognized by cytosolic TLR9 that senses unmethylated CpG sequences in pneumococcal DNA. In a model of pneumococcal pneumonia, TLR9-deficient mice had a higher mortality rate and reduced bacterial clearance but unaltered cytokine production compared to wild-type mice (189). Importantly, mice deficient for MyD88, which acts as an adaptor molecule for most TLRs as well as the IL-1 and IL-18 receptors (15), were highly susceptible towards pneumococcal infections. In these studies, *MyD88*$^{-/-}$ mice had increased mortality, enhanced bacterial replication and dissemination and impaired cytokine production as compared to WT animals in models of pneumonia, sepsis and meningitis (190–192). The milder phenotypes observed in mice deficient in only TLR2, -4 or -9 might be explained by the redundant functions of TLRs in pneumococcal infections. Furthermore, the absence of IL-1 and IL-18 signaling may also contribute to the strong phenotype of *MyD88*$^{-/-}$ mice.

Apart from TLRs, members of the NLR family play an important role in pneumococcal recognition. NOD2 is stimulated by internalized *S.pneumoniae* and triggers the activation of NF-κB and the subsequent production of proinflammatory cytokines (193). Moreover, it has been reported that NOD2 mediates inflammatory responses during pneumococcal meningitis (194). In 2011, Davis *et al*. showed that pneumococcal peptidoglycans are recognized by NOD2, which was crucial for macrophage-mediated clearance of colonizing pneumococci (195). In addition to NOD2, NLRP3 is activated by *S.pneumoniae* which leads to the formation of inflammasomes and the production of mature IL-1 by macrophages and DCs (188, 196–198). Two different groups reported that this activation was dependent on PLY (188, 196). NLRP3 deficiency increased lung permeability and the severity of pneumococcal pneumonia (196). In contrast, *Nlrp3*$^{-/-}$ mice showed improved clinical scores in a model of pneumococcal meningitis (197). Other important PRRs during pneumococcal infection include the C-type lectin SIGN-R1 and the scavenger receptors MARCO and SR-1 (199–202). A recent study further

demonstrated that pneumococcal DNA is detected by a yet unidentified cytosolic DNA receptor in dependency of the adaptor molecule STING and the transcription factor IRF3, which mediates the production of type 1 IFN in macrophages (203).

1.3.4 THE ANTIBACTERIAL IMMUNE RESPONSE TO *S. PNEUMONIAE*

The innate immune system provides a vast array of mechanisms to sense and eliminate invading pneumococci. The nasopharyngeal colonization is a prerequisite for pneumococcal disease (160). However, the entering of bacteria into the nasal cavity is counteracted by the production of viscous mucus, which traps invading pathogens and contains antimicrobial peptides (204). In addition, *S.pneumoniae* is recognized by PRRs in the nasopharynx and thereby initiates the innate immune response. For example, recognition of pneumococci by TLR2 has been shown to stimulate the generation of pneumococcal-specific IL-17-expressing CD4+ T cells, and the subsequent recruitment of antibacterial monocytes/macrophages and neutrophils (205). In addition, the sensing of pneumococcal peptidoglycan fragments by NOD2 is critical for the production of the monocyte chemotactic protein 1 (MCP1), the recruitment of macrophages and the clearance of pneumococci (195).

Several preexisting or acquired factors, such as viral infections, can weaken the immune system and damage the epithelial barrier so that higher numbers of pneumococci are aspirated or invade the circulation. If pneumococci reach the lower respiratory tract (LRT), some are cleared by resident phagocytotic AMΦs. When the killing capacity of AMΦs is exhausted, the cells undergo apoptosis which involves the production of ROS and nitric oxide and helps to kill ingested bacteria (206, 207). The complement system is another important arm of the innate host defense that mediates the opsonization of bacteria promoting phagocytosis, activation of neutrophil chemotaxis, and the direct killing of microbes. During pneumococcal infection, this requires mainly the activation of the classical pathway (208). In parallel, invading pneumococci are recognized by PRRs expressed on resident AMΦs, DCs and alveolar epithelial cells (177). PRRs stimulate the activation of NF-κB and IRF transcription factors in these cells and induce the production of proinflammatory cytokines including TNFα, IL-1β, IL-6, IL-12, CXCL1 (KC), CXCL2 (Mip2α) and CCL2 (MCP1) (177, 209). The chemokines directly stimulate recruitment of neutrophils and subse-

quently of macrophages. IL-1 which is mainly produced by macrophages additionally activates non-hematopoetic cells such as epithelial cells to booster the chemokine production in those cells and enhance the leukocyte recruitment. Neutrophils are the main phagocytic cells that eliminate pneumococci in the airways (210). Furthermore, they contain granules with antimicrobial peptides and proteases that are released during infection. It has been shown, that neutrophil killing of *S. pneumoniae* does not require NADPH oxidase-dependent generation of ROS, but is dependent on the generation of the neutrophil serine proteases cathepsin G and neutrophil elastase (211, 212). Another killing mechanism of neutrophils is the release of NETs, although *S. pneumoniae* has been reported to escape the entrapment by NETs through the action of the endonuclease A (213). Importantly, neutrophils are a double edged sword during pneumococcal infection. Whereas their recruitment and activation is vital for eliminating the bacteria, an overwhelming accumulation of neutrophils can also result in excessive tissue damage and lung injury. It is therefore important that the proinflammatory response is delicately balanced and down-regulated at the appropriate time to ensure the resolution of neutrophilic inflammation (210).

Resident and recruited DCs play a critical role in antigen presentation and the initiation of adaptive immune responses. The activation of Th1, Th17, CTLs, and NKT cells contributes to the elimination of the bacteria, and B cells are crucial for antibody responses to naturally occurring infection and immunization (214–218).

1.4 Aim of the study

The innate immune system mediates the first line of defense against *S. pneumoniae*. The invading pathogen is sensed by PRRs, which leads to the activation of a proinflammatory immune response. An appropriate innate immune response is indispensable to eliminate invading pneumococci. However, an overwhelming immune response can also cause necrotic cell death, which might result in excessive tissue damage and lung injury and is thus detrimental for the host (168, 219). Therefore, the innate immune response must be tightly regulated. The molecular mechanisms mediating this negative regulation are yet incompletely characterized.

The induction of necrotic cell death is accompanied by the release of DAMPs, which can also be sensed by the innate immune system. While the role of DAMPs in sterile inflammation has been fairly well characterized (39), there is little knowledge if and how DAMPs and their receptors influence the immune response during infections. Based on these considerations, this study was set out to test the hypothesis that DAMPs are released during infection with *S. pneumoniae* and that they regulate the antibacterial innate immune response.

2 — Material and Methods

2.1 Bacteria

In vivo, mice were infected with *S.pneumoniae* serotype 3 strain PN36; NCTC7978. *In vitro*, the serotype 2 strain D39 and an isogenic capsule-deficient mutant (D39Δ*cps*) was used. All strains were kindly provided by Prof. Sven Hammerschmidt (Ernst-Moritz-Arndt-Universität Greifswald, Germany).

Bacteria were cultured as described before (220). They were stored at -80°C in THY media (30 g/l Todd-Hewitt Broth, 5 g/l yeast extract) containing 10% glycerol. Before use, pneumococci were cultured on Columbia blood agar + 5% sheep blood for 8 h (strain PN36; NCTC7978) or 12 h (strain D39, D39Δ*cps*). The mutant strain D39Δ*cps* and wildtype D39 additionally required antibiotics on the agar plate (2 mg kanamycin per plate). Single colonies were transferred into THY media to yield $OD_{600} = 0.03\text{-}0.04$. Liquid cultures were incubated at 37°C and 5% CO_2 for 2.5-4 h until the bacteria reached a phase of logarithmic growth ($OD_{600} = 0.2\text{-}0.4$). Bacteria were pelleted at 2,700 g for 10 min at 4°C. The pellet was resuspended in PBS or media and appropriate dilutions were prepared. $OD_{600} = 0.1$ equates to 10^8 CFU/ml.

2.2 Mice

All animal experiments were approved by institutional (Charité – Universitätsmedizin Berlin) and governmental animal welfare committees (LAGeSo Berlin; approval IDs G0210/11, G0365/12, T0013/11, T0014/13). All mice used were on C57BL/6J background, 8 - 10 weeks old and female.

St2$^{-/-}$ (also known as *Il1rl1*$^{-/-}$) (221), *Il33*$^{-/-}$ (101), and wild-type (WT) control mice were bred in the animal facility of the „Forschungseinrichtungen für experimentelle Medizin (FEM), Charité - Universitätsmedizin Berlin“. *St2*$^{-/-}$ mice were kindly provided by Andrew McKenzie, Ph.D. (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom) and *Il33*$^{-/-}$ mice were kindly provided by Yasuhide Furuta, Ph.D. (RIKEN Center for Developmental Biology, Kobe, Japan). *Fcer1g*$^{-/-}$ (222), *Mincle*$^{-/-}$ (53) and WT control mice were bred in the animal facility of the “Universitätsklinikum Erlangen” and were kindly provided by Prof. Roland Lang (Universitätsklinikum Erlangen, Germany). *P2xr7*$^{-/-}$ (223) and *P2yr6*$^{-/-}$ (224) were bred in the animal facility of the “Universitätsklinikum Freiburg” and were kindly provided by Prof. Marco Idzko (Universitätsklinikum Freiburg, Germany). WT control mice (*P2xr7*$^{-/-}$, *P2yr6*$^{-/-}$) and intratracheally treated WT mice were obtained from Charles River Laboratories (Germany).

2.3 Murine pneumococcal pneumonia model

2.3.1 INFECTION OF MICE

Infection of mice was performed as described before (196). Mice were anesthetized by intraperitoneal (i.p.) injection of 80 mg/kg ketamine and 25 mg/kg xylazine and transnasally inoculated with 7.5×10^4 or 5×10^6 CFU *S.pneumoniae* serotype 3 (PN36; NCTC7978) in 20 µl PBS per mouse. The control groups were sham-infected with 20 µl PBS. For analysis, mice were sacrificed (12, 24, and 48 hours post infection (h p.i.) or 4, 6, 8, and 12 days post infection (d p.i.)) or survival was recorded every 12 h for 10 days. All mice analyzed at 4, 6, 8 or 12 d p.i. were treated with ampicillin (0.4 mg in 200 µl 0.9% NaCl, i.p.) 30 h p.i., 36 h p.i. and then every 12 h until sacrification.

Before analysis, mice were anesthetized (160 mg/kg ketamine, 75 mg/kg xylazine), heparinized (60 µl 12,500 I.E.) and sacrificed by final blood withdrawal. The blood was centrifuged (1500 g, 10 min, 4°C) and the serum was collected. All samples and organs of interest were collected and used for further analyses or were frozen in liquid nitrogen and stored at -80°C.

2.3.2 INTRATRACHEAL TREATMENT OF MICE

WT mice were intratracheally treated with PBS (control), uricase (0.073 U/per mouse), apyrase (0.32 U/per mouse), suramin (32 µM/per mouse) or pyridoxalphosphate-6-azophenyl-2‘,4‘-disulfonic acid (PPADS) (32 µM/per mouse) dissolved in 25 µl PBS immediately before *S.pneumoniae* infection (0 h p.i.) and 24 h p.i. Concentrations used, were described before in other models (225, 226). Mice were anesthetized by i.p. application of 80 mg/kg ketamine and 25 mg/kg xylazine (0 h p.i.) or were shortly sedated by isoflurane (24 h p.i.) and orotracheally intubated with a laryngoscope. The solution was then applied into the lungs with a microsprayer. Mice were sacrificed 48 h p.i.

2.3.3 BRONCHOALVEOLAR LAVAGE OF MOUSE LUNGS

After sacrifice, mice were tracheotomized and ventilated and the lungs were perfused with sterile 0.9% NaCl via the pulmonary artery for 2 min. Lungs were lavaged twice with 650 µl PBS containing protease inhibitors (1 tablet per 10 ml PBS) and both fractions of the bronchoalveolar lavage (BAL) were centrifuged (425 g, 10 min, 4°C). The supernatants were collected and the cell pellets were combined and used for immune cell recruitment analysis (see 2.3.7).

2.3.4 DETERMINATION OF BACTERIAL LOAD

The bacterial load was determined in bronchoalveolar lavage fluid (BALF), blood and spleen samples of each mouse. Serial dilutions up to $1:10^5$-fold were prepared from the first lavage, the whole blood or the spleen directly after isolation. The spleen was passed through a 70 µm cell strainer and 10 ml PBS were added. Serial dilutions were plated on blood agar and incubated at 37°C overnight before colonies were counted.

2.3.5 DETERMINATION OF LUNG MICROVASCULAR LEAKAGE

Human serum albumin (HSA) (1 mg in 75 µl 0.9% NaCl) was intravenously injected into the mouse tail 1 h before BAL. Lung microvascular leakage was quantified by the amount of HSA diffused into the BALF from the blood through the epithelial-endothelial barrier. The HSA concentration in serum and BALF (first and second lavage were mixed 1:1) was measured by ELISA according to the manufacturer's instructions, and the HSA BALF/serum ratio was calculated.

2.3.6 HISTOPATHOLOGICAL ANALYSIS OF THE LUNG

For histopathological analysis, lungs were harvested without flushing and fixed in 4% formalin (pH 7.0). Following, samples were dehydrated through ascending graded ethanol and embedded in paraffin. Tissue sections were cut and deparaffinized and rehydrated sections were then stained with hematoxylin and eosin and analysed by microscopy. Histopathological staining and evaluation of the samples was kindly performed by Dr. Olivia Kershaw (Institut für Tierpathologie, Freie Universität Berlin, Germany).

2.3.7 IMMUNE CELL RECRUITMENT ANALYSIS FROM THE BALF

The cell pellets from both lavages (see 2.3.3) were pooled in 1 ml PBS. Following, cells in the BALF were counted by hemocytometer and differentiated by flow cytometry (see 2.5.2). Polymorphonuclear neutrophils (PMN) were defined as CD45+Gr1+ cells and macrophages as CD45+F4-80+ cells. The relative amount of PMNs and macrophages was then related to the absolute number of cell influx.

2.3.8 ANALYSIS OF ILC2 RECRUITMENT AND ACTIVATION IN THE LUNG

Lungs were perfused, isolated, cut into small pieces, and incubated in 5 ml RPMI 1640 containing collagenase (2 mg/ml) and DNAse (0.5 mg/ml) for 45 min at 37°C while shaking. After digestion, the lung samples were passed through a 100 µm filter and centrifuged (300 g, 8 min, 4°C). The cells were incubated for 1 min on ice in RBC (red blood cell) lysis buffer, PBS-washed and centrifuged (300 g, 8 min, 4°C).Following, leukocytes were purified by density gradient centrifugation. The cells were resuspended in 3 ml high-density percoll (70% in RPMI 1640)

and were put below 3 ml low-density percoll (30% in RPMI 1640) in a 15 ml falcon. The gradient was centrifuged at 400 g for 20 min at 25°C without brake. The interphase was carefully transferred into a new falcon with a pasteur pipette. Cells were washed in RPMI 1640, centrifuged (300 g, 8 min, 4°C), and resuspended in 2 ml RPMI. The total leukocyte number was determined by hemocytometer. Cells were stained and analyzed by flow cytometry. ILC2 were identified as described before by Monticelli *et al*. (112) by gating on Lineage-negative (CD3-, CD11b-, CD11c-, CD19-, NK1.1-), CD25+, CD90.2+, CD127+ and ST2+ cells (see 2.5.2). For further analysis, ILC2 were defined as Linage-negative, CD25+CD90.2+ cells. Activation of ILC2s was quantified by intracellular staining for IL-5. The relative amount of ILC2s was then related to the total number of leukocytes in the lung.

2.3.9 RNA-ISOLATION FROM THE LUNG

Perfused lungs were mechanically homogenized on ice. Each lung was transferred into 1 ml of TRIzol® reagent frozen in liquid nitrogen, and then stored at -80°C. For RNA isolation, the samples were thawed and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred into a new tube and 200 µl chloroform was added. Tubes were mixed 10 times and incubated at room temperature for 5 min. After centrifugation (12,000 g, 15 min, 4°C), the upper phase was carefully transferred into a new tube containing 500 µl isopropanol. The tubes were mixed and incubated for 20 min at -20°C. Afterwards, tubes were centrifuged (12,000 g, 20 min, 4°C) and the supernatant was discarded. The RNA pellet was washed two times by vortexing in 500 µl 70% ethanol followed by centrifugation (12,000 g, 8 min, 4°C). The pellet was resuspended in 100 µl RNase-free H_2O and the RNA concentration was determined using Nanodrop. 100 ng/µl RNA were used for transcription to complementary DNA (cDNA) (see 2.6.2). RNA was stored at -80°C.

2.4 Cell culture

2.4.1 ISOLATION OF MURINE ALVEOLAR EPITHELIAL CELLS

Alveolar epithelial cell (AEC) isolation was performed as described before (220). In brief, mice were sacrificed and each lung was perfused with HBSS. Then, 1.5 ml dispase and 500 µl low melting point agarose were injected into the lung through the trachea. After 10 min, lungs were transferred into 4.5 ml dispase (5 U/ml) and were incubated at room temperature for further 30 min. This was followed by 10 min of DNase digestion (0.1 mg/ml) in AEC-medium (DMEM + 2.5% HEPES + 10% FCS + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep) and dissociation of the lung into a cell suspension. The suspension was passed through cell strainers (100 µm, 40 µm, 20 µm) and centrifuged twice (100 g, 8 min, 4°C). The cell pellet was resuspended in 2 ml medium and incubated with biotinylated anti-mouse antibodies (anti-CD45: 0.45 µg/1×10^6 cells, anti-CD31: 0.2 µg/1×10^6 cells, anti-CD16/32: 0.34 µg/1×10^6 cells) at 37°C + 5% CO_2 for 30 min. Cells were washed twice in AEC-medium without FCS and incubated with magnetic Dynabeads® Biotin Binder (4.3×10^6 beads/10^6 cells) for 30 min with gentle shaking. The cell suspension was placed on a magnet for 10 min and the epithelial cells were separated from cells bound to the magnetic beads.

