Hit the brakes!

Inhibitory receptors as therapeutic target for neutrophil-driven pathologies

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Inhibitory immune receptors as therapeutic target for neutrophil-driven pathologies

Trap de rem in!

Remmende immuunreceptoren als therapeutisch doelwit voor de behandeling van ziekten met neutrofielen als drijvende kracht

(met een samenvatting in het Nederlands)

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1

Introduction

Preface

What is life? According to physicist and Nobel Prize laureate Erwin Schrödinger – of cat-in-a-box fame – it is to "negate entropy", or, to maintain a system with low entropy in a universe that marches ever on towards greater chaos.[1] In biological terms, this would equate to maintaining homeostasis, with the required energy (to keep entropy low) provided by consuming organic matter (heterotroph), or by utilising sunlight or the oxidation of inorganic compounds (photo- and lithoautotroph, respectively). Considering its etymology, the term 'homeostasis' could be construed as misleading. With 'homeo-' from Greek *homoios* (ὅμοιος), meaning 'similar', and '-stasis' from Greek *stasis* (στάσις), meaning 'standing still', it suggests an unchanging or 'static' state. However, active regulation is continuously required to keep bodily parameters at values conducive to life. Parameters fluctuate around a target value; active regulation keeps these parameters within the 'homeostatic range'. Perhaps combining *homoios* with *dunamis* (δύναμις), from Greek meaning 'ability' or 'potential', to yield homeodynamis, would more accurately represent the dynamic regulation taking place in organisms to sustain life. Yet, far be it for me to upend decades of scientific terminology, and for the remainder of this thesis, I will yield to the established use of term 'homeostasis'.

Preserving homeostasis

The maintenance of homeostasis is crucial to, if not the defining feature of, all life. In the struggle for survival, perturbation of homeostasis by outside agents is a constant threat. Pathogens seek to invade their respective hosts to appropriate the hosts' resources in order to facilitate their own propagation. In addition to the foreign menace posed by pathogens, multicellular life faces a domestic threat to homeostasis as well, i.e., malignancies. To preserve homeostasis, all known organisms have some form of immune system to fend off would-be pathogenic invaders. For example, prokaryotes (including both eubacteria and archaea) use restriction enzymes and clustered regularly interspaced palindromic repeats (CRISPRs) to degrade the genetic material of invading viruses,[2] and plant cells express transmembrane pattern recognition receptors (PRRs) and cytosolic nucleotide oligomerisation domain (NOD)-like receptors to detect and respond to pathogens.[3] Unique to animals, however, are dedicated, motile immune cells. In particular, vertebrates have evolved a lymphocyte-based adaptive immune system. Gnathostomes (i.e., jawed vertebrates, including humans) employ somatic recombination to produce a vast diversity of antigen-specific T cell receptors (TCRs), B cell receptors (BCRs), and

antibodies from a limited number of VDJ gene segments. Working in conjunction with TCRs, the major histocompatibility complex (MHC) is also unique to gnathostomes. Conversely, agnathans (i.e., jawless vertebrates) lack the MHC and generate variable lymphocyte receptors (VLRs) through a template-mediated combinatorial assembly of various leucine-rich repeat sequences.[4,5]

For an immune response to be optimally effective at protecting homeostasis, a number of prerequisites must be met: (i) the elicited immune response must rapidly follow the insult; (ii) it must be proportional and specific to the threat; (iii) and it must be sustained just long enough to eliminate the threat and then be abrogated posthaste. An inadequately potent or delayed immune response gives free rein to pathogens or malignancies to propagate at detriment to the host. Whereas excessive, untimely, or misdirected immune activation may result in autoimmunity and inflammatory disease. Immune regulation is therefore of vital importance.[6]

A large variety of activating immune receptors recognises the presence of potential pathogens to facilitate a swift response. Key amongst these are the innate PRRs, which recognise evolutionarily conserved pathogen-associated molecular patterns (PAMPs). These include several classes, including, but not limited to, Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), and NOD leucine-rich repeat-containing receptors (NLRs). Activation of PRRs induces an inflammatory milieu that informs and facilitates the subsequent antigen-specific lymphocyte-driven response.[7]

Inhibitory receptors

It is conceivable for an immune system to be regulated solely by activating receptors. In this case, the stimulation of activating receptors initiates the response, the amount of stimulation (determined both by the presence of ligands and their respective receptors) dictates the strength of the response, and elimination of the perturbation abrogates the response due to the absence of continued stimulation. However, our immune system features a large number of inhibitory receptors to counteract activating receptors. Indeed, the human genome encodes more than 300 predicted transmembrane inhibitory receptors with cytosolic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), of which around 60 have been functionally characterised. A recent literature study and mathematical analysis published by our group highlights the advantage of inhibitory receptors to immune regulation.[8] These advantages include: (i) enabling context-dependent immune regulation by varying inhibitory receptor and

ligand expression in different tissues; (ii) setting thresholds to prevent unnecessary activation and ensure responses of an appropriate (non-trivial) magnitude; and (iii) hastening abrogation of the elicited immune responses to prevent lingering activation after elimination of the perturbation.

ITIM-bearing inhibitory receptors do not act independently to lower the overall activation state of a cell, rather they directly counteract stimulatory signals relayed by activating receptors. TLRs, cytokine receptors, and a variety of receptors with immunoreceptor tyrosine-based activation motifs (ITAMs), such activating Fc receptors, CD3, and the ζ-chains of the TCR complex, are among those activating receptors counteracted by ITIM-bearing receptors. Signalling by both ITIM- and ITAM-bearing receptor requires the phosphorylation of the cytosolic tyrosine residue in their respective motifs by Src-family kinases. Phosphorylated (p)ITAMs recruit and activate Syk kinases to relay their stimulatory signal by phosphorylating downstream effectors, whereas phosphorylated (p)ITIMs recruit SHP-1, SHP-2, and/or SHIP phosphatases to dephosphorylate the pITAM-bearing receptors and their downstream effectors to abate or abrogate their signalling. While downstream effectors of ITAM-bearing receptors include 'free-floating' cytosolic proteins, ITIM-bearing receptors are only capable of dephosphorylating targets in their direct vicinity (ca. 20 nm radius) and must therefore co-localise with ITAM-bearing receptors in order to function.[8,9] For instance, SHP-1 tethered to the pITIM of a canonical inhibitory receptor on T cells, programmed death 1 (PD-1), has an effective reach of 13.0 nm.[10]

Importantly, the counteracting activity of inhibitory receptors on activating receptors is not absolute. Inhibitory receptors can provide a threshold for activation or abate activation in a gradual manner, but do not abrogate it outright.[9] Rather than an on/off switch, inhibitory receptors should be viewed as volume control for the intensity of the immune response. The outcome of integrating stimulatory and inhibitory signals, i.e., the strength of the elicited immune response, depends on the potency of both signals.

Immune checkpoint blockade therapy

The importance of inhibitory receptors for maintaining immune homeostasis is illustrated by the genetic association of inhibitory receptors with autoimmunity. This association is particularly strong for PD-1, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and the inhibitory Fc receptor FcγRIIB.[11–13] PD-1 and CTLA-4 are also the targets of the latest breakthrough in cancer treatment: immune checkpoint blockade therapy. This entails blocking the interaction of these inhibitory receptors with their respective

ligands by the administration of antibodies that either target the receptor directly (PD-1 or CTLA-4) or its ligand (PD-1L). By preventing inhibitory signalling, anti-tumour immune responses are strengthened to yield unprecedented clinical effects in cancer treatment. Immune checkpoint blockade therapy illustrates the vast potential inhibitory receptors have for the development for future immune regulatory therapies.[14]

However, immune checkpoint blockade evokes side effects that follow from perturbed immune regulation known as immune-related adverse effects (irAEs). By releasing the natural brakes on T cells to fight malignancies, latent auto-reactivity is also unleashed. Auto-reactive T cells that have escaped central tolerance in the thymus, but are kept in check by peripheral tolerance can become activated to deleterious effect; in mice ca. 30% of T cells in the periphery are auto-reactive to some degree.[15] As a result, irAEs resemble the early stages of auto-immune diseases. Severe irAEs are reported in more than 50% of patients treated with combination anti-PD-1 and anti-CTLA-4 antibodies, yet the vast majority of patients completely recover from irAEs after (temporary) cessation of treatment or with the administration of immunosuppressive drugs.[14,16]

Inhibitory immune receptor ligation therapy

Conversely, the autoimmune disease-like irAEs elicited by immune checkpoint blockade imply that targeted activation of inhibitory receptors represents a novel therapeutic avenue for the treatment of autoimmunity and other immune-mediated pathologies. Considering their role in abating stimulatory signalling by a variety of activating receptors, any of the >300 predicted and >60 characterised ITIM-bearing inhibitory receptors are potential therapeutic targets.

Crucially, ligating inhibitory receptors as treatment may suppress the excessive immune activation of leukocytes that cannot be targeted by current therapeutics, including neutrophils. Neutrophils are the most numerous leukocyte in the human body and are vital to antimicrobial defence. Indeed, reduced reactive oxygen species (ROS) production by neutrophils due to a genetic defect leads to chronic granulomatous disease with severe recurrent infections. However, neutrophil effector mechanisms such as ROS production, protease secretion, and extracellular trap (NET) formation are promiscuous in their effects and deployment hereof results in collateral damage to surrounding tissues. Excessive neutrophilic inflammation is thought to be a prominent cause of immune-mediated pathology in a variety of diseases, ranging from respiratory syncytial virus (RSV) infection-induced bronchiolitis to ischaemia-reperfusion injury.[17,18]

The lack of therapeutics that can effectively ameliorate excessive neutrophilic inflammation is a major obstacle in the treatment of these diseases and represents a significant unmet clinical need. However, neutrophils express a multitude of inhibitory receptors that could be targeted for ligation therapy. In this thesis, we endeavour to explore the potential of inhibitory immune receptors on neutrophils as targets for novel immunosuppressive treatments.

Aim and scope

In **Chapter 2** we review why neutrophils are a promising target for therapeutic intervention in RSV infection-induced bronchiolitis. RSV bronchiolitis is the second-most common cause of death among infants in developing nations (second only to malaria) and strongly associated with the development of wheezing and asthma later in life. While neutrophils are crucial to antimicrobial defence, neutrophil effector mechanisms lack specificity and excessive neutrophilic inflammation leads to immune pathology. At present, no therapeutics exist that can effectively suppress neutrophil activity. However, neutrophils express a range of inhibitory receptors at different stages of activation which may provide new therapeutic avenues. In **Chapter 3**, we set out to identify immune checkpoints that can negatively regulate neutrophilic inflammation in children with acute bronchiolitis. We discover that the inhibitory collagen receptor leukocyte-associated immunoglobulin-associated receptor 1 (LAIR-1) is expressed at the cell surface by activated, but not resting neutrophils. The latter store preformed LAIR-1 in intracellular granules. Further, by ex vivo ligating LAIR-1 with agonistic antibodies on neutrophils obtained from the airways of RSV bronchiolitis patients, we demonstrate that LAIR-1 is capable of suppressing extracellular trap formation by neutrophils. In **Chapter 4**, we further investigate the role of LAIR-1 in regulating neutrophilic airway inflammation using in vivo mouse models. By employing mice genetically deficient for LAIR-1, we find that LAIR-1 suppresses neutrophilic airway inflammation resulting from RSV infection as well as exposure to cigarette smoke. To rule out a developmental defect due to LAIR-1 deficiency, we also administered LAIR-1-Fc chimeric protein, which acts as a decoy to block LAIR-1/ligand interaction, and similarly found exacerbated neutrophilic airway inflammation during RSV infection in mice. In **Chapter 5**, we attempt to obtain proof of concept for targeting LAIR-1 with agonists as a therapeutic target for neutrophil-mediate immune pathology. We generated novel antibodies against mouse LAIR-1 and obtained an agonistic antibody, 2C7, capable of ligating LAIR-1 in solution, and an antagonistic antibody, 5F8, that blocks LAIR-1 binding to collagen.

However, administration of 2C7 prior to RSV inoculation did not ameliorate subsequent disease severity nor reduce neutrophil recruitment. Nevertheless, the novel anti-mouse LAIR-1 antibodies may prove useful tools for future studies. In **Chapter 6**, we examine another inhibitory receptor expressed by neutrophils: Allergin-1. Despite employing the same downstream effectors as LAIR-1 (phosphatases SHP-1 and -2), genetic ablation of Allergin-1 in mice did not exacerbate disease severity or enhance neutrophil airway infiltration in response to RSV infection. These results highlight the functional diversity between inhibitory receptors. In **Chapter 7**, we discuss the findings described in this thesis.

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Chapter

2

Neutrophils in respiratory syncytial virus infection: A target for asthma prevention

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Abstract

Lower respiratory tract infections by respiratory syncytial virus (RSV) are the foremost cause of infant hospitalization and are implicated in lasting pulmonary impairment and the development of asthma. Neutrophils infiltrate the airways of pediatric patients with RSV-induced bronchiolitis in vast numbers: approximately 80% of infiltrated cells are neutrophils. However, why neutrophils are recruited to the site of viral respiratory tract infection is not clear. In this review we discuss the beneficial and pathologic contributions of neutrophils to the immune response against RSV infection. Neutrophils can limit viral replication and spread, as well as stimulate an effective antiviral adaptive immune response. However, low specificity of neutrophil antimicrobial armaments allows for collateral tissue damage. Neutrophil-induced injury to the airways during the delicate period of infant lung development has lasting adverse consequences for pulmonary architecture and might promote the onset of asthma in susceptible subjects. We suggest that pharmacologic modulation of neutrophils should be explored as a viable future therapy for severe RSV-induced bronchiolitis and thereby prevent the inception of subsequent asthma. The antiviral functions of neutrophils suggest that targeting of neutrophils in patients with RSV-induced bronchiolitis is best performed under the umbrella of antiviral treatment.

Introduction

Respiratory syncytial virus (RSV) is a ubiquitous seasonal human pathogen that, on infection of the upper respiratory tract, causes cold-like symptoms in most healthy adults and children. At 2 years of age, nearly all children will have been infected with RSV at least once. Lower respiratory tract infections (LRTIs) by RSV are a major cause of morbidity and mortality among infants. Annual RSV-associated mortality is estimated at a quarter million deaths per year, of which 99% occur in developing countries, and it accounts for approximately 7% of deaths among infants younger than 1 year of age.[1] In the developed world 1% to 2% of infants are admitted to the hospital with RSV-induced bronchiolitis; therefore it is the foremost cause of infant hospitalization during the winter season.[2,3] Severe RSV-induced bronchiolitis in infancy is linked to impaired lung function in adulthood and causally implicated in the onset of recurrent wheezing and asthma.[4-7]

Massive pulmonary neutrophil infiltration is observed in pediatric patients with RSV-induced bronchiolitis.[8-10] In the lower airways neutrophils account for a median of 76% of infiltrated cells and a median of 93% in the upper airways, as measured in bronchoalveolar lavage fluid and nasopharyngeal aspirates, respectively.[9] Similarly, postmortem histopathologic analyses of fatal RSV-related LRTI cases reveal extensive neutrophil infiltration of the airway wall and lumen, as well as the alveoli.[11] This begs the question of why neutrophils are recruited *en masse* to the site of viral respiratory tract infection. A wide variety of inflammatory stimuli purposefully attract neutrophils to the lungs, such as to clear invading bacteria, apoptotic debris, or foreign substances. Could neutrophils also function protectively during RSV infection?

Neutrophils and RSV-induced bronchiolitis disease severity

Neutrophils, the most abundant leukocytes in human circulation, are classically portrayed as unsophisticated, first-line foot soldiers with a role limited to the engulfment and subsequent elimination of invading extracellular bacteria and fungi. In this view lung-infiltrated neutrophils seem out of place during RSV infection. The promiscuous cytotoxicity of neutrophil antimicrobial armaments might even potentiate virus-induced lung injury.[12] Indeed, the degree of neutrophilic inflammation correlates with disease severity in patients with RSV-induced bronchiolitis.[13] Moreover, a common single nucleotide polymorphism just upstream of the IL-8–encoding gene that is tentatively associated with increased production of IL-8, a potent neutrophil chemoattractant, is more frequent among infants with severe RSV-induced bronchiolitis, in particular among infants who lack other known

risk factors.[14] Increased IL-8 levels in the airways of patients with bronchiolitis are linked to increased disease severity, as measured based on oxygen saturation, Silverman score, and respiratory rate.[15,16] Genes related to neutrophil function, such as those encoding α-defensin-1 and elastase, are overexpressed in the blood of patients with RSV-induced bronchiolitis, and expression levels positively correlate with disease severity.[17] Thus neutrophils are thought to contribute to lung injury in patients with severe RSV-induced bronchiolitis, but their exact role in pathogenesis is still unclear.

Clinical link between RSV-induced bronchiolitis and asthma inception

A large body of epidemiologic evidence, including prospective case-control and cohort studies, implicates RSV-induced bronchiolitis during infancy in the inception of recurrent wheeze and lasting lung function impairment.[5- 7,18-31] However, the association between wheeze and a history of RSV-induced bronchiolitis during infancy partially subsides with age.[7,32] It is not clear to what extent RSV-induced bronchiolitis is related to increased risk of asthma at school age or beyond. Nonetheless, nearly half of children with severe RSV-induced bronchiolitis present with asthma at 6 years of age.5 Estimates indicate that early-life RSV-induced bronchiolitis is responsible for up to 13% of childhood asthma cases.[24] Moreover, smoking adults with RSV-induced bronchiolitis during infancy, but not those without LRTIs in early life, have an increased risk of persistent asthma.[26] However, association does not necessarily imply causation. Severe RSV-induced bronchiolitis could occur mainly in infants with pre-existing susceptibility to asthma development. Common genetic and/or environmental factors appear to underlie predisposition to both diseases.[21]

We recently addressed the issue of causality in a randomized, double-blind, placebo-controlled clinical trial with RSV immunoprophylaxis of healthy preterm infants, in which we demonstrated a causal relationship between RSV-induced bronchiolitis and the development of recurrent wheeze.[4] Although wheezing in early life is a strong risk factor for asthma in (early) adulthood,[33] asthma trials are needed to provide conclusive evidence that RSV disease is causally related to persistent asthma.

Neutrophilic lung inflammation and asthma inception

Neutrophils play an important role in asthma exacerbations by inducing mucus hypersecretion and airway remodelling, which result in acute reversible and progressive irreversible airway obstruction, respectively.[34,35] Although neutrophils are thought to affect lung function decrease, their role in asthma inception during viral bronchiolitis is understudied. Nonetheless,

the severity of infant bronchiolitis shows a dose-response relationship with the risk and morbidity of childhood asthma,[22] and as discussed above, the degree of neutrophilic inflammation correlates with the severity of RSV-induced bronchiolitis.[13,15,16] Therefore severe neutrophilic lung inflammation during infancy could tenably predispose children to subsequent wheezing and onset of asthma. Indeed, a functional IL-8 polymorphism more frequent among infants with severe RSV-induced bronchiolitis is also overrepresented in infants who had postbronchiolitis wheezing compared with patients with bronchiolitis who did not have wheezing afterward.[14,36] Neutrophilic inflammation–induced collateral tissue damage and airway sensitization could mediate the development of asthma susceptibility.

Transient damage to development of infant lungs can have far-reaching and lasting adverse consequences for pulmonary anatomy and function; this is particularly true if the critical process of alveolar multiplication by septation is disrupted.[37] Elastin fibers, which are present in alveolar duct and septal walls, provide elasticity to the lungs and transmit cues of mechanical stress to stimulate alveolarization. Neutrophil elastase, which is released on neutrophil recruitment to the lungs, degrades elastin fibers and thereby demolishes lung tissue structure.[38] Through elastase release and other lung-damaging mechanisms discussed below, the massive pulmonary neutrophil infiltrate of pediatric patients with RSV-induced bronchiolitis might thus cause irreparable disruption of lung development that predisposes these infants to subsequent asthma inception (Fig 1). In addition to inducing airway remodeling through collateral tissue damage, neutrophils might sensitize the airways to asthma through mast cell recruitment. IL-9 is detected in the bronchoalveolar lavage fluid of patients with RSV-induced bronchiolitis, and airway-infiltrated neutrophils are the principal producers.[39] In response to pulmonary expression of IL-9, long-lived mast cells migrate to and accumulate in lung tissue.[40,41] Lung-resident mast cells, specifically those located in the bronchial smooth muscle bundles, contribute to airway hyperresponsiveness and are considered crucial to the pathogenesis of (allergic) asthma (Fig 1).[42]

Dampening excessive neutrophilic inflammation during RSV-induced bronchiolitis might protect lung function, curtail airway sensitization, and reduce the risk of recurrent wheezing and asthma. A proof-of-concept, randomized, double-blind, placebo-controlled trial of macrolide treatment in patients with RSV-induced bronchiolitis provides tentative clinical support in favor of dampening neutrophilic inflammation.[43] Macrolides have antineutrophilic activities in vitro and attenuate neutrophilic airway inflammation in patients with refractory asthma and during RSV infection in

mice.[44-46] In comparison with placebo-treated patients, macrolide-treated patients with RSV-induced bronchiolitis showed reduced nasal lavage IL-8 levels, experienced fewer days after bronchiolitis with respiratory symptoms, tended toward fewer subsequent wheezing episodes, and exhibited a delayed third wheezing episode.[43]

RSV immunoprophylaxis is costly and available only to high-risk infants, for whom it reduces RSV-related hospitalization by approximately 50%.[47] For patients, supportive care is the sole treatment regimen available. Clearly, new treatment options for severe RSV-induced bronchiolitis are required to ameliorate disease and reduce sequelae, asthma in particular. The prominent neutrophilic inflammation in patients with RSV-induced bronchiolitis represents a promising target.

Below, we examine the role of neutrophils in patients with RSV infection and discuss the relevance thereof for therapy and asthma prevention. Because studies performed with RSV in this regard are scarce, we will also draw information from the literature on other viruses, in particular influenza virus, infections with which also exhibit severe pulmonary neutrophil infiltration.

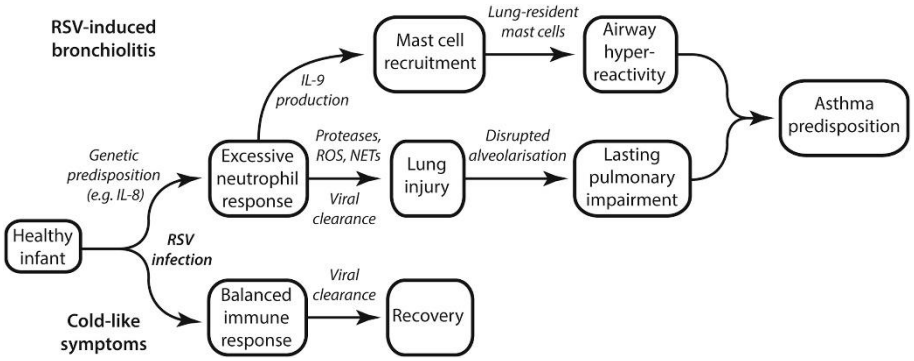

Figure 1. Schematic representation of the tentative relationship between neutrophil-induced lung damage and airway sensitization during RSV-induced bronchiolitis and subsequent susceptibility to asthma.

Neutrophil-induced lung injury

Neutrophils can injure the lungs in multiple ways: (1) release of proteolytic enzymes, including elastase, into the microenvironment through degranulation; (2) production of reactive oxygen species (ROS); (3) formation of neutrophil extracellular traps (NETs) by the cell death program known as NETosis; and (4) stimulation of mucus production (Fig 2).

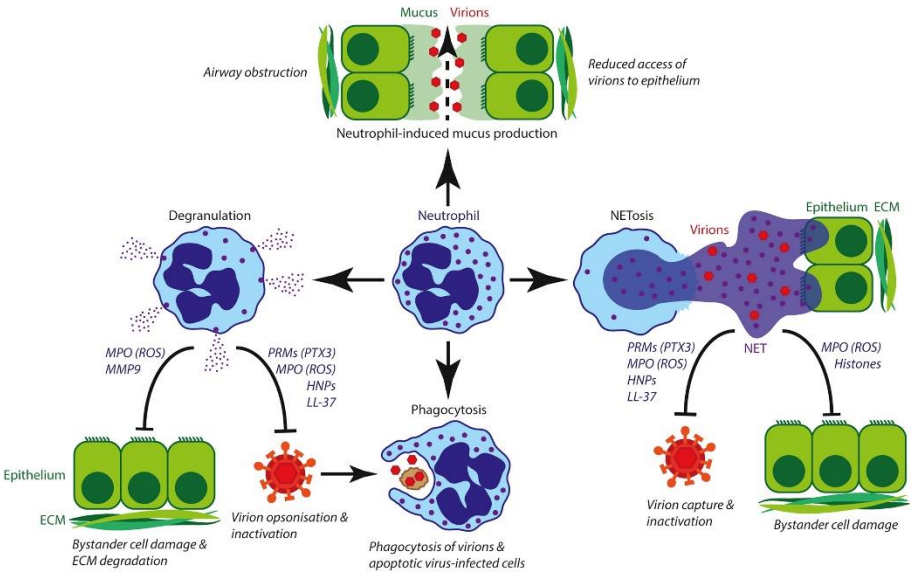

Figure 2. Protective and lung-injurious roles of neutrophils during respiratory tract viral infection. Neutrophil-induced mucus production limits access of viral particles to the epithelium but obstructs airflow. Degranulation releases antimicrobial mediators that are also cytotoxic to host cells. Phagocytosis of (opsonized) viral particles and virus-infected cells limits viral spread. NETs capture and deactivate viral particles but also damage healthy bystander cells. ECM, Extracellular matrix; PRMs, pattern recognition molecules.

Neutrophil migration into the airways during influenza infection in mice depends on the neutrophil tertiary granule-expressed matrix metalloproteinase (MMP) 9, which digests extracellular matrix. MMP9-mediated neutrophil influx into the airways is required for the control of influenza virus replication in mouse lungs.[48] However, excessive MMP9 proteolytic activity in response to high viral loads exacerbates pathology.48 Pulmonary inflammation caused by noninfectious agents, such as cigarette smoke, implicate other neutrophil proteolytic enzymes, including cathepsin G, neutrophil elastase, and proteinase 3, in lung tissue destruction as well.[49]

The inflammatory milieu in the lungs during RSV infection induces the neutrophil oxidative burst.[50] The abundantly produced ROS indiscriminately oxidize biomolecules, which compromises their function. Therefore, ROS are potently antimicrobial but also damage host cellular structures. Oxidative stress during RSV infection promotes lung injury.[51,52] Thus neutrophil-derived ROS are likely to contribute to lung damage in patients with RSV infection.

NETs are DNA-based meshes decorated with histones and granular antimicrobial proteins and peptides, including elastase, myeloperoxidase (MPO), and α-defensins. By trapping microbes in NETs, microbial spread can be minimized and microbe killing optimized by high local concentrations of antimicrobial proteins and peptides.[53] Exposure of neutrophils to RSV particles in vitro induces NET formation through specific interaction of RSV fusion (F) protein with neutrophil-expressed Toll-like receptor (TLR) 4.[54] Data on the in vivo antiviral activity of NETs are conflicting.[55] Genetic ablation of peptidylarginine deiminase 4, which is required for NET formation, did not affect viral replication or mortality in mice infected with influenza virus.[56] Instead, NETs are located in areas of alveolar damage in the lungs of influenza virus–infected mice.[57] In vitro NETs directly damage alveolar epithelial and endothelial cells. The histone component of NETs is chiefly responsible for this cytotoxic effect.[58] This suggests that NETs induce lung injury rather than suppressing viral replication. However, the absence of NETs in peptidylarginine deiminase 4–deficient mice does not reduce lung pathology during influenza infection either.[56] In this mouse model of human influenza virus infection, NETs appear to play neither a beneficial nor a pathologic role. The contribution of NETs during viral infection might be virus specific and depend on the degree of NET induction.

In addition to directly damaging the respiratory tract, neutrophils can contribute to airway obstruction by inducing mucus production. Mucus forms a protective barrier to viral infection by limiting access of viral particles to the pulmonary epithelium. However, excessive mucus production might cause airway obstruction. Mouse neutrophils promote the expression of mucin, the chief component of mucus, during RSV infection.[59] Thus the extensive neutrophil infiltration in the lungs might contribute to the airway obstruction and resultant wheezing observed in patients with severe RSV-induced bronchiolitis.

Pulmonary IL-17 enhances neutrophil recruitment to the lungs early in RSV infection by inducing IL-8 production in airway epithelial cells.[60] The amount of IL-17 in the lungs of pediatric patients with RSV-induced bronchiolitis correlates to the degree of subsequent pulmonary neutrophil influx.[60] Additionally, IL-17 induces the production of obstructive mucus in the airways.[61] This suggests that IL-17 plays a pathogenic role during LRTIs by RSV. The association of a polymorphism in the gene encoding IL-17 with severe RSV-induced bronchiolitis supports this notion.[62] Moreover, the response of TH17 cells, noted producers of IL-17, is enhanced in infants with RSV-induced bronchiolitis.[63] An RSV mouse model also links IL-17 and TH17 responses to RSV disease severity: pulmonary neutrophil recruitment and

lung pathology are exacerbated in RSV-infected mice, which have increased TH17 responses caused by genetic ablation of the IL-27 receptor.[64] Moreover, genetic ablation or antibody-mediated blocking of IL-17 in RSV-infected mice reduces neutrophilic inflammation, mucus production, and viral loads and improves the cytotoxic T-cell response against RSV.[65] Nonetheless, it is not clear whether local IL-17 levels are protective or harmful. IL-17 reduces airway reactivity of RSV-infected mice presensitized with ovalbumin,[66] and mice that received a novel attenuated *Bordetella pertussis* vaccine were protected from RSV infection in an IL-17–dependent fashion.[67] It is possible that a limited IL-17 response is protective, whereas excessive IL-17 production induces immune pathology through unrestrained neutrophil recruitment. However, the early recruitment of neutrophils to the lungs of patients with RSV-induced bronchiolitis is unlikely to be mediated by an adaptive TH17 response because RSV antigen–driven clonal expansion and differentiation of TH17 cells require several days. Instead, there might be a role for natural TH17 cells that acquire effector functions during thymic development and regulate early-phase airway neutrophil responses.[68,69] Thus (natural) TH17 cells and IL-17 might promote a pathogenic neutrophil-dominated response to RSV infection.

Control of viral replication by neutrophils

The neutrophil antimicrobial arsenal, although damaging to the lungs if deployed excessively, has also shown efficacy against viruses in addition to its traditional bacterial and fungal targets. Based on this, a contribution of neutrophils to antiviral defense is suggested.[55,70,71] Indeed, a beneficial role for neutrophils in patients with viral respiratory tract infections has been reported for influenza virus.[72-75] Neutrophil depletion in mice by neutrophil-specific anti-Ly6G antibody treatment results in enhanced viral replication and lung damage.[72] Therefore neutrophil recruitment to the airways during RSV infection could also be a protective response. Neutrophils mediate direct antimicrobial effects through, in essence, 3 known effector mechanisms, namely (1) phagocytosis, (2) degranulation, and (3) NET formation (Fig 2 and Table 1).[76-86]

By means of phagocytosis, neutrophils can eliminate RSV-infected cells, thereby preventing further viral replication, and engulf virions to limit infection of new cells. Blocking the recognition and phagocytosis of apoptotic cells by local Annexin V administration 1 day after influenza virus inoculation increases mortality among mice.[76] Both mouse macrophages and neutrophils phagocytose influenza virus–infected apoptotic cells in vivo.[77] This process is amplified by TLR4,[77] which recognizes RSV F protein expressed on the

surfaces of virions and infected cells.[87] Moreover, as shown in vitro for herpes simplex virus, antibody- and complement-mediated opsonization facilitates the phagocytosis of viral particles and virus-infected cells by neutrophils.[88]

Neutrophil granules contain antimicrobial proteins and peptides, including α-defensins 1 to 4 (or human neutrophil peptides [HNPs] 1-4), cathelicidins (ie, LL-37), pentraxin (PTX) 3, and MPO, which are released into the microenvironment on degranulation.[89] Neutrophils degranulate in response to in vitro stimulation with RSV particles.[90] HNPs are small, cationic amphipathic peptides that demonstrate antiviral activity. For instance, multiple steps of cellular entry by HIV-1 are inhibited by HNP-1,[78] and HNPs promote influenza virus aggregation and uptake by neutrophils.[79] Whether the antiviral activity of HNPs extends to RSV is not currently known, but the broad antiviral activities of HPNs against multiple enveloped and nonenveloped viruses hints at the possibility.[91]

Recent in vitro work demonstrates that the cationic antimicrobial peptide LL-37 possesses direct antiviral activity against RSV virions, protects infected epithelial cells against RSV-induced cell death, inhibits production of new viral particles, and reduces the susceptibly of epithelial cells to RSV infection.[80] Low serum levels of LL-37 precursor (ie, hCAP-18) are associated with severe infantile RSV-induced bronchiolitis.[81]

PTXs are soluble pattern recognition molecules that recognize pathogen-associated molecular patterns and mediate innate humoral immunity, functionally resembling antibodies of the adaptive immune system. PTX3 can activate compliment through recruitment of C1q (ie, the classical pathway) and act as opsonins by interacting with the FcγRIII (CD16) and FcγRII (CD32) receptors, which are expressed by phagocytes, including neutrophils.[92] Mature neutrophils represent a major reservoir of preformed ready-to-use PTX3, as well as other pattern recognition molecules.[93] Direct evidence of PTX3-mediated antiviral activity against RSV is lacking but has been demonstrated for influenza virus and mouse coronavirus. PTX3 reduces the infectivity of both viruses. Moreover, PTX3-deficient mice inoculated with either virus fare worse than their wild-type counterparts but can be rescued by administration of exogenous PTX3.[82,83]

MPO catalyzes the production of hypochlorous acid, which is a potent antimicrobial ROS, because of its ability to chlorinate and oxidize a great variety of biomolecules. Secreted and NET-bound MPO deactivates HIV-1 particles in vitro.[84,85] The RSV F protein induces formation of MPO-coated NETs through TLR4,[54] and HIV-1 triggers NETosis through a TLR7/TLR8- and ROS-dependent pathway. NET-bound MPO and HNPs abolish the infectivity of HIV-1 particles. In coculture neutrophils reduce the infection of CD4+ T

cells by HIV-1 in a NET-dependent manner.[85] Thus NETs are elicited by stimulation with virus and possess direct antiviral activity in vitro.

