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„Allergen- and ICAM-1-specific antibodies for prevention of seasonal allergies and infection by human rhinovirus“

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List of abbreviations

AIT	Allergen-specific immunotherapy
AP	Alkaline phosphatase
BAT	Basophil activation test
BSA	Bovine serum albumin
CDHR3	Cadherin-related family member 3
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcεRI	Fc receptors specific for ε heavy chains
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LDLR	Low-density lipoprotein receptor
LFA-1	Leukocyte function associated molecule-1
LRP-1	Low-density lipoprotein receptor-related protein 1
Mac-1	Macrophage-1 antigen
MHC	Major histocompatibility complex
OD	Optical density
P5/ICAM1	Phl p 5-specific human IgG1/ICAM-1-specific mouse IgG1
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline containing 0.05% (v/v) Tween-20
PFIE	*Plasmodium falciparum*-infected erythrocytes
RBL	Rat basophilic leukemia
RTCA	Real Time Cell Analysis
RV	Human rhinovirus
sICAM-1	Soluble intercellular adhesion molecule 1
ssRNA	Single-stranded-RNA
$TCID_{50}$	50% tissue culture infective dose
TCR	T cell receptor
TEER	Transepithelial electrical resistance
T_H2	T helper 2
TNF	Tumor necrosis factor
VP	Viral protein

1. Introduction

More than 25% of the population in industrialized countries are affected by Type I allergy, also called immunoglobulin E (IgE)-mediated hypersensitivity. During spring and summer, grass pollen represent one of the major sources of airborne allergens depending on climate and geographical conditions (reviewed in Kleine-Tebbe and Davies 2014). Immediate symptoms of grass pollen allergy are caused by cross-linking of Fc receptors specific for ε heavy chains (FcεRI) on effector cells (e.g. mast cells) by IgE-allergen immune complexes resulting in the release of allergic mediators (reviewed in Valenta 2002). One of the most severe symptoms of allergy is bronchial asthma. Beside allergy, infection by respiratory viruses, such as human rhinoviruses (RVs), also plays an important role in the pathogenesis of asthma suggesting that RVs and allergens may act synergistically to promote inflammation of the airway epithelium (reviewed in Heymann et al. 2005). Therefore, a successful treatment of Type I allergy as well as RV infection is crucial. In the case of allergy, there are several strategies to improve allergic symptoms including allergen-specific immunotherapy (AIT) (reviewed in Valenta et al. 2018, reviewed in Larché et al. 2006), whereas no approved medications for prophylaxis of RV infection are available so far (reviewed in Jacobs et al. 2013).

The aim of this project was to develop a non-invasive topical treatment, e.g. in form of nasal sprays or inhalators, for preventing inflammatory immune responses caused by exposure of grass pollen allergic individuals to Phl p 5, a major grass pollen allergen (Valenta et al. 1992, Vrtala et al. 1993), and by RV infection. We hypothesize that immobilization of the allergen on respiratory epithelia via binding to an allergen-specific antibody reduces activation and degranulation of effector cells and thus allergic symptoms. To locate the allergen-specific antibody on the epithelial surface, it was conjugated with another antibody that binds to the glycoprotein intercellular adhesion molecule 1 (ICAM-1). In addition, ICAM-1 is utilized as cellular receptor by the major group of RVs (Staunton et al. 1989, Greve et al. 1989, Tomassini et al. 1989). Therefore, we hypothesize further that the anti-ICAM-1 antibody blocks virus-receptor interactions and consequently inhibits infection by RV. To proof these hypotheses, we generated a recombinant antibody conjugate specific for Phl p 5 and human ICAM-1 and investigated its potential to prevent allergen uptake as well as infection by RVs and thus reduce inflammatory symptoms occurring during season of grass pollen and RVs.

2. Background

2.1 Type I allergy

Type I allergy, also termed immediate hypersensitivity, is an IgE-mediated disease causing immediate symptoms, e.g. allergic rhinoconjunctivitis, allergic bronchial asthma or even anaphylactic shock, in atopic individuals after repeated exposure to the sensitizing allergen (Kay 2008). In 1921, groundbreaking experiments performed by Prausnitz and Küstner showed for the first time that a transferable humoral factor (IgE) plays an important role in the pathological mechanisms of allergy. Additionally, a disease-eliciting antigen (allergen) and a component in the tissue of allergic and healthy individuals (effector cells) are required for Type I allergy (Prausnitz and Küstner 1921). The cross-linking of at least two allergen-specific IgE antibodies (Segal et al. 1977) bound to their receptors on effector cells by environmental antigens activates the effector cell (e.g. mast cell) resulting in IgE-mediated degranulation of biological mediators and hence allergic inflammation (reviewed in Valenta 2002).

2.1.1 Sensitization and memory

For developing Type I allergies, atopic individuals are sensitized after initial allergen contact resulting in production of allergen-specific IgE antibodies (**Figure 1**). First contact at mucosal surfaces with a soluble allergen, which is released from allergen bearing particles e.g. pollen, might lead to allergen uptake by dendritic cells (DCs) in the epithelia (reviewed in Valenta 2002, reviewed in Hammad and Lambrecht 2008). After capturing allergens, DCs transport them to draining lymph nodes, where allergens are processed. Thus, allergen peptides are presented by major histocompatibility complex (MHC) class II on DCs to naïve T cells causing the differentiation of CD4+ T cells to the T helper 2 (T_H2) cell phenotype. T_H2 cells then activate allergen-specific B cells and secrete cytokines, mainly interleukin (IL)-4 and IL-13, promoting heavy chain isotype switching towards IgE in activated B cells. As a result of sensitization, activated B cells differentiate into plasma cells producing allergen-specific IgE antibodies that are then secreted into the plasma to bind to FcεRI on tissue mast cells as well as circulating basophils and eosinophils. Therefore, bound IgE acts as receptor for the allergen on the surface of sensitized effector cells

(Abbas et al. 2005). Beside cytokines, several other factors control the T cell differentiation to T_H2 cells. In this context, the genetic predisposition of the individual plays an important role, as well as the site of allergen contact, the allergen dose and conformation, biological mediators, number and nature of antigen-presenting cells and the extent of T cell receptor (TCR) ligation (reviewed in Valenta 2002).

In addition to IgE secreting plasma cells, memory B and T cells are formed (reviewed in Palm and Henry 2019). Memory B and T cells allow a rapid and enhanced immune response to subsequent allergen contact and may survive even many years after sensitization. Some of these cells migrate to lymph nodes, where they can be activated immediately after allergen presentation and thus proliferate and differentiate into plasma cells. Other memory cells are found in mucosal tissues or in circulation in the blood in order to be rapidly recruited to the site of inflammation. In the case of B lymphocytes, the memory cells produce certain isotypes of immunoglobulins including the allergen-specific IgE, but also IgG and IgA. In contrast, naïve B cells synthesize only IgM and IgD. Memory T lymphocytes differ from their naïve form regarding the expression levels of adhesion molecules, which are higher in memory T cells. Those adhesion molecules, such as integrins and CD44, on the cell surface facilitate the migration of memory cells to the infection site (Abbas et al. 2005).

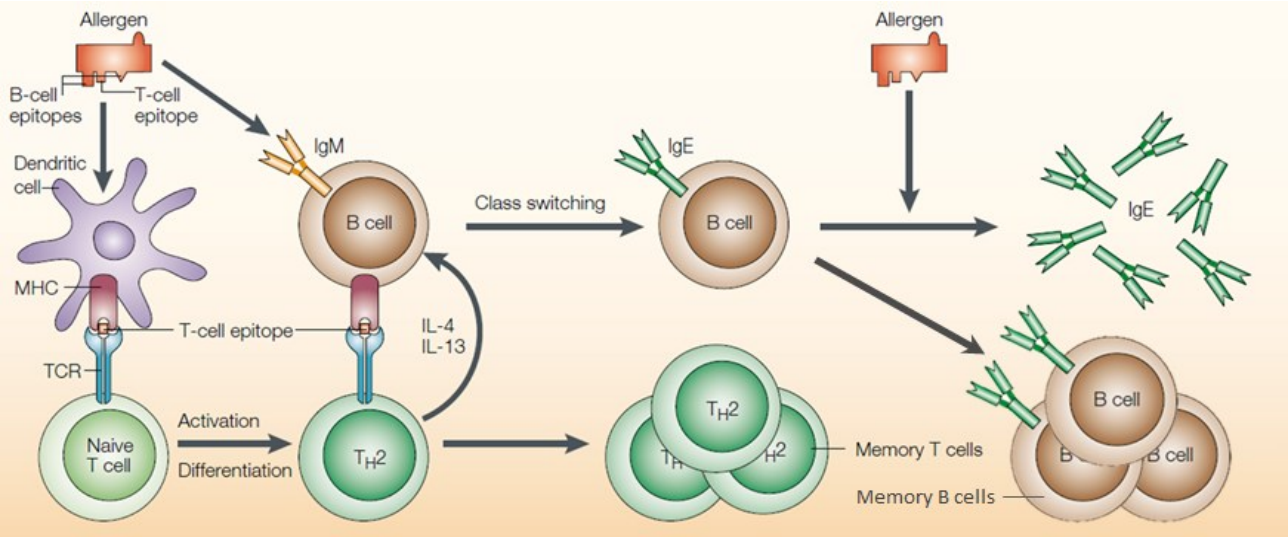

Figure 1 | Sensitization and memory of IgE-mediated allergies. [adapted from Valenta, Nature Reviews Immunology (2002) 2:446-453]

2.1.2 Immediate-phase reaction

In sensitized individuals, allergen-specific IgEs are produced upon repeated allergen contact. Circulating IgEs then bind with high affinity to FcεRI on effector cells, e.g. mast cells in submucosal tissue or basophils in blood. As soon as allergens migrate through the mucosal epithelium, they are able to cross-link FcεRI by binding to at least two receptor-bound IgEs. More precisely, the allergen binding site of the antibody (paratope) specifically recognizes regions of the allergen (epitope). Consequently, the effector cells release vasoactive mediators, such as histamine and leukotrienes, causing the immediate symptoms of allergic inflammation (**Figure 2**). These symptoms including rhinitis, bronchial asthma and conjunctivitis occur within minutes after allergen penetration (reviewed in Valenta 2002).

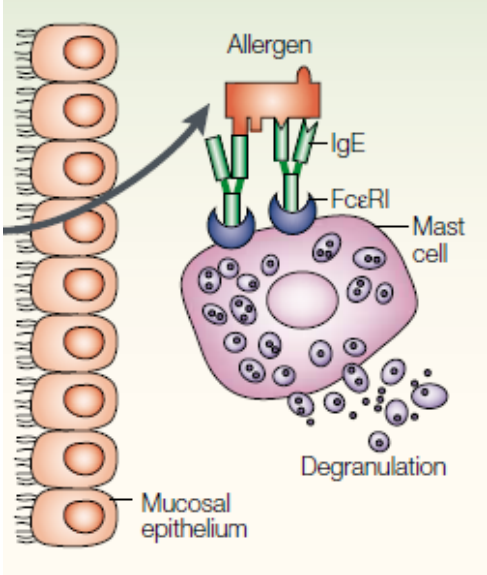

Figure 2 | Degranulation of effector cells upon FcεRI crosslinking via IgE-allergen complexes. [Valenta, Nature Reviews Immunology (2002) 2:446-453]

2.1.3 Effector cells in Type I allergy

Typical symptoms of Type I allergy are mediated by several vasoactive substances, which are released by effector cells due to IgE crosslinking by allergens. The most relevant effector cells in Type I allergy are mast cells, but basophil granulocytes also play an important role. Both cell types derived from progenitors in the bone marrow. While mast cells maturate after migration to peripheral tissues, basophils are already mature when they are leaving the bone marrow. Mature mast cells are present mainly near blood vessels and nerves as well as underneath epithelia and lymphoid organs. In contrast, basophils are

usually circulating in the blood, but also can be recruited to inflammatory tissues. Mast cells as well as basophils show high levels of FcεRI expressed on their surface. This high affine receptor is consisting of one α chain that mediates IgE binding, one β chain and two γ chains responsible for signaling. The extracellular domain of the α chain forms the binding site for the Fc region of IgE, whereas the cytoplasmic portions of β and γ chains contain immunoreceptor tyrosine-based activation motifs (Abbas et al. 2005).

Upon crosslinking of FcεRI due to allergen binding to receptor-bound IgEs, effector cells get activated and mediators, either preformed or new produced, are released consequently. Mast cells and basophils synthesize four important types of mediators including biogenic amines (e.g. histamine), lipid mediators (e.g. prostaglandins and leukotrienes) as well as cytokines (e.g. tumor necrosis factor (TNF), IL-4 and IL-5) and enzymes (e.g. tryptase). Preformed mediators, such as histamine and enzymes, are stored in cytoplasmic granules, while other substances (lipid mediators and cytokines) are newly synthesized after transcriptional activation. The release of histamine and lipid-derived substances leads to vascular leakage, bronchoconstriction and intestinal hypermotility causing symptoms of the immediate response. Enzymes secreted from granules and cytokines released by effector cells or T_H2 cells are mainly responsible for the late-phase reaction and contributing to tissue damages. More precisely, IL-4 and IL-5 recruit and activate another type of effector cells (eosinophils) resulting once more in the release of inflammatory substances (Abbas et al. 2005).

2.1.4 Allergens: Timothy grass pollen

Antigens, also termed allergens, eliciting Type I allergy are commonly environmental proteins and chemicals, which are usually harmless in most individuals. However, in genetically predisposed (atopic) individuals those allergenic particles can cause severe reactions by the immune system. It remains unclear why some antigens are able to induce strong T_H2 responses and thus cause the development of Type I allergy, but others do not. In atopic patients, the repeated exposure to typical allergens including proteins in pollen, animal products, house dust mites, foods or chemicals, e.g. the antibiotic penicillin, leads to allergic reactions. While it`s not yet possible to predict the allergenicity of a protein, there are some, mostly chemical, properties shared in common allergens. These include

glycosylation, low molecular weight as well as a high solubility of the antigen in body fluids (Abbas et al. 2005).

Allergens derived from grass pollen are mostly present during spring and summer and are affecting people all over the world. In the case of timothy grass pollen (*Phleum pratense*), Phl p 1, Phl p 2, Phl p 4 and Phl p 5 were characterized as major allergens and are responsible for most of the sensitizations in patients allergic to timothy grass (reviewed in Andersson and Lidholm 2003). Further, grass pollen allergens are divided into groups according to their protein structure and function. For example, Phl p 5 consists of a α-helical secondary structure and shows a molecular weight of 27-35 kDa (Flicker et al. 2000). The biological function of Phl p 5 is still unknown, even though it is found also in other grass species of the subfamily *Pooideae* (reviewed in Kleine-Tebbe and Davies 2014). However, group 1 (Phl p 1) and especially group 5 (Phl p 5) allergens show the highest potency to induce sensitization in atopic individuals (reviewed in Kleine-Tebbe and Davies 2014). In fact, Phl p 5 is recognized by IgEs in about 80% of patients allergic to timothy grass pollen and up to 60% of patients with total grass pollen allergies react with Phl p 5 (Vrtala et al. 1993). In addition, IgE directed against Phl p 5 was demonstrated to cross-react with other group 5 allergens from grass species including Kentucky Blue-grass, rye grass as well as rye and with less reactivity with common reed and wheat. The reason therefore may be the fact that the domain, which contains the epitope, shows a highly cross-reactive structure (Flicker et al. 2000).