AECs were washed in AEC-medium and seeded at a density of 4×10^5 cells/ml. After 2-3 days medium was changed. After 5 days cells were differentiated into type I AECs and were used for experiments. For infection, DMEM + 2.5% HEPES + 4.5 mM L-glutamine was used and cells were cultured at 37°C and 5% CO_2. Samples were collected 16 h p.i. for mRNA and protein quantification.

2.4.2 ISOLATION OF MURINE MICROVASCULAR ENDOTHELIAL CELLS

Mice were sacrificed and lungs were isolated without flushing. The lungs were washed in HBBS and cut into small pieces followed by incubation in 5 ml HBSS + DNAse (0.5 mg/ml) + Dispase (5 U/ml) per lung for 1 h at 37°C while shaking. Meanwhile Dynabeads® Sheep Anti-Rat IgG were washed 3 times with 500 µl HBBS+++ (HBBS + Ca^{2+} + Mg^{2+} + 0.5% BSA). Per lung, 3 µl beads were coated with 0.65 µl rat-anti-mouse-CD144 and incubated for 1 h at 37°C. Before usage,

beads were washed again 3 times with HBBS+++. The lung digestion was stopped by adding 5 ml FCS. The cell suspension was mixed several times by being pipetted up and down and passed through a cell strainer (70 µm). Cells were centrifuged (300 g, 5 min, RT) and washed with HBBS+++. The cell pellet was resuspended in 700 µl HBBS and anti-CD144-coated beads were added. After incubation for 45 min at 37°C, cells were placed on a DynaMag™ Spin Magnet and the supernatant containing the unlabeled cells was discarded. The magnetically labeled CD144+ cells were washed 4 times with HBBS+++ before they were resuspended in endothelial cell medium (Endothelial Cell Growth Medium MV2 + Supplement + 15 % FCS + 100 µg/ml Pen/Strep).

Microvascular endothelial cells (MVECs) of 2 lungs were seeded in fibronectin-coated (100 µg/ml, 1 h at RT) 35 mm tissue culture dishes. The medium was changed the next day and cells were splitted after reaching confluence 2 or 3 days later. Cells were trypsinized (5 min, 37°C), washed with medium, and seeded in fibronectin-coated well plates. The confluent cells were infected 2 days later in endothelial cell infection medium (Endothelial Cell Growth Medium MV2 + Supplement + 15 % FCS). Cells were cultured at 37°C and 5% CO_2. Samples were collected 5 h p.i. for mRNA and protein quantification.

2.4.3 ISOLATION OF MURINE NEUTROPHILS FROM BONE MARROW

Neutrophils were isolated from the bone marrow by magnetic-activated cell sorting (MACS) using the Anti-Ly-6G MicroBead Kit (Milteny Biotech) according to manufacturer's instructions. In brief, bone marrow was isolated from the femurs and tibiae of sacrificed mice. It was resuspended in 15 ml RPMI 1640 and passed through a 40 µm filter. After centrifugation (300 g, 6 min, 4°C), cells were washed with 10 ml MACS buffer (PBS + 0.5% FCS + 2 mM EDTA) and centrifuged again. The cell pellet was resuspended in 200 µl MACS buffer per 10^8 total cells and 50 µl of anti-Ly-6G-biotin antibodies were added. The cells were incubated for 10 min at 4°C. Following, 150 µl MACS buffer and 100 µl of anti-biotin microbeads were added per 10^8 total cells and incubated for 15 min at 4°C. After incubation, cells were washed with 10 ml MACS buffer, centrifuged (300 g, 6 min, 4°C) and resuspended in 500 µl MACS buffer.

Afterwards, magnetic cell separation was performed. MS MACS Columns were placed in the magnetic field of a MACS separator and columns were prepared by rinsing with 500 µl MACS buffer. The cell suspension was applied

onto the column and the flow through contained the unlabeled cells. The column was washed 3 times with 500 µl MACS buffer. Subsequently, the column was removed from the separator and 1 ml MACS buffer was applied onto the column. The magnetically labeled cells were flushed out by firmly pushing the plunger into the column. The collected Ly-6G-positive cells were washed with 10 ml HBSS, centrifuged (300 g, 6 min, 4°C) and immediately used for opsonophagocytic neutrophil killing assays or were infected in RPMI 1640 for 4 h.

2.4.4 ISOLATION OF MURINE ALVEOLAR MACROPHAGES

Mice were sacrificed and each lung was lavaged ten times with 500 µl PBS + 2 mM EDTA. Lavages were pooled and centrifuged at 300 g for 8 min at 4°C. Afterwards, cells were resuspended in RPMI 1640 + 10% FCS + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep and seeded at a concentration of 4×10^5 cells/ml. For infection, RPMI 1640 + 2% FCS + 4.5 mM L-glutamine was used. Cells were cultured at 37°C and 5% CO_2. Samples were collected 12 h p.i (mRNA) or 16 h p.i. (protein).

2.4.5 ISOLATION AND CULTURE OF MURINE BONE MARROW-DERIVED MACROPHAGES

Isolation and culturing of bone marrow-derived macrophages (BMMs) was conducted as described before (220). Bone marrow was isolated from the femurs and tibiae of sacrificed mice. The bones were washed once in 70% ethanol and then once in RPMI 1640. Afterwards, the bones were crushed in a sterilized mortar in 20 ml RPMI 1640. The suspension was passed through a 70 µm cell strainer and the cells were centrifuged at 150 g for 10 min. The pellet was resuspended in FCS + 10% DMSO and then frozen at -80°C in aliquots of 10^7 cells/ml. Cells were stored in liquid nitrogen.

For culturing of bone marrow-derived macrophages (BMMs), bone marrow aliquots were thawed and transferred into “BMM growth medium” (RPMI 1640 + 20% FCS + 30% L929 fibroblast supernatant + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep). Cells were centrifuged (230 g, 10 min), resuspended in 20 ml BMM growth medium and divided into two Optilux petri dishes. For medium preparation, L929 fibroblast supernatant was harvested from macrophage colony-stimulating factor (M-CSF) producing L929 cells and sterile-filtered (0.2 µm). L929 were cultured in RPMI 1640 + 10% FCS + 4.5 mM L-glutamine for 10 days (37°C and 5% CO_2).

After 4 days of BMM culture, 10 ml growth medium per dish were added. After 10 days, confluent cells were scraped from the petri dish with 7 ml PBS + 2 mM EDTA. Cells were washed and resuspended in "BMM replating medium" (RPMI 1640 + 10% FCS + 15% L929 fibroblast supernatant + 4.5 mM L-glutamine). Cells were seeded at a concentration of 4×10^5 cells/ml. After overnight incubation, the medium was replaced by RPMI 1640 medium and the cells were used for experiments. Cells were cultured at 37°C and 5% CO_2. Samples were collected 5 h p.i (mRNA) or 16 h p.i. (protein).

2.4.6 CULTURE OF HUMAN LUNG TISSUE

Fresh lung explants were obtained from patients undergoing lung resection at local thoracic surgery clinics. Written informed consent was obtained from all patients and the study was approved by the ethics committee at the Charité - Universitätsmedizin Berlin (protocol numbers EA2/050/08 and EA2/023/07). Tumor-free normal lung tissue was prepared as described previously (227). Briefly, lung tissue was stamped into small cylinders and weighed. Lung specimens were incubated in RPMI 1640 + 10% FCS for 24 h before being infected. 200 µl of prepared control or infection (1×10^6 *S.pneumoniae* strain D39) medium per 100 mg lung tissue was then injected and 24 h p.i. samples were collected. Culture and infection of human lung tissue was performed by Andreas Hocke and Christoph Machnik (Charité - Universitätsmedizin Berlin, Germany) and cell supernatants were kindly provided.

2.5 Immunological methods

2.5.1 ELISA

Cytokines (IL-6, TNF-α, IL-1β, IL-33), chemokines (KC, MIP-2α, CXCL-5), ST2 and HSA were quantified by commercially available sandwich ELISA Kits (eBioscience; R&D systems; Biomol) in cell-free supernatants or in the BALF and serum of mice. ELISAs were performed according to manufacturer's instructions. In brief, 96-well plates were incubated with the respective coating-antibody

solution over night at 4°C. Plates were washed tree times with PBS + Tween20 and were subsequentially incubated with blocking solution (assay diluent or PBS + 1% BSA) for at least 1 h at RT. After washing the plates tree times, samples and standard were transferred to the wells and incubated for 2 h at RT. Blocking solution was used for dilution if required. This was followed by 5 times washing and incubation with the respective biotinylated detection antibody solution for 1 h or 2 h at RT. After washing the plates 5 times, the wells were incubated for 30 min with streptavidin-horseradish peroxidase (HRP)-solution at RT in the dark and washed again 7 times. Substrate solution was added and incubated for 10-30 min before the reaction was stopped by adding H_2SO_4. The optical density of each well was determined in a microplate reader (OD_{450}-Ref:OD_{570}).

2.5.2 FLOW CYTOMETRY

Cells were washed with FACS buffer (PBS + 3% FCS + 2 mM EDTA) and centrifuged (300 g, 6 min, 4°C). For the extracellular staining of surface markers, a mastermix was prepared by diluting Fc-block and all antibodies used (see Tab. 1) in FACS buffer. The pellet was resuspended in 50 µl mastermix. Cells were incubated for 10-15 min at 4°C in the dark. Subsequentially, cells were washed with FACS buffer and centrifuged

TABLE 1: Antibodies

ANTIBODY	CLONE	COMPANY	DF
Anti-mouse CD45 – Biotin	30-F11	BD Bioscience	
Anti-mouse CD16/CD32 – Biotin	2.4G2	BD Bioscience	
Anti-mouse CD31 – Biotin	MEC 13.3	BD Bioscience	
Anti-mouse CD144 BD Pharmingen	11D4.1	BD Bioscience	
Anti-mouse CD3 – APC-eFluor® 780	17A2	eBioscience	1:75
Anti-mouse CD11b – PE-Cy7	M1/70	eBioscience	1:75
Anti-mouse CD11c – APC-eFluor® 780	N418	eBioscience	1:75
Anti-mouse CD16/CD32 purified (Fc-block)	2.4G2	BD Bioscience	1:100
Anti-mouse CD19 – PE-Cy7	eBio1D3(1D3)	eBioscience	1:75
Anti-mouse CD25 – PE	PC61.5	eBioscience	1:75
Anti-mouse CD45 – FITC	30-F11	eBioscience	1:75
Anti-mouse CD90.2 – eFluor® 450	53-2.1	eBioscience	1:300
Anti-mouse CD127 – FITC	A7R34	eBioscience	1:75
Anti-mouse F4/80 – APC	BM8	eBioscience	1:75
Anti-mouse/human IL-5 – PE	TRFK5	eBioscience	1:75
Anti-mouse Ly-6G (Gr-1) – PE	RB6-8C5	eBioscience	1:75
Anti-mouse NK1.1 – PE-Cy7	PK136	eBioscience	1:75
Anti-mouse ST2 (IL-33R) – APC	RMST2-2	eBioscience	1:75

(300 g, 6 min, 4°C). The cells were immediately used for flow cytometry analysis or fixed with 2% PFA in PBS for 15 min at RT in the dark followed by washing and centrifugation (300 g, 6 min, 4°C).

For intracellular staining, fixed cells were washed with FACS-saponin buffer (PBS + 0.05% saponin + 3% FCS + 2 mM EDTA) and centrifuged (300 g, 6 min, 4°C). Fc-block and IL-5 antibody were diluted in FACS-saponin buffer and 50 µl were added to the cells. Cells were incubated 10-15 min at 4°C in the dark. Afterwards cells were washed in FACS buffer, centrifuged (6 min, 1200 rpm, 4°C) and analyzed. Cell pellets were resuspended in 100-300 µl FACS-buffer and analyzed on a FACSCanto or FACSCalibur. Analysis of FACS data was performed using FlowJo 7.6.3.

2.6 Molecular biology

2.6.1 RNA PURIFICATION FROM CELLS

RNA was extracted from cells using the "PerfectPure™ RNA Cultured Cell Kit" (5'Prime) according to the manufacturer's instructions including digestion of genomic DNA. In brief, cells were lysed by adding 400 µl lysis buffer and stored at -20°C until further purification. The lysate was pipetted 10 times up and down and loaded onto a purification column. The columns were centrifuged (16,000 g, 1 min, RT) and 400 µl "Wash 1 solution" was added per column. After another centrifugation step (16,000 g, 1 min, RT), 50 µl of DNAse were added to the columns and incubated for 15 min at RT. Following, the columns were washed twice with 200µl "DNase wash solution" and twice with 200 µl "Wash 2 solution". The columns were centrifuged after each washing step (16,000 g, 1 min, RT; last step: 2min). The columns were transferred into a new collection tube and the RNA was eluted by adding 50 µl ddH_2O and centrifugation at 16,000 g for 1 min. 10 µl RNA was used for transcription to cDNA. The remaining RNA was stored at -80°C.

2.6.2 RNA TRANSCRIPTION TO cDNA

RNA was transcribed to cDNA using the "High Capacity Reverse Transcription Kit" (Applied Biosystems). A mastermix was prepared on ice containing 4.2 µl ddH_2O,

2 µl Reverse Transcription buffer, 2 ml Random Primers, 0.8 µl dNTPs, and 1 µl Reverse Transcriptase per reaction. 10 µl mastermix were mixed with 10 µl RNA yielding a final volume of 20 µl. The reaction mixture was incubated in a thermo cycler for 10 min at 25°C, then for 2 h at 37°C, and lastly for 5 sec at 85°C. After this incubation, the samples were diluted with 60 µl ddH_2O per tube and stored at -20°C.

2.6.3 QUANTITATIVE REAL TIME PCR

The mRNA expression was measured using quantitative real time (RT)-PCR. A mastermix was prepared containing 10 µl Gene Expression Master Mix (Applied Biosystems), 4 µl ddH_2O and 1 µl Taqman Assay (self-designed or purchased from Applied Biosystems) per reaction (see Tab. 2). The self-designed oligonucleotides were diluted with ddH_2O yielding a concentration of 18 nmol/ml (forward-

TABLE 2: Primer- and Probesequences

TARGET GENE	FORWARD	REVERSE	PROBE (5'-FAM, 3'-TAMRA)
Ccl2	TGT GTC CGT CGT GGA TCT GA	CCT GCT TCA CCA CCT TCT TGA	CCG CCT GGA GAA ACC TGC CAA GTA TG
Cxcl1	TGT GTC CGT CGT GGA TCT GA	CCT GCT TCA CCA CCT TCT TGA	CCG CCT GGA GAA ACC TGC CAA GTA TG
Gapdh	TGT GTC CGT CGT GGA TCT GA	CCT GCT TCA CCA CCT TCT TGA	CCG CCT GGA GAA ACC TGC CAA GTA TG
Il5	TGA GTC TCC CTG TCC	CTC ATG TTC ACC ATC	AAT CAG GCG ACG GTG TG
Il6	TGT GTC CGT CGT GGA TCT GA	CCT GCT TCA CCA CCT TCT TGA	CCG CCT GGA GAA ACC TGC CAA GTA TG
Il33	TGA GTC TCC CTG TCC	CTC ATG TTC ACC ATC	AAT CAG GCG ACG GTG TG
St2	TGG AAA TAG GAA AAC CAG CAA GTA T	TTT GTT AAT CTG CCA CAG GAC ATC	TTT GGC AAA GGC TCT CAC TTC TTG GC
	ASSAY-ID (APPLIED BIOSYSTEMS)		
Camp	Mm00438285_m1		
Cxcl2	Mm00436450_m1		
Gcsf	Mm00438334_m1		
Il10	Mm00439614_m1		
Il23	Mm00518984_m1		
Lcn2	Mm01324470_m1		
S100a8	Mm00496696_g1		
S100a9	Mm00656925_m1		

and reverse-primer) or 5 nmol/ml (6-FAM-labeled probe). For each reaction, 15 µl of the reaction mastermix and 5 µl of cDNA were transferred into a 96 well. The mixture was incubated for 2 min at 50°C, then for 10 min at 95°C followed by 40 amplification cycles (15 sec at 95°C, then 1 min at 60°C). The expression of *Gapdh* was used as an internal reference for normalization of the data.

2.7 Cell biology

2.7.1 LACTATE DEHYDROGENASE ASSAY

Cell death was quantified with the CytoTox 96® Assay (Promega) which quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. To analyze cell death induction, cells were infected with *S.pneumoniae* D39 (MOI=10) and cell-free supernatants were harvested 16 h p.i. As a positive control, cells were lysed by adding Triton X-100 to determine the maximum of LDH release. The spontaneous LDH was determined in the uninfected control. LDH Assay was performed according to manufacturer's instructions. In brief, 50 µl of substrate reaction were added to 50 µl cell supernatant in a 96-well plate and incubated for 30 min in the dark at RT. Then, 50 µl stop solution were added to the reaction and the absorbance was measured at 490nm in a plate reader. The percentage of specific LDH release was calculated by using the following formula: specific LDH release [%] = $((OD_{Target} - OD_{Control}) / (OD_{Maximum}-OD_{Control})) \times 100$.

