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Targeting Antiviral Mechanisms in Asthma: Pharmacological Modulation of Epithelial Viral Sensing

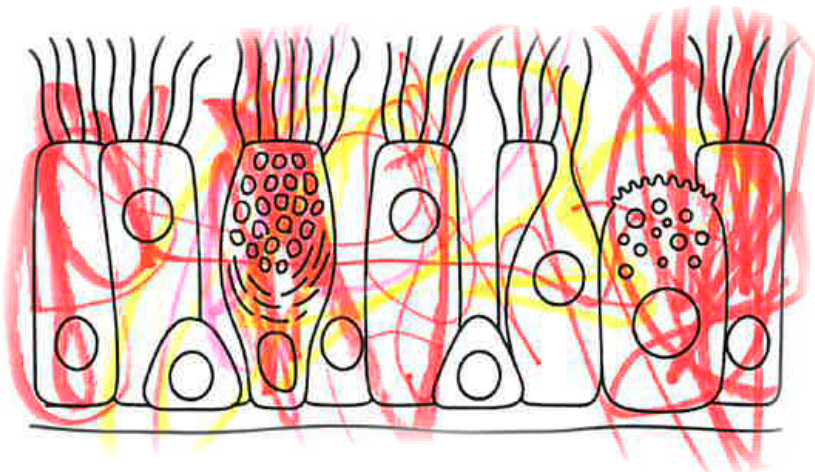
Malm Tillgren, Sofia

2026

Targeting Antiviral Mechanisms in Asthma

Pharmacological Modulation of Epithelial Viral Sensing

SOFIA MALM TILLGREN
EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on Thursday 12th of February 2026 at 09.00 in Segerfalksalen, BMC, Lund

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We demonstrate that imiquimod, a TLR7 agonist, suppresses the poly(I:C) (a viral replication mimic)-induced pro-inflammatory and alarmin responses, while enhancing antiviral signaling in bronchial epithelial cells (BECs) from patients with asthma. We also demonstrate that following three days of intranasal poly(I:C) administration, BALB/c and C57Bl/6J, but not C57Bl/6N, mice show upregulation of general inflammatory markers in the lung, identifying them as suitable models for studying antiviral immune responses in the lung. Furthermore, we show that azithromycin, a macrolide antibiotic, enhances antiviral signaling in BECs from patients with asthma regardless of clinical asthma phenotype, via the TBK1/IKKε-IRF3/IRF7 axis. Interestingly, a macrolide with negligible antimicrobial activity showed similar effects, suggesting that the antiviral effects of azithromycin are independent of its antimicrobial activity.

This thesis sheds light on different pharmacological approaches to normalizing altered immune mechanisms in the asthmatic bronchial epithelium and broadens our understanding of the immunomodulatory mechanisms of macrolide antibiotics.

Key words: asthma, asthma exacerbations, rhinovirus, imiquimod, azithromycin, antiviral defense

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”Det du inte gör idag, slipper du göra om imorgon”

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II. **Malm Tillgren S**, Nieto-Fontarigo JJ, Cerps S, Ramu S, Menzel M, Mahmutovic Persson I, Meissner A, Akbarshahi H, Uller L. C57Bl/6N mice have an attenuated lung inflammatory response to dsRNA compared to C57Bl/6J and BALB/c mice. J Inflamm (Lond). 2023 Feb 21;20(1):6. doi: 10.1186/s12950-023-00331-4. PMID: 36810092; PMCID: PMC9942641.

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Papers not included in the thesis

1. Cerps S, Sverrild A, Ramu S, Nieto-Fontarigo JJ, Akbarshahi H, Menzel M, Andersson C**, Tillgren S**, Hvidtfeldt M, Porsbjerg C, Uller L. House dust mite sensitization and exposure affects bronchial epithelial anti-microbial response to viral stimuli in patients with asthma. Allergy. 2022 Aug;77(8):2498-2508. doi: 10.1111/all.15243. Epub 2022 Feb 11. PMID: 35114024; PMCID: PMC9546181.
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Abstract

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Selected Abbreviations

Azm	azithromycin
BEC	bronchial epithelial cell
COVID-19	coronavirus disease 2019
DC	dendritic cell
dsRNA	double-stranded RNA
FeNO	fractional exhaled nitric oxide
FEV1	forced expiratory volume in 1 second
FVC	forced vital capacity
ICS	inhaled corticosteroids
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
MDA5	melanoma differentiation-associated protein 5
NF-κB	nuclear factor kappa-light chain enhancer of activated B-cells
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene I
RV	rhinovirus
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
ssRNA	single-stranded RNA
T2	type 2
TLR	toll like receptor

Introduction

Asthma

Asthma is a heterogeneous chronic respiratory disease affecting millions of people worldwide. In 2019, an estimated 262 million people suffered from asthma, and in the same year, asthma caused 455,000 deaths (1). The social and economic burden of asthma is high, both due to direct costs, such as hospitalization and medication, and indirect costs, including loss of work and school time. Moreover, the prevalence of asthma has increased over the past decades, which further contributes to the global burden (2). Effective treatment is still lacking for some patients, underscoring the importance of asthma research.

Asthma definition and diagnosis

Asthma is characterized by airway inflammation, mucus hypersecretion, airway hyperresponsiveness and airway remodeling. Airway hyperresponsiveness refers to the exaggerated tendency of the airways to constrict in response to various stimuli, leading to reversible and variable airflow limitation. This, in turn, causes symptoms such as wheezing, cough, shortness of breath and chest tightness (3).

Airway remodeling comprises structural changes and overproduction of extracellular matrix (ECM) proteins in the basement membrane, lamina propria and submucosa. In addition, airway smooth muscle cell hyperplasia and hypertrophy are features of airway remodeling in asthma. Together with fibroblasts and epithelial cells, smooth muscle cells contribute to ECM protein overproduction, promoting airway wall thickening and stiffness thereby contributing to symptoms such as wheezing, dyspnea and airflow limitation (4).

Mucus hypersecretion is a result of goblet cell hyperplasia, which contributes to mucus plugging, airflow obstruction and cough (5, 6).

A major feature of asthma is that symptoms vary both over time and in intensity. These fluctuations often manifest as exacerbations, defined as acute or subacute changes in symptoms and lung function from baseline, that require additional medication (7). Exacerbations are commonly triggered by environmental factors such as respiratory viral infections, bacterial infections, aeroallergens, air pollution or smoke, but can also be triggered by exercise (1, 8). Exacerbations are also

associated with increased hospitalizations and can be life threatening if not managed properly. Indeed, the majority of asthma-related health care costs are attributed to asthma exacerbations (8, 9).

Asthma diagnosis relies on a multifactorial evaluation of clinical, familial and functional parameters. First, asthma presents with a characteristic pattern of respiratory symptoms, including wheezing, shortness of breath, chest tightness and cough together with a variable airflow limitation. Although these symptoms can be caused by other conditions, in asthma they are typically worse at night or in the morning, and, as mentioned, vary in intensity over time. Second, family history is assessed, since having relatives with asthma increases the likelihood of developing the disease. Finally, lung function and variability in airflow limitation are assessed using spirometry before and after treatment with a bronchodilator or a bronchial challenge with, for example, mannitol. Forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) are measured and compared pre- and post-test. An increase in FEV1 or FVC of more than 12% after bronchodilation, and/or a fall in FEV1 of more than 20% after bronchial challenge, supports a diagnosis of asthma. The FEV1/FVC ratio is also examined to assess the degree of airflow limitation (7).

Asthma heterogeneity: defining phenotypes and endotypes

The complexity of asthma is being increasingly understood, and asthma is now regarded as an umbrella term rather than a single disease. In recent years, two important pillars have been highlighted: (i) the clinical and inflammatory *phenotypes* and (ii) the underlying *endotypes.*

A phenotype describes an observable clinical, biological or physiological trait, such as eosinophilia, age of onset, allergic sensitization, atopy or obesity. The endotype describes the underlying causes of the phenotypes at a cellular and molecular level, and can broadly be categorized into type 2 (T2)-high or T2-low inflammation (figure 1) (10).

The molecular mechanisms underlying T2-high and T2-low asthma will be discussed in more detail later, but briefly, T2-high asthma is characterized by elevated levels of T2 inflammation, involving type 2 helper T-cells (Th2), type 2 innate lymphoid cells (ILC2s) and the production of interleukin (IL)-4, IL-5 and IL-13 with airway eosinophilia. This endotype is more often linked to atopy, aspirin-exacerbated respiratory disease or exercise-induced asthma. Clinical markers include high fractional exhaled nitric oxide (FeNO), serum IgE and blood and sputum eosinophils. These patients often benefit from biological therapies targeting specific T2 cytokines (11).

In contrast, T2-low asthma is less well described, and reliable biomarkers are currently lacking. This endotype is instead defined by the absence of T2-associated

markers and may involve either neutrophilia or paucigranulocytic airway inflammation. Th17 cells and their cytokines, including IL-17 and TNF-α, have been implicated in driving T2-low inflammation and promoting neutrophil recruitment to the airways (12). In addition, Th1 cells have been suggested to contribute to this endotype by producing high levels of IFN-γ, which may drive airway hyperresponsiveness (13). The T2-low endotype is typically associated with non-atopic, obese or smoking patients.

In precision medicine, both phenotyping and endotyping are important for achieving treatment success, as targeting a phenotypic trait may not be effective if the endotype is not considered. It is also important to note that both endotypes and phenotypes are dynamic and may change over time (10).

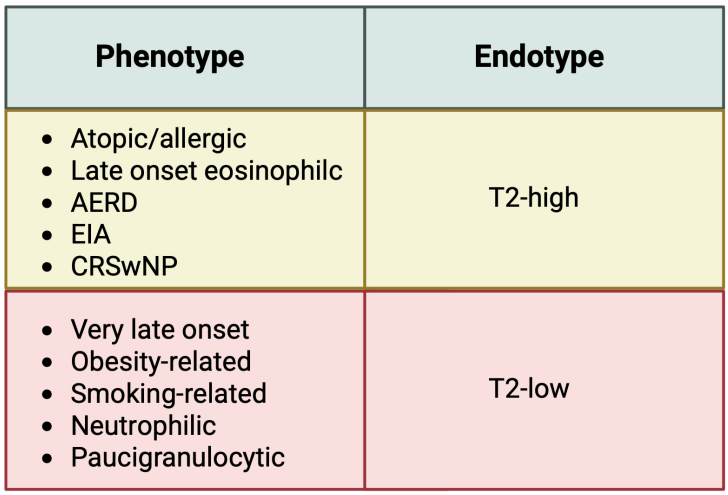

Figure 1. Asthma phenotypes and endotypes. AERD: aspirin-exacerbated respiratory disease. CRSwNP: chronic rhinosinusitis with nasal polyps. EIA: exercise-induced asthma. T2: type 2. The figure was adapted from Wenzel, S. et al. *Nat Med.* 18, 716–725 (2012), and created in https://BioRender.com.

Genetic and environmental drivers of asthma

There is no single cause of asthma, rather, a combination of hereditary and environmental factors contributes to its development. Early-life risk factors include low birth weight, premature birth, exposure to tobacco smoke or other air pollutants and respiratory viral infections. Later in life, exposure to air pollution, allergens, molds and occupational chemicals, as well as obesity and overweight status, further increases the risk. Asthma is also more common in individuals with allergic conditions such as eczema, food allergy and rhinitis (14).

Furthermore, a family history of asthma increases the risk of developing asthma, highlighting the contribution of genetic factors (14). Asthma is a polygenic disorder,

with genome-wide association studies identifying more than 400 different genetic variants linked to an increased risk. Notably, single nucleotide polymorphisms (SNPs) in genes such as interleukin-1 receptor-like 1 (IL1RL1), thymic stromal lymphopoietin (TSLP), IL-13, nedd4 family interacting protein 1 (NDFIP1), human leukocyte antigen-DR isotype (HLA-DR) and IL-33 are strongly associated with asthma susceptibility (15, 16).

Asthma management: a stepwise approach

There is no cure for asthma, and treatment is therefore aimed at reducing symptoms and preventing exacerbations. Asthma treatment is based on a stepwise approach, described by the Global Initiative for Asthma (GINA), an independent organization of leading experts and patient representatives worldwide. Together, they work to increase awareness, improve management and decrease asthma-related morbidity and mortality. The GINA guidelines consist of five main steps, ranging from mild to severe disease.

For mild to moderate asthma, most patients receive mainstay or as-needed low-medium doses of inhaled corticosteroids (ICS) as controller medications. These are usually combined with so-called relievers, drugs that give quick relief, such as short- or long-acting β2-agonists (SABA and LABA, respectively). If asthma control is not achieved, the ICS dose can be increased and a long-acting muscarinic antagonist (LAMA) may be introduced. If these measures are still insufficient, the asthma phenotype is assessed, and biological drugs such as anti-IL-4, anti-IL-5 or anti-TSLP may be prescribed. The GINA guidelines also mention other alternatives for difficult-to-treat asthma, including either the macrolide antibiotic azithromycin (azm) or a low dose of oral corticosteroids (7, 17).

Despite the available treatment options, approximately 3-10% of patients have severe asthma and require high doses of inhaled corticosteroids along with a second controller medication to prevent exacerbations, or they continue to experience frequent exacerbations despite therapy (18). Having frequent exacerbations has been linked to a decline in lung function over time and is a major risk factor for future exacerbations. Although this patient group is small, it accounts for the majority of asthma-related costs and mortality, which highlights the urgent need for new treatment strategies for these patients (19-22).

The airway epithelium

The airway epithelium as a physical barrier

The airway epithelium lines the airways and functions as a barrier to the external environment, and is our first line of defense against airborne agents. Through cell-cell interactions via tight junctions, gap junctions, adherens junctions and desmosomes, the epithelial layer forms a physical barrier that prevents inhaled agents from entering our body (23). The epithelium consists of several different cell types that vary throughout the respiratory system. In the conducting airways, stretching from the trachea to the bronchi, no gas exchange occurs. Instead, air is transported, humidified and heated, and any inhaled pathogen is cleared. Here, ciliated, goblet, club and basal cells dominate, together with the rarer pulmonary neuroendocrine cells, brush cells and pulmonary ionocytes (figure 2) (24, 25). The central airway cell layer is pseudostratified, meaning that even though it may appear multi-layered, all cells have contact with the basement membrane. Goblet cells secrete mucus, a gel-like secretion composed mainly of highly charged glycoproteins called mucins, mixed with antiviral and antibacterial molecules such as IgA, cytokines, antioxidants and antimicrobial peptides. The mucus creates a barrier that traps pathogens and is then cleared from the airway by ciliated cells, whose hair-like structures beat to move the mucus up the airway tree. Basal cells are the main stem cells of the airway epithelium, generating new cells in response to injury. Club cells are also multipotent, and can act as stem cells in response to injury. They also secrete uteroglobin, a protein with anti-inflammatory and immunosuppressive properties (figure 2) (26).

After passing through the conducting zone, the air reaches the respiratory zone and the alveoli. Here, the epithelium is no longer pseudostratified, and the predominant cell types are the squamous alveolar epithelial cell type 1 (AEC1) and the cuboidal alveolar epithelial cell type 2 (AEC2) cells. AEC1 facilitate gas exchange, while AEC2 produce surfactant that reduces surface tension (figure 2) (27).

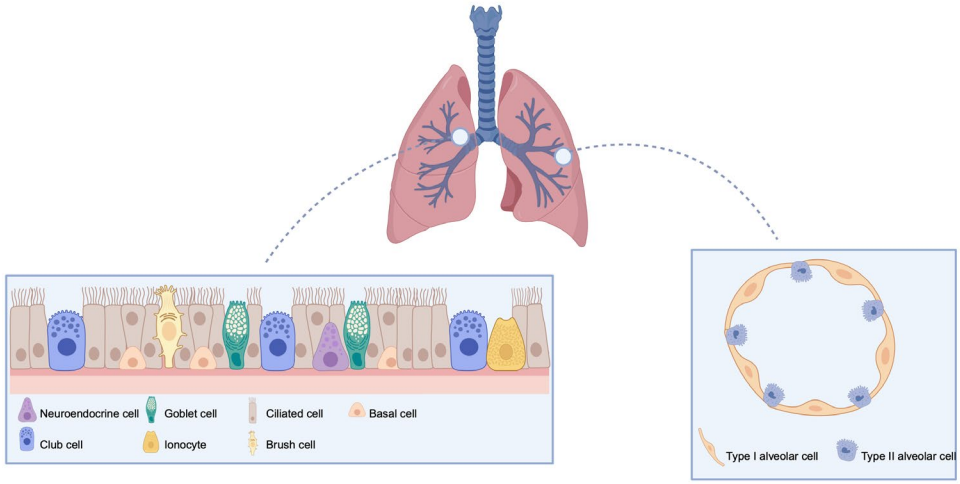

Figure 2. The structural composition of the central (left) and the peripheral (right) airway epithelium. Figure was created in https://BioRender.com.

The airway epithelium as an immunological barrier

Pattern recognition receptors

Another important function of the airway epithelium is to provide an immunological barrier towards inhaled agents. Epithelial cells act as innate immune cells by sensing the local environment and signal to other cells when necessary. This occurs through pattern recognition receptors (PRRs). PRRs share a common structure, typically consisting of three domains: a ligand-recognition domain, an intermediate domain and an effector domain. The ligand-recognition domain recognizes pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) and virus-derived RNA or DNA. It also recognizes danger-associated molecular patterns (DAMPs), which are released from apoptotic or necrotic cells. Upon recognition, the intermediate domain transmits the signal to the effector domain, which recruits adaptor proteins that relay downstream signaling.

Toll-like receptors

The first group of PRRs that was discovered were the toll-like receptors (TLRs). To date, 10 different TLRs (TLR1-10) have been identified in humans, each specialized to recognize specific PAMPs or DAMPs. For example, TLR5 recognizes bacterial flagellin, TLR4 bacterial lipopolysaccharides, TLR3 viral double-stranded RNA (dsRNA) and TLR7 viral single-stranded RNA (ssRNA). The localization of the TLRs within the cell reflects the type of pathogen they are designed to detect. TLR4 and TLR5 are therefore positioned at the cell membrane, where they can sense

extracellular bacteria, while TLR3 and TLR7 are localized to endosomes, enabling recognition of replicating viruses inside the cell.

C-type lectin receptors, NOD-like receptors and RIG-I-like receptors

In addition to TLRs, three other groups of PRRs have been identified: C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs). Similar to TLR4 and TLR5, NLRs are specialized in recognizing bacterial components, such as diaminopimelic acid from the cell wall of gram-negative bacteria. In addition, certain NLRs can recognize viral ssRNA. CLRs are characterized by their C-type lectin domain, and bind to a variety of pathogen structures, such as mycobacteria. RLRs comprise melanoma differentiation-associated protein 5 (MDA5), retinoic acid-inducible gene I (RIG-I) and laboratory of genetics and physiology 2 (LGP2), which play key roles in the recognition of intracellular RNA viruses such as rhinoviruses (RV) (28).

Pattern recognition receptor signaling following rhinovirus infections

In the context of asthma, RV infections are the most common cause of exacerbations (29-32). RV is primarily recognized by the cytosolic MDA5 and RIG-I, which recognize dsRNA produced during viral replication, as well as by the endosomal TLR3, which also recognizes dsRNA (33-35). Upon dsRNA binding, MDA5 and RIG-I interact with mitochondrial antiviral signaling protein (MAVS) via their caspase recruitment domains (CARDs), which translocate the complex to the mitochondria or peroxisomes. The signal is then relayed downstream to the kinases TANK-binding kinase 1 (TBK1) and IκB kinase ε (IKKε), which phosphorylate the transcription factors interferon regulatory factor (IRF) 3 and/or 7, as well as nuclear factor kappa-light chain enhancer of activated B cells (NF-κB). Activated IRFs and NF-κB translocate to the nucleus and promote transcription of interferons (IFNs) and immunomodulatory cytokines to orchestrate the antiviral immune response (figure 3) (36).

TLR3 is anchored to an endosomal membrane and recognizes dsRNA from within endosomes. Signaling occurs through a TIR-domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathway, which can signal through both tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) and TRAF6, NF-κB-activating kinase associated protein 1 (NAP1) and TBK1/IKKε, or receptor-interacting protein-1 (RIP-1) and transforming growth factor-β-activated kinase 1 (TAK1) and IKK-α/β. Similar to MDA5 and RIG-I signaling, these pathways converge to activation of NF-κB or IRF3/7, ultimately driving transcription of IFN and pro-inflammatory cytokines. In addition, TLR3 signaling can activate activator protein 1 (AP-1), further contributing to IFN and cytokine transcription (figure 4) (37).