2.1.5 Pollen-related food allergies

Cross-reactivity of IgE antibodies directed against pollen allergens can also lead to food allergy. Over the last few decades, food allergy as cause for inflammatory reactions has become more and more important. Primary food allergy is more frequent in young children, while pollen-related food allergy mainly affects adolescents and adults (Worm et al. 2014). In the case of pollen associated food allergy, individuals are sensitized by inhalation of pollen allergens that show a high degree of similarity in the amino acid sequence with food allergens. More precisely, IgE antibodies, which are produced after sensitization, cross-react with food allergens because of their homologous protein structures. Therefore, exposure to the dietary allergen can cause allergic reactions due to cross-reactivity of the

specific IgE antibodies (reviewed in Bohle 2007). Allergic patients show symptoms immediately or at least within 2h after uptake of the respective food. The immune response can affect several organs including the skin, oral mucosa, gastro-intestinal and respiratory tract as well as the cardiovascular system (Worm et al. 2014).

A common example for pollen-related food allergies is the “birch-fruit-vegetable-syndrome”. Over 70% of individuals who are allergic to birch (*Betula verrucosa*) pollen show allergic reactions after consumption of nuts, stone-fruits or certain vegetables. In this context, the most relevant inhalant allergen, which causes the sensitization, is the major birch pollen allergen Bet v 1 (Breiteneder et al. 1988, Kazemi-Shirazi et al. 2000). In more than 95% of birch pollen-allergic individuals, IgE against Bet v 1 can be detected in serum (Jarolim et al. 1989). Minor allergens including Bet v 2, Bet v 5 and Bet v 6 are also playing a role in the development of this allergic syndrome, but are not as important as Bet v 1. Bet v 1 belongs to a protein family with conserved structural features present in vegetables of *Apiaceae* (e.g. Api g 1 in celery or Dau c 1 in carrot), fruits of *Rosaceace* (e.g. Pru a 1 in cherry, Mal d 1 in apple or Pyr c 1 in pear), soybean (Gly m 4), mungbean (Vig r 1), hazelnut (Cor a 1) and peanut (Ara h 8). These proteins partially share the amino acid sequence and IgE antibodies specific for the major birch pollen allergen frequently cross-react with Bet v 1-related proteins. Uptake of these cross-reactive dietary allergens can cause allergic reactions in Bet v 1-sensitized patients. In most patients, allergic inflammation occurs in parts of the oropharynx summarized as “oral allergy syndrome”. Occasionally, patients also experience asthma, urticarial or even anaphylactic shock (reviewed in Bohle 2007).

However, oral allergy symptoms found in Bet v 1- sensitized patients were also reported for grass pollen allergic individuals of northern Europe after consumption of certain fruits and vegetables (Ghunaim et al. 2005). Since almost 75% of grass pollen allergic patients in northern Europe also show reactions to Bet v 1, these pollen-related food allergies may not be caused due to exposure to grass pollen allergens but Bet v 1. In fact, grass pollen-allergic individuals show nearly no clinically relevant IgE reactivity to food plants. Sensitization with grass pollen allergens, e.g. Phl p 5, seems to be not as clinically relevant as birch pollen in the development of pollen-related food allergy (Ghunaim et al. 2005).

A common way to reduce symptoms caused by pollen-related food allergies is to avoid the potentially cross-reactive nutrients. Nevertheless, the prevention of penetration by inhalant

allergens or allergen therapy can also reduce the prevalence of pollen associated food allergies.

2.1.6 Therapeutic approaches against Type I allergy

2.1.6.1 Allergen-specific immunotherapy

Nowadays, there are different concepts for the treatment of allergy, but the only causative strategy is still AIT. It is based on repeated subcutaneous or sublingual administration of small amounts of the allergen, which caused the sensitization, with the aim to induce a protective immune response (reviewed in Zhernov et al. 2019).

In 1911, first experiments of active immunization using subcutaneous injections of grass pollen extract were performed and published by Noon (Noon 1911). In clinical trials, it was confirmed that AIT improves symptoms and furthermore, these effects were sustained even one 1 year after the therapy. Later, injected allergen extracts were encased by adjuvants to increase the efficacy of AIT and to reduce side effects. Additionally, the allergenic activity of allergen extracts was decreased either by chemically modifications or by cleavage to obtain short peptides (Marsh et al. 1970, Briner et al. 1993, Focke et al. 2001). In the 1980s, new technologies made it possible to generate recombinant allergens as well as synthetic peptide epitopes (reviewed in Larché et al. 2006). Since then, several clinical trials were performed indicating that molecular AIT strategies including the administration of recombinant and synthetic allergen derivatives have many advantages over AIT based on injections of allergen-extracts (reviewed in Valenta et al. 2018, reviewed in Zhernov et al. 2019).

It is well accepted that the production of blocking antibodies (IgG1, IgG4 and IgA) as result of AIT is responsible for the improvement of symptoms. The immunological mechanism behind AIT is the modification of humoral and cellular responses to allergens. Subsequently, immunoglobulin class switching to IgG1, IgG4 and IgA in B cells is induced, while the IgE production is suppressed. The secreted blocking antibodies then compete with IgE for binding to allergens and hence reduce cross-linking of FcεRI on effector cells. At the same time, number as well as function of effector cells (mast cells and basophils) that are involved in allergic reactions are decreased (reviewed in Larché et al. 2006).

These disease modifying effects of AIT are unique among the strategies of allergy treatment. It was shown that a reduction of allergic responses to the allergen was lasting even beyond the therapy and a progression from mild-to-severe manifestations (e.g. allergic asthma) could be prevented. In addition, this strategy is highly efficient and cost-effective, but also time consuming (reviewed in Zhernov et al. 2019).

2.1.6.2 Passive immunization with blocking antibodies

Another strategy to help patients to reduce allergic reactions is passive administration of blocking antibodies. Therefore, allergen-specific blocking antibodies (e.g. IgG) are injected to compete with IgE for allergen binding and thus block the cross-linking of FcεRI by IgE-allergen-complexes (reviewed in Flicker and Valenta 2003, reviewed in Flicker et al. 2011).

Already in 1890, Emil von Behring introduced the idea of passive vaccination by performing experiments to neutralize bacterial toxins (Behring 1890). However, blocking antibodies in the field of allergy were first described in 1935 by Cooke et al. while investigating the protective mechanisms of AIT (Cooke et al. 1935). Five years later, Mary H. Loveless found that blocking antibodies specifically bind to the same allergen as the sensitizing antibodies (Loveless 1940), which were identified as IgE antibodies by the work of Ishizaka and Ishizaka as well as the group of Johansson not before 1967 (Ishizaka and Ishizaka 1967, Johansson et al. 1968). Further, Loveless managed to differentiate the blocking and sensitizing antibodies by heating the sera to 56°C, whereby the sensitizing antibodies were destroyed (Loveless 1943). In 1968, it was demonstrated by Lichtenstein et al. that the histamine release from basophils of patients allergic to ragweed was inhibited when the major ragweed allergen was pre-incubated with the blocking antibody fraction (Lichtenstein et al. 1968). Additionally, they analyzed sera from patients undergoing immunotherapy over a 2-year period and found a significant correlation between increased allergen-specific IgG levels and decreased IgE levels (Lichtenstein et al. 1973). Over 10 years later, Gleich et al. were able to confirm these findings by using new technologies and demonstrated again that the blocking antibodies reach a plateau level after 2 years of AIT (Gleich et al. 1982). Since then, few studies gave evidence that passive administration of blocking antibodies can be useful in allergy therapy. For example, the pre-treatment of patients allergic to bee venom with IgG from beekeepers in order to prevent side effects of AIT showed the

potential of blocking antibodies to reduce allergic reactions (Muller et al. 1986, Bousquet et al. 1987). Ever since, a lot of effort was put in the production of allergen-specific therapeutic antibodies or antibody derivatives generated by combinatorial library technology, hybridoma techniques or single cell Reverse Transcription Polymerase Chain Reaction (reviewed in Flicker et al. 2011). Recently, a proof of principle study showed for the first time that monoclonal allergen-specific antibodies are suitable for allergy treatment. A single injection of two human monoclonal IgG4 antibodies directed against the major cat allergen Fel d 1 significantly reduced allergic symptoms over the tested time period of 3 months in patients suffering from cat allergy (Orengo et al. 2018).

Passive administration of allergen-specific blocking antibodies may act in three ways to reduce allergic immune responses. The first one is the competition for allergen binding with IgE bound to FcεRI on effector cells. The resulting inhibition of degranulation and release of biological mediators may also occur due to co-cross-linking of receptor-bound IgE and IgG by allergens. Second, the blocking IgG antibodies may prevent T cell activation by competing with IgE, which is bound to antigen presenting cells, and third, may inhibit the initial IgE production by capturing allergens (reviewed in Flicker et al. 2011).

The strategy of passive immunization with allergen-specific antibodies may be convenient especially for patients suffering from seasonal allergies such as grass pollen allergy. Since the half-life of a human IgG antibody is approximately 21 days, the treated patients could be protected for a whole pollen season with a single injection of allergen-specific blocking antibodies (reviewed in Flicker et al. 2011).

2.2 Link between allergic inflammation and human rhinovirus infection

A successful therapy is crucial for improving the quality of life for allergic patients suffering from typical symptoms including rhinoconjunctivitis or even bronchial asthma, which is one of the most severe manifestations of allergic reactions. Beside allergy, infection by respiratory viruses, such as RV, plays also an important role in the pathogenesis of asthma (Edlmayr et al. 2009). In fact, allergic patients show more severe and longer-lasting symptoms in the lower respiratory tract after infection by RV compared to non-atopic individuals (Corne et al. 2002), suggesting that RV infection and exposure to inhalant

allergens may act synergistically to enhance inflammatory reactions in the airway epithelium (reviewed in Heymann et al. 2005). Upon RV infection, the epithelial barrier function is disrupted and therefore less protection against intrusion of allergens is given (reviewed in Mattila et al. 2011). In addition, the expression of ICAM-1, the receptor of the major group of RVs, on respiratory epithelial cells is upregulated under allergic conditions (reviewed in Hua 2013, Ciprandi et al. 1994).

Since the respiratory mucosa is the main site of entry for both, aeroallergens and RVs, it plays an important role in host defense and regulation of inflammation in airway tissues. The respiratory epithelium is consisting of columnar, ciliated epithelial cells, basal cells as well as mucous-secreting goblet cells (reviewed in Blaas and Fuchs 2016). To build a functional barrier, the epithelium requires complex networks formed by tight junctions, adherens junctions and desmosomes that link epithelial cells to each other (reviewed in Mattila et al. 2011). The barrier function of epithelial cells is responsible for the accessibility of inhalant allergens to DCs and therefore determines the adaptive immune response **(Figure 3)**. Moreover, airway epithelium is also playing a role in controlling the immune response to allergens by producing cytokines and chemokines. These cytokines then attract, activate and polarize DCs, which then present the allergen to T cells resulting in differentiation to T_H2 cell (reviewed in Hammad and Lambrecht 2008).

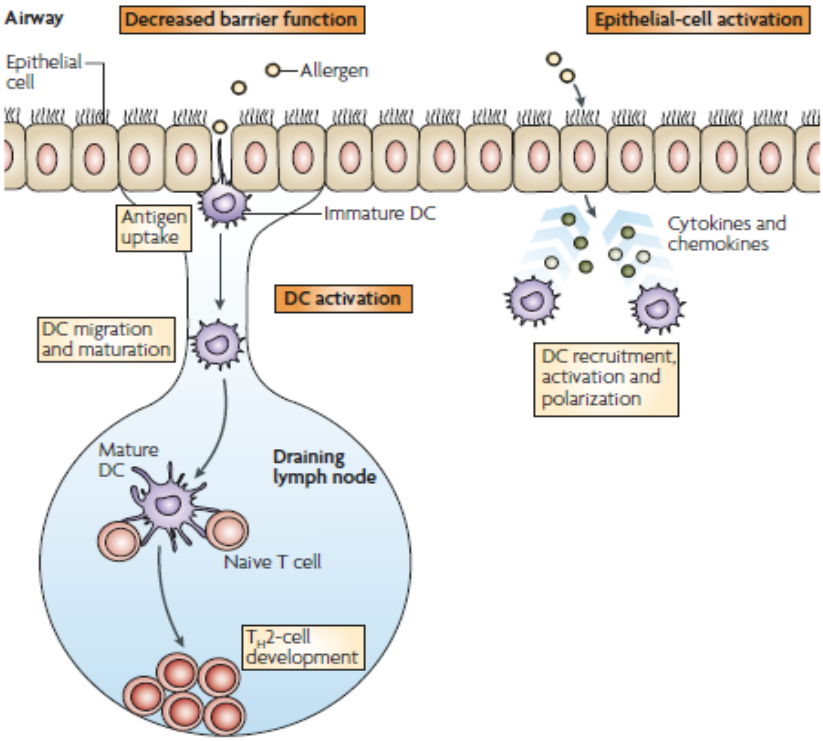

Figure 3 | Interaction between allergens and airway epithelial cells. [Hammad and Lambrecht, Nature Reviews Immunology (2008) 8:193-204]

While allergens migrate through the epithelial barrier because of their own proteolytic potential or via internalization by DCs (reviewed in Hammad and Lambrecht 2008, reviewed in Mattila et al. 2011), RVs require the binding to receptors on the respiratory epithelium, e.g. ICAM-1, to induce infection.

2.3 Human rhinovirus

2.3.1 Intercellular adhesion molecule 1

ICAM-1, also known as CD54, is a surface glycoprotein and a member of the Ig superfamily consisting of five consecutively linked extracellular domains (D1-D5). D1 forms the N-terminus followed by D2-D5 as well as a transmembrane region and a short C-terminal cytoplasmic domain. Expressed by a number of different cell types including leukocytes, endothelial and epithelial cells, ICAM-1 promotes adhesion in inflammatory and immunological reactions. More precisely, ICAM-1 acts as ligand for leukocyte function associated molecule-1 (LFA-1) and macrophage-1 antigen (Mac-1) and thus plays an important role in the leukocyte migration from the peripheral circulation to the tissue during inflammation (reviewed in Basnet et al. 2019). It was shown that LFA-1 primarily binds to domain 1 of the extracellular Ig-like structure of ICAM-1, whereas Mac-1 binds to domain 3 (Staunton et al. 1990, Diamond et al. 1991). Domain 1 also contains the binding site of *Plasmodium falciparum*-infected erythrocytes (PFIE) (Berendt et al. 1992) as well as fibrinogen, which mediates leukocyte adhesion to the vascular endothelium (Bella et al. 1998). More importantly, ICAM-1 functions as cellular receptor for the major group of RVs, what was first identified in 1989 (Staunton et al. 1989, Greve et al. 1989, Tomassini et al. 1989). Mutational analysis of ICAM-1 have shown that members of the major group of RVs also attach to domain 1 (Olson et al. 1993). However, the binding sites of LFA-1, PFIE and RV in domain 1 of ICAM-1 are not identical, but are overlapping **(Figure 4)** (Bella et al. 1998).