2.7.2 QUANTIFICATION OF URIC ACID RELEASE

Uric acid release was determined after infection with *S.pneumoniae* D39 using the Amplex® Red Uric Acid/Uricase Assay Kit (Life technologies) according to manufacturer's instructions. In brief, 50 µl cell supernatant or uric acid standard were added to 50 µl working solution containing 5 µl Amplex® Red reagent (10 mM), 2 µl HRP (100 U/ml), 2 µl uricase (100 U/ml), and 49.1 µl 1× reaction buffer per reaction. The reaction was incubated for 30 min at 37°C in the dark, while uric acid being converted to allantoin, hydrogen peroxide (H_2O_2) and carbon

dioxid by uricase. The H_2O_2 then, in the presence of HRP, reacts with Amplex® Red reagent to generate the resorufin. The optical density of resorufin (OD_{590}) was measured in a microplate reader.

2.7.3 QUANTIFICATION OF ATP RELEASE

After infection with *S.pneumoniae* D39, ATP release was quantified using ATP Determination Kit (Molecular probes) according to manufacturer's instructions. Briefly, 10 µl cell supernatant or ATP standard were gently mixed with 100 µl reaction solution containing 1 µl dithiothreitol (100 mM), 5 µl D-luciferin (10 mM), 0.025 µl firefly luciferase (5 mg/ml), 5 µl 20× reaction buffer and 89 µl dH_2O per reaction. The assay is based on luciferase's requirement for ATP in producing light by catalyzing the oxidation of D-luciferin. After mixing all reagents, luminescence was immediately measured using a luminometer.

2.7.4 BACTERIAL UPTAKE BY BMMS

To determine the phagocytosis of pneumococci by BMMs, cells were infected with *S.pneumoniae* (D39, D39Δ*cps*) (MOI = 2.5) followed by centrifugation (190 g, 37°C, 5 min) and incubation for 30 min at 37°C + 5% CO_2. Following, cells were washed 3 times with PBS and incubated with 50 µg/ml gentamycin in RPMI 1640 for 30 min to kill extracellular bacteria. Then, BMMs were washed 3 times with PBS and lysed with 0.1% saponin in PBS for 10 min. The cell lysate (10 µl) was plated on a blood agar plate and incubated overnight. The number of CFUs was counted the next day reflecting the number of internalized pneumococci.

2.7.5 OPSONOPHAGOCYTIC NEUTROPHIL KILLING ASSAY

Opsonophagocytic killing assays were mainly conducted as described as described before (212). In brief, bacteria (D39Δ*cps*) were grown to mid-log phase, PBS-washed, and resuspended in HBSS++ solution (HBSS with Ca^{2+} and Mg^{2+}). The bacteria (10^3 CFU in 10 µl) were preopsonized with infant rabbit serum (40 µl per 10^3 CFU) for 30 min at 37°C. Isolated murine bone marrow derived neutrophils (see 2.4.3) were added to the reaction (4×10^5 cells in 150 µl HBSS++) and incubated for 1 h at 37°C. The solution was plated on a blood agar plate and incubated overnight. The number of viable bacteria was quantified the next day. Percent survival was determined relative to control reactions lacking neutrophils.

2.8 Statistical analysis

Data was analyzed using the GraphPad Prism software, Version 4.02. For comparison of two groups, Mann-Whitney U Test was used. If more than two groups were compared, Kruskal-Wallis Test followed by a Dunn's post-hoc test was used. Survival was analyzed by Log-rank test.

2.9 Reagents and kits

TABLE 3: Kits

KIT	COMPANY
Amplex® Red Uric Acid/Uricase Assay Kit	Life technologies
Anti-Ly-6G MicroBead Kit, mouse	Miltenyi Biotec
ATP Determination Kit	Molecular probes
Human Albumin ELISA Quantitation Set	Biomol
Human IL-33 ELISA	PeproTech
Mouse CXCL1/KC DuoSet	R&D Systems
Mouse CXCL2/MIP-2 DuoSet	R&D Systems
Mouse CXCL5	R&D Systems
Mouse IL-1 beta ELISA Ready-SET-Go!®	eBioscience
Mouse IL-6 ELISA Ready-SET-Go!®	eBioscience
Mouse IL-33 DuoSet	R&D Systems
Mouse ST2/IL-1 R4 DuoSet	R&D Systems
Mouse TNF alpha ELISA Ready-SET-Go!®	eBioscience
PerfectPure RNA Cultured Cell Kit	5 PRIME
PerfectTaq™ Plus DNA Polymerase	5 PRIME

TABLE 4: Reagents

REAGENT	COMPANY
Ampicillin	Ratiopharm
Ampuwa® (RNase-free H2O)	Fresenius Kabi
Apyrase	Sigma-Aldrich
Bacto Twodd Hewitt Broth	BD Biosciences
Bacto Yeast extract	BD Biosciences
Collagenase	Serva
Columbia Agar + 5% sheep blood	BD Biosciences
Complete protease inhibitor cocktail tablets	Roche
Dispase	BD Biosciences
DMEM high glucose	Gibco
DMSO	Sigma-Aldrich
DNase	Serva
Dynabeads® Biotin Binder	Invitrogen
Dynabeads® Sheep Anti-Rat IgG	Invitrogen
EDTA	Roth
Endothelial Cell Growth Medium MV2 (+ Supplement)	PromoCell
Ethanol	Merck
FCS	PAA
Fibronectin	Sigma
Gentamycin	PAA
Glycerol	Roth
HBSS	PAA
Heparin	Ratiopharm
HEPES	Biochrom
Human serum albumin	Baxter
Isopropanol	Sigma-Aldrich
Kanamycin	Sigma
Ketamine (Ketavet)	Sigma
L-glutamine	PAA
Low-melt agarose	Bio-Rad
LPS	Alexa Biochemicals
NaCl (0.9%)	B. Braun
Pancoll	PAN Biotech GmbH
Paraformaldehyde	Sigma
PBS	Gibco
Penicillin/Streptomycin	PAA
PPADS	Sigma-Aldrich
RPMI 1640	Gibco
RBC Lysis Buffer 10X	BioLegend
Saponin	Sigma-Aldrich
Suramin	Sigma-Aldrich
TaqMan® Gene Expression Master Mix	Applied Biosystems
TRIzol®	Invitrogen
Tween-20	Sigma-Aldrich
Uricase (Fasturtec®)	Sanofi
Xylazine (Rompun 2%)	Bayer

2.10 Instruments and consumables

TABLE 5: Instruments

INSTRUMENT	COMPANY
7300 Real-Time PCR System	Applied Biosystems
BD FACSCalibur™	BD Biosciences
BD FACSCanto™	BD Biosciences
FilterMax™ F5 Multimode Microplate Reader	Molecular Devices
Heracell™ 240i CO2 Incubator	Thermo Scientific
Herasafe™ KS	Thermo Scientific
Lumat LB9501	Berthold
MACS® Manual Separators	Miltenyi Biotec
Mastercycler Gradient	Eppendorf
Microcentrifuge 5417R	Eppendorf
NanoDrop 2000	Thermo Scientific
Photometer	Eppendorf
Rotanta 460 R	Hettich
Vortex mixer, VV3	VWR

TABLE 6: Consumables

CONSUMABLE	COMPANY
Cell culture flask	BD Biosciences
Cell strainers (100 µm, 70 µm, 40 µm)	BD Biosciences
Cell culture tubes	Falcon
Cuvettes	Fisher Scientific
ELISA plates	Thermo Scientific
non-tissue culture treated dishes Optilux	Thermo Scientific
Serological pipets	Thermo Scientific
Sterile filters	Millipore

3 — Results

3.1 *S. pneumoniae* induced cell death and the release of DAMPs upon infection

The first aim of this study was to examine whether *S. pneumoniae* induces cell death after infection accompanied by the release of DAMPs. Therefore, murine bone marrow derived macrophages (BMMs), alveolar epithelial cells (AECs), human lung tissue (HuLu), and human peripheral blood mononuclear cells (PBMCs) were infected with *S. pneumoniae*. Cell death induction was determined by quantifying the specific release of LDH, which is liberated after lytic cell death. Furthermore, the release of the DAMPs uric acid, ATP and IL-33 was quantified. Indeed, *S. pneumoniae* infection led to cell death in infected BMMs and -to a lesser extent- in AECs (Fig. 2A) as well as in human PBMCs and HuLus (data not shown). In line with these findings, the release of DAMPs after infection was detected. The amount of extracellular uric acid was significantly or by trend enhanced in infected BMMs or AECs and HuLus, respectively, compared to uninfected controls (Fig. 2B). Furthermore, significant ATP release was measured after infection in BMMs and AECs (Fig. 2C) and IL-33 was released by *ex vivo* infected HuLus (Fig. 2 D). In contrast, no IL-33 release in BMMs and AECs was found (data not shown). Taken together, these data demonstrate that *S. pneumoniae* induces cell death and DAMP release upon infection.

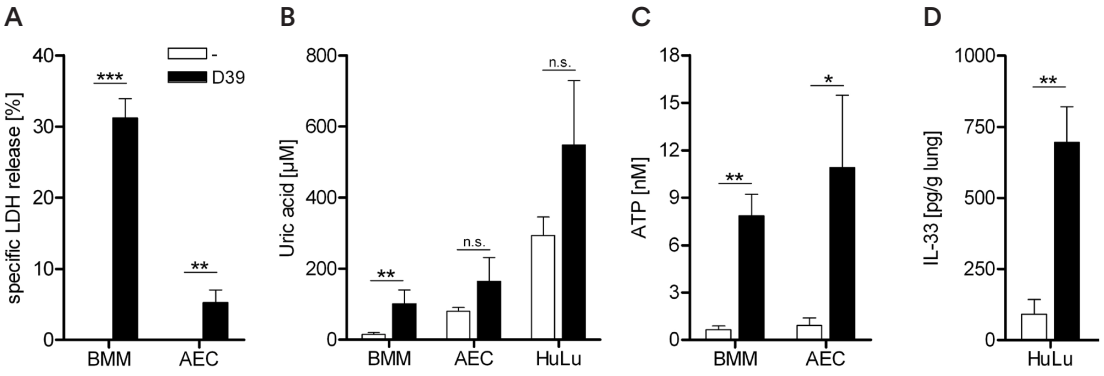

FIGURE 2: *S. pneumoniae* induces cell death and the release of DAMPs upon infection. BMMs and AECs, isolated from C57BL/6 WT mice, or HuLus were left untreated or infected with 1×10^6 *S. pneumoniae* strain D39 for 16 h (BMM, AEC) or 24 h (HuLu). (A) Cell death was quantified by specific LDH release. (B) Uric acid and (C) ATP release were measured in cell-free supernatants. (D) IL-33 release was determined by ELISA. Data are shown as mean ± SEM of (D) eight, (A, BMM) four, (A, AEC; B) three or (C) two independent experiments carried out in duplicates or triplicates. Statistical analysis was performed as described; * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.001$.

3.2 *St2*$^{-/-}$ mice displayed reduced bacterial loads in the acute phase of pneumococcal pneumonia, whereas inhibition of uric acid, ATP and purinergic P2 receptors and deficiency for P2X7, P2Y6, FcRγ and Mincle had no impact on bacterial burden

It has been shown that recognition of DAMPs by specific receptors can induce sterile inflammation (39). Considering that DAMPs are also released in response to pneumococcal infection, it was hypothesized that DAMPs would also affect the antibacterial immune response and susceptibility of mice during *S. pneumoniae* infection. Therefore, the functions of the DAMPs uric acid and ATP, the DAMP recognizing purinergic P2 receptor family, and the DAMP receptors Mincle and ST2 were examined in a murine model of pneumococcal pneumonia. Wild-type (WT) mice were treated with the uric acid-degrading enzyme uricase, the ATP-hydrolyzing enzyme apyrase, the broad-spectrum P2 receptor antagonist suramin or the selective P2X receptor antagonist PPADS. Mice were treated before and 24 h after infection with 5×10^6 *S. pneumoniae* per mouse. In addition, respective DAMP receptor knock-out mice were infected with the same dosage.

After 48 h of infection, bacterial loads were determined in the BALF and blood of infected mice. At this time point, all mice suffered from severe pneumococcal pneumonia, characterized by the loss of body temperature and body weight, excessive pulmonary neutrophil influx, and elevated inflammation parameters (data not shown). Mice treated with the enzymes uricase or apyrase or the P2 receptor antagonists suramin or PPADS had almost equal amounts of bacteria in the BALF and blood compared to PBS-treated control mice (Fig. 3A, B). Similarly, deficiency of the purinergic receptors P2X7 and P2Y6, which are preferentially activated by ATP or UDP, respectively, did not affect bacterial loads of mice after *S. pneumoniae* infection (Fig. 3C, D). Furthermore, mice deficient for

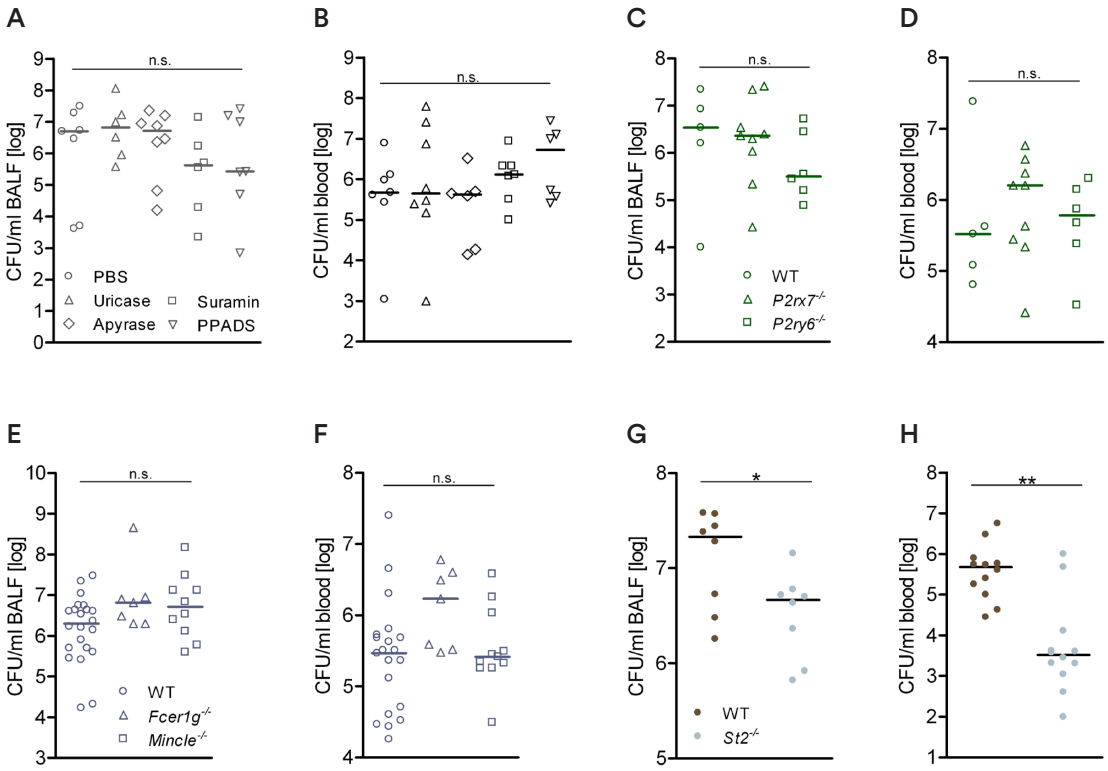

FIGURE 3: Role of different DAMPs and potential DAMP receptors in pneumococcal pneumonia. C57BL/6 mice were transnasally infected with 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). (A-H) Bacterial loads were determined in the BALF and blood 48 h p.i. (A, B) Infected WT mice were intratracheally treated with PBS, uricase (0.073 U/mouse), apyrase (0.32 U/mouse), suramin (0.32 µM/mouse) or PPADS (0.32 µM/mouse) before infection and after 24 h. (C, D) WT, *P2xr7*$^{-/-}$ and *P2yr6*$^{-/-}$ or (E, F) WT, *Fcerg1*$^{-/-}$ and *Mincle*$^{-/-}$ or (G, H) WT and *St2*$^{-/-}$ mice were infected. Single experimental data are depicted as individual symbols, lines represent median. (A, B) n = 6-8; (C, D) n = 5-8; (E, F) n = 5-22; (G, H) n = 8-12 mice each group. Statistical analysis was performed as described; * = $p < 0.05$, ** = $p < 0.01$.

the C-type lectin receptor Mincle, known to detect the DAMP SAP130 (49), or the downstream signaling adaptor molecule FcRγ (encoded by *Fcer1g*) had unaltered bacterial loads compared to WT mice (Fig. 3E, F). However, significantly reduced bacterial loads in the BALF and blood of mice deficient for the IL-33 receptor ST2 compared to WT mice were detected (Fig. 3G, H).

Overall, the data indicate that ST2 regulates the antibacterial innate immune response during pneumococcal pneumonia, which might be dependent on the detection of the DAMP IL-33. They further suggest that the DAMPs uric acid and ATP as well as DAMP recognizing receptors from the purinergic receptor family, in particular P2X7 and P2Y6, and Mincle have no impact on the bacterial clearance in *S. pneumoniae* infected mice in the acute phase of pneumonia.