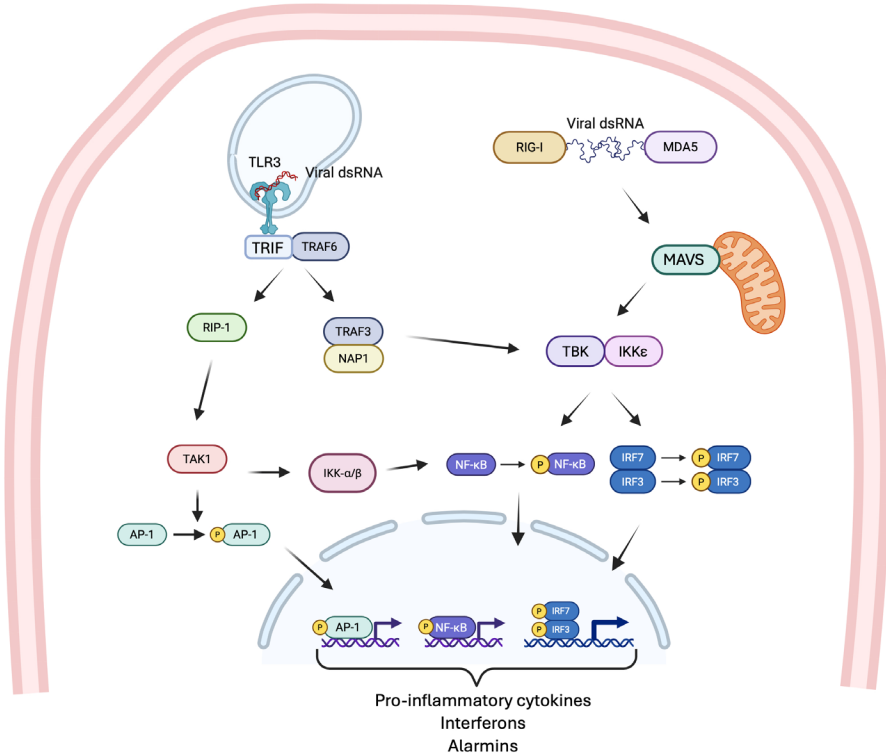

Figure 3. TLR3 and MDA5/RIG-I signaling upon viral infection. Figure was adapted from Chen Y, et al. J Zhejiang Univ Sci B. Aug 15;22(8):609-632 (2021) and Rehwinkel, J. et al. Nat Rev Immunol 20, 537–551 (2020). Figure was created in https://BioRender.com.

The following sections will outline the major classes of molecules that are transcribed and translated upon PRR recognition of viral particles:

Interferons

IFNs are a diverse group of cytokines involved in antiviral defense and immune regulation. They are classified into three different types: type I, type II and type III. Type I IFNs, including IFN-β and IFN-α, are produced mainly by innate immune cells, but are also expressed by airway epithelial cells. Type II IFNs consist solely of IFN-γ, which is expressed by activated immune cells, such as natural killer (NK) and T cells. Type III IFNs, or IFN-λ, are mostly expressed at epithelial and endothelial barriers, where they act locally. The transcription of IFNs is mainly governed by IRFs, but can also be regulated by NF-κB and AP-1 (38, 39).

The three different IFN classes signal through three different IFN receptors: the IFN-α receptor (IFNAR), the IFN-γ receptor (IFNGR) and the IFN-λ receptor (IFNLR). Both IFNAR and IFNLR activate janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), leading to phosphorylation of signal transducers and activators of

transcription (STAT) 1 and 2. Together with IRF9, they form the IFN-stimulated gene factor 3 (ISGF3) complex, which translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) on the promoter region of interferon-stimulated genes (ISGs), promoting their transcription (40). ISGs encode proteins that restrict viral replication and spread. For instance, they block viral entry, target viral proteins for proteasomal degradation and inhibit transcription and translation of viral genomes (41). The IFNGR, on the other hand, signals through both JAK1 and JAK2, resulting in STAT1 homodimerization and translocation to the nucleus (40). Once in the nucleus, STAT1 binds to IFN-γ activated site (GAS) elements located in the promoter regions of specific ISGs, thereby driving their transcription (figure 4) (42).

Cytokines and chemokines

The activation of PRRs and subsequent activation of NF-κB and AP-1 leads to transcription of a wide range of pro-inflammatory cytokines. For instance, NF-κB activates transcription and release of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β (43). These cytokines are very diverse, and exert various effects on both inflammation and immunity. IL-6 promotes synthesis of acute-phase proteins, enhances B-cell antibody production and supports CD4+ T-cell development (44). TNF-α possesses antiviral properties by interfering with viral replication and boosting cytotoxic activity of natural killer (NK) cells (45, 46). Similarly, IL-1β exhibits antiviral activity, and enhances CD4 and CD8+ T-cell responses (47, 48).

In addition, activation of NF-κB induces the transcription of chemokines, such as C-X-C motif chemokine ligand 10 (CXCL10), CXCL8, chemokine (C-C motif) ligand 2 (CCL2) and CCL5. The primary function of chemokines is to recruit innate and adaptive immune cells such as T-lymphocytes, neutrophils, dendritic cells (DCs) and monocytes to the site of infection, to enhance the immune response. By producing cytokines and chemokines, airway epithelial cells play a crucial role in both initiating and sustaining inflammatory responses to pathogens, serving as a bridge between innate and adaptive immunity (figure 4) (43).

Alarmins

IL-33, IL-25 and TSLP belong to a group of cytokines called alarmins, which are epithelial-derived cytokines that are pre-stored within the cells, enabling a rapid release in response to cellular damage or stress. They regulate infection, inflammation and metabolic homeostasis, and are released upon PRR activation, mechanical injury, other cytokines or proteases (49, 50). Alarmins act on a broad range of cells, and generally promote T2 inflammation by acting on DCs, Th2 and ILC2s. In addition, mast cells, macrophages and eosinophils express alarmin receptors, highlighting the wide impact of alarmins on immune regulation (figure 4) (49).

IL-33, TSLP and IL-25 all play key roles in host defense against helminths, bacteria and fungi (51-53). Unlike other pro-inflammatory cytokines, alarmins are constitutively expressed, allowing their rapid release. However, their expression can also be further enhanced during inflammation (51).

IL-33

IL-33 belongs to the IL-1 superfamily and is expressed in the nuclei of epithelial, endothelial and stromal cells. Its receptor, ST2, is expressed on Th2 cells, mast cells, ILC2s, and regulatory T cells (Tregs). Binding of IL-33 to ST2 leads to the production of T2 cytokines from both Th2 cells and ILC2s, thereby amplifying T2 inflammation. IL-33 also activates mast cells, further aggravating any ongoing allergic response (54). The ST2 receptor also exists in a soluble isoform, which acts as a decoy receptor (55).

TSLP

TSLP is a multifunctional cytokine expressed by various cell types, with epithelial and stromal cells in the lungs, skin and gastrointestinal tract being the main sources. TSLP exists in both a short form and a long form. The short form is constitutively expressed and is not induced by inflammation, whereas the long form is expressed following TLR stimulation (50).

Its receptor is a heterodimeric complex composed of the TSLP receptor (TSLPR) and the IL-7 receptor α-chain (IL-7Rα). This receptor complex is expressed on DCs, macrophages, mast cells, basophils and ILC2s. Binding of TSLP to its receptor triggers JAK1 and JAK2 signaling, which leads to production of IL-4, IL-5, IL-9 and IL-13 that drive T2 inflammation (50). In addition, TSLP promotes T-cell proliferation and upregulates co-stimulatory molecules on DCs necessary for Th2 differentiation (56).

TSLP has also been described to drive non-T2 inflammatory responses by promoting differentiation of Th17 cells and recruitment of neutrophils to the airways (57). Supporting this, anti-TSLP treatment in asthma has proven effective even in patients with non-T2-inflammation (58).

IL-25

IL-25, also known as IL-17E, belongs to the IL-17 family and, similar to IL-33 and TSLP, is a multifunctional alarmin. In addition to epithelial cells, IL-25 is produced by Th2, CD8+ T-cells, mast cells, alveolar macrophages, DCs, eosinophils, basophils and airway endothelial cells. Its receptors, IL-17RB and IL-17RA, are expressed on T-cells, ILC2s, fibroblasts, epithelial cells and endothelial cells. Binding of IL-25 to its receptor activates several downstream signaling mediators, including the JAK/STAT and NF-κB pathways. Like the other alarmins, IL-25

induces proliferation and cytokine production in receptor-expressing cells, thereby contributing to T2 inflammation (59).

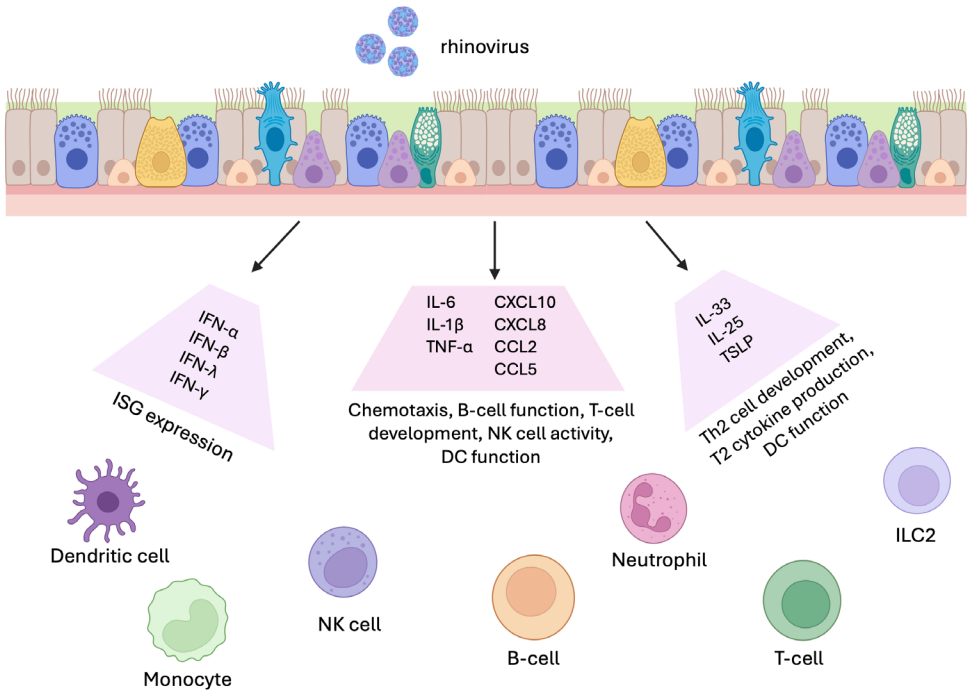

Figure 4. Outcomes of epithelial PRR signaling following rhinovirus infection. ISG; interferon stimulated gene. NK; natural killer. DC; dendritic cell. ILC2; innate lymphoid cell 2. Figure was created in https://BioRender.com.

The airway epithelium in asthma

Disruption of the structural barrier in asthma

The structural barrier of the epithelium is frequently compromised in asthma, with disruption of tight and adherens junction proteins such as E-cadherin, zonula occludens-1 (ZO-1) and occludin (60, 61). Allergens, pollutants and pathogens have all been shown to disrupt tight junction proteins by various mechanisms, but genetic factors also likely contribute to an increased susceptibility of barrier disruption (62, 63). When the barrier is compromised, pathogens, allergens and pollutants are more accessible to DCs, which promotes an immune response and/or allergic sensitization and inflammation (64). In addition, the subepithelial space becomes more exposed to pathogens, thereby facilitating infection (62).

Furthermore, a common feature of the bronchial epithelium in asthma is goblet cell hyperplasia, and as a consequence, overproduction of mucus. This leads to mucus plugging with narrowing of the airways, which contributes to symptoms such as cough and shortness of breath (65).

The airway epithelium shapes inflammatory endotypes

As discussed above, asthma is a heterogeneous disease with different molecular endotypes driving the disease. Two major endotypes are typically described: T2-low and T2-high. The T2-high phenotype can be further subdivided into allergic asthma, driven primarily by Th2 cells, and non-allergic asthma, mainly driven by ILCs (figure 5).

Epithelial responses are central to the development of the distinct endotypes in asthma. A healthy epithelium responds to environmental triggers with the so-called alarmins: TSLP, IL-33 and IL-25. As described above, alarmins act as upstream initiators of T2 immune responses by signaling danger and orchestrating immune cell activation. In asthma, alarmin expression has been shown to be increased, which mainly results in excessive T2 inflammation. For instance, TSLP expression is elevated in the airway epithelium and lamina propria in patients with asthma, and correlates with the severity of airflow obstruction (66, 67). Moreover, in mice with ovalbumin-induced allergic airway inflammation, TSLP is increased in the airways, and removal of the TSLP receptor alleviates the inflammation, further suggesting that TSLP is a key mediator in asthma (68). Similarly, IL-33 and sST2 levels are higher in induced sputum and in serum from patients with asthma, and rhinovirus (RV)-induced IL-33 correlates with asthma symptom severity *in vivo* (69, 70).

Allergic T2-high asthma

In allergic T2-high asthma, TSLP primes antigen presenting cells such as DCs, promoting their ability to prime naïve T-cells into Th2 differentiation during antigen presentation and activation. Th2 cells produce the typical T2 cytokines IL-4, IL-5 and IL-13. IL-4 and IL-13 promote B-cell maturation and class switching to IgE production. Upon allergen exposure, cross-linking of IgE on mast cells triggers degranulation and release of inflammatory mediators. IL-5 promotes eosinophilia by stimulating differentiation, maturation and survival of eosinophils from the bone marrow (10). IL-13 contributes to goblet cell hyperplasia and drives excessive mucus production. It also drives airway remodeling by inducing smooth muscle cell proliferation and collagen synthesis in fibroblasts. Lastly, IL-13 contributes to airway hyperresponsiveness (71).

Non-allergic T2-high asthma

Non-allergic T2-high asthma is mainly driven by ILC2s. These cells respond directly to epithelial-derived alarmins, including IL-33, IL-25 and TSLP, and produce large amounts of IL-5 and IL-13. This induces a T2 inflammatory response,

characterized by eosinophilia, mucus production, airway remodeling and airway hyperresponsiveness. Although ILC2s are the main drivers of non-allergic T2-high asthma, they also contribute to T2 inflammation in the allergic T2-high asthma endotype (72).

T2-low asthma

T2-low asthma is characterized by the absence of T2 inflammatory markers, such as eosinophils. Instead, the inflammation is thought to be driven by neutrophils or is paucigranulocytic (i.e., low or normal levels of inflammatory cells). The concept of T2-low asthma has been widely debated. Many patients diagnosed with T2-low asthma may in fact have an underlying T2-high phenotype that is masked by treatment. Patients with "true" T2-low asthma are thought to be relatively rare, and this endotype is often linked to obesity, smoking and a late onset of the disease (73). The molecular drivers of T2-low asthma are less understood, but TSLP may play a role, as it has been shown to induce Th17 polarization by activating DCs. IL-17 from Th17 cells then induces epithelial release of IL-8 and GM-CSF, which recruit neutrophils to the airways (74, 75).

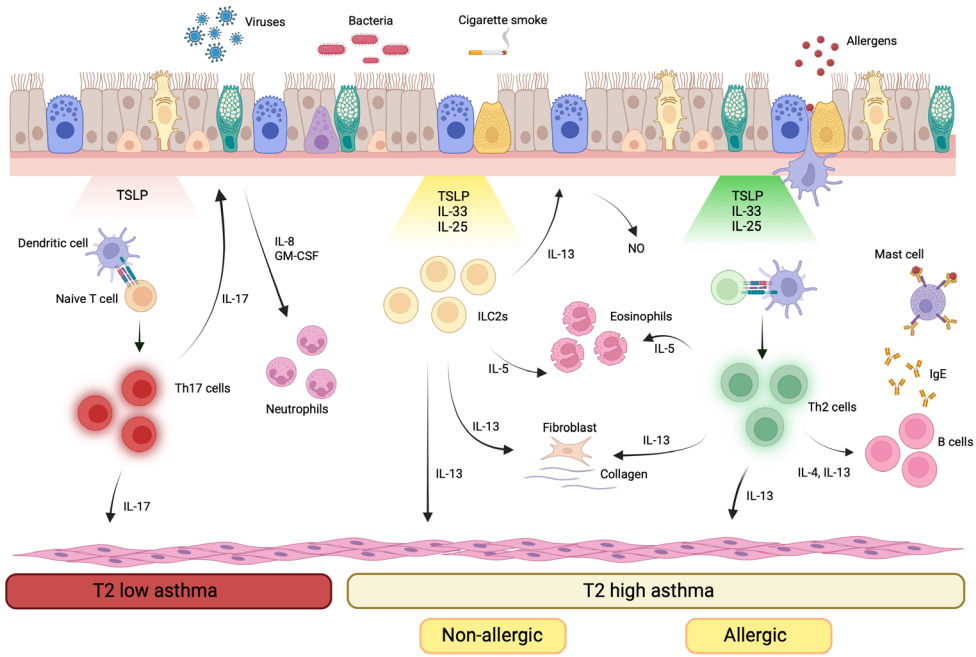

Figure 5. Molecular mechanisms underlying asthma endotypes. The airway epithelium can directly shape T2-low and T2-high asthma through its release of alarmins. Figure was adopted from Gauvreau, G. M., et al. Expert Opin Ther Targets, 24(8), 777–792 (2020), and created in https://BioRender.com.

Viral-induced asthma exacerbations

Rhinovirus is a major driver of asthma exacerbations

An asthma exacerbation is defined as an episode of worsening of symptoms and lung function, where airway inflammation is increased. Exacerbations are triggered by a variety of environmental agents, with the most frequent cause being RV infections, which are mostly known to cause common cold (29, 76). RV is a small (approximately 30 nm) non-enveloped, single-stranded (ss)+ RNA virus that belongs to the *Picornaviridae* family and the *Enterovirus* genus. It can be classified into three different species: RV-A, RV-B and RV-C.

Rhinovirus uses different receptors for binding and entry

RV-A and RV-B use either the "major" receptor Inter-Cellular Adhesion Molecule 1 (ICAM-1) or the "minor" receptor low-density lipoprotein receptor (LDL receptor) to bind and enter respiratory epithelial cells. In contrast, RV-C uses the cadherin-related family member 3 (CDHR3) protein as entry receptor (figure 6) (77, 78).

After receptor binding, RV is internalized via clathrin-dependent or clathrin-independent endocytosis. The acidic pH of the endosome triggers uncoating and release of viral RNA into the cytosol, where it is translated into a single polyprotein. Autolytical cleavage then processes the polypeptide into structural and non-structural proteins. The RNA is replicated by the viral RNA-polymerase, and new viral particles are assembled and released from infected cells.

Rhinovirus infects both the upper and lower respiratory tract

Epithelial cells of the respiratory system are the main targets of RV infection and replication. RV can replicate in nasal epithelial cells, which is most often the starting point of the infection, but replication can also occur within the lower respiratory tract, in bronchial epithelial cells (BECs) (79). In healthy individuals, RV replication mainly occurs in the upper airways, while in patients with asthma, infection may also extend to the lower airways, leading to more severe symptoms and asthma exacerbations (80, 81). Several studies have demonstrated that the main cell type infected by RV is the ciliated cells (82-84). However, *in vitro,* after separation of basal cells and suprabasal cells, basal cells appear more prone to RV infection, which may be because they express higher levels of ICAM-1 (85).

Pathological mechanisms during rhinovirus infections in asthma

Multiple mechanisms interplay during an RV-induced exacerbation. First of all, RV infection directly damages the epithelium with the ability to induce cytopathic cell death. This triggers activation of wound healing mechanisms which, in asthma

patients, tend to result in airway remodeling rather than repair. Pre-existing goblet cell hyperplasia is also exacerbated, with mucin-related genes being upregulated in response to RV infection. This leads to excessive mucus production and plugging, contributing to airway obstruction (86, 87).

RV is also known to trigger increased release of TSLP, IL-25 and IL-33 in asthma, thereby amplifying T2 pathways as described above (87-89). In addition, BECs from asthma patients have been shown to respond with elevated levels of IL-8, CCL5 and CXCL10 in response to a viral mimic or RV infection (86, 88). This further amplifies the already ongoing airway inflammation, driving tissue damage and remodeling rather than effective viral clearance and repair.

Several lines of evidence also suggest that BECs from asthma patients respond with reduced or delayed IFN-β following RV infection and/or viral mimics compared to BECs from healthy patients (89-91). This notion has been debated during recent years, as some studies have reported no difference in IFN production between healthy and asthma BECs (92, 93). Several factors may explain the conflicting evidence. Veerati et al. have demonstrated that *in vitro,* IFN-β expression is significantly induced in BECs from healthy individuals, but not from individuals with asthma, at 24 hours post virus infection. However, at 72 and 96 hours post infection, IFN-β expression was induced also in asthma-derived BECs (91). This suggests that the expression of IFN-β may be delayed rather than impaired in asthma BECs.