Furthermore, the release of NETs by neutrophils during intravenous myxoma poxvirus challenge in mice protects against viral infection of liver cells.[86] Therefore NETs can benefit antiviral defense in vivo. As discussed above, NETosis deficiency does not affect the course of disease in human influenza virus-inoculated mice.[56] A contribution, protective or damaging, of NETs to antiviral defense might be virus and organ (eg, liver vs lung) specific.

Table 1. Neutrophils possess direct antiviral activity

Mechanism or molecular mediator	Effect and function	Virus/model	References
Phagocytosis	Eliminates virus-infected cells and virionss	Influenza virus/*in vivo*	76, 77
HNP-1	Inhibits viral entry into cell	HIV/*in vitro*	78
HNP-1 and HNP-2	Promotes virion aggregation and phagocytosis	Influenza/*in vitro*	79
LL-37	Inactivates virions, protects epithelial cells from infection and cell death, inhibits virion production	RSV/*in vitro* and clinical association	80, 81
PTX3	Reduces virion infectivity	Influenza virus and MHV-1/*in vitro* and *in vivo*	82, 83
MPO	Inactivates virions through HClO production	HIV-1/*in vitro*	84
NETosis	Reduces viral infectivity and spread, inactivates virions	Influenza virus and myxoma poxvirus/*in vitro* and *in vivo*	85, 86

Neutrophil effector mechanisms, neutrophil-derived molecules, and their respective antiviral activities are listed. The supporting lines of evidence and related references are included. HClO, Hypochlorous acid; MHV-1, mouse hepatitis virus strain 1.

Neutrophil-mediated immune modulation

Recent studies demonstrate multiple immune regulatory functions for neutrophils. In addition to their role as effector cells in patients with RSV infection described above, neutrophils can also act as immune regulatory cells (Fig 3). Although neutrophil-mediated immune regulation in the context of RSV infection has yet to be examined directly, general concepts can be derived from studies with other viruses and guide further research into the

severely understudied role of neutrophils in patients with RSV infection. Here we focus on regulatory roles potentially involved in RSV infection. For a more complete overview, the reader is referred to a number of recent reviews.[89,93-95]

Neutrophils can promote antiviral CD8+ T-cell responses. Influenza virus antigen–displaying neutrophils stimulate lung-infiltrated CD8+ T cells to produce IFN-γ, whereas infected epithelial cells only elicit their own cytolysis.[96,97] Stimulation of IFN-γ production by neutrophils can enhance antiviral defense against RSV infection.[98] The CD8+ T-cell response to influenza in the airways of mice is maintained by infiltrated neutrophils.75 Neutrophils thus regulate CD8+ T-cell effector function at the site of viral infection. Neutrophils efficiently cross-present phagocytosed antigen to naive CD8+ T cells in vivo to stimulate proliferation, IFN-γ production, and cytolytic activity.[99] By acting as viral antigen shuttles, neutrophils can indirectly induce an antiviral CD8+ T-cell response. Mouse neutrophils take up modified vaccinia Ankara virus in skin, transport viral antigen to the bone marrow, and there, through local phagocytic antigen-presenting cells, trigger virus-specific CD8+ T-cell proliferation and establishment of memory.[100] Moreover, neutrophils can indirectly stimulate T-cell responses by recruiting immature dendritic cells (DCs) to a site of infection through CCL3 secretion,[101] and by inducing DC maturation through direct cell-cell contact, which depends on altered glycosylation patterns of Mac-1 (CD11b/CD18) and carcinoembryonic antigen-related cell adhesion molecule 1 (CAECAM1) that are specific to activated neutrophils.[102,103]

The homeostasis of innate cytotoxic lymphocytes (ie, natural killer [NK] cells) is neutrophil dependent.[104-106] Neutropenic patients and neutrophil-depleted mice present with immature hyporesponsive NK cells.[104] Neutrophils stimulate NK cells through IL-18 production and the interaction of CD18 with NK cell–expressed intercellular adhesion molecule 3. Moreover, neutrophils stimulate DCs to produce NK cell–stimulatory IL-12 through CD18/intercellular adhesion molecule (ICAM) 1 interaction.[105,106] IFN-γ produced by activated NK cells potentiates the interaction between neutrophils and DCs, thus creating a neutrophil-dependent positive feedback loop that supports NK cell maturation, survival, and IFN-γ production.[105]

The adaptive humoral response is stimulated by neutrophils as well. Through cytokine secretion (ie, B cell–activating factor of the TNF family [BAFF], a proliferation inducing ligand [APRIL], and IL-21), splenic neutrophils induce immunoglobulin class-switch recombination, somatic hypermutation, and antibody production by marginal zone B cells.[107]

Neutrophils can also suppress adaptive immune responses. Systemic inflammation in human subjects induces a neutrophil subset with T cell–suppressive capabilities. These suppressive neutrophils form Mac-1–dependent immunologic synapses with T cells and locally produce bursts of membrane-associated hydrogen peroxide (H2O2) that suppress T-cell proliferation.[108] Furthermore, neutrophil-derived thromboxane limits T-cell cytokine production in response to protein-in-adjuvant vaccination of mice and suppresses the spread of the T-cell response from the draining lymph nodes of the injection site to distal lymph nodes.[109] Neutrophils can indirectly suppress T-cell responses through deposition of MPO in lymph nodes, which impairs DC function.[110]

In a more passive manner neutrophils might contribute to the resolution of inflammation. Depending on environmental stimuli, macrophages can acquire a proinflammatory or proresolution phenotype. The phagocytosis of apoptotic neutrophils by macrophages induces a regulatory phenotype in macrophages, which might contribute to the resolution of inflammation.[111] In RSV-infected cotton rats, proresolution macrophages limit lung pathology.[112]

Neutrophils, RSV-induced bronchiolitis, and asthma: Remaining caveats

Is it conceivable that neutrophils are abundantly present in the airways during severe RSV infection without playing a major role in either the pathogenesis of bronchiolitis or the subsequent inception of asthma? Although indicators of neutrophilic inflammation, including airway IL-8 levels and neutrophil elastase activity, correlate with the severity of bronchiolitis, cell counts of airway-infiltrated neutrophils, which are invariably high, are not associated with RSV disease severity.[10,13, 15,16,113] Similar to RSV-induced bronchiolitis, extensive neutrophil infiltration of the airways and alveoli is observed during bacterial pneumonia, usually in the absence of wheezing. If neutrophils contribute to the inception of asthma after bronchiolitis, an increased risk of asthma should also be observed after bacterial respiratory tract infections in early childhood. Evidence in support of this is limited to observations in a high-risk birth cohort.[31] Future studies should attempt to validate the relationship between bacterial respiratory tract infection during infancy and the subsequent development of reactive airway disease.

Concluding remarks and implications for therapy

The vast number of lung-infiltrating neutrophils in patients with RSV-induced bronchiolitis appears to be the principal instigator of pulmonary immunopathology. The secretion of proteolytic enzymes, such as elastase,

the production of ROS, and NET release can clearly injure the lungs. This might disrupt the delicate process of lung development in infancy and have lasting adverse consequences for lung anatomy, function, and susceptibility to chronic disease, including asthma. However, through direct antiviral activity of these and other neutrophil mechanisms, as well as through regulation of adaptive immune responses, neutrophils can also contribute to a protective antiviral response. In patients with severe RSV-induced bronchiolitis, the balance between neutrophil antimicrobial activity and collateral tissue damage appears to have shifted toward a pathologic response. Hence pharmacologic suppression of neutrophil activity could curtail lung injury. Possible approaches include (1) neutrophil-inhibitory macrolides; [43,46] (2) inhibiting damaging neutrophil-derived products, such as elastase;[114] (3) antagonizing neutrophil-modulatory adenosine;[115] (4) neutrophil chemokine receptor blockade;[116,117] (5) and targeting neutrophil-expressed inhibitory receptors.[118,119] We eagerly await the results of future clinical trials for the treatment of RSV infection with pharmacologic agents that can effectively dampen neutrophilic inflammation, such as the selective small-molecule CXCR2 (IL-8 receptor) antagonist danirixin (ClinicalTrials.gov identifier: NCT02201303).[117]

The relationship between extensive neutrophil infiltration of the airways in early life and later asthma development might not be limited to RSV infection. Lower respiratory tract illnesses in infants induced by other viruses are also associated with subsequent wheeze and asthma.[7,31,120] Limited evidence also hints at a causal association between bacterial invasion of the respiratory tract and subsequent asthma development.[121-123] Colonization of neonatal airways by pathogenic bacteria induces neutrophil response–associated cytokines and is associated with development of persistent wheezing and childhood asthma in a prospective birth cohort.[124,125] Moreover, among prospectively followed high-risk children, respiratory tract infections by pathogenic bacteria in early life were associated with an increased risk of school-age asthma.[31] In fact, rather than a specific viral or bacterial pathogen, the total number of early respiratory tract infections correlated with the risk of childhood asthma in this study.[31]

Although injurious when present in excess, neutrophils are essential to antimicrobial defense. The safety of treating RSV-induced bronchiolitis with neutrophil suppression could improve with the administration of RSV antivirals and prophylactic antibiotics. Recently, a newly developed RSV antiviral was successfully tested in a clinical trial wherein healthy adult subjects were challenged with RSV.[126] By itself, however, an RSV antiviral might confer limited benefit when administered several days after onset of

symptoms. For influenza, oseltamivir is only effective when administered within 36 to 48 hours after symptom onset.[127] Similarly, antiviral treatments against RSV should ideally be administered as close to initial infection as possible to be at their most effective, yet RSV loads are already on the decline when patients are admitted to the intensive care unit.[128] Nonetheless, neutrophils are still abundantly present in the lungs,[9-11] and thus neutrophil-mediated damage to the airways persists (Fig 2). Therefore, we propose that suppressing neutrophil activity under the umbrella of RSV antivirals and prophylactic antibiotics offers a promising treatment strategy to improve the outcomes of infants with RSV-induced bronchiolitis and reduce the risk of subsequent asthma.

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Chapter

3

LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis

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To the Editor:

Respiratory syncytial virus (RSV) infection-induced bronchiolitis is the second most important cause of infant death, following malaria. An estimated 253,000 children die each year of RSV-related disease.[1] We have previously shown that severe RSV bronchiolitis leads to recurrent episodes of wheezing long after the infection is cleared.[2]

Rapid and efficient clearance of viral respiratory infections requires a potent immune response. However, the intricate lung tissue architecture that facilitates gas exchange is fragile and, in addition to direct virus-induced pathology, excessive inflammation can cause damage that disrupts this vital function. The immune response must therefore be tightly regulated to balance pathogen eradication with immune-induced collateral tissue damage. Inhibitory immune receptors, or immune checkpoints, play a crucial role in preventing the latter. The inflammatory response in the airways of RSV bronchiolitis patients is characterized by extensive neutrophil infiltration: neutrophils comprise more than 80% of infiltrated leukocytes. We have recently reviewed how airway-infiltrated neutrophils may contribute to the control of viral replication, while concurrently inducing immune injury due to the promiscuous cytotoxicity of antimicrobial armaments.[3]

We therefore set out to identify inhibitory receptors that regulate the inflammatory neutrophil response during viral respiratory infection. In infants mechanically ventilated for RSV bronchiolitis, we determined the expression of six inhibitory receptors (four shown) on airway-infiltrated neutrophils obtained by bronchoalveolar lavage (BAL) and circulating neutrophils in peripheral blood by flow cytometry (Fig 1A-C). Of these receptors, only leukocyte-associated immunoglobulin-like receptor (LAIR)-1 was undetectable on blood neutrophils but highly expressed on the airway-infiltrated neutrophils (Fig 1B). Similarly, neutrophils in nasal lavages of adult acute rhinitis patients expressed LAIR-1, but their blood counterparts did not (Fig E1). Airway-infiltrated neutrophils of children intubated for reasons unrelated to RSV also express LAIR-1 (Fig E2). Thus, we find that LAIR-1 is expressed on airway-infiltrated neutrophils, but absent on circulating neutrophils, during upper or lower viral respiratory tract infection. While expression of the inhibitory collagen receptor LAIR-1 on mononuclear immune cells is well documented, its expression on mature neutrophils *in vivo* has not previously been reported.[4]

Further analysis revealed that LAIR-1 expressing, airway-infiltrated neutrophils were highly activated, as indicated by increased CD11b expression and shedding of CD62L, when compared to blood neutrophils (Fig 1C). To study the role of activation versus tissue migration in the induction of

LAIR-1 expression, we analyzed peripheral blood neutrophils of four pediatric patients with severe, blood culture-positive sepsis by Gram-negative (*H. influenza*) or Gram-positive bacteria (*S. capitis*, *S. pneumoniae*) for LAIR-1 surface expression at several time points. Circulating neutrophils in all four sepsis patients expressed high levels of LAIR-1, which decreased with clinical improvement (Fig 1D). This was not explained by the recruitment of immature neutrophils from bone marrow (Fig E3). Thus, activation per se – in absence of migration into tissue – was sufficient to induce LAIR-1 expression on neutrophils ***in vivo*.**

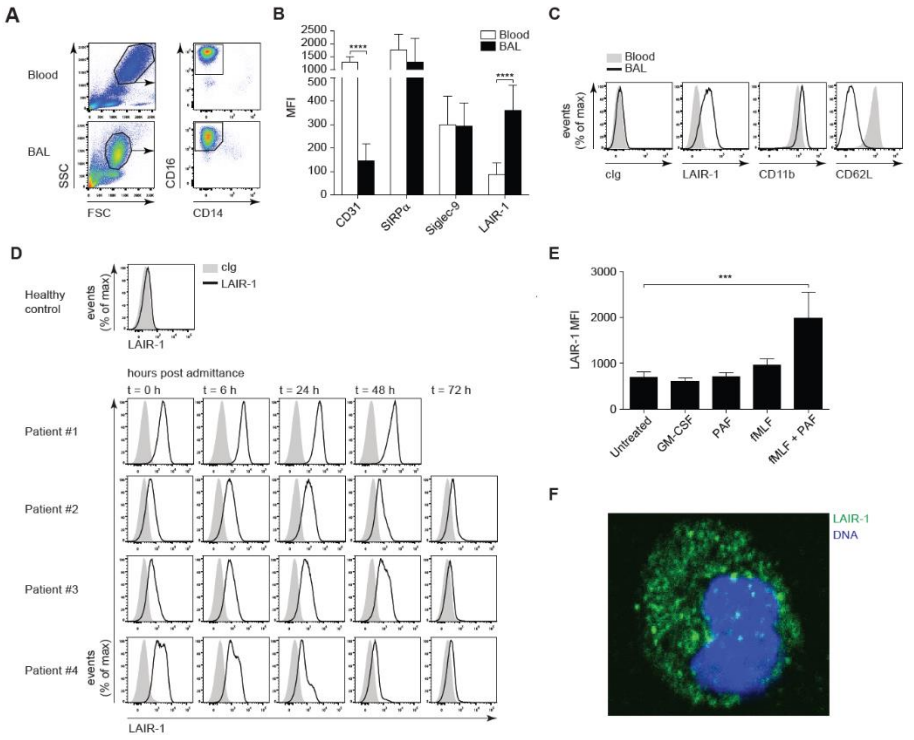

Figure 1. *In vivo* activated human neutrophils express LAIR-1.
Representative example of the gating strategy for neutrophils from blood and BAL of RSV bronchiolitis patients (n = 9) (A), the quantified expression of inhibitory receptors as MFI (n = 9) (B), and a representative example of activation markers (C) on these neutrophils (n = 9). (D) LAIR-1 expression on peripheral blood neutrophils of sepsis patients (n = 4) and a representative healthy control child. (E) Surface expression of LAIR-1 after in vitro stimulation (n = 3). (F) Representative image of intracellular LAIR-1 expression by resting neutrophils (n = 3). *** $p < 0.001$. BAL, bronchoalveolar lavage; cIg, isotype-matched control antibody; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; MFI, median fluorescence intensity.

LAIR-1 expression was induced *in vitro* on healthy isolated peripheral blood neutrophils by combined stimulation with formyl-methionyl-leucyl phenylalanine (fMLF) and platelet-activating factor (PAF) (Fig 1E). The rapid (≤10 min) induction of LAIR-1 surface expression argued against *de novo* protein production. Indeed, confocal microscopy revealed that LAIR-1 is present in the granules of resting neutrophils (Fig 1F). LAIR-1 expression is similarly induced on primed neutrophils exposed to the conditioned culture medium of RSV-infected pulmonary epithelial cells (Fig E4).

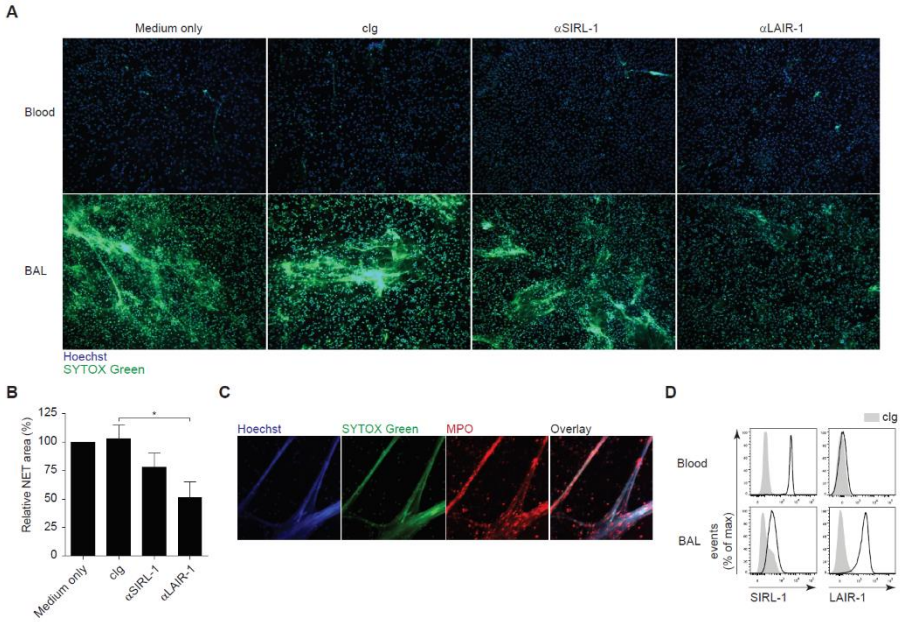

Figure 2. LAIR-1 suppresses NET formation by airway-infiltrated neutrophils obtained from RSV bronchiolitis patients.

(A) Representative example of SYTOX Green-visualized NETs produced by neutrophils from blood and BAL of RSV bronchiolitis patients (n = 6) incubated *in vitro* with an isotype-matched control antibody (cIg), anti-SIRL-1 antibody, anti-LAIR-1 antibody, or medium only. (B) Quantified NET area normalized to medium only for each patient (n = 6). (C) Co-staining of Hoechst 33342- and SYTOX Green-positive NET DNA with MPO (n = 6). (D) Representative example of surface expression of LAIR-1 and SIRL-1 on neutrophils from blood and BAL of RSV bronchiolitis patients (n = 9). * $p < 0.05$. BAL, bronchoalveolar lavage; cIg, isotype-matched control antibody; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; MFI, median fluorescence intensity; MPO, myeloperoxidase; SIRL-1, signal inhibitory receptor on leukocytes-1.

To investigate the functional relevance of LAIR-1 surface expression on activated neutrophils, we studied neutrophil extracellular trap (NET) formation. Although antiviral properties of NETs have been reported, NETs can also damage the lungs during viral infection and cause airway obstruction.[3] Neutrophils isolated from the blood of RSV bronchiolitis patients showed little to no NET formation – measured as DNA release using fluorescent microscopy – in the absence of *ex vivo* stimulation (Fig 2A). In contrast, airway-infiltrated neutrophils readily formed NETs during 90 minutes of *ex vivo* culture (Fig 2A-B). Co-staining of DNA with myeloid peroxidase (MPO) confirmed the presence of NETs (Fig 2C). Correspondingly, a previous study found that RSV particles and fusion protein induce NET formation.[5] Ligation of LAIR-1 with agonistic antibodies significantly reduced NET formation by airway neutrophils during *ex vivo* culture compared to isotype-matched control antibodies (Fig 2B). In contrast, antibody-mediated ligation of SIRL-1 – which suppresses NET formation by peripheral blood neutrophils[6] – only modestly reduced NET formation. This corresponds to the lower surface expression of SIRL-1 on airway-infiltrated compared to circulating neutrophils – the mirror image of LAIR-1 (Fig 2D). Thus, LAIR-1 ligation inhibits NET formation by airway-infiltrated neutrophils in response to RSV infection. This is in line with a prior study where silencing of LAIR-1 in *ex vivo*-differentiated mouse neutrophils enhanced NET formation.[7]

Recently, two novel RSV antivirals were successfully tested in clinical trials with experimentally infected adults.[8] Treating naturally infected infants with similar timing – within 12 hours of detecting RSV in nasal washes – may prove difficult. In fact, RSV loads are already declining when patients are admitted into intensive care.[9] Parallels might be drawn with oseltamivir, which needs to be administered within 36-48 hours of the onset of symptoms to be effective against influenza. Despite declining viral loads, large numbers of neutrophils persist in the airways of RSV bronchiolitis patients.[9] Ligation of LAIR-1 may offer a potential immunotherapeutic strategy to limit neutrophil-induced immune injury.

We acknowledge several potential limitations. First, the total patient sample size is limited. Nonetheless, all patients showed the same pattern of inhibitory receptor expression and ligation of LAIR-1 restricted NET formation in all cases. Second, our patient cohort does not include a control population of non-ventilated children with mild RSV infection, because we cannot sample the airways of non-ventilated infants. Third, although antibody-mediated ligation of LAIR-1 reduces NET formation by airway-derived neutrophils *ex vivo*, it is unclear whether LAIR-1 ligation reduces NET formation *in vivo* or how much NET formation needs to be reduced *in vivo* to

decrease disease severity. Finally, LAIR-1-mediated regulation of neutrophil functions other than NET formation were not studied.

In conclusion, we provide the first evidence that airway neutrophils of infants with RSV bronchiolitis are regulated by the immune inhibitory receptor LAIR-1. We propose that suppressing neutrophil activity via LAIR-1 ligation under the umbrella of newly available RSV antivirals may provide a novel strategy to treat one of the most important infections during infancy.

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Supplementary methods and materials

Patients

The RRR study was initiated to study the expression of LAIR-1 on blood and airway specimens during acute respiratory infection (protocol 10-247). We included a cohort of previously healthy term or otherwise healthy preterm children gestational age >32 weeks, aged less than one year who needed mechanical ventilation for RSV bronchiolitis. Airway-infiltrated cells were obtained from infants with PCR-proven RSV bronchiolitis by tracheobronchial aspiration within 48-72 hours after intensive care unit admission. For the current study, we recruited three control populations. First, to study whether LAIR-1 expression on neutrophils was specific to RSV bronchiolitis, we also recruited adults with simple rhinorrhea (EXIRA study, protocol 11-264). Second, to distinguish whether neutrophil migration or *in vivo* immune activation was sufficient for LAIR-1 expression, we studied children mechanically ventilated for severe culture-proven sepsis (KIRS study, protocol 13-032). Third, neutrophils were isolated from peripheral blood of healthy volunteers for other in vitro experiments. The ethical review board approved all studies and participants or their parents (legal guardians) provided written informed consent.

Flow cytometry

Flow cytometry was performed on red blood cell-depleted single-cell suspensions incubated with fluorochrome-conjugated antibodies using a BD Canto II or LSR Fortessa; cells were sorted with a BD FACSAria III. Acquisitions were analyzed using FlowJo software (version 10.0.7, Treestar).

Neutrophil isolation

Human neutrophils were isolated from heparinized venous blood of patients or healthy donors by density gradient centrifugation with Ficoll (Amersham Biosciences) followed by lysis of red blood cells with ammonium chloride buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA).

***In vitro* neutrophil stimulation**

To study the *in vitro* induction of LAIR-1 expression, neutrophils from healthy donors were isolated and stimulated at 37 °C with granulocyte-macrophage colony-stimulating factor (GM-CSF, 1 μM) for 30 min, platelet-activating factor (PAF, 1 μM) for 10 min, formyl-methionyl-leucyl phenylalanine (fMLF, 1 μM) for 10 min, or PAF (1 μM) and fMLF (1 μM) for 10 min. In other

experiments, neutrophils were primed for 15 min with GM-CSF (1 µM) and stimulated with conditioned culture medium of RSV-infected cells or conditioned culture medium of non-infected cells as control. For this, A549 cells were infected with RSV-A2 at a multiplicity of infection of 1. Culture medium was collected at 2 days post infection and stored at -80 °C until used. After stimulation, LAIR-1 surface expression was determined by flow cytometry.

Intracellular LAIR-1 expression

For detection of intracellular LAIR-1, neutrophils were fixed with 1% paraformaldehyde (PFA) in PBS, permeabilized with 0.1% saponin (Sigma) in PBS and stained for LAIR-1 expression with the 8A8 monoclonal antibody (mouse IgG1, own production), which was detected with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Life Technologies). After staining, the cells were plated on 0.001% (v/v) poly-L-lysine-coated coverslips. The coverslips were embedded with Fluoromount-G (Southern Biotech), supplemented with 1 µM Hoechst 33342 (Life Technologies) and analyzed by confocal microscopy (Zeiss LSM710) at 630-fold magnification (Plan-Apochromat 63x/1.40NA objective) with ZEN imaging software (Zeiss).

NET formation by BAL neutrophils

NET formation by airway and blood neutrophils of RSV bronchiolitis was analyzed by fluorescence microscopy as previously described.[1] Cells obtained from RSV bronchiolitis patients by BAL and neutrophils isolated from heparinized venous blood of patients were treated with anti-LAIR-1 antibody (10 µg/mL, clone 8A8, mouse IgG1, own production), anti-SIRL-1 antibody (10 µg/mL, clone 1A5, mouse IgG1, own production), an isotype-matched control antibody (10 µg/mL, clone MOPC-21, mouse IgG1, BD Biosciences), or culture medium only at 4 °C for 30 min. Anti-LAIR-1, anti-SIRL-1, and isotype-matched control antibodies were cross-linked with secondary F(ab')2 goat anti-mouse IgG fragments (20 µg/mL, SouthernBiotech) for 15 min at 4 °C. Cells were suspended in RPMI-1640 with 2% fetal calf serum (FCS), transferred onto glass coverslips coated with 0.001% (v/v) poly-L-lysine (Sigma), and incubated at 37 °C with 5% CO_2 for 90 min with 0.5 µM of SYTOX Green (Life Technologies) present for the last 15 min of incubation. Cells were fixed with 2% (v/v) PFA in PBS and stained with 1 µM of Hoechst 33342 (Life Technologies) in PBS. In some experiments, coverslips were incubated with polyclonal rabbit antibodies against myeloid peroxidase (MPO; Abcam) for 16 h at 4 °C, which were visualized with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibodies (Life Technologies). Coverslips were mounted

with Fluoromount-G (SouthernBiotech). To assess the extent of NET formation, SYTOX Green fluorescence was measured with wide-field microscopy at 100-fold magnification (Pathway 855 Bioimaging System, BD) using a Hamamatsu Orca High Resolution 155 CCD camera. The system was controlled with BD AttoVision software (version 1.7/855). Quantification of NETs was performed using nine random fields-of-view per sample that were analyzed with Fiji software.[2] The same contrast and fluorescence thresholds were applied to the images from all conditions. SYTOX Green-positive pixel counts were divided by the total number of pixels of thresholded 8-bit images, and expressed as the percentage of image area covered by positive fluorescence staining. Additionally, SYTOX Green-permeable neutrophils that retained regular nuclear morphology were excluded from the NET quantification using ImageJ particle analysis. To examine MPO localization in NETs, coverslips were examined by confocal microscope (Zeiss LSM710) at 200-fold magnification (Plan-Apochromat 20x/0.8NA objective) using ZEN imaging software (Zeiss).

Statistics

Differences between inhibitory receptor expression on airway and blood neutrophils of RSV patients were analyzed using a paired 2-tailed Student's t-test. The statistical significance of differences between two groups of patients was calculated with the unpaired Mann-Whitney test, more than two groups were compared with the Kruskal-Wallis test. Statistical analyses were performed using Prism 6 (GraphPad) software. P values <0.05 were considered significant.

Supplementary figures

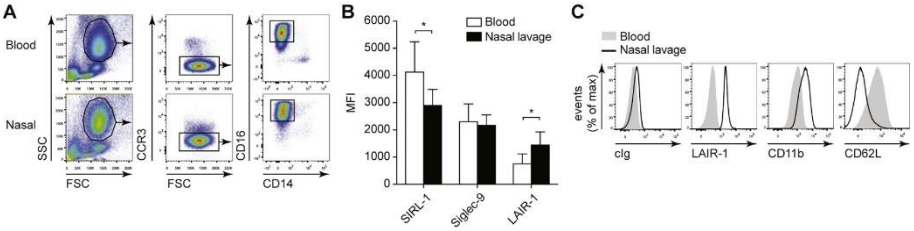

Figure E1. Upper airway neutrophils of acute rhinitis patients express LAIR-1.
Blood and BAL cells obtained from rhinitis patients were stained for cell lineage and activation status, as well as a panel of inhibitory receptors, for analysis by flow cytometry. Neutrophils were gated based on their characteristic forward- and side-light scatter properties and further identified by negative CD14 and CCR3 staining, as well as positive CD16 staining. Blood and BAL neutrophils as gated in (A) are compared for their expression of the inhibitory receptors CD31, SIRL-1, SIRPα, Siglec-9, and LAIR-1 in (B). Data are presented as means ± SD of eight (B) patients. Staining for activation markers (CD11b and CD62L), LAIR-1, and isotype-matched control Ig on BAL versus blood neutrophils of a representative patient are depicted in (C). * $p < 0.05$. BAL, bronchoalveolar lavage; cIg, isotype-matched control antibody; LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; SIRL-1, signal inhibitory receptor on leukocytes-1; Siglec-9, sialic acid-binding immunoglobulin-like lectin 9.

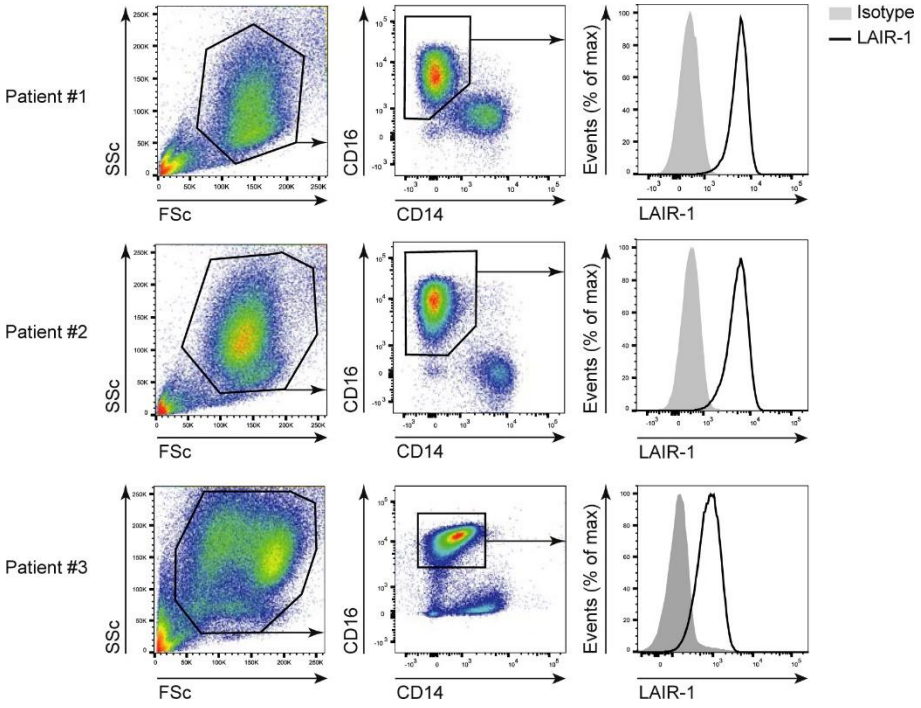

Figure E2. Airway-infiltrated neutrophils of ventilated, RSV-negative pediatric patients express LAIR-1.