3.3 ST2 and IL-33 deficiencies enhanced bacterial clearance and integrity of the epithelial-endothelial barrier during progression of pneumococcal pneumonia

To further investigate the function of ST2 and its ligand IL-33 in pneumococcal pneumonia, WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally infected with 5×10^6 *S. pneumoniae* per mouse and bacterial loads in the BALF, blood and spleen were analyzed over time. All mice showed similar amounts of bacteria in the BALF and no systemic bacterial dissemination after 12 h of infection (Fig. 4A-C). However, as early as 24 h p.i. a significant increase of bacterial burden in the blood and spleen of WT mice compared to knock-out mice was detected (Fig. 4B, C). At this time point, bacterial loads in the BALF were not significantly different, but slightly diminished in *Il33*$^{-/-}$ mice (Fig. 4A). As already shown before (Fig. 3G, H), reduced bacterial loads in the BALF and blood and additionally in the spleen of *St2*$^{-/-}$ mice compared to WT mice after 48 h were observed (Fig. 4A-C). Importantly, the same observation was made when comparing *Il33*$^{-/-}$ mice and WT mice at this time point (Fig. 4A-C). Next, the integrity of the epithelial-endothelial barrier in these mice was analyzed by quantifying the amount of HSA, which was diffused into the BALF from the blood after intravenous HSA injection. The permeability of the barrier in WT mice was increased compared to knock-out mice after 24 h (Fig. 4D). This is in accordance with the finding, that WT mice showed

increased bacterial dissemination 24 h after infection. In addition, a significantly higher permeability in WT mice compared to *St2*$^{-/-}$ or *Il33*$^{-/-}$ mice after 48 h was detected (Fig. 4D), which, however, might be related to the increased bacterial burden in these animals.

Next, transcriptional regulation of *St2* and *Il33* in the lungs of PBS-treated and *S.pneumoniae* infected mice was investigated over time. ST2 and IL-33 are known to be constitutively expressed in the lung (70, 71). Both proteins were not regulated during the early phase of infection (Fig. 5A, B), but 48 h after

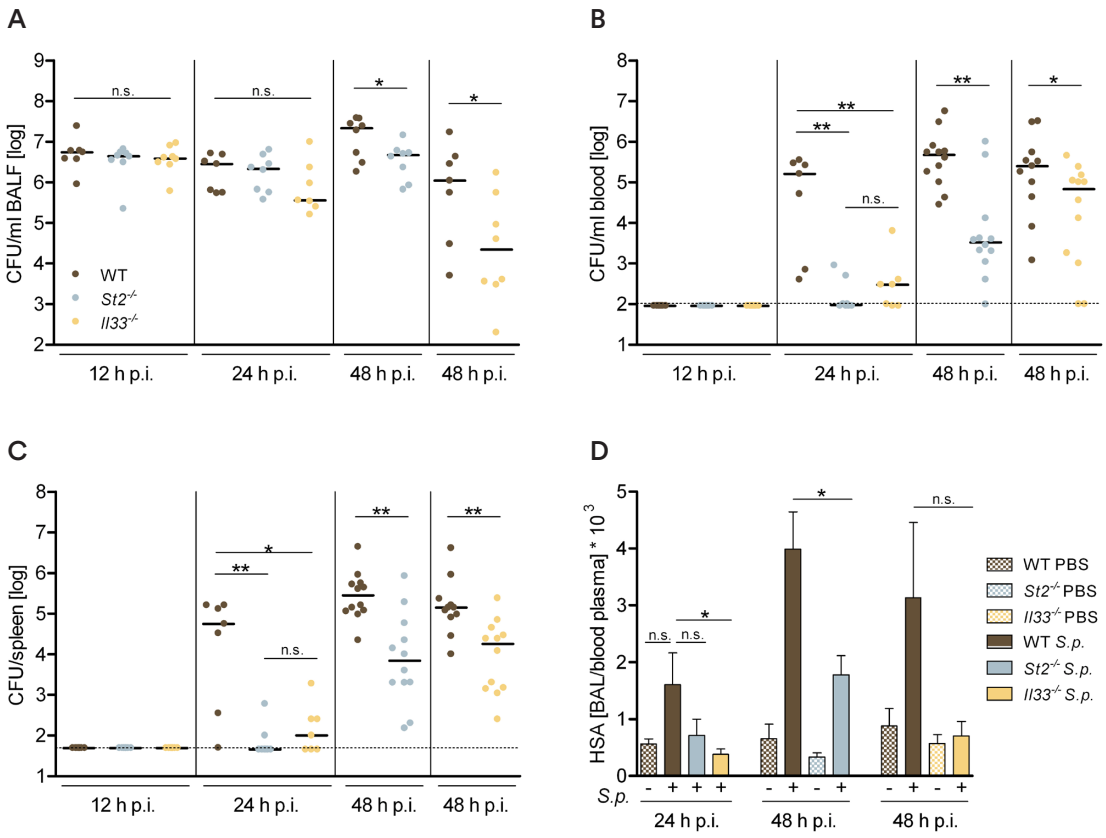

FIGURE 4: ST2 and IL-33 deficiencies increase the bacterial clearance and the integrity of the epithelial-endothelial barrier during the progression of pneumococcal pneumonia. (A-D) C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally infected 5×10^6 CFU/mouse of *S. pneumoniae* (PN36) or (D) sham-infected with PBS. Bacterial loads were determined 12, 24 or 48 h p.i. in the (A) BALF, (B) blood and (C) spleen. (D) HSA was intravenously infused 1 h before BALF and lung permeability was quantified by determining the HSA BALF/blood plasma ratio by ELISA 24 and 48 h p.i. (A-C) Single experimental data are depicted as individual symbols, lines represent median and the dotted line represents the lower detection limit. Data already shown in figure 1 (G, H) were included for clarity. (D) Data shown in are mean ± SEM; (A-D) n = 7-8 or (B, C 48 h p.i.) n = 11-12 mice each group. Statistical analysis was performed as described; * = $p < 0.05$, ** = $p < 0.01$.

infection the relative expression of *St2* was markedly decreased in the lungs of infected mice compared to uninfected controls (Fig. 5A). Moreover, the IL-33 decoy receptor sST2 was released into the BALF after 48 h (Fig. 5C). However, the IL-33 protein could not be detected in the BALF (data not shown), which might have been based on the low sensitivity of the ELISA used (and the low amount of IL-33 protein in the BALF). Alternatively, IL-33 might not be released during murine pneumococcal pneumonia. In summary, the data suggest that ST2 and IL-33 have detrimental effects on bacterial clearance and the integrity of the epithelial-endothelial barrier during the acute phase of pneumococcal pneumonia.

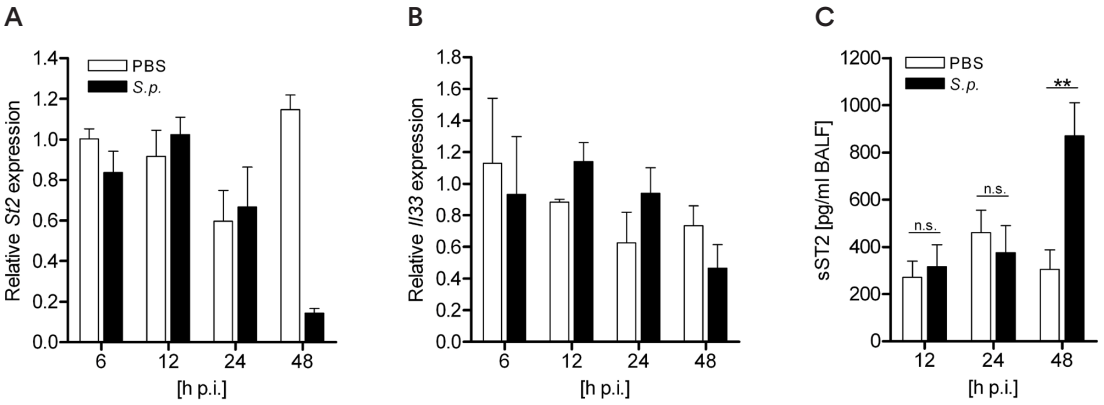

FIGURE 5: Transcriptional regulation of *St2* and *Il33* in the lung and release of sST2 during the progression of pneumococcal pneumonia. C57BL/6 WT mice were transnasally sham-infected with PBS or infected with 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). Mice were sacrifised 6, 12, 24 or 48 h p.i. RNA was isolated from the lung and relative expression of (A) *St2* and (B) *Il33* was determined by quantitative RT-PCR. (C) Release of sST2 into the BALF was determined by ELISA. Data are shown as mean ± SEM; (A, B) n = 3 or (C) n = 6-14 mice each group. Statistical analysis was performed as described; ** = $p < 0.01$.

3.4 ST2 and IL-33 deficiencies differentially affected the resistance of mice infected with *S. pneumoniae*

Following the observation that *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice showed enhanced bacterial clearance, it was expected that ST2 and IL-33 are crucial for the survival during pneumococcal pneumonia. In previous experiments, mice were infected with a lethal dose of *S. pneumoniae* resulting in a severe progress of the disease in all

infected mice. Despite the fact that knock-out mice had reduced bacterial burden, they were, like WT mice, not able to clear all bacteria to restrict the progression of the disease. For this reason, each mouse was infected with a sublethal dose of 7.5×10^4 *S.pneumoniae* to study the survival. Indeed, ST2- and IL-33-deficient mice were more resistant to pneumococcal infection than WT mice (Fig. 6A). Only 33% of WT mice compared to 67% of *Il33*$^{-/-}$ and 95% of *St2*$^{-/-}$ mice survived the infection. Interestingly, the resistance of *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice was significantly different, suggesting a function of ST2 or IL-33 which is independent of the receptor-ligand interaction.

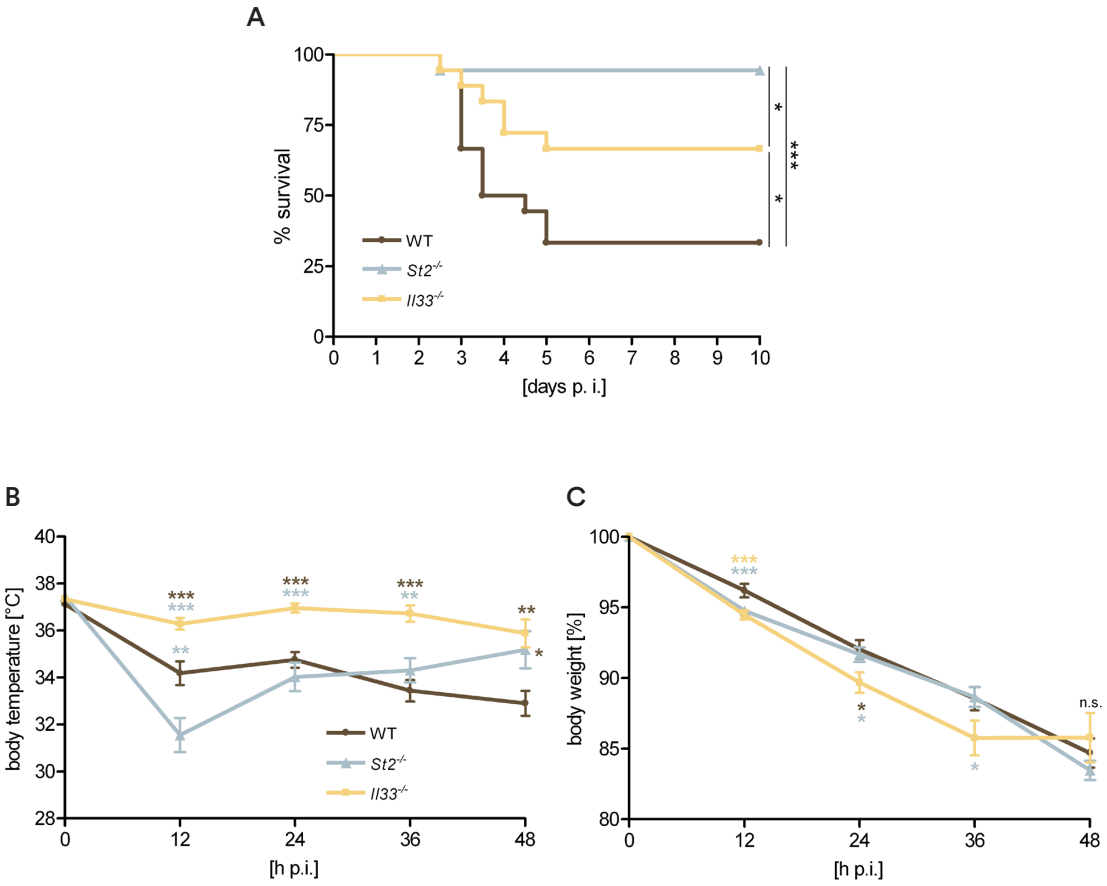

FIGURE 6: ST2 and IL-33 deficiencies improve survival and differentially affect the resistance of infected mice towards *S. pneumoniae*. C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally infected with (A) 7.5×10^4 (B, C) or 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). (A) Survival was monitored every 12 h for 10 days. (B) Body temperature and (C) body weight loss were assessed every 12 h until sacrification. (A) n = 18 mice each group; (B, C) Data are shown as mean ± SEM; (12 h p.i.) n = 26-33; (24 h p.i.) n = 20-27; (36, 48 h p.i.) n = 12-19 mice each group. Statistical analysis was performed as described; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Next, the influence of ST2 and IL-33 on the loss of body temperature and body weight was examined, which are both important clinical signs of severe pneumococcal pneumonia. Therefore, mice were infected with a lethal dose of 5×10^6 of *S. pneumoniae*. Notably, the loss of body temperature was again differentially affected by the deficiencies of ST2 and IL-33, supporting the idea that both proteins have independent functions from each other (Fig. 6B). The body temperature of *St2*$^{-/-}$ mice strongly declined after 12 h from 37°C to 31.5°C and increased in the following course of the disease to 35°C, whereas the body temperature of *Il33*$^{-/-}$ mice was only slightly reduced to 36°C during pneumococcal pneumonia. Still, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice displayed a higher body temperature after 48 h of infection as compared to WT mice, which supports the previous findings showing improved bacterial clearance (Fig. 4A-C) and survival in the two knock-out mouse strains (Fig. 6A). On the contrary, only a moderate impact of ST2 and IL-33 on the body weight loss during the course of infection was found. All mice reduced their initial body weight around 15% until sacrifice and only *Il33*$^{-/-}$ mice displayed a marginal diminished body weight compared to WT and *St2*$^{-/-}$ mice until 36 h p.i. (Fig. 6C). Moreover, all mice inoculated with PBS maintained their initial body temperature and weight over time (data not shown). Collectively, the data suggest that (i) expression of ST2 and IL-33 enhances the susceptibility of mice towards pneumococcal pneumonia, and that (ii) ST2 and IL-33 have receptor-ligand independent functions during pneumococcal pneumonia.

3.5 ST2 deficiency protected from severe pleurisy and edema during pneumococcal pneumonia

To examine the impact of ST2 and IL-33 on the histopathology of the lung during pneumococcal pneumonia, hematoxylin and eosin stained, paraffin-embedded lung tissue sections were prepared. In contrast to PBS-treated mice (Fig. 7A), all infected mice showed typical characteristics of a catarrhal purulent and necrotizing pneumonia, with lesions mainly located close to the hilus after 48 h of infection (Fig. 7B). Even though there were no clear differences between the groups regarding the quality and quantity of pneumonia, ST2 and IL-33 deficiencies affected the occurrence of pleurisy and perivascular edemas (Fig. 7C-J). WT mice suffered from purulent and necrotizing pleurisy with transition towards the

mediastinal fatty tissue (Fig. 7C), whereas *St2*$^{-/-}$ mice displayed no pleurisy at all (Fig. 7D). The role of IL-33 deficiency for the development of pleurisy was not clear, because two out of four mice exhibited an inflamed pleura (Fig. 7E) while the other two showed no signs of pleurisy (Fig. 7F). In addition, histological analysis revealed that all mice suffering from pleurisy had prominent perivascular