Furthermore, we recently showed that the IFN response to a viral mimic in BECs from mild-to-severe asthma did not differ from the IFN response in BECs from healthy patients. However, when patients were stratified into atopic vs. non-atopic, eosinophilic vs. non-eosinophilic, and by disease severity (mild, moderate and severe), it became evident that IFN responses varied across asthma phenotypes and severity. Notably, within both atopic and eosinophilic patients, severe patients exhibited a lower IFN response compared to mild patients. Interestingly, the same patterns, but reversed (i.e., higher release in severe patients), could be seen for IL-33 and TSLP in our study (88). These findings highlight the importance of considering asthma phenotypes and severity when assessing *in vitro* responses. Moreover, varying degree of severity and phenotypes may therefore explain the conflicting results observed in previous studies.

The role of SARS-CoV-2 in asthma exacerbations

The emergence of the pandemic severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) led to a rapid increase in research related to this novel virus. SARS-CoV-2 is a ss (+) RNA enveloped virus, approximately 100 nm in size, belonging to the *Coronaviridae* family and the *Betacoronavirus* genus (figure 6). It causes an acute respiratory disease that is called coronavirus disease 2019, or COVID-19.

SARS-CoV-2 uses ACE2 as an entry receptor in epithelial cells

SARS-CoV-2 is protected by a helical capsid surrounded by an envelope with embedded spike (S) proteins. The S proteins bind to angiotensin converting enzyme 2 (ACE2) on the surface of epithelial cells and are then cleaved by the host proteases furin and transmembrane serine protease 2 (TMPRSS2), enabling viral entry (94, 95). Similar to RV, SARS-CoV-2 infects epithelial cells of both the upper and the lower respiratory tract (96).

After entry, SARS-CoV-2 RNA is released into the cytosol. Host ribosomes are then recruited to translate RNA into two polyproteins, which are processed by viral proteases into a replication-transcription complex. This complex replicates the viral genome, which occurs within double membrane vesicles. The newly synthesized RNA is then released into the cytosol and new viral proteins are produced so that viral particles can be assembled using endoplasmic reticulum-Golgi intermediate compartments. Finally, newly formed virions are released from infected cells (97).

SARS-CoV-2 infection and COVID-19

The nature of SARS-CoV-2 infection and the following COVID-19 varies widely between individuals. Some individuals experience only mild symptoms, whereas others progress to acute respiratory distress syndrome (ARDS) accompanied by the life threating cytokine release syndrome (CRS). CRS is characterized by a severe inflammation with an excessive release of IL-6, IL-1β, TNF, IL-17 as well as chemokines CXCL2, CXCL8, CXCL9 and CXCL16 (98, 99). This "cytokine storm" contributes to damage not only in the lung, but also in other organs such as the liver and kidneys (99).

Similar to observations in asthma, severe COVID-19 has been linked to a failure to produce early IFNs (98). In fact, both genetic mutations affecting IFN signaling and autoantibodies against IFN have been identified in patients with severe COVID-19 (100, 101). Hence, several clinical trials have investigated the efficacy of exogenous IFN-β in COVID-19 patients. Interim results from the world health organization (WHO) solitary randomized trail however showed no effect on overall mortality, initiation of ventilation and duration of hospital stay and IFN-β therapy were later discontinued due to futility (102, 103).

Asthma is not a major risk factor for developing severe COVID-19

Whether asthma is a risk factor for developing severe COVID-19 has been a subject for discussion. Early in the pandemic, evidence suggested that asthma patients were not at increased risk of developing severe disease. However, more recent large-scale studies have indicated a slightly elevated risk of hospitalization or death from COVID-19 in patients with severe or uncontrolled asthma (104). Historically, coronaviruses have not been strongly associated with asthma exacerbations (32, 105). Thus, in the context of asthma and COVID-19, the disease burden for asthma

patients appears to be more related to the severity of the COVID-19 itself rather than the triggering of an asthma exacerbation.

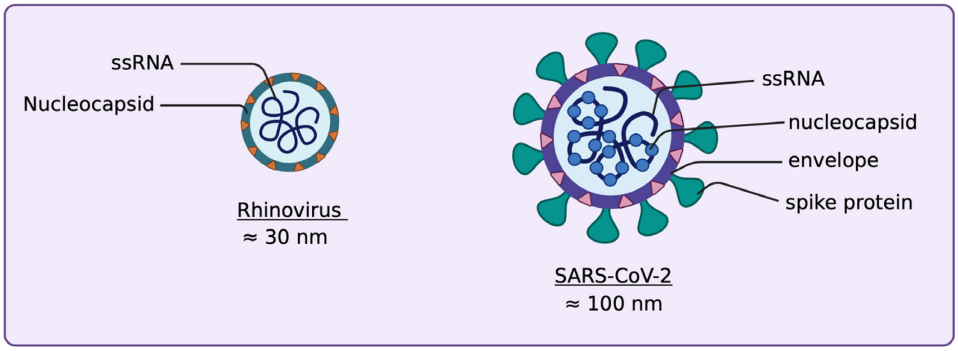

Figure 6. Simple schematic illustration of Rhinovirus and SARS-CoV-2 structures. Created in https://BioRender.com.

Novel treatment strategies for asthma

Targeting inflammatory pathways with biologics

The discovery of the underlying molecular pathways driving asthma (i.e., phenotypes) sparked the development of a new class of drugs - biologics. These are monoclonal antibodies initially designed to target specific cytokines of the T2 inflammatory pathway, such as IL-4, IL-5 and IL-13. Although these therapies have been shown to be efficient in many ways, they only reduce exacerbations in approximately 50% of patients, and are primarily effective in patients with T2-high asthma (74). However, new generation biologics targeting alarmins upstream of the classical T2 cytokines, such as anti-TSLP, appear to be more promising for reducing asthma exacerbations also in other phenotypes. In fact, anti-TSLP has been shown to reduce asthma exacerbations in patients with severe uncontrolled asthma independent of the T2 status of the patient (106-108).

Shaping the immune system with immunotherapy

Since asthma can be an allergen-driven disease, it is not surprising that allergen immunotherapy (AIT) is effective in reducing asthma symptoms in allergic patients (109). In contrast to other asthma medications, AIT is considered to be disease-modifying, and has been shown to have persistent effects even after treatment completion (110). In AIT, small amounts of allergen are administered to patients repeatedly over several years via sublingual, subcutaneous or oral routes. This

induces a protective IgG response toward the given allergen, which competes with allergen-specific IgE antibodies, preventing their binding to the allergen and inhibiting an allergic reaction (111).

Macrolide antibiotics as modulators of inflammation and antiviral responses

Macrolides is a group of antibiotics that exert their antibacterial action by binding to the 50S large ribosomal unit of bacteria to prevent protein synthesis. The first macrolide to be discovered was erythromycin, which consists of a 14-membered macrocyclic lactone ring attached to two sugar moieties. Erythromycin does, however, have a short half-life, results in low blood levels due to its instability in the stomach and leads to gastric irritation. Azithromycin (azm) is a modified version of erythromycin, that instead of a 14-membered macrocyclic ring, has a 15-membered ring and an added nitrogen, which improves half-life, acidic stability and reduces gastric irritation (112, 113).

Interestingly, not long after their first discovery, it was also discovered that macrolides have immunomodulatory properties. Specially, it was first discovered that erythromycin improved survival in patients with diffuse panbronchiolitis (DPB). This led to the development of new macrolides as well as studies of their non-antibiotic effects in other chronic respiratory lung diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and asthma (114). In 2019, Gibson *et al.* reported in the AMAZES study that, in patients with severe asthma, azm improves quality of life and reduces exacerbations (115). In the 2024 update of the GINA guidelines, add-on azm is recommended for patients with persistent asthma despite high-dose ISC-LABA, but only after specialist consultation due to the risk of antibiotic resistance (7).

The immunomodulatory actions of azm have been studied during the last years. In humans, azm reduces sputum neutrophils, IL-1β and IL-6 (116, 117). *In vitro,* it polarizes macrophages into an M2-phenotype, and under TLR4-stimulation by LPS, it reduces the activation of NF-κB and STAT1 (118). Similarly, azm inhibits LPS-induced neutrophilia, CCL2, GM-CSF, IL-1β, TNF-α and sE-selectin in lung homogenates from mice (119). In BECs, azm inhibits IL-13-induced MUC5AC, and enhances barrier integrity *in vitro* (120, 121). In addition, azm increases the expression of IFN and antiviral responses, and reduces viral load of RV-infected BECs (122-124). It remains unclear to what extent these effects contribute to the exacerbation-reducing properties of azm, and the underlying mechanisms are largely yet to be elucidated.

Non-antibiotic macrolides to minimize antimicrobial resistance

The off-label and continuous use of macrolides such as azm may lead to increased antimicrobial resistance (125). Hence, efforts are made to develop non-antibiotic macrolides. To date, there are no approved non-antibiotic macrolides for clinical use, but a number of erythromycin and azithromycin derivatives have been tested preclinically. For instance, an erythromycin derivative, EM900, has been shown to reduce IL-1β-induced IL-8, TNF-α and mucin expression in airway epithelial cells (126). Similarly, EP395, an azm derivative, has been shown to enhance airway epithelial barrier integrity, reduce neutrophilic airway inflammation in mice and be well tolerated and reduce lipopolysaccharide (LPS)-induced pro-inflammatory cytokines in humans (127-129).

Aim of thesis

The primary aim of this thesis was to explore immune mechanisms through which pharmacological agents modulate viral sensing and antiviral and pro-inflammatory responses in the bronchial epithelium in asthma. We also aimed to optimize a mouse model of viral mimic-induced lung inflammation as a tool for studying these mechanisms. Specifically, this thesis focused on the following objectives:

I. To explore the effects of imiquimod on bronchial epithelial antiviral and inflammatory responses to viral mimics (paper I).

II. To evaluate the pulmonary immunological response to a viral mimic in three commonly used inbred mouse strains (paper II).

III. To investigate whether BECs from patients with different asthma phenotypes respond differently to treatment with azm, and to elucidate the molecular mechanisms underlying the antiviral effects of azm (papers III and IV).

Methods

In this section, the main methods are described and discussed, including advantages and disadvantages of the different approaches that can be utilized to study asthma and asthma exacerbations. More details about the exact methodologies can be found in the materials and methods sections of each individual publication or manuscript.

Primary bronchial epithelial cells

The use of primary bronchial epithelial cells (BECs) is central to this thesis. All papers except paper II include work conducted using BECs from healthy and asthma patients. A key advantage of primary BECs is that they retain disease-specific phenotypes *in vitro*, allowing studies of asthma-related mechanisms (88). This makes them more physiologically relevant to asthma research compared to immortalized cell lines, such as BEAS-2B cells. The benefit of using primary BECs is particularly evident in paper III, where we assessed phenotype-dependent immunological responses to RV *in vitro.* However, a major drawback of the use of primary BECs is the limited number of passages they can be cultured and thus sustained.

In the papers included in this thesis, BECs were cultured under submerged conditions, i.e., on a plastic surface and covered in medium. A limitation of this method is that it does not allow for differentiation of BECs into all cell types that make up the respiratory tract. To obtain a differentiated epithelium, air-liquid interface (ALI) cultures are required. These cultures allow the study of key epithelial functions such as cilia beating, mucus production and barrier integrity. Although ALI cultures provide a more physiologically relevant (differentiated) epithelium, the differentiation process is artificial, and the microenvironment is simplified, lacking immune, stromal and vascular cells, meaning that the resulting epithelium does not fully recapitulate the characteristics of the *in vivo* epithelium (130). In addition, ALI cultures are both time- and resource-consuming, as full differentiation takes 4 weeks to complete. Conversely, submerged cultures are both time- and resource-efficient, and remain a suitable model for studying immunological responses to viruses, as they retain disease- and phenotype-specific expression patterns that closely resemble those observed during *in vivo* viral infection (88, 131). Hence, for studies involving larger cohorts, as in paper III and IV, submerged

cultures are more feasible to conduct. In conclusion, the submerged and ALI cultures are best used as complementary systems, and should be chosen depending on the specific research question. During the work with this thesis, we have established protocols for culturing of BECs at ALI, to enable validation of our results in a differentiated epithelium.

Preparation and culturing

BECs were obtained during bronchoscopy using sterile-sheared nylon cytology bronchial brushings. Four brushes were obtained from each patient, and collected in 15 ml tubes containing RPMI supplemented with 10% fetal bovine serum (FBS). The tubes were then vortexed for 1 minute and the RPMI from the four tubes was pooled to one tube, which was centrifuged for 5 minutes at 180 rcf. The supernatant was then removed, the cell pellet was resuspended in 6 ml of bronchial epithelial growth medium (BEGM) and taken up by a syringe and passed through an 18-gauge needle into 6 collagen IV-coated T25 flasks containing BEGM medium. The next day, the flasks were rinsed with PBS to remove any residual blood from the bronchoscopy, and new BEGM was added. The cells were then cultured at 37°C and 5% CO2 and the medium was refreshed every other day until confluency, when cells were frozen down in BEGM medium containing 20% FBS and 10% DMSO and stored in liquid nitrogen until use.

At the time of the experiments, 800,000-1,000,000 cells were added to a collagen IV-coated T75 flask containing BEGM. The cells were allowed to attach for 24 hours, and the medium was then changed to fresh BEGM. The medium was changed every other day for 7-10 days, and the cells were passaged into collagen IV-coated 12-well plates. The cells were then cultured for 2-3 days until 80% confluency was reached, after which the experiments were started (figure 7).

Cell stimulations

The main focus of this thesis has been on viral-induced asthma exacerbations. In order to model a viral infection in BECs, there are two main options: either infect the BECs with live RV, or use a synthetic viral mimic (figure 7). In the case of RV, poly(I:C) is widely used as a mimic, which is a synthetic dsRNA analogue that mimics the dsRNA that is produced during viral replication. Poly(I:C) binds to TLR3, MDA5 and RIG-I. There are pros and cons to both methods. Of course, RV infection is more biologically relevant, but it also introduces biological variation, as the viral infectivity in primary cells may vary between asthma patients and between experiments. In that sense, poly(I:C) stimulations produce more stable and reproducible results, as an exact amount can be administered to the cells in each experiment. Hence, this method is valuable if immunological responses are to be

compared between, for instance, different patient groups. We therefore use both methods continuously in the lab, depending on the specific research question.

We used 10 µg/ml poly(I:C), a dose previously established in our lab to induce an antiviral and pro-inflammatory response without causing cytotoxicity. For live RV infections, we determined the tissue culture infective dose 50 (TCID50) in HeLa cells, and then evaluated the multiplicity of infection (MOI) required to elicit an antiviral and pro-inflammatory response with minimal cytotoxicity in BECs.

We have also investigated how two different pharmacological agents, imiquimod and azithromycin, modulate the BEC immune response to poly(I:C) or RV. A dose of 10 µg/ml imiquimod was selected based on previous dose-response studies in the lab. We also confirmed that the effects of imiquimod were not dependent on pre-treatment of the cells. In contrast, we have previously established that pre-treatment with azithromycin is required for its effects in BECs. A concentration of 10 µM was used, as this dose is clinically relevant and has been shown to elicit antiviral activity in BECs (123, 124).

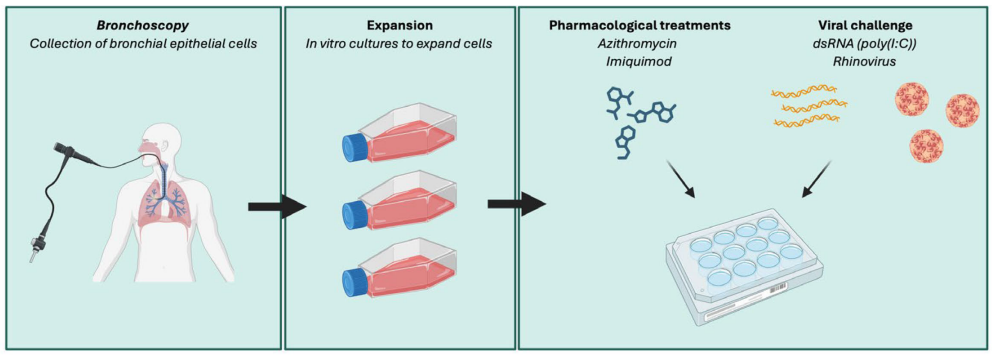

Figure 7. Overview of the work flow of bronchial epithelial cell cultures. Created in https://BioRender.com.

Animal models

Although *in vitro* methods can provide valuable insights into disease mechanisms, they do not represent the complex interaction of different cell types and tissues *in vivo*. Hence, combining *in vitro* and *in vivo* studies is important. Mice cannot develop asthma, but allergic sensitization can be introduced, and the resulting airway inflammation resembles allergic asthma. The mice can then be inoculated with poly(I:C) or live virus to evoke an "exacerbation" (132). In asthma research, the most commonly used mouse strains are C57Bl/6 and BALB/c. The immune

response to allergens such as house dust mice and viruses such as pneumonia virus of mice is however strain-dependent (133, 134). In addition, within the C57Bl/6 strain, several substrains exist, most notably the C57Bl/6J and C57Bl/6N, which show both genetic and phenotypic differences (135).

In paper II, we therefore evaluated the immunological response to poly(I:C) in these three different mouse strains, in order to determine which strain that is best suited to mimic human RV infections. Poly(I:C) was administered intranasally to C57Bl/6N, C57Bl/6J and BALB/c mice once per day for three consecutive days, and lactate dehydrogenase (LDH) activity, total protein concentration and inflammatory cells were analyzed in bronchoalveolar lavage fluid (BALF). In addition, protein and mRNA expression was analyzed by RT-qPCR, western blot or ELISA in lung homogenates. This paper demonstrates one of the major limitations of animal studies, since we show that the immunological response to poly(I:C) varies not only between the different mouse strains, but also between substrains. Therefore, it is essential to select strain according to the specific aims of the study.

Gene expression analyses

The majority of the work included in this thesis is based on gene expression analyses. The main method used in our lab is reverse transcription (RT)-quantitative polymerase chain reaction (qPCR). RNA is extracted from cells and reversely transcribed into complementary (c)DNA, which is then amplified by specific primers for the targets of interest. The amount of cDNA is measured using fluorescence, and relative quantities of RNA transcripts can be determined using the ΔΔCt method. This method is quick and relatively affordable, while also providing high specificity and sensitivity, depending on the quality of primers, amplification efficiency and RNA quality. We have evaluated primer efficiency for all primers, and always check RNA quality before proceeding with cDNA transcription.

A major drawback of RT-qPCR is that each gene must be analyzed one by one. When several genes are of interest, or if the aim is to analyze molecular pathways, other methods are therefore better suited. One option is RNA sequencing, which provides sequencing data for all genes in the sample. This is, however, quite expensive and time-consuming, so for research questions where only certain parts of the genome are of interest, multiplex mRNA analyses with a fixed panel of genes can be used instead. NanoString's nCounter system utilizes unique molecular barcodes for each gene in a pre-defined panel of genes, and counts for each gene are provided and compared between different samples. This method provides a large output with a very small input, and in contrast to RT-qPCR, where the RNA is converted to cDNA before analysis, the nCounter method is based on direct

detection of RNA, which enhances specificity and lowers the amount of sample needed.

Protein expression analyses

In general, gene expression only becomes functionally meaningful when the transcribed mRNA is successfully translated into protein. Hence, analysis of protein expression is equally important as gene expression analyses. Throughout the papers included in this thesis, different methods for protein expression have been used depending on the protein of interest. For intracellular proteins, we have used western blot. For proteins that are released into supernatants, we have used both single-protein ELISAs and Luminex or MSD for multiplex analyzes. In paper I, we also used proteomic analysis using mass spectrometry of all peptides in the samples. This method is more expensive and time-consuming than antibody-based methods such as western blot and ELISA, but generally provides more specificity, as it is not dependent on the quality of the antibody.

Artificial intelligence (AI)

The generative AI tool ChatGPT has been used to improve and refine sentences and sections in this thesis. I generated text that was used as input, and ChatGPT gave suggestions on improvements to the text. I have processed the generated text and take full responsibility for the content.

Summary of results and discussion

The following section presents an integrated overview and discussion of the key findings from the studies included in this thesis.

Imiquimod has dual effects on antiviral and pro-inflammatory responses in BECs from asthma patients (paper I)

Imiquimod is a TLR7 agonist that is clinically approved for topical treatment of viral and tumoral skin conditions, and has been shown to reduce viral replication, inflammation and lung dysfunction in Influenza A-infected mice (136). Given these antiviral and immunomodulatory properties, we aimed to evaluate whether imiquimod could exert dual effects in BECs, acting both on the pro-inflammatory response and the antiviral response simultaneously.

We found that imiquimod alone decreased the baseline gene expression of the entry receptor for SARS-CoV-2, ACE2, as well as the alarmin IL-33 and pro-inflammatory IL-1β. It also increased IFN-β, MDA5, CCL5 and TNF-α mRNA. Proteome pathway analysis showed a downmodulation of pathways relating to infectious diseases and influenza infection, metabolism of amino acids and RNA, the cell cycle, as well as angiotensin metabolism. These pathways included proteins implicated in viral mRNA and protein synthesis, such as the ribosomal proteins RPS16, RPLP0 and RPS15.