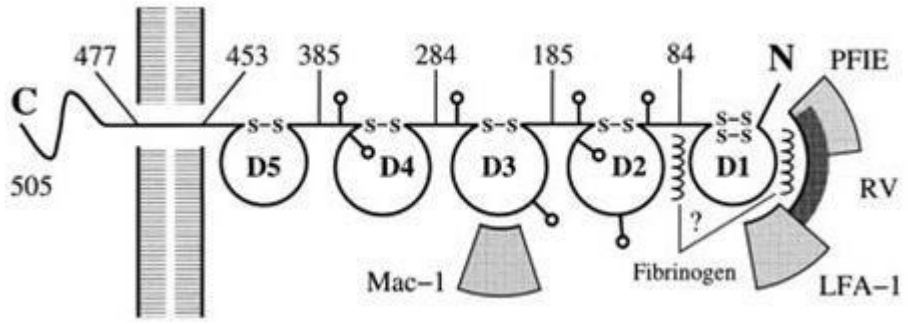

Figure 4 | **Schematic diagram of an ICAM-1 molecule showing the approximate location of binding sites for Mac-1, fibrinogen, LFA-1, RV and PFIE.** [Bella et al., Biochemistry (1998) 95:4140-4145]

While ICAM-1 is constitutively expressed at low levels under healthy conditions, it is upregulated on several cell types by cytokines during inflammatory processes such as allergic immune responses. These cytokines, which upregulate the ICAM-1 expression, include IL-1, TNF-α as well as interferon-γ (IFN-γ). Upon inflammation, the strongest expression of ICAM-1 is observed within 24h. Additionally, after induction of ICAM-1 upregulation it remains on the cell surface for more than 48h (reviewed in Hua 2013, Ciprandi et al. 1994, Scholz et al. 1996). In the case of ICAM-1 upregulation due to RV infection of respiratory epithelial cells, the receptor expression is increased via NF-kB p65-mediated transcription. Interestingly, not only the major group of RVs induces upregulation of ICAM-1, but also representors of the minor group, even though they do not utilize ICAM-1 as their receptor (Papi and Johnston 1999).

ICAM-1 also exists in a soluble form (sICAM-1) e.g. in nasal secretion or serum. sICAM-1 as well as the surface-bound ICAM-1 mediate the migration of leukocytes to the site of inflammation via interactions with its ligand LFA-1. When the concentrations of sICAM-1 in nasal mucosa of allergic and non-allergic individuals were compared, no significant difference was detected. The amount of sICAM-1 was only elevated in patients with chronic non-allergic sinusitis. Since infection by RVs, also known as cause for sinusitis, promotes upregulation of ICAM-1, this might explain the increased concentrations of sICAM-1 in nasal secretions of patients with chronic non-allergic sinusitis (Kramer et al. 2000).

2.3.2 Classification and virion structure

RVs are the most common cause of infections of the upper respiratory tract. When RVs were first isolated in the 1950s by Dr. Winston Price at Johns Hopkins University, they were associated with relatively benign common cold (Price 1956). Typical symptoms such as nasal stuffiness, sneezing, coughing and a sore throat usually resolve after 5-7 days. However, RVs recently got linked to asthma development, exacerbation of chronic pulmonary disease and fatal pneumonia in immune-compromised adults and elderly as well as severe bronchiolitis in infants and children. Even though there are a number of potentially harmful events due to RV infection, there are currently no approved antiviral agents for the prevention of RV infection (reviewed in Jacobs et al. 2013).

RVs belong to the genus *Enterovirus* in the family *Picornaviridae* and are positive-sense, single-stranded-RNA (ssRNA) viruses of approximately 7,200 bp. As all members of the family *Picornaviridae*, RVs are composed of a capsid consisting of four viral proteins (VP1, VP2, VP3 and VP4) to encase the RNA genome. VP1, VP2 and VP3 are responsible for the antigenic diversity of the virus, whereas VP4 anchors the RNA core to the capsid. All four capsid proteins are copied 60 times and assembled as an icosahedron with a canyon in VP1 to build a binding site for cell surface receptors (reviewed in Jacobs et al. 2013). Nowadays, there are more than 160 RV serotypes, which are classified as species A, B and C (RV-A, RV-B and RV-C) based on phylogenetic sequence criteria (reviewed in Basnet et al. 2019). Further, RV can be categorized according to their cellular receptors. Twelve members of RV-A are forming the minor group of RVs, which binds the low-density lipoprotein receptor (LDLR). The remaining serotypes of RV-A and all RV-B types belong to the major group of RVs, which utilizes ICAM-1 as their receptor (reviewed in Blaas and Fuchs 2016). Members of RV-C interacts with the recently identified cadherin-related family member 3 (CDHR3) (Bochkov et al. 2015).

The overall capsid structure of RV-A and RV-B consists of star-shaped plateaus at the 12 fivefold axes of symmetry, each surrounded by a canyon **(Figure 5 A)** (reviewed in Basnet et al. 2019). In the case of RV-A16, a member of the major group of RVs, the binding site of ICAM-1 is located in the hydrophobic canyon of VP1. To enable the binding to ICAM-1 it is necessary to have access to the contact residues of VP1 and VP2, which are found at the base of the canyon close to the pocket pore leading to a non-surface hydrophobic

pocket (Oliveira et al. 1993). This pocket is partially filled with cell-derived lipids (pocket factors) in serotypes of RV-A, while in RV-B the pocket is unoccupied (reviewed in Basnet et al. 2019). Since the binding sites for ICAM-1 and for pocket factors are overlapping, those two compete with each other, because the attachment of the one component interferes with the binding of the other. Thus, if the pocket is filled, the conformation of the canyon is changed so that the attachment to ICAM-1 is blocked. Pocket factors increase the stability of the virus, which is required during the transit from one host to another. However, to enable the virus entry into the host cell, the blocking pocket factors are displaced in the presence of ICAM-1 and the virus can interact with its receptor. Consequently, the virus is destabilized and therefore the viral RNA is released during the receptor-mediated uncoating process (Oliveira et al. 1993).

In contrast, the viral surface of RV-C differs from the one of RV-A and RV-B **(Figure 5 B)**. First, the fivefold plateau is much smaller and specific residues of VP1 and VP2 are forming spiky protrusions (fingers). Second, the canyon is non-continuous and narrow and therefore might not contain the binding site for its receptor. Although the VP1 proteins show a small internal pocket, they have no external pores what makes it inaccessible for pocket factors (reviewed in Basnet et al. 2019).

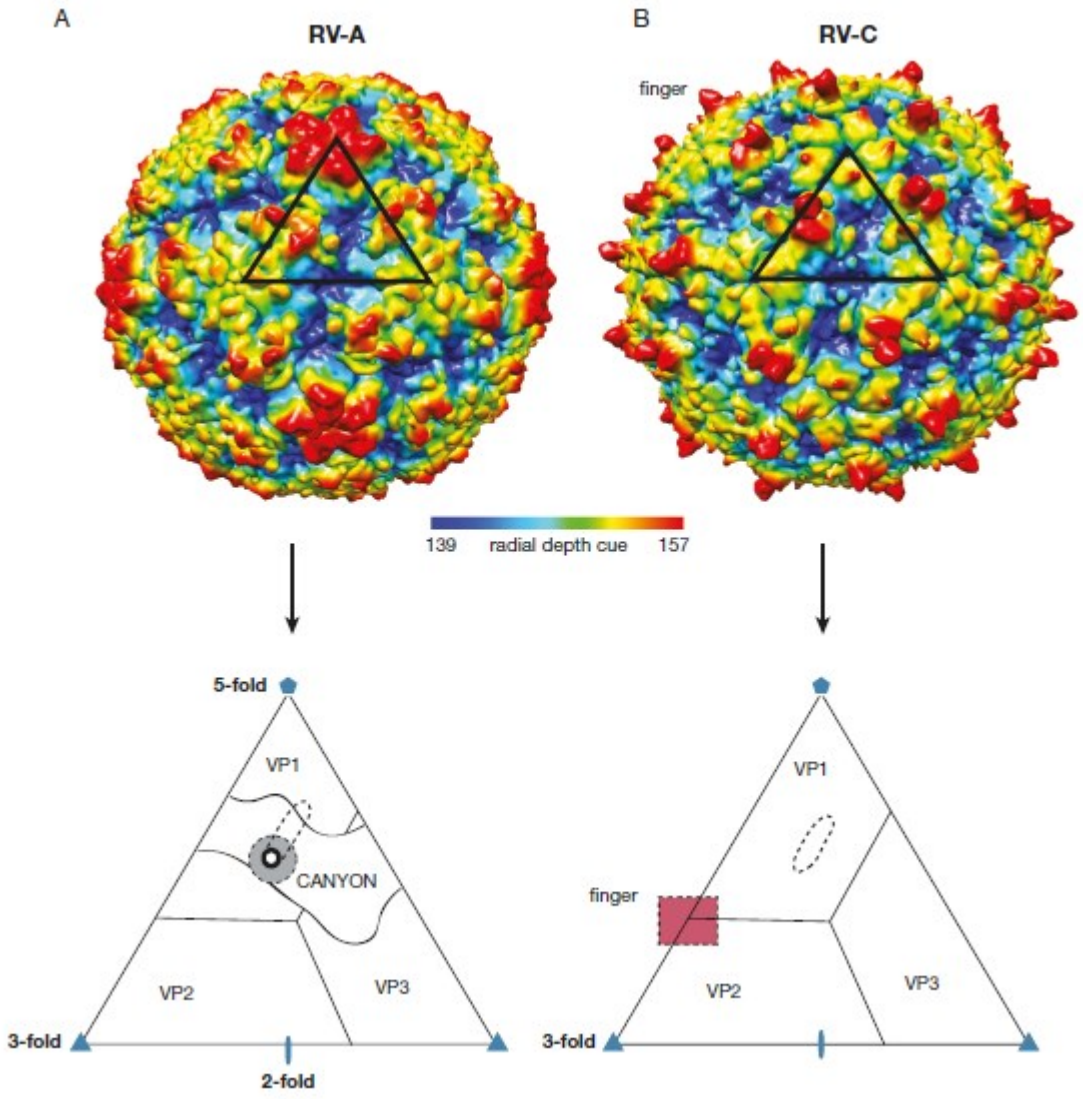

Figure 5 | **Characteristics of the virion surface of RV-A and RV-C.** [Basnet et al., Chest (2019) 155(5):1018-1025]

2.3.3 Viral entry and replication

For viral uptake, the respective receptors depending on the serotype, have to be accessible on the site of virus entry, e.g. at the apical surface of ciliated respiratory epithelium. LDLR is located at the basolateral plasma membrane of intestinal, hepatic, renal as well as polarized airway cell lines. However, LDLR and LDLR-related protein 1 (LRP-1) are also found at the apical side of the nasal epithelium, suggesting that LDLR is available to serve as receptor for the minor group of RVs (reviewed in Blaas and Fuchs 2016). ICAM-1, as the receptor for the major group of RVs, is expressed on the apical side of several cell types including epithelial and endothelial cells, e.g. in the respiratory tract.

Moreover, ICAM-1 is also found on the basolateral side of endothelial cells. Additionally, the expression of ICAM-1 is up-regulated in the nasal mucosa upon inflammation (reviewed in Hua 2013, Ciprandi et al. 1994). CDHR3 was recently identified as receptor for RV-C and therefore facilitates the viral entry into the host. High rates of gene expression of CDHR3 are found in the airway epithelium, fallopian tubes as well as the brain (reviewed in Basnet et al. 2019).

Since the mechanisms of entry and receptor-mediated signaling of RV-C are still unknown, the following information is limited to the RV species A and B. Both, RV-A and RV-B, enter cells by receptor-mediated endocytosis **(Figure 6)**. When the virus binds to its receptor at the ciliated surface, the virus-receptor complex is internalized and then transported into the early endosomes. In the case of minor group of RVs, e.g. RV-A2, the dissociation of LDLR and the virus occurs due to the mildly acidic pH in early endosomes and LDLR is then recycled back to the plasma membrane. The ß-propeller domain of LDLR and LRP-1 also influence the virus release and thereby allow the transport of virus into late endosomes (pH ≤ 5.6). At this point, the virus converts into subviral A particles, which are no longer containing the capsid protein VP4 but still carrying the RNA genome. Subsequently, the viral RNA is released into the cytoplasm during the uncoating process and the empty capsids, also called subviral B particles, are directed to lysosomes for degradation.

In contrast, members of the major group of RVs depending on the serotype, show differences in conditions required for conversion from ICAM-1 bound RV into subviral A particles. For example, RV-A89 is transported together with ICAM-1 to the perinuclear recycling endosomes. The acidic pH is similar to the pH conditions in late endosomes leading to conversion from native RV-A89 into subviral A particles and thus resulting in the transmigration of viral RNA into the cytoplasm. In the case of RV-A89, the conversion is facilitated at 20 °C, while the majority of ICAM-1 binding RVs require a temperature higher than 26 °C. After release of the viral genome, the remaining empty capsids are most probably returned to the apical mucus layer. However, RV-B14 utilizes another route after binding to ICAM-1. The virus-receptor complex is directed from the early endosomes to the late endosomes, where the virus is undergoing ICAM-1-dependent conformational modifications. This causes a rupture of the membrane of late endosomes by the virus leading to transmigration of RNA into the cytoplasm. The conversion of RV-B14 into subviral A particles is promoted at 20 °C or above as well as by acidic pH conditions and

receptor-dependent manner (reviewed in Blaas and Fuchs 2016). Once the positive sense RNA genome is released into the cytoplasm, it is translated into a polyprotein, which is proteolytic cleaved into structural and non-structural proteins. Subsequently, the resulting viral RNA polymerase generates negative strand copies of the RV genome which then serve as templates for replication of positive strand genomes. The structural gene products are forming the capsid consisting of all four viral proteins (VP1-VP4) to pack the new synthesized viral ssRNA (Kennedy et al. 2012, Warner et al. 2019).