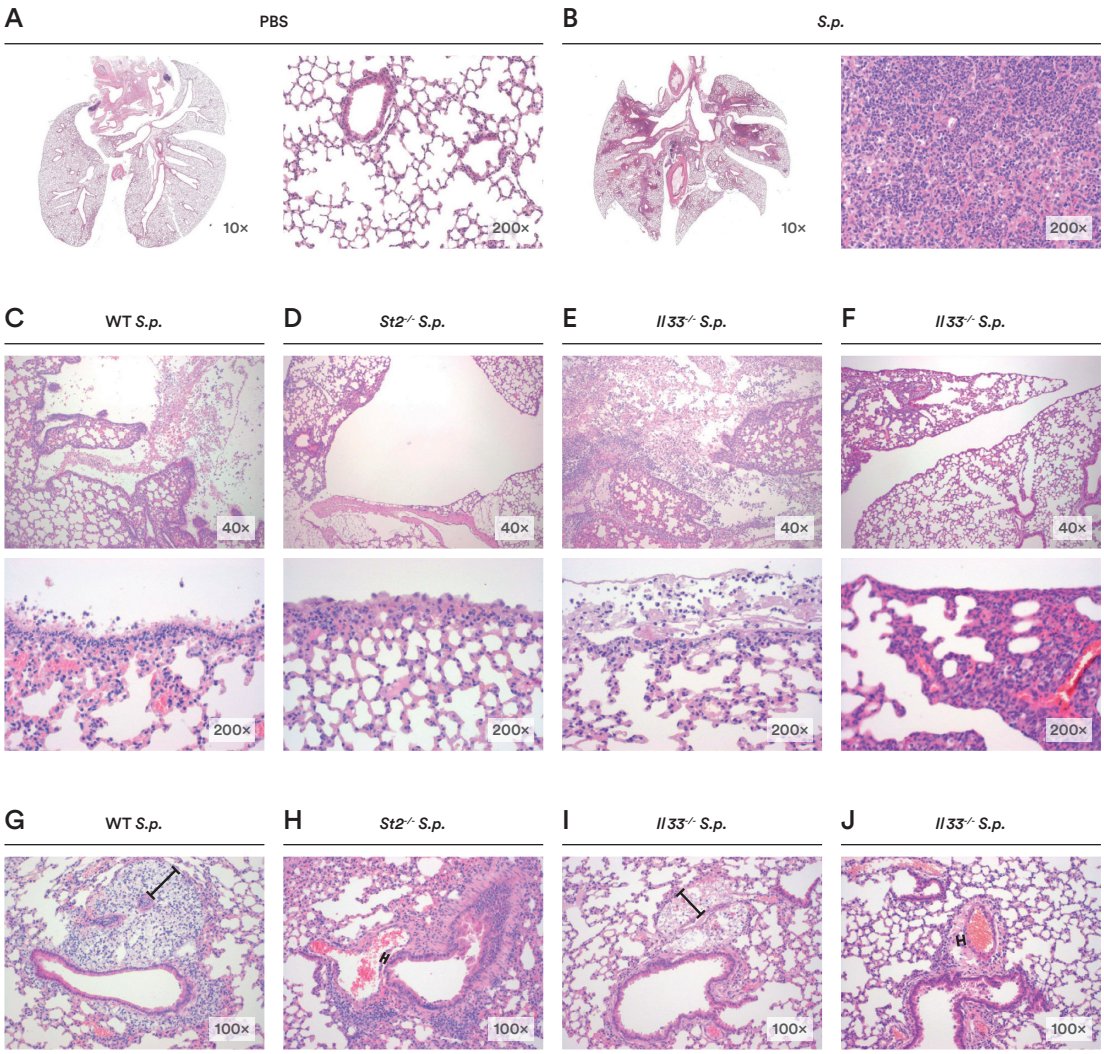

FIGURE 7: ST2 deficiency protects from severe pleurisy and edema during pneumococcal pneumonia. C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally (A) sham-infected with PBS or (B-J) infected with 5×10^{6} CFU/mouse of *S. pneumoniae* (PN36) and sacrifisied 48 h p.i. Lungs were fixed and embedded in paraffin followed by hematoxylin and eosin staining of dewaxed tissue sections. (C-F) Appearance of pleurisy and (G-J) perivascular edema was depicted and black lines represent the size of edemas. Magnifications are depicted in each graph. Pictures are representative for (A) n = 4 WT mice, (B) all infected mice (WT, *St2*$^{-/-}$, *Il33*$^{-/-}$), (C, D, G, H) n = 4 or (E, F, I, J) n = 2 mice each group.

edemas (Fig. 7G-J). In summary, lack of ST2 protected mice from severe pleurisy and edema during pneumococcal pneumonia, whereas the effect of IL-33 deficiency was less pronounced. These findings are in accordance with previous results of this study, showing an intermediate survival of *Il33*$^{-/-}$ mice compared to WT and *St2*$^{-/-}$ mice.

3.6 ST2 and IL-33 were highly expressed in AECs and MVECs

Based on the *in vivo* findings, it was hypothesized that ST2 and IL-33 would differentially regulate the anti-bacterial innate host defense in lung cells towards pneumococcal infection. To test this hypothesis, the basal expressions of *St2* and *Il33* and their transcriptional regulation upon infection were investigated in various cell types, which play an important role for the innate immune response against

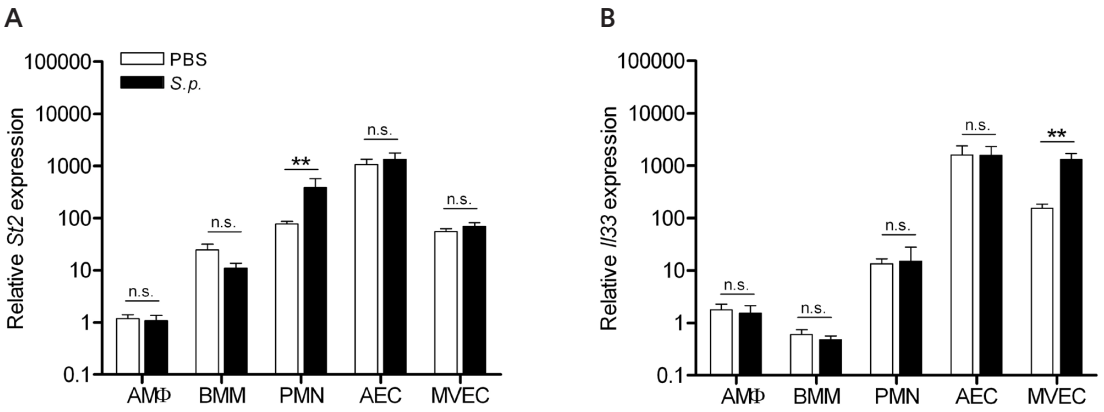

FIGURE 8: Basal expression *St2* and *Il33* in different cell types and transcriptional regulation of upon *S. pneumoniae* infection. AMΦs, AECs, and MVECs were isolated from the lung, and PMNs and BMMs from the bone marrow of C57BL/6 WT mice. Cells were left untreated or infected with *S. pneumoniae* D39 and total RNA was isolated. (AMΦ, BMM, PMN: MOI=1; AEC, MVEC: MOI=10). Relative expression of (A) *St2* and (B) *Il33* was determined by quantitative RT-PCR. Data are shown as mean ± SEM of (BMM, AEC) seven or (AMΦ, MVEC, PMN) three independent experiments carried in duplicates. Statistical analysis was performed as described; ** = $p < 0.01$.

invading pneumococci (168). The highest amounts of *St2* and *Il33* transcripts were detected in AECs as compared to the other cell types analyzed. Both molecules were further highly expressed in MVECs. *St2* levels in PMNs (polymorphonuclear neutrophils) were similar to that of MVECs, whereas *Il33* levels were lower. AMΦs and BMMs displayed the lowest expression of *St2* and *Il33* (Fig. 8A, B).

Upon infection with *S. pneumoniae*, the expression of *St2* was upregulated in PMNs as compared to the control, but *St2* was not regulated in macrophages, AECs and MVECs (Fig. 8A). With respect to IL-33, no regulation in macrophages, AECs and PMNs, but enhanced expression in infected MVECs compared to untreated cells was observed (Fig. 8B). Taken together, *St2* and *Il33* are most abundantly expressed in AECs and MVECs, but showed an intermediate or low expression in PMNs or macrophages, respectively.

3.7 ST2 negatively regulated KC production in *S. pneumoniae* infected AECs, independently of IL-33

To study whether ST2 and IL-33 affect the innate immune response towards *S. pneumoniae* in AECs and MVEC, primary AECs and MVECs from murine lungs were infected with *S. pneumoniae*. LPS treatment was performed as positive control, because Brint *et al*. reported that ST2-deficient macrophages produced more cytokines after LPS stimulation as compared to WT cells (92). Production of KC and IL-6 was determined, since both cytokines were shown to be important mediators of the antibacterial defense during pneumonia (177). In the present study, ST2-deficient AECs secreted significantly more KC after *S. pneumoniae* infection and LPS treatment compared to WT cells. However, levels of KC were similar in IL-33-deficient and WT cells (Fig. 9A). Thus, the inhibitory effect of ST2 on the pneumococci-induced KC production seemed to be independent of the ligand IL-33. Similarly, KC production was slightly elevated in $St2^{-/-}$ MVECs as compared to WT and $Il33^{-/-}$ cells after infection. However, this trend was not significant and less pronounced in cells infected with a higher dose (MOI=10). The secretion of IL-6 was not affected by ST2 and IL-33 deficiencies in AECs and MVECs (Fig. 9B, D). In summary, ST2 negatively regulates *S. pneumoniae* induced KC, but not IL-6 production in AECs, independently of IL-33.

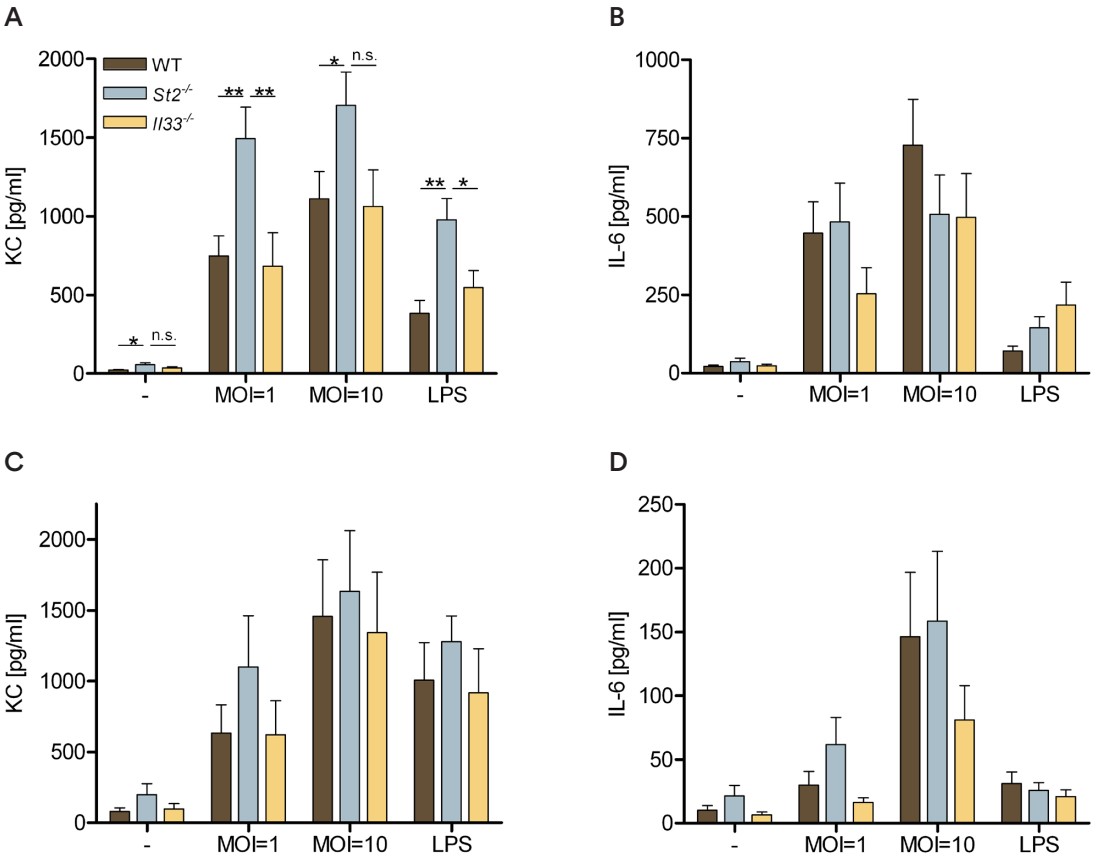

FIGURE 9: ST2 negatively regulates KC production in *S. pneumoniae* infected AECs. (A, B) AECs and (C, D) MVECs were isolated from lungs of C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice. Cells were left untreated, infected with *S. pneumoniae* (D39) (MOI=1; 10) or stimulated with 100 ng/ml LPS for (A, B) 16 h or (C, D) 5 h. (A, C) KC and (B, D) IL-6 secretion was quantified by ELISA. Data are shown as mean ± SEM of (A, B) six or (C, D) five independent experiments carried out in duplicates. Statistical analysis was performed as described; * = $p < 0.05$, ** = $p < 0.01$.

3.8 ST2 and IL-33 did not affect the innate immune response of macrophages to *S. pneumoniae* and the anti-pneumococcal killing capacity of neutrophils

Considering the weak expression of ST2 and IL-33 in AMΦs and BMMs, a minor impact of these proteins on the innate immune response in macrophages was suggested. Indeed, the secreted amounts of KC, IL-6, TNF-α, and IL-1β were almost equal in WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ BMMs upon *S. pneumoniae* infection

(Fig. 10A, B and data not shown). Moreover, it was not possible to reproduce the data from Brint *et al*. (92) showing that LPS-stimulated macrophages from *St2*$^{-/-}$ mice secrete more IL-6 than WT macrophages.

A major function of macrophages is the phagocytosis of bacteria. To test whether ST2 and IL-33 influence the ability of macrophages to phagocytose *S. pneumoniae*, BMMs were infected with WT D39 bacteria and the unencapsulated mutant D39Δ*cps*. The bacterial uptake was determined by quantifying the

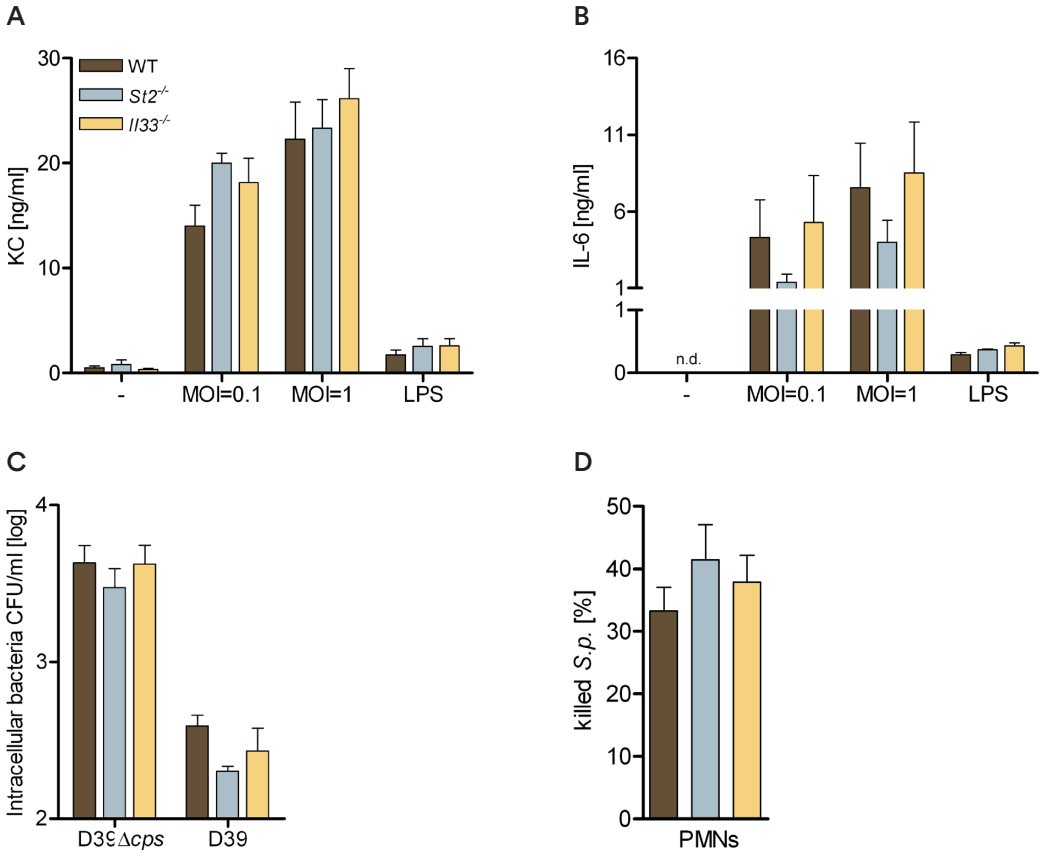

FIGURE 10: ST2 and IL-33 do not affect the innate immune response of macrophages to *S. pneumoniae* and the anti-pneumococcal killing capacity of neutrophils. BMMs and PMNs were isolated from bone-marrow of C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice. (A-B) BMMs were left untreated, infected with *S. pneumoniae* (D39) (MOI=1; 10) or stimulated with 100 ng/ml LPS. (A) KC and (B) IL-6 secretion was quantified by ELISA after 16 h. (C) BMMs were infected with *S. pneumoniae* D39Δ*cps* and D39 (MOI = 2.5) and were treated with gentamicin (50 mg/ml) 30 min p.i. Intracellular bacteria were quantified 30 min after treatment. (D) PMNs were incubated with preopsonized *S. pneumoniae* D39Δ*cps* and neutrophil-mediated killing was assessed after 1 h incubation. Data are shown as mean SEM of (A, B) four, (D) three (C) or two independent experiments carried out in (A-C) duplicates or (D) quadruplicates. Statistical analysis was performed as described.

amount of engulfed bacteria after 1h. As expected, *S.pneumoniae* was protected from phagocytosis by the capsule, and less viable intracellular bacteria were found after infection with D39 compared to D39Δ*cps* (Fig. 10C). However, the capability of macrophages to phagocytose *S.pneumoniae* was not influenced by the deficiency of ST2 or IL-33.

Because neutrophil-mediated killing is crucial for the innate host defense against *S.pneumoniae* (168, 219), and *St2* and *Il33* were also expressed in these cells, the function of ST2 and IL-33 deficiencies on neutrophil killing capacity was analyzed. Therefore, neutrophils were incubated with preopsonized unencapsulated pneumococci (D39Δ*cps*) and the percentage of killed bacteria was determined after 1 h of incubation. Neutrophils were able to kill around 30-40% of *S.pneumoniae*, however, the killing capacity was not influenced by the deficiencies of ST2 and IL-33. Overall, ST2 and IL-33 do not seem to affect innate anti-bacterial defense mechanisms of macrophages and neutrophils in response to *S.pneumoniae*.

3.9 Production of inflammatory cytokines, chemokines and antimicrobial peptides appeared to be independent of ST2 and IL-33 *in vivo*

To elucidate whether ST2 and IL-33 also modulate the inflammatory immune response *in vivo*, the concentration of several cytokines and chemokines was quantified in the BALF of mice inoculated with *S.pneumoniae* or treated with PBS as control. The chemokines KC, MIP-2α and CXCL-5 initiate neutrophil recruitment during bacterial pneumonia (210). Other important innate immune response mediators are the proinflammatory cytokines TNF-α, IL-1β and IL-6 (177).