Patient #1: eleven months-old pneumonia patient requiring mechanical ventilation with positive sputum culture for *S. aureus*, *S. pneumonia*, and *M. catarrhalis*, but negative for RSV. Patient #2: one month-old patient requiring mechanical ventilation for central hypoventilation. Patient #3: sixteen months-old patient requiring mechanical ventilation for upper respiratory tract obstruction. Neutrophils in tracheal aspirates of patients were analyzed for LAIR-1 expression by flow cytometry. LAIR-1, leukocyte-associated immunoglobulin-like receptor-1.

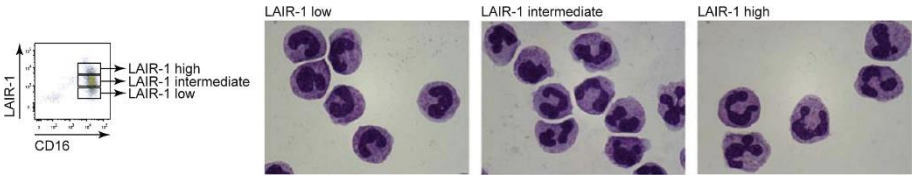

Figure E3. LAIR-1 expressing circulating neutrophils in sepsis patients are mature. Neutrophils from peripheral blood of a sepsis patient (n = 1) were sorted based on phenotypical neutrophil markers and LAIR-1 expression. Low, intermediate and high LAIR-1 expressing neutrophils were sorted and subjected to Giemsa staining to examine nuclear morphology. LAIR-1, leukocyte-associated immunoglobulin-like receptor-1.

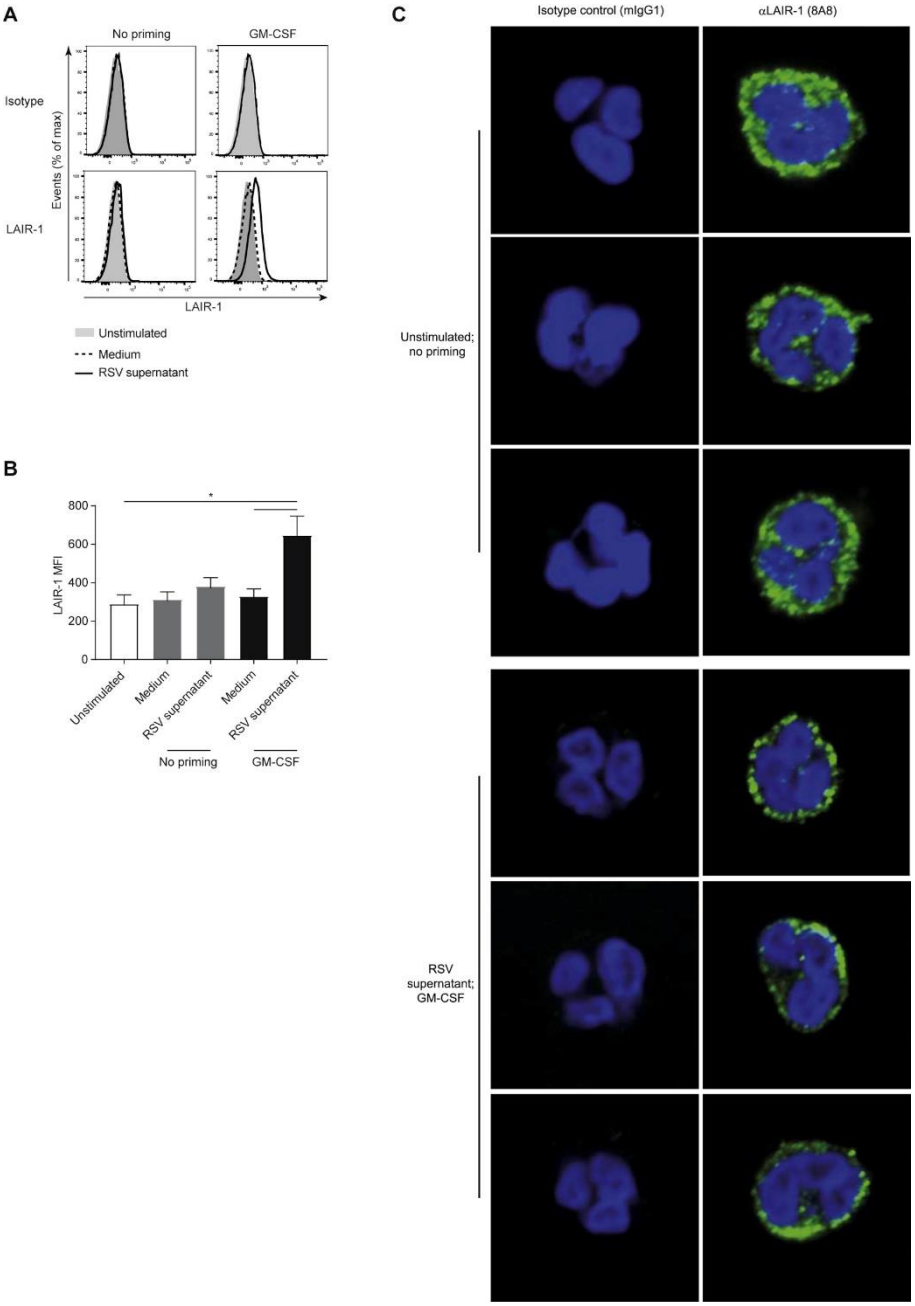

Figure E4. Conditioned culture medium of RSV-infected A549 cells induces LAIR-1 expression on GM-CSF-primed neutrophils.

(A) A representative example of LAIR-1 expression on healthy donor neutrophils (n=5) induced by 30 min incubation with conditioned culture medium of RSV-infected

A549 cells (RSV supernatant) or conditioned culture medium of uninfected A549 cells (medium) following prior 15 min priming with granulocyte-macrophage colony-stimulating factor (GM-CSF). (B) Quantified LAIR-1 median fluorescence for 5 healthy donors. (C) Confocal microscopy for LAIR-1 (green) and DNA (blue) of unstimulated versus RSV supernatant-stimulated neutrophils (n=3). * $p < 0.05$. LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; RSV, respiratory syncytial virus.

Supplementary references

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Chapter

4

LAIR-1 Limits Neutrophilic Airway Inflammation

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Abstract

Neutrophils are crucial to antimicrobial defense, but excessive neutrophilic inflammation induces immune pathology. The mechanisms by which neutrophils are regulated to prevent injury and preserve tissue homeostasis are not completely understood. We recently identified the collagen receptor leukocyte-associated immunoglobulin-like receptor (LAIR)-1 as a functional inhibitory receptor on airway-infiltrated neutrophils in viral bronchiolitis patients. In the current study, we sought to examine the role of LAIR-1 in regulating airway neutrophil responses in vivo. LAIR-1-deficient (*Lair1*$^{-/-}$) and wild-type mice were infected with respiratory syncytial virus (RSV) or exposed to cigarette smoke as commonly accepted models of neutrophil-driven lung inflammation. Mice were monitored for cellular airway influx, weight loss, cytokine production, and viral loads. After RSV infection, *Lair1*$^{-/-}$ mice show enhanced airway inflammation accompanied by increased neutrophil and lymphocyte recruitment to the airways, without effects on viral loads or cytokine production. LAIR-1-Fc administration in wild type mice, which blocks ligand induced LAIR-1 activation, augmented airway inflammation recapitulating the observations in *Lair1*$^{-/-}$ mice. Likewise, in the smoke-exposure model, LAIR-1 deficiency enhanced neutrophil recruitment to the airways and worsened disease severity. Intranasal CXCL1–mediated neutrophil recruitment to the airways was enhanced in mice lacking LAIR-1, supporting an intrinsic function of LAIR-1 on neutrophils. In conclusion, the immune inhibitory receptor LAIR-1 suppresses neutrophil tissue migration and acts as a negative regulator of neutrophil-driven airway inflammation during lung diseases. Following our recent observations in humans, this study provides crucial in-vivo evidence that LAIR-1 is a promising target for pharmacological intervention in such pathologies.

Introduction

The lungs are constantly exposed to potential pathogens and other harmful agents. To protect against sudden incursions, neutrophils patrol the lung capillaries. A rapid and robust neutrophil response is crucial to antimicrobial defense (1, 2). Neutrophilic inflammation is a common trait of some respiratory diseases. We have recently reviewed literature showing that due to the promiscuous cytotoxicity of neutrophils, excessive neutrophilic inflammation induces immune injury in viral infection (3). Therefore, balancing pathogen eradication with neutrophil-induced tissue injury is of the utmost importance to preserve tissue homeostasis. However, the mechanisms that regulate neutrophilic inflammation in the airways are still unclear.

Leukocyte-associated Ig-like receptor (LAIR)-1, also known as CD305, is an ITIM-bearing inhibitory receptor expressed on majority of immune cells (4). Mouse and human LAIR-1 share ~40% homology, potent inhibitory capacity and bind to collagen and collagen-like molecules (5–8). Circulating neutrophils do not express LAIR-1 on the cell surface, but surface expression can be induced by in vitro stimulation, suggesting that LAIR-1 is involved in the regulation of activated, tissue-infiltrated neutrophils (9). We recently identified the collagen receptor LAIR-1 as functional inhibitory receptor on airway neutrophils obtained from RSV bronchiolitis patients (10). Activated airway-infiltrated neutrophils, but not their resting circulating counterparts, express LAIR-1 at the cell surface. Resting neutrophils store LAIR-1 intracellularly in granules, which allows for rapid surface upregulation upon activation. Agonistic antibody-mediated ligation of LAIR-1 on patient airway neutrophils suppresses neutrophil extracellular traps (NET) formation ex-vivo. Ligands for LAIR-1 are abundant in the lungs, including collagen in the extracellular matrix and surfactant protein-D (SP-D, which contains a collagen-like domains, in the airway lumen (11, 12). We, therefore, hypothesized that LAIR-1 regulates neutrophilic inflammation in lung diseases to minimize tissue injury. However, patient studies do not allow for experimental settings required to investigate the in-vivo role of LAIR-1. To test this hypothesis, we used mouse models and examined two distinct lung diseases in which neutrophilic inflammation plays a key role, namely, acute viral bronchiolitis caused by RSV infection, and lung inflammation induced by short-term smoke-exposure.

Materials and Methods

Animals

Lair1$^{-/-}$ mice were generated on the C57BL/6 background by Taconic Artemis as described (13). BALB/c mice were procured from Harlan (Horst, the Netherlands). All animal studies were approved by the Institutional Animal Care and Use Committee and carried out in accordance with the national and institutional guidelines.

Mouse RSV Infection

Eight to Twelve-week-old female C57BL/6 *Lair1*$^{-/-}$ mice or their wild-type littermates were intranasally infected with 1 × 107 PFU of RSV-A2 in 50 µl PBS. RSV-A2 preparation, quantitative assay for RSV-A2 titration and RSV-A2 infection of mice, including, intranasal inoculation, termination, and sample collection, was performed as described previously (14, 15). Mice were sacrificed on day 2 or 5 post-infection.

LAIR-Fc Administration

For LAIR-1 blocking experiments, recombinant mouse LAIR-1 fused with the Fc portion of mouse IgG2a was produced in-house. The Fc tail was mutated to prevent binding to Fc receptors and complement as described previously (16).

Eight to twelve-week-old female BALB/c wild-type mice were injected intraperitoneally with 200 or 400 µg of mouse LAIR-1-Fc chimeric protein in 100 µl PBS or PBS alone as control, 1 day before and 2 days after RSV infection. Intranasal RSV infection was performed as described above.

Bronchoalveolar Lavage Collection and Processing

Bronchoalveolar lavage (BAL) fluid collection was performed by flushing the lungs 2 times with 1.0 ml of ice-cold PBS. BAL fluid was centrifuged; supernatants were stored at −80°C for further analyses. Total cell counts in BAL were determined using a Bürker-Türk hemocytometer. BAL cells were analyzed by flow cytometry or examined by light microscopy.

For differential cell analysis by light microscopy, at least 200 cells were counted to assign relative quantities of macrophages, lymphocytes, and neutrophils based on morphology after May–Grünwald–Giemsa stain.

RSV-A2 concentrations in BAL fluid supernatants were analyzed by real-time PCR as described previously (15). Mouse CXCL1 (KC) and IL-6 levels

in BAL fluid supernatants were measured by ELISA (Peprotech, London, UK) according to the manufacturer's instructions.

Cigarette-Smoke Exposure

Male, 8–12 weeks old, wild-type and *Lair1*$^{-/-}$ C57BL/6 mice were randomly assigned to undergo cigarette smoke (CS) or control air exposure. Mice were exposed to whole body mainstream CS generated from standard research cigarettes (3R4F; 9.4 mg tar/0.726 mg nicotine, University of Kentucky) using a Watson-Marlow roller pump (323 E series, speed 35 RPM; Watson Marlow, Rotterdam, The Netherlands) that directed the CS into the exposure chamber (25 L of air volume). Carbon monoxide and oxygen levels in the exposure chamber were measured using a gas analyzer from Bacharach (PCA-3 series; Bacharach, New Kensington, PA, USA) carbon monoxide concentrations were held at 150–300 ppm and oxygen levels were kept at 20%. Mice were exposed to CS twice daily for a maximum of 30 min with a 5-h smoke-free interval, for 10 consecutive days. On the first day of CS exposure, mice were exposed to CS from 2 pairs of cigarettes, followed by CS of two times 3 cigarettes on the second day, CS of 4 and 5 cigarettes on the third day, CS of 5 and 6 cigarettes on the fourth day, and CS of two times 6 cigarettes on fifth and remaining days. Mice were weighed daily. On the final day, mice were sacrificed and BAL fluid was collected.

Intranasal Instillation of CXCL1

Eight- to Sixteen-week-old male and female *Lair1*$^{-/-}$ mice (on a C57BL/6 background) or their wild-type littermates were randomly assigned to be instilled with 0.1 µg or 0.5 µg CXCL1 (R&D Systems, Minneapolis, MN, USA) in 50 µl PBS or PBS alone. After 4 h, mice were sacrificed and BAL fluid was collected. Cells were counted and analyzed by flow cytometry as described below.

Single-Cell Suspension Preparation

Spleen and lymph nodes were mechanically dissociated and filtered sequentially through 100 and 70 µm cell strainers (BD Biosciences, San Jose, CA, USA). Bone marrow was flushed from femurs and tibiae and filtered through 70 µm cell strainers. Lungs were mechanically dissociated using the gentleMACS Dissociator (Miltenyi Biotec, Leiden, the Netherlands) as recommended by the manufacturer, and were enzymatically digested for 30 min at 37 °C with 0.13 Wünch U/ml of LiberaseTM (Roche, Basel, Switzerland) and 200 µg/ml DNAse I (Roche, Basel, Switzerland) in RPMI-1640. Subsequently, the digested lung cell suspension was passed through 70 µm

cell strainers. Red blood cells were lysed by 10 min incubation at 4°C in ammonium chloride carbonate buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA. Single-cell suspensions were analyzed by flow cytometry.

Flow Cytometry

Single-cell suspensions from tissues and cells from BAL fluid were stained for surface markers for 30 min at 4 °C in PBS containing 0.01% (m/v) sodium azide and 1% (m/v) BSA. Propidium iodide (0.3 µg/mL, Sigma, St. Louis, MO, USA) in PBS was used to distinguish vital cells. Samples were treated with rat anti-mouse CD16/CD32 antibody (clone 2.4G2, BD Biosciences, Basel, Switzerland) to block FcR-mediated non-specific antibody binding prior to incubation with fluorochrome-conjugated antibodies. Acquisitions were made with a BD Canto II or LSR Fortessa and analyzed using FlowJo software (version 10.0.7, Treestar).

Statistics

The statistical significance of differences between two groups was calculated with the unpaired Mann-Whitney or Student's t test where appropriate and more than two groups were compared with the 2-way ANOVA or Kruskal-Wallis test as mentioned in the legends. Statistical analyses were performed using Prism 6 (GraphPad) software. P-values < 0.05 were considered significant and are marked in the graphs where applicable. All unmarked differences are non-significant.

Results

LAIR-1 Negatively Regulates Neutrophil Recruitment During RSV Infection

We recently demonstrated that LAIR-1 is a functional inhibitory receptor on airway-infiltrated neutrophils of RSV infection-induced bronchiolitis patients in an ex-vivo setting (10). We therefore hypothesized that LAIR-1 regulates the neutrophil response in vivo during viral bronchiolitis. To test this hypothesis, wild-type and *Lair1*$^{-/-}$ C57BL/6 mice were intranasally inoculated with the RSV-A2 strain. Mice were sacrificed on day 2 or 5 post-infection and BAL was performed to assess the cellular airway infiltrate, cytokine levels, and viral load.

Total leukocyte influx into the airways was notably increased in LAIR-1-deficient compared with wild-type mice at both the time points (Figure 1A). Early in infection, the increase in cell numbers was mostly contributed by

enhanced neutrophil recruitment in LAIR-1-deficient mice, while at day 5 post-infection lymphocytes mainly constituted the infiltrating population (Figures 1B,C). There were no differences in macrophage recruitment between genotypes (Figure 1D). Thus, LAIR-1 limits neutrophil and lymphocyte recruitment during RSV infection in vivo.

Despite the enhanced leukocyte recruitment, concentrations of the major neutrophil chemoattractant CXCL1 and the inflammatory cytokine IL-6 were not increased in the BAL fluid of RSV-infected *Lair1*$^{-/-}$ mice compared with wild-type mice (Figures 1E,F), nor were there differences in the viral load (Figure 1G). Thus, the data suggest that LAIR-1 negatively regulates neutrophil and lymphocyte recruitment during RSV infection without directly affecting the local inflammatory milieu or viral replication.

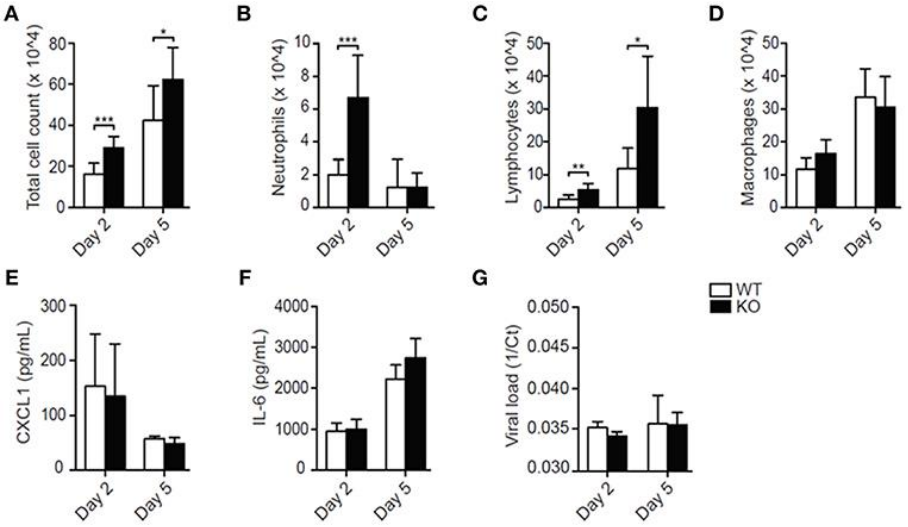

Figure 1. LAIR-1 regulates neutrophil and lymphocyte recruitment during RSV-A2 infection in mice.

(A–G) Mice were inoculated with RSV-A2 and sacrificed on day 2 or 5. Total (A) and differential (B–D) BAL cell counts, BAL CXCL1 (E) and IL-6 (F) concentrations, and viral loads (G) were determined. Data are presented as means ± SD and represent 8 mice per group in 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001; unpaired Student's t-test with Welch's correction. WT, wild-type and KO, *Lair1*$^{-/-}$ on C57BL/6 background.

Tissue-Infiltrated Neutrophils Express LAIR-1

To rule out basal differences, we performed immunophenotyping of unchallenged *Lair1*$^{-/-}$ C57BL/6 mice and confirmed that there were little to no differences in the composition of immune cell populations (Supplemental

Figures S1, S2) as described before (13, 17). Moreover, we demonstrate that unchallenged wild-type and *Lair1*$^{-/-}$ mice did not differ in neutrophil numbers and activation state—indicated by the CD11b, CD62L, and CD182 markers—in either blood or bone marrow (Figure 2A). In unchallenged wild-type mice, circulating neutrophils did not express LAIR-1, whereas tissue-infiltrated neutrophils did (Figure 2B). However, upon infection with RSV, circulating neutrophils started to express LAIR-1 (Figure 2C). In line with the observation regarding tissue infiltrated neutrophils in unchallenged mice, airway-infiltrated neutrophils also expressed LAIR-1 and were highly activated—indicated by upregulation of CD11b and shedding of CD62L (Figure 2C). Thus, RSV infection induced LAIR-1 expression on circulating and airway-infiltrated neutrophils which may directly regulate the function/adhesion of neutrophils.

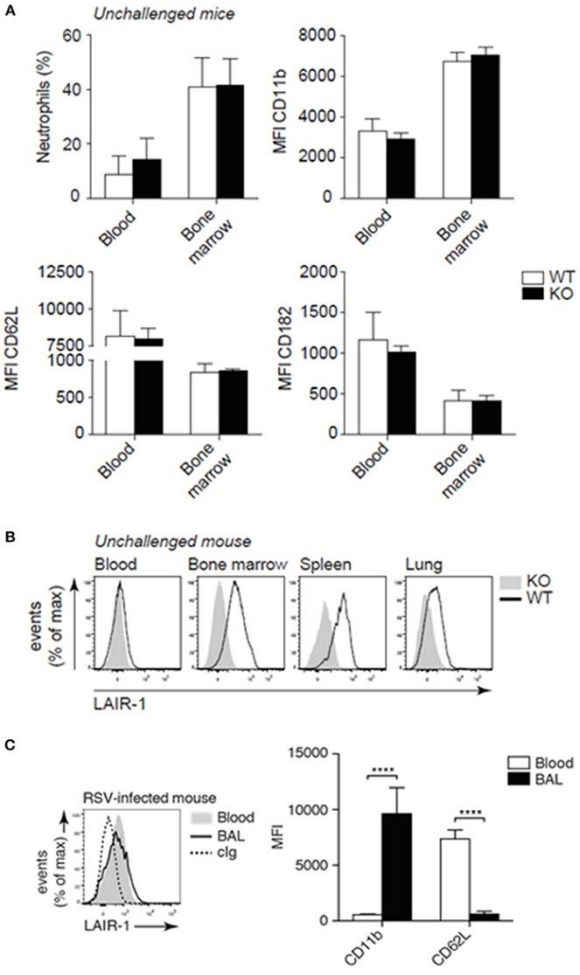

Figure 2. LAIR-1 expression and deficiency in mice.
(A–C) Mouse leukocytes were examined for lineage and activation markers and LAIR-1 expression by flow cytometry. Neutrophils were identified based on characteristic forward- and side-light scatter properties and the expression of Ly-6G. (A) The percentage neutrophils (Ly-6G+) among total live leukocytes in blood and bone marrow as well as the expression of activation markers (CD11b, CD62L, and CD182) were compared by flow cytometry. (B) Flow cytometric analysis of LAIR-1 expression on blood and tissue (bone marrow, spleen, and lung) neutrophils. (C) Wild-type mice were inoculated with RSV and sacrificed 2 days post-infection. Expression of LAIR-1 and activation markers (CD11b and CD62L) was measured on BAL and blood neutrophils. ****$p < 0.0001$; 2-way ANOVA with Holm-Šídák multiple comparison correction. Data are representative of eight mice (A, B) or three independent experiments with at least three mice (C). Error bars in (A) and (C) represent mean ± SD. WT, wild-type and KO, *Lair1*$^{-/-}$ on C57BL/6 background; cIg, isotype-matched control antibody; MFI, mean fluorescence intensity.

Blocking LAIR-1-Ligand Interaction Enhanced Neutrophil Recruitment During RSV Infection

To further rule out developmental differences in *Lair1*$^{-/-}$ mice as cause of the observed phenotype, the interaction between endogenous LAIR-1 and its ligands was blocked during RSV infection by injecting wild-type mice with LAIR-1-Fc chimeric protein. Here, BALB/c mice rather than C57BL/6 mice were used as the former are more sensitive to RSV infection (18, 19). The results obtained with RSV-infected BALB/c mice in which endogenous LAIR-1-collagen interactions were blocked, mimic those of the RSV-infected *Lair1*$^{-/-}$ C57BL/6 mice, when compared to their respective vehicle-treated or wild-type mice. The total cell count in the BAL of RSV-infected LAIR-1-Fc chimeric protein-treated mice was increased (Figure 3A). This was due to enhanced neutrophil and lymphocyte recruitment, whereas macrophage numbers remained unaffected (Figures 3B–D). Concentrations of CXCL1 and IL-6 as well as viral load were comparable between vehicle- and LAIR-1-Fc-treated mice (Figures 3E–G). The data further confirm that LAIR-1 negatively controls neutrophil and lymphocyte recruitment during RSV infection with no direct effect on the local inflammatory milieu or viral replication.

LAIR-1 Limits Disease Severity and Neutrophil Airway Recruitment in Response to Cigarette-Smoke Exposure

Despite the enhanced neutrophil recruitment during RSV infection in mice that lack LAIR-1 signalling, there was no effect on disease severity as measured by weight loss (data not shown). However, RSV pathophysiology in humans and mice is notably different. While neutrophils dominate the

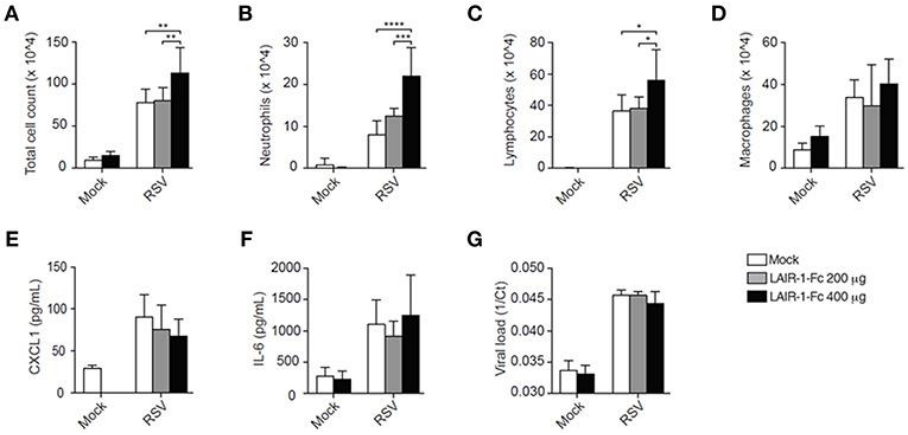

Figure 3. LAIR-1-Fc chimeric protein administration effects pulmonary neutrophil recruitment during RSV infection.

(A–G) Wild-type BALB/c mice were inoculated intranasally with RSV and sacrificed on day 5. One day before RSV inoculation and 2 days after inoculation mice were treated intraperitoneally LAIR-1-Fc fusion protein or PBS (vehicle). Total (A) and differential (B–D) BAL cell counts, BAL CXCL1 (F) and IL-6 concentrations (G), and viral load (G) were determined. Data are presented as means ± SD and represent 6 mice per group for panels (A–G) in 2 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. 2-way ANOVA with Holm-Šídák multiple comparison correction.

cellular inflammatory response in RSV bronchiolitis patients, with neutrophils comprising ≥80% of infiltrating leukocytes (20–22), lymphocytes are more prominent in the airways of mice during experimental RSV infection (Figures 1A–D, 3A–D). We hypothesized that in a genuine neutrophil-driven disease model the loss of LAIR-1-mediated immune regulation and the corresponding increase in neutrophil infiltration would exacerbate disease severity. Therefore, we employed another model of neutrophilic airway inflammation and exposed wild-type and *Lair1*$^{-/-}$ C57BL/6 mice to cigarette smoke. Smoke-exposed *Lair1*$^{-/-}$ mice lost more body weight and showed delayed and significantly attenuated recovery as compared to their wild-type counterparts (Figure 4A). In line with this, BAL fluid analysis revealed that *Lair1*$^{-/-}$ mice had significantly higher lung immune infiltration in response to smoke-exposure as compared to the wild-type (Figure 4B). This increase in total cell influx was contributed by increased recruitment of neutrophils, macrophages and lymphocytes to *Lair1*$^{-/-}$ lungs (Figures 4C–E). The increase in BAL cell counts in *Lair1*$^{-/-}$ mice was highest for neutrophils (ca. 3-fold) followed by macrophages (ca. 2-fold), while the increase in lymphocytes was modest and failed to reach statistical significance. We confirmed that airway-

infiltrated neutrophils of smoke-exposed mice expressed LAIR-1 and were highly activated (Supplemental Figure S2). Thus, LAIR-1 limits neutrophil recruitment to the airways during cigarette smoke-induced lung inflammation and regulates disease severity.

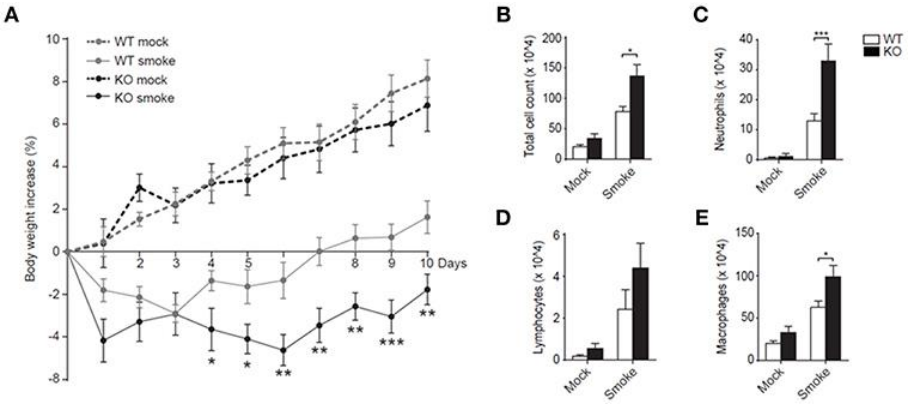

Figure 4. LAIR-1 regulates neutrophilic airway inflammation during cigarette-smoke exposure.

(A–E) Mice were exposed to cigarette smoke or mock (air) in whole-body chambers twice daily for 10 consecutive days. (A) Body weight of mice was measured daily and percentage change relative to day 0 was calculated. Total (B) and differential counts (C–E) in BAL were determined after 10 days of cigarette-smoke exposure. Data are representative of at least 10 mice per group in 2 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The Mann-Whitney U test was used to calculate the statistical significance of differences between WT and KO mice (A) and a 2-way ANOVA with Holm-Šídák multiple comparison correction was used for the cell counts (B,C). WT, wild-type and KO, *Lair1*$^{-/-}$ on C57BL/6 background.

LAIR-1 Directly Suppresses Neutrophil Migration

The local inflammatory milieu in the lung, as reflected by CXCL1 and IL-6 production, was not directly regulated by LAIR-1 (Figures 1E,F, 3E,F). Since the lung is enriched in LAIR-1 ligands such as collagen, SP-D and airway-infiltrated neutrophils in both the RSV infection and the smoke exposure model express LAIR-1 (Figure 2C; Supplemental Figure S3), we hypothesized that LAIR-1 can directly limit the migratory capacity of neutrophils. To examine this hypothesis, the lungs of wild-type and *Lair1*$^{-/-}$ C57BL/6 mice were instilled with the neutrophil chemoattractant CXCL1 by intranasal administration and analyzed for the BAL fluid cellular infiltrates. We observed significantly higher total BAL cell influx in *Lair1*$^{-/-}$ mice as compared to wild-type mice in response to 0.5 µg of CXCL1 (Figure 5A). Flow cytometry confirmed that CXCL1

specifically attracted neutrophils, as they constitute ca. 88% of the total cell population in BAL fluid (Figure 5B), which were highly activated and expressed LAIR-1 (in wild-type mice) (Figure 5C). These data demonstrate that LAIR-1 intrinsically limits neutrophil infiltration of the airways, thereby controlling neutrophilic airway inflammation.