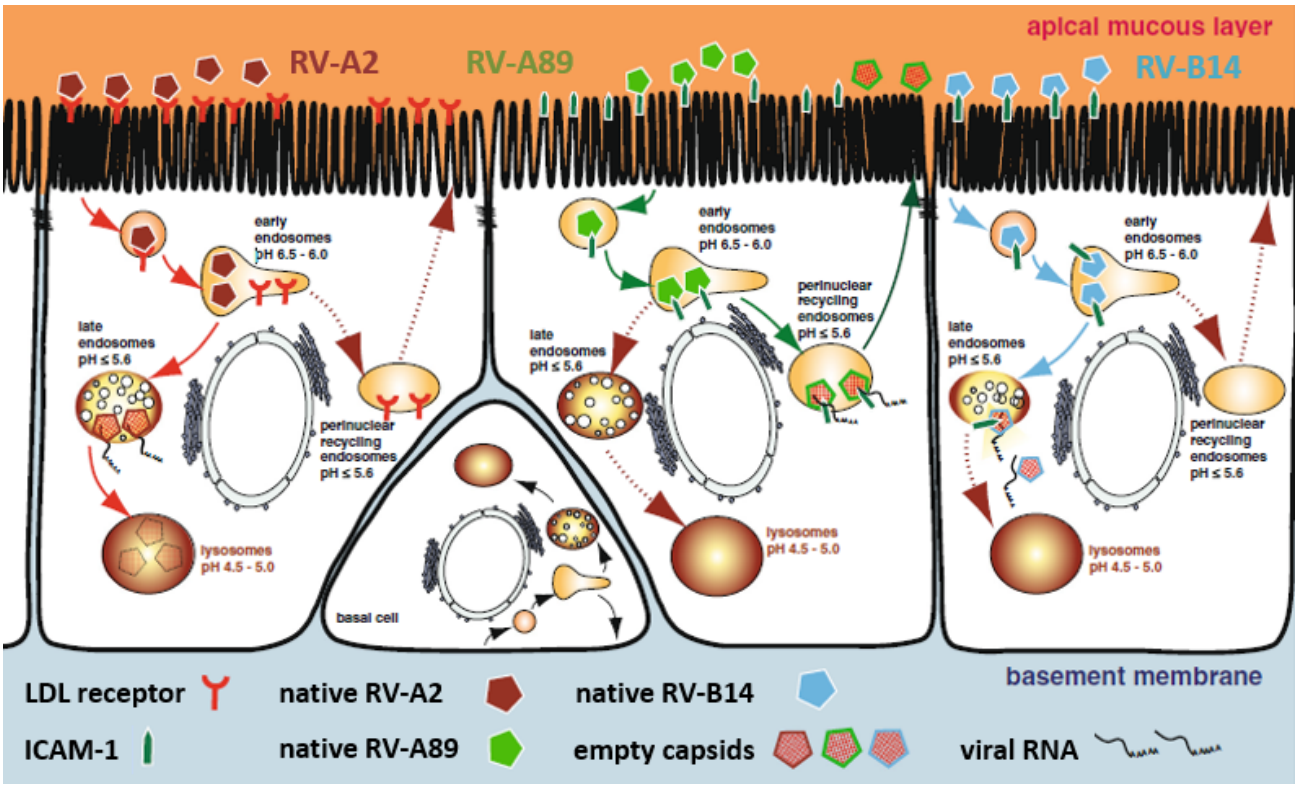

Figure 6 | **Mechanisms of viral entry and uncoating of RV-A2, RV-A89 and RV-B14 in ciliated nasal epithelial cells.** [adapted from Blaas and Fuchs, Molecular and Cellular Pediatrics (2016) 3:21]

2.3.4 Pathological mechanism and host response

Signaling pathways are activated after viral replication in ciliated epithelial cells resulting in release of cytokines (IFN-γ, IL-1ß, IL-6, IL-11), chemokines (RANTES, IL-8), growth factors (G-CSF) as well as vasoactive peptides (bradykinin). Hence, inflammatory cells, such as leukocytes, granulocytes and monocytes, are recruited to the submucosa leading to tissue inflammation and symptoms typical for the common cold. Controversy, it was shown that RV itself has no cytopathic effects like e.g. influenza viruses but disrupts epithelial barrier

function. More precisely, RV infection leads to increased vascular leakage and mucus secretion as well as disruption of tight junctions. Consequently, the permeability of the epithelial mucosa increases whereby the translocation of various pathogens and their produced soluble substances as well as allergens is facilitated (Kennedy et al. 2012). Interestingly, the host response to RV infection is mediated by the innate as well as the adaptive immune system. Type-I interferons are the early mediators and part of the innate immune response, while neutralizing IgA and IgG in serum and secretions observed one or two weeks after infection are consequences of the adaptive immune system (reviewed in Blaas and Fuchs 2016). It is being discussed that not the pathology caused by the infection itself, but the extent of the immune responses to RV determines the symptom profile (Kennedy et al. 2012).

However, the severity of symptoms of RV infection can vary among individuals. While non-asthmatic patients show mostly mild symptoms limited to the upper respiratory tract, RV infection in individuals with underlying asthma or other chronic lung diseases involves the lower respiratory tract. Symptoms of lower respiratory illness include shortness of breath, chest tightness, cough and wheezing (Kennedy et al. 2012). This might cause life-threatening conditions in people at risk, e.g. immune-compromised adults, elderly or children and infants with severe bronchiolitis. Especially for those patients, who are suffering from severe symptoms due to RV infection, the development of antiviral agents or vaccines for prevention of RV infection is necessary.

2.3.5 Therapeutic approaches against human rhinovirus infection

Several therapeutic strategies are currently under investigation (reviewed in Basnet et al. 2019). For example, it was recently shown that administration of the steroid Betamethasone protects the respiratory epithelium against damage induced by RV infections *in vitro* and thus is potentially useful in prevention of asthma (Waltl et al. 2018). Another strategy is to block the binding of the major group of RVs to its receptor using monoclonal ICAM-1-specific antibodies. The intranasal administration of a single monoclonal antibody was proven to be effective in reducing symptoms but not in preventing infection by RV presumably due to its insufficient avidity (reviewed in Fang and Yu 2004). However, it was demonstrated that topical or systemic application of one

monoclonal antibody specific for domain 1 of human ICAM-1 can inhibit inflammation induced by representor of the major group of RVs in mice (Traub et al. 2013). In addition, a lot of effort is put into developing vaccines against RVs. Recently, immunodominant epitopes of RV-A16 were identified and equivalent recombinant polypeptides were synthesized for immunization of mice. Even though, the observed IgG antibodies target only a small and highly-serotype specific region of the viral capsid, they bind to at least four other serotypes indicating a cross-serotype recognition (Sam Narean et al. 2019).

Nevertheless, the generation of proper medications is challenging because of several reasons, e.g. the large number of RV serotypes and their variety of pathological mechanisms, and thus there are only treatments to relieve the symptoms on the market so far (reviewed in Basnet et al. 2019).

3. Aim of the thesis

Since there are no treatments for preventing RV infections and allergen penetration, the aim of this project is to develop a topical, non-invasive application to inhibit trans-epithelial allergen migration as well as receptor binding by the major group of RVs. To achieve this goal we generated antibody conjugates bi-specific for allergens and ICAM-1 to stop allergen and RV entry through the epithelial barrier. Applications, such as nasal sprays or inhalators, are easy and independent to use and thus very convenient especially for patient suffering from symptoms caused by airborne allergens, e.g. grass pollen, and RV infection during season.

For this proof of principle study, we choose a Phl p 5-specific IgG1 antibody to investigate its potential to immobilize the major grass pollen allergen Phl p 5 (Valenta et al. 1992, Vrtala et al. 1993) on the respiratory epithelium. First, we hypothesize that the Phl p 5-specific IgG1 antibody captures Phl p 5 and thus prevents trans-epithelial allergen migration. Consequently, penetration by Phl p 5 as well as the following cross-linking of specific IgE bound to its receptor on effector cells (e.g. mast cells or basophils) is inhibited. Hence, degranulation and release of biological mediators by effector cells and allergic reactions to Phl p 5 are prevented. The second part of the bi-specific conjugate is the ICAM-1-specific IgG1 antibody with the aim to anchor the allergen-specific antibody on the

epithelial surface. Since ICAM-1 is highly expressed on the cell surface in allergic patients (reviewed in Hua 2013, Ciprandi et al. 1994), the anti-ICAM-1 antibody is very suitable for locating the Phl p 5-specific IgG1 antibody on the airway epithelium. In addition, ICAM-1 is the cellular receptor of the major group of RVs (Staunton et al. 1989, Greve et al. 1989, Tomassini et al. 1989) and thus we hypothesize that the ICAM-1-specific IgG1 blocks the virus-receptor interactions and hence inhibits the uncoating of the major group of RVs.

Therefore, we generated a bi-specific conjugate consisting of Phl p 5-specific IgG1 and ICAM-1-specific IgG1 antibodies by streptavidin-biotin-coupling **(Figure 7 A)** and analyzed its potential to prevent both, allergic reactions and RV infection **(Figure 7 B)**, *in vitro*.

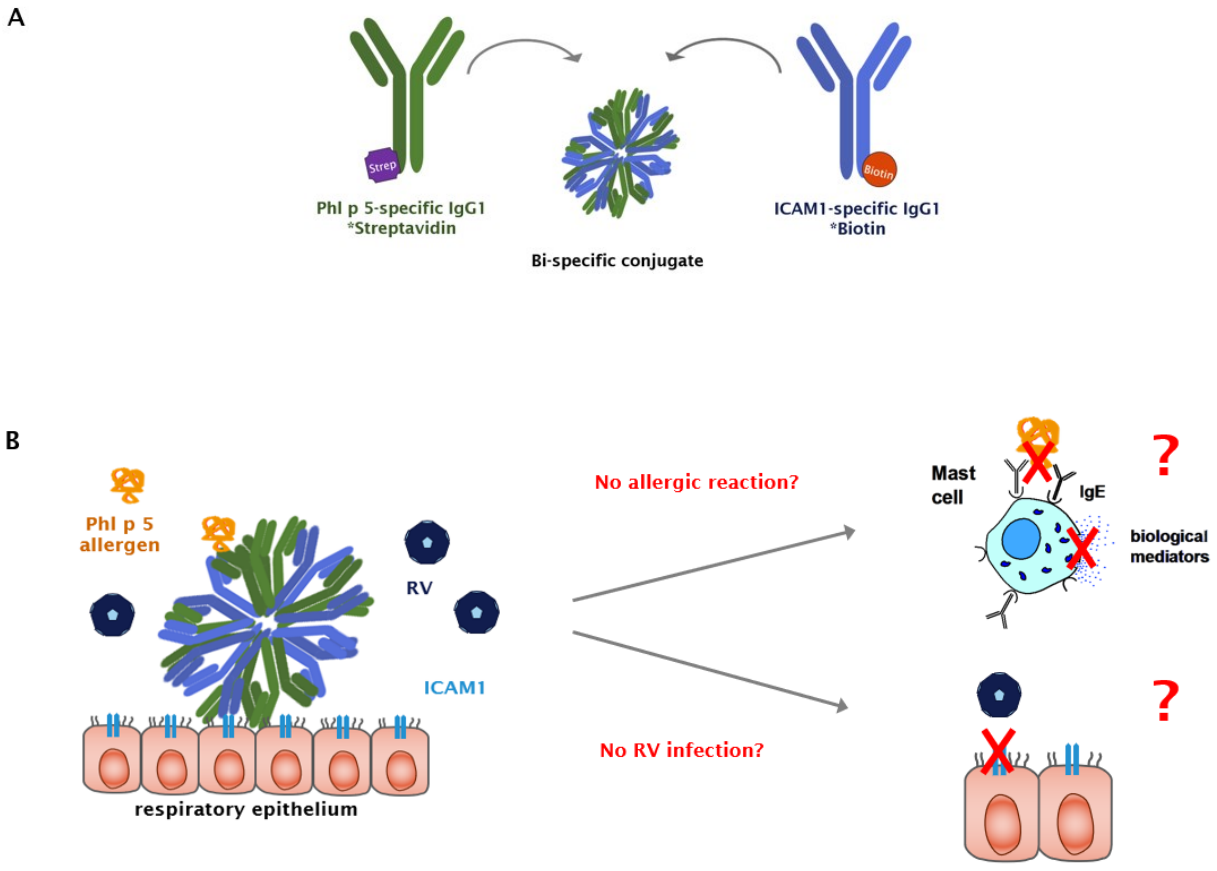

Figure 7 | **Concept and hypothesis.** Bi-specific antibody conjugates are generated via streptavidin-biotin coupling of Phl p 5-specific IgG1 and ICAM-1-specific IgG1 **(A)**. Phl p 5-specific IgG1 may capture Phl p 5 and hence may inhibit allergen migration through the respiratory epithelium. In addition, ICAM-1-specific IgG1 may block the binding of RV to its cellular receptor ICAM-1. Thus, bi-specific antibody conjugates may prevent allergic reactions as well as RV infections **(B)**.

4. Methods and Materials

4.1 P5/ICAM1 conjugates specifically bind Phl p 5 and human ICAM-1

4.1.1 Antibodies and antibody conjugation

A Phl p 5-specific IgE Fab-fragment was previously isolated from an allergic patient as described (Steinberger et al. 1996) and was then converted into a fully human IgG1 antibody. After stable transfection, Phl p 5-specific IgG1 were expressed in CHO-K1 cells (Flicker et al. 2000) and purified via Protein G affinity chromatography (Thermo Fisher Scientific, Waltham, USA). The Phl p 5-specific IgG1 antibody was conjugated with Lightning-Link® Streptavidin (Expedeon Inc., San Diego, USA) according to manufactures' advice. ICAM-1-specific mouse IgG1 antibodies labelled with Biotin were purchased either from LifeSpan BioSciences (Seattle, USA) or Abcam (Cambridge, UK). To generate bi-specific antibody conjugates, termed P5/ICAM1, the Phl p 5-specific human IgG1 and the ICAM-1-specific mouse IgG1 in a ratio of 1:0.25 were incubated together for at least 1.5h at room temperature and in addition overnight at 4°C **(Figure 8)**. Each streptavidin monomer can bind four biotin molecules leading to high affine and stable complexes (Laitinen et al. 2006). As described previously (Madritsch et al. 2015), bi-specific antibody conjugates act similarly in different ratios (1:1, 1:0.5 and 1:0.25) and thus a ratio of 1:0.25 was used for the following experiments for financial reasons.

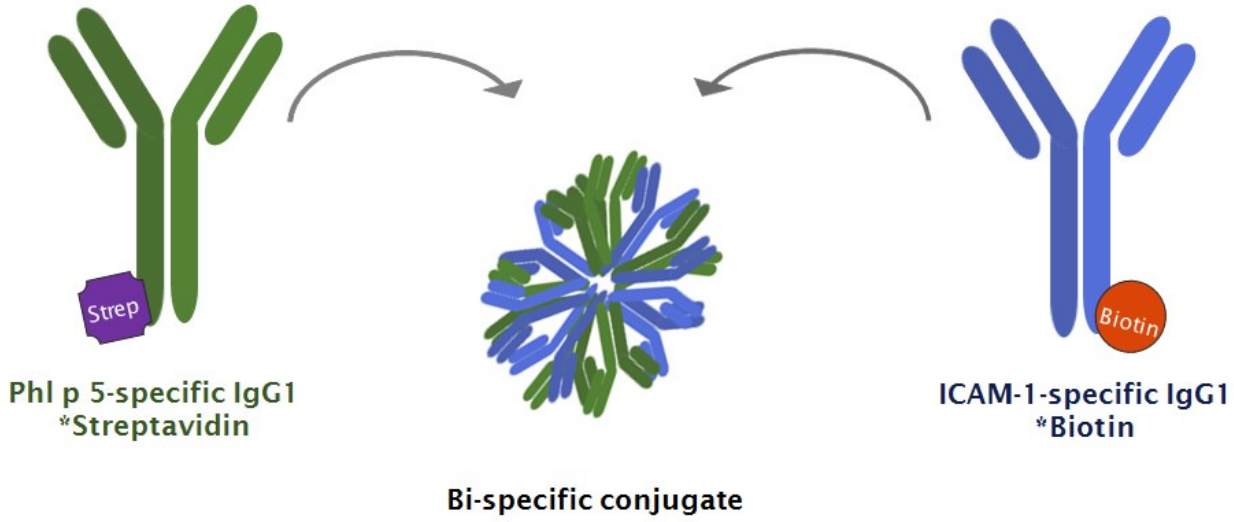

Figure 8 | Generation of bi-specific conjugate through streptavidin-biotin-coupling of Phl p 5-specific human IgG1 and ICAM-1-specific mouse IgG1.