KC production was indistinguishable in all three mouse strains at 12 h and 24 h p.i. (Fig. 11A). This might appear unexpected considering that ST2 negatively regulated the secretion of KC in AECs and MVEC (see Fig. 9A, C). However, this can be most probably explained by the fact that KC is mainly produced by leukocytes and not by epithelial or endothelial cells during pneumococcal pneumonia (228, 229). Nevertheless, the production of CXCL-5, which is predominantly produced by epithelial cells (229), was also not influenced by ST2 and IL-33 at 12 h and 24 h p.i. (Fig. 11B). Likewise, *S.pneumoniae*-stimulated

production of MIP-2α, IL-6, IL-1β and TNF-α was not altered by the expression of ST2 or IL-33 at these early time points (Fig. 11C-F). The production of

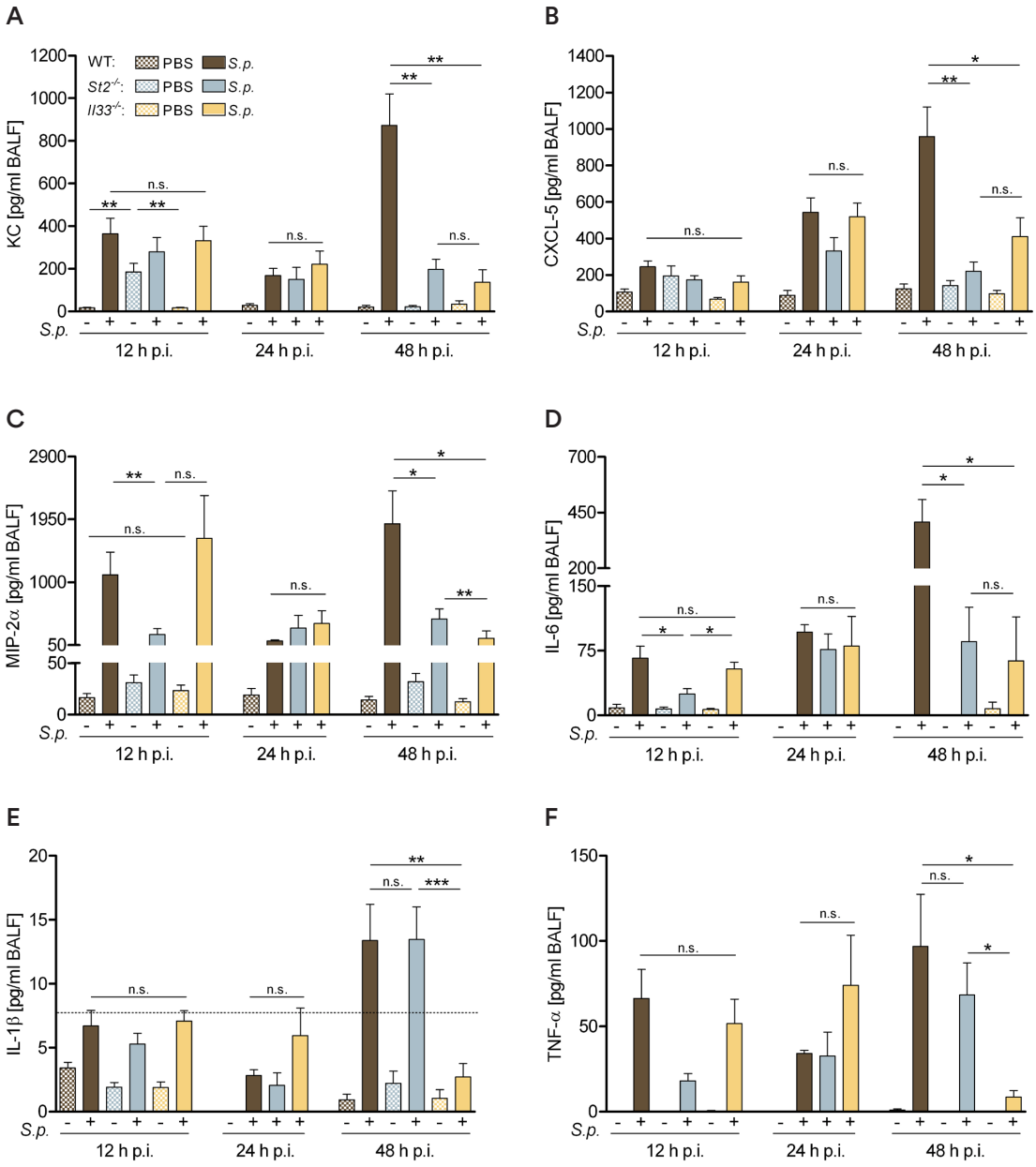

FIGURE 11: ST2 and IL-33 deficiencies have a minor impact on cytokine secretion into the BALF during pneumococcal pneumonia. C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally sham-infected with PBS or infected with 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). (A) KC, (B) CXCL-5, (C) MIP-2α, (D) IL-6, (E) IL-1β and (F) TNF-α secretion into the BALF was quantified by ELISA 12, 24 or 48 h p.i. Data are shown as mean ± SEM; n = 7-8 or (WT 48 h p.i.) n= 14-16 mice each group. The dotted line represents the lower detection limit. Statistical analysis was performed as described; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

MIP-2α, IL-6 and TNF-α was even slightly diminished in *St2*$^{-/-}$ mice compared to WT and *Il33*$^{-/-}$ mice after 12 h. However, at 48 h p.i. KC, CXCL-5, MIP-2α, and IL-6 secretion was significantly higher in WT mice as compared to the knock-out mice (Fig. 11A-D), which is most likely a result of the enhanced bacterial burden in these mice (see Fig. 4A-C). With regard to IL-1β and TNF-α production, WT mice showed significantly higher levels than *Il33*$^{-/-}$-, but not *St2*$^{-/-}$ mice, in the BALF after 48 h (Fig. 11E-F). Furthermore, PBS-treated control mice showed a moderate secretion of inflammatory cytokines in the BALF (Fig. 11A-E). This was not surprising since transnasal inoculation can lead to small irritations in the lung, which triggers an inflammatory response. It is important to note that PBS-treated *St2*$^{-/-}$ mice had significantly increased KC levels in the BALF after 12 h compared to the other groups (Fig. 11A) suggesting a role of ST2 in regulating basal production of KC.

Besides the quantification of cytokines in the BALF, the transcriptional regulation of various cytokines in the total lung was examined 12 h after infection. At this time point, bacterial loads were similar in all mouse strains examined (see Fig. 4A). Consistent to the presented protein data, *Cxcl1* and *Cxcl2* mRNA transcripts, which encode for KC and MIP-2α respectively, were significantly increased in the lungs of PBS-treated *St2*$^{-/-}$ mice compared to WT mice, but there were no differences between the groups of infected mice (Fig. 12A, B). The expression of the proinflammatory cytokines *Gcsf, Il23, Il1β* and anti-inflammatory *Il10* was also not affected by the deficiency of ST2 or IL-33 (Fig. 12C-E and data not shown). Transcription of the Th2 cytokine *Il5* was, however, diminished in the lungs of infected *Il33*$^{-/-}$ mice in comparison to *St2*$^{-/-}$ but not to WT mice (Fig. 12F). Importantly, *Ccl2* and *Il6* transcripts in the lungs of infected *Il33*$^{-/-}$ mice were more than twofold higher compared to WT and *St2*$^{-/-}$ mice (Fig. 12G, H). *Ccl2* encodes for MCP1 and is crucial for monocyte/macrophage recruitment in response to *S.pneumoniae* (195). Controversially, IL-6 protein levels were not increased in the BALF of *Il33*$^{-/-}$ mice as compared to the other groups after 12 h or 24 h of infection, which might be due to the different sources analyzed (BALF vs. total lung) or due to differential posttranscriptional regulation.

Finally, the impact of ST2 and IL-33 on the expression of antimicrobial peptides (AMPs) during pneumococcal pneumonia was studied, since enhanced expression of AMPs could explain the lower bacterial loads in *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice compared to WT controls (see Fig. 4A-C). *S.pneumoniae* infection induced upregulation of lipocalin-2 (*Lcn2*), cathelicidin (*Camp*) and the S100 calcium binding proteins A8 and A9 (*S100a8, S100a9*) after 12 h (Fig. 13A-D).

Moreover, an insignificant trend towards enhanced expression of *Lcn2* in *St2*$^{-/-}$ mice as compared to WT and *Il33*$^{-/-}$ animals was observed (Fig. 13A). Expression of the other AMPs examined was comparable in the infected WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ animals (Fig. 13B-D). Notably, *Lcn2* expression was significantly enhanced in PBS-treated *St2*$^{-/-}$ mice compared to WT mice, suggesting higher basal levels of *Lcn2* in these mice (Fig. 13A). In summary, the production of several inflammatory cytokines, chemokines and AMPs appears to be largely independent of ST2 and IL-33 *in vivo*. However, the expression of *Ccl2* and *Il6* seems to be negatively regulated by IL-33 after *S. pneumoniae* infection.

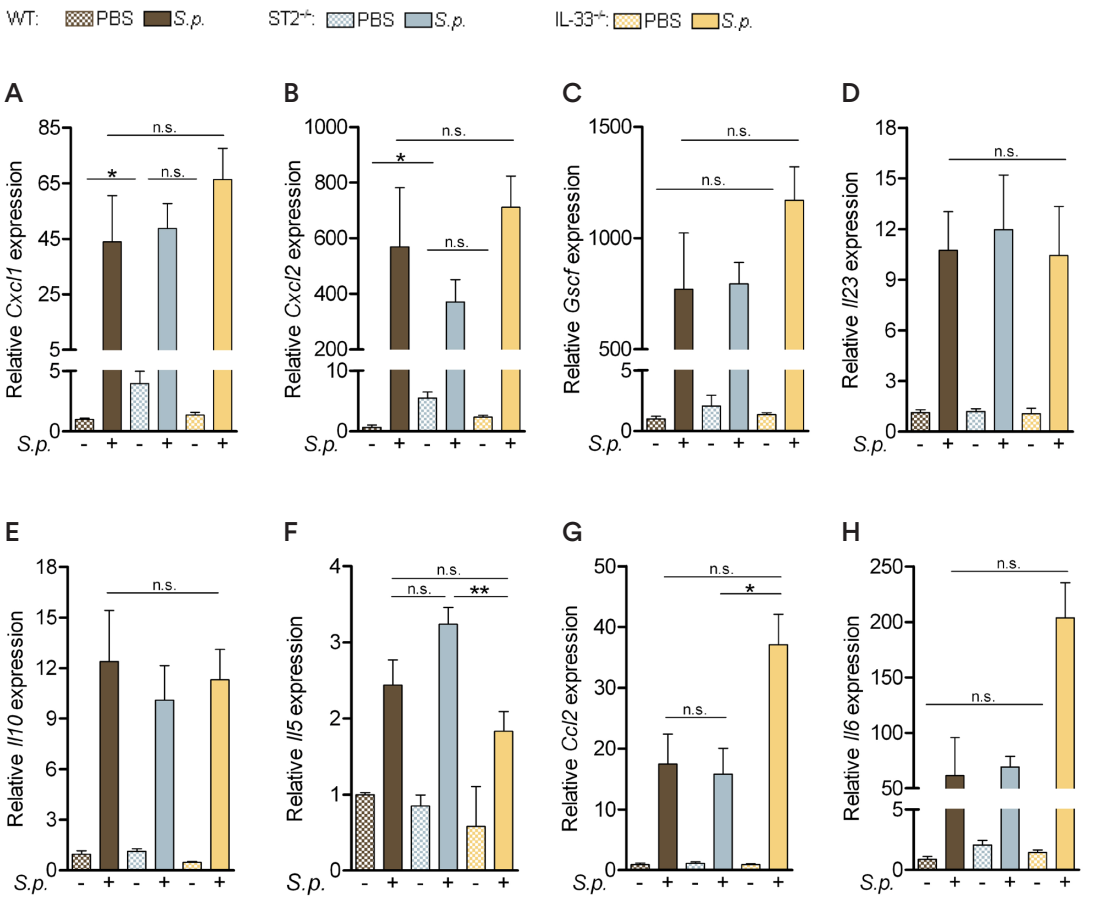

FIGURE 12: *Ccl2* and *Il6* expression is negatively regulated by IL-33 after *S. pneumoniae* infection by trend. C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally sham-infected with PBS or infected with 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). Mice were sacrifised 12 h p.i. and RNA was isolated from the total lung. Relative expression of (A) *Cxcl1*, (B) *Cxcl2*, (C) *Gcsf*, (D) *Il23*, (E) *Il-10*, (F) *Il5*, (G) *Ccl2* and (H) *Il6* was determined by quantitative RT-PCR. Data are shown as mean ± SEM; n = 5 mice each group. Statistical analysis was performed as described; * = $p < 0.05$.

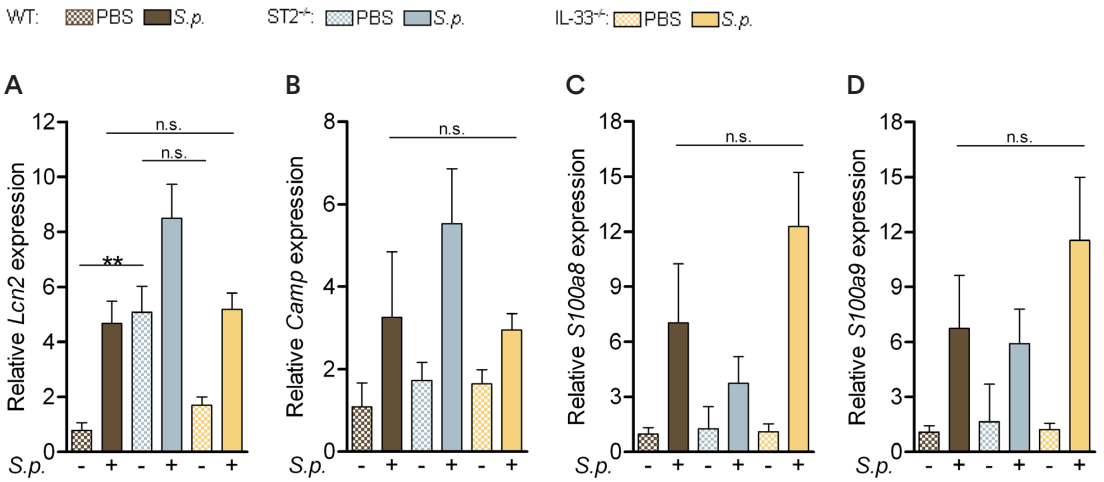

FIGURE 13: Expression of antimicrobial peptides during pneumococcal infection is not affected by ST2 and IL-33. C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally sham-infected with PBS or infected with 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). Mice were sacrifised 12 h p.i. and RNA was isolated from the total lung. Relative expression of (A) *Lcn2*, (B) *Camp*, (C) *S100a8* and (D) *S100a9* was determined by quantitative RT-PCR. Data are shown as mean ± SEM, n = 5 mice each group. Statistical analysis was performed as described; ** = $p < 0.01$.

3.10 Recruitment of neutrophils and/or macrophages was negatively regulated by ST2 and/or IL-33

To investigate if the enhanced bacterial clearance in *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice compared to WT animals is related to an enhanced recruitment of antibacterially acting leukocytes such as neutrophils and macrophages, their recruitment into the BALF in *S. pneumoniae*-infected or PBS-treated control mice was analyzed. The number of neutrophils was elevated in all infected mice compared to the controls after 12 h and further increased over time. The highest number of neutrophils was detected after 48 h in WT and *St2*$^{-/-}$ mice, whereas in *Il33*$^{-/-}$ mice neutrophil recruitment peaked at 24 h p.i. and declined thereafter (Fig. 14A). With regard to macrophages, only a slightly increased number was found in the infected groups compared to the control after 12 h (Fig 14B). This can, however, been explained by the high number of resident AMΦs, which are already present in the lung to combat invading pathogens (168). Newly recruited exudate macrophages, which mature into alveolar macrophages, accumulated in the lung after 24 h and declined again after 48 h (Fig. 14B).

The recruitment of neutrophils and macrophages was comparable in all mouse strains examined at 12 h p.i. Neutrophil counts were solely enhanced by trend in PBS-treated *St2*$^{-/-}$ mice compared to the other groups after 12 h (Fig. 13A), which is in line with the higher basal KC levels detected in these mice (see Fig. 11A). Interestingly, significantly more neutrophils were found in the BALF of infected *St2*$^{-/-}$ mice compared to WT and *Il33*$^{-/-}$ mice after 24 h (Fig. 14A). This provides a plausible explanation for the reduced bacterial load observed in ST2-deficient mice compared to the WT counterparts at 48 h p.i (see Fig. 4A-C). In addition, the number of neutrophils was still slightly higher in *St2*$^{-/-}$ mice than in WT mice after 48 h although the latter had augmented bacteria in the BALF. However, neutrophil recruitment was not affected by the deficiency of IL-33 suggesting another mechanism leading to enhanced bacterial clearance in these mice. Previous results showing elevated *Ccl2* transcripts in the lungs of *Il33*$^{-/-}$ mice (see Fig. 12G) suggest that IL-33 might negatively regulate the recruitment of monocytes and macrophages. Indeed, macrophage counts were significantly increased in *Il33*$^{-/-}$ mice and by trend also in *St2*$^{-/-}$ mice at 24 h p.i. compared to WT mice (Fig. 14B).