In the presence of the viral mimics SARS-CoV-2 spike protein 1 (SP1) or poly(I:C) (dsRNA), imiquimod further potentiated expression of IFN-β, while still decreasing ACE2 expression. With siRNA silencing, we could show that the imiquimod-mediated enhancement of IFN-β expression during poly(I:C) stimulation depends on MDA5 and RIG-I. We also found that complement C1q binding protein (C1QBP), which is a negative regulator of MDA5 and RIG-I signaling, was downregulated by imiquimod during poly(I:C) stimulation (figure 8). These data indicate that stimulation of TLR7 may enhance the RIG-I-like receptor response to poly(I:C) stimulation. Such interplay has been implicated in plasmacytoid DCs before (137).

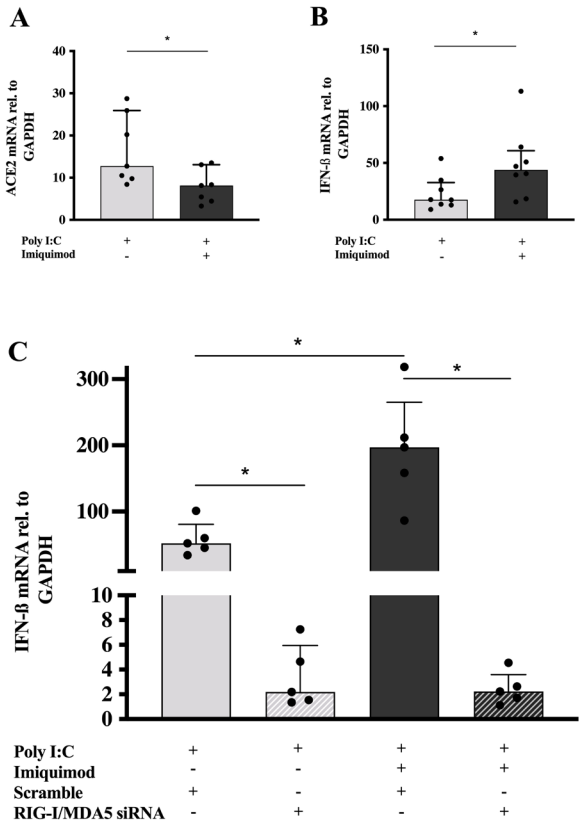

Figure 8. Imiquimod decreases poly(I:C)-induced ACE2 while enhancing IFN-β. BECs from patients with asthma were stimulated with poly(I:C) without or with imiquimod for 24 hours and gene expression of ACE2 (A) and IFN-β (B) was measured using RT-qPCR. Wilcoxon signed rank test, n=7-8. BECs were pre-incubated with siRNA against MDA5 and RIG-I and stimulated with poly(I:C) without or with imiquimod and gene expression of IFN-β was measured using RT-qPCR (C). One-way ANOVA followed by Holm-Šídák's multiple comparisons test, n=5.

Furthermore, the protein release of several poly(I:C)-induced pro-inflammatory cytokines, such as IL-8, IL-6, IL-1β, was also downmodulated by imiquimod treatment. We further identified single Ig IL-1-related receptor (SIGIRR), a negative regulator of IL-1R signaling, as upregulated by imiquimod during poly(I:C) stimulation (138). However, whether this protein is responsible for the observed downregulation of pro-inflammatory signaling by imiquimod remains to be determined. Figure 9 summarizes the main findings from paper I.

These findings are relevant to both asthma and COVID-19, since a deficiency in IFN-β has been described for both (89-91, 98). Imiquimod has previously shown antiviral activity in murine models of respiratory syncytial virus (RSV) and Influenza A infection (136, 139), an action that could be attributed to its IFN-

boosting effect. Moreover, our exploratory proteome analysis indicates that imiquimod may have antiviral effects beyond IFN expression, with a downmodulation of proteins and pathways that are involved in viral replication. This has been suggested before by Salinas et al. in a study where they showed that imiquimod interferes with RSV RNA and protein synthesis independently of the innate response (139).

The potential of imiquimod to downmodulate excessive activation of a pro-inflammatory response is also important to highlight, as both COVID-19 and asthma exacerbations can be characterized by hyperinflammation (140). Given the broad actions of imiquimod, acting both to strengthen the antiviral defense and to hamper excessive immune activation, we believe that TLR7 agonists are of interest for further development as broad-spectrum antivirals.

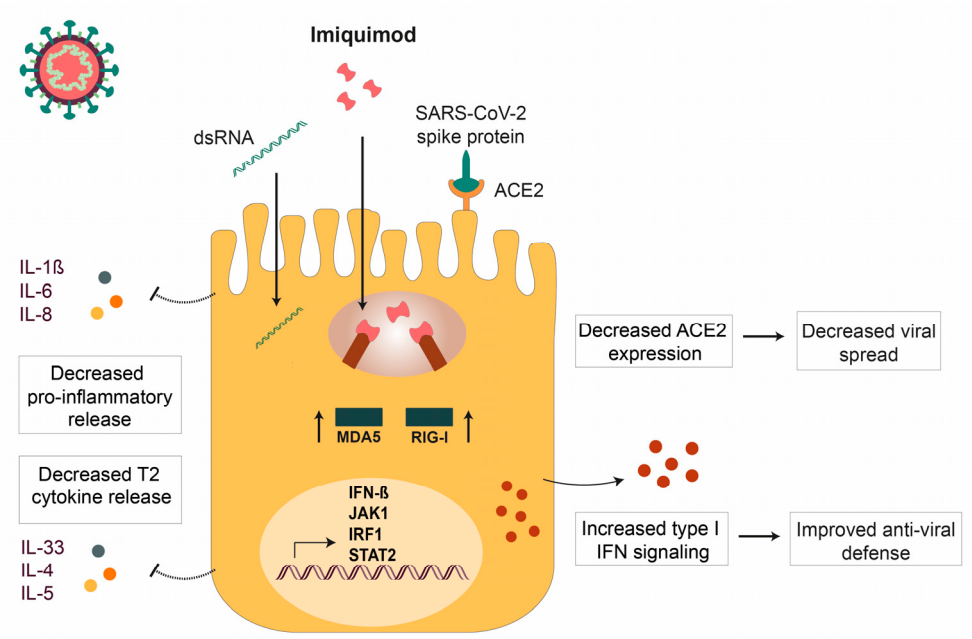

Figure 9. Summary of the main findings in paper I. Imiquimod increases type I IFN signaling, while decreasing pro-inflammatory and alarmin release.

C57Bl/6J, C57Bl/6N and BALB/c mice show immunological discrepancies in their response to intranasal poly(I:C) (paper II)

In vivo mouse models are a valuable tool in preclinical research. In paper II, we aimed to build upon paper I and optimize a mouse model of poly(I:C)-induced lung inflammation. Hence, we compared the immunological response to poly(I:C) in three commonly used inbred mouse strains: C57Bl/6J, C57Bl/6N and BALB/c. The mice were given 100 µg poly(I:C) intranasally (i.n.) once per day for three consecutive days.

First, we evaluated general inflammation markers, including cell concentration and composition in bronchoalveolar lavage fluid (BALF). BALB/c mice showed a significantly higher cell concentration in BALF after poly(I:C) stimulation compared to C57Bl/6N mice. Furthermore, only BALB/c and C57Bl/6J mice had a significant increase in neutrophils, total protein concentration (figure 10) and lactate dehydrogenase (LDH) activity in BALF following poly(I:C) stimulation.

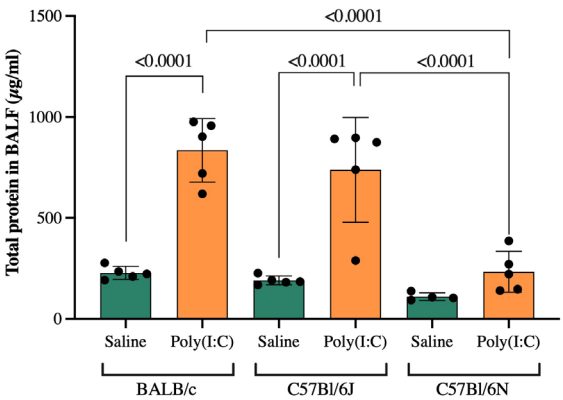

Figure 10. Total protein concentration is increased in bronchoalveolar lavage fluid (BALF) in response to i.n. poly(I:C) in BALB/c and C57Bl/6J mice. Concentration of total protein (µg/ml) in BALF in response to three days of i.n. administration of saline or 100 µg of poly(I:C) (dsRNA) in BALB/c, C57Bl/6J and C57Bl/6N mice. Data are presented as mean +- SD. Two-way ANOVA with Tukey's multiple comparisons test. N=4-5.

We next evaluated how gene expression of the PRRs TLR3, MDA5 and RIG-I were affected by poly(I:C) stimulation. Only BALB/c and C57Bl/6J mice significantly upregulated the gene expression of these genes. For MDA5 and RIG-I, the same pattern was found on protein level.

Downstream of PRR signaling, we also analyzed how antiviral IFN-β, IFN-λ and viperin, as well as pro-inflammatory TNF-α, IL-1β and CXCL1 were affected by

poly(I:C) stimulation. Of the antiviral genes, only viperin was upregulated in response to poly(I:C), and only significantly in C57Bl/6J mice. TNF-α was also significantly upregulated in C57Bl/6J, and almost significantly in BALB/c ($p=0.0517$). CXCL1 was only significantly upregulated in BALB/c mice. On the contrary, IL-1β was only upregulated in C57Bl6/N mice (figure 11).

Immunological differences between BALB/c and C57Bl/6 mice have previously been demonstrated in response to both pneumonia virus of mice (PVM) and SARS-CoV-2 (133, 141). However, the striking differences between C57Bl/6N and C57Bl/6J observed here are underreported. Warden et al., have, however, previously shown that the innate immune response to poly(I:C) in the brain is shorter in C57Bl/6N mice compared to C57Bl/6J mice, as it occurs already at 3 hours post poly(I:C) administration, and is not sustained at 24 hours after administration. These results could explain our observations at 24 hours post poly(I:C) challenge, and future studies should compare the kinetics of the innate inflammatory response to poly(I:C) also in the lung (142).

Our study suggests that, at 24 hours after the last poly(I:C) administration, BALB/c and C57Bl/6J mice display a distinct inflammatory response, while C57Bl/6N mice show an attenuated response, which, as mentioned, may be due to alternative kinetics of the inflammatory response. BALB/c mice showed the most prominent inflammation with regard to all the investigated parameters, but the C57Bl/6J mice were the only strain to significantly upregulate the antiviral gene viperin. C57Bl/6N mice will likely fail to recapitulate key features of viral infection, including lung infiltration of immune cells, elevated pro-inflammatory mediator levels and antiviral responses, whereas C57Bl/6J mice may provide a more representative model. Hence, our observations highlight the importance of both substrain *selection* and *reporting* when performing *in vivo* studies, and will serve as an important guide for our further studies with viral-induced asthma exacerbation mouse models.

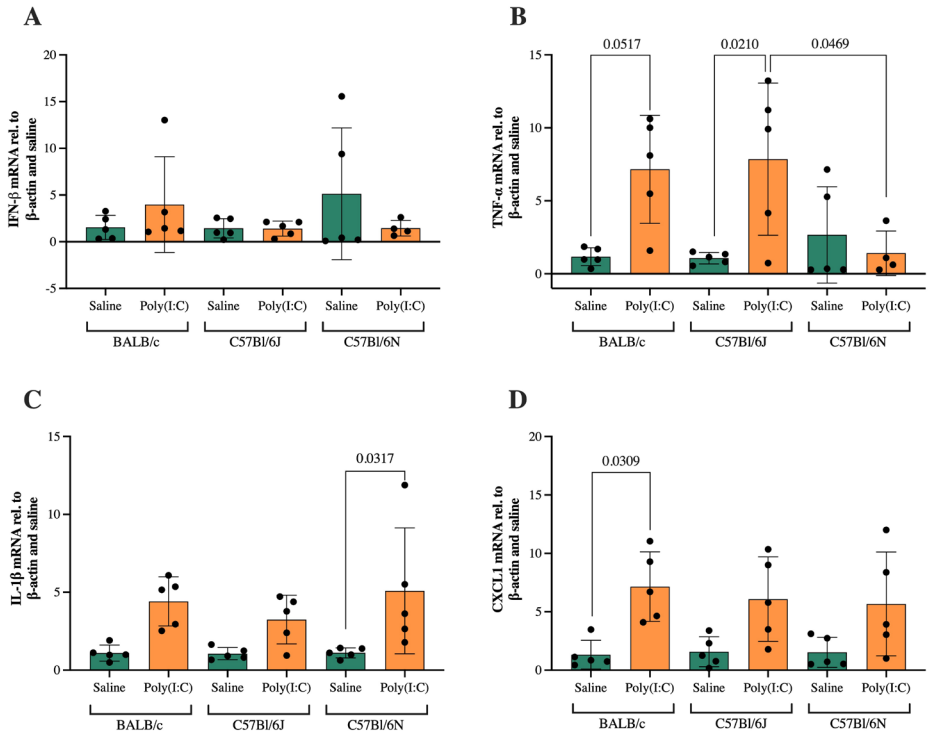

Figure 11. Differential lung gene expression patterns in response to i.n. poly(I:C) in BALB/c, C57Bl/6J and C57Bl/6N mice. Gene expression of IFN-β (A), TNF-α (B), IL-1β (C) and CXCL1 (D) in lung homogenates in response to three days of i.n. administration of saline or 100 µg of poly(I:C) (dsRNA) in BALB/c, C57Bl/6J and C57Bl/6N mice. Data are presented as mean +- SD. Two-way ANOVA with Tukey's multiple comparisons test. N=4-5.

Azm increases the antiviral response to rhinovirus in BECs from asthma patients, regardless of clinical phenotype (paper III)

Azm is known to increase viral-induced IFN-β and reduce viral load in BECs (122, 124). In addition, it reduces asthma exacerbations clinically and is now included as an add-on treatment in GINA step 5. The response to treatment among patients is, however, variable, and it remains unclear which patient groups benefit most from azm. Previous studies by our lab have shown that BECs from asthma patients exhibit differential responses to poly(I:C) *in vitro*, depending on clinical phenotype (88). Therefore, to broaden our understanding of phenotype-dependent responses to azm, we treated BECs from patients with eosinophilic, non-eosinophilic, atopic or non-

atopic asthma with azm, infected them with RV1B and evaluated their response to treatment *in vitro*.

Azm augmented RV-induced release of IFN-β and λ in both the eosinophilic and non-eosinophilic groups (figure 12A, C). Further, significant increases in both IFN-β and IFN-λ were observed only in the non-atopic group, while the atopic group showed similar, but not significant, trends (figure 12B, D). We also measured viral infectivity with a 50% tissue culture infectious dose (TCID50) assay, which showed the same patterns as IFN, but reversed: viral infectivity was significantly reduced by azm regardless of eosinophilia, but only in the non-atopic group. The atopic group showed a similar trend, but it did not reach statistical significance. The lack of significance in the non-atopic group for both IFN and viral load could be attributed to low statistical power, given that each group only included 10 patients. Thus, whether patients with non-atopic asthma respond less to azm is a subject for further investigation.

In addition, we analyzed the release of TSLP, IL-33, IL-6, IL-8 and IL-1β. Notably, BECs derived from non-eosinophilic patients showed a significant increase in both TSLP and IL-6 following azm treatment, while no change was observed when all phenotypes were analyzed together. While azm has previously been shown to downregulate TSLP in a T2-high environment, no study has reported an azm-induced increase in TSLP expression (143). Similarly, consistent with our findings, others have reported no effect of azm on IL-6 and IL-8 when asthma phenotypes are not considered, but our observation that IL-6 is increased in non-eosinophilic patients has not previously been reported (122). Further research is required to determine the clinical relevance of these findings, and whether the observed effects are beneficial or detrimental.

Nevertheless, our results support the findings from the AMAZES clinical trial, where azm reduced asthma exacerbations in both eosinophilic and non-eosinophilic asthma (115). Overall, we believe that these results highlight the broad applicability of azm in reducing viral-induced asthma exacerbations, and that its actions are independent of T2 inflammatory pathways, which is in contrast to the majority of available biologic treatments for asthma.

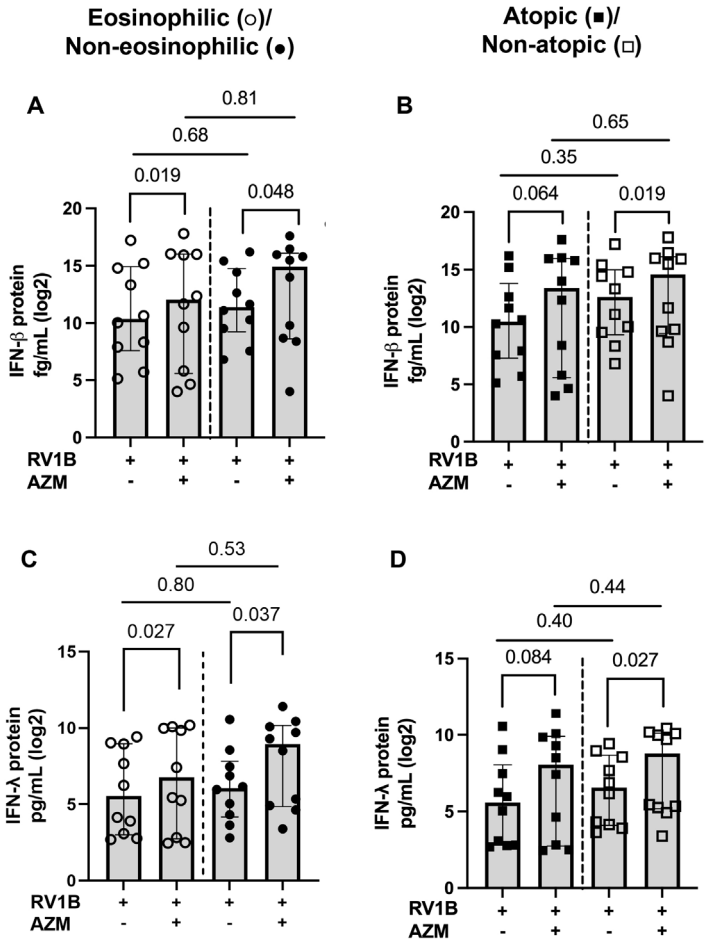

Figure 12. Azm increases viral-induced IFN-β and IFN-λ in BECs from both eosinophilic and non-eosinophilic asthma. BECs were pre-treated with 10 μM azm for 24 hours, followed by 48 hour RV infection. Protein release of IFN-β (A, B) and IFN-λ (C, D) was analyzed. Within-group comparisons were performed using Wilcoxon signed rank test and between-group comparisons using the Mann-Whitney U test. N=10 for each phenotype.

Azm increases antiviral signaling in asthma BECs via TBK1 and IKKε activity (paper IV)

Using the same patient cohort as paper III, we further investigated the molecular mechanisms of azm in BECs. By using a multiplex mRNA analysis, we aimed to unravel broad effects of azm and identify key pathways and genes involved in its mechanism of action. Since azm is administered preventatively to patients with asthma, we sought to investigate its effects both in the absence (azm alone) and in

the presence of RV1B infection, thereby modeling both steady state and viral-induced exacerbations. Hence, BECs were either treated with azm continuously for 30 hours or pre-treated with azm for 24 hours and infected with RV1B for an additional 24 hours.

Both in the absence and presence of RV, gene set enrichment analysis (GSEA) of differentially expressed genes (DEGs) indicated that azm most strongly enriched IFN and antiviral signaling pathways, including upregulation of ISGs such as IFIT1 and MX1, but with little or no effect on pro-inflammatory signaling. This confirms and expands previous findings from Gielen et al., who found no effect of azm on viral-induced IL-8 or IL-6 in BECs (122). Furthermore, azm alone downmodulated "Response to elevated platelet cytosolic Ca^{2+}" and "extracellular matrix organization", both of which are associated with airway remodeling. An increase in intracellular Ca^{2+} is also known to increase mucus production in BECs (144). Consistent with our observation, Pu et al. have demonstrated that azm prevents TGF-β-induced epithelial-mesenchymal transition in BECs. This suggests that azm may have effects on both remodeling and mucus production at steady state, which are important aspects of asthma pathogenesis. How much these actions contribute to the overall exacerbation-sparing effect of azm *in vivo* remains to be determined (figure 13).

Considering that azm had similar effects on the antiviral response both in the absence and presence of infection, we further focused on dissecting the mechanism underlying the enhanced antiviral response during RV infection. First, we performed an upstream regulatory analysis by conducting transcription factor enrichment on the DEGs, which identified STAT1 as the most likely upstream regulator. However, IRF7 was also identified, and given that IRF7 regulates IFN transcription while STAT1 functions downstream of the IFN receptor, we focused our further analyses on IRF7 (145-147).