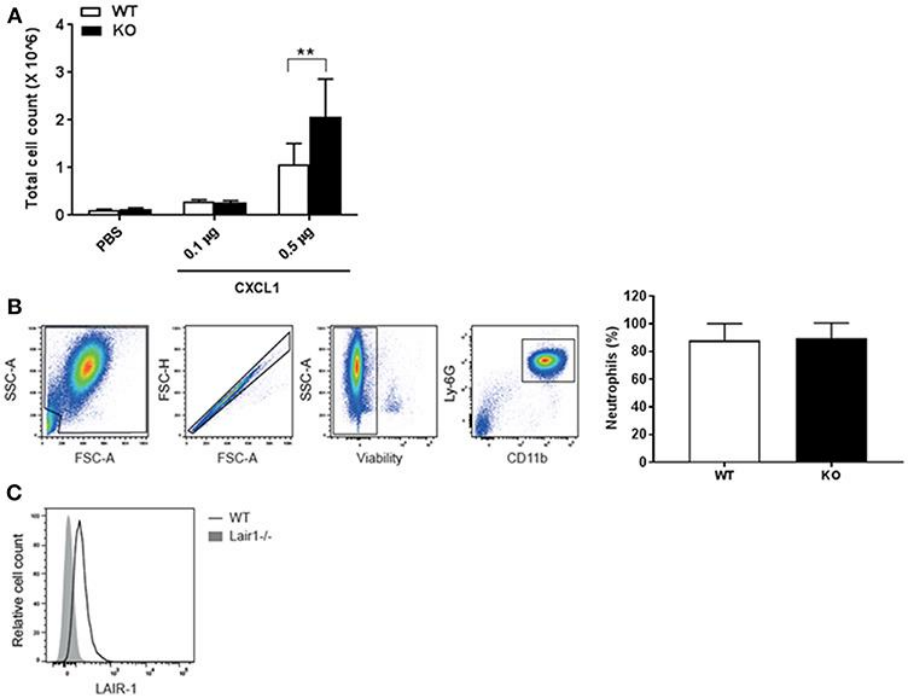

Figure 5. LAIR-1 directly controls neutrophil migration.
(A–C) Mice were intranasally instilled with CXCL1 or vehicle (PBS). After 4 h mice were sacrificed and BAL was performed. (A) Total cell counts in BAL fluid were determined and represented as mean ± SD (n = 4–8 mice per group per genotype; 2 independent experiments). Differences between WT and KO in cell influx at CXCL1 (0.5 µg) were consistent in both experiments. **p < 0.01; 2-way ANOVA with Tukey's multiple comparison test. (B,C) Cells in BAL fluid were analyzed by flow cytometry. (B) Neutrophils were identified by concurrent Ly-6G and CD11b expression; graph represents neutrophils as percent of total live leukocytes in BAL fluid after 0.5 µg CXCL1 administration in WT and KO mice, mean ± SD and, (C) a representative histogram of LAIR-1 expression on BAL fluid neutrophils. WT, wild-type and KO, *Lair1*$^{-/-}$ on C57BL/6 background.

Discussion

The pulmonary immune response must protect against the ever-present threat of pathogens, while limiting immune-induced tissue damage to allow for gas exchange. Neutrophils are crucial for antimicrobial defense but such responses must be tightly regulated to prevent bystander damage. The mechanisms underlying the regulation of neutrophil activation and responses are incompletely understood. We have recently identified LAIR-1 as an inhibitory receptor on activated airway neutrophils which limits NET formation during RSV bronchiolitis (10). In the current study, we investigated the role of LAIR-1 in regulating airway inflammation using two different models of neutrophil pre-dominant lung diseases. Using a mouse model of RSV bronchiolitis, we demonstrate that LAIR-1 functions as a negative regulator of airway inflammation as LAIR-1 deficiency or administration of Lair1-Fc chimeric protein led to enhanced recruitment of neutrophils and lymphocytes. Similar results were obtained in the cigarette smoke exposure model where LAIR-1 deficient mice show marked increased neutrophilia. Our study, thus, underlines a key regulatory role of LAIR-1 in limiting neutrophilic inflammation in lung diseases.

Ligands for LAIR-1 are abundant in the lungs. En route to the airways, for instance in response to RSV infection or irritants in smoke, neutrophils will traverse the extracellular matrix, which contains collagen. In the airway lumen, neutrophils will encounter pulmonary surfactant-associated protein-D (SP-D) and C1q, which possess a collagen-like domain (11, 12). The interaction of LAIR-1 with the ligands present in the extracellular matrix may limit the recruitment of neutrophils to the airways. In support hereof, we observed an increased neutrophil recruitment to airways of RSV-infected and smoke-exposed mice that lack functional LAIR-1. Interestingly, this enhanced neutrophilia in *Lair1*$^{-/-}$ mice was not associated with augmented chemokine production—for example, by LAIR-1-expressing alveolar macrophages—that attracts more neutrophils. There were no changes in the abundance of CXCL1, a potent neutrophil chemoattractant (23) and IL-6, a major pro-inflammatory cytokine, among wild-type and *Lair1*$^{-/-}$ mice. This is suggestive for an enhanced intrinsic cellular migratory capacity in the absence of an inhibitory interaction of LAIR-1 with extracellular matrix. Indeed, in response to an intrapulmonary challenge with the neutrophil chemoattractant CXCL1 (23), neutrophil recruitment was strongly enhanced in *Lair1*$^{-/-}$ mice compared with wild-type mice, thereby demonstrating a neutrophil-intrinsic role for LAIR-1 in migration to the airways. Thus, during an active inflammation and

in response to chemoattractant stimuli, LAIR-1 interaction with its ligands impedes neutrophil migration in wild-type mice but not in *Lair1*$^{-/-}$ mice.

A prior study of *Lair1*$^{-/-}$ mice did not reveal an overt clinical phenotype in multiple lymphocyte-driven disease models (13, 17). Similar to our study (Supplemental Figure S3), LAIR-1 deficiency had little effect on the composition of immune cell populations or neutrophil activation state in unchallenged mice. However, in the previous studies neutrophils were not extensively studied. In contrast, we examined two different models of lung diseases where neutrophils are a dominant contributor, namely RSV bronchiolitis and smoke-induced inflammation. In both cases, LAIR-1 regulated neutrophilic lung inflammation. We observed no differences in disease severity between RSV-infected *Lair1*$^{-/-}$ and wild-type mice. A possible explanation for this is that while in RSV bronchiolitis patients, the immune response to RSV is characterized by massive infiltration of neutrophils into the airways—≥80% of infiltrating leukocytes are neutrophils (20–22)—the contribution of airway infiltrating neutrophils on disease severity is notably less pronounced in mice (24). However, in response to cigarette smoke exposure, a bona fide neutrophil-driven lung inflammation model in mice, the enhanced neutrophilic inflammation in LAIR-1-deficient mice was accompanied by worsened weight loss and retarded recovery underlining the critical role of LAIR-1.

Neutrophils are crucial to antimicrobial defense, but cytotoxic effector mechanisms such as protease secretion, reactive oxygen species production, and NET formation cause bystander tissue damage (1–3, 25). Therefore, excessive neutrophilic inflammation is harmful. Major lung diseases, including RSV bronchiolitis and chronic obstructive pulmonary disease (COPD), are characterized by a massive neutrophilic inflammation (26). However, the regulatory mechanisms hereof are not yet fully elucidated. A better understanding of how neutrophilic inflammation is regulated could reveal potential targets for pharmaceutical intervention.

In human neutrophils, LAIR-1 is stored in intracellular granules and is rapidly recruited to the surface upon activation (10), presenting a plausible mechanism that would ensure a proper balance between neutrophil function and tissue injury. Under steady state, a low or lack of expression of LAIR-1 would ensure neutrophil activation against invading pathogens whereas rapid recruitment of LAIR-1 from intracellular stores would promptly impede the neutrophil influx and limit the tissue injury during neutrophilic inflammation. Whereas, our study shows that LAIR-1, indeed, impedes neutrophil influx during active inflammation in mouse models, the

mechanisms of LAIR-1 surface expression on mouse neutrophils remains to be investigated.

Our study sheds light on a novel regulatory mechanism involved in neutrophilic inflammation but has several limitations. First, we cannot rule out the contribution of other LAIR-1-expressing immune cell populations, such as lymphocytes and macrophages, to the observed phenotypes. In addition to increased neutrophil infiltration, we also see increased recruitment of lymphocytes after RSV infection and increased number of macrophages after smoke-exposure in the airways of LAIR-1-deficient mice. Possibly, the augmented neutrophil response directly contributes to the subsequent increase in lymphocyte and macrophage influx. Indeed, neutrophils, by depositing CXCL12-containing vesicular trails during migration, are critical to the recruitment of T cells to the airways of influenza virus-infected mice (27). Whereas, both lymphocytes and macrophages could possibly contribute to increased disease severity, increased airway infiltration of neutrophils remains a consistent observation in LAIR-1-deficient mice in both RSV and smoke-exposure models.

Second, we measured a limited number of cytokines. Also, we did not investigate a direct effect of LAIR-1 on mouse neutrophil functions such as NET formation and migration in-vitro. Which aspect of the observed increased airway infiltration of neutrophils aggravates disease severity, remains unresolved. These questions require further investigation. The strength of our study lies in being the first to discern the role of LAIR-1 in neutrophil-predominant airway diseases in vivo. We show that LAIR-1 acts as a crucial regulator of neutrophils and is therefore a potential target for pharmacological intervention in neutrophil-driven lung diseases.

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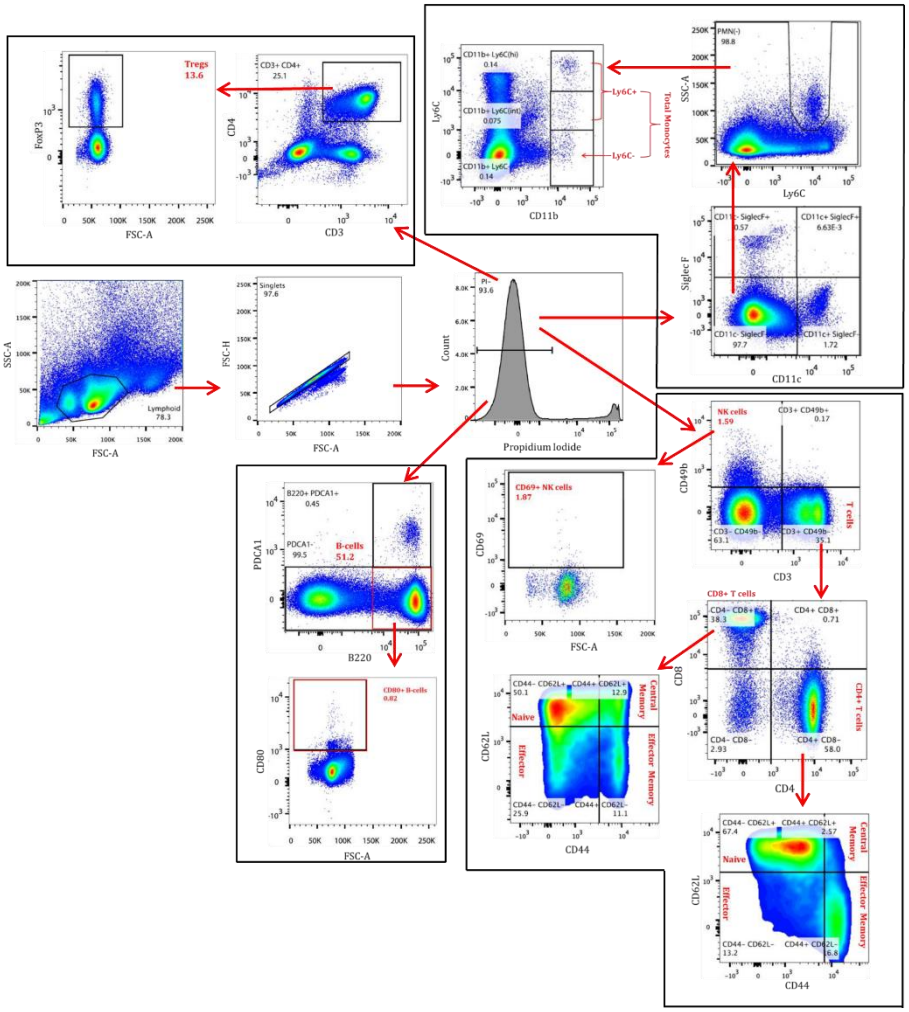
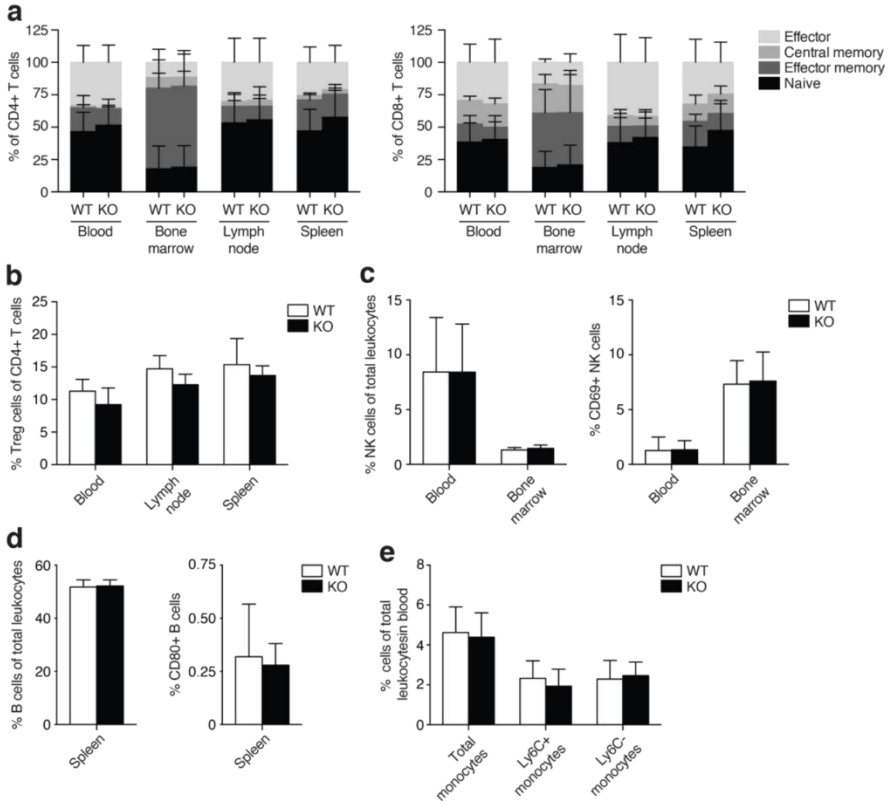
Supplementary figures

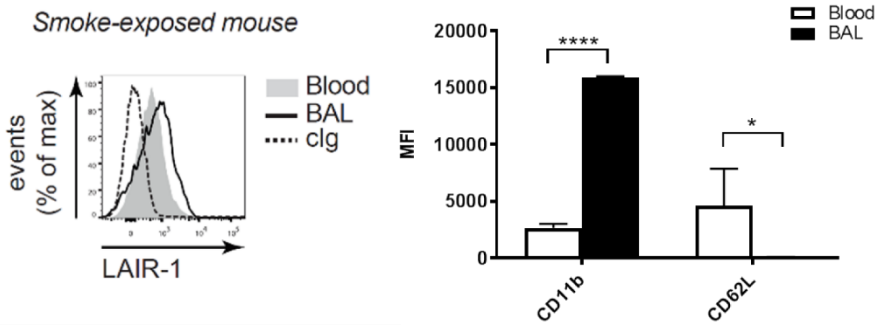


Supplemental Figure S1.
Representative flow cytometry gating strategy for immunophenotyping shown in Supplemental Figure S2.



Supplemental Figure S2. No immunological differences between unchallenged mice.

(a-e) Blood and tissue leukocytes of unchallenged WT and KO mice were stained for lineage and activation markers and analyzed by flow cytometry. (a) The percentages of naive (CD44-CD62L+), effector memory (CD44+CD62L-), central memory (CD44+CD62L+), and effector (CD44-CD62L-) cells among CD4+and CD8+T cell (CD3+) populations were determined in blood, bone marrow, lymph nodes, and spleen. (b) The percentage of T_{reg} cells in the CD4+T cell population of blood, lymph nodes, and spleen of unchallenged WT and KO mice were determined by intracellular staining of FoxP3. (c) The percentage of CD49b+ natural killer (NK) cells present among total leukocytes and the expression of activation marker CD69 on NK cells in blood and bone marrow. (d) The percentage of B cells present among total leukocytes from spleen and the percentage of splenic B cells that express the activation marker CD80. (e) The percentages of total, classical (Ly-6C+), and non-classical (Ly-6C-) monocytes present among blood leukocytes. WT, wild-type and KO, *Lair1*$^{-/-}$on C57BL/6 background.



Supplemental Figure S3. Repeated cigarette smoke exposure induces LAIR-1 expression on mouse neutrophils.
Wild-type C57BL/6 mice were repeatedly exposed to cigarette smoke during 10 days. Expression of LAIR-1 and activation markers (CD11b and CD62L) was measured on BAL and blood neutrophils by flow cytometry. Bar graph represents mean ± SD. * $p<0.01$ and **** $p<0.001$; 2-way ANOVA with Holm-Šídák multiple comparison correction. cIg, isotype-matched control antibody; MFI, mean fluorescence intensity

Chapter

5

Novel agonistic anti-mouse LAIR-1 antibodies for preclinical studies on RSV-induced airway inflammation

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Work in progress

Abstract

RSV infection-induced bronchiolitis is a major cause of child mortality and morbidity worldwide. Airways of paediatric RSV bronchiolitis patients are infiltrated by activated neutrophils in large numbers and contribute to the immune pathology of the disease. Airway-infiltrated neutrophils, but not their blood counterparts, express the inhibitory collagen receptor LAIR-1. We previously demonstrated that LAIR-1 has a critical role in limiting neutrophilic airway inflammation. Therefore, LAIR-1 represents a potential therapeutic target to ameliorate neutrophil-induced immune pathology. To test LAIR-1 ligation as a therapy in our RSV disease mouse model, we required potent LAIR-1 agonists for in vivo use. We generated novel agonistic mouse anti-mouse LAIR-1 antibodies in *Lair1*$^{-/-}$ mice and compared these to a commercially available clone. After extensive testing, the 2C7 antibody clone was selected for further in vivo use because of soluble agonistic potency, lack of LAIR-1-collagen interaction blocking, and minimal induction of immune cell depletion. However, in our initial experiments, intraperitoneal administration of 2C7 to mice prior to inoculation with RSV did not alter disease severity or leukocyte airway infiltration. Therefore, although no proof of concept for LAIR-1 as therapeutic target to ameliorate immune pathology was obtained, the newly generated antibodies may proof useful in other disease models.

Introduction

Respiratory syncytial virus (RSV) infection-induced bronchiolitis is the second-largest cause of child mortality in the developing world.[1] While symptomatic treatment, including mechanical ventilation, is available in developed nations, and usually sufficient to prevent death, severe RSV disease is nonetheless associated with sequalae, such as wheezing and asthma, later in life.[2] In addition to cytological damage caused by the virus itself, excessive neutrophilic inflammation is thought to exacerbate tissue damage in the lungs.[3] The inflammatory response in the airways of paediatric RSV bronchiolitis patients is dominated by neutrophils, which comprise more than 80% of total infiltrating leukocytes.[4] Neutrophil effector mechanisms, such as reactive oxygen species (ROS) production, proteolytic enzyme release via degranulation, and extracellular trap (NET) formation, are highly effective against microbial pathogens, but lack specificity. Hence, excessive deployment hereof induces immune pathology.[3]

Currently, no effective treatment exists that can specifically suppress acute neutrophilic inflammation. Targeting inhibitory receptors with agonists may represent a novel therapeutic approach to limit excessive inflammation. The reverse, antagonising inhibitory receptors in immune checkpoint blockade therapy to potentiate anti-tumour responses, has proven to be highly effective in the treatment of cancer.[5] Neutrophils express multiple inhibitory immune receptors that could be targeted. We have previously demonstrated that leukocyte-associated immunoglobulin receptor (LAIR)-1, an ITIM-bearing inhibitory collagen receptor, is expressed on the activated, airway-infiltrated neutrophils of RSV bronchiolitis patients, but not their blood counterparts. Importantly, ligating LAIR-1 on these airway neutrophils ex vivo using agonistic antibodies inhibited NET formation.[6] Moreover, the genetic ablation of LAIR-1 exacerbated disease severity and enhanced neutrophil recruitment to the airways in mice experimentally infected with RSV.[7] Combined, LAIR-1 limits neutrophilic airway inflammation by suppressing neutrophil recruitment as well as neutrophil effector mechanisms.

Therefore, LAIR-1 is potentially a promising therapeutic target to reduce neutrophil-induced immune pathology. To test this hypothesis, agonists for LAIR-1 that can be used in vivo for administration in animal models are required. Inhibitory signalling by ITIM-bearing receptors such as LAIR-1 requires the clustering of receptor molecules on the cell membrane.[8] High-affinity antibodies could represent potent agonists by crosslinking

receptor molecules. More so, potentially, than endogenous ligands. Either because the affinity of the antibodies for the receptor is higher than the affinity of the receptor for its ligand, or by facilitating crosslinking in environments where endogenous ligands do not. For instance, when the ligands are not or scarcely present or receptor-ligand interactions are blocked by endogenous decoys. We set out to generate and characterise novel agonistic mouse antibodies against mouse LAIR-1 with characteristics superior to the commercially available hamster anti-mouse LAIR-1 antibody clone 113, and to use the novel antibodies in our preclinical RSV infection model in mice.

Materials and Methods

Antibody generation

Mouse anti-mouse LAIR-1 antibody-producing hybridomas were generated by immunizing *Lair1*$^{-/-}$ mice with a LAIR-1 expressing cell line in collaboration with the Utrecht Monoclonal Antibody Facility (UMAB) according to previously published protocols.[9] Mouse-anti mouse LAIR-1 antibodies were generated by immunizing *Lair1*$^{-/-}$ mice with mLAIR-1 expressing cells. After fusion of mouse spleen cells with sp2 cells, 18 hybridomas tested positive for LAIR-1 staining. From these clones, 10 clones were subcloned, resulting in 20 surviving subclones, derived from 6 individual hybridomas. These clones were tested for agonistic activity using our mLAIR-1 reporter system.[10] This functional screen initially yielded four independent agonist-producing hybridomas, two of which stopped producing antibody.

Cells

2B4 NFAT-GFP reporter cells expressing a mLAIR-1-CD3ζ chimera, which produce GFP upon LAIR-1 ligation, were used as described previously.[10] Receptor ligation induced by agonistic anti-mLAIR-1 monoclonal antibodies (mAbs) that are cross-linked with secondary anti-mouse Ig antibodies results in the expression of GFP by reporter cells that express the mLAIR-1-CD3ζ chimera, but not by the parental reporter cells.

Flow cytometry

Mouse bone marrow and spleens were passed through 100 µm cell strainers (Corning) to generate single-cell suspensions. Erythrocytes were depleted using ammonium chloride buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). Single-cell suspensions were incubated with viability dye followed by

fluorochrome-conjugated antibodies, or non-conjugated primary antibodies and secondary fluorochrome-conjugated specific anti-Ig antibodies sequentially and analysed on a LSR Fortessa (BD Biosciences). Acquisitions were analysed using FlowJo software (version 10.0.7, Treestar). To determine absolute cell counts, Trucount Absolute Counting Tubes (Becton Dickinson Biosciences) were used according to the manufacturer's instructions.

Animals

Mice of the BALB/c background were bred under specific pathogen–free conditions at the animal facility of Utrecht University. All animal studies were approved by the Institutional Animal Care and Use Committee and carried out in accordance with national and institutional guidelines.

Mouse RSV infection

Eight to twelve-week-old female BALB/c mice were intranasally inoculated with 1 × 10^6, 2 x 10^6, or 10 x 10^6 PFU of RSV-A2 in 50 µl of PBS, or PBS only. RSV-A2 preparation, quantitative assay for RSV-A2 titration and RSV-A2 infection of mice, including, intranasal inoculation, termination, and sample collection and analysis, was performed as described previously.[7] One day prior to inoculation (day -1), mice were injected intraperitoneally with 200 µg of 2C7 anti-mouse LAIR-1 antibody, isotype-matched control antibody, or PBS only. Mice were sacrificed on day 5 post-infection.

Results and Discussion

Novel mouse anti-mouse LAIR-1 antibody-producing hybridomas

After generating a pool of antibody-producing hybridomas, we selected two clones, designated 2C7 and 5F8, based on their agonistic potential in our initial reporter assay screen for further characterisation. These were compared to the previously described anti-mouse LAIR-1 antibody clone 113, which is of hamster origin.[11] Cells transfected with mouse LAIR-1 were incubated with either one of the newly generated antibodies or mAb 113, followed by fluorochrome-conjugated mouse- or hamster-specific secondary antibodies, and analysed by flow cytometry. Clear signal was obtained when compared isotype-matched control antibodies (Fig 1A). No signal was observed on non-transfected cells (data not shown). Genetic sequencing was used to determine that 2C7 and 5F8 are of the IgG2a and IgG1 isotypes, respectively (data not shown).

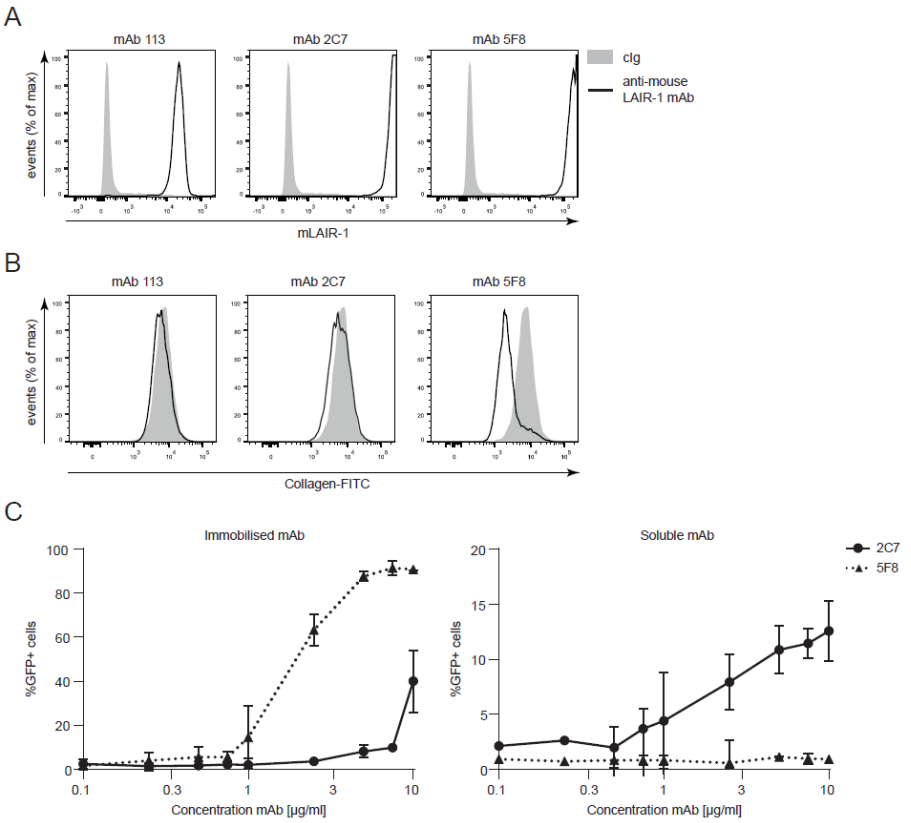

Figure 1. Characterisation of two novel agonistic anti-mouse LAIR-1 antibodies.
(A) Two novel mouse anti-mouse (m)LAIR-1 monoclonal antibodies (mAb 2C7, 5F8) were tested for LAIR-1 binding on mLAIR-1 transfected cells and compared to the commercially available clone 113, a hamster-derived antibody, using flow cytometry. LAIR-1 expression is shown in black and isotype controls are shown in grey. (B) Collagen-FITC binding to mLAIR-1-transfected cells was assessed in the presence of anti-LAIR-1 antibodies or isotype controls (in grey) using flow cytometry. (C) Agonistic activity of the newly generated mAb 2C7, 4E4 and 5F8 was examined by measuring GFP expression by mLAIR-1 reporter cells with flow cytometry after incubation with either immobilised or soluble mAb present. The percentage of GFP-positive cells at varying concentrations mAb (2C7, solid; 5F8, dotted) is plotted.

The newly generated mouse monoclonal antibodies against mouse LAIR-1 demonstrate agonistic activity in soluble and immobilized state

To test whether the novel antibodies interfered with the collagen-binding site on LAIR-1, we examined whether the antibodies were able to block binding of FITC-labelled collagen I or III (collagen-FITC) to mouse LAIR-1-transfected

cells. The 5F8 clone blocked the binding of collagen to mouse LAIR-1 expressing cells, but clone 2C7 and 113 did not (Fig 1B).

Subsequently, we titrated the agonistic activity of the novel antibody clones in soluble and immobilised (to a plastic cell-culture plate) state using our LAIR-1 reporter cell system (Figure 1C). The 2C7 clone demonstrated agonistic activity in both soluble and immobilized state. The 5F8 clone, however, had no agonistic activity when presented as a soluble antibody. The agonistic activity of both antibody clones was higher when immobilised. Plate-bound 5F8 antibodies induced more receptor signalling than 2C7 antibodies at lower concentrations. Immobilised antibodies may induce receptor clustering at a higher rate than soluble antibodies, since it recruits receptors to the cell-plate interface, resulting in an accumulation of receptors. Soluble antibodies would induce smaller clusters of receptors distributed over the entirety of the cell membrane, with one antibody molecule only being able to link two receptor molecules.

The endogenous activating ligands of inhibitory receptors nearly exclusively contain multiple moieties that can be bound to induce clustering, more akin to the immobilised antibody situation. In the case of LAIR-1, one collagen fibril can be bound by a multitude of LAIR-1 molecules. Nevertheless, when administered in vivo, the antibodies are present in a soluble state or cross-linked by Fc receptors. We therefore determined that the 2C7 clone was the most promising LAIR-1 agonist for in vivo application. This is further strengthened by the fact that the 2C7 mAb does not block the interaction with collagen, which could prohibit interaction with endogenous LAIR-1 ligands in vivo. The 5F8 clone, however, with its ability to block LAIR-1 binding to collagen and a complete lack of agonistic potential even at high soluble antibody concentrations, may represent a useful antagonist for future studies into LAIR-1 biology. To this end, the Fc tail of 5F8 would need to be mutated to prevent Fc receptor-mediated effects. Table 1 summarises the characteristics of the anti-mouse LAIR-1 antibodies.

Intraperitoneal administration of the newly generated anti-mouse LAIR-1 antibodies does not induce substantial leukocyte depletion

To ensure that any effects observed by the administration of the novel agonistic anti-mouse LAIR-1 can be ascribed to agonistic function, the potential depletion of leukocytes by these mAbs was assessed 3 and 6 days after intraperitoneal injection (Fig 2). On day 3 after treatment with 200 µg of either the 2C7 or 5F8 antibody clone, the cell counts of all examined blood leukocyte populations were comparable to PBS-treated mice. On day 6, decreased leukocyte cell counts were found in the blood of the 5F8-treated

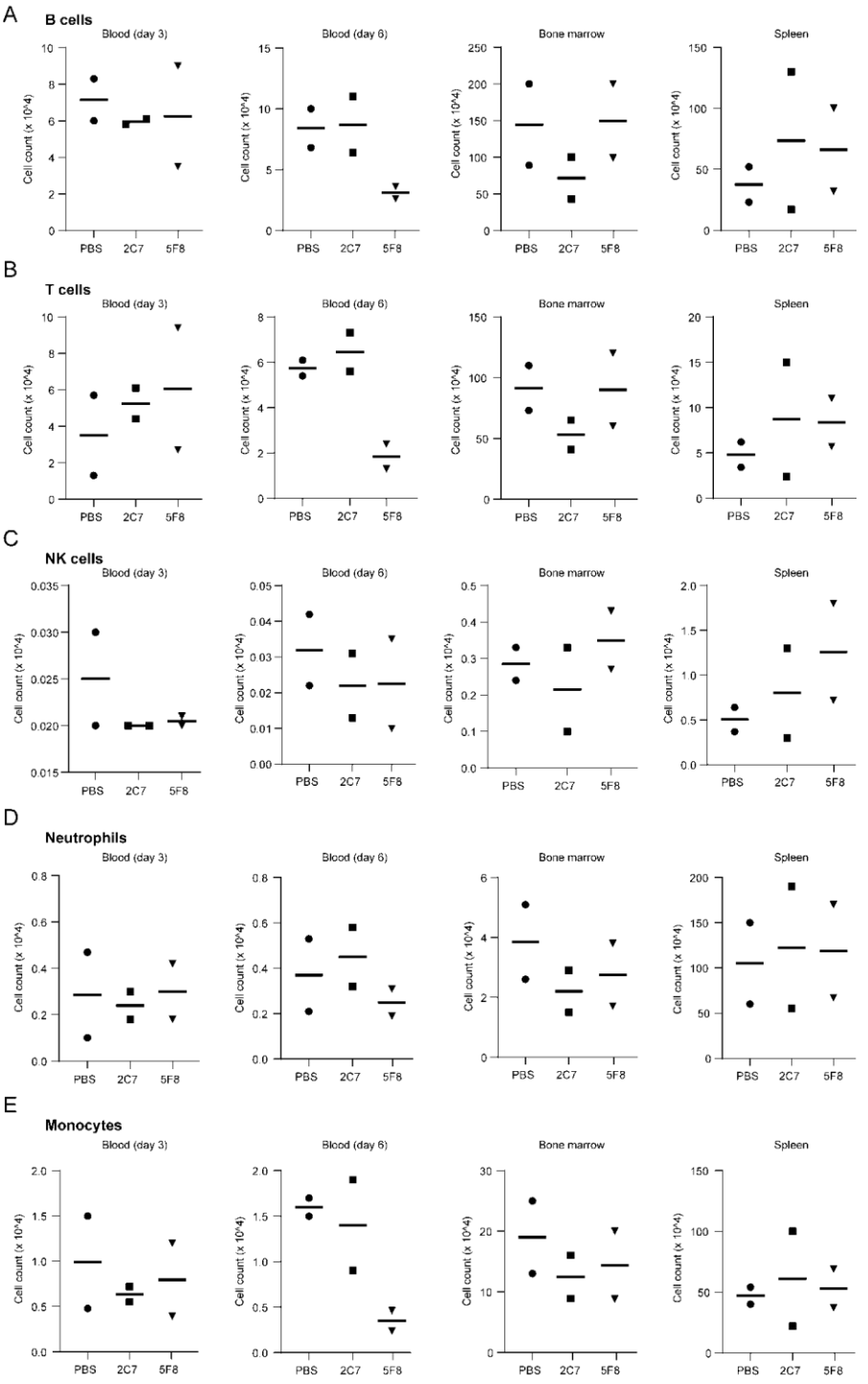
A
B cells
Blood (day 3)
Blood (day 6)
Bone marrow
Spleen
B
T cells
C
NK cells
D
Neutrophils
E
Monocytes
Cell count (x 10^4)
PBS
2C7
5F8

Figure 2. Antibody-induced immune cell depletion.
Wild-type C57Bl/6 mice (n=2, per treatment) were injected with one of the anti-mLAIR-1 antibody clones 2C7 or 5F8 to determine whether immune cell depletion was induced. Antibodies, or PBS as control, were injected intraperitoneally. Blood was drawn on day 3 and 6 after antibody injection; spleens and bone marrow from hind legs were harvested on day 6. After incubation with fluorochrome-conjugated antibodies, single-cell suspensions of blood and tissues were analysed by flow cytometry to determine cell subsets: (A) B cells (B220+ CD3-), (B) T cells (B220- CD3+), (C) natural killer cells (B220- CD3- Ly6-G- CD11b- CD49b+), (D) neutrophils (B220- CD3- Ly6-G+ CD11b+), and (E) monocytes (B220- CD3- Ly6-G- CD11b+). The Trucount Absolute Counting Tubes (Becton Dickinson Biosciences) were used to determine the cell counts of these subsets.

mice, while spleen cell numbers were comparable to PBS-treated mice. The blood and spleen cell numbers of mice treated with the 2C7 antibody clone are similar at both time points for all cell populations, except for NK cells, and are comparable to PBS-treated mice.