Overall, ST2 and IL-33 differentially regulated the recruitment of anti-bacterially acting innate immune cells into the lung during pneumococcal pneumonia. ST2 negatively regulated the infiltration of neutrophils independently of its ligand IL-33. The recruitment of macrophages was negatively regulated by IL-33 and possibly also by ST2.

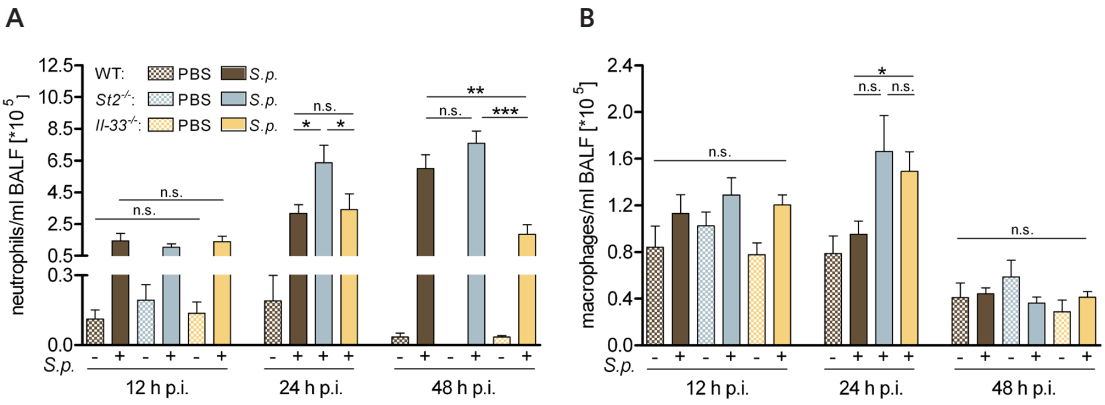

FIGURE 14: Recruitment of neutrophils and macrophages is negatively regulated by ST2 and/or IL-33 during pneumococcal pneumonia. C57BL/6 WT, *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were transnasally sham-infected with PBS or infected with 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). (A) Neutrophils and (B) macrophages (B) in the BALF were quantified by flow cytometry 12, 24 or 48 h. p.i. Data are shown as mean SEM; n = 7-8 or (WT 48 h p.i.) n= 14-16 mice each group. Statistical analysis was performed as described; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.11 ILC2s were located in the lung during resolution of pneumococcal pneumonia

It has been reported that ILC2 are recruited into the lung dependent on IL-33 and contribute to tissue repair after influenza virus infection (112). Considering that *S. pneumoniae* infection led to cell death induction *in vitro* (see Fig. 2A), lung tissue damage *in vivo* (see Fig. 7B and (210)), and IL-33 release in human lungs *ex vivo* (see. Fig. 2C), it was hypothesized that ILC2s might be also recruited during the resolution phase of pneumococcal pneumonia to mediate tissue repair. To test this, mice were infected with a lethal dose of 5×10^6 of *S. pneumoniae* and after 30 h p.i., when mice showed clinical signs of severe pneumococcal pneumonia, they were treated with ampicillin to resolve the infection. The antibiotic treatment was continued every 12 h from 36 h p.i. until mice were analyzed after 4, 6, 8 or 12 days p.i. After treatment, the body temperature of infected mice rapidly increased, mice gained weight and recovered from pneumonia (Fig. 15B and data not shown).

Using flow cytometry, ILC2s were detected in the lungs of infected mice (Fig. 15A). Moreover, the percentage of ILC2s related to the total amount of leukocytes in the lung was twofold higher in *S. pneumoniae* infected mice compared to the sham-infected control after 6 days (Fig. 15B). To analyze the kinetics of ILC2 recruitment during the resolution phase after *S. pneumoniae* infection, the total number of lung ILC2s was determined after 4, 6, 8 and 12 d p.i. ILC2s number slightly increased over time and peaked after 8 days (approximately 3900 ILCs), followed by a decline. In addition, the intracellular production of the Th2 cytokine IL-5 was quantified, which is a marker of ILC2 activation (150). Indeed, IL-5+ ILC2 were detected in the lungs of infected mice. Around 15% of ILC2s were positive for IL-5 after 6 days, but less than 5% were positive after 8 d and 12 d p.i. Taken together, these data demonstrate that ILC2 are present in the lungs of *S. pneumoniae* infected mice suggesting that ILC2 might be recruited during the resolution phase after infection.

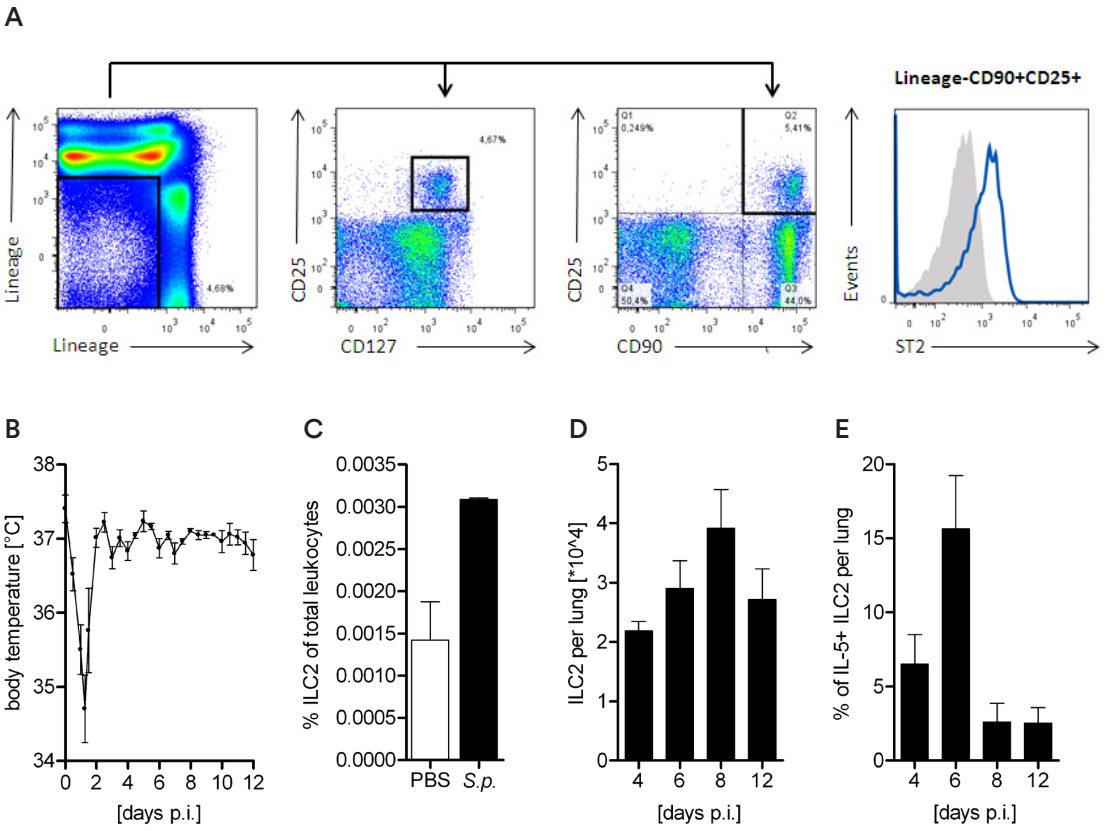

FIGURE 15: ILC2s are detected in the lung after pneumococcal pneumonia. C57BL/6 WT mice were transnasally (C) sham-infected with PBS or (B-E) infected with 5×10^6 CFU/mouse of *S. pneumoniae* (PN36). Mice were treated with ampicillin (0.4 mg/mouse) after 30 h and from 36 h p.i. every 12 h until sacrification. (A, C-E) Lungs were homogenisiezed and analysied by flow cytometry. (A) Gating strategy for the identification of Lineage-negative (CD3, CD11b, CD11c, CD19, NK1.1) CD90+CD25+CD127+ST2+ ILC2s in the lung. (B) Body temperature was assesed every 12 h. (C) % ILC2 of total leucocytes was determined after 6 d. (D) Total numbers of ILC2s and (E) % activated IL-5+ ILC2s of total ILC2s were quantified after 4, 6, 8 or 12 days. (B-E) Data are shown as mean ± SEM; n=2-3 mice each group.

4 — Discussion

This study examines for the first time the role of different DAMPs and receptors previously implicated in DAMP recognition in pneumococcal pneumonia. The results show that *S. pneumoniae* induces the release of the DAMPs uric acid, ATP and IL-33 from macrophages and epithelial cells or lung tissue. The anti-bacterial immune response was found to be regulated by the IL-33 receptor ST2, but not by uric acid, ATP and nucleotide receptors, or Mincle.

In-depth characterization of $St2^{-/-}$ and $Il33^{-/-}$ mice revealed that both molecules negatively regulate the anti-pneumococcal innate defense and the integrity of the epithelial-endothelial barrier in the lung. Mechanistically, *St2* and/or *Il33* were found to be expressed by AECs, MVECs and neutrophils, to reduce KC production in AECs, and to regulate neutrophil and macrophage recruitment, respectively. Furthermore, the data suggest that ST2-expressing ILC2s are recruited during the resolution phase of pneumococcal pneumonia.

4.1 Cell death and the release of DAMPs

In recent years, many studies demonstrated that DAMPs are released following sterile cell damage and tissue injury (39). *S. pneumoniae* expresses virulence factors such as PLY and the H_2O_2-producing enzyme pyruvate oxidase (*spxB*) (230, 231), which can induce cell damage. In addition, innate immune responses to bacterial infections are characterized by a massive recruitment of neutrophils, which eliminate bacteria but also cause tissue damage (210). It was therefore plausible to suggest that DAMPs are released during infection with *S. pneumoniae*. Indeed, the release of uric acid, ATP and IL-33 upon infection was observed in the present study, whereas the Mincle-ligand SAP130 could not be unambiguously detected in the supernatant of infected cells. It can be assumed that other DAMPs such as HMGB1, S100 proteins and mitochondrial DNA might also be released during bacterial infection, which, however, was not addressed in this study.

This study as well as other studies (171, 230, 232) clearly demonstrated that *S. pneumoniae* induces cell death, which was dependent on PLY (data not shown). However, the present study could not prove that the release of DAMPs was the result of cell death. Indeed, ATP can be also released by living cells via non-lytic mechanisms, involving maxi-anion channels, anion transporters, hemichannels, or exocytosis (233), and IL-33 might be actively released in an ATP-dependent manner by some cells (75, 78, 234). In line with the fact that DAMPs can be liberated both passively after cell death and perhaps actively via other mechanisms, the release of uric acid and ATP was PLY-dependent in some cells and PLY-independent in others (data not shown). IL-33 was released by human lung tissue upon infection with WT and PLY-deficient pneumococci, but it could not be detected in supernatants of *S.pneumoniae*-infected cells *in vitro* and in the BALF of infected mice *in vivo*. However, as the levels of released murine IL-33 in other studies were very low (104, 110, 235), it is possible that IL-33 was released during pneumococcal pneumonia in mice, but the sum signal in the cell supernatants and BALF was so low, that the applied detection assay was not able to detect it. Future studies should therefore continue to examine which DAMPs are released by specific cell types during bacterial infections, and how this is mechanistically linked to various forms of cell death.

4.2 Role of DAMPs during bacterial infections

The role of DAMPs in sterile inflammation following tissue damage has been well established (39). For example, it has been reported that ATP is released by injured epithelial cells and contributes to wound repair (236), and that extracellular ATP acts as a proinflammatory danger signal in the pathogenesis of lung fibrosis, chronic obstructive pulmonary disease and graft-versus-host disease (60, 237, 238). Moreover, uric acid has been implicated to promote an acute inflammatory response to sterile cell death (226) and bleomycin-induced lung injury in mice (239). In contrast, only few studies examined the function of DAMPs in bacterial infections. It has been shown that the S100 protein Mrp14 is released during *Klebsiella pneumoniae* infection and contributes to protective immunity (240). Other reports demonstrated that HMGB1 concentrations are elevated during pneumonia (241), and that deficiency in the HMGB1 receptor RAGE improves the host defense in pneumococcal pneumonia in mice (242). Furthermore, ATP and its receptor P2X7 have been implicated in anti-bacterial defense against *Mycobacterium tuberculosis* (243). In a pilot study, metabolic analysis of the blood plasma from African children who suffered from pneumonia revealed that uric acid levels are elevated in the pneumonia group compared to the control group (244).

Because both the pathogen itself and the immune system can cause tissue damage, it was hypothesized that DAMPs regulate the immune response to bacterial infections. Therefore, the role of uric acid, Mincle, ATP and nucleotide receptors, as well as IL-33 and its receptor ST2 were examined during acute pneumococcal pneumonia in the current study. There was no effect of the degradation of extracellular uric acid and ATP, the inhibition or lack of nucleotide-recognizing purinergic receptors, and the deficiency in Mincle on the anti-bacterial innate immune response during pneumococcal pneumonia. Thus, the data strongly suggest that the investigated pathways do not play an important role in *S. pneumoniae* infections. However, it cannot be excluded that the degrading enzymes and inhibitors used completely abrogated the DAMP-induced signaling, although our conclusion regarding ATP involvement is based on different experimental approaches (degradation of ATP, broad chemical inhibition of nucleotide receptors, usage of specific knock-out mice). Moreover, considering that the anti-bacterial response is initially and primarily induced by the recognition of PAMPs, and that PAMP-recognizing pathways play a partly redundant role during infections *in vivo* (168, 177), it is conceivable that the lack of one type of DAMP can be compensated by PAMP-recognizing receptors or other DAMP-detecting molecules. In contrast

to uric acid, nucleotide recognition and Mincle, ST2 and IL-33 were shown to negatively regulate the anti-bacterial innate immune response to *S. pneumoniae* infection. Therefore, further investigations were focused on these two molecules.

4.3 Different functions of ST2 and IL-33

The ST2/IL-33 axis regulates various immune pathways in many diseases. Although IL-33 is generally considered as a type 2 cytokine, the outcome of IL-33 signaling appears to largely depend on the cytokine milieu. In addition, intracellular and extracellular IL-33, which is released upon necrotic cell death, can have different or even opposing effects on the immune response. Whereas intracellular IL-33 has been suggested to inhibit NF-κB-dependent proinflammatory gene expression, extracellular IL-33 activates ST2L-dependent signaling leading to NF-κB activation. The activity of extracellular IL-33 can, however, be inhibited by the decoy receptor sST2 (94). In addition, ST2L has been identified as a negative regulator of IL-1R and TLR signaling. This might explain the differential roles of ST2 and IL-33 during pneumococcal pneumonia.

4.3.1 ROLE OF ST2 IN *S. PNEUMONIAE* INFECTION

ST2 is broadly expressed in various immune cells such as granulocytes, mast cells, Th2 cells, and ILC2s, as well as different non-hematopoietic cells (94). It was shown that ST2 negatively regulates TLR/IL-1R signaling in macrophages, although it is unknown whether this was dependent or independent of ligand recognition (92, 93). ST2L is the receptor for IL-33 and predominantly induces Th2 responses, which are characterized by the release of IL-4, IL-5 and IL-13, tissue eosinophilia, goblet-cell hyperplasia and tissue repair (94, 102). Previous studies revealed that ST2 plays a minor role in pneumococcal sepsis (245) and post-influenza pneumococcal pneumonia *in vivo* (246). Moreover, it has been described that *St2*-deficient mice are more resistant to secondary *Pseudomonas aeruginosa* pneumonia following CLP-induced sepsis (128).This study showed that ST2 is highly expressed in AECs and moderately expressed in MVECs and neutrophils. ST2-deficient AECs produced enhanced amounts of KC upon *S. pneumoniae* infection and LPS stimulation compared to WT and *Il33*$^{-/-}$ cells. This might be exp-

lained by a ST2-dependent but IL-33-independent inhibition of TLR-2 signaling through the sequestration of the TLR-signaling molecules MyD88 and TIRAP (92, 177). To prove this assumption, future studies should investigate the impact of ST2 on *S. pneumoniae*-stimulated NF-κB activation in AECs.

The present data further demonstrate that *St2*$^{-/-}$ mice were more resistant towards pneumococcal pneumonia compared to WT animals, showing reduced bacterial loads in the lung and blood, no appearance of pleurisy and edema, and an increased integrity of the epithelial-endothelial barrier. The enhanced antibacterial defense in *St2*$^{-/-}$ mice might be explained by the increased neutrophil influx and augmented macrophage recruitment into the lung. However, no effect of ST2 on the production of the neutrophil-recruiting chemokines KC, MIP-2α, and CXCL-5 was observed at 12 h and 24 h p.i. in the lung. Importantly, *Il33*$^{-/-}$ mice showed no increased neutrophil recruitment, although they were also more resistant to *S. pneumoniae* infection compared to WT animals. Overall, these data suggest that ST2 negatively regulates the bacteria-induced chemokine production in AECs *in vitro*, and the recruitment of neutrophils into the lung *in vivo* independently of its ligand IL-33. Since there was no influence of ST2 on the chemokine production during pneumococcal pneumonia, the data further indicate that ST2 regulates neutrophil recruitment also independently of KC, MIP-2α, and CXCL-5. It is thus conceivable that ST2 controls other steps of neutrophil recruitment from the vasculature to the tissue. These might include rolling, adherence, crawling or transmigration, the regulation of chemokine receptor expression on neutrophils, or the action of other chemokines involved in neutrophil recruitment. Further studies should therefore examine the impact of ST2 on the expression of adhesion molecules such as E-selectin, ICAM1 (intercellular adhesion molecule 1), or VCAM1 (vascular cell adhesion molecule 1) on endothelial cells, and the expression of CXCR2 on neutrophils (8). Taken together, the data suggest that the ST2 receptor has functions independent of its ligand IL-33, and that ST2 negatively regulates the anti-pneumococcal immune response in the lung (see Fig. 16).