4.1.2 ELISA evaluation of the binding specificities of P5/ICAM1 conjugates

Enzyme-linked immunosorbent assay (ELISA) plates (Nunc Maxi-Sorp, Roskilde, Denmark) were coated with 5 µg/ml recombinant Phl p 5 (Biomay AG, Vienna, Austria) or recombinant human ICAM-1 (R&D Systems, Minneapolis, USA) in bicarbonate buffer (100 mM NaHCO3, pH = 9.6). Plates were incubated overnight at 4°C, washed twice with 1x phosphate-buffered saline (1xPBS) containing 0.05% (v/v) Tween-20 (PBST) and saturated with PBST containing 3% (w/v) bovine serum albumin (BSA) for 3h. Then, 1 µg/ml and 5 µg/ml P5/ICAM1 conjugates as well as 1 µg/ml Phl p 5-specific human IgG1 (Streptavidin) and anti-ICAM-1 mouse IgG1 (Biotin) were applied overnight at 4°C. Bound bi-specific antibody conjugates were detected either with alkaline phosphatase (AP)-conjugated goat anti-human F(ab´)2 antibodies (Thermo Fisher Scientific, Waltham, USA) diluted 1:500 in PBST containing 0.5% (w/v) BSA (PBST/0.5% BSA) or with horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG1 antibodies (BD Bioscience, San Jose, USA) diluted 1:5000. Optical density (OD) measurements were carried out on ELISA reader Infinite F50 (Tecan, Männedorf, Switzerland) at 405 nm with a reference wavelength at 490 nm.

4.2 Phl p 5 can be immobilized via P5/ICAM1 conjugates onto respiratory epithelial cells

4.2.1 Cell culture

The epithelial cell line 16HBE14o- was derived from human bronchial surface epithelial cells (Prof. D. C. Gruenert, University of California, San Francisco, USA) and is used as a surrogate for the respiratory epithelium showing properties of differentiated airway epithelial cells (Wan et al. 2000). In cultivation, cells form tight junctions, apical microvilli as well as cilia and grow in polarized monolayers. 16HBE14o- cells were cultured in Minimum Essential Medium (Thermo Fisher Scientific, Waltham, USA) including 10% fetal bovine serum (FBS) (HyClone; GE Healthcare, Buckinghamshire, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, Waltham, USA) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were cultured in flasks coated with collagen-fibronectin (BD Biosciences, San Jose, USA).

4.2.2 Flow cytometry

4.2.2.1 Detection of ICAM-1 on 16HBE14o- cells by anti-human ICAM-1 antibody

For fluorescence-activated cell sorting (FACS) analyses, approximately $2x10^5$ 16HBE14o- cells per well were stained with Fixable Viability Dye eFlour 780 (Thermo Fisher Scientific, Waltham, USA) to exclude dead cells from the analysis. Cells were incubated with 1 µg anti-ICAM-1 mouse IgG1 Biotin (LifeSpan BioSciences, Seattle, USA) or 1 µg anti-human IgA1/A2 (BD Bioscience, San Jose, USA) as isotype control in 50 µl FACS buffer (1xPBS supplemented with 0.1% w/v BSA). After incubation for 20min on ice and in darkness, cells were stained with Streptavidin DyLight ®488 (Thermo Fisher Scientific, Waltham, USA) and analyzed on a Canto II Cytometer (BD Bioscience, San Jose, USA) counting at least 50.000 cells per sample and were evaluated with FlowJo Software (version 10, FlowJo LCC, Ashland, USA).

4.2.2.2 Detection of Phl p 5 immobilized via cell bound P5/ICAM1 conjugates onto the surface of 16HBE14o- cells

For this experiment, 16HBE14o- cells were treated as described above. $2x10^5$ cells per well were incubated with 1 µg P5/ICAM1 in 50 µl FACS buffer for 30min on ice. After washing, aliquots of 1 µg, 2 µg or 5 µg Phl p 5 in 50 µl FACS buffer were added and cells were again incubated for 30min on ice. After another washing step, Phl p 5-specific rabbit antibodies or pre-immune rabbit serum as isotype control were added and cells were stained with Alexa Fluor ®405-labelled goat anti-rabbit antibody (Thermo Fisher Scientific, Waltham, USA). Cells were sorted by a Canto II Cytometer (BD Bioscience, San Jose, USA) counting at least 50.000 cells per sample and were evaluated with FlowJo Software (version 10, FlowJo LCC, Ashland, USA).

4.2.3 Immunofluorescence Microscopy

16HBE14o- cells ($2x10^4$ cells per well) were seeded on ibiTreat tissue culture-treated µ-dishes (Ibidi GmbH, Munich, Germany) and grown overnight. Cells with approximately 90% confluency were washed three times with 1xPBS. Complexes of bi-specific conjugates and allergen were formed by incubation of 5 µg P5/ICAM1 and 1 µg Phl p 5 in 300 µl

1xPBS for 1h at room temperature. In a next step, cells were incubated with the complexes at 37°C for 24h and unbound complexes were removed by washing with 1xPBS. Then, fixation of cells was performed with 4% paraformaldehyd solution applied for 20min at room temperature. After another washing step, remaining aldehyde groups were quenched with 50 mM ammonium chloride in 1xPBS for 10min. Cells were washed tree times with 1xPBS containing 0.05% (v/v) saponin (1xPBS/0.05% saponin) to induce permeability of cell surfaces. This is necessary to provide access to intracellular P5/ICAM1 or Phl p 5 for following reagents. Fixed cells were incubated with Phl p 5-specific rabbit antibodies diluted 1:1000 in 1xPBS/0.05% saponin, washed and then 10% goat serum in 1xPBS was applied overnight at 4°C to block unspecific binding sites. To detect P5/ICAM1 bound onto surfaces of 16HBE14o- cells, Alexa Fluor ®488 goat anti-mouse IgG (Molecular Probes, Eugene, USA) at a dilution of 1:1000 in 1xPBS/0.05% saponin containing 10% goat serum was added to cells for 2h at room temperature. Additionally, Phl p 5 bound to P5/ICAM1 was visualized by using Alexa Fluor ®568 goat anti-rabbit IgG (Molecular Probes, Eugene, USA) diluted 1:1000 in 1xPBS/0.05% saponin containing 10% goat serum applied for 2h at room temperature. Finally, nuclei were stained with 1 µg/ml DAPI (Molecular Probes, Eugene, USA) in 1xPBS. For control purposes, samples were included omitting either P5/ICAM1 or Phl p 5. Wide-field fluorescence imaging of fixed cells was performed with a Zeiss Observer Z1 Axio inverted fluorescence microscope equipped with an oil immersion 40x objective (Pan Apochromat, 1.4 NA, Carl Zeiss Inc., Oberkochen, Germany) and the Zeiss Axio Software package.

4.3 P5/ICAM1 conjugates prevent migration of Phl p 5 through respiratory epithelial cell monolayers

4.3.1 Transwell migration assay

Approximately $2x10^5$ 16HBE14o- cells per well were seeded and cultivated on permeable membranes of apical chambers of the Transwell system **(Figure 9)**. Transepithelial electrical resistance (TEER) baseline was measured with an ohm voltmeter (Millipore, Bedford, USA or World precision instruments, Inc. Sarasota, USA) and ranged consistently from 130 to 140 Ω/cm² for wells with medium without cells. 50 ng/ml IFN-γ (Pepro Tech Inc., Rocky Hill, USA) was added in each basolateral well when wells with cells reached a

TEER value of approximately 500 Ω/cm² to allow detectable allergen migration through the cell monolayer (Madritsch et al. 2015). Once TEER values were lower than 200 Ω/cm², cells were incubated with aliquots of 10 µg/ml P5/ICAM1 for 3h at 37°C. Apical supernatants of all wells were removed and cell monolayers were washed once with fresh medium to avoid soluble P5/ICAM1 in apical chambers. Then, cells were loaded with different concentrations (10 ng/ml or 20 ng/ml) of Phl p 5 for 24h or 72h at 37°C. The effect of P5/ICAM1 on Phl p 5 apical-to-basolateral migration after 24h or 72h was measured by comparing wells loaded with P5/ICAM1 (+ P5/ICAM1 conjugates) and untreated control wells (- P5/ICAM1 conjugates).

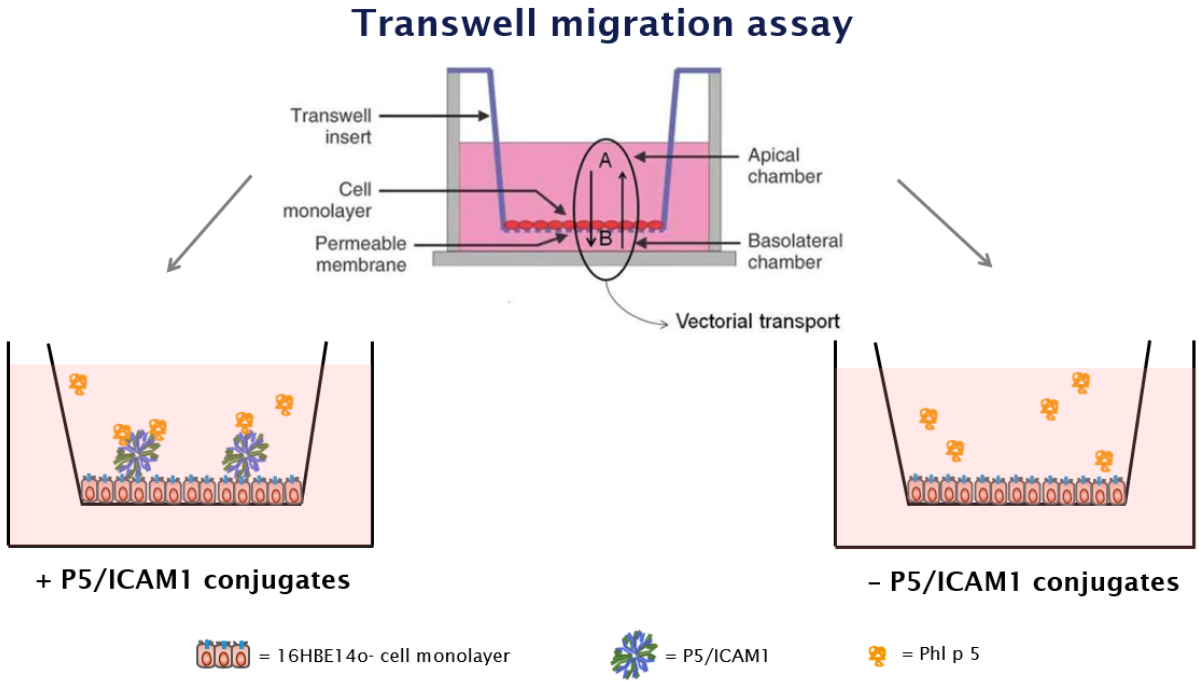

Figure 9 | Principle and design of Transwell migration assay.

4.3.2 ELISA measuring free Phl p 5 in apical and basolateral supernatants of Transwell migration assay

The amount of free Phl p 5 in apical and basolateral supernatants from two independent Transwell migration assays was determined by ELISA experiments. Therefore, ELISA plates were coated with Phl p 5-specific human IgG1 or IgG4 (1 µg/ml in 100mM NaHCOP3, pH= 9.6) for 1h at 37°C and washed twice with PBST. After plates were saturated with PBST containing 3% (w/v) BSA for 3h at 37°C, apical and basolateral

supernatants, collected at two different time points (24h or 72h after Phl p 5 addition), were applied overnight at 4°C. In a next step, free Phl p 5 was incubated with rabbit anti-Phl p 5 antibodies diluted 1:1000 in PBST/0.5% BSA. Then, rabbit antibodies bound to Phl p 5 were visualized with HRP-labelled donkey anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:2000 in PBST/0.5% BSA. OD measurements at 405 nm with a reference wavelength at 490 nm were performed on ELISA reader Infinite F50 (Tecan, Männedorf, Switzerland).

4.3.3 ELISA measuring Phl p 5 bound to P5/ICAM1 conjugates in apical and basolateral supernatants of Transwell migration assay

To detect whether there is P5/ICAM1-complexed Phl p 5 in apical or basolateral samples from Transwell cultures, ELISA plates were coated with 2.5 µg/ml recombinant human ICAM-1 (Sino Biological Inc., Beijing, China) diluted in bicarbonate buffer (100mM NaHCOP3, pH= 9.6). After incubation for 1h at 37°C, plates were washed two times with PBST and den saturated with PBST/3% BSA for 3h at 37°C. Supernatants of apical and basolateral compartments of a Transwell migration experiment were applied overnight at 4°C. For control purposes, Phl p 5-specific human IgG1 labelled with Streptavidin and biotinylated ICAM-1-specific mouse IgG1 in a ratio of 1:0.25 were incubated together for 1h at room temperature and then a total amount of 5.6 µg P5/ICAM1 were co-incubated with 1 µg Phl p 5 for again 1h at room temperature. P5/ICAM1 with bound Phl p 5 were added in different concentrations (1 µg/ml, 0.1 µg/ml and 0.01 µg/ml P5/ICAM1-Phl p 5) in parallel to the culture samples. Then, Phl p 5 bound to P5/ICAM1 was detected with Phl p 5-specific rabbit antibodies (1:1000 in PBST/0.5% BSA) and HRP-labelled donkey anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:2000 in PBST/0.5% BSA. OD measurements at 405 nm with a reference wavelength at 490 nm were carried out on ELISA reader Infinite F50 (Tecan, Männedorf, Switzerland).

4.4 P5/ICAM1 conjugates reduce activation and degranulation of effector cells

4.4.1 Basophil activation test

Basophil activation was determined *in vitro* by measuring upregulation of CD203c expression on the cell surface of human basophils (Hauswirth et al. 2002). Heparinized

blood samples from patients allergic to grass pollen and therefore containing Phl p 5-specific IgE antibodies were obtained after informed consent and the approval of the ethics committee (Medical University of Vienna, Austria, EK 1641/2014). Respectively, 90 µl of blood aliquots were incubated with apical as well as basolateral supernatants from the Transwell experiments for 15min at 37°C. As controls, monoclonal anti-IgE antibody (Immunotec Inc., Vaudreuil-Dorion, Canada) with an end concentration of 1 µg/ml or 1xPBS were included. Upregulation of CD203c on the cell surface induced by Phl p 5 was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFIstim) and unstimulated (MFIcontrol) cells and expressed as stimulation index (SI) (MFIstim /MFIcontrol) (Hauswirth et al. 2002). Cells were analyzed by 2-color flow cytometry on a FACScan (BD Biosciences, San Jose, USA).

4.4.2 Rat basophilic leukemia cell mediator-release assay

Humanized rat basophilic leukemia (RBL) cells transfected with the human high affinity IgE receptor FcεRI (RS-ATL8) (Nakamura et al. 2010) were cultured in Minimum Essential Medium (Thermo Fisher Scientific, Waltham, USA) containing 10% FBS (GE Healthcare, Buckinghamshire, UK), 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine and 0.2 µg/ml hygromycin B as well as 0.2 µg/ml geneticin (Thermo Fisher Scientific, Waltham, USA) at 37°C in a humidified atmosphere containing 5% CO_2. Cells at 80% confluency were transferred from tissue culture flasks into microplates (Corning, New York, USA) ($1.5x10^5$ cells per well) and loaded with specific IgEs from serum of grass pollen allergic patients diluted 1:10 and incubated overnight. After washing 3 times with Tyrode's buffer (Sigma-Aldrich, St. Louis, USA) cells were exposed to different concentrations (1:10 – 1:100) of supernatants from Transwell cultures containing Phl p 5 for 1h at 37°C. IgE-loaded cells were incubated with Tyrode's buffer only to measure spontaneous release and total β-hexosaminidase content was determined after lysis of cells by the addition of 10% Triton-X100 (Sigma-Aldrich, St. Louis, USA). For control purposes, RBL cells were not pre-incubated with patients' sera but buffer and then challenged with supernatants in the highest concentration, respectively. Controls omitting supernatants but not patients' sera were included. Release of ß-hexosaminidase induced by allergens is given as percentage of total mediator content.