Azm enhanced both gene and protein expression of IRF7 during RV infection. Its binding partner IRF3 was, however, unchanged by azm treatment. Analysis of a publicly available RNA sequencing data set generated from BECs from healthy individuals and asthma patients, differentiated at air-liquid interface and infected with RV, further demonstrated that IRF7 induction is delayed in asthma-derived BECs compared to healthy BECs.

To determine if IRF7 was required for the azm-enhanced antiviral signaling, we performed siRNA-mediated knockdown of IRF7. This abolished the RV- and azm-induced expression of the ISGs IFIT1 and MX1, however, a small but significant effect of azm remained. Inhibition of the upstream kinases TBK1 and IKKε with BX795, similarly attenuated the RV- and azm-mediated upregulation of IFIT1 and MX1, but no significant residual effect of azm remained. Together, these findings suggest that IRF7 is an important mediator of the azm-mediated enhancement of the RV-induced antiviral response, as its knockdown reduced both RV- and azm-

induced expression of IFIT1 and MX1. However, the persistence of a small residual azm effect following IRF7 knockdown suggests that IRF7 is not the sole contributor. In contrast, given that inhibition of TBK1 and IKKε eliminated the residual response, activation of both IRF3 and IRF7 is most likely required. Thus, the azm-mediated enhancement of antiviral signaling appears to depend on the coordinated activity of IRF3 and IRF7.

Other studies have indicated a role for IRF7 in asthma: IRF7 DNA methylation and single nucleotide polymorphism (SNP) variants have been linked to asthma exacerbation risk and IL-17A regulation (148). Children with low expression of IRF7 have also been shown to be more likely to be hospitalized following an asthma exacerbation, compared to children with high expression of IRF7 (149). Our findings indicate that azm might function to normalize the antiviral response by promoting an earlier or enhanced induction of antiviral and IRF7 signaling in asthma patients.

In addition, TBK1 activation by azm has been shown in previous studies: in combination with a STING agonist, Petcharat et al. demonstrated that azm promoted TBK1 phosphorylation, and Li et al. observed similar effects of azm in fibroblasts and alveolar A549 cells (150, 151). To our knowledge, our study is the first to show functional dependence on TBK1 and IKKε for azm-mediated antiviral signaling in BECs. We have previously shown that MDA5 is required for azm-induced IFN-β expression during RV infection in asthma-derived BECs. Together with the results from this study, we identify the MDA5-TBK1/IKKε-IRF7/IRF3 axis as a central signaling pathway underlying the azm-induced antiviral signaling in BECs (figure 13).

Prolonged use of macrolide antibiotics may lead to antimicrobial resistance (125, 152). EP395 is a novel derivative of azm, which has been modified to reduce antimicrobial activity. In tests against 117 bacteria from 17 different species, EP395 had negligible antimicrobial activity (128). In paper IV, we studied the effects of EP395 on IRF7, IFIT1 and MX1, and found that, similar to azm, EP395 significantly upregulated the RV-induced expression of these genes. We have also confirmed that EP395 augments RV-induced gene expression of IFN-β and MDA5 and reduces viral load in BECs from asthma patients, with a potency comparable to azm (unpublished observations).

These findings are novel, as antiviral effects of a non-antibiotic macrolide in BECs have not been demonstrated previously. Similar effects have only been reported in human tracheal or nasal epithelial cells using an erythromycin derivative (153).

EP395 has previously been shown to share other effects with azm, including increased barrier integrity of BECs and suppressed neutrophilic airway inflammation in mice (127, 128). Given that the use of azm is controversial and should be limited due to the risk of antimicrobial resistance, the development of non-antibiotic macrolides with comparable efficacy would be beneficial, offering a

broad and cost-effective alternative to targeted biologics. This study also provides additional evidence that the immunomodulatory effects of azm are independent of its antibacterial activity, and that these effects can be de-coupled in non-antibiotic macrolides.

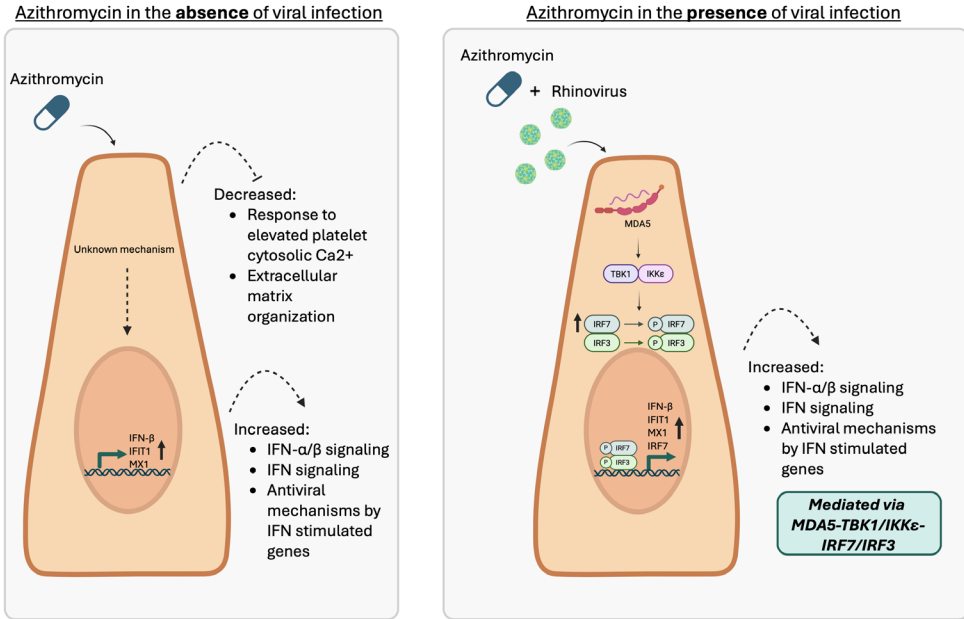

Figure 13. Summary of findings in paper IV. Azm enhances IFN and antiviral signaling in both absence and presence of RV infection. Created in https://BioRender.com.

In addition to paper III and IV, we have conducted a randomized, placebo-controlled clinical trial to investigate whether azm enhances RV-induced IFN responses in BECs from moderate-to-severe asthma patients *in vivo* (AZIMUNE, EudraCT number: 2019-003768-31). In this study, patients received 500 mg azm or placebo three times per week for 12 weeks, and BECs were obtained by bronchoscopy at baseline and at the end of treatment. BECs were cultured *in vitro* and infected with RV, and IFN, alarmin and pro-inflammatory responses were assessed using MSD assays. Compared to baseline, azm increased the RV-induced BEC release of IFN-β and IFN-λ, while no change was observed in the placebo group. Furthermore, RV-induced IL-33 release was reduced only in the azm-treated group (manuscript in preparation).

Although our studies cannot conclusively establish that the azm-mediated reinforcement of BEC antiviral mechanisms contributes to its exacerbation-sparing effects *in vivo*, results from both clinical and *in vitro* IFN-β treatment support the

hypothesis that one of the beneficial effects of azm is to strengthen the antiviral defense of the bronchial epithelium: Djukanovic et al. investigated the effect of inhaled IFN-β on viral-induced asthma exacerbations, but found no significant effect on asthma symptoms. However, morning peak expiratory flow recovery was improved, and in a sub-analysis of patients with step 4-5 asthma, IFN-β did reduce the proportion of patients with worsening of symptoms during viral infection (154). In this trial, IFN-β was administered to patients within 24 hours of developing cold symptoms. By contrast, in an *in vitro* study using monocyte-derived macrophages and BECs and with RSV infection, IFN-β administration reduced viral infection only when administered prior to infection, but not after (155, 156). Collectively, these findings suggest that IFN-β is important for preventing viral-induced exacerbations, but its efficacy may benefit from prophylactic administration. In this regard, azm is particularly promising, as we have shown both that it primes BECs with an enhanced antiviral response prior to and during viral infection (paper III and IV), and that *in vivo* azm treatment enhances the IFN response to RV in BECs (manuscript in preparation).

Conclusions

In this thesis, we have investigated strategies to modulate altered bronchial epithelial immune responses in asthma, and explored underlying molecular mechanisms. Asthma is a complex disease involving altered alarmin, inflammatory and antiviral bronchial epithelial responses. Hence, an ideal therapy would normalize several of these alterations. Current standard treatment with corticosteroids and biologics targets inflammatory pathways, but does not enhance antiviral resistance. We therefore explored drugs with potential effects on both inflammatory and antiviral immunity. Furthermore, we have unravelled important immunological differences between inbred mouse strains that are of relevance when modeling viral infections *in vivo.* From the four different studies included in this thesis, we can conclude the following (summarized in figure 14):

- Imiquimod has dual effects on BECs from asthma patients. It enhances both the baseline and poly(I:C)-induced antiviral defense by upregulating IFN-β and downregulating the SARS-CoV-2 entry receptor ACE2. Moreover, it suppresses pro-inflammatory and alarmin expression at baseline and during poly(I:C) stimulation, potentially preventing excessive immune activation during viral infections.
- There are notable differences in the lung immune response to three days of intranasal poly(I:C) administration in BALB/c, C57Bl/6J and C57Bl/6N mice. BALB/c mice exhibit the most pronounced response, with a marked increase in neutrophils, LDH activity and CXCL10 protein expression in BALF, along with an induction of PRR, TNF-α and CXCL1 gene expression in lung homogenates. C57Bl/6J mice show a similar, albeit less pronounced response, but also upregulate the antiviral gene viperin. In contrast, C57Bl/6N mice exhibit a significant increase *only* in IL-1β gene expression.
- Azithromycin enhances antiviral mechanisms of BECs independently of asthma phenotype. In the absence of viral infection, it enhances expression of several antiviral genes. This priming results in an augmented antiviral response upon RV infection, including an increased expression of the interferon regulatory factor 7 (IRF7). Furthermore, inhibition of TBK1 and IKKε abrogates the upregulation of antiviral genes. EP395, a macrolide with negligible antimicrobial activity, also enhances antiviral and IRF7 gene expression during RV infection. These findings indicate that azm

enhances antiviral signaling during RV infection through the TBK1/IKKε-IRF3/IRF7 axis, and that the same effects can be achieved with non-antibiotic macrolides.

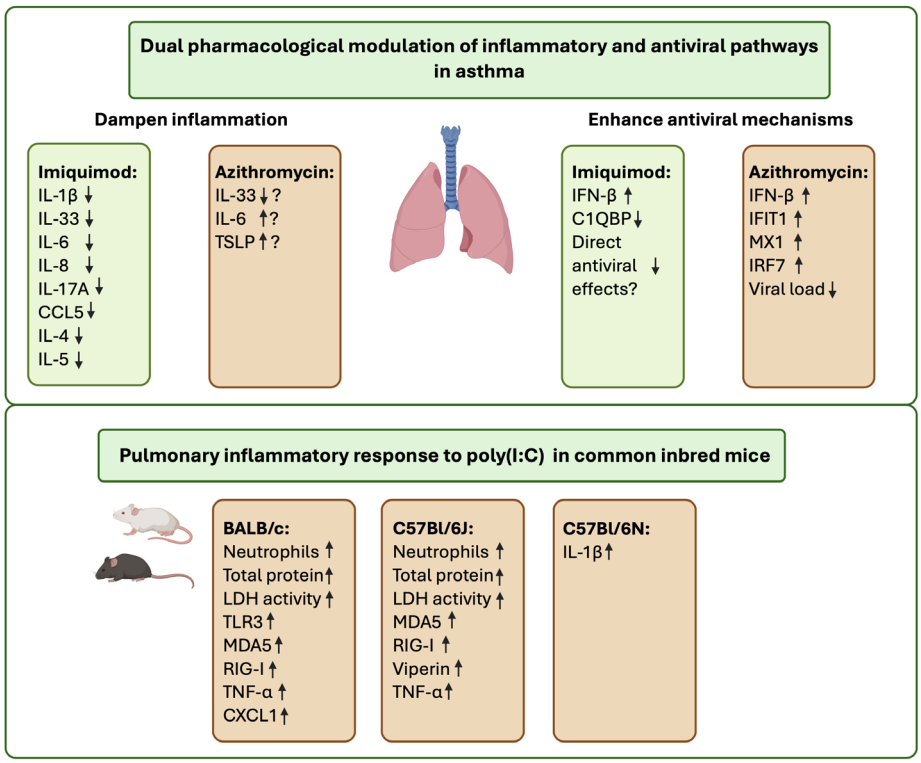

Figure 14. Summary of the main findings from the papers included in the thesis. Created in https://BioRender.com.

Future perspectives

Although this thesis has provided several important insights, our findings also open new avenues for research. The following sections highlight key questions that remain to be addressed in future studies.

To further explore dual-action therapeutic strategies in asthma

In paper I, we show that a single drug (imiquimod) can both enhance the antiviral defense and dampen inflammatory and alarmin responses in BECs. Thus, the activation of TLR7 (by imiquimod or other molecules) represents a promising avenue for future studies. Several TLR7-agonists are currently commercially available, including resiquimod and AZD8848. AZD8848 has been shown to reduce symptoms of allergic rhinitis and airway hyperresponsiveness in patients with allergic asthma (157, 158). Further studies should explore the effects of TLR7 activation during live RV infection, rather than poly(I:C) stimulation, to better evaluate the effects on antiviral defense and inflammatory responses in BECs from asthma patients. It would also be relevant to perform such studies in an ALI system, to investigate potential effects on a differentiated epithelium as well as on barrier integrity. Furthermore, assessing the antiviral potential of TLR7 activation against other viruses could increase preparedness for future pandemics.

Although azm primarily affected the antiviral response in BECs in our studies (paper III and IV), it may be seen as a dual-action drug due to its effects on inflammation *in vivo* and on other cell types (116-118). Evidence showing that azm reduces asthma exacerbations in patients with moderate-to-severe asthma further supports the concept that the development of dual-action drugs is feasible and warrants greater attention (115). Moreover, since the concept of deficient antiviral immunity in asthma remains debated, it would be interesting to elucidate the relative contribution of the antiviral and anti-inflammatory properties of azm to its ability to reduce asthma exacerbations. This may provide valuable insights into the further development of exacerbation-sparing drugs.

To further elucidate the mechanism of action of azm and non-antibiotic macrolides

In paper III, we concluded that azm enhances the antiviral defense of BECs *in vitro*, regardless of eosinophilic status. However, BECs from patients with atopic asthma appeared less responsive to azm, as the azm-mediated increases in IFN-β and IFN-λ reached only near significance in this group (p=0.064 and 0.084, respectively). Similarly, the reduction of viral load was also nearly significant (p=0.054). To our knowledge, no other study has investigated the impact of atopy on the response to azm treatment. Given the relatively low sample size in our study (n=10 per group), larger studies are needed to determine whether atopy influences treatment response. Larger clinical studies should also be conducted to investigate whether our findings translate to treatment response in terms of the reduction of clinical symptoms, and not only the BEC antiviral response.

Furthermore, the results from the clinical study AZIMUNE, a randomized double-blind, placebo-controlled trial of 12-week azm treatment in patients with moderate-to-severe asthma, are planned to be published. This study has generated a conference abstract (159), but a full manuscript is currently under revision. Completion of this study enables valuable post-hoc analyses, such as assessing the impact of eosinophilia and atopy on the epithelial responses to clinically administered azm, thereby complementing and extending the findings in paper III. It would also be relevant to investigate whether the expression of IFIT1, MX1 and IRF7, found to be upregulated after *in vitro* azm treatment in paper IV, is increased in BECs by clinical azm treatment. Furthermore, the observation that RV-induced IL-33 was reduced by clinical azm treatment is intriguing and warrants further investigation, as this indicates that prolonged azm exposure may exert additional effects not captured by short *in vitro* exposure.

Paper IV, which explored the molecular mechanisms underlying the effects of azm on BECs, opens up several avenues for future research. Although we demonstrated that the TBK1/IKKε-IRF3/IRF7 axis is involved in the azm-mediated enhancement of antiviral signaling during RV infection, the mechanisms through which azm induces antiviral signaling alone, in the absence of infection, remain unknown. It would also be of interest to determine whether azm, for instance, enhances the TBK1/IKKε pathway through direct activation of PRRs, or by modulating other transcriptional processes.

In paper IV we primarily focused on the antiviral effects of azm. However, we also found that, in the absence of RV infection, azm downregulated pathways related to remodeling and mucus production, two central processes in asthma pathogenesis. The observation that these pathways were affected only under non-infected conditions may reflect a “steady state” effect of azm that might contribute to its clinical effect. These findings warrant further investigation in ALI cultures, where

remodeling and mucus production can be evaluated. For instance, we have previously studied crosstalk between bronchial smooth muscle cells (BSMCs) and BECs using conditioned medium from BECs (160). With ALI models, co-cultures of azm-treated BECs and BSMCs could be established to investigate the effects of azm on BSMC proliferation, a major trait of airway remodeling in asthma (161).

Finally, what is perhaps most important for further macrolide-related studies is the continued development of non-antibiotic macrolides. Given the global issue of antimicrobial resistance, research should focus on finding non-antibiotic alternatives to azm, such as EP395. Future studies should compare the mechanisms of action of EP395 and azm, to determine whether they also involve TBK1 and IKKε signaling. Furthermore, the effects of EP395 and azm on antiviral immunity should be confirmed in ALI cultures, where co-culture models could be used to capture more complex interactions with other cell types in the lung.

To optimize mouse models of asthma exacerbations

Paper II describes a mouse model of viral infections. To increase its relevance for asthma research, an allergic background should be introduced by sensitizing mice with house dust mite (HDM) or other relevant allergens via intranasal administration. Exacerbations can then be evoked by subsequent administration of poly(I:C) as described in paper II. Our group has previously published a model with HDM sensitization and poly(I:C)-induced exacerbation, but with C57Bl/6 mice that were bred in-house with unknown substrain identity (162). We now aim to establish this model with commercially obtained mice with known substrain identity. Since the C57Bl/6N strain did not display enhanced inflammatory responses to poly(I:C) in our model, future use of the HDM-poly(I:C) exacerbation model will focus on BALB/c or C57Bl/6J strains. Furthermore, we aim to include additional sensitization or exacerbation agents that are relevant to asthma. Increased air pollution with particulate matter (PM) is a growing concern worldwide, and PM is a known asthma trigger. Hence, we will also develop models of PM-induced exacerbations to further increase the translational relevance of this mouse model.

The use of these models is a valuable tool for investigating virus-induced asthma exacerbations *in vivo* and for evaluating novel therapeutic strategies in a setting that models the complex interaction of the several cell types in the lung which cannot be fully recapitulated *in vitro*.

To establish air-liquid interface mono- and co-cultures of BECs

As previously mentioned, a future goal is also to fully establish ALI models of BECs in our lab. While submerged cultures are highly useful for investigating intracellular signaling, other aspects of epithelial biology, such as barrier function, morphology, cell composition and mucus production are best studied in ALI cultures. ALI systems also allow for more complex co-cultures with other cell types such as BSMC, T-cells, B-cells and monocytes, thereby enhancing the translational relevance of findings. As mentioned, these models will be particularly valuable for extending the findings presented in this thesis, for example by investigating the effects of imiquimod and azm on epithelial barrier integrity and mucus production, but also by validating results in a more physiologically relevant model system. Both azm and imiquimod have been shown to affect other cell types beyond BECs, but how treatment influences the interactions between BECs and, for example, T-cells and monocytes is less well described. Gaining these insights is crucial for understanding the complexity of how pharmacological interventions may reduce asthma exacerbations, given the involvement of multiple cell types in the disease pathogenesis.

Populärvetenskaplig sammanfattning

De flesta känner till att astma är en relativt vanlig lungsjukdom som gör det svårt att andas ibland, men färre vet att astma är en komplex sjukdom som kan vara svår att kontrollera, och att den kan indelas i flera kliniska undergrupper. I grunden handlar astma om en inflammation i luftvägarna som förvärras av olika ”triggers” - till exempel träning, pollen, kvalster, luftföroreningar och infektioner. Vad som orsakar astma är inte helt klarlagt, men både genetiska och miljöfaktorer spelar in.

Hosta, pipande eller väsande andning, ökad slemproduktion och andfåddhet är typiska symptom för astma. När en person utsätts för en trigger kan symptomen snabbt förvärras i en så kallad astmaattack. Den allra vanligaste orsaken till en attack är en infektion. En enkel förkylning som för friska individer är en bagatell kan för en person med astma leda till så svår andnöd att vardagliga aktiviteter blir omöjliga – och kan i värsta fall kräva akut vård.

De allra flesta som lever med astma får god hjälp av antiinflammatoriska kortikosteroider och luftvägsvidgande läkemedel. Men för en grupp patienter räcker inte behandlingen till, och de drabbas av återkommande attacker. En viktig orsak är att astma inte är en enda sjukdom, utan det finns flera olika varianter med olika bakomliggande mekanismer. Hos vissa dominerar allergi och en typ av vita blodkroppar som kallas eosinofiler, medan andra typer inte alls är kopplade till allergi. Detta gör att patienter svarar olika på behandling - och understryker behovet av fler läkemedelsalternativ.