4.3.2 ROLE OF IL-33 IN *S. PNEUMONIAE* INFECTION

IL-33 is constitutively expressed in a variety of epithelial barrier tissues (70, 71), as well as in vascular endothelial cells and other cells types (59). The dual-function cytokine can be either released as a bona-fide DAMP to activate ST2L on immune cells (59, 91, 100, 113, 143, 247), or act as a nuclear transcriptional regulator (65, 66). Extracellular IL-33 has been shown to regulate the immune response during e.g. asthma, parasite infections and other Th2 cytokine-mediated diseases (94). In contrast, nuclear IL-33 has been suggested to dampen NF-κB stimulated gene transcription (67).

In line with previously published studies (70, 71), IL-33 was mainly expressed by AECs. The current data further demonstrated that IL-33 (similar to ST2) negatively affects the bacterial clearance and the lung barrier integrity during pneumococcal pneumonia. Moreover, *S. pneumoniae*-stimulated macrophage recruitment was diminished by IL-33 (and also by trend by ST2) suggesting that IL-33 acts as a DAMP to regulate macrophage recruitment and possibly anti-pneumococcal innate immune responses in general through ST2. To prove this assumption it would be indispensable to use more sensitive asssays for IL-33 protein quantification in the BALF. It would be further interesting to investigate the effect of IL-33 treatment on pneumococcal pneumonia.

Interestingly, the susceptibility of mice toward *S. pneumoniae* appeared to be differentially affected by ST2 and IL-33, with *St2*$^{-/-}$ being more resistant than *Il33*$^{-/-}$ mice. In addition, *Ccl2* and *Il6* expression seemed to be negatively regulated by IL-33, but not by ST2 during lung infection, although this finding was only observed on a transcriptional level. The effect of IL-33 on MCP1 (encoded by *Ccl2*) might at least partly explain its influence on macrophage recruitment (195). Considering that ST2 does not regulate *Ccl2*, one may speculate that nuclear rather than extracellular IL-33 transcriptionally regulates the expression of *Ccl2* and perhaps other genes, as previously suggested (65–69). To prove this hypothesis, further studies should measure MCP1 on a protein level in addition to the transcripts, and compare the transcriptome of *St2*$^{-/-}$/*Il33*$^{-/-}$ and *St2*$^{-/-}$ cells. In summary, the data presented here suggest that IL-33 regulates the anti-pneumococcal immune response in two different ways, as an extracellular DAMP through its receptor ST2 and as an intracellular transcriptional regulator (see Fig. 16).

4.4 Role of ILC2 during pneumococcal pneumonia

Recent studies have highlighted the important role of innate lymphoid cells in regulating immunity and tissue repair at barrier surfaces (141). ILC2s are dependent on the transcription factors GATA3 and RORα and characterized by the production of typical type 2 cytokines IL-5, IL-13, and IL-9 in response to IL-33 and IL-25 or TSLP. In the lung, they have been implicated in the immune response to parasites (100), the repair phase following influenza infection (112), and the pathogenesis of allergic asthma (151) and pulmonary fibrosis (248). Since ILC2s highly express ST2 and respond to IL-33, and because repair mechanisms after *S. pneumoniae* infections are poorly understood, the role of ILC2s in the lung during/after pneumococcal pneumonia was investigated in the present study.

The pneumococcal pneumonia mouse model used in most experiments of this study is characterized by an acute inflammation and high mortality rate. Therefore, infected animals were treated with antibiotics to allow a longer survival for analysis of ILC2s during the later resolution and/or repair phases (249). Moreover, this model of pneumococcal pneumonia with antibiotic treatment reflects a common clinical situation in human patients. The data presented here provide first evidence for a recruitment of ILC2s into the lung in the resolution phase of pneumococcal pneumonia, i.e. after bacterial killing by antibiotics. However, future studies based on a larger sample size and extended postinfection time interval are needed to extend these investigations. Further, it would be interesting to examine the role of ST2 and IL-33 (e.g. by using respective knock-out mice) in *S. pneumoniae*-induced ILC2 recruitment. Moreover, the function of other epithelial cell-derived cytokines such as IL-25 and TSLP in ILC2 recruitment should be tested in parallel. The function of ILC2s in pneumococcal infection could be investigated by measuring type 2 cytokines and assessing repair processes (histopathology, lung barrier function) following antibody-mediated cell depletion and cell transfer experiments. Taken together, ILC2s appear to be recruited to the lung at the later phase of pneumococcal pneumonia, and future studies should investigate their impact on lung tissue repair.

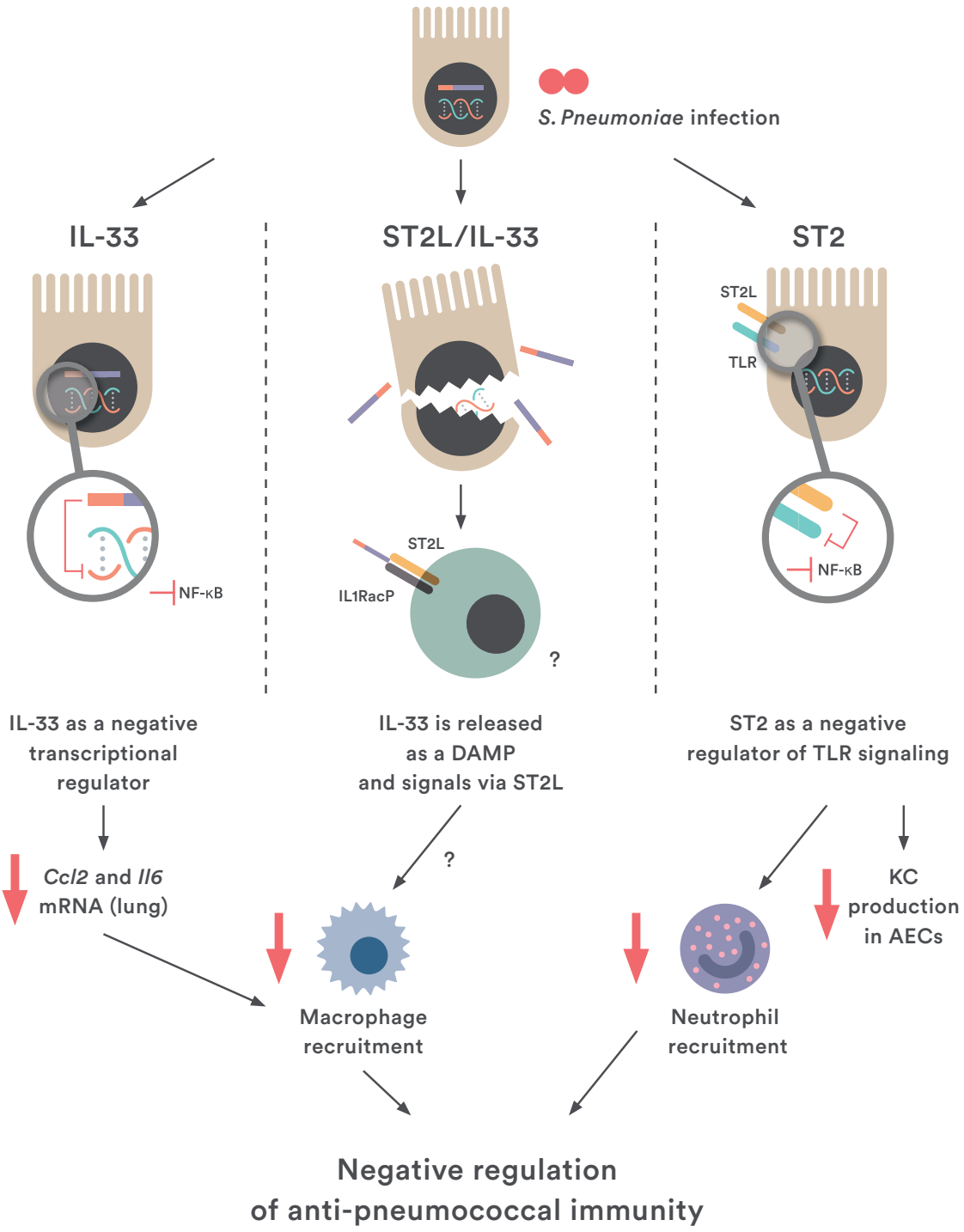

FIGURE 16: IL-33 and ST2 differentially regulate the anti-pneumococcal innate immune response. The scheme presents a possible model for the role of ST2 and IL-33 during pneumococcal pneumonia. In response to *S. pneumoniae* infection IL-33 (i) can act as a nuclear transcriptional regulator that negatively regulates *Ccl2* and *Il6* in the lung and (ii) is possibly released as a DAMP that signals via ST2L. Nuclear IL-33 and perhaps ST2L/IL-33 signaling mediates decreased macrophage recruitment. Independent of IL-33, (iii) ST2 may act as a negative regulator of TLR signaling, which results in decreased neutrophil recruitment and diminished KC production by AECs. Both, IL-33 and ST2 are negative regulators of the anti-pneumococcal immune response.

4.5 Concluding remarks

Although the PRR-stimulated proinflammatory immune response is required for fighting invading pathogens, overwhelming and temporally unrestricted inflammation can lead to excessive tissue damage and the development of acute lung injury and acute respiratory distress syndrome (ARDS) (250). Much progress has been made in our understanding of the initiation of innate immune responses by the recognition of PAMPs, however, the impact of DAMP sensing on the course of lung infections is poorly understood.

This study revealed that ST2 and IL-33 differentially regulate the anti-pneumococcal innate immune response, whereas other DAMPs and DAMP receptors investigated seem to play a minor role during pneumococcal pneumonia. Interestingly, the effects of ST2 and/or IL-33 were shown to be only partly dependent on IL-33 acting as a DAMP. Future studies should further discriminate and characterize i) the ST2-dependent functions of the DAMP IL-33, ii) the receptor-independent functions of intracellular IL-33, and iii) the ligand-independent role of ST2. A better understanding of those pathways might help to develop novel strategies to treat severe pneumonia.

Abstract

The innate immune response to pathogens in the lung is initiated after detection of microbial molecules (pathogen-associated molecular patterns, PAMPs) by pattern recognition receptors (PRRs). This immune response is vital for preserving lung function during pneumonia as it fights the invading microbes. An unrestricted PRR-mediated inflammation, however, can also lead to excessive tissue damage and the development of acute lung injury. Cellular injury leads to the release of so-called damage-associated molecular patterns (DAMPs), which play an important role in the regulation of sterile inflammation. In this study, the hypothesis was tested that DAMPs are released during *Streptococcus pneumoniae* infection and influence the antibacterial immune response during pneumonia.

The data demonstrate that *S. pneumoniae* infection leads to the release of several DAMPs including uric acid, ATP, and IL-33 by macrophages, alveolar epithelial cells (AECs) and/or lung tissue. Experiments with specific knock-out mice, inhibitors and degrading enzymes revealed that uric acid, ATP, as well as several DAMP-recognizing receptors play a minor role in pneumococcal pneumonia. In contrast, IL-33 and its receptor ST2 negatively regulated the immune response to *S. pneumoniae*. *St2*$^{-/-}$ and *Il33*$^{-/-}$ mice were more resistant to pneumococcal pneumonia and showed increased bacterial clearance, reduced mortality, and enhanced integrity of the lung epithelial-endothelial barrier compared to wild-type animals. *St2* and *Il33* were found to be mainly expressed by AECs and endothelial cells. Interestingly, ST2 but not IL-33 negatively regulated KC production in AECs as well as neutrophil influx into the lung, whereas both molecules seemed to affect the recruitment of macrophages. Finally, ST2-expressing type 2 innate lymphoid cells appeared to be recruited to the lung during the resolution phase of pneumococcal pneumonia.

In summary, this study demonstrates that IL-33 and ST2 differentially regulate the anti-pneumococcal innate immune response. The data suggest that IL-33 can act as a DAMP via ST2, and that both molecules can also have independent functions from each other.

Zusammenfassung

Die angeborene Immunantwort auf Infektionen in der Lunge basiert auf der Erkennung von konservierten mikrobiellen Molekülen (*pathogen-associated molecular patterns*, PAMPs) durch Mustererkennungsrezeptoren. Diese Immunantwort ist zur Bekämpfung eindringender Pathogene und zur Aufrechterhaltung der Lungenfunktion essentiell. Eine überschießende Entzündungsreaktion kann jedoch das Gewebe schädigen und ein akutes Lungenversagen hervorrufen. Durch zelluläre Schädigungen werden endogene, sogenannte *damage-associated molecular patterns* (DAMPs) freigesetzt, die eine wichtige Rolle in der Regulation von sterilen Entzündungen spielen. In dieser Arbeit wurde die Hypothese getestet, dass DAMPs während der Pneumokokkeninfektion freigesetzt werden und die antibakterielle Immunantwort beeinflussen.

Die Ergebnisse zeigen, dass die Infektion von Makrophagen, alveolären Epithelzellen und/oder Lungengewebe mit *Streptococcus pneumoniae* die Freisetzung der DAMPs Harnsäure, ATP und IL-33 bewirkt. In Experimenten mit spezifischen knock-out-Mäusen, Inhibitoren und degradierenden Enzymen zeigte sich, dass Harnsäure, ATP und verschiedene DAMP-erkennende Rezeptoren nur eine geringe Rolle in der Pneumokokkenpneumonie spielen. Im Gegensatz dazu wurde die antibakterielle Immunantwort gegen *S.pneumoniae* durch IL-33 und dessen Rezeptor ST2 negativ reguliert. $St2^{-/-}$ und $Il33^{-/-}$ Tiere waren resistenter gegenüber *S.pneumoniae* und zeigten eine verstärkte Bakterienelimination, eine reduzierte Sterblichkeit, sowie eine gesteigerte Integrität der Lungenbarriere im Vergleich zu Wildtyp-Mäusen. *St2* und *Il33* wurden vorrangig in alveolären Epithelzellen und Endothelzellen exprimiert. Interessanterweise zeigte sich eine negative Regulation der KC-Produktion in alveolären Epithelzellen, sowie der Neutrophilenrekrutierung in die Lunge durch ST2, jedoch nicht durch IL-33. Im Gegensatz dazu schien die Rekrutierung von Makrophagen durch beide Moleküle beeinflusst zu werden. Ferner zeigten die Ergebnisse, dass ST2-exprimierende *type 2 innate lymphoid cells* während der Resolutionsphase der Pneumokokkenpneumonie in die Lunge rekrutiert werden.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass die angeborene Immunantwort gegen *S. pneumoniae* durch ST2 und IL-33 differenziell reguliert wird. Die Ergebnisse deuten darauf hin, dass IL-33 als ein DAMP über ST2 wirken kann, und dass beide Moleküle auch voneinander unabhängige Funktionen ausüben können.

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List of abbreviations

6-FAM	6-Carboxyfluorescein
ADP	adenosine diphosphate
AHR	airway hyper-reactivity
AIDS	acquired immunodeficiency syndrome
AMΦ	alveolar macrophage
APC	Allophycocyanin
ATP	adenosine triphosphate
BAL	broncho-alveolar lavage
BMM	bone marrov-derived macrophage
BPL	bacterial lipoprotein
CARD	caspase recruitment domain
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony-forming unit
cGAMP	cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthase
CLP	cecal ligation puncture
CLR	C-type lectin receptors
CpG	cytidine-phosphate-guanosine DNA sequence
CTL	cytotoxic T cells
CXCL-5	chemokine (C-X-C motif) ligand 5
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FcRγ	Fc receptor γ chain
FCS	fetal calf serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazi-neethanesulfonic acid hematopoietic
HMGB1	high-mobility-group-protein B1
HRP	horseradish peroxidase
HuLu	human lung tissue
IFN	interferon
IL	interleukin
IL-1R	interleukin-1 receptor
ILC2	type 2 innate lymphoid cells
IPD	invasive pneumococcal diseases
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
KC	keratinocyte-derived chemokine
LDH	Lactate dehydrogenase
LPS	lipopolysaccharide
LRT	lower respiratory tract
LTA	lipoteichoic acid
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinases
MCP1	monocyte chemotactic protein 1

MDA5	melanoma differentiation associated gene 5
Mincle	Macrophage-inducible C-type lectin
MIP-2α	macrophage inflammatory protein 2-alpha
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MSU	monosodium urate
MVEC	microvascular endothelial cell
MyD88	myeloid differentiation primary response 88
n. s.	not significant
NADPH	nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NF-κB	nuclear factor ‚kappa-light-chain-enhancer' of activated B-cells
NK cell	natural killer cell
NKT cell	natural killer T cell
NLR	NOD-like receptors
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain
p.i.	post infection
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PE	Phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PLY	pneumolysin
PMN	Polymorphonuclear neutrophil
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PRR	pattern recognition receptors
RAGE	receptor for advanced glycation endproducts
RIG-I	retinoic acid-inducible gene-I
RIPK	receptor-interacting protein kinase
RLRs	RIG-I-like receptors
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
S.p.	*Streptococcus pneumoniae*
S. pneumoniae	*Streptococcus pneumoniae*
SAP130	subunit 3 of the splicing factor 3b protein complex
SP	surfactant proteins
ssRNA	single-stranded dsRNA
sST2	soluble ST2
ST2L	ST2 long
STING	stimulator of IFN genes
Syk	spleen tyrosine kinase
Tab.	table
TAMRA	5-carboxytetramethyl-rhodamine
Th-cells	T helper-cells
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-containing adaptor protein
TLR	Toll-like receptors
TNF-α	tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
WT	wild-type

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