4.5 P5/ICAM1 conjugates prevent damage to respiratory epithelial cells by human rhinovirus infection

4.5.1 Human rhinovirus preparation

HeLa cells (Ohio strain; Flow Laboratories, McLean, USA) were infected with RV-B14 (ATCC, The Global Bioresource Center, Manassas, USA) for 40h in suspension cultures. RV-B14 was prepared by precipitation with polyethylene glycol and then resuspended in 1xPBS as described previously (Gangl et al. 2015). Purified RV-B14 was used for infection of confluent 16HBE14o- cell monolayers in e-plates of the xCELLigence DP system. To determine the concentration of RV-B14, 50% tissue culture infective dose ($TCID_{50}$) measurements were performed.

4.5.2 Real-time monitoring of 16HBE14o- cells with xCELLigence system

At 70-90% confluency, $2x10^4$ 16HBE14o- cells per well were seeded into collagen-fibronectin (BD Biosciences; San Jose, USA) coated wells of e-plates 16 of the xCELLigence Real Time Cell Analysis (RTCA) DP system (ACEA Biosciences; San Diego, USA). The xCELLigence RTCA DP system monitors non-invasive impedance-based cell responses at physiological conditions and the outcome is complementary to for example changes in cell morphology and proliferation as well as cytotoxicity. Cell Index is expressed as an arbitrary unit and is calculated from impedance measurements between cells and sensors of the xCELLigence DP system. Confluence of cell monolayers was confirmed by phase contrast microscopy (Olympus IX73, Tokyo, Japan) and is equivalent to Cell Index values of 13-15, which was reached approximately after 20h of cultivation. Visualization of changes in cell responses starts from this value onwards and were measured every 30min. At this time point the Cell Index values were normalized (Normalized Cell Index; NCI = 1) (Waltl et al. 2018). Then, 0.5 µg/ml and 1.5 µg/ml P5/ICAM1 conjugates or fresh medium were added to 16HBE14o- cell monolayers. After 3h of incubation, various conditions were infected with 150 $TCID_{50}$/ cell of RV-B14. In control wells 1xPBS was added instead of virus.

4.6 Statistics

Differences in amount of free Phl p 5 (ELISA experiments) or in percentage of allergen-induced ß-hexosaminidase release of RBL cells (RBL mediator-release assays) among the two groups (w/o treatment of Transwell cultures with P5/ICAM1 conjugates) were analyzed with Mann-Whitney U tests. Results with a p-value ≤ 0.05 were considered significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Statistical analyses were performed with IBM SPSS Statistics 25 software (IBM, Armonk, USA).

5. Results

5.1 P5/ICAM1 conjugates specifically bind Phl p 5 and human ICAM-1

Bi-specific antibody conjugates, termed P5/ICAM1, are consisting of Phl p 5 (P5)- and ICAM-1 (ICAM1)-specific monoclonal antibodies. Since the Phl p 5-specific human IgG1 is conjugated with Streptavidin (αP5-IgG1*Strep) and the anti-ICAM-1 mouse IgG1 is biotinylated (αICAM1-IgG1*Bio), bi-specific conjugates were generated via streptavidin-biotin coupling of the respective components. P5/ICAM1 conjugates were tested for their potential to recognize their antigens (Phl p 5 and ICAM-1) even after the formation. In ELISA experiments it was demonstrated that P5/ICAM1 antibody conjugates bind specifically to recombinant Phl p 5 **(Figure 10 A)** or human ICAM-1 **(Figure 10 B)**, when the antigens were immobilized on ELISA plates. The bi-specificity of P5/ICAM1 conjugates was demonstrated by using either detection antibodies specific for Phl p 5-specific human IgG1 (AP-labelled anti-human F(ab)2) or ICAM-1-specific mouse IgG1 antibodies (HRP-labelled anti-mouse antibodies) and results are mean values of duplicates. One representative of three independent ELISA experiments is shown.

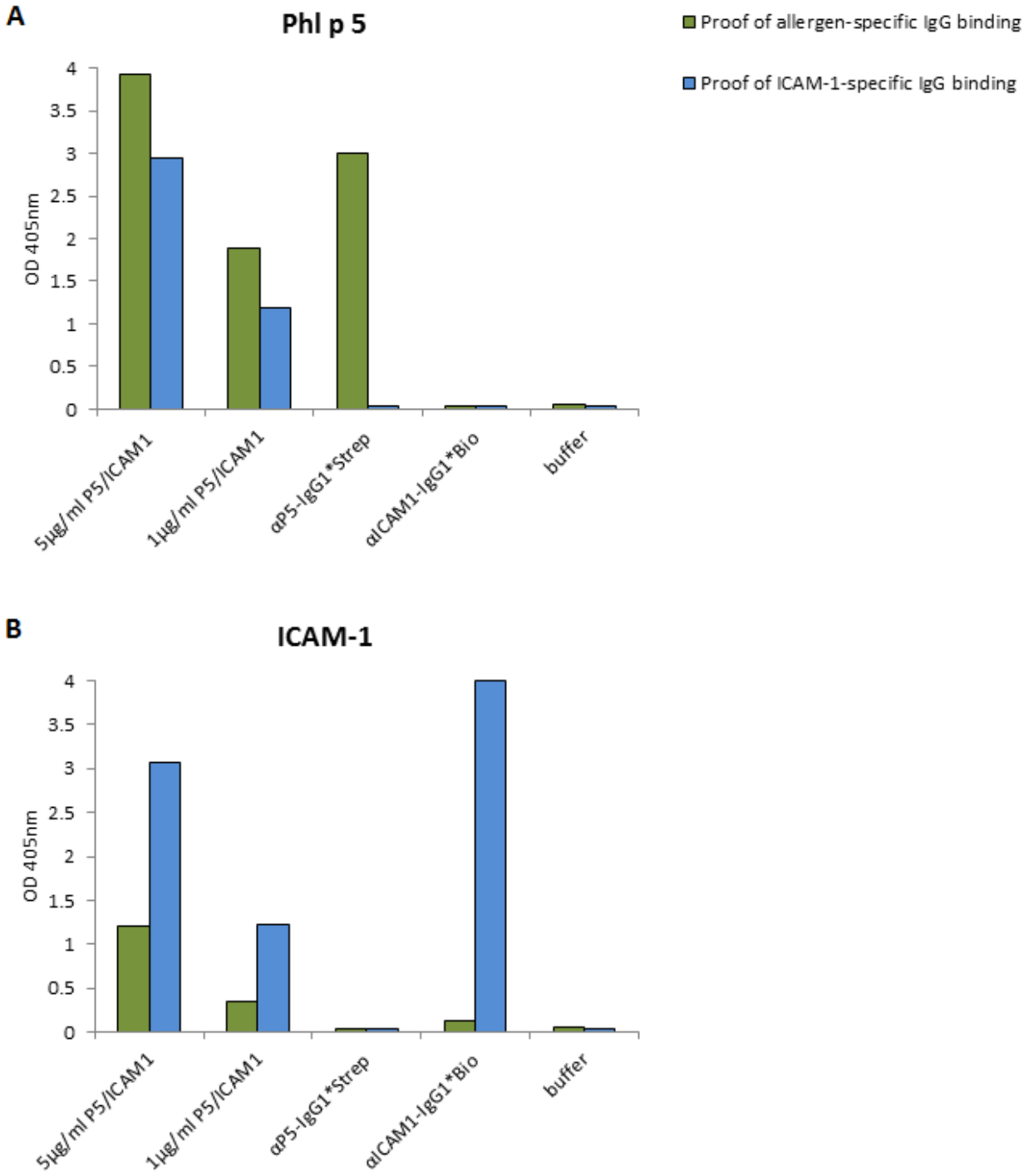

Figure 10 | **P5/ICAM1 conjugates still recognize Phl p 5 and human ICAM-1 after formation.** ELISA plates were coated with recombinant Phl p 5 **(A)** or human ICAM-1 **(B)**. Then, 1 µg/ml and 5 µg/ml P5/ICAM1 or, for control purposes, 1 µg/ml anti-Phl p 5 IgG1 (αP5-IgG1*Strep) and anti-ICAM-1 IgG1 (αICAM1-IgG1*Bio) were added. Bi-specificity of P5/ICAM1 conjugates was proofed by binding of AP-labelled anti-human F(ab)2 detection antibody to Phl p 5-specific human IgG1 and HRP-labelled anti-mouse IgG detection antibody to ICAM-1-specific mouse IgG1. OD was determined at 405 nm and data are shown as means of duplicates.

5.2 Human ICAM-1 is expressed on 16HBE14o- cells and Phl p 5 can be immobilized via P5/ICAM1 conjugates onto the cell surface

To examine whether the 16HBE14o- cell line, which is commonly used as surrogate for investigations of the nasal epithelium, expresses human ICAM-1 on the surface and is therefore suitable to immobilize the bi-specific conjugate, and further to proof the immobilization of Phl p 5 via cell bound P5/ICAM1, FACS analyses were performed **(Figure 11a**). 16HBE14o- cells were pre-incubated with 1 µg biotinylated anti-ICAM-1 mouse IgG1 antibody or an isotype control (biotinylated mouse IgG1) and then stained with Streptavidin DyLight ®488. Stained cells were sorted and anti-ICAM-1 antibody bound to ICAM-1 on the cell surface was detected proofing that human ICAM-1 is expressed on the surface of 16HBE14o- cells. Therefore, the anti-ICAM-1 antibody, as one part of the bi-specific conjugate, is suitable to anchor Phl p 5-specific IgG onto respiratory cells **(Figure 11a A)**.

In a next experiment, cells were pre-incubated with 1 µg P5/ICAM1 conjugates for 30min and then 1 µg, 2 µg or 5 µg Phl p 5 was added. Immobilization of Phl p 5 onto the cell surface via P5/ICAM1 could be demonstrated by using Phl p 5-specific rabbit antibodies, which were then stained with Alexa Fluor ®405 Goat Anti-rabbit **(Figure 11a B)**. In this assay, rabbit pre-immune serum was used as isotype control. There is a small positive population in the isotype control, which seems to occur due to auto-fluorescence and therefore has no impact on the analysis. Results are means of triplicates and one representative of two independent experiments is shown.

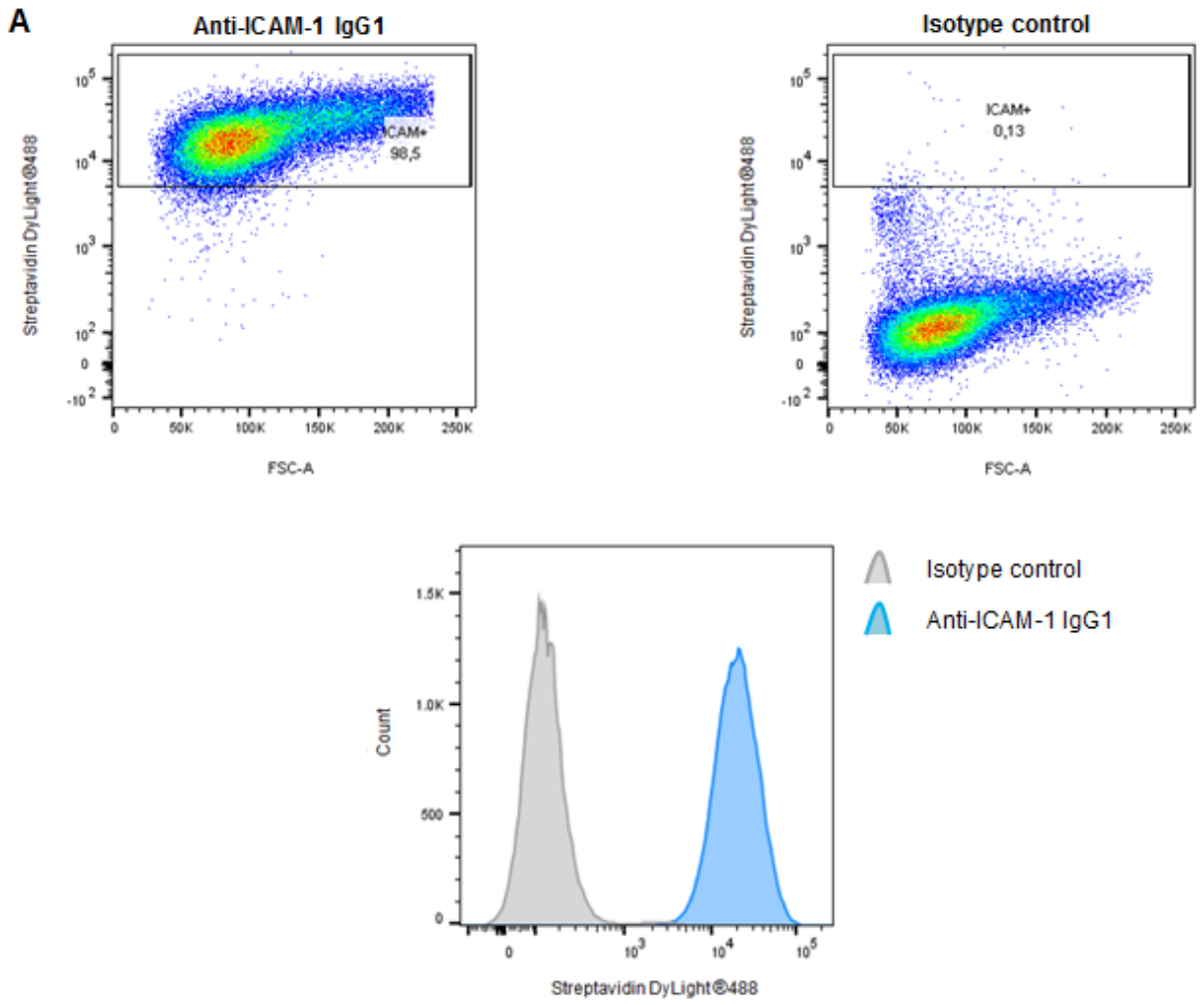

Figure 11a A | **Detection of human ICAM-1 on the surface of 16HBE14o- cells by using flow cytometry.** 16HBE14o- cells were pre-incubated with anti-ICAM-1 antibody or isotype control. Anti-ICAM-1 antibody bound to ICAM-1 on the cell surface was then detected. Results are shown as means of triplicates.