För att kunna utveckla nya läkemedel måste vi förstå vad som skiljer en förkylning hos en frisk person från en förkylning hos någon med astma, särskilt vad gäller immunförsvaret i luftvägarna. När ett virus kommer ner i lungorna är det så kallade epitelceller som det möter först. Dessa celler skapar en barriär som dels är fysisk, med slem som fångar upp virus och små flimmerhår som transporterar bort det, men också immunologisk, då de fungerar som kroppens larmcentral genom att skicka ut signaler som sätter igång immunförsvaret vid en pågående infektion.

Immunresponsen mot virus kan delas in i två delar: den antivirala responsen, som hämmar viruset, och den pro-inflammatoriska responsen, som lockar dit fler immunceller för att bekämpa infektionen. Balansen mellan dessa är avgörande. Om den antivirala responsen blir för svag eller inflammationen för stark kan kroppen ta mer skada än vad själva viruset orsakar. Just detta sker ofta i luftvägarna hos personer med astma. Epitelcellerna producerar för lite antivirala ämnen, samtidigt

som inflammationen blir överdriven. Resultatet blir värre symptom, sämre virusbekämpning och en långsiktig försämring av astman.

Det virus som oftast utlöser en astmaattack är rhinovirus, ett av våra vanligaste förkylningsvirus. Under COVID-19-pandemin diskuterades mycket om astmapatienter löpte särskild risk vid infektion med SARS-CoV-2. Samlade bevis tyder nu på att det finns en liten ökad risk för astmapatienter att bli inlagda eller avlida till följd av COVID-19, men den främsta risken är relaterat till svårighetsgraden av COVID-19, och inte triggandet av en astmaattack.

I den här avhandlingen har vi undersökt hur man på olika sätt kan stärka epitelcellernas antivirala försvar och samtidigt dämpa den överdrivna inflammationen. Vi har bland annat visat att imiquimod, ett läkemedel som idag används för behandling av vårtor och vid viss hudcancer, både ökar det antivirala svaret och minskar inflammationen i epitelceller från astmapatienter. Dessa data visar att det är möjligt att med ett och samma läkemedel både öka det antivirala svaret och minska inflammation, och gör imiquimod till en lovande kandidat för framtida behandling av astma.

Vidare har vi studerat det vanliga antibiotikumet azitromycin, som vid klinisk behandling visats minska risken för astmaattacker, en effekt som vi tror är oberoende av dess anti-bakteriella effekt. Azitromycin har också visats öka det antivirala svaret i epitelceller från astmapatienter, något vi har undersökt närmare. Vi har kunnat visa att behandling med azitromycin förstärker det antivirala svaret i epitelceller från astmapatienter, oavsett astmatyp, en observation som är viktig för riktlinjer vad gällande dess användande. Vi har också kunnat identifiera att denna förstärkning sker via en specifik signalväg, där proteinerna TBK1, IKKε, IRF3 och IRF7 ingår.

Eftersom antibiotika bör användas med försiktighet undersökte vi också ett derivat av azitromycin som saknar antibakteriell effekt. Våra resultat visar att även detta ämne stärker det antivirala svaret på samma sätt som azitromycin. Detta öppnar för möjligheten att utveckla behandling som kan hjälpa astmapatienter utan att bidra till antibiotikaresistens.

Sammanfattningsvis har vi visat att det är möjligt att påverka både den antivirala förmågan och inflammationen i luftvägarna med ett och samma läkemedel, något som kan vara avgörande för att minska risken för astmaattacker. Av de två läkemedel vi undersökt används redan azitromycin i kliniken, medan imiquimod behöver studeras mer innan det kan bli aktuellt som behandling vid astma. Fortsatta studier av hur azitromycin fungerar är också viktiga för att utveckla säkrare varianter som saknar antibakteriella egenskaper och därmed inte riskerar att driva antibiotikaresistens. Då azitromycin har visats ha effekt oberoende av astmatyp hoppas vi att vår forskning kan bidra till att fler med svårbehandlad astma kan få ett bättre liv.

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Paper I

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Imiquimod Boosts Interferon Response, and Decreases ACE2 and Pro-Inflammatory Response of Human Bronchial Epithelium in Asthma

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Background: Both anti-viral and anti-inflammatory bronchial effects are warranted to treat viral infections in asthma. We sought to investigate if imiquimod, a TLR7 agonist, exhibits such dual actions in *ex vivo* cultured human bronchial epithelial cells (HBECs), targets for SARS-CoV-2 infectivity.

Objective: To investigate bronchial epithelial effects of imiquimod of potential importance for anti-viral treatment in asthmatic patients.

Methods: Effects of imiquimod alone were examined in HBECs from healthy (N=4) and asthmatic (N=18) donors. Mimicking SARS-CoV-2 infection, HBECs were stimulated with poly(I:C), a dsRNA analogue, or SARS-CoV-2 spike-protein 1 (SP1; receptor binding) with and without imiquimod treatment. Expression of SARS-CoV-2 receptor (ACE2), pro-inflammatory and anti-viral cytokines were analyzed by RT-qPCR, multiplex ELISA, western blot, and Nanostring and proteomic analyses.

Results: Imiquimod reduced ACE2 expression at baseline and after poly(I:C) stimulation. Imiquimod also reduced poly(I:C)-induced pro-inflammatory cytokines including IL-1β, IL-6, IL-8, and IL-33. Furthermore, imiquimod increased IFN-β expression, an effect potentiated in presence of poly(I:C) or SP1. Multiplex mRNA analysis verified enrichment in type-I IFN signaling concomitant with suppression of cytokine signaling pathways induced by imiquimod in presence of poly(I:C). Exploratory proteomic analyses revealed potentially protective effects of imiquimod on infections.

Conclusion: Imiquimod triggers viral resistance mechanisms in HBECs by decreasing ACE2 and increasing IFN-β expression. Additionally, imiquimod improves viral infection tolerance by reducing viral stimulus-induced epithelial cytokines involved in severe COVID-19 infection. Our imiquimod data highlight feasibility of producing pluripotent drugs potentially suited for anti-viral treatment in asthmatic subjects.

Keywords: asthma, anti-viral drug, SARS – CoV – 2, imiquimod, COVID-19, TLR7 agonist

INTRODUCTION

Asthma is a chronic inflammatory disease affecting more than 300 million people worldwide (1). Acute exacerbations of asthma are the major cause of disease worsening and morbidity, and they are responsible for increasing health care burden (2). Rhinovirus infection is the major trigger for asthma exacerbation (2). However, other respiratory viruses such as respiratory syncytial virus (RSV), influenza A or coronaviruses have been also related to an aggravation of the disease (2). For example, intensive care admissions and mortality of patients with H1N1 influenza A was higher in patients with asthma during 2009 H1N1 pandemic (3).

In December 2019, a novel coronavirus disease, named coronavirus disease-19 (COVID-19), emerged in the Hubei province of China (4). This disease is caused by severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) (5). Although the first epidemiologic studies could not find an association of asthma with an increase risk to suffer from COVID-19, novel data from large cohorts evidenced a higher prevalence of asthma in patients with COVID-19 compared to the general population (6). Asthma was also associated to a longer duration of severe COVID-19 disease (7). Furthermore, several studies demonstrated that severe asthma is a risk factor for developing severe COVID-19 (8, 9). The high infection and mortality rates, in combination with the high economic impact of COVID-19, underpinned an unprecedented global search for efficient treatments and successful developments of vaccines. However, there is still a need for new drug opportunities to treat SARS-CoV-2 infection, especially in patients with a high risk to develop a severe sickness such as asthmatic subjects.

Similar to rhinoviruses, SARS-CoV-2 is a single stranded RNA (ssRNA) virus (10). SARS-CoV-2 mainly infects human cells through the high affinity interaction of the receptor binding domain (RBD) of the spike protein 1 (SP1) with the angiotensin converting enzyme II (ACE2) protein on host cells (11, 12). ACE2 is widely expressed in different cell types and tissues including bronchial and upper airway epithelial cells, representing the main port of entry by SARS-CoV-2 (11, 13–15). In addition to ACE2, the host serine protease TMPRSS2, responsible for fusion of the viral and host cell membranes by cleaving S protein at the S1/S2 and S2′sites, is essential for viral entry (11). Hence, investigating the expression of both ACE2 and TMPRSS2 in the bronchial epithelium may contribute to understand the infectivity and actions of SARS-CoV-2 and assist in the search of pharmacological opportunities to combat COVID-19.

Following cell entry, both rhinovirus and SARS-CoV-2 virus replicate and produce intermediate double stranded RNA (dsRNA) molecules (16, 17). dsRNA molecules activate pattern recognition receptors (PRRs) in host cells including Toll-like Receptor (TLR)3, Melanoma Differentiation-Associated protein 5 (MDA5) and Retinoic Acid-Inducible Gene I (RIG-I) (17, 18). Activation of PRRs results in the activation of two important downstream transcriptional factors, NF-κB and Interferon Regulatory Factors (IRFs) leading to production of cytokines or chemokines (such as IL-6, TNF-α, IL-8) and type-I and type-III interferons (IFN; IFN-β and IFN-λ), respectively (17). These molecules represent early responses to viral infection, followed later by anti-viral T-cell responses (17, 19). In COVID-19, a low induction of type-I and type-III IFNs has been described (20, 21). A dysregulation of IFN pathways at early stages of COVID-19 is thought to contribute to uncontrolled viral replication and subsequent dramatic immune responses at later stages (20–23). In addition, asthmatic patients have been described to have an impaired anti-viral IFN response (24, 25), which could predispose these patients to be more susceptible to viral infections. Therefore, one of the desired treatment strategies in order to improve the outcome of viral infection in these patients is the increase of type I IFN responses in the bronchial epithelium.

A second common feature of asthma exacerbations after viral infection and severe COVID-19 is the exaggerated pro-inflammatory response of the bronchial epithelium to virus (17, 26). In this regard, glucocorticoids are an acknowledged treatment of both asthma and severe COVID-19 due to their broad anti-inflammatory effects (27–29). Indeed, inhaled budesonide administered in the early stages of COVID-19 reduced the need for urgent medical care and improved the recovery time (30). However, due to a potential to decrease the anti-viral type I interferon response (31–34), a high dose of glucocorticoids could be harmful if they are given at the peak of viral replication (34). It is, therefore, when the immunopathological features of the illness become more evident that the glucocorticoid effect could be most beneficial, creating a window of opportunity vital to clinical effect (27, 34). The question arises whether drugs can be found that both improve anti-viral responses and reduce severity of pro-inflammatory responses.

Previous attempts to discover pharmacological opportunities to improve both anti-viral defense and reducing cytokine inflammation have been proven to be difficult to combine in one single molecule [(35), and unpublished observations]. Literature data, however, suggest that imiquimod, an imidazo-quinoline used as an immune response modifier, could be an interesting compound to explore further in regards of combined anti-viral and anti-inflammatory actions. Imiquimod is a TLR7 agonist clinically approved as topical treatment of viral and tumoral skin conditions. Imiquimod may activate the production of type-I and III interferons as well as the NF-κB pathway (36), although results vary (37, 38) and effects on human airway epithelium seem unexplored. Imiquimod has been considered as treatment for allergic diseases like asthma due to its inhibition of allergic type 2 inflammation and favoring of type 1 responses (39, 40). Focusing on potential anti-allergic efficacy, local airway treatment with other TLR7 agonist have been employed in clinical trials in asthma and allergy (41, 42). Interestingly, intranasal administration of imiquimod has been demonstrated to protect against Influenza A virus in mice, reducing viral replication, neutrophil infiltration, and lung dysfunction (37). Based on this observation, and observations

Abbreviations: ACE2, Angiotensin Converting Enzyme II; BEGM, Bronchial Epithelial Growth Medium; COVID-19, Coronavirus Disease-19; dsRNA, Double Stranded RNA; HBECs, Human Primary Bronchial Epithelial Cells; ICS, Inhaled Corticosteroids; IRFs, Interferon Regulatory Factors; MDA-5, Melanoma Differentiation-Associated protein 5; PRRs, Pattern Recognition Receptors; RBD, Receptor Binding Domain; RIG-I, Retinoic Acid-Inducible Gene I; SARS-CoV-2, Severe Acute Respiratory Syndrome-Related Coronavirus 2; SP1, Spike Protein 1; STRING, Search Tool for the Retrieval of Interacting Genes; ssRNA, Single Stranded RNA; TLR, Toll-Like Receptor; WHO, World Health Organization.

on potential anti-viral actions in a variety of cell systems, imiquimod has already been suggested as a potential therapy in early phase COVID-19 disease (43–45). However, studies have not focused on major target cells for primary site of infectivity of SARS-CoV-2, the human airway epithelium. Little is thus known about effects of imiquimod on human primary bronchial epithelial cells (HBECs). Considering the occurrence of reduced anti-viral resistance in asthma, demonstrated as reduced interferon production at viral infection (46), and the increasing evidence demonstrating that severe asthma is a risk factor for COVID-19 related admission to intensive care (8, 9), studies of imiquimod in HBECs from asthmatic patients are of particular interest.

Our study explores effects of imiquimod on ACE2 and TMPRSS2 expression in HBECs stimulated with both the TLR3 agonist poly(I:C) (SARS-CoV-2 replication mimic) or the recombinant SP1 from SARS-CoV-2 (RBD). In addition, effects of imiquimod on anti-viral and anti-inflammatory responses were examined by using multiplex ELISA and gene expression analyses, and non-targeted proteomics. We also used siRNA technology to shed light on the potential mechanisms of action of imiquimod in HBECs.

MATERIAL AND METHODS

Primary Human Bronchial Epithelial Cells (HBECs)

HBECs from two different study populations of adult patients with asthma (study population 1, N = 9; study population 2, N = 9), as well as from a small reference population (N = 4) of healthy subjects (**Table 1**), were obtained by bronchial brushings and expanded *in vitro*, as previously described (47). Asthma diagnosis was confirmed as defined by Global Strategy for Asthma Management and Prevention criteria, (GINA 2017; https://ginasthma.org). Asthma study population 1 was made up of steroid-free mild asthmatics, whereas study population 2 included mild to severe and uncontrolled asthmatic patients treated (N = 5) or not (N = 4) with inhaled corticosteroids (ICS). Both study populations consisted of a mix of patients with T2-high/T2-low and atopic/non-atopic phenotypes. A positive allergen specific IgE test against the 10 most common aeroallergens was used to define atopic asthma. A fractional exhaled nitric oxide (FeNO) > 25 ppb was used to define T2-high asthma. T2-low asthma is defined as FeNO < 25 ppb.

TABLE 1 | Clinical and demographic characteristics of asthma patients.

	Healthy population	Study population 1	Study population 2	P-value
N	4	9	9	
Demographic characteristics				
Age [mean (range)]	22.51 (19-29)	28.56 (19-57)	42.78 (19-70)	0.217
Sex (M/F)	1/3	6/3	6/3	0.309
BMI (Kg/m^2)	22.56 (5.01)	22.32 (3.32)	23.92 (5.06)	0.555
Asthma phenotype				
Atopy (Yes/No)	0/4	5/4	4/5	0.164
T2-high/T2-low	–	4/5	6/3	0.343
GINA severity*	–	9/0	4/5	0.008
Lung function and asthma control				
FEV1 (%)	108.5 (29.7)	96.0 (19.0)	83.0 (27.0)	0.121
FVC (%)	110.5 (37.0)	103.0 (16.5)	102.0 (26.5)	0.531
FEV1/FVC (%)	96.4 (7.0)	75.0 (13.5)	72.0 (14.5)	0.002
PD_{15} (mg)	635 (0)	178.0 (217.0)	176.4 (323.8)	0.003
ACQ_6	–	1.50 (1.58)	1.50 (1.51)	0.616
ICS treatment (Yes/No)	–	0/9	5/4	0.008
Blood biomarkers				
FeNO (ppb)	–	19.0 (58.2)	25.0 (26.1)	0.730
IgE (IU/mL)	–	46.0 (162.0)	98.0 (112.5)	0.546
Blood differential cell count				
Leukocytes ($10^3/\mu L$)	–	6.00 (3.20)	6.00 (2.20)	0.948
Neutrophils ($10^3/\mu L$)	–	3.50 (1.30)	3.60 (2.30)	>0.999
Lymphocytes ($10^3/\mu L$)	–	1.70 (0.80)	1.60 (0.85)	0.714
Monocytes ($10^3/\mu L$)	–	0.40 (0.30)	0.60 (0.25)	0.570
Eosinophils ($10^3/\mu L$)	–	0.10 (0.28)	0.12 (0.19)	0.812
Basophils ($10^3/\mu L$)	–	0.04 (0.05)	0.05 (0.03)	0.502
Sputum differential cell count				
Sputum Eosinophils (%)	–	0.75 (8.05)	4.50 (4.50)	0.798
Sputum Neutrophils (%)	–	42.75 (64.35)	38.50 (47.25)	0.955
Sputum Macrophages (%)	–	50.63 (57.35)	46.25 (37.25)	0,955
Sputum Lymphocytes (%)	–	0.00 (0.41)	1.00 (1.00)	0,029

BMI, Body mass index; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; PD_{15}, Dose of mannitol required to reduce the FEV1 by 15% of the baseline value; ACQ_6, Asthma Control Questionnaire; FeNO, fractional exhaled nitric oxide.

T2-high is defined as FeNO > 25 ppb.

Data are presented as median value (Interquartile range), unless otherwise expressed.

**GINA Severity: mild/moderate/severe.*

Kruskal-Wallis or U-Mann Whitney test for continuous variables and chi squared test for categoric variables.

The study was conducted in accordance with the Helsinki II declaration and with permission from the Danish Committee on Health Research Ethics (Ethics: H-16043663 and H-16002008), and all patients have given informed consent.

Design, Expression and Purification of Recombinant SARS-CoV-2 Spike Protein (SP1)

The boundaries for SP1 sequence (ID: QIA20044.1) covered aa. 325-561, surrounding the RBD. The codon-optimized gene sequence (synthesized by Geneart©) had a N-terminal BiP secretion signal and a C-terminal (x10) poly-histidine tag used for purification. SP1 was expressed using the ExpreS2 platform. Briefly, Schneider-2 insect cells were transfected (ExpreS2 Insect TRx5, ExpreS2ion Biotechnologies) and cells were grown at 25°C in shake flasks for 3 days. The supernatant was then centrifuged (5000 rpm; 10 min; 4°C) and filtered through a 0.22 µm vacuum filter (PES). Recombinant SP1 was further purified on a 5 mL HisTrap HP column (GE healthcare), and bound protein was eluted with 500 mM Imidazole in PBS buffer, pH 7.4.

Imiquimod, Poly(I:C) and SP1 Treatment

HBECs ($3x10^4$ cells/mL) were cultured in bronchial epithelial growth medium (BEGM, Clonetics, San Diego, CA, USA) until 80% confluence in type-I bovine collagen-coated (Advanced BioMatrix, San Diego, USA) 12-wells plates (Nunc Technolgies, Carlsbad, CA, USA) at 37°C, 5% CO_2 in air. Cells were treated with 10 µg/mL imiquimod (Tocris Bioscience, Bristol, UK) with or without 10 µg/mL polyinosine-polycytidylic acid (poly(I:C)) (InvivoGen, San Diego, US) or SARS-CoV-2 SP1 for 3, 24 or 48 hours. Cell-free supernatants were collected for protein release analysis and cell lysates were collected for protein or gene expression analysis.

Western Blot Analysis

Quantification of protein expression from cell lysates was performed by Western blot, as previously described (46). For that, primary rabbit anti-human antibodies from Bio-Rad, Stockholm, Sweden (ACE2 #AHP888) and Cell Signaling, Danvers, Massachusets, USA (GAPDH #5174S) were used at dilution 1:1000, and an anti-rabbit secondary antibody #09/2029 lot. 28 (Cell Signaling, Danvers, Massachusets, USA) conjugated to HRP was used. Optical density was detected using a LI-COR odyssey Fc imager system (LI-COR, Lincoln, USA). Optical density ratio between samples and GAPDH were calculated and normalized towards poly(I:C) stimulated samples.