Moderately decreased (compared to PBS-treated mice) cell numbers were observed in the bone marrow of 2C7-treated mice, but not in 5F8-treated mice. The range of bone marrow cell numbers of 2C7-treated mice overlapped with the range observed for PBS-, 4E4, and 5F8-treated mice, suggesting that the cell number reduction is only moderate. We selected 2C7 for continued testing because of the overall lack of noteworthy immune cell depletion and its advantageous characteristics as determined in vitro (Table 1).

Table 1. Novel anti-mouse LAIR-1 antibodies.
The experimentally determined characteristics of the two newly generated anti-mouse LAIR-1 monoclonal antibodies are summarized below. (++ versus + represents a higher relative potency).

Clone	Isotype	LAIR-1 agonist soluble	LAIR-1 agonist immobilized	Collagen block	Immune cell depletion
113	Hamster IgG1	-	+	-	not tested
2C7	Mouse IgG2b	++	+	-	-
5F8	Mouse IgG1	-	++	+	+

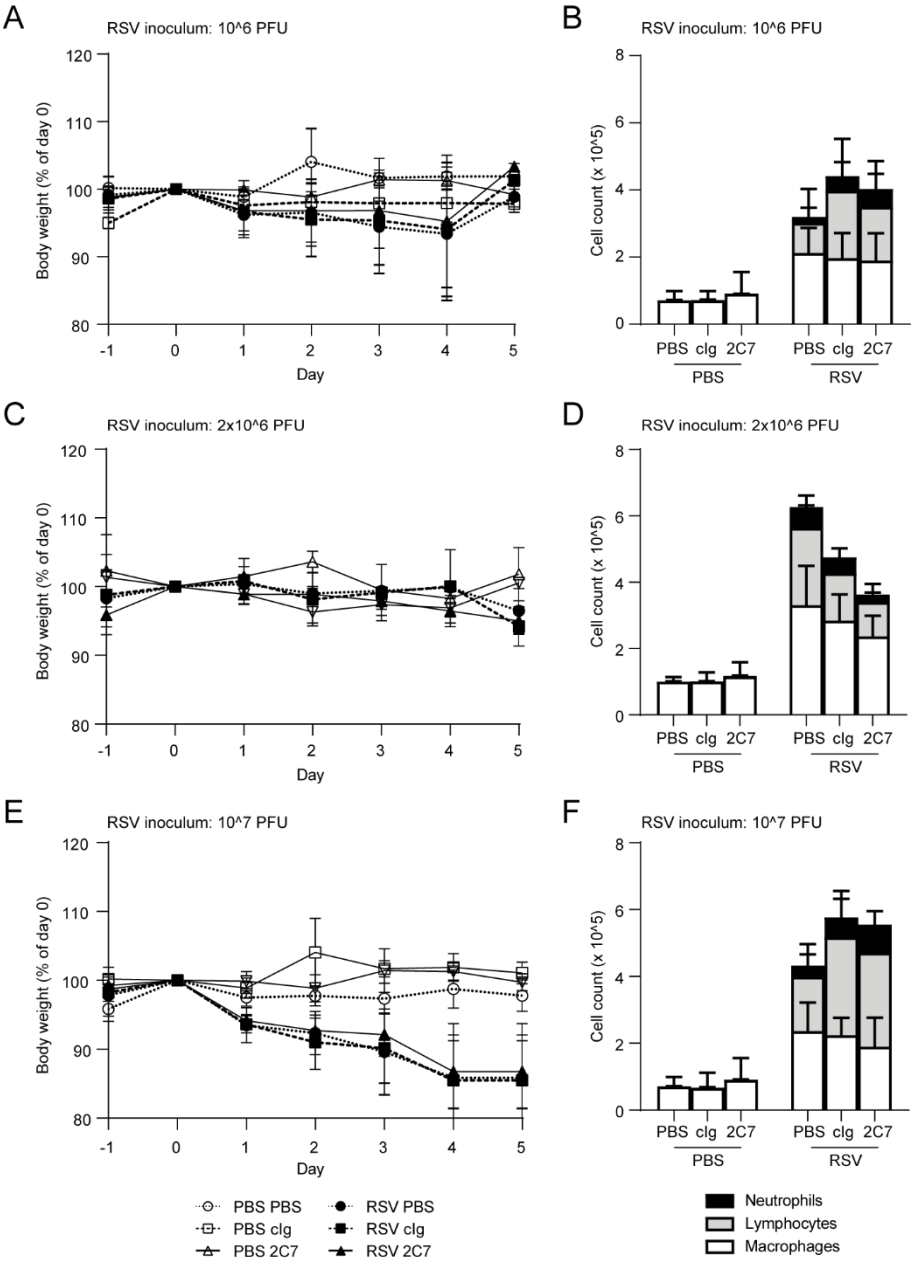

Figure 3. Administration of agonistic anti-mLAIR-1 antibody 2C7 does not affect experimental RSV infection in mice.

Mice were intraperitoneally injected with 200 µg of 2C7, isotype-matched control antibody, or PBS only on day -1, intranasally inoculated with 1 x 10^6 PFU RSV-A2 (A-

B) (n=8, per treatment group), 2 x 10^6 PFU RSV-A2 (C-D) (n=8, per treatment group), 1 x 10^7 PFU RSV-A2 (E-F) (n=8, per treatment group), or PBS only (A-F) (n=4, per treatment group) on day 0 and sacrificed on day 5. Mice were weighed each day; weight is expressed as percentage body weight relative to day 0 (A, C, E). Total and differential cell counts were determined in bronchioalveolar lavage; differential cell counts are shown stacked (white, macrophage; grey, lymphocytes; black, neutrophils) (B, D, F).

Agonistic LAIR-1 antibody does not decrease disease severity or leukocyte airway infiltration induced by RSV infection

We administered 2C7 in the context of RSV infection with the goal of suppressing neutrophil responses and ameliorating disease. One day prior to infection (day -1), mice were injected intraperitoneally with 200 µg of 2C7, 200 µg of isotype-matched control antibody, or PBS only. Mice were infected with 3 different doses of RSV inoculum (on day 0), namely 1 x 10^6 (Fig 3A-B), 2 x 10^6 (Fig 3C-D), or 10 x 10^6 PFU (Fig 3E-F), or inoculated with PBS, and sacrificed 5 days post infection (day 5). As expected, the higher RSV doses induced greater cellular influx into the airways, and only the highest dose (10 x 10^6 PFU, Fig 3E-F) resulted in weight loss. However, there was no clear effect of 2C7 administration on total or differential cell counts, nor on weight loss. With an inoculum of 2 x 10^6 PFU, a trend could be observed towards lower cellular infiltrate when mice are treated with 2C7, but this did not reach statistical significance.

Therefore, in this mouse model of RSV infection and at this concentration of antibody, we were unable to demonstrate a therapeutic effect of targeted LAIR-1 ligation. In part, this may be due to the limitations of the mouse model. Compared to RSV bronchiolitis in patients, neutrophil numbers relative to macrophages and lymphocytes are low in RSV-inoculated mice. Indeed, others have shown that antibody-mediated neutrophil depletion or enhanced neutrophil recruitment by administration of the neutrophil chemoattractant CXCL-1 during RSV infection does not affect disease severity or viral load in mice.[12]

The concentration of 2C7 antibodies necessary to evoke a response in vivo is unknown and therefore the administered dose of antibody may have been insufficient. Rather than a single dose of antibody prior to infection, multiple doses before and during infection may also promote a stronger response. Moreover, we did not assess the local concentration of 2C7 antibody in the airways, which may have been too low to optimally ligate LAIR-1 at the site of infection. Local administration of agonistic anti-LAIR-1

antibody, e.g., by nasal inoculation, may prove to be more effective in future studies.

Agonists for LAIR-1 ligation

A mono-specific anti-LAIR-1 antibody, such as 2C7, may not be the most efficacious agonist. Clustering of LAIR-1 induces signalling,[8] and a mono-specific antibody can only link one pair of receptors. Hence the lower absolute response of soluble antibody in our LAIR-1 activity reporter assay compared to immobilised antibody (Fig 1C). The latter allows larger signalling clusters to form at the interface with the antibody-coated plastic. Inducing further clustering by utilising a secondary antibody to link the individual antibody-LAIR-1 pairs enhances reporter activity (data not shown). This approach is unfortunately not applicable in the in vivo situation, since it would result in inflammatory antibody complexes. A secondary anti-mouse antibody would not only target our 2C7 antibody, but all endogenous antibodies as well. Alternatively, antibodies could be covalently linked to allow for more binding sites; perhaps modelled after the secretory IgA dimer.

The biology of inhibitory receptors provides challenges to the development of an effective agonist. An ITIM-bearing inhibitory immune receptor does not act independently to signal an overall lower activation state or responsiveness of the entire cell, rather it directly modulates the signal of an adjacent activating receptor. In particular, the cytosolic SHP-1 and SHP-2 phosphates are recruited to the cytoplasmic tail of an inhibitory receptor which dephosphorylate the activating receptor to reduce or abrogate its signal. Therefore, not only must the agonist orchestrate sufficiently large clusters of inhibitory receptors to induce adequate signalling, it must also allow for proximity to the activating receptor. For instance, if the size of the agonist bound to the inhibitory receptor sterically hinders the proximity (at the nanometre scale) to an activating receptor, which may be located in an size-exclusive immunological synapse, the potency of the agonist will be greatly reduced.[12] One avenue currently being pursued is the development of bispecific antibodies that directly link inhibitory and activating receptors together.[13]

Nonetheless, the newly generated agonistic 2C7 antibody may prove useful in subsequent studies. RSV bronchiolitis is hardly the only condition in which LAIR-1-expressing neutrophils play a deleterious role, others include sterile inflammations such as ischemia reperfusion injury and systemic lupus erythematosus.[14,15] Further studies using 2C7 may provide proof of concept for LAIR-1 ligation as a therapeutic target.

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Chapter

6

Differential isoform expression of Allergin-1 during acute and chronic inflammation

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Abstract

Introduction
Neutrophils are crucial to antimicrobial defence, but excessive neutrophilic inflammation elicits immune pathology. Currently, no effective treatment exists to curb neutrophil activation. However, neutrophils express a variety of inhibitory receptors which may represent potential therapeutic targets to limit neutrophilic inflammation. Indeed, we previously showed that the inhibitory collagen receptor leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) regulates neutrophilic airway inflammation and inhibits neutrophil extracellular trap formation. The inhibitory receptor Allergin-1 is expressed by myeloid cells and B cells. Allergin-1 supresses mast cell and basophil activation, but a potential regulatory role on neutrophils remains unexplored. We aimed to demonstrate regulation of neutrophils by Allergin-1.

Methods
We examine Allergin-1 isoform expression on human neutrophils during homeostatic (healthy donors) and chronic inflammatory (systemic lupus erythematosus patients) conditions in comparison to other circulating leukocytes by flow cytometry**.** To reveal a potential role for Allergin-1 in regulating neutrophilic inflammation, we experimentally infect wild-type and Allergin-1-deficient mice with respiratory syncytial virus (RSV) and monitor disease severity and examine cellular airway infiltrate. Flow cytometry was used to confirm Allergin-1 expression by airway-infiltrated neutrophils in RSV infection-induced bronchiolitis patients.

Results
Only the short 1 (S1) isoform, but not the long (L) or S2 isoform could be detected on blood leukocytes, with the exception of non-classical monocytes, which exclusively express the S2 isoform. Allergin-1 expression levels did not vary significantly between healthy individuals and patients with systemic inflammatory disease on any interrogated cell type. Airway-infiltrated neutrophils of paediatric RSV bronchiolitis patients were found to express Allergin-1S1. However, Allergin-1-deficient mice experimentally infected with RSV did not show exacerbated disease or increased neutrophil airway infiltration compared to wild-type littermates.

Conclusion
Allergin-1 isoform expression is unaffected by chronic inflammatory conditions. In stark contrast to fellow inhibitory receptor LAIR-1, Allergin-1 does not regulate neutrophilic inflammation in a mouse model of RSV bronchiolitis.

Introduction

Neutrophilic granulocytes are vital to antimicrobial defence and other immune functions. However, neutrophil effector mechanisms, such as reactive oxygen species (ROS) production, release of proteolytic enzymes, and neutrophil extracellular trap (NET) formation, damage host tissues as well as pathogens.[1, 2] Excessive neutrophilic inflammation thereby induces immune pathology in conditions as diverse as respiratory syncytial virus (RSV) infection-induced bronchiolitis and ischemia-reperfusion injury.[3] By supressing neutrophil activation, immune pathology may be prevented in a myriad of conditions. However, no effective treatment is currently available to limit neutrophilic inflammation.

Neutrophils express a multitude of inhibitory receptors that negatively regulate their activation status, by providing an activation threshold and/or counteracting activating signals.[4, 5] The efficacy and potency of targeting inhibitory receptors for therapy has been demonstrated by checkpoint blockade immunotherapy in cancer.[6] Possibly, activating inhibitory receptors to suppress excessive inflammation, rather than blocking inhibitory receptor to strengthen the anti-tumour response, may provide new avenues of treatment for neutrophil-driven immune pathology.

Allergy inhibitory receptor 1 (Allergin-1) is an ITIM-bearing immunoglobulin (Ig)-like receptor that is primarily expressed on B cells (in humans) and myeloid cells (in humans and mice), including all types of granulocytes.[7] The inhibitory function of Allergin-1 on mast cells and basophilic granulocytes and a regulatory role in allergic diseases has been demonstrated.[8-12] A potential regulatory function of Allergin-1 on neutrophilic granulocytes and monocytes has received less scrutiny, however. Since neutrophils highly express Allergin-1 and are regulated by other ITIM-bearing inhibitory receptors, such as leukocyte-associated Ig-like receptor 1 (LAIR-1) and signal inhibitory receptor on leukocytes 1 (SIRL-1), an inhibitory function of Allergin-1 on neutrophils seems plausible.[13, 14] Additionally, Allergin-1 promotes the clearance of apoptotic debris by enhancing phagocytosis by macrophages, which suppresses auto-antibody production.[15]

Human Allergin-1 has three isoforms, namely Allergin-1S1 (short form), -S2, and -L (long form), defined by the inclusion or exclusion of its two extracellular Ig domains. The S1 and S2 isoforms each contain one of the two Ig domains, whereas the L isoform contains both. Most attention has gone to

the Allergin-1S1 and/or Allergin-1L isoforms, the former of which is homologous to the mouse Allergin-1.[7]

In the current study, we investigate possible changes in Allergin-1 isoform expression due to chronic systemic inflammation as well as regulation by Allergin-1 of acute neutrophilic inflammation. In particular, we examine neutrophilic airway inflammation, since Allergin-1 on mast cells has been reported to supress allergic airway inflammation. We compare the isoform expression of Allergin-1 on various leukocyte subsets in blood during homeostatic conditions and in chronic systemic inflammation, i.e., in healthy donors and systemic lupus erythematosus (SLE) patients. To examine a potential regulatory role for Allergin-1 in acute neutrophilic airway inflammation, we employ an experimental mouse model of RSV-induced bronchiolitis.

Materials and Methods

Flow cytometry

The red blood cells in heparinized venous blood samples obtained from healthy donors and SLE patients were lysed with an ammonium chloride buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). Erythrocytes in blood samples of paediatric RSV patients were lysed by treatment with hypotonic distilled water. Aspirated sputum samples of intubated paediatric RSV patients were resuspended in phosphate-buffered saline solution (PBS) and passed through 100 µm-pore filters twice to obtain a single-cell suspension. Erythrocyte-depleted single-cell suspensions were incubated with viability dye followed by fluorochrome-conjugated antibodies and analysed on a LSR Fortessa (BD Biosciences). Acquisitions were analysed using FlowJo software (version 10.0.7, Treestar). See Supplementary Figure 1 for the employed gating strategy and Supplementary Table 1 for an overview of the antibodies used in this study. For an overview of the origin of samples, in which experiments they were used, and in which Figures their data is presented, see supplementary Figure 2.

Animals

Mirl1$^{-/-}$ mice were generated on the BALB/c background by Hitomi *et al.* as described.[7] *Mirl1*$^{-/-}$ mice and their wild-type littermates were bred under specific pathogen–free conditions at the animal facility of Utrecht University. All animal studies were approved by the Institutional Animal Care and Use

Committee and carried out in accordance with national and institutional guidelines.

Mouse RSV infection

Eight to twelve-week-old female BALB/c *Mirl1*$^{-/-}$ mice or their wild-type (WT) littermates were intranasally inoculated with 1×10^7 PFU of RSV-A2 in 50 µl of PBS. RSV-A2 preparation, quantitative assay for RSV-A2 titration and RSV-A2 infection of mice, including, intranasal inoculation, termination, and sample collection, was performed as described previously. Mice were sacrificed on day 2 or 5 post-infection.[14] For the quantification and differentiation of airway-infiltrated cells whole-lung broncheoalveolar lavage (BAL) was performed using 1.0 mL of PBS. Cells obtained by BAL were quantified using a haemocytometer and differentiated based on morphology following Giemsa staining.

Healthy donors & patients

Peripheral venous blood was obtained from healthy adult volunteers (n=8), SLE patients (n=13), and paediatric RSV bronchiolitis patients (n=3). Disease characteristics of SLE patients can be viewed in Supplementary Table 2. Aspirated sputum from intubated, mechanically ventilated RSV bronchiolitis patients was obtained by instillation and re-aspiration of physiological saline in the endotracheal tube. The infants were aged less than one year and had PCR-proven RSV bronchiolitis, but were otherwise healthy with no detectable co-infections. All subjects or their caretakers gave written informed consent and protocols were approved by the institutional review board.[13]

Statistical analyses

To determine the statistical significance of differences in Allergin-1 isoform expression between healthy donors and SLE patients, the unpaired Mann-Whitney U test was used. The Kruskal-Wallis test was employed to determine whether there were statistically significant differences between the four groups of mice (WT and *Mirl1*$^{-/-}$ with PBS or RSV) in the RSV infection model.

Results and discussion

Differential expression of Allergin-1S1 and -S2 isoforms on healthy blood leukocytes

We determined the expression of the S1 and S2 isoforms of Allergin-1 on the blood leukocytes of healthy volunteers by flow cytometry. Numerous

leukocyte subsets were interrogated, namely: neutrophils and basophils (Fig 1A); naïve B cells and plasmablasts (Fig 1B); both CD4+ and CD8+ naïve, central memory, effector memory, and terminal effector T cells, natural killer (NK) cells, and NKT cells (Fig 1C-E); and classical, intermediate, and non-classical monocytes, myeloid DCs (mDCs), and plasmoid DCs (pDCs) (Fig 1F-G). For gating strategies see supplementary figure 1.

Allergin-1S1 was highly expressed on blood neutrophils and basophils, whereas Allergin-1S2 was expressed at low or undetectable levels (Fig 1A). Similar results were obtained for B cells, with notable Allergin-1S1 expression on naïve B cells and plasmablasts, but minor or non-existent Allergin-1S2 expression. Differentiation of naïve B cells into plasmablasts did not appear to affect the expression of Allergin-1 isoforms (Fig 1B). None of the assayed CD4+ and CD8+ T lymphocyte sub-populations expressed either Allergin-1S1 or Allergin-1S2 (Fig 1C-D). Among innate lymphocytes, however, there appeared to be a sub-population of NK cells that expressed Allergin-1S2 (Fig 1E), this population was not defined by high CD56 expression (data not shown). NKT(-like) cells did not express Allergin-1 (Fig 1E). The expression of Allergin-1 on both pDCs and mDCs was restricted to the S1 isoform (Fig 1G).

We detected expression of Allergin-1S1, but not Allergin-1S2, on classical and intermediate monocytes (Fig 1F). In contrast, Allergin-1S2, but not Allergin-1S1, was detected on non-classical monocytes. Differentiation of classical monocytes into non-classical monocytes coincided with the loss of Allergin-1S1 and the upregulation of Allergin-1S2. On the whole, the expression of the S1 and S2 isoforms appeared to be largely mutually exclusive on all cell types. In fact, Allergin-1S2 could only be conclusively detected on non-classical monocytes. Since surface expression of the L isoform would result in simultaneous detection of both the S1 and S2 Ig domains, this rules out the *in vivo* expression of the L isoform on blood leukocyte during homeostasis. In contrast, the initial identification of Allergin-1 was made by the detection of the cDNA for Allergin-1L in a cDNA library derived from the human bone marrow stromal cell line HAS303.[7] Possibly, Allergin-1L is not expressed by blood leukocytes, but is expressed by cells in the bone marrow or other tissues.

Interestingly, classical monocytes expressed Allergin-1S1 but lost this expression when they differentiated into non-classical monocytes, which in turn started to express Allergin-1S2. While the ligand for Allergin-1 is currently not known, an isoform shift may also impact ligand interaction or specificity. The biological relevance of this change in Allergin-1 isoform expression would be an interesting subject for further inquiry.

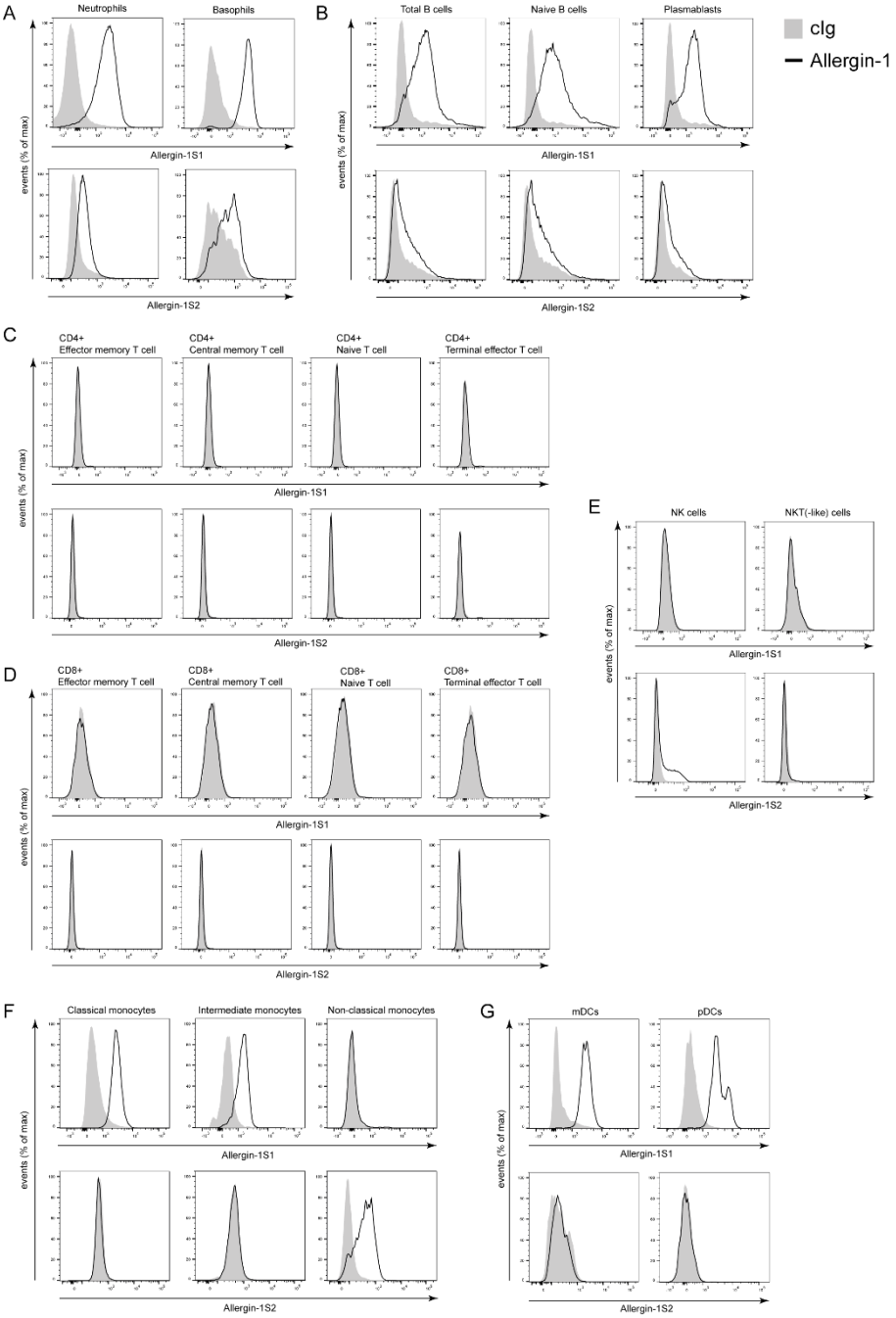

Figure 1. Differential expression of Allergin-1 isoforms on blood leukocytes of healthy donors

Blood leukocytes obtained from healthy donors (n=8) were stained for cell lineage markers and Allergin-1S1 and Allergin-1S2 isoforms. All leukocyte populations were gated based on forward- and side-light scatter. Doublets and non-vital cells were excluded from analysis. Black lines indicate Allergin-1 expression and grey plots represent isotype-matched control Ig (cIg); representative plots are shown. Leukocyte subsets were identified as follows: (A) neutrophils, CD14- CD16+, basophils CD14- CD16- CD117- FcεRI+; (B) total B cells, CD19+, naïve B cells, CD19+ CD27- IgD+, plasmablasts, CD19+ CD27+ IgD-; (C) CD4+ effector memory T cells, CD3+ CD4+ CD27- CD45R0+, CD4+ central memory T cells, CD3+ CD4+ CD27+ CD45R0+, CD4+ naïve T cells, CD3+ CD4+ CD27+ CD45R0-, CD4+ terminal effector T cells, CD3+ CD4+ CD27- CD45R0-; (D) CD8+ effector memory T cells, CD3+ CD8+ CD27- CD45R0+, CD8+ central memory T cells, CD3+ CD8+ CD27+ CD45R0+, CD8+ naïve T cells, CD3+ CD8+ CD27+ CD45R0-, CD8+ terminal effector T cells, CD3+ CD8+ CD27- CD45R0-; (E) natural killer (NK) cells, CD3- CD56+, NKT-like cells, CD3+ CD56+; (F) classical monocytes, CD3- CD19- CD56- HLA-DR+ CD14++ CD16-, intermediate monocytes, CD3- CD19- CD56- HLA-DR+ CD14+ CD16+, non-classical monocytes, CD3- CD19- CD56- HLA-DR+ CD14- CD16++; (G) myeloid DCs (mDCs), CD3- CD19- CD56- HLA-DR+ CD11c+ BDCA1+, plasmacytoid DCs (pDCs), CD3- CD19- CD56- HLA-DR+ CD11c- BDCA2+. See supplementary figure 1 for the employed gating strategy.

Moreover, while neutrophils represent a homogeneous population where Allerin-1 expression is concerned (Fig 1A), other cell types, including plasmablasts, pDCs, basophils, and NK cells (Fig 1B, E, G), comprise more heterogeneous populations. Possibly, this represents subpopulations within these cell types or differences in activation state that our current flow cytometry panel was unable to distinguish.

Expression of Allergin-1 on blood leukocytes of SLE patients

Next, we assessed whether an *in vivo* inflammatory milieu would affect Allergin-1 isoform expression. To this end, we investigated blood samples of patients with a systemic inflammatory disease, namely systemic lupus erythematosus (SLE). Neutrophils and B cells expressed Allergin-1S1 (Fig 1A-B), and both are suggested to be intimately involved in the pathogenesis of SLE.[16, 17] Moreover, by promoting the phagocytosis of apoptotic debris by macrophages, Allergin-1 supresses the production of auto-antibodies, e.g., against double-stranded DNA, which are characteristic of SLE.[15]

Although there was a tendency for higher Allergin-1S1 expression on, in particular, monocytes, mDCs and pDCs from SLE patients, no statistically significant differences in Allergin-1 isoform expression were observed between healthy donors and SLE patients (Fig 2). However, the heterogeneity of the SLE patient population may have obscured the fact that some

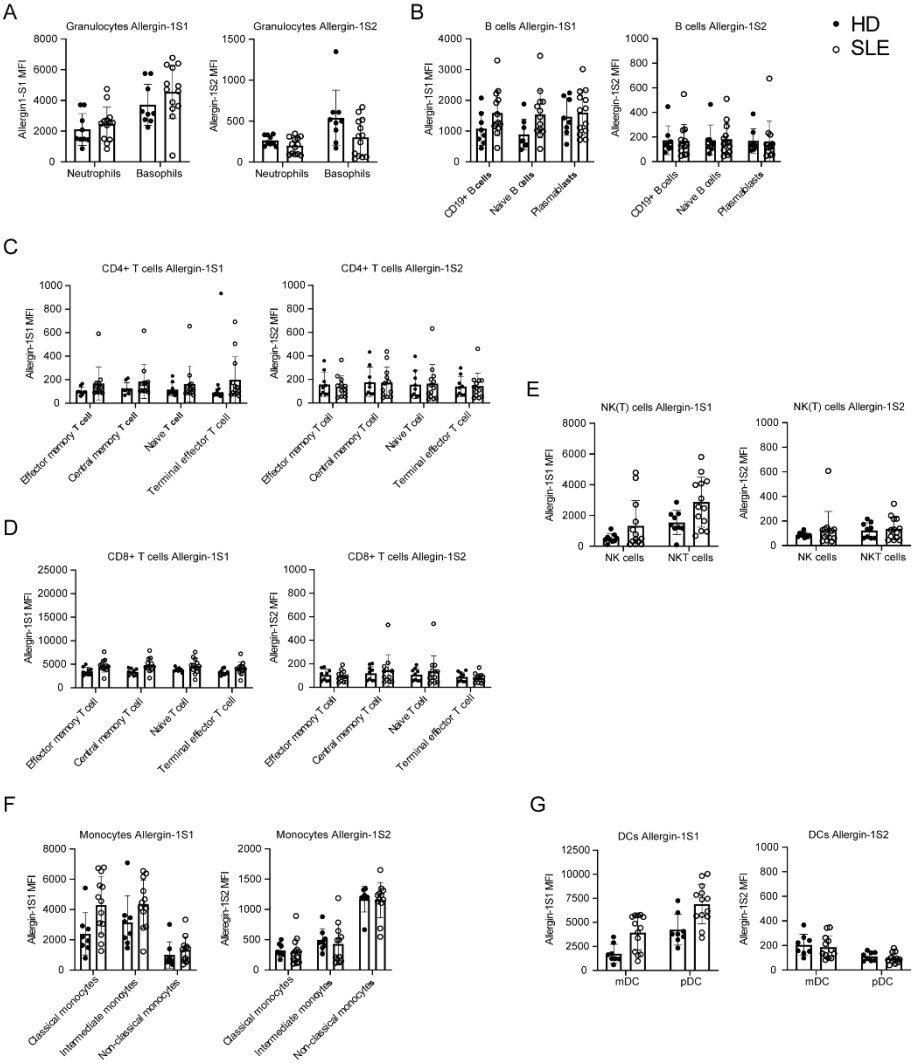

Figure 2. Allergin-1 isoform expression on blood leukocytes of healthy donors and SLE patients.

Blood leukocytes obtained from healthy donors (n=8) and SLE patients (n=13) were stained for cell lineage markers and Allergin-1S1 and Allergin-1S2 isoforms. All leukocyte populations were gated based on forward- and side-light scatter. Doublets and non-vital cells were excluded from analysis. Cell populations were identified as previously described; see also supplementary figure 1. Median fluorescence intensity (MFI) of the Allergin-1S1 and -S2 isoforms on the following cell populations are shown: (A) neutrophils and basophils; (B) total B cells, naïve B cells, and plasmablasts; (C) CD4+ effector memory T cells, central memory T cells, naïve T cells, and terminal effector T cells; (D) CD8+ effector memory T cells, central memory T cells, naïve T

cells, and terminal effector T cells; (E) NK cells and NKT(-like) cells; (F) classical monocytes, intermediate monocytes, and non-classical monocytes; and (G) myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). There were no statistically significant differences ($p < 0.05$) as determined by Mann-Whitney U test.

individual patients expressed notably higher levels of Allergin-1S1 (Fig 2A, G). Expression levels of Allergin-1S2 were wholly comparable between the two populations (Fig 2). Additionally, while the absolute cell counts of SLE patients' blood leukocytes and their relative cell type percentages may differ from healthy controls, e.g., due to neutropenia, the percentage of Allergin-1 isoform expressing cells among these cell types remained unchanged.