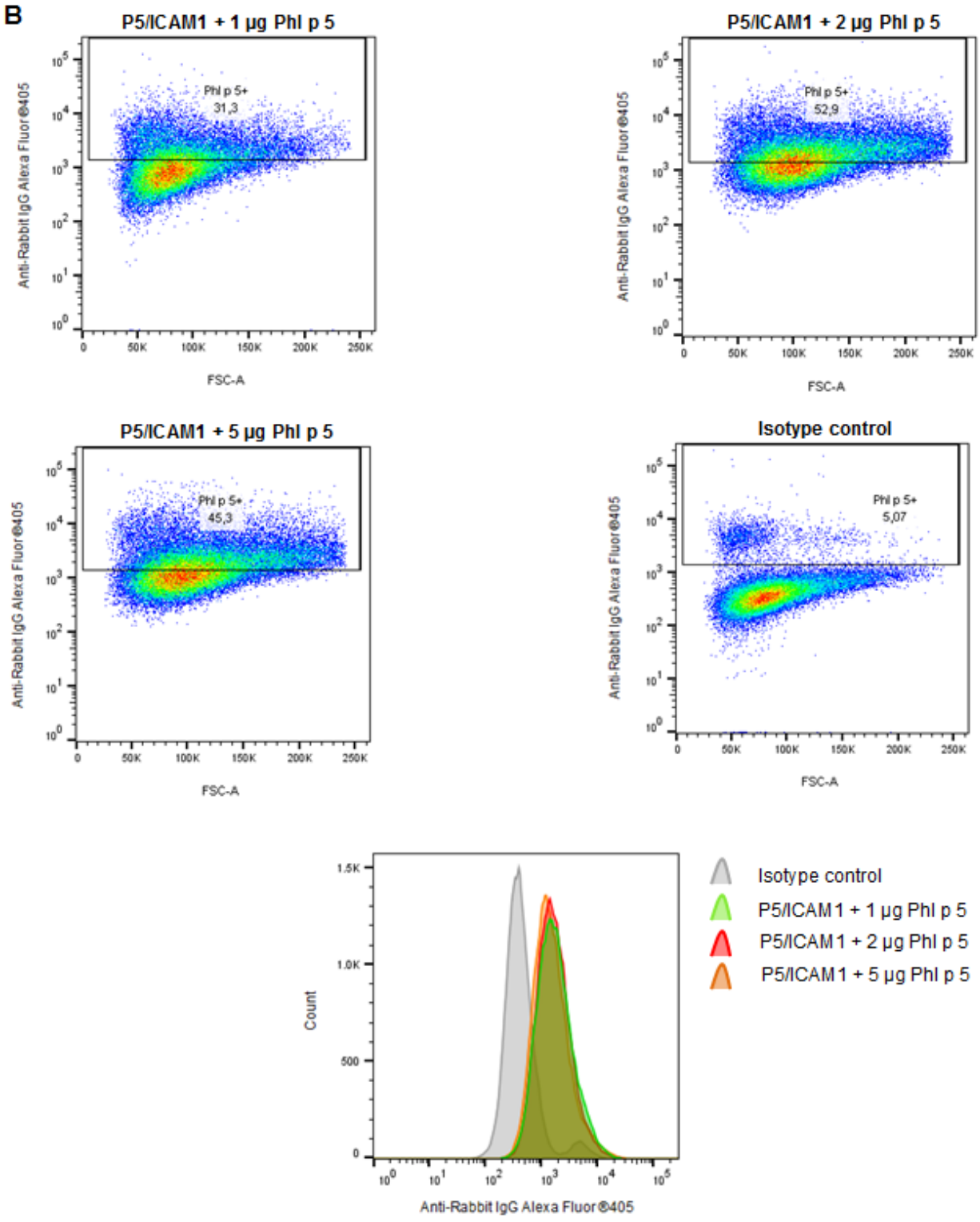

Figure 11a B | **Detection of Phl p 5 immobilized via P5/ICAM1 onto the surface of 16HBE14o- cells by using flow cytometry.** 16HBE14o- cells were pre-incubated with P5/ICAM1 conjugates and different concentrations of Phl p 5 (1 µg, 2 µg or 5 µg) were sorted by the ability to immobilize Phl p 5 via binding to P5/ICAM1 on the cell surface. Results are shown as means of triplicates.

In parallel, immunofluorescence microscopy experiments were performed to confirm these findings, and further to visualize the immobilization of Phl p 5 via cell bound P5/ICAM1 **(Figure 11b**). Co-localization of Phl p 5 (Alexa Fluor ®568) and P5/ICAM1 (Alexa Fluor ®488) on the cell surface was seen in images (**Figure 11b A,** merge). When Phl p 5 was omitted, no binding of Alexa Fluor ®568 anti-rabbit IgG was observed, but P5/ICAM1 binding to human ICAM-1 on the epithelial cells was detected by Alexa Fluor ®488 anti-mouse IgG (**Figure 11b B**). When no P5/ICAM1 was applied, detection antibodies did not bind at all (**Figure 11b C**). Shown results are means of duplicates.

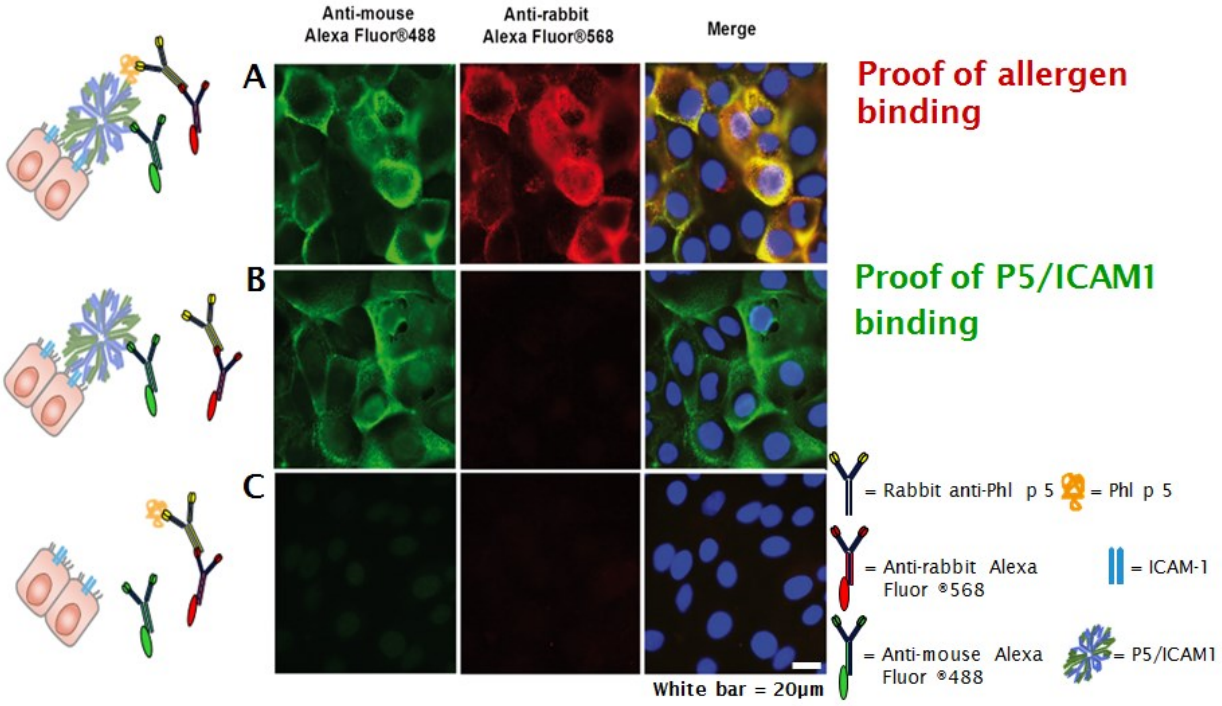

Figure 11b | **Visualization of P5/ICAM1 conjugate and Phl p 5 on 16HBE14o- cells by means of immunofluorescence microscopy**. Images **(A-C)** show 16HBE14o- cells incubated with different combinations of reactants, which where stained with Alexa Fluor ®488-labeled anti-mouse antibodies (green) and Alexa Flour ®568-labeled anti-rabbit antibodies (red) to visualize mouse anti-ICAM-1 IgG1 and Phl p 5, respectively. Nuclei were stained with 4', 6-Diamidino-2-Phenylinodole, Dihydrochloride (blue), and merged images are shown in the right column. Results are shown as means of duplicates.

5.3 P5/ICAM1 conjugates prevent migration of Phl p 5 through 16HBE14o- cell monolayers

In a next step, it was investigated whether P5/ICAM1 conjugates are able to prevent allergen migration from apical to basolateral sides through a layer of cultured human bronchial epithelial cells. For this purpose, Transwell migration assays were performed. The 16HBE14o- cell monolayer in apical chambers was pre-incubated w/o P5/ICAM1 conjugates, washed and then exposed to Phl p 5. After 24h or 72h, supernatants of all apical and basolateral compartments were collected and then analyzed for the extent of Phl p 5 penetration through the cell layer into the basolateral chamber. Thus, ELISA experiments were performed to measure the amount of free Phl p 5 in the supernatants of Transwell migration assays **(Figure 12a)**. Incubation of cell monolayers with P5/ICAM1 significantly reduced the trans-epithelial migration of Phl p 5 at the tested time points (24h + and 72h +) when compared to no treatment with P5/ICAM1 (24h - and 72h -). In apical chambers without cell monolayer, Phl p 5 migrated through the permeable membrane from the apical to the basolateral compartments, regardless of the presence of P5/ICAM1 (no cells, 24h +/ 24h -; no cells, 72h +/ 72h -). Results are shown as means of triplicates and error bars indicate SDs. Two representatives of repeated ELISA experiments are shown, respectively. Differences in amount of free Phl p 5 among the two groups (w/o treatment with P5/ICAM1 conjugates) were analyzed with Mann-Whitney U tests. Results with a p-value ≤ 0.05 were considered significant.

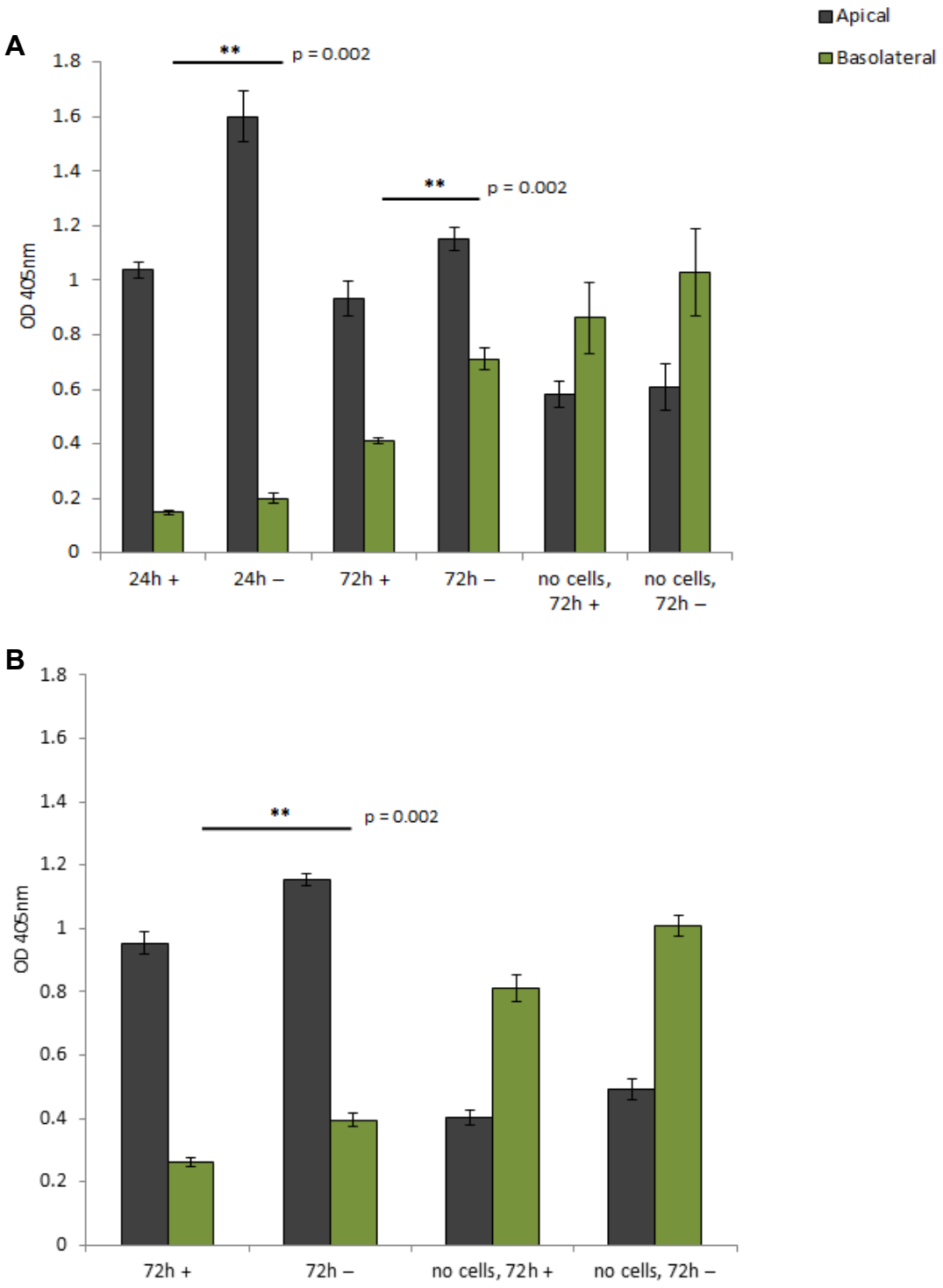

Figure 12a | **Detection of free Phl p 5 in apical and basolateral supernatants.** ELISA plates were coated with Phl p 5-specific IgG4 and then incubated with apical and basolateral supernatants from two independent Transwell migration assays **(A+B)**. For control purposes, supernatants of wells without cell monolayer but w/o addition of P5/ICAM1 were also analyzed for free Phl p 5 (no cells, 24h +/ 24h -; no cells, 72h +/ 72h -). OD-values (y-axis) corresponding to concentrations of free Phl p 5 are shown as means of triplicates and error bars indicate SDs. Significant differences between groups w/o conjugate at two points (24h +/ 24h -; 72h +/ 72h -) were calculated with Mann-Whitney U tests.

Further ELISA experiments were performed to analyze apical as well as basolateral supernatants to detect Phl p 5 bound to P5/ICAM1 conjugates, which are not attached to the cell surface **(Figure 12b)**. Phl p 5 complexed with P5/ICAM1 was detected in none of the supernatants, but positive controls (P5/ICAM1-Phl p 5) showed binding depending on the concentration. Results are mean values of duplicates.