Sample Preparation for Mass Spectrometry

Cells were lysed in ice cold 5% SDS in 100 mM Tris (pH=7.55) sonication using a Branson Digital Sonifier® 250-D (Branson Ultrasonics Corporation, Danbury, USA), at amplitude 10%, with 10s pulse on and 20s off, for a total of 36 cycles. The lysates were centrifuged at 15,871 xg for 8 minutes to remove insoluble material. 60 µg protein per cell lysate supernatant per sample was processed on HILIC Microspheres (ReSyn Biosciences, Gauteng, South Africa) in a King-Fisher Flex (Thermo Fisher Scientific, Bremen). The following steps were performed in 96-well plates in the system: magnetic microspheres (1:10 protein:beads ratio) were incubated in equilibration buffer (15% ACN, 100 mM NH_4Ac, pH=4.5); protein samples were incubated in binding buffer (30% ACN, 200 mM NH_4Ac, pH=4.5) for binding of proteins to HILIC beads; beads were washed twice in 95% ACN; digestion of proteins for 1h at 37°C with trypsin (20:1 protein:trypsin ratio) dissolved in 50 mM AMBIC. Peptides were recovered from the plate and dried in a Speedvac (Thermo Fisher Scientific, Germany) prior to C_{18} desalting. Peptide desalting was performed using BioPureSPN Mini, PROTO 300 C_{18} (The Nest Group, Inc., MA, USA). Briefly, columns were equilibrated with 100 µl 70% ACN, 5% FA and conditioned using 100 µl 5% FA. Peptide samples were resuspended in 100 ul 5% FA and loaded on the column. Columns were washed with 5% FA and before elution of peptides using 100 µl 50% ACN, 5% FA. The resulting peptide solution was dried by vacuum centrifugation and stored at -20°C until analysis.

Mass Spectrometry

Samples were resuspended in 10 µl 0.1% FA and 6 µl were loaded onto an EASY-nano LC system (Thermo Fisher Scientific, Germany). The analytical column was a silica capillary (75 µm* 16 cm Pico Tip Emitter, New Objective, USA) packed in house with C18 ReproSil-Pur 1.9 µm (Dr. Maisch GmbH, Germany). Peptides were separated using a 60 min LC gradient from 5% to 25% solvent B (80% ACN, 0.1% FA) and continuously sampled by a Q-Exactive HF-X Mass Spectrometer (Thermo Fisher Scientific, Germany) through an electrospray interface. Data were acquired using data-dependent acquisition (DDA) in positive ion mode. Precursor spectra (375 to 1500 m/z) were acquired at 120,000 resolution with automatic gain control (AGC, MS1 target $3x10^6$) and a maximum injection time of 50 ms. The 20 most abundant ion peptides were continuously selected for fragmentation. Fragmentation spectra were acquired at 15,000 resolution with an AGC target of $1x10^5$ ions and a maximum injection time of 20 ms. Isolation width for fragmentation was set to 1.2 m/z.

Knockdown of RIG-I and MDA5

For siRNA-mediated down-regulation of MDA5 and RIG-I expression, HBECs were transfected with 10 nM siRNA targeting MDA5, RIG-I or non-specific siRNA (scramble) (Ambion, Thermo Scientific, Waltham, NA, USA) using Lipofectamine RNAiMAX (*Invitrogen*, Thermo Scientific, Waltham, NA, USA), as previously described (48).

RNA Isolation and Gene Expression Analysis Using RT-qPCR and NanoString

Total RNA was isolated from cell lysates using RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol and 1 µg RNA was reversely transcribed into cDNA with Precision Nanoscript Reverse Transcription kit (PrimerDesign, Southampton, UK). RT-qPCR gene expression analysis was performed on an Mx3005P qPCR system (Stratagen, La Jolla, USA) with standard cycling parameters and primers from

Qiagen (Stockholm, Sweden) and PrimerDesign (Chandler's Ford, UK). GAPDH was used as reference. A full list of the primers used in qPCR analyses is shown in **Supplementary Table 1**. Gene expression was analyzed following the ΔΔCt method.

For multiplex mRNA expression analysis, the NanoString nCounter system (NanoString technologies, Seattle, USA) was used. First, RNA quality was measured using a bioanalyzer (Agilent technologies, Santa Clara, USA). Then a panel of 579 mRNAs involved in the human immune response (NanoString Immunology Human V2 panel) was analyzed. Data was normalized to seven house-keeping genes, selected using the geNorm algorithm, and differential gene expression and pathway analysis were performed. All analysis was performed with nSolver software (NanoString technologies, Santa Clara, USA) and PANTHER classification system.

Multiplex ELISA Analysis of Released Proteins

Protein levels of IFN-β, IL-1β, TNF-α, IL-8, IL-6, IL-33, CCL-5, IL-4, IL-5, IL-13 and IL-17A were measured using a Luminex immunoassay (R&D Systems, Abingdon, UK) in cell-free supernatants on a MAGPIX instrument (R&D Systems, Abingon, UK) according to manufacturer's instructions. Lower limit of detection was 3.86 pg/mL for IFN-β, 3.39 pg/mL for IL-1β, 11.77 pg/mL for TNF-α, 2.10 pg/mL for IL-8, 9.19 pg/mL for IL-6, 15.25 pg/mL for IL-33, and 10.12 pg/mL for IL-17A.

Statistical Analysis

The data are presented as median (interquartile range). The normality of the data was assessed with Shapiro-Wilk test. Paired analysis of difference between groups was made using the Wilcoxon test for two group comparisons, and RM-one way ANOVA with Holm-Sidák´s *post hoc* test or Friedman with Dunn´s multiple comparisons test for more than two groups. Unpaired analyses were performed using Mann-Whitney U-test for two groups comparisons, or Kruskal-Wallis tests when multiple groups were assessed. P-values of < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism version 8.0 software (GraphPad Software). Multiple t-test analyses were carried out for the detection of differentially expressed genes and proteins after Nanostring and proteomic analyses. Gene Ontology and pathway analyses were performed using the PANTHER classification system (version 13.1) and the Search Tool for the Retrieval of Interacting Genes (STRING) (Version: 11.0, www.string-db.org).

RESULTS

Imiquimod Decreases ACE2 Receptor Expression and Increases IFN-β Expression in HBECs From Patients With Asthma

The bronchial epithelial expression of SARS-CoV-2 receptors, as well as the IFN response to virus are two key aspects of anti-viral resistance in the light of SARS-CoV-2 infection. Hence, we studied the direct effect of imiquimod on the expression of ACE2, TMPRSS2, and IFN-β signaling mediators. Treatment with imiquimod for 24 hours resulted in a clear decrease of ACE2 expression ($P<0.0001$) in HBECs from asthmatics compared to non-treated cells (**Figure 1A**). A slight increase in TMPRSS2 expression ($P=0.0174$) was, however, observed after imiquimod treatment in asthmatic epithelial cells (**Figure 1B**). Since imiquimod is known to increase IFN-β in cell systems beyond the lung (49, 50), we determined whether such an effect occurred also in HBECs. Imiquimod increased IFN-β expression ($P=0.0214$) in HBECs from asthmatic patients compared with untreated cells (**Figure 1C**). We also studied the effects of imiquimod on PRRs involved in dsRNA recognition (TLR3, MDA5 and RIG-I). Compared with untreated cells, imiquimod decreased TLR3 expression ($P=0.0043$) (**Figure 1D**). However, in accordance with the increased IFN-β expression MDA5 expression was clearly up-regulated (90% increase; $P=0.0002$) with imiquimod (**Figure 1E**), and no change was found in RIG-I expression (**Figure 1F**).

Imiquimod Displays Mixed Effects on Pro-Inflammatory Response of HBECs From Steroid-Free Mild Asthmatics

Another aspect of importance in the bronchial epithelial response to virus is the viral infection tolerance. Due to potential cofounding epithelial effects of anti-inflammatory steroid treatment in asthma, the effect of imiquimod was first studied in HBECs from steroid-free mild asthmatics (Study population 1; **Table 1**). Particularly important in asthma pathophysiology, imiquimod treatment of HBECs from steroid-free asthmatic patients reduced ($P<0.01$) the gene expression of the allergic disease-related alarmin IL-33 (**Figure 2A**). By some contrast, the eosinophilic chemoattractant CCL5 was augmented at gene level after 3h and 24h ($P<0.05$) treatment with imiquimod (**Figure 2B**). However, no change was found at CCL5 protein level (data not shown). Similarly, imiquimod increased ($P<0.05$) TNF-α only at gene level and only after 3h imiquimod treatment (**Figure 2C**). Interestingly, imiquimod decreased ($P<0.01$) the gene and protein levels of IL-1β (**Figures 2D, E**), and a similar trend was found for IL-6 and IL-8 at protein level (**Figure 2E**).

Imiquimod Treatment Modifies the Proteome of HBECs From Steroid-Free Asthmatics

To further explore the effect of imiquimod treatment on the bronchial epithelium immune response, a quantitative proteomic analysis was performed comparing imiquimod treated *vs* untreated HBECs from 3 steroid-free asthmatics. In this exploratory analysis, 75 out of 1266 proteins were found down-modulated with imiquimod treatment (log2 fold-change > 1.5; $P < 0.05$) (**Figure 3A** and **Supplementary Table 2**). Pathway analyses of these proteins indicated an enrichment of the protein pathways "*HSA-5663205: infectious diseases*" and "*HSA-168254: influenza infection*" (**Figure 3B** and **Supplementary Table 3**), which included proteins implicated in viral mRNA and protein

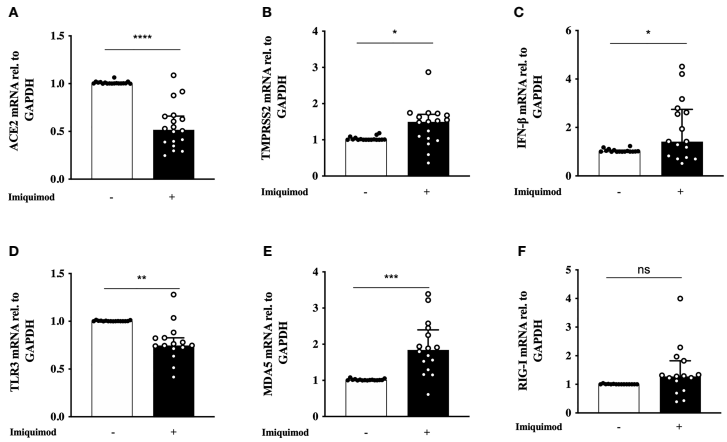

FIGURE 1 | Effect of imiquimod treatment on SARS-CoV-2 receptor expression and IFN response in human bronchial epithelial cells (HBECs) from asthmatics. Imiquimod effect on the mRNA expression of ACE2 **(A)** and TMPRSS2 **(B)** in HBECs from asthmatic patients. **(C–F)** Imiquimod effect on the anti-viral response of bronchial epithelium. IFN-β mRNA expression **(C)**, as well as the pattern recognition receptors TLR3 **(D)**, MDA5 **(E)**, and RIG-I **(F)** are shown. HBECs from study population 1 and 2 were used, N = 16-18. Wilcoxon Signed Rank Test. ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

synthesis (e.g., ribosomal proteins RPS16, RPLP0, or RPS15), highlighting a potential protective effect of imiquimod against viral infections. In line with the decreased ACE2 expression in response to imiquimod treatment, the pathway "*HSA-2022377: Metabolism of Angiotensinogen to Angiotensins*", was also found overrepresented between the proteins most down-regulated by imiquimod (**Figure 3B** and **Supplementary Table 3**). Finally, several pathways related to metabolism of RNA, metabolism of amino acids, and cell cycle were also found enriched within down-modulated proteins in response to imiquimod treatment (**Figure 3B** and **Supplementary Table 3**). On the other hand, only 2 proteins, BLMH and PRRC2A were found up-regulated in HBECs from asthmatic patients in response to imiquimod treatment (**Supplementary Table 2**).

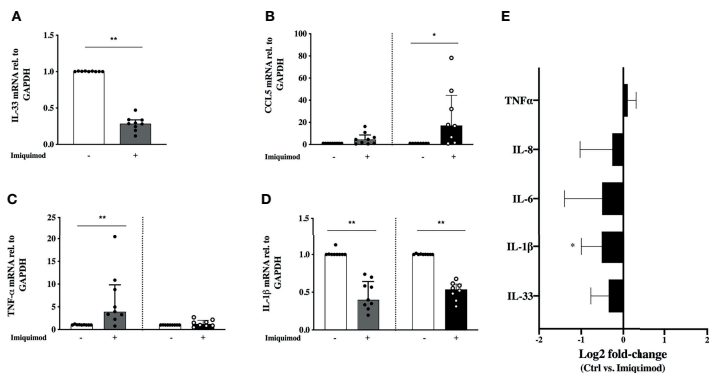

FIGURE 2 | Effects of imiquimod treatment on pro-inflammatory response of human bronchial epithelial cells (HBECs) from steroid-free asthmatic patients. Imiquimod effect on the mRNA expression of the pro-inflammatory mediators IL-33 **(A)**, CCL-5 **(B)**, TNF-α **(C)** and IL-1β **(D)** is depicted. **(E)** Log2 fold-change expression of TNF-α, IL-8, IL-6, IL-1β, and IL-33 in cell culture supernatants from HBECs after 24 hours of imiquimod treatment measured by multiplex ELISA (Luminex). *p < 0.05; **p < 0.01, Wilcoxon Signed Rank Test. HBECs from study population 1 were used, N = 7-9.

Imiquimod Boosts the Interferon Response in the Presence of SP1

Furthering potential relevance of the present results with regard to COVID-19, HBECs from a study population of asthmatic patients with different severity degrees were exposed to SP1 in this study (study population 2; **Table 1**). In addition, a small reference population of healthy subjects (N = 4) was included.

The cellular entry of SARS-CoV-2 depends on the binding of SP1 to ACE2 and the subsequent priming by the protease TMPRSS2. In order to mimic, in part, the earliest phase of SARS-CoV-2 infection, we stimulated HBECs from asthma patients with SP1. We did not find any direct effect of SP1 stimulation alone (at concentrations ranging from 5nM to 100 nM) on the gene levels of ACE2, TMPRSS2, TNF-α or IFN-β in HBECs (**Supplementary Figure 1**).

In order to address the effect of SP1 stimulation in the imiquimod treatment response of HBECs, we used a combination of SP1 with imiquimod for 24 h. There was no additional effect with imiquimod on ACE2 or TMPRSS2 gene expression in the presence of SP1 (**Figures 4A, B**). In addition, a similar trend to a decreased expression of ACE2 after imiquimod treatment (and no additional effect of SP1) was found after dividing the patients in treatment categories (i.e., ICS treated *vs.* ICS untreated) (**Supplementary Figure 2A**). However, there was a significantly higher (P<0.05) IFN-β gene expression in cells that were treated with both imiquimod and SP1 compared with cells that were only treated with imiquimod (**Figure 4C**), especially in patients treated with ICS (**Supplementary Figure 2B**). Adding SP1 did not alter the effects of imiquimod on TLR3 expression (**Figure 4D**). There was, however, a small trend to an increase of both MDA5 and RIG-I in cells treated with SP1 in combination with imiquimod compared with cells that were only treated with imiquimod (**Figures 4E, F**). Moreover, the effect of imiquimod on these parameters may not be dependent on underlying disease, as similar trends were shown for different asthma phenotypes (Atopic *vs.* Non-atopic and T2-high *vs.* T2-low; **Supplementary Figures 2C–F**) and healthy subjects (**Supplementary Figures 3A–F**).

Imiquimod Attenuates Poly(I:C)-Induced ACE2 Upregulation and Pro-Inflammatory Response, and Boosts Poly(I:C)-Induced IFN Signaling in HBECs From Asthma Patients

To further characterize the effects of imiquimod on primary HBECs from study population 2 (**Table 1**) and from a small reference population of healthy subjects (N = 4), we studied its interaction with poly(I:C), a SARS-CoV-2 replication mimic that would represent biological effects of infection without being subject to variation in infection rates (18). Poly(I:C) stimulation resulted in a 10-20-fold upregulation of ACE2 mRNA expression in HBECs from asthmatic donors (**Figure 5A**). More interesting, imiquimod significantly decreased (P<0.05) poly(I:C)-induced ACE2 mRNA expression in asthmatic HBECs (**Figure 5A**), and a similar decrease was found at protein level (**Figure 5B**). Similar effects of imiquimod on ACE2 expression were shown in HBECs from healthy subjects (**Supplementary Figure 4A**). In addition, a 5-fold increase of TMPRSS2 expression was found in patients with asthma after poly(I:C) stimulation, but no effect of imiquimod was found on TMPRSS2 expression in unstimulated asthmatic or healthy HBECs (**Figure 5C** and **Supplementary Figure 4B**).

Imiquimod further increased (P<0.05) the poly(I:C)-induced IFN-β gene expression in asthmatic HBECs (**Figure 5D**). Following siRNA mediated down-modulation of MDA5 and RIG-I mRNAs (**Supplementary Figures 5A, B**), poly(I:C)-induced IFN-β was inhibited and the imiquimod-dependent effect on IFN-β expression was absent (**Figure 5E**), suggesting a role of these PRRs in poly(I:C) and imiquimod mediated IFN upregulation.

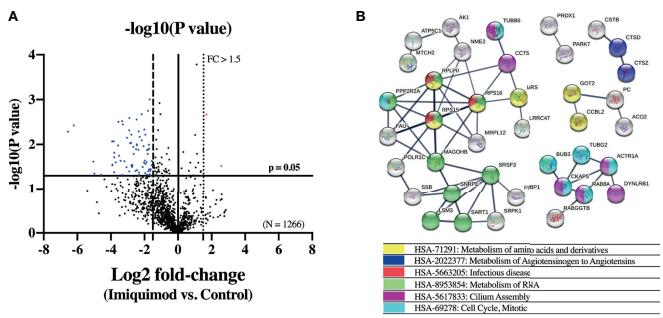

FIGURE 3 | Imiquimod treatment modifies the proteome of human bronchial epithelial cells (HBECs) from patients with asthma. **(A)** Volcano plot showing differentially expressed proteins between imiquimod treated and control unstimulated HBECs from patients with steroid-free asthma (N = 3). Proteins down-regulated (blue dots; log2 fold-change < -1.5, p < 0.05) and up-regulated (red dots; log2 fold-change > 1.5, p < 0.05) are depicted. **(B)** STRING interaction network of the proteins down-regulated by imiquimod treatment. Only proteins interacting with high confidence score (0.700) are shown. Colors represent the most relevant enriched Reactome pathways.

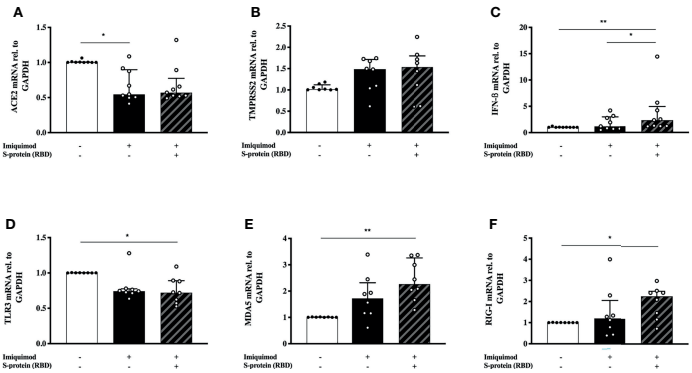

FIGURE 4 | S-protein 1 (SP1) from SARS-CoV-2 in combiantion with the anti-viral drug imiquimod decreases the expression of ACE2 receptor and increases the anti-viral response in human bronchial epithelial cells (HBECs) from asthma patients. mRNA expression of ACE2 **(A)**, TMPRSS2 **(B)**, IFN-β **(C)**, and the pattern recognition receptors TLR3 **(D)**, MDA5 **(E)**, and RIG-I **(F)** in HBECs from patients with asthma (study population 2; N = 8-9) in response to imiquimod treatment alone (black bars) or in combination with SP1 (striped bars). *p < 0.05, **p < 0.01, Friedman test followed by Dunn´s multiple comparison test.

The imiquimod-dependent down-regulation of ACE2 expression did not differ between ICS treated and untreated patients, or between different asthma phenotypes (**Supplementary Figures 6A–C**). No changes in the imiquimod-dependent upregulation of IFN-β expression were found between ICS treated and untreated patients, or between different asthma phenotypes (**Supplementary Figures 6D–F**).

Expanding gene expression data, we wanted to investigate the effect of imiquimod on the release of poly(I:C)-induced pro-inflammatory cytokines involved in COVID-19 pathogenesis. Importantly, in the presence of the viral infection mimic, imiquimod significantly decreased the majority of the cytokines evaluated and none were increased (**Figure 5F**).