Similar to healthy donors, classical and intermediate monocytes of SLE patients expressed Allergin-1S1 but did not express Allergin-1S2, whereas non-classical monocytes only expressed the Allergin-1S2 isoform. Therefore, the L isoform was also not detected under systemic inflammatory conditions. Whether the Allergin-1 expression in tissues is impacted remains to be determined.

Allergin-1 does not limit RSV disease severity in mice

An inhibitory function of Allergin-1 on two types of granulocytes, namely mast cells and basophils, has previously been reported for allergic conditions, including anaphylaxis and allergic airway inflammation.[10-12] To investigate a possible regulatory function of Allergin-1 on neutrophilic granulocytes, we examined respiratory syncytial virus (RSV) infection, an infectious disease with prominent neutrophilic airway inflammation. We previously reported that inhibitory receptor expression patterns differ between blood and airway-infiltrated neutrophils.[13, 14] Therefore, we initially determined whether Allergin-1 is expressed on the blood and airway-infiltrated neutrophils of intubated paediatric RSV bronchiolitis patients by flow cytometry. Allergin-1S1, but not -S2, was expressed by both blood and airway-infiltrated neutrophils (Fig 3). Of note, we only enlisted 3 patients at a single time point for this part of the study (see Supplementary Figure 3 for the additional patients). We can therefore not exclude that Allergin-1 expression may vary with disease severity throughout the course of disease.

To determine a possible limiting role of Allergin-1 in neutrophilic airway infiltration, we inoculated wild-type (WT) and Allergin-1-deficient (*Milr1*$^{-/-}$, KO) BALB/c mice with RSV, or PBS as control, and followed weight loss as a marker of disease severity over time. Additionally, we assessed leukocyte airway infiltration by terminal bronchioalveolar lavage (BAL) at 2- and 5-days post infection (Fig 3B-D). RSV-infected mice lost weight compared

to control PBS-inoculated mice, but no differences were observed between WT and *Milr1*$^{-/-}$ mice (Fig 4A). Experimental RSV infection resulted in leukocyte airway infiltration on both day 2 and day 5 post infection. However, total cell counts, neutrophil counts, macrophage counts, and lymphocyte counts did not differ between WT and *Milr1*$^{-/-}$ mice (Fig 4B-C). Thus, Allergin-1 did not limit disease severity or leukocyte airway infiltration in this experimental mouse model of RSV infection. However, we did not assess a role for Allergin-1 in limiting viral replication by measuring viral loads in the lungs of the mice; this represents a limitation of our study. Unfortunately, a potential role for the Allergin-1 isotype switch from S1 to S2 observed in human classical versus non-classical monocytes could not be examined in our mouse model, since mice only express the S1 isoform.

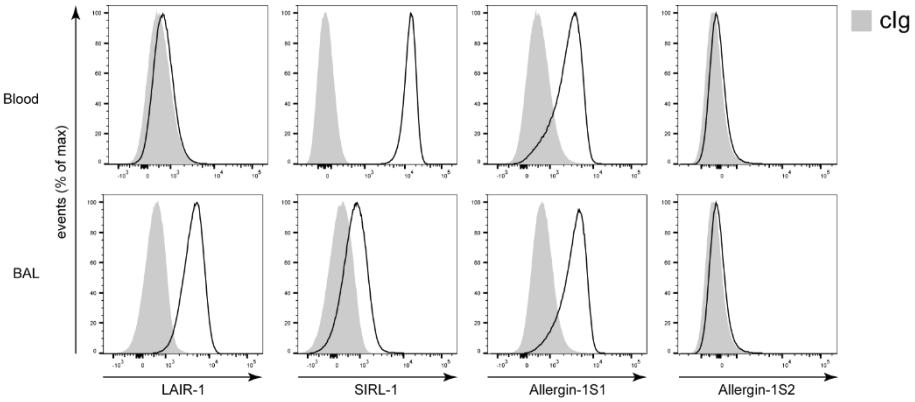

Figure 3. Allergin-1S1 is expressed on both blood and airway-infiltrated neutrophils of RSV bronchiolitis patients.
Blood and aspirated airway-infiltrated leukocytes obtained from intubated paediatric RSV bronchiolitis patients (n=3) were stained for cell lineage markers, LAIR-1, SIRL-1, and the Allergin-1S1 and Allergin-1S2 isoforms. Grey plots represent isotype-matched control Ig (cIg); representative graphs are shown.

By using LAIR-1-deficient mice in the same model of RSV disease, we previously demonstrated that the inhibitory collagen receptor LAIR-1 plays a critical role in regulating neutrophil airway influx and limiting disease severity.[14] Both LAIR-1 and Allergin-1 are ITIM-bearing inhibitory receptors that recruit the down-stream phosphatases SHP-1 and SHP-2 upon ligation. Nevertheless, their role in the regulation of neutrophil migration and/or activity appears to differ greatly. Possibly, the availability of the respective ligands of LAIR-1 and Allergin-1 in the lungs is responsible. While collagen(-

like) molecules are known ligands of LAIR-1, the ligand of Allergin-1 is unknown. However, airway hyperresponsiveness and inflammation in response to house dust mite are suppressed by Allergin-1 on mast cells and lung-resident CD11b+ DCs, respectively.[10, 12]

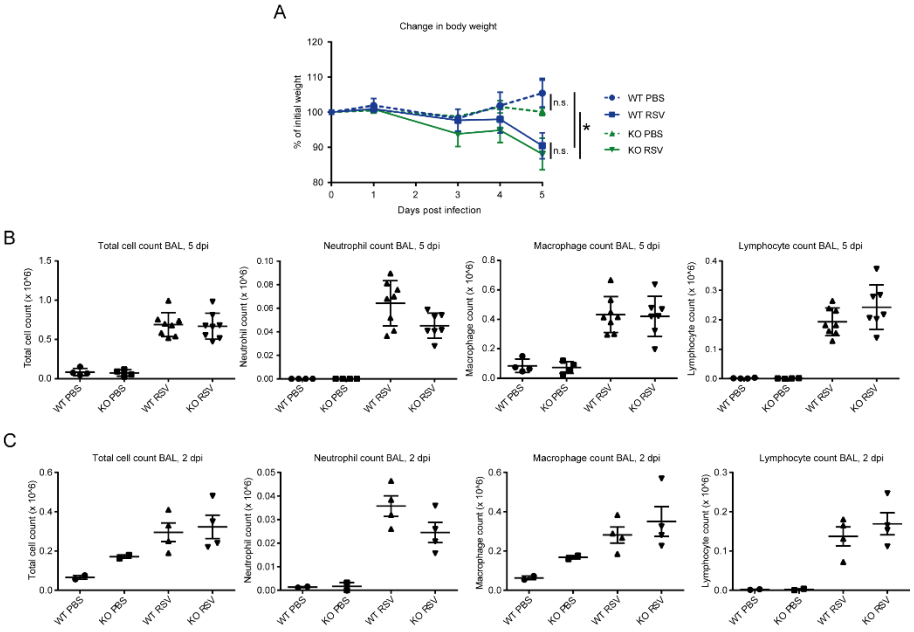

Figure 4. Allergin-1 deficiency does not affect experimental RSV infection in mice. (A) BALB/c *Mirl1*$^{-/-}$ mice (KO) and wild-type littermates (WT) inoculated with RSV (n=8) or PBS (n=4) were weighed daily; data is represented as weight change in percentage relative to day 0. Terminal bronchioalveolar lavage (BAL) was performed on mice on day 5 (B) and day 2 (C) post infection (dpi). Total cell counts and differential (neutrophil, macrophage, lymphocyte) cell counts, based on cellular morphology, are shown. Data at 5 dpi is representative of two independent experiments, each with comparable group sizes (n=8 for WT/KO RSV, n=4 for WT/KO PBS); data at 2 dpi represents one experiment (n=4 for WT/KO RSV, n=2 WT/KO for PBS). Statistical significance was determined by Kruskal-Wallis test. *, $p < 0.05$; n.s., not significant.

This suggests that ligands for Allergin-1 are present in lung tissue. Whether or not neutrophils can interact with these ligands in a manner that results in Allergin-1-mediated inhibition, remains to be determined. The development of agonists, e.g., agonistic antibodies, or the identification of

the ligand(s) of Allergin-1 would facilitate further investigation into the function of Allergin-1 on neutrophils.

In conclusion, we compared the expression of Allergin-1 isoforms on human blood leukocytes in homeostatic and systemic inflammatory conditions and found no significant differences. A potential caveat, however, is that the SLE patient population is rather heterogeneous and we enlisted a limited number (n=13) of patients. This may obscure individual differences or fail to reveal the existence of possible subpopulations with altered expression. The Allergin-1S1 isoform was expressed by every interrogated myeloid cell, as well as B lymphocytes. In contrast, the Allergin-1L isoform could not be detected on any subset of blood leukocyte, and the S2 isoform was only detected on non-classical monocytes after their transition from classical monocytes, which solely express the S1 isoform. While airway-infiltrated neutrophils expressed Allergin-1, genetic ablation of Allergin-1 did not affect disease severity or leukocyte infiltration in a mouse model of RSV disease. The biological relevance of the Allergin-1S1 to -S2 isoform switch on classical and non-classical monocytes, respectively, as well as inhibitory potential of Allergin-1S1 on neutrophils are prime targets for further investigation.

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Supplementary tables

Supplementary Table 1. Antibodies used in the study.
This table compiles the antibodies used in the current study. Listed are the target of the antibody, its clone, the conjugated fluorochrome, and wherefrom it was purchased or by whom it was produced.

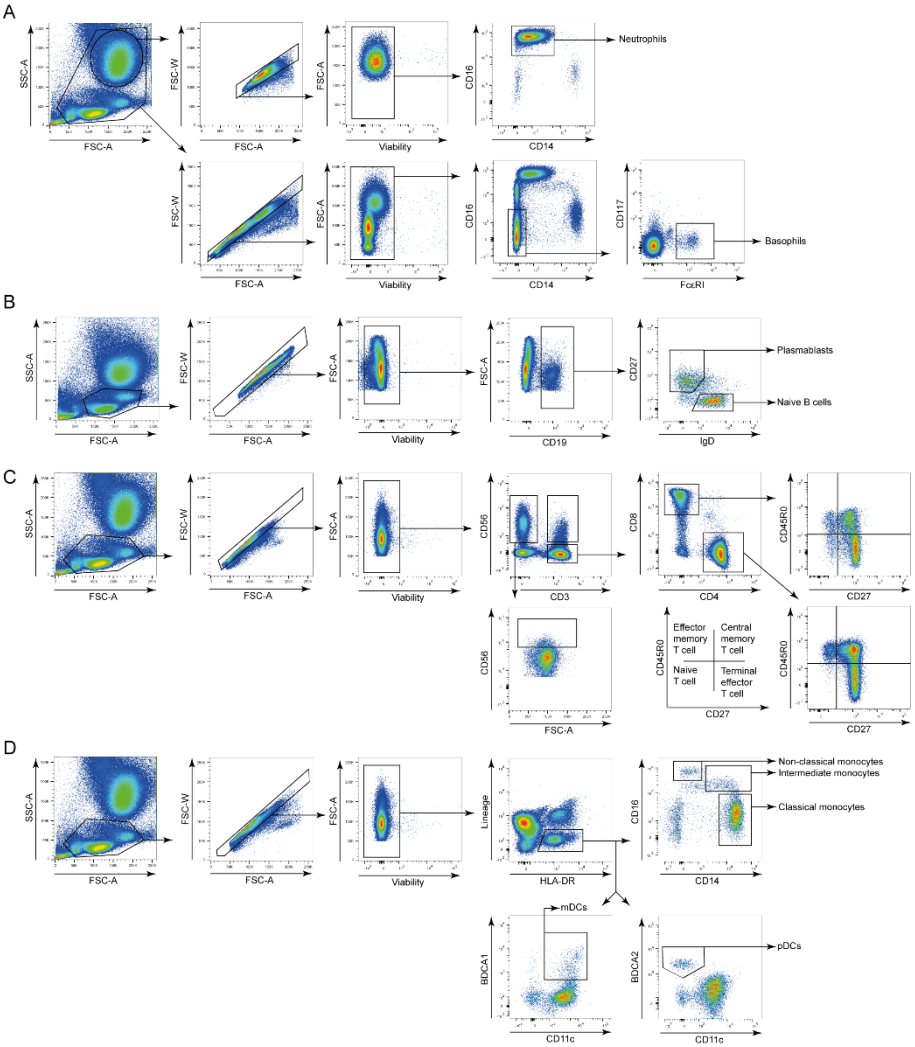
	Target	Clone	Fluorochrome	Origin
Primary A	Control Ig rIgG2a	R35-95 (rIgG2a)	purified	BD Biosciences 553927
	Control Ig mIgG2a	G155-178 (mIgG2a)	purified	BD Biosciences 553454
Primary B	Allergin-1S1	EX32 (rIgG2a)	purified	ONO Pharmaceutical
	Allergin-1S2	EX29 (mIgG2a)	purified	ONO Pharmaceutical
Secondary	rat IgG	donkey polyclonal	AF647	Jackson ImmunoResearch 712-605-153
	mouse IgG	donkey polyclonal	AF488	Jackson ImmunoResearch 715-545-151
Panel 1	CD19	HIB19	eF450	eBioscience 48-0199-42
	CD27	L128	BV510	BD Biosciences 563092
	IgD	3G8	AF700	BD Biosciences 558122
	CD24	ML5	PerCP-Cy5.5	BD Biosciences 561647
	CD38	HIT2	PE-Cy7	eBioscience 25-0389-42
	CXCR3	G025H7	BV605	BioLegend 353728
Panel 2	CD3	OKT3	eF450	eBioscience 48-0037-42
	CD4	OKT4	BV711	Sony Biotech 2187200
	CD8	B9.11	AF700	Beckman Coulter B49181
	CD27	L128	BV510	BD Biosciences 563092
	CD45R0	UCHL1	PE-Cy7	BD Biosciences 337168
	CXCR5	J252D4	PErCP-Cy5.5	BioLegend 356910
	CD56	B159	PE-CF594	BD Biosciences 562289
Panel 3	CD14	RMO52	ECD	Beckman Coulter IM2707U
	CD16	3G8	BV785	BioLegend 302046
	CD11b	ICRF44	AF700	BD Biosciences 557918
	CD62L	DREG-56	BV650	BioLegend 304832
	CD203c	NP4D6	PE-Cy7	eBioscience 25-2039-41
	CD117	104D2	PerCP-Cy5.5	BioLegend 313214
	FCεRI	AER-37	PB	BioLegend 334618

Panel 4	-CD3 (Lineage)	SP34-2	V500	BD Biosciences 560770
	-CD19 (Lineage)	HIB19	BV510	BioLegend 302242
	-CD56 (Lineage)	HCD56	BV510	Sony Biotechnology 2191700
	HLA-DR	G46-6	BV605	BD Biosciences 562845
	BDCA1 (CD1c)	L161	BV421	BioLegend 331526
	BDCA2 (CD303)	201A	PerCP-Cy5.5	Sony Biotechnology 2371050
	BDCA3 (CD141)	1A4	BV711	BD Biosciences 563155
	CD14	RMO52	ECD	Beckman Coulter IM2707U
	CD16	3G8	BV785	BioLegend 302046
	CD11C	3.9	AF700	eBioscience 56-0116-42
Additional mAbs	LAIR-1	DX26	PE	BD Biosciences 550811
	SIRL-1	1A5	FITC	own production

Supplementary Table 2. Patient characteristics and ongoing treatment.
SLEDAI: SLE disease activity index, dsDNA: double-stranded DNA, ENA: extractable nuclear antigens, Sm: Smith antigen, SS-A: Sjoegren Syndrome Antigen A, SS-B: Sjoegren Syndrome Antigen B, PM-Scl: Polymyositis-Scleroderma, RibP: Ribosomal antigen P, HCQ: Hydroxychloroquine, Aza: Azathioprine, Pred: Prednisone, MMF: Mycophenolate Mofetil, MTX: Methotrexate

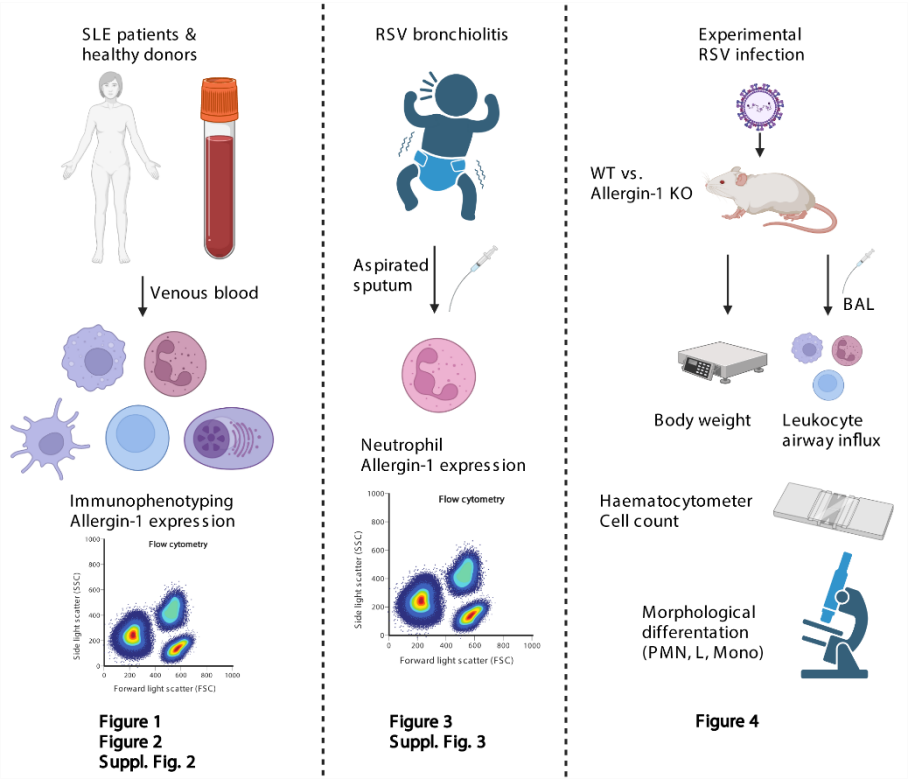
Age	Sex	SLEDAI	dsDNA (IU/mL)	ENA	Medication	History of nephritis
37	F	2	90	SS-A, nRNP/Sm, histones, nucleosomes, PM-Scl	HCQ, Pred (7,5 mg), MMF	yes
37	F	0	7.2	none	HCQ, Pred (17,5 mg)	yes
44	F	0	<0,5	PM-Scl, AMA-M2	HCQ, Pred (5 mg), Aza	no
49	F	0	<0.5	Sm, nRNP/Sm, nucleosomes, PM-Scl	HCQ Pred (7,5 mg), Aza	yes
33	F	0	1.6	SSA-60, nRNP/Sm	HCQ Pred (7,5 mg), Aza	yes
47	M	0	1.7	SS-A	HCQ	no
52	F	0	2.9	SS-A, SS-B	Pred (2,5 mg), MMF	yes
20	F	8	121	Sm, nRNP/Sm, nucleosomes, RibP	HCQ, Pred (10 mg), Aza	no
46	F	0	<0.5	SS-A	HCQ, Pred (2,5 mg), Aza	yes
54	F	0	58	none	HCQ, Pred (5 mg), MMF	yes
22	F	0	18	SS-A, RibP	HCQ, Pred (5 mg), Aza	no
55	F	0	31	none	HCQ	no
48	F	2	0.8	SS-A	HCQ	no

Supplementary figures

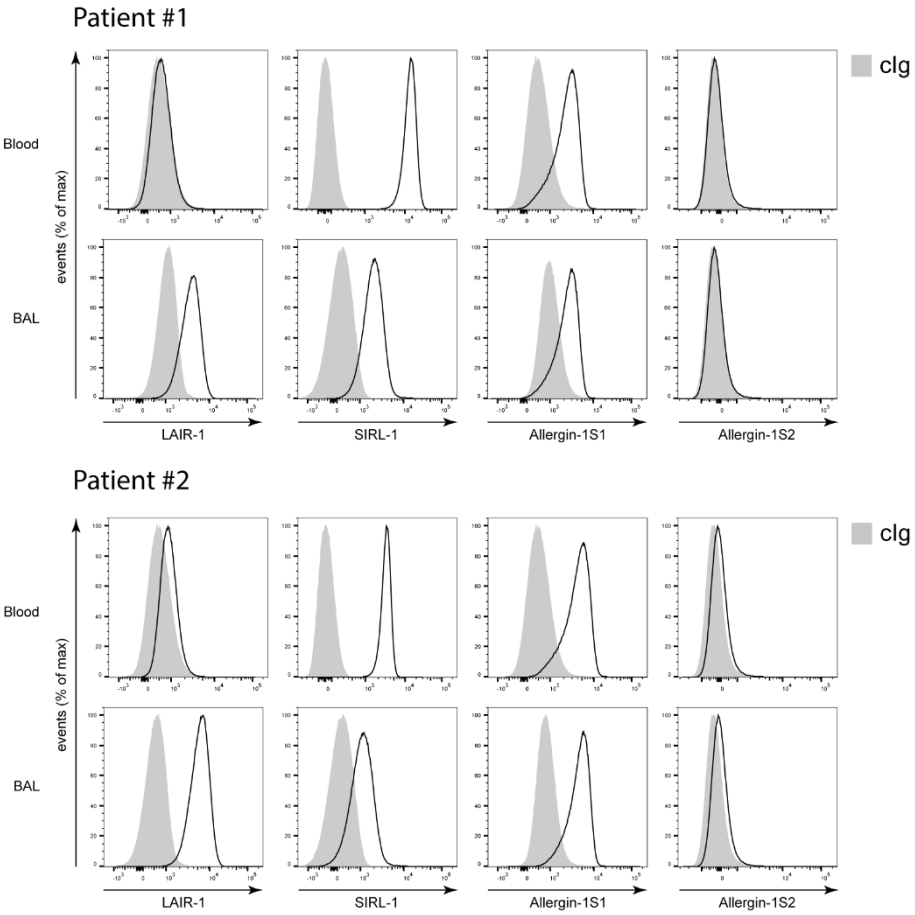


Supplementary Figure 1. Gating strategy for immunophenotyping.
The gating strategy that was used to identify leukocyte subsets in the flow cytometry data is shown for a representative healthy donor. Leukocyte subsets were identified as follows: (A) neutrophils, CD14- CD16+, basophils CD14- CD16- CD117- FcεRI+; (B) total B cells, CD19+, naïve B cells, CD19+ CD27- IgD+, plasmablasts, CD19+ CD27+ IgD-; (C) CD4+ effector memory T cells, CD3+ CD4+ CD27- CD45R0+, CD4+ central memory T cells, CD3+ CD4+ CD27+ CD45R0+, CD4+ naïve T cells, CD3+ CD4+ CD27+ CD45R0-, CD4+ terminal effector T cells, CD3+ CD4+ CD27- CD45R0-; (D) CD8+ effector memory T cells, CD3+ CD8+ CD27- CD45R0+, CD8+ central memory T cells,

CD3+ CD8+ CD27+ CD45R0+, CD8+ naïve T cells, CD3+ CD8+ CD27+ CD45R0-, CD8+ terminal effector T cells, CD3+ CD8+ CD27- CD45R0-; (E) natural killer (NK) cells, CD3- CD56+, NKT-like cells, CD3+ CD56+; (F) classical monocytes, CD3- CD19- CD56- HLA-DR+ CD14++ CD16-, intermediate monocytes, CD3- CD19- CD56- HLA-DR+ CD14+ CD16+, non-classical monocytes, CD3- CD19- CD56- HLA-DR+ CD14- CD16++; (G) myeloid DCs (mDCs), CD3- CD19- CD56- HLA-DR+ CD11c+ BDCA1+, plasmoid DCs (pDCs), CD3- CD19- CD56- HLA-DR+ CD11c- BDCA2+.



Supplementary Figure 2. Overview of samples used in this study.
This figure illustrates the origin of the samples and the data generated with them as used in the current study. Also listed are the Figures in which the data is presented. Created with BioRender.



Supplementary Figure 3. Allergin-1 isotype expression on blood and airway neutrophil of two additional RSV bronchiolitis patients.

Blood and aspirated airway-infiltrated leukocytes obtained from two intubated paediatric RSV bronchiolitis patients (in addition to the patient presented in Fig 3) were stained for cell lineage markers, LAIR-1, SIRL-1, and the Allergin-1S1 and Allergin-1S2 isoforms. Grey plots represent isotype-matched control Ig (cIg).

Chapter

7

Discussion

Overview

In this thesis, I investigated agonistic ligation of immune inhibitory receptors as a potential future therapy for neutrophil-driven inflammatory diseases. Specifically, in **Chapter 2** I review literature to propose that reducing neutrophilic inflammation in respiratory syncytial virus (RSV)-induced bronchiolitis patients could ameliorate acute immune pathology and help prevent sequelae. **Chapter 3-5** focus on the inhibitory collagen receptor leukocyte-associated immunoglobulin (Ig)-like receptor (LAIR)-1. I demonstrate that LAIR-1 directly inhibits airway-derived neutrophils and limits neutrophilic airway inflammation. In **Chapter 6** I show that another inhibitory receptor, Allergin-1, fails to control neutrophilic inflammation in the same model. This highlights the functional diversity between inhibitory immune receptors expressed on the same cell. Here, I discuss further the role of neutrophils in the pathology of diseases and by what means inhibitory immune receptors may be targeted to ameliorate neutrophil-driven inflammation.

As discussed extensively in **Chapter 2**, neutrophils are crucial to antimicrobial defence. Not only do neutrophils phagocytose (opsonized) microbes and release potent antimicrobial components, they also have immuno-modulatory functions that can instruct the adaptive immune response. The importance of neutrophils in the defence against pathogens is most strikingly illustrated by the recurrent and severe bacterial and fungal infections suffered by chronic granulomatous disease (CGD) patients, whose neutrophils are impaired in their production of reactive oxygen species (ROS) due to a genetic defect. In recent years, much attention has also been directed at the deleterious aspects of (excessive) neutrophil activation. The expulsion of neutrophil extracellular traps (NETs), in a process of programmed cell death termed NETosis, has been of particular interest to the scientific community. NETs are composed of decondensed chromatin decorated with anti-microbial peptides and proteins, including neutrophil elastase (NE), cathelicidin LL-37, and myeloperoxidase (MPO). A detrimental contribution of NETs has been attributed to a diverse array of pathologies, ranging from autoimmunity and ischaemia reperfusion injury to cancer progression and viral infections.

Neutrophils and IL-17 in viral respiratory infection

Since the publication of **Chapter 2**, it has been uncovered that neutrophils are not only key players in the immune response during RSV infection-induced bronchiolitis, but also play an instrumental role in setting the stage for RSV infection.[1] Neutrophil activation in the airway mucosa prior to

experimental inoculation with RSV predisposes adult volunteers to the development of symptomatic disease, i.e., common cold symptoms. In absence of prior airway neutrophil activation, an interleukin 17 (IL-17)-based immune response during the pre-symptomatic phase shortly after RSV exposure protects against infection. Other lines of evidence also point towards a protective role for IL-17 in the defence against respiratory viruses. Administration of secukinumab, a human anti-IL-17 monoclonal antibody (mAb), for the treatment of psoriasis, increases the incidence of respiratory infections compared to placebo-treated patients in clinical trials.[2,3] A candidate vaccine for *Bordetella pertussis*, the causative agent of pertussis or whooping cough, protects mice from experimental infection with RSV in an IL-17-dependent manner.[4] Moreover, intranasal administration of IL-17 shortly after the inoculation of mice with RSV reduced subsequent disease severity.[5] Combined, these studies reveal a critical role for IL-17 in the early, pre-symptomatic stages of viral respiratory infection, likely by preventing the establishment of productive viral replication.

In the early stages of infection, the protection provided by IL-17 may be attributable to its role in the barrier function of mucosa, where it contributes to the maintenance of barrier integrity and induces the production of antimicrobial proteins and peptides by epithelial cells.[6] Activated neutrophils, however, release proteases that can comprise barrier function, digest antimicrobial peptides, and process IL-1-family cytokines (both negatively and positively modulating their activity).[7] An initial disturbance of the barrier integrity by activated neutrophils at the time of viral exposure, may provide a foothold for invading viruses to initiate productive replication. The cause of the deleterious, pre-existing neutrophil activation has yet to be determined, but could be attributed to non-symptomatic bacterial or fungal infections.

Interestingly, despite the apparent dichotomy between protective IL-17 response and deleterious neutrophilic inflammation, IL-17 is strongly associated with and induces neutrophilic inflammation; it is even secreted by neutrophils themselves.[8] One might assume that an IL-17 response to RSV exposure would induce neutrophilic airway inflammation, but does not appear to be the case. The mechanism behind this disconnect between IL-17 and neutrophils in this setting has yet to be unravelled.

Although IL-17 appears to have a protective role at the time of exposure and immediately thereafter, a pathological role has been attributed to IL-17 in later stages of viral respiratory infection. Indeed, antibody-mediated neutralization of IL-17 in mouse models of influenza virus, rhinovirus, and RSV infection resulted in faster resolution of airway

hyperresponsiveness, reduced neutrophil airway infiltration, and ameliorated immunopathology.[9] Moreover, a pathological role for IL-17 has also been described in non-respiratory viral infections, such as the mosquito-borne alphaviruses chikungunya and Ross River virus,[10,11] and human papillomavirus.[12] Taken together, this suggest that the timing of IL-17 release is critical to whether it induces a protective (early stages) or pathological (later stages) immune response to viral respiratory infection.

Neutrophils and COVID-19

In discussing the role of neutrophils in viral respiratory infections, it would be remiss not to mention the ongoing pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the coronavirus disease 2019 (COVID-19) affliction that it can elicit in infected individuals. Neutrophils are strongly suggested to play a major pathological role in severe cases of COVID-19. Indeed, a proteomic signature indicative of neutrophil activation in blood predicts exacerbation of disease severity, intensive care unit admittance, and increased mortality for hospitalized COVID-19 patients. This neutrophil-activation signature was present as early as the first day of hospitalization and predicted progression to critical disease.[13] Total amounts of developing and mature neutrophils were also higher in COVID-19 patients that suffered worse outcomes.[13]

Histopathological comparison of influenza virus- and SARS-CoV-2-infected lungs revealed that vascular recruitment of neutrophils, the formation of NETs, and immunothrombosis are key features of COVID-19, but less prominent in influenza. Thus, neutrophils may play a central role in the pathology of severe COVID-19.[14] NETs in particular appear to directly cause substantial tissue damage and elicit immunothrombosis.[15] SARS-CoV-2 directly induces healthy neutrophils to release NETs, which in turn promote pulmonary epithelial cell death.[16] Moreover, the NETosis-inducing capacity of sera obtained from hospitalized COVID-19 patients correlates to severity of disease.[17] It has even been suggested that NETs could be a driving force behind post-acute COVID-19 syndrome, mainly by exposing auto-antigens for immunization.[18]

Neutrophilic inflammation: a matter of timing?

Taken together, these recent studies show that neutrophilic inflammation during viral respiratory infections contributes to immune pathology. Interestingly, an initial neutrophil-driven immune response to the virus upon exposure and during the first days of infection appears to be a key determinant for the subsequent course and severity of disease. Pre-existing

neutrophilic inflammation at the time of viral exposure may compromise the barrier function of the mucosa and grant the virus a foothold to jumpstart viral replication. In later stages, the numerous and highly activated neutrophils directly evoke tissue damage through their promiscuous effector mechanisms, with NETs chief among them. Neutrophilic inflammation is therefore a promising target for novel therapies to prevent viral respiratory disease from exacerbating and to ameliorate severe viral respiratory disease. Possibly, suppressing the pre-existing neutrophilic inflammation may even act as a prophylaxis to prevent infection all together. Of course, this may also give free rein to bacteria and fungi to propagate in the lungs and should not be taken lightly.

The cause of the initial neutrophilic inflammation is still unclear, but a likely candidate is exposure to bacteria and/or fungi, which elicit a neutrophil-driven response. After all, the airways are, by necessity, constantly exposed to microbes. The human challenge model for RSV infection quite definitively demonstrated that pre-existing neutrophil activation facilitates viral infection.[1] I speculate that this is not unique to RSV, but rather a general feature of viral respiratory infections. Future studies on this subject with other respiratory viruses would be of great interest.

Neutrophils in ischaemic injury

As first responders, neutrophils are also quick to arrive at the scene of tissue injury caused by trauma, where cell death results in the release of damage-associated molecular patterns (DAMPs) that attract neutrophils. Case in point, neutrophils congregate at sites of ischaemic injury following reperfusion, such as after ischaemic stroke, myocardial infarction, or organ transplant.[19] In recent years, numerous reports have been made of neutrophils, and in particular NETs, contributing to ischaemia-reperfusion injury in a variety of organs, including the brain, lungs, kidneys, and heart.[20–22] Moreover, in the brain, the presence of NETs was found to contribute to the breakdown of the blood-brain barrier and diminish vascular repair and neovascularisation, thereby impairing recovery.[22,23] In this setting, the deposition of NETs peaked at 3-5 days post ischaemic event. This suggest there is a therapeutic window wherein neutrophil inhibition could prevent additional tissue damage and facilitate vascular repair. However, neutrophils are also involved in tissue repair following ischaemic injury. For instance, neutrophil-derived proteases degrade the extracellular matrix (ECM) around necrotic cells to facilitate their clearance. Neutrophils also clear out necrotic tissue and dismantle damaged blood vessels to allow neovascularisation. Therefore, a complete suppression of neutrophil activity, even if located solely to the ischaemic site, may be

undesirable. Rather, specific inhibition of certain neutrophil functionalities, such as NETosis, may represent the most promising way forward.

Neutrophils in chronic inflammatory diseases

Neutrophils are primarily known as rapid-deployment immune cells: first on the scene in response to a pathogenic threat. In this context, a role of neutrophils in the anti-viral immune response, whether beneficial or detrimental, is to be expected. However, neutrophils have also been implicated as pathological players in chronic and sterile inflammatory diseases, including the autoimmune diseases rheumatoid arthritis (RA), anti-neutrophil cytoplasmic antibodies (ANCA) vasculitis (AAV), and systemic lupus erythematosus (SLE). These chronic diseases have distinct clinical presentations: RA is characterized by joint inflammation and destruction of bone tissue; in AAV, inflammation leads to the destruction of the small vasculature in various organs; and SLE manifestations can vary from mild joint and skin inflammation to severe lupus nephritis, which can compromise kidney function.[24]

NETs are present in the inflamed joints and synovium of RA patients, in the small-vessel lesions of AVV patients, and the inflamed tissues of SLE patients. Here, NETs can wreak havoc directly on the endothelium and other tissues by action of, among others, its bound proteases and the cytotoxicity of its histones.[24] Common to all three autoimmune diseases is the presence of antibodies targeted at autoantigens that are found in NETs. RA patients produce auto-antibodies against post-translationally modified proteins, including the citrullinated histones of NETs; antibodies against MPO and the neutrophil serine protease proteinase 3 (PR3) are characteristic of AAV; and anti-double stranded DNA (dsDNA) antibodies are a defining feature of SLE. Additionally, the immune complexes formed by NETs and auto-antibodies can stimulate the activation of the complement system, thereby further enhancing inflammation and depositing complement factors on NETs. In turn, the enhanced inflammation and auto-antibody complexes induce more NET formation, advancing the destructive cycle.

Under homeostatic conditions, the extracellular endonuclease deoxyribonuclease (DNase) I digests self-DNA, including the degradation of DNA in NETs, to prevent unnecessary immune activation. Mice genetically deficient for DNase I spontaneously develop SLE-like disease with kidney involvement.[25] Moreover, dysfunctional DNase I is associated with the development of lupus nephritis by SLE patients and higher levels of autoantibodies.[26,27] Possibly, the impaired clearance of NETs by DNase I contributes to the exacerbated autoimmunity, although other sources of

extracellular self-DNA such as apoptosis or necrosis cannot be fully eliminated. Whether or not impaired clearance of NETs plays a causal role in the aetiology of SLE, a contribution of NETs to the progression and pathology of the disease seems highly probable.

NETs bound by complement factors and auto-antibodies, as well as DNA of which the deoxyribose backbone has been oxidized by ROS (produced, e.g., by the MPO in NETs), are more resistant to degradation. Partially digested fragments of NETs can be phagocytosed by macrophages and other myeloid cells. By an unknown, NE-dependent mechanism, phagocytosed NET fragments can translocate from the phagolysosome into the cytosol and activate the innate DNA sensor cyclic guanosine 5'-monophosphate (GMP)–adenosine 5'-monophosphate (AMP) synthase (cGAS) to stimulate type I interferon (IFN) production.[28] In addition to cGAS, NET fragments can also stimulate plasmacytoid dendritic cells (pDCs) to produce large quantities of IFN via the endosomal Toll-like receptor 9 (TLR-9), which recognizes self-DNA complexed with LL-37.[29] DNA-LL37 complexes can similarly stimulate memory B cells to produce auto-antibodies by interaction with TLR-9 and the B cell receptor.[30] Type I IFNs, which derive their name from their ability to interfere with viral replication, and IFN-stimulated genes (ISGs) are indispensable to antiviral defence and have wide-ranging stimulatory effects on both innate and adaptive immunity. The majority of SLE patients display increased expression of ISGs, the so-called 'interferon signature'.[31] The stimulation of IFN production by NETs may be in part to blame for this hallmark of SLE. Moreover, IFN-stimulated neutrophils have an increased propensity to form NETs, thereby further stoking the fires that drive SLE pathology; see Figure 1 for a schematic overview of the role of NETS in driving autoimmunity.

It stands to reason that reducing neutrophil activity, in particular NETosis, by pharmaceutical means could provide new treatment avenues for various autoimmune diseases. Breaking the vicious circle of NETs inducing pathological immune activation that in turn promotes the deposition of more NETs, could ameliorate disease and induce remission in patients. Of note, however, another variety of programmed cell death, ferroptosis, has recently come under scrutiny in the context of SLE. Ferroptosis is morphologically, genetically and biochemically separate from other cell death programmes, such as apoptosis, necroptosis and NETosis; it does not require caspase activation, and is characterized by an accumulation of lipid-ROS and iron ions.[32] Circulating neutrophils from both lupus-prone mice and SLE patients undergo ferroptosis and a neutrophil-specific haploinsufficiency of a key ferroptosis inhibitor (i.e., glutathione peroxidase 4, which shields against membrane lipid peroxidation) results in lupus-like symptoms in mice that

recapitulate human disease. Conversely, treatment with a specific ferroptosis inhibitor ameliorates disease in lupus-prone mice.[33] The role of neutrophils in the aetiology and pathology of autoimmune diseases such as SLE may therefore be broader and more involved than currently understood; more research is highly advisable.

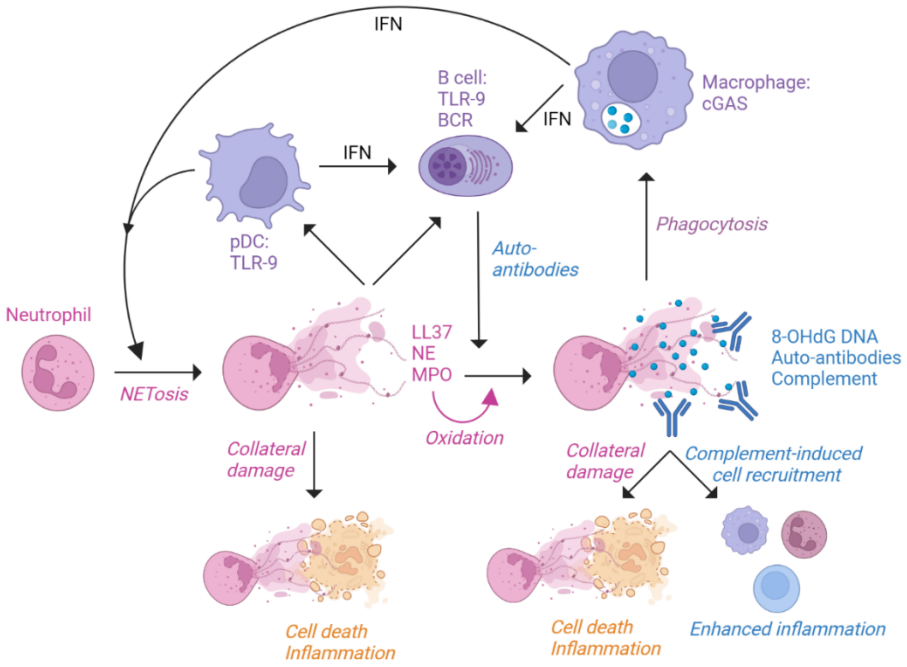


Figure 1. NETs in autoimmunity.
In addition to inducing direct tissue damage and inflammation, NETs can stimulate the production of IFN by macrophages and pDCs, as well as the production of auto-antibodies by memory B cells. IFN in turn potentiate neutrophils to release NETs, thereby creating a vicious cycle.

Neutrophils and tumour metastases

Multiple deleterious roles for neutrophils in tumour initiation and progression have been described through their actions in the tumour micro-environment, including stimulation of angiogenesis, promoting tumour cell proliferation, and inhibition of anti-tumour immunity, among others.[34] Recent reports have added the promotion of metastasis by NETs to that appalling list.[35] Earlier reports demonstrated that tumour-induced NETs are found in circulation and promote metastasis, particularly in the lung and liver. Initially, this was presumed to be a passive effect whereby circulating tumour cells were trapped by NETs functioning as their non-capitalized namesakes

(i.e., nets), allowing adhesion to, e.g., liver sinusoids.[36,37] More recently, however, it was shown that circulating tumour cells can express a transmembrane receptor, CCD25, that recognizes NET-DNA. Specifically, CCD25 has a preference for 8-hydroxy-2'-deoxyguanosine (8-OHdG)-enriched DNA, which is a predominate form of ROS-induced oxidised DNA, present in NETs. Binding of CCD25 to NET-DNA enhanced the motility of tumour cells, thereby promoting tissue infiltration and the establishment of metastases.[38] Further, high levels of circulating NET-DNA is a biomarker for progressive disease in lung, oesophageal, and gastric adenocarcinoma; and the presence of NET-DNA in serum predicts the occurrence of liver metastases in patients with early-stage breast cancer.[38,39] Therefore, NETs seem to play a crucial role in the foremost cause of cancer-related mortality, i.e., metastasis. Disrupting NET formation, or the interaction between tumour cells and NETs, may lead to new immunotherapeutic breakthroughs in the treatment of cancer.

NETs: does size matter?

Despite the plethora of potential pathological roles played by NETs, as discussed above, the ability of neutrophils to form NETs is highly conserved amongst animals.[40] This suggests that, from an evolutionary point of view, the advantages provided by NETs have outweighed any harmful effects. The propensity to form NETs can differ widely among species. For example, in response to phorbol 12-myristate 13-acetate (PMA) stimulation, around 40-60% of human circulating neutrophils will form NETs, compared to only 1-2% for mouse neutrophils.[41] However, it should be noted that PMA is a highly artificial stimulus and its mechanism of action differs wildly from more physiological stimuli, such as opsonised bacteria, immune complexes, or the monosodium urate (MSU) crystals found in gout.[42]

There is evidence to suggest that the foremost intended target of NETs are pathogens whose size exceed the neutrophil's capacity to phagocytose. In the case of the dimorphic fungus *Candida albicans*, neutrophils form NETs in response to large hyphae, but not small yeast cells. Similarly, aggregated clusters of *Mycobacterium bovis*, but not a multitude of singular bacterial cells in similar numbers, induce NETosis.[43] Upon the phagocytosis of small microbes by neutrophils, azurophilic granules are rapidly recruited to fuse with the phagosome to deliver their microbicidal payload, which includes NE. In the absence of phagocytosis, and sequestering of NE in the phagolysosome, NE will slowly transfer from azurophilic granules to the cytosol of activated neutrophils. In turn, this allows for the translocation of NE to the nucleus where it promotes chromatin decondensation, which is required for NET formation.[43]

Recent research on neutrophils and NETs in zebrafish – again illustrating the broad evolutionary conservation of NETosis among animals, even beyond mammals – revealed that NET components are instrumental to the induction of neutrophil swarms.[44] During swarming, large groups of neutrophils coordinate their efforts to synergistic effect, enhancing their antimicrobial activity beyond merely the sum of comparable numbers of independently acting neutrophils. Further, NET release within neutrophil swarms was shown to restrict the growth of *Candida albicans* clusters.[45]

Helminths represent another class of pathogens that are too large for neutrophils to phagocytose. Nonetheless, by releasing NETs, neutrophil can contribute to the anti-parasite response. NETs released by neutrophils of their respective species can kill hookworm larvae (*Nippostrongylus brasiliensis, Necator americanus*) that prey on humans and mice, and nematodes (*Ostertagia ostertagi*) that infect the bovine gastrointestinal tract.[46,47] Both human and mouse hookworms secrete DNase that degrade NETs and impair NET-mediated killing of larvae.[46] The presence of this immune evasion strategy suggests that NETs are important to the control of parasitic helminth infections.

Mix and match: NET looking for pathogen

Interestingly however, grey platelet syndrome (GPS) patients, who display a markedly reduced capacity for NET formation, do not appear to suffer from excessive infections. GPS is primarily defined as an autosomal recessive bleeding disorder with progressive myelofibrosis caused by rare loss-of-function variants in neurobeachin-like 2 (NBEAL2) that result in the absence or marked reduction of α-granules in platelets. The reduced capacity of NET formation by GPS patient neutrophils appears linked to specific granule irregularities. GPS patient neutrophils showed no abnormalities in ROS production, chemotaxis, or bacterial and fungal killing. Yet, NET formation in response to PMA, MSU crystals, and opsonised *C. albicans* was strongly impaired. There was, however, a minor decrease in the neutrophil-mediated killing of *Aspergillus fumigatus*, which may be causally linked to impaired NET formation since *A. fumigatus* forms large hyphae.[48]

The fact that a specific and potent reduction in NET forming capacity does not lead to increased infection rates among patients, suggests that NETs may not be important to preventing or fighting off infections. However, GPS is exceedingly rare with less than 100 reported cases world-wide; diagnosed cases are likely to originate from developed countries.[49] As a result, known GPS patients may simply not be exposed to the type of pathogens, such as helminths, against which NETs are equipped to offer protection. Additionally,

it suggests that reducing NETs through pharmaceutical means would not expose patients to opportunistic infections.

Inhibitory receptor-mediated regulation of NETosis

Taking into account the potential collateral damage that NETs can elicit, it should come as no surprise that NET release is subject to regulation by inhibitory receptors. Indeed, in **Chapter 3** I show that the inhibitory collagen receptor LAIR-1 is capable of suppressing NET expulsion ex vivo by in-vivo activated, airway-infiltrated neutrophils. Ligation of signal inhibitory receptor on leukocytes 1 (SIRL-1), another inhibitory receptor discovered and characterised by our group, is similarly able to suppress NET release in response to multiple stimuli, even though the varying stimuli signal via different pathways. Notably, intracellular bacterial killing was unaffected by SIRL-1 ligation.[50,51]

The extensive involvement of NETs in a plethora of diseases and the unmet clinical need for effective inhibitors of neutrophil activity, represents an opportunity for the development of agonistic inhibitory receptor ligation therapeutics. However, the biology of inhibitory immune receptors represents a challenge to the development of effective agonists. Whereas current immune checkpoint blockade therapeutics need only to disrupt the interaction between an inhibitory receptor and its ligand, which can be accomplished with a 'simple' blocking antibody, agonistic therapeutics for inhibitory receptors have more demanding requirements, key among them: inducing clustering. For instance, fibrillar collagen in the ECM is bound by LAIR-1, which results in clustering of receptors, and induces inhibitory signalling. When collagen is degraded, however, the resulting soluble peptides can still bind to LAIR-1, but no longer induce signalling (unpublished data). In contrast, immobilised collagen peptides (e.g., on a plastic plate) are capable of inducing signalling.[52]

Inhibitory receptor signalling

There are two – likely complimentary – hypotheses that explain the requirement for clustering: local concentration and kinetic-segregation. When receptors cluster together on a membrane, the local concentration skyrockets. Higher concentrations increase the stochastic probability of repeated interaction with freely diffusible, cytosolic partners, in the case of ITIM-bearing receptors: the phosphatases SHP-1, SHP-2, and/or SHIP. The recruitment of phosphatases to inhibitory receptors initially requires the phosphorylation of the tyrosine residue in their ITIM by Src-family kinases. Constitutively active phosphatases counteract this. An example hereof is the

CD45 phosphatase, expressed by all haematopoietic cells and commonly used as a pan-leukocyte marker. In T lymphocytes, the Src-family kinase Lck constitutively phosphorylates ITAMs in the TCR, but is counteracted by the constitutive phosphatase activity of CD45. Upon interaction of the TCR with MHC molecules on a target cell, an immunological synapse can be formed in which membranes of both cells are brought in close proximity. At which point, the space between the two membranes is insufficient to accommodate the large extracellular domain of CD45, leading to its physical exclusion from the synapse. As a result, CD45 can no longer act to dephosphorylate the TCR, which enables the TCR to signal.[53,54] The exclusion of CD45 from the PD-1-PD-1L complex by clustering is similarly required for signalling; PD-1-PD-1L interaction by itself is enough to orchestrate CD45 exclusion.[54] The induction of clustering by a potential agonist is therefore required to allow the initial phosphorylation of an inhibitory receptor, which enables it to bind its phosphatase(s), and to improve the odds that it will encounter said free-floating phosphatase(s).

Indeed, as recently reviewed by our group, virtually all known ligands for inhibitory receptors are capable of inducing clustering.[55] Such ligands vary from membrane-associated ligands, ECM-associated ligands, to ligands that become de facto multimeric structures after being immobilised on a scaffold (e.g., multiple opsonin molecules bound to a single bacterium) or forming aggregates.

In order for an activated cluster of inhibitory receptors to relay an inhibitory signal, it must be in close proximity to activating receptors. Inhibitory receptors do not produce freely diffusing secondary mediators, but directly counteract activating receptors. Tethered to the cytoplasmic tail of PD-1 or LAIR-1, SHP-1 can effectively dephosphorylate targets within a radius of 13.0 and 23.0 nm, respectively.[56,57] A potential inhibitory receptor agonist therapeutic must therefore also allow for the co-localisation of activating and inhibitory receptors, if not induce it outright.

Inhibitory receptor agonist design

The prospect that a monomeric, mono-specific antibody could fulfil these above-mentioned criteria is poor. With two identical binding sites per antibody, receptor pairs can be formed, but not larger chains or clusters. Although, if the target inhibitory receptor forms dimers or larger multimers, more cross-linking may be possible. This highlights the increased complexity and difficulty in designing/generating an agonist for inhibitory receptor ligation therapy, compared to immune checkpoint blockade therapy. Possible

approaches for developing inhibitor receptor agonists include: artificial ligands, bispecific antibodies, and multimeric antibodies.

Artificial ligands would have to comprise multiple binding domains to induce receptor clustering. This would likely result in ligands with a high molecular mass, which may encounter difficulties where delivery is concerned. Moreover, the affinity of the receptor for the artificial ligand would likely need to be higher than that for the endogenous ligand, which represents a significant design challenge. Bispecific antibodies can link inhibitory and activating receptors together. This could allow for a certain amount of specificity by preferentially inhibiting a specific stimulatory signal. Moreover, inducing co-localisation could induce the phosphorylation of the inhibitory receptor by the activating receptor, thereby enhancing inhibitory signalling. Still, whether this would be sufficient to overcome constitutive dephosphorylation by CD45-like phosphatases remains to be seen, as such a bispecific antibody would not directly induce clustering. Multivalent antibodies, such as the IgM pentamer or hexamer with 10 or 12 antigen-binding sites, respectively, could theoretically bind together larger clusters of inhibitory receptors. However, the bulky nature of an IgM-style pentamer/hexamer could result in distant spacing of the bound receptors, thereby not effectively clustering them close together and failing to exclude CD45-like phosphatases.

Further research into inhibitory receptor biology and technological developments in the generation of antibodies for therapy could open up new avenues for agonist design. Novel techniques, such as the generation of trispecific antibodies for anti-cancer treatment,[58] may also be appropriated for the development of agonistic antibodies for inhibitory receptors.

Concluding remarks and future directions

Neutrophils: a blessing in disguise or a curse masquerading as a boon? As the cliché goes: there is a time and place for everything. The severe bacterial and fungal infections that recur frequently in CGD patients show, without a shadow of a doubt, that neutrophils are critical to antimicrobial defence. Yet, as thoroughly discussed in this chapter, neutrophils, and in particular their NETs, can contribute to a wide range of pathologies. Although the broad evolutionary conservation of NET release is suggestive of its importance, the notable lack of excessive infections in GPS patients, who are deficient in NET release, raises doubts on the relevance of NETs to the protection against microbes – at least against those that are prevalent in developed countries.

RSV infection-induced bronchiolitis is characterised by a large influx of neutrophils into the airways; their pathological contributions are discussed

above and in **Chapter 2**. Treatment of RSV bronchiolitis patients is currently solely supportive and mostly focused on facilitating gas exchange. The lack of therapeutics to effectively reduce neutrophilic inflammation represents a large unmet clinical need. In this thesis, I identified the inhibitory collagen receptor LAIR-1 as a key regulator of neutrophilic inflammation in RSV bronchiolitis. Antibody-mediated ligation of LAIR-1 on airway-infiltrated neutrophils suppressed NET release. Interestingly, Allergin-1, another inhibitory immune receptor expressed by neutrophils that, similar to LAIR-1, relays it inhibitory signal by recruiting the SHP-1 phosphatase to its pITIM, did not regulate neutrophils during RSV disease in mice. This highlights a functional diversity amongst inhibitory immune receptors which is not yet fully understood. I attempted to demonstrate that ligating LAIR-1 in vivo using a newly generated agonistic antibody would ameliorate disease in RSV-infected mice, but failed to observe an effect of the antibody. However, this monospecific IgG isoform antibody is most likely not an ideal inhibitory receptor agonist for in vivo use; future development of multivalent and/or polyspecific antibodies could offer more effective alternatives.

The development of immune checkpoint blockade has rightly been hailed as a breakthrough therapy for cancer. The effectiveness of these treatments, as well as their unfortunate immunological side effects, illustrate the therapeutic potential of inhibitory receptors. Now, the time has come to harness the power of inhibitory receptors more directly by targeted activation. Our current understanding of inhibitory receptor biology indicates that ligation and clustering are required for efficient signal transduction. Therefore, the development of agonists should focus on molecules that can ligate multiple receptor molecules together to induce cluster formation. Such agonists may take the shape of, e.g., multimeric ligands or multivalent and/or polyspecific antibodies. Currently, it is beyond our capabilities to accurately estimate the in-vivo efficacy of novel agonists a priori; trial and error will be required. Nonetheless, multiple actors within the pharmaceutical industry have shown interest in the development of inhibitory receptors agonists as a therapeutic strategy to treat immune-mediated diseases, such as atopic dermatitis, RA, and SLE. This includes the development of PD-1 agonists by Eli Lilly, Janssen Pharmaceuticals, MiroBio, and Anaptys Bio for the treatment of various inflammatory diseases; the clinical testing of a CD200R agonist by Eli Lilly for the treatment of chronic spontaneous urticaria; and the development of agonists for B- and T-lymphocyte attenuator (BTLA) by Eli Lilly, MiroBio, and Anaptys Bio for a variety of inflammatory conditions.

For a potential inhibitory immune receptor agonist to be effective in the treatment of an immune-mediated disease, a number of requirements

can be surmised (Table 1). Foremost, the inhibitory receptor must be present on the target cell at the site where it induces pathology (e.g., LAIR-1 on the airway-infiltrated but not blood neutrophils of RSV bronchiolitis patients). There are still many (>200) predicted ITIM-bearing receptors that remain uncharacterised. This represents more than twice the total investigated to date (ca. 60). Their characterisation will require major effort, but may result in the discovery of new potential targets for therapy as well as enhancing our overall understating of inhibitory immune receptor biology and immunology in general. Inhibitory receptor expression patterns across different cell types in various tissues could allow for localised rather than systematic immune suppression. Localised administration of, e.g., agonistic antibodies into the airways by nebulisation will require different pharmacological formulations than those used for intravenous administration, possibly adding to the challenge of developing localised agonists.[59]

Based on the biology of inhibitory immune receptors discussed above, effective agonists will have to induce receptor clustering. Currently, the majority of agonists in development are monoclonal antibodies that rely on Fc receptor (FcR)-mediated crosslinking to induce signalling; the cell membrane of adjacent FcR-expressing immune cells act as a scaffold to bring antibody-bound target inhibitory receptors in close proximity. This necessitates careful selection, and possible optimisation, of the agonistic antibody's Fc tail. IgG1 isotype antibodies are efficient at inducing clustering due to the high affinity of their Fc domain for IgG-Fc-receptors (FcγR). However, the interaction of cell-bound IgG1 with FcγR IIIa in particular also triggers natural killer (NK)-cell mediated antibody-dependent cell cytotoxicity (ADCC), which may result in depletion of the cells that express the targeted inhibitory receptor. Low-depleting isotypes, i.e., IgG2 and IgG4, may therefore represent a more appropriate choice of Fc domain, although their ability to induce FcR-mediated crosslinking is comparatively poor. Nonetheless, studies on costimulatory receptors suggest that the crosslinking potential of the IgG2 and IgG4 isotypes are sufficient to induce clustering.[60]

Another approach would be to design agonists that induce clustering by themselves without the need for FcRs (or other receptors) on adjacent cells. The high affinity of antibodies may give them an edge over natural ligands as agonists, as well as bypass competition for ligand binding. In the case of LAIR-1, for example, soluble LAIR-2 competes for the binding of collagen(-like domains) and collagen breakdown products can bind LAIR-1 (thereby occupying the ligand binding site) without inducing signalling. Generating novel agonistic antibodies will pose technical challenges. As opposed to activating receptors, which can directly elicit a measurable

response upon stimulation by an agonist, the effect of a stimulated inhibitory receptor can only be measured by a reduced response in the context of an activating receptor or through the introduction of an exogenous reporter mechanism. Nevertheless, reporter systems, such as the one we employed in **Chapter 5**, are available and could be adapted for high-throughput screening; new antibody techniques for the generation of, e.g., trispecific antibodies provide promising platforms for the production of agonistic antibodies.

Table 1. Overview of design considerations for inhibitory immune receptor agonists.

	Requirements	Opportunities	Challenges
Receptor	Expressed on target cell Expressed at site of insult Preferably, highly expressed	Targeting of specific cell type(s) Site/tissue specific target	Many uncharacterised IRs
Screening	In vitro: reporter cells In vivo: disease model	High-throughput reporter systems	No direct measurement of IR activity possible without reporter or in relation to activation/disease
Pharmacology	Reach site of insult	Localised administration	Immune suppression Dosage (too low, insufficient inhibition; too high, no clustering, no inhibition)
Target disease	Initially: acute, localised No existing effective treatment	New therapy options	Adverse events (infection, malignancies) due to immune suppression in chronic treatment
Agonist	Induces clustering	Novel mAb techniques (bi-, trispecific mAbs); multiple, combined mAbs	Multivalent binding needed for clustering; singular mono-specific mAbs likely insufficient without cross-linking via FcRs on adjacent cells Isotype and Fc domain selection of agonistic mAbs to avoid ADCC

ADCC, antibody-dependent cellular cytotoxicity; FcR, Fc receptor; IR, inhibitory receptor; mAb, monocolonal antibody

Determining the dosage of inhibitory receptor agonists also represents a somewhat unique challenge, as both overly low and high doses may result in a lack of response. Too low a dose would induce too little clustering and subsequent signalling by inhibitory receptors to counteract activating receptors, whereas too high a dose could result in inhibitory receptors being bound individually by an agonist, which would fail to induce receptor clustering. Additionally, too high an effective dose or sustained

administration of inhibitory receptor agonists for chronic disease could result in immune suppression, which may be accompanied by excessive infections or the outgrowth of malignancies. Initially, it may therefore be preferable to focus on acute diseases in which the cause, e.g., viral infection, will dissipate. However, by breaking the vicious circle in auto-immune diseases like SLE (Fig. 1), even a short-term treatment with inhibitory receptor agonists may result in long-lasting remission. Although this remains contentious, it warrants further study. Table 1 summarises opportunities and challenges in design of agonists for inhibitory receptors.

Clearly, significant distance remains between bench and bedside for inhibitory immune receptor agonists and challenges, technical and experimental, still lie ahead. However, despite challenges, the vast potential of inhibitory immune receptors as therapeutic targets for inflammatory and autoimmune diseases warrants the effort. As was the case for immune checkpoint blockade therapy in 2013, inhibitory immune receptor ligation therapy may well be declared *Science*'s breakthrough of the year in the not-too-distant future.

Appendix

A

Summary

We live in a world absolutely teeming with microbial life; you'd be hard pressed to find a single spot on earth devoid of microbes. Indeed, even within our own bodies, our cells are outnumbered by microbes ten to one. While these microbial passengers are mostly harmless, and in fact usually helpful, others are less benign. Rather than living together in harmony as our commensal microbial guests, pathogenic microbes seek to invade our bodies for their own gain at our expense. To thwart such vile machinations, our bodies have an intricate collection of cells, organs, and molecules at their disposal, known as the immune system, to protect ourselves.

The most numerous of the immune cells, also known as white blood cells or leukocytes, is the so-called neutrophilic granulocyte, or neutrophil. Neutrophils are the immune system's first responders and quick to arrive at the site of a potential incursion by a pathogen. Certain organs are at greater risk of pathogen exposure than others. The airways, for instance, are by necessity constantly exposed to potential pathogens. After all, we need to breath to survive, but just by doing so we inhale millions of microbes each day. In most cases, neutrophils make quick work of any would-be invaders. How effective neutrophils are at this, is starkly illustrated by patients with chronic granulomatous disease (CGD) whose neutrophils are defective due to a genetic mutation. CGD patients suffer repeatedly from severe infections, which, more often than not, present in the lungs.

The need for immune regulation

In order to fight infections, the immune system has powerful armaments at its disposal. However, the more powerful the weapons, the more dangerous friendly fire becomes. Neutrophils, for instance, can produce and release substances that are lethal to microbes, but their specificity is limited. Neutrophil-derived proteases can digest proteins, reactive oxygen species (ROS) can bind and disrupt a variety of molecules, and anti-microbial peptides can insert into and disrupt the membranes that encompass (microbial) cells. Moreover, neutrophils can sacrifice themselves by releasing neutrophil extracellular traps (NETs), which involves neutrophils decorating their DNA with antimicrobial proteins and peptides and then expelling it as a web to trap and kill microbes. While such methods are highly effective in the fight against many pathogens, they also result in collateral damage to surrounding tissue. ROS, for instance, can disrupt the functioning of both microbial as well as our own proteins. It is therefore of the utmost importance that the

immune system is tightly regulated to ensure a quick and targeted, but also measured response.

If the immune response is too weak or slow, pathogens are given free rein to invade and wreak havoc. Additionally, it will allow malignancies to grow unchecked; our bodies, after all, face threats both foreign and domestic. However, an immune response that is too strong or long lasting will excessively damage surrounding healthy tissue in the resulting inflammation. It may even develop into life-long auto-immune diseases. Damage to the body that is induced by the immune system itself is referred to as immunopathology.

Respiratory syncytial virus

One such disease wherein excessive inflammation is a key pathological feature is respiratory syncytial virus (RSV) infection-induced bronchiolitis (i.e., inflammation of the lower airways). RSV is a ubiquitous pathogen that in most instances causes nothing more than a cold (i.e., inflammation of the upper airways). The vast majority of people will have been exposed to RSV at least once before the age of two, and will continue to be exposed and infected by RSV throughout their lives. However, particularly in very young infants, bronchiolitis may develop with potentially dire consequences. RSV bronchiolitis is second only to malaria as the foremost cause of infant mortality world-wide. Treatment is supportive and revolves mainly around the mechanical ventilation of severely ill patients. Children who suffered from RSV bronchiolitis are at higher risk of developing wheezing and asthma later in life.

A key characteristic of RSV bronchiolitis is a massive infiltration of neutrophils into the patients' airways. In **Chapter 2** we review the role that neutrophils play in the immune response to RSV infection. While some anti-viral properties can be ascribed to neutrophils, we conclude that presence of neutrophils in the airways is mostly pathological. In particular, neutrophil-derived ROS and proteases directly damage the epithelial lining of the airways. Moreover, the web-like NETs that neutrophils extrude can clump together mucus into plugs that block off airflow in small bronchioles. We speculate that neutrophil-induced damage to the lungs results in a higher risk of future sequelae, such as wheezing and asthma. Logically, we therefore propose that reducing neutrophil activity in the lungs could ameliorate disease and prevent long-lasting damage. Unfortunately, however, there are currently no medications available that can effectively reduce neutrophil activity.

Activating and inhibitory receptors

The lack of neutrophil inhibitors represents a great unmet clinical need. In **Chapter 3**, therefore, we set out to identify possible modulators of neutrophil activity during RSV disease. In particular, we focus on so-called inhibitory immune receptors. Cells must make decisions on how to act based on cues from the local environment, e.g., a neutrophil will act differently in a bacterially infected wound compared to the sterile blood stream. Information about the environment is relayed into cells (i.e., past the outer cell membrane) by proteins termed receptors, which are activated by binding to their target ligand. The immune system is regulated by both stimulatory and inhibitory receptors, the interplay of which determine the type and potency of an immune response. We hypothesised that inhibitory receptors modulate neutrophil activity during RSV disease. There are, however, a multitude of inhibitory immune receptors expressed differentially across a plethora of immune cells. As such, we endeavoured to identify which inhibitory receptors were capable of restricting neutrophil activity directly.