Imiquimod Treatment of Poly(I:C)-Stimulated HBECs Resulted in Upregulation of Multiple Anti-Viral Gene Expression Pathways

Finally, we explored the impact of imiquimod treatment on the HBECs response to poly(I:C) by performing a multiplex mRNA expression analysis of a total number of 579 genes involved in the human immune response. 68 of these genes were found differentially expressed in response to imiquimod treatment with an adjusted P-value < 0.1 (**Figure 6A** and **Supplementary Table 4**). Reactome pathways overrepresentation of the 25 most up-regulated and down-regulated genes (P < 0.05) against human whole genome is shown in **Table 2**. In line with our previous findings, differential expression analyses revealed a major effect of imiquimod treatment in several genes from innate immune pathways (**Figure 6B** and **Table 2**). Importantly, several genes involved in type-I IFN signaling pathway (GO:0060337), including the IRF1, JAK1, and STAT2, were up-regulated (P < 0.05) in poly(I:C)-stimulated HBECs after imiquimod treatment (**Figure 6B**). Other IFN-stimulated genes, such as IFN alpha/beta receptor (IFNAR1, IFNAR2), positive regulators of IFN-beta production (e.g., IRF5), and other anti-viral genes (e.g., STAT1, STAT2) also tend to increase (adjusted P < 0.1) after imiquimod treatment (**Supplementary Table 4**). In the same line, C1QBP, encoding a negative regulator of DDX58- and IFIH1-mediated signaling pathways (gC1qR), was found down-regulated with imiquimod treatment in HBECs stimulated with poly (I:C) (**Figure 6B**). On the other hand, SIGIRR, encoding a negative regulator of IL-1R signaling, was shown to be up-regulated by imiquimod treatment (**Figure 6B**), potentially explaining the imiquimod-mediated down-modulation of IL-1ß and IL-33 shown in **Figures 2**, **5**. Finally, both SIGIRR expression and C1QBP expression effects of imiquimod were further validated by using RTqPCR in HBECs from study population 2 (**Figures 6C, D** and **Table 1**).

DISCUSSION

There is an urgent need of finding new drugs for the treatment of respiratory viral infections in asthma. In this study, several pharmacological effects of the TLR7 agonist imiquimod on HBECs from asthmatics, of interest for the treatment of both viral induced asthma exacerbations and COVID-19, have been demonstrated (**Figure 7**). On one hand, imiquimod boosts IFN-β response and decreases ACE2 expression in HBECs both at baseline and in the presence of the viral infection mimics poly(I:C) and the spike protein of the SARS-CoV-2, SP1. On this note, multiplex gene expression profiling of poly(I:C)-stimulated HBECs subjected to imiquimod treatment demonstrated change of multiple genes involved in anti-viral immune response. We further observed a decrease of C1QBP, a

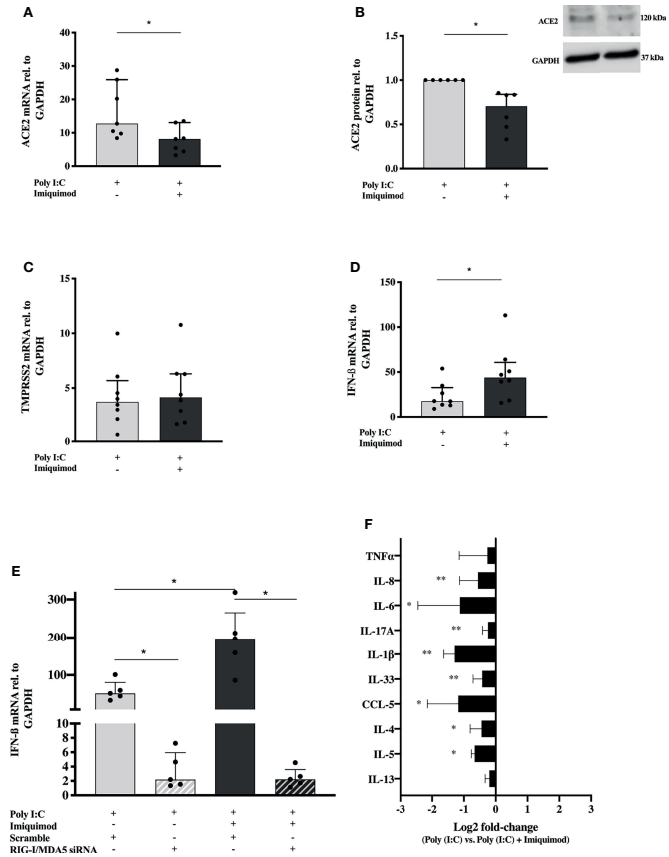

FIGURE 5 | Imiquimod decreases the expression of ACE2 receptor, and several pro-inflammatory cytokines in response to poly(I:C) stimulation, but increases the IFN response in human bronchial epithelial cells (HBECs) from asthmatics. ACE2 expression in HBECs from patients with asthma after treatment with poly(I:C) alone or in combination with imiquimod at gene **(A)** and protein (N = 6) **(B)** level. TMPRSS2 **(C)**, and IFN-β **(D)** mRNA expression in HBECs from asthmatic subjects in response to poly(I:C) or in combination with imiquimod. **(E)** Imiquimod effect on poly(I:C)-induced IFN-β mRNA expression in HBECs after siRNA-mediated downmodulation of MDA5 and RIG-I expression (N = 5; RM one-way ANOVA followed by Holm-Šídák's *post hoc* test). **(F)** Log2 fold-change [poly(I:C) *vs*. poly(I:C) + imiquimod] expression of several pro-inflammatory mediators in cell culture supernatants from HBECs after 24 hours poly(I:C) and imiquimod treatment, measured by multiplex ELISA (Luminex). Asthma (study population 2), N = 7-9 unless otherwise is expressed. Wilcoxon matched-pairs signed rank test for comparisons between two different treatment conditions. *p < 0.05; **p < 0.01.

negative regulator of MDA5 and RIG-I signaling. Proteomic analysis of HBECs also highlighted that the potential protective effect of imiquimod on viral infections involved a down-modulation of proteins implicated in viral mRNA and protein synthesis. On the other hand, our results evidenced a down-modulation of several pro-inflammatory mediators implicated in both viral-induced asthma exacerbations and COVID-19 pathophysiology following imiquimod treatment. This was in part supported by the up-regulation of SIGIRR, a negative regulator of TLR signaling, in poly (I:C)-stimulated HBECs treated with imiquimod.

Increasing evidence demonstrate that severe asthma is a risk factor for COVID-19 related admission to intensive care and death (8, 9). Indeed, a deficient anti-viral interferon response in asthma patients has been previously described, potentially leading to more severe virus-induced exacerbations (25). Yuen et al. have also shown that SARS-CoV-2 directly suppresses the interferon response during infection *in vitro* (51). Treatment

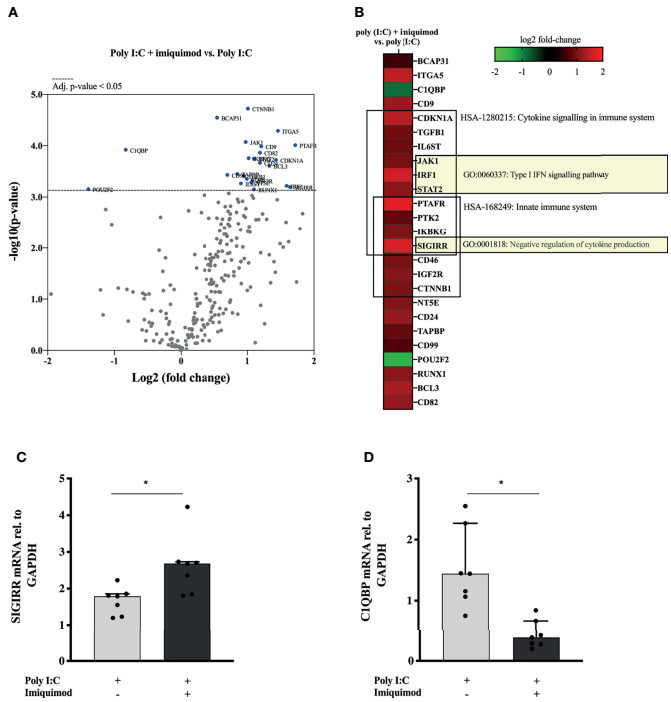

FIGURE 6 | Imiquimod induces the upregulation of multiple anti-viral gene expression pathways in human bronchial epithelial cells (HBECs) exposed to the viral replication mimic poly(I:C). **(A)** Volcano plot showing differentially expressed genes between poly(I:C) + imiquimod treated *versus* poly(I:C)-stimulated HBECs, N = 3. Multiple t-test analyses with the corrected method of Benjamini and Yekutieli were used. An adjusted p-value < 0.05 was considered significant. **(B)** Heat map representation of the 25 most down-regulated and up-regulated genes in response to imiquimod treatment. The most relevant overrepresented Gene Ontology (GO) terms are depicted. N = 3. SIGIRR **(C)** and C1QBP **(D)** mRNA expression in HBECs from asthmatic subject (N = 7) in response to poly(I:C) or in combination with imiquimod. Wilcoxon matched-pairs signed rank test for comparisons between two different treatment conditions. *p < 0.05.

TABLE 2 | Reactome pathways overrepresentation against whole human genome.

	Observed	Expected	Fold Enrichment	FDR
Immune System (R-HSA-168256)	16	2.62	6.11	2.89E-02
Cytokine Signaling in Immune system (R-HSA-1280215)	10	1.00	10.01	4.07E-07
Hemostasis (R-HSA-109582)	7	.81	8.62	4.00E-02
Innate Immune System (R-HSA-168249)	7	1.34	5.22	5.17E-03
Transcriptional Regulation by RUNX3 (R-HSA-8878159)	4	.11	35.43	4.43E-02
Interferon Signaling (R-HSA-913531)	4	.24	16.81	2.49E-02
Apoptosis (R-HSA-109581)	4	.21	19.27	3.60E-02
Interferon Gamma Signaling (R-HSA-877300)	3	.11	27.16	1.51E-02
Interleukin-4 and Interleukin-13 signaling (R-HSA-6785807)	3	.13	22.26	1.26E-02
Transcriptional regulation by RUNX2 (R-HSA-8878166)	3	.14	21.12	4.27E-02
Interleukin-27 Signaling (R-HSA-9020956)	2	.01	>100	3.08E-03
Interleukin-35 Signaling (R-HSA-8984722)	2	.01	>100	2.36E-05
IL-6-type Cytokine Receptor Ligand Interactions (R-HSA-6788467)	2	.02	96.92	1.56E-02
Interleukin-20 Family Signaling (R-HSA-8854691)	2	.03	65.90	1.71E-02
Antigen Presentation: Folding, Assembly and Peptide Loading of Class I MHC (R-HSA-983170)	2	.03	63.37	2.72E-02

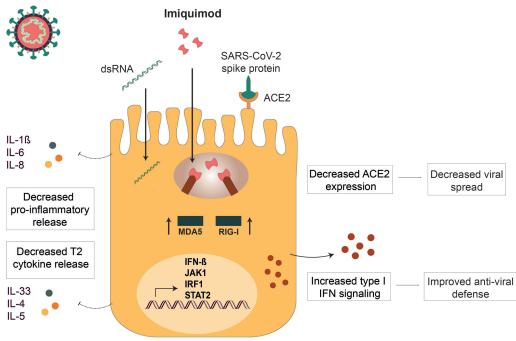

FIGURE 7 | Imiquimod treatment exerts anti-viral actions in human bronchial epithelium. Imiquimod exert dual actions on human bronchial epithelium: 1) Improves viral resistance by decreasing ACE2 expression (decreased viral spread) and increasing IFN-β expression (restored viral defense) on human bronchial epithelial cells (HBECs). 2) Potentiates viral infection tolerance by reducing viral stimulus-induced epithelial cytokines involved in severe COVID-19 infection (Prevention of cytokine storm).

with nebulized IFN-β in COVID-19 patients led to greater odds of improvement compared with patients receiving placebo control (52). The same treatment in asthma patients has been shown to reduce need of additional treatment while boosting innate immunity (53). However, IFN-β therapy was mainly beneficial in patients with severe asthma, whereas side effects occurred in milder asthmatics (53). It is possible that drugs boosting endogenous production of IFN-β may hold the advantage of potentially avoiding flu-like symptoms normally associated with exogenous interferon treatment (54). The present data extend previous observations on imiquimod-induced type I IFN in diverse experimental models to primary HBECs (37, 55, 56). Of particular interest, our data support the feasibility of obtaining drugs that improve airway IFN-β levels both at baseline and during a viral infection. mRNA expression profiling of imiquimod´s effect on poly(I:C)-stimulated HBECs revealed upregulation of multiple genes involved in type-I IFN pathway including STAT1, STAT2, IRF1, and JAK1. In addition, a decrease in C1QBP mRNA expression, a negative regulator of MDA5/RIG-I signaling (57), was found after imiquimod treatment of HBECs also exposed to poly (I:C). Further, siRNA mediated inhibition of MDA5/RIG-I expression in HBECs inhibited the imiquimod-induced effect on IFN-β expression, thus highlighting a potential role of these PRRs in imiquimod mediated IFN upregulation. A similar RIG-I and STAT1-dependent upregulation of type I IFN in response to imiquimod has been previously described in plasmacytoid dendritic cells (pDCs) (56).

Apart from the impaired IFN response in asthma and COVID-19 patients, another possible factor for developing a severe respiratory SARS-CoV-2 infection is the rapid increase of ACE2 expression in the first hours of SARS-CoV-2 infection, probably leading to an exaggerated replication and a rapid spread (58). Supporting the relevance of the present work, we demonstrated that poly(I:C) also increased ACE2 in bronchial epithelial cells from asthma and healthy donors. Given the importance of ACE2 levels in initiation and development of the SARS-CoV-2 infection, it is of interest that pharmacological attenuation of ACE2 expression levels may decrease the infection rates of the virus (58). Finney et al. have shown a glucocorticoid-induced lowering of the levels of ACE2 in the bronchial epithelium, strongly arguing that this action is caused by steroid-induced suppression of type I interferon (28). Previous studies have further shown that ACE2 could be an interferon-stimulated gene in airway epithelium (59). By contrast, our study demonstrates that imiquimod both reduces poly(I:C)-induced ACE2 expression and increases IFN-β in HBECs. These distinct observations are not readily explained by different sources of HBECs, COPD (28) and asthma (this study). Further work is clearly warranted to explain the dual effects on infection resistance of imiquimod, increasing IFN-β and decreasing ACE2, which would co-operate in reducing SARS-CoV-2 infectivity.

Both SARS-CoV-2 infection and viral-induced asthma exacerbations display hyperinflammatory immune responses, indicating a dysfunctional host tolerance to infection (60). In this study, imiquimod decreased gene expression of cytokines of special interest in asthma pathogenesis such as IL-33 and IL-1β. Furthering potential relevance of imiquimod treatment in asthma, Maazi et al. have previously demonstrated that TLR7 activation on pDCs induces the release of IFN-α, which in turn will inhibit the proliferation and cytokine production in ILC2 cells, key mediators of asthma pathogenesis (61). Imiquimod was also suggested to reduce allergen-induced airway inflammation in mice, potentially triggering T1 responses and decreasing T2 inflammation (62). Moreover, IL-33 protein release was also reduced by imiquimod in HBECs after poly (I:C) stimulation in the present study. Stanczak et al., have recently demonstrated that IL-33 expression is increased in COVID-19 seropositive patients after stimulation with SARS-CoV-2 peptides (63). They also evidenced that disease severity and T-cell activation in SARS-CoV-2 infected subjects is

correlated with IL-33 production (63). Indeed, IL-33 has been purposed as a key player in the pathogenesis of COVID-19, possibly being involved in early activation of innate immune responses, but also in later stages of the disease by inducing lung fibrosis (64). Other cytokines and chemokines such as IL-1β, IL-6, IL-8, or TNF-α have also been implicated in the pathogenesis of viral-induced exacerbations of asthma and severe COVID-19 (17, 65). We demonstrated that IL-1β, IL-6 and IL-8 protein expression were reduced by imiquimod in HBECs at poly(I:C) stimulation. Imiquimod transiently increased baseline gene expression of TNF-α, but this action was not reflected by protein release; in presence of poly(I:C), TNF-α protein was rather reduced by imiquimod. Taken together, the present data suggest that imiquimod-like drugs targeting SARS-CoV-2 infected airway epithelium are of interest for further development towards tentative treatment of viral induced exacerbation of asthma and COVID-19 both at early stages to avert infection and at later stages associated with airway hyperinflammation. It is not known to what extent the present profile of action of imiquimod is shared with other TLR7 agonists, including two drugs that already have been subjected to clinical trials to determine anti-allergic efficacy by local airway administration in asthma and rhinitis (41, 42).

Imiquimod-induced attenuation of the pro-inflammatory cytokines IL-6 and TNF-α, the neutrophilic chemokine IL-8, or different TH-type cytokines including IL-4, IFN-β, or IL-17, has been previously shown in airway epithelial cells or mouse models of RSV and Influenza A virus infections (37, 38). Of note, Salinas et al. suggested the interference of imiquimod with viral macromolecular synthesis as one potential mechanism of imiquimod-dependent down-regulation of pro-inflammatory cytokines after RSV infection (38). This possibility is in line with our exploratory proteomic analysis on the direct effect of imiquimod on HBECs; our data revealed a possible protective effect of imiquimod on viral infections through down-modulation of proteins implicated in viral mRNA and protein synthesis, including several ribosomal proteins. In addition, multiplex mRNA expression analyses in this study demonstrated an increased expression of the negative regulator of IL-1R signaling SIGIRR in imiquimod treated HBECs, highlighting another potential mechanism of the imiquimod-mediated down-modulation of IL-1β and IL-33 expression (66).

Imiquimod is currently available as a drug, Aldara, for topic treatment of skin conditions, including genital warts, actinic keratosis, and superficial basal cell carcinoma. Delivery of imiquimod has previously been employed in mice against influenza A infection (37), and for reducing airway inflammation in rats (62). Moreover, efficacy, as well as safety profile of intranasal application of imiquimod have been also demonstrated in rhinovirus infection in primates (67). However, this study has demonstrated an unexpected profile of action of imiquimod indicating drug feasibility rather than suggesting that imiquimod would be an ideal drug for nasal or inhalational treatment in COVID-19 or future viral pandemics.

A limitation in this study is the use of poly(I:C) and SP1 as a mimic of SARS-CoV-2 and rhinovirus infection, but not the real virus. Poly(I:C) is a dsRNA analogue and TLR3 agonist that mimics several biological effects of viral infection without being subject to the variation in intensity that may apply to actual infections. Poly(I:C) stimulation of bronchial epithelial cells induces a similar cytokine profile compared to rhinovirus and SARS-CoV-2 infection in bronchial epithelial cells (18, 68, 69). Moreover, poly(I:C) and SP1 administration to mice has been proven to induce cytokine storm syndrome and ARDS (70). Although studies on interaction between imiquimod and actual infection by SARS-CoV-2 are now warranted we argue that our approach employing poly(I:C) has strong guiding merits. In this study we have used submerged and not differentiated human bronchial epithelial cells. Ravindra et al. have previously demonstrated that basal bronchial epithelial cells are infected over the course of SARS-CoV-2 infection (71). We further demonstrate that our undifferentiated bronchial basal cell-like monolayer harbors significant ACE2 receptors and respond well to poly(I:C). Moreover, infectivity by rhinovirus is also particularly pronounced in human bronchial basal cells (72). Furthermore, undifferentiated epithelium commonly occurs in asthmatic bronchi, which is a focus in our study. It reflects both preferential shedding of columnar differentiated cells and actual denudation where the naked basement membrane is promptly covered by basal cell-like repair cells.

In conclusion, we demonstrate that imiquimod increases IFN-β expression, in part targeting MDA5 and RIG-I receptors and involving activation of several anti-viral immune pathways. In addition, we demonstrate, for the first time, that treatment with imiquimod reduced ACE2 expression in the bronchial epithelium. Finally, the drug´s profile would be additionally beneficial through its anti-inflammatory effect, reducing several epithelium-derived cytokines and chemokines implicated in the hyper-inflammatory response in viral-induced asthma exacerbation and severe COVID-19 cases. Taken together, the present demonstration of several epithelial actions of imiquimod in HBECs of potential importance for human respiratory viral infections is both promising and challenging. The promise lies in demonstration of feasibility of finding drugs with multiple desirable actions in virus-induced asthma exacerbations and COVID-19. The challenge is manifold but in part lies in further delineation of mechanisms of action of a drug with the present profile of action.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Proteomic data have been uploaded to the PRIDE database (73) under accession no: PXD029135; Nanostring data has been uploaded to NCBI GEO under accession no: GSE181281.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Danish Committee on Health Research Ethics

(Ethics: H-16043663 and H-16002008). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JJN-F contributed to the design, acquisition, analysis, and interpretation of data, have drafted and revised the work. ST contributed to the design, acquisition, analysis, and interpretation of data, have drafted and revised the work. SC contributed to the design, acquisition, analysis, and interpretation of data, have drafted and revised the work. AS contributed to the acquisition, analysis, and interpretation of data, and have revised the work. MH contributed to the acquisition, analysis, and interpretation of data, and have revised the work. SR contributed to the acquisition, analysis, and interpretation of data, and have revised the work. MM contributed to acquisition, analysis, and interpretation of data, and have revised the work. AFS contributed to the design of the work, interpretation of data, and revised the work. CP contributed to the design, analysis, and interpretation of data, have drafted and revised the work. LU contributed to the design, acquisition, analysis, and interpretation of data, have drafted and revised the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.743890/full#supplementary-material

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