LAIR-1 in RSV disease

By examining the blood and aspirated sputum from the airways of paediatric RSV bronchiolitis patients, we discovered that neutrophils in the airway of patients display the leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) at their cell surface, ready to receive and relay signals. Comparatively, blood neutrophils stored pre-made LAIR-1 intracellularly, from where it can translocate to the cell surface upon activation. Prior to that however, LAIR-1 would not be able to transmit outside signals. LAIR-1 relays an inhibitory signal upon binding to its ligand: collagen. Since collagen is found abundantly in tissue, it may signal neutrophils not to go overboard with their destructive armaments in fragile tissues. Indeed, we found that stimulating LAIR-1 on neutrophils obtained from the airways of RSV bronchiolitis patients significantly reduced the release of NETs.

In **Chapter 4**, we further investigate the role of LAIR-1 in RSV disease. A commonly used tool in biomedical research to investigate the role of a particular gene (and the protein it encodes) in a disease process are so-called ‘knock-out mice’, in which the gene of interest has been deleted or otherwise rendered inoperable. By infecting LAIR-1 knock-out mice with RSV, we showed that LAIR-1 limits neutrophilic airway inflammation. In the absence of LAIR-1, more neutrophils infiltrate RSV-infected lungs and weight loss (a measure of disease severity) was increased. Similar results were obtained in mice in which the interaction between LAIR-1 and collagen was blocked by administrating soluble LAIR-1 protein just prior to infection with RSV. This

soluble LAIR-1 protein cannot signal, but can bind collagen. By preventing cell-bound LAIR-1 from interacting with its ligand, no inhibitory signal will be relayed. The demonstrates a direct effect of LAIR-1 during the infection, rather than the absence of LAIR-1 resulting in developmental defects in knock-out mice.

However, neutrophils are not the only cells that express LAIR-1. In fact, LAIR-1 was originally identified as an inhibitory receptor on immune cells known as 'natural killer (NK) cells'. To strengthen our case that LAIR-1 acts directly on neutrophils, we intranasally administered a compound that specifically attracts neutrophils, i.e., CXCL1, to both LAIR-1 knock-out and regular mice. The increased recruitment of neutrophils to the lungs of mice that lack LAIR-1, supports the notion that LAIR-1 directly inhibits neutrophil migration and function.

LAIR-1 as a therapeutic target

The results presented in **Chapters 3 and 4** suggest that LAIR-1 is a promising target to ameliorate neutrophil-driven pathologies. If we increase inhibitory signalling via LAIR-1, damage inflicted by neutrophils could be reduced. In order to obtain proof-of-concept data in support hereof, in **Chapter 5** we set out to generate antibodies that were able to bind to LAIR-1 and activate inhibitory signalling, with the goal of administering these antibodies to RSV-infected mice to reduce disease severity. Antibodies are proteins that can bind their targets with high specificity and affinity. However, depending on the specific binding site, an antibody may activate a target receptor or, conversely, block or antagonise it. Alternatively, and most commonly, an antibody may bind its target without notably impacting functionality. Moreover, cells that are bound by antibodies may become targets of the immune system itself, resulting in their depletion. Since it is not yet possible to predict in advance how to build an antibody with all desired characteristics, we generated a large number of different antibodies against LAIR-1 and selected those with the most favourable features.

We selected an antibody, designated 2C7, for its agonistic potential (i.e., the ability to induce signalling by LAIR-1), its non-interference with the binding of LAIR-1 to collagen, and minimal induction of immune cell depletion upon injection in mice. Unfortunately, intraperitoneal injection of the 2C7 antibody prior to inoculation of mice with RSV did not alter disease severity or leukocyte airway infiltration. The precise reason why the desired reduction in disease severity was not observed is hard to pinpoint. Possibilities include that the 2C7 antibody is not a potent enough stimulator of LAIR-1 activity or that the effective concentration of 2C7 antibody at the

required site (i.e., the lungs) was insufficient. While after intraperitoneal injection the 2C7 antibody will disperse systemically, including to the lungs, intranasal administration, for instance, may result in higher effective local concentrations. Further research into LAIR-1 as therapeutic target is ongoing.

Allergin-1: another target?

In **Chapter 6**, we investigate a different inhibitory immune receptor: Allergin-1. As opposed to LAIR-1, the ligand(s) of Allergin-1 are as of yet unknown. Inside the cell, however, Allergin-1 relays signals in a manner highly similar to LAIR-1. Moreover, previous research has demonstrated that Allergin-1 is able to suppress allergic airway inflammation. The fact that LAIR-1 and Allergin-1 signal in similar manners, and both are able to reduce inflammation in lungs (be it allergic or neutrophilic), spurred us on to investigate a potential regulatory role for Allergin-1 on neutrophils.

Allergin-1 has three different shapes it can take, known as isoforms. There is a 'long form' as well as two 'short forms', where the two short isoforms each lack different part of the long isoform. The significance of the different isoforms is not currently understood, but it may impact ligand specificity or affinity. We characterised isoform expression of Allergin-1 on a wide range of immune cells and found that the S1 short form was by far the most commonly expressed isoform. This included neutrophils in blood as well as neutrophils obtained from the airways of RSV bronchiolitis patients. Using the same mouse model of RSV disease that we employed to identify LAIR-1 as a regulator of neutrophilic inflammation, we attempted to demonstrate the same for Allergin-1. However, knock-out mice that lack Allergin-1 were wholly comparable to regular mice when infected with RSV; no differences in neutrophil infiltration or disease severity were observed. Therefore, despite their similarities, Allergin-1 and LAIR-1 are functionally distinct. The large variety of inhibitory receptors – more than 300 inhibitory immune receptors are predicted to exist – suggests that targeting specific inhibitory receptors for different diseases may be a future therapeutic strategy.

In this thesis we have identified LAIR-1 as a promising therapeutic target to limit neutrophil-driven disease. At current, the only pharmaceuticals that target immune inhibitory receptors aim to block inhibitory signalling. Doing so releases a brake from the immune system that strengthens its response. Known as immune checkpoint blockade, it has revolutionised anti-cancer treatments. However, whereas in the case of malignancies a more potent anti-tumour response is desirable, there exists a multitude of diseases that call for a softened response from an overeager immune system. Here,

targeting inhibitory receptors in a stimulatory manner may offer solace. The development of agonists to activate inhibitory receptors may prove more challenging than their blocking counterparts. Certain requirements, such as the clustering of multiple inhibitory receptor molecules, rather than simply blocking their ligand binding site, need to be met. See Figure 1 for a simplified illustration of antibodies targeting inhibitory receptors for immune-related therapies. Further research into basic inhibitory receptor biology will reveal how to best accomplish this. Moreover, there are hundreds of potential inhibitory receptors that have yet to be characterised. Despite similarities, their regulatory roles and prospects as therapeutic target may differ drastically. Clearly, it would be a worthwhile endeavour to uncover their hidden potential. The insights gained in this thesis will contribute to the development of therapeutics that target inhibitory receptors to ameliorate, or even cure, hitherto therapy-resistant immune-mediated diseases.

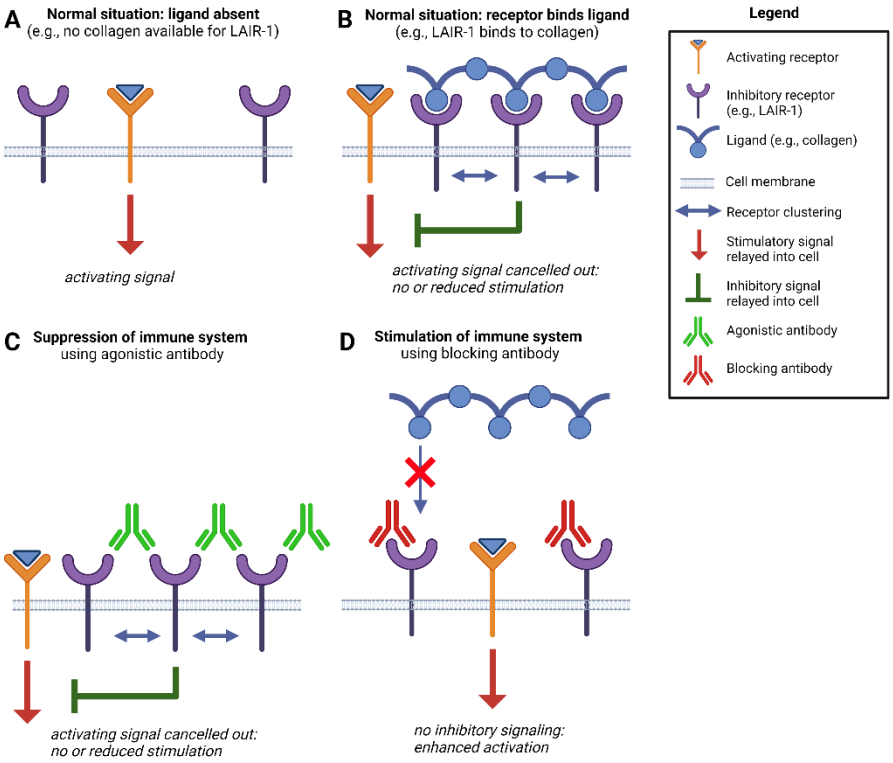


Figure 1. Agonistic and blocking antibodies for inhibitory immune receptors.
Under normal conditions, an inhibitory immune receptor will not relay an inhibitory signal into the cell in the absence of its ligand (**A**). Only when multiple inhibitory

receptors cluster together, e.g., a number of LAIR-1 molecules binding the same chain of collagen, will they transmit an inhibitory signal (**B**). Our goal is to mimic the situation in **B** by generating an agonistic antibody that induces the clustering of inhibitory immune receptors and causes them to signal, in order to dampen excessive inflammation (**C**). Where a strengthened immune response is desired, e.g., in the treatment of cancer, the interaction between inhibitory immune receptors and their ligands can be prevented by using blocking antibodies. Thusly blocked inhibitory immune receptor will not relay an inhibitory signal despite the availability of ligands (**D**).

Samenvatting

Onze planeet barst van het leven, waarvan het meeste met het blote oog niet eens is te zien. Het is vrijwel onmogelijk om een enkele plek op aarde te vinden zonder microscopisch leven. Onze eigen lichamen zijn ook gekoloniseerd door talloze microben. We dragen zelfs tien keer meer bacteriën met ons mee dan dat we lichaamscellen hebben. De overgrote meerderheid van deze microscopische passagiers zijn buitengewoon behulpzaam en bevinden zich vooral op de huid en in de darmen. Echter, een vocale minderheid bestaat uit zogenaamde pathogenen: microben die kwaad in de zin hebben. Pathogenen streven ernaar om onze lichamen binnen te dringen voor hun eigen gewin, wat voornamelijk neerkomt op vermeerdering en verdere verspreiding, ten koste van onze gezondheid. Om dergelijke aanvallen af te slaan beschikken onze lichamen over een uitgebreid samenwerkingsverband van verschillende cellen en organen die het immuun- of afweersysteem wordt genoemd.

Cellen die deel uitmaken van het immuunsysteem worden witte bloedcellen, leukocyten (afgeleid van Oudgrieks λευκός [leukos], wit) of simpelweg immuuncellen genoemd. De meest voorkomende immuuncel is de zogenaamde neutrofiele granulocyt of neutrofiel. Neutrofielen vormen de eerste verdedigingslinie van het immuunsysteem; zij zijn als eerste ter plaatse als pathogenen binnenvallen. Niet elk orgaan ondervindt evenveel blootstelling aan pathogenen. De hersenen zijn bijvoorbeeld veilig afgebakend, maar de longen worden hier dagelijks mee geconfronteerd. We moeten immers ademhalen en inhaleren zodoende elke dag miljoenen bacteriën, schimmels en virussen die in de lucht dwarrelen. In de meeste gevallen maken onze neutrofielen en ander immuuncellen hier korte metten mee. De hoge doelmatigheid en het belang van neutrofielen wordt overduidelijk als men patiënten bestudeert wier neutrofielen gedeeltelijk defect zijn door een genetische afwijking. Patiënten met chronisch granulomateuze ziekte (CGD, vanuit Engels: chronic granulomatous disease) hebben te lijden onder herhaaldelijke, zeer ernstige infecties, veelal van de luchtwegen.

Immuunregulatie is noodzakelijk

Om infecties af te slaan, beschikt het immuunsysteem over een krachtig scala aan wapens. Echter, des te sterker de wapens, des te meer schade ze ook onder eigen gelederen kunnen aanbrengen. Neutrofielen produceren moleculen die dodelijk zijn voor microben, maar ook schadelijk zijn voor onze

eigen lichaamscellen. Zo maken neutrofielen uiterst reactieve moleculen op basis van zuurstofradicalen (ROS, vanuit Engels: reactive oxygen species), zoals waterstofperoxide (een veelvuldig gebruikt ontsmettingsmiddel), en proteasen, enzymen (biologische katalysatoren of 'reactieversnellers') die eiwitten afbreken. Als ultieme zelfopoffering kunnen neutrofielen zelfs zogenaamde extracellulaire vallen (NETs, vanuit Engels: neutrophil extracellular trap) vormen. Dit houdt in dat neutrofielen hun eigen DNA met antimicrobiële moleculen bedekken om het vervolgens de cel uit te gooien als een soort plakkerig net of spinnenweb om pathogenen mee te vangen en uit te schakelen. Deze methodes zijn zeker effectief in het doden van microben, maar brengen ook schade toe aan omringende lichaamscellen en weefsel. Het is dus van groot belang dat de reactie van het immuunsysteem streng gecontroleerd wordt.

Als het immuunsysteem te zwak of te laat reageert, kunnen pathogenen vrij hun gang gaan en schade berokkenen. Daarnaast zou het kunnen leiden tot tumorgroei, aangezien het immuunsysteem ook verantwoordelijk is voor het detecteren en opruimen van kankercellen. Echter, een te sterke immuunreactie kan door aanhoudende ontsteking weefsel- en orgaanschade tot gevolg hebben. Auto-immuunziektes, zoals diabetes type 1 en reumatoïde artritis, waarbij het immuunsysteem het eigen lichaam aanvalt, zijn duidelijke voorbeelden van foutief gereguleerde immuunreacties.

Respiratoir syncytieel virus

Een veelvoorkomende infectieziekte waarbij een overdadige immuunreactie tot schade kan leiden, is bronchiolitis (ontsteking van de kleine luchtwegen; longontsteking) veroorzaakt door respiratoir syncytieel (RS) virus. Vrijwel iedereen is voor zijn of haar tweede levensjaar aan het RS-virus blootgesteld en zal gedurende zijn of haar leven herhaaldelijk geïnfecteerd worden. Meestal leidt dit tot een milde (neus)verkoudheid. Echter, de ziekte kan ook aanzienlijk ernstiger verlopen, voornamelijk onder prematuren en erg jonge kinderen. Wereldwijd veroorzaakt alleen malaria namelijk meer kindersterfte dan RS-virus bronchiolitis. In het geval van ernstige ziekte bestaat de behandeling voornamelijk uit ondersteunende zorg zoals kunstmatige beademing. Hoewel met gepaste zorg de sterfte door RS-virus bij kinderen vrijwel nihil is, kunnen langetermijneffecten, zoals astma, een gevolg zijn van ernstige RS-virusinfectie.

Kenmerkend aan het ziekteverloop van RS-virus bronchiolitis is de aanwezigheid in groten getale van neutrofielen in de luchtwegen van patiënten. In **Hoofdstuk 2** bespreken we de rol van neutrofielen in de

immuunreactie tegen RS-virus in detail. Wij concluderen dat, hoewel neutrofielen in beperkte mate uitgerust zijn om tegen RS-virus te vechten, neutrofielen voornamelijk schade aan de luchtwegen veroorzaken. De van neutrofielen afkomstige ROS en proteasen beschadigen de luchtwegen en het longweefsel. Terwijl de plakkerige NETs het mucus ('slijm') in de longen kunnen doen samenklonteren en daarmee de kleine luchtwegen blokkeren. Wij speculeren dat de schade die neutrofielen aan de longen toedragen het risico op langetermijneffecten, zoals astma, vergroten. Het remmen van neutrofiel in de longen zou volgens ons daarom de ernst van de ziekte kunnen verminderen en toekomstige aandoeningen voorkomen. Helaas is er echter tot op heden geen medicatie beschikbaar die effectief neutrofielen kan remmen.

De activerende en remmende receptoren van het immuunsysteem

Het gebrek aan effectieve farmaceutische remmers van neutrofielen is een groot klinisch gebrek. In **Hoofstuk 3** gaan wij daarom op zoek naar moleculen op neutrofielen zelf die hun activiteit kunnen verminderen. Hierbij richten wij ons op zogenaamde remmende immuunreceptoren. Receptoren zijn eiwitten aan de buitenkant van cellen die belangrijk zijn voor hun onderlinge communicatie. Cellen moeten hun gedrag namelijk aanpassen aan de omgeving. Een neutrofiel gedraagt zich bijvoorbeeld anders in de bloedbaan dan in een geïnfecteerde wond. Een receptor draagt een signaal van buiten de cel over het celmembraan (de buitenste laag van de cel) heen de cel in, waar het tot veranderingen in gedrag kan leiden. Verschillende receptoren binden hun bijbehorende signaalmoleculen. In deze context worden zulke signaalmoleculen 'liganden' genoemd; een receptor en ligand vormen zodoende een paar. Immuuncellen bezitten zowel activerende en remmende receptoren die hun activiteit reguleren. Een optelsom van activerende en remmende signalen die deze receptoren doorgeven bepaalt het uiteindelijke gedrag van de immuuncel: op welke manier en hoe krachtig deze reageert.

Er zijn een groot aantal verschillende remmende (en ook activerende) receptoren. Op basis van onze genetische code valt te voorspellen dat er circa 300 remmende immuunreceptoren zijn, waarvan tot op heden circa 60 in verschillende mate van detail zijn onderzocht. Ons doel was het identificeren van een remmende immuunreceptor die neutrofielen kan beteugelen ten tijde van RS-virus bronchiolitis.

LAIR-1 en RS-virusinfectie

Door het bloed en aspiraat uit de beademingsbuis van mechanisch geventileerde RS-virus bronchiolitis patiënten te bestuderen hebben we in

Hoofstuk 3 ontdekt dat een remmende receptor genaamd LAIR-1 (Engelse afkorting van leukocyte-associated immunoglobulin-like receptor 1) aanwezig is op het celmembraan van neutrofielen die de luchtwegen zijn ingekropen. Bij neutrofielen in het bloed daarentegen is LAIR-1 niet aanwezig op het celoppervlak maar ligt het opgeslagen in granulen (blaasjes) binnenin de cel. Wij laten zien dat wanneer neutrofielen gestimuleerd worden, LAIR-1 op het celmembraan terecht komt doordat deze granulen met het celmembraan versmelten (vergelijkbaar met hoe twee zeepbellen kunnen samensmelten). LAIR-1 kan pas signalen van buiten doorgeven aan de cel, als het zich op het celoppervlak bevindt. Onze onderzoeksgroep heeft eerder bepaald dat collageen het ligand van LAIR-1 is. Collageen is rijkelijk aanwezig in weefsel, en mogelijk dient LAIR-1 daarom om een remmend signaal aan neutrofielen af te geven dat ze zich in weefsel bevinden en niet te veel schade moeten berokkenen. We tonen dan ook aan dat het aanzetten van LAIR-1 het vormen van NETs door neutrofielen kan remmen.

Door middel van vervolgonderzoek in muizen, die hun eigen variant van LAIR-1 hebben en ook door RS-virus geïnfecteerd kunnen worden, laten we in **Hoofdstuk 4** zien dat LAIR-1 de neutrofiele longontsteking bij RS-virusinfectie kan remmen. De longontsteking en het ziekteverloop zijn ernstiger in muizen waarbij LAIR-1 is verwijderd of niet kan signaleren. Blootstelling aan sigarettenrook leidt ook tot een neutrofiele ontsteking in de longen die wordt verergerd als LAIR-1 afwezig is. Het intranasaal (via de neus) toedienen van CXCL1 (een molecuul dat specifiek neutrofielen aantrekt) leidt soortgelijk tot een grotere hoeveelheid neutrofielen in de luchtwegen bij muizen die LAIR-1 moeten missen. Wij concluderen hieruit dat LAIR-1 de ernst van neutrofiele longontstekingen beperkt.

LAIR-1 als therapeutisch doelwit

In **Hoofdstuk 3 en 4** laten we zien dat de remmende immuunreceptor LAIR-1 in staat is om neutrofielen te beteugelen en om longontstekingen waarbij neutrofielen een leidende rol spelen te dempen. In **Hoofstuk 5** stelden we daarom als doel om aan te tonen dat het stimuleren van LAIR-1 ten tijde van RS-virusinfectie met een antilichaam het ziekteverloop in muizen zou verbeteren. Een antilichaam is een eiwit dat door het immuunsysteem wordt geproduceerd en heel specifiek aan bepaald molecuul, zoals een receptor, kan binden. Afhankelijk van de manier waarop een antilichaam aan een receptor bindt, kan het de receptor activeren of juist blokkeren. Bijvoorbeeld, als het antilichaam bindt op of nabij de plek waar de receptor zijn ligand bindt, voorkomt dit dat de twee elkaar kunnen bereiken. Momenteel is het nog niet mogelijk om een antilichaam zodanig te ontwerpen dat we van tevoren

weten of deze een receptor zal activeren, blokkeren, of simpelweg geen effect heeft. We hebben daarom een aantal verschillende antilichamen tegen LAIR-1 geproduceerd en vervolgens getest welke het beste LAIR-1 kon stimuleren.

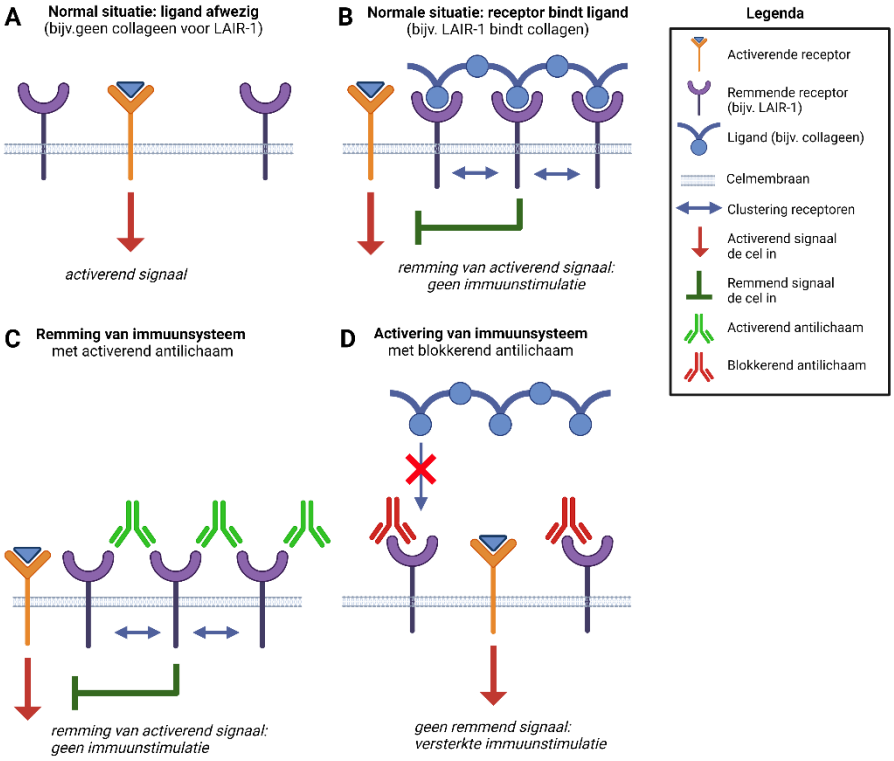
Uiteindelijk is de keuze gevallen op een antilichaam met de aanduiding 2C7. We hebben het 2C7 antilichaam intraperitoneaal (via injectie in de buikholte) toegediend alvorens muizen met RS-virus te infecteren. Dit had echter geen effect op het ziekteverloop. Mogelijk was de stimulerende werking van het antilichaam ontoereikend om een effect te bewerkstelligen. Ook zou de concentratie van het antilichaam in de longen onvoldoende kunnen zijn geweest. Hoewel het antilichaam zich na intraperitoneale injectie over het lichaam verspreidt, zou een lokale (intranasale) toediening mogelijk tot hogere concentraties in de luchtwegen kunnen leiden. Verder onderzoek naar LAIR-1 als therapeutisch doelwit is nog gaande.

Allergin-1: nieuw doelwit, nieuwe kansen?

In **Hoofdstuk 6** bestuderen we een andere remmende immuunreceptor genaamd Allergin-1. Uit eerder onderzoek is gebleken dat Allergin-1 op eenzelfde manier als LAIR-1 signalen doorgeeft aan de cel. Het ligand van Allergin-1 is nog niet ontdekt, maar wel is uit muizenonderzoek gebleken dat Allergin-1 in staat is om allergische ontstekingen in de longen te dempen. Wij onderzochten daarom of Allergin-1 ook neutrofiele longontstekingen zou kunnen remmen. Omdat er drie varianten van Allergin-1 zijn, die isovormen genoemd worden, hebben we ook in kaart gebracht welke variant van Allergin-1 op welk soort immuuncel voorkomt, zowel in gezonde mensen als in patiënten met een chronische ontsteking en kinderen met RS-virus bronchiolitis. Hieruit bleek o.a. dat een ontsteking, chronisch of acuut, geen invloed heeft op welke variant van Allergin-1 aanwezig is op immuuncellen. In muizen leidt een afwezigheid van Allergin-1 niet tot een ernstigere longontsteking of ziekteverloop. In tegenstelling tot LAIR-1 lijkt Allergin-1 daarom geen wezenlijke rol te spelen bij de regulering van neutrofielen in de longen. Hieruit blijkt dat remmende immuunreceptoren op dezelfde immuuncel sterk uiteenlopende rollen kunnen hebben.

In dit proefschrift hebben we vastgesteld dat de remmende immuunreceptor LAIR-1 veel belofte vertoont als therapeutisch doelwit voor het verminderen van schade die door overenthousiaste neutrofielen wordt veroorzaakt. Momenteel zijn er wel medicijnen op de markt die zich richten op remmende immuunreceptoren, maar dit zijn enkel antilichamen met als doel om de signalering van remmende immuunreceptoren te blokkeren. Dit versterkt de

immuunreactie, wat buitengewoon wenselijk is voor de behandeling van kanker. Deze blokkerende antilichamen vormen dan ook de basis van de baanbrekende 'immune checkpoint blockade' therapie, die remissie van voorheen onbehandelbare tumoren mogelijk heeft gemaakt. Er zijn echter ook ziektebeelden, zoals RS-virus bronchiolitis en auto-immuunziektes, waarbij het juist wenselijk is om de immuunreactie te dempen. Ook hier zouden remmende immuunreceptoren een revolutie voor de behandeling kunnen bewerkstelligen. Een voorwaarde hiervoor is dat er effectieve agonisten (moleculen die een receptor stimuleren) ontwikkeld worden. Dit zou meer moeilijkheden met zich mee kunnen brengen dan de nu al zo succesvolle blokkerende antilichamen. Een effectieve agonist voor remmende immuunreceptoren moet namelijk o.a. de clustering van meerdere receptormoleculen faciliteren, terwijl een blokkerend antilichaam 'alleen maar' de bindingsplek van het ligand hoeft af te schermen. Zie ook Figuur 1 voor een overzicht van hoe activerende en blokkerende antilichamen voor remmende immuunreceptoren werken. Desalniettemin is de belofte van remmende immuunreceptoren als therapeutisch doelwit in onze optiek dusdanig groot dat het aangaan van deze uitdaging de moeite dubbel en dwars waard zal zijn. De inzichten die zijn opgedaan bij het tot stand komen van dit proefschrift zullen bijdragen aan de ontwikkeling van agonisten voor remmende immuunreceptoren die tot op heden beperkt behandelbare of zelfs onbehandelbare immuun-gerelateerde ziektes kunnen verminderen in ernst dan wel genezen.



Figuur 1. Activerende en blokkerende antilichamen voor remmende immuunreceptoren.
Onder normale omstandigheden stuurt een remmende receptor geen signaal de cel in als er geen ligand is om aan te binden (**A**). Pas als meerdere remmende receptoren samenkomen door aan een ligand te binden (bijv. meerdere LAIR-1 moleculen die aan keten van collageen binden), zullen de geclusterde remmende receptoren een signaal afgeven de cel in (**B**). Wij streven de situatie in **B** na te bootsen door antilichamen te maken die remmende receptoren kunnen clusteren zodat deze gaan signaleren, om zo overdreven ontstekingen te remmen (**C**). Voor de behandeling van o.a. kanker is het juist wenselijk om de immuunrespons tegen de tumor te versterken. Dit kan worden bewerkstelligd door ervoor te zorgen dat remmende immuunreceptoren geen signaal kunnen afgeven door de binding met hun ligand te blokkeren (**D**).

Dankwoord

Fin!
Eindelijk is het dan zover: mijn 'boekje' (ik zet mijn vraagtekens bij de gebruikelijke verwijzing naar een proefschrift met een verkleinwoord) is af! In mijn eentje had ik deze berg nooit kunnen beklimmen. Zonder steun in, op, rond en buiten het lab had ik deze tocht niet tot een succesvol einde kunnen brengen. Mijn dank aan iedereen voor jullie steun, begrip, aanmoediging en advies is dan ook onmeetbaar groot.

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A big thank you as well to all Bonte Meyaardjes, past and present, and all CTI colleagues at large. Together, you provided the scientifically stimulating and socially supportive environment that facilitated the completion of this thesis.

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vasthouden. Dankzij jullie steun, door dik en dun, ben ik zover gekomen. Ontzettend bedankt!

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Iedereen, bedankt!

Curriculum Vitae

Ruben Joost Geerdink was born on the 11th of July 1990 in Rotterdam, the Netherlands. He obtained his Bachelor of Science degree in Biomedical Sciences *cum laude* in 2011 at the University of Utrecht. In 2014 he completed the Master's programme 'Infection & Immunity' at Utrecht University and obtained his Master of Science degree *cum laude*. His first Master's internship was completed in the research group of prof. dr. E.J.H.J. Wiertz, under supervision of dr. M.E. Ressing at the University Medical Centre Utrecht (the Netherlands). His second and final research internship was performed in the research group of prof. dr. A. Shibuya, under supervision of dr. S. Tahara-Hanaoka at the University of Tsukuba (Japan).

During his second internship, he collaborated with prof. dr. Linde Meyaard and prof. dr. Louis Bont to write a research proposal for the NWO graduate programme 2012. The proposal was accepted and provided the necessary funds for him to start working on inhibitory immune receptors on neutrophils as a Ph.D. candidate in 2014 under the combined supervision of prof. dr. Linde Meyaard and prof. dr. Louis Bont.

List of Publications

This thesis

1. **Geerdink RJ**, Pillay J, Meyaard L, Bont L. Neutrophils in respiratory syncytial virus infection: A target for asthma prevention. J Allergy Clin Immunol. 2015 Oct;136(4):838-47. doi: 10.1016/j.jaci.2015.06.034. Epub 2015 Aug 12. PMID: 26277597; PMCID: PMC7112351.
2. **Geerdink RJ**, Hennus MP, Westerlaken GHA, Abrahams AC, Albers KI, Walk J, Wesselink E, Janssen R, Bont L, Meyaard L. LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis. J Allergy Clin Immunol. 2018 Feb;141(2):811-814. doi: 10.1016/j.jaci.2017.08.031. Epub 2017 Oct 16. PMID: 29050972.
3. Kumawat K*, **Geerdink RJ***, Hennus MP, Roda MA, van Ark I, Leusink-Muis T, Folkerts G, van Oort-Jansen A, Mazharian A, Watson SP, Coenjaerts FE, Bont L, Meyaard L. LAIR-1 Limits Neutrophilic Airway Inflammation. Front Immunol. 2019 Apr 26;10:842. doi: 10.3389/fimmu.2019.00842. PMID: 31080449; PMCID: PMC6497752.
4. **Geerdink RJ**, Pascoal Ramos MI, Van den Hoogen, LL, Radstake TRDJ, Shibayama S, Shibuya A, Bont L, Meyaard L. Differential isoform expression of Allergin-1 during acute and chronic inflammation. Immun Inflamm Dis. 2022 Dec;10(12):e739. doi: 10.1002/iid3.739. PMID: 36444625; PMCID: PMC9695092.

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Other publications

1. Horst D, **Geerdink RJ**, Gram AM, Stoppelenburg AJ, Ressing ME. Hiding lipid presentation: viral interference with CD1d-restricted invariant natural killer T (iNKT) cell activation. Viruses. 2012 Oct 23;4(10):2379-99. doi: 10.3390/v4102379. PMID: 23202469; PMCID: PMC3497057.
2. van Gent M, Gram AM, Boer IGJ, **Geerdink RJ**, Lindenbergh MFS, Lebbink RJ, Wiertz EJ, Ressing ME. Silencing the shutoff protein of Epstein-Barr virus in productively infected B cells points to (innate) targets for immune evasion. J Gen Virol. 2015 Apr;96(Pt 4):858-865. doi: 10.1099/jgv.0.000021. Epub 2014 Dec 12. PMID: 25502648.
3. Pascoal Ramos MI, Keşmir C, Stok JE, **Geerdink R**, Satravelas N, Westerlaken GHA, Meyaard L, van der Vlist M. CD200R1L is a functional evolutionary conserved activating receptor in human neutrophils. J Leukoc Biol. 2022 Feb;111(2):367-377. doi: 10.1002/JLB.2A0520-334R. Epub 2021 Apr 22. PMID: 33884657; PMCID: PMC9291319.