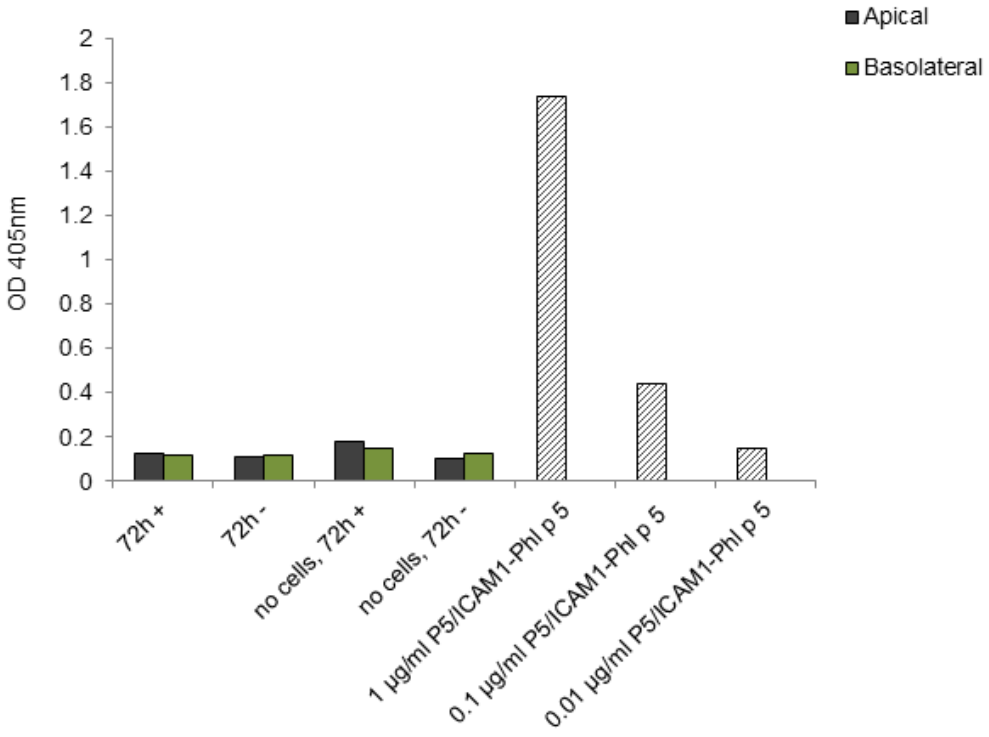

Figure 12b | **Detection of Phl p 5 bound to P5/ICAM1 conjugates in apical and basolateral supernatants.** ELISA plates were coated with recombinant human ICAM-1 and then incubated with supernatants from apical and basolateral compartments from Transwell cultures collected 72 h after addition of Phl p 5. Wells without cell monolayer but w/o addition of P5/ICAM1 were also analyzed for complexed Phl p 5 (no cells, 72h +; no cells, 72 h -). As positive control, different concentrations of complexes of P5/ICAM1 and Phl p 5 were applied. OD-values (y-axis) corresponding to concentrations of Phl p 5 complexed with P5/ICAM1 conjugates are shown as means of duplicates.

5.4 P5/ICAM1 conjugates reduce activation and degranulation of basophils

Further, to examine whether a reduction of allergen migration through the epithelial monolayer by P5/ICAM1 conjugates has an effect on effector cells, basophil activation tests (BATs) were performed. Full blood samples taken from patients allergic to Phl p 5 were incubated with apical and basolateral supernatants. The upregulation of CD203c on the surface of human basophils due to IgE-crosslinking caused by the amount of allergen

in the supernatants was measured as marker for basophil activation. When the extent of basophil activation induced with basolateral supernatants was compared in the groups with (+) or without (-) P5/ICAM1 treatment, the P5/ICAM1 conjugate was not only able to reduce the allergen amount in the basolateral compartments but also to decrease the activation of basophils **(Figure 13a)**. This effect was observed 24h and 72h after the allergen was added to the apical side.

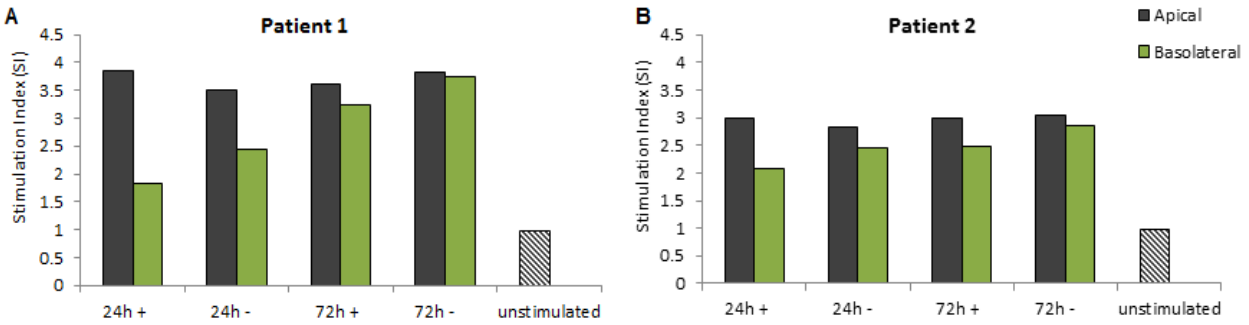

Figure 13a | Inhibition of trans-epithelial migration of Phl p 5 through allergen binding to P5/ICAM1 conjugates results in reduced basophil activation. 16HBE14o- cell monolayers were loaded with 10 µg/ml P5/ICAM1 conjugates for 3h. Then, 20 ng/ml Phl p 5 was added and apical as well as basolateral samples were obtained after 24h or 72h. Basophils obtained from allergic patients were incubated with apical and basolateral supernatants and up-regulation of CD203c expression as marker for basophil activation due to allergen penetration is displayed as stimulation indices (SI).

Similar results were obtained when basolateral samples were analyzed for their potential to induce degranulation of humanized RBL cells. Humanized RBL cells, which are expressing the human FcεRI on their surface, were loaded with specific IgE from patients proven to be allergic to Phl p 5 and then incubated with basolateral supernatants of independent Transwell experiments. The ability of supernatants to induce degranulation of basophils was determined by measuring the release of ß-hexosaminidase. In this experiment, the ß-hexosaminidase release of humanized RBL cells was significantly reduced, when P5/ICAM1 was applied to the cell monolayer in the Transwell system compared to wells without P5/ICAM1 treatment **(Figure 13b)**. OD values corresponding to percentage of ß-hexosaminidase release are means of triplicates and error bars indicate SDs. Results are shown as representatives of independent experiments. Differences in ß-hexosaminidase release among the two groups (+ conjugate/ - conjugate) were analyzed with Mann-Whitney U tests and results with a p-value ≤ 0.05 were considered significant.

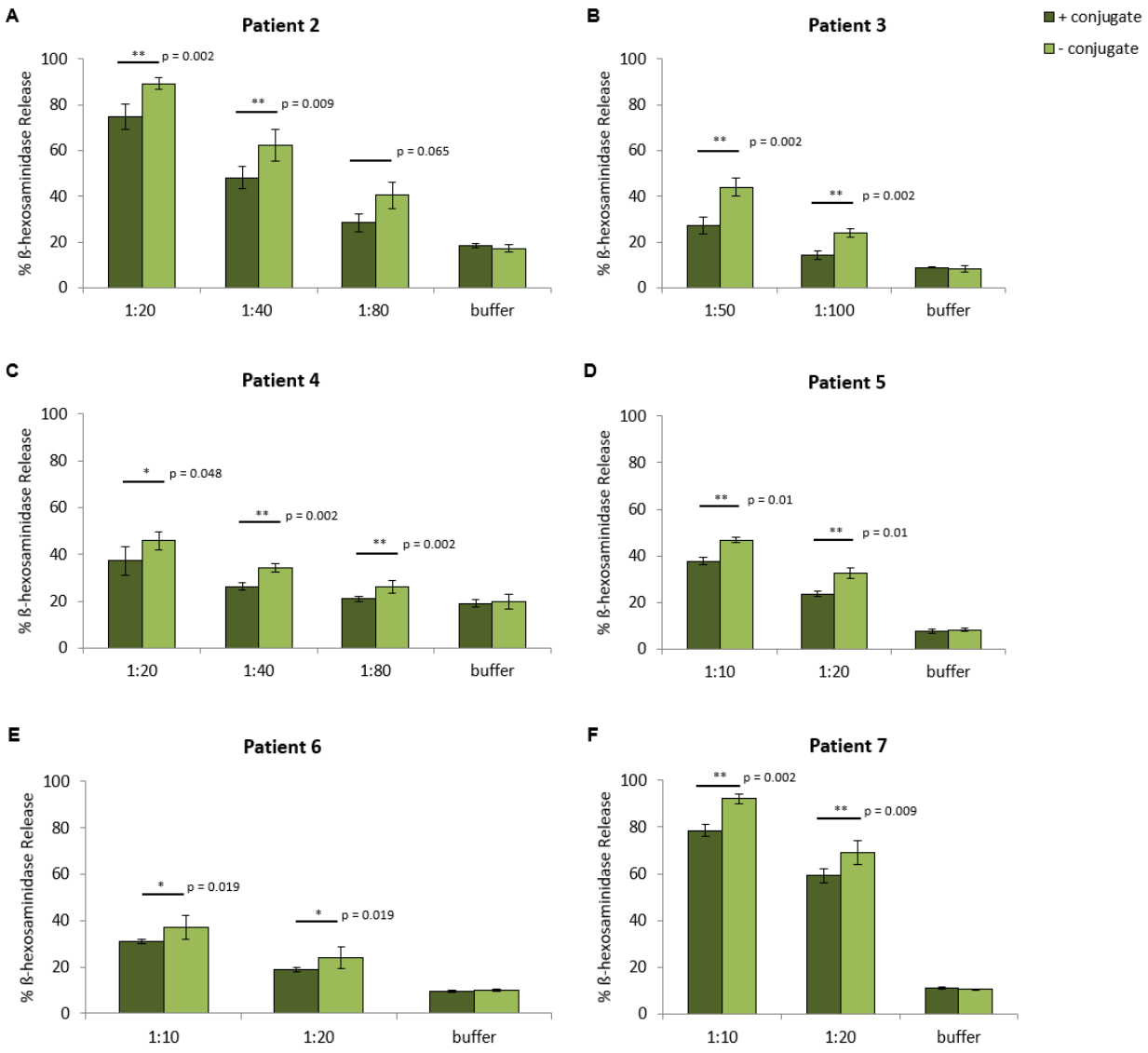

Figure 13b │ Inhibition of trans-epithelial migration of Phl p 5 through allergen binding to P5/ICAM1 conjugates results in decreased release of ß-hexosaminidase of humanized RBL cells. 16HBE14o- cell monolayers were loaded with 10 µg/ml P5/ICAM1 conjugates for 3h. Then, 10 ng/ml **(A-C)** or 20 ng/ml Phl p 5 **(D-F)** was added and basolateral samples were analyzed after 72h. Humanized RBL cells were pre-incubated with patients' sera containing Phl p 5-specific IgE antibodies, and supernatants from basolateral compartments of Transwell cultures in different dilutions (1:10 – 1:100) or buffer were added. Basophil degranulation was then determined by measuring ß-hexosaminidase release after incubation with basolateral supernatants. Results are means of triplicates and error bars indicate SDs. Significant differences between groups with or without conjugate (+/− conjugate) were calculated with Mann-Whitney U tests.

5.5 P5/ICAM1 conjugates prevent damage to 16HBE14o- cells by human rhinovirus infection

Since ICAM-1 is the cellular receptor for the major group of RVs, further analyses with the xCELLigence RTCA DP system were performed to investigate the protective effect of P5/ICAM1 conjugates on RV infection. Therefore, 16HBE14o- cells were incubated w/o P5/ICAM1 conjugates at two different concentrations (0.5 µg/ml or 1.5 µg/ml) and then exposed to 150 $TCID_{50}$/cell of RV-B14 representative for the major group of RV. When cells were pre-incubated with P5/ICAM1 conjugates, the cells were protected against infection by RV-B14 at a concentration of 150 $TCID_{50}$/cell, whereas cells incubated with medium were infected by the virus (**Figure 14**). The lower concentration (0.5 µg/ml) of P5/ICAM1 conjugates only partially inhibited damage of the epithelial barrier caused by RV-B14 infection, whereas the higher concentration (1.5 µg/ml) fully protected the epithelium from damage by the virus even after 4 days. Additionally, it was observed that the P5/ICAM1 conjugate applied in the higher concentration itself did not disrupt the cell barrier integrity. Results are figured as means of duplicates and one representative analysis of repeated experiments is shown.

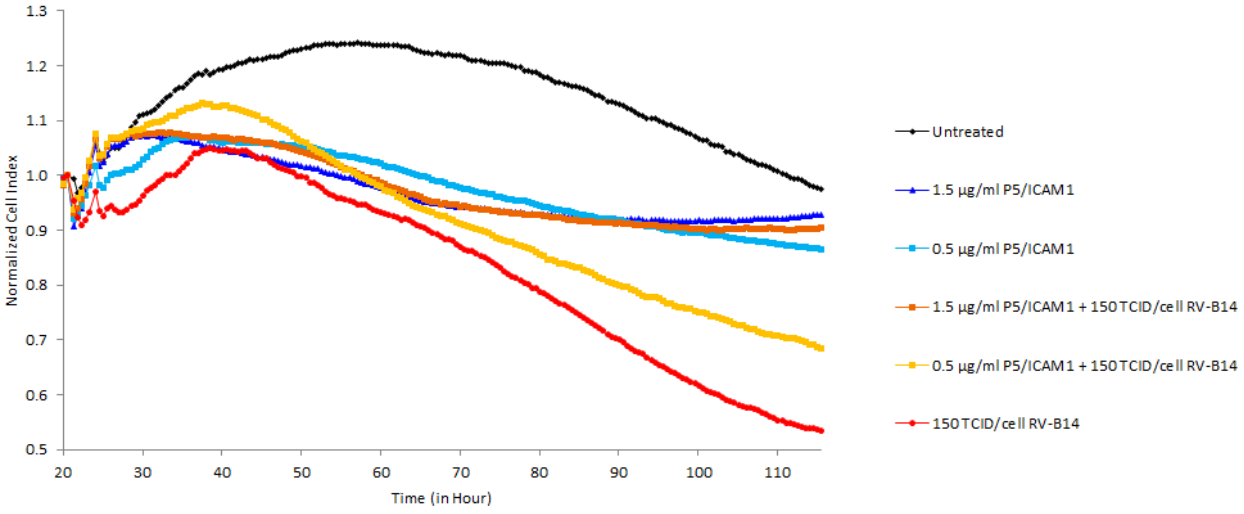

Figure 14 | Cell bound P5/ICAM1 conjugates prevent infection of 16HBE14o- cells by RV-B14. 16HBE14o- cells were cultured on e-plates of the xCELLigence DP system for 20h and then incubated with 0.5 µg/ml or 1.5 µg/ml P5/ICAM1 conjugates or medium control. Three hours thereafter, cells were infected with 150 $TCID_{50}$/cell of RV-B14. Controls without virus treatment were included. Impedance values were automatically measured every 30min by the xCELLigence DP system for 120h and are expressed as normalized Cell Index (NCI; y-axis). Data are shown as mean values of duplicates for each measuring point.

6. Conclusion and discussion

These findings demonstrate that our generated bi-specific antibody conjugate prevents allergic immune responses and additionally blocks the binding to ICAM-1 by RVs *in vitro*. First, we showed by performing ELISA experiments that the Phl p 5-specific human IgG1 as well as the ICAM-1-specific mouse IgG1 were able to recognize their antigens, recombinant Phl p 5 and recombinant human ICAM-1, even after formation of bi-specific conjugates via streptavidin and biotin coupling. We proved with FACS analyses that the human bronchial epithelial cell line 16HBE14o-, which is commonly used as surrogate for investigations of the respiratory epithelium, is expressing human ICAM-1 on its cell surface and proved therefore that the 16HBE14o- cells are suitable for our experiments. Further, we were able to demonstrate the binding of Phl p 5 to the bi-specific conjugate on the surface of 16HBE14o- cells with FACS experiments and immunofluorescence microscopy. In a next series of experiments, we showed that the incubation of 16HBE14o- with bi-specific conjugates in Transwell migration assays immobilizes Phl p 5 on cell surfaces and significantly reduces the migration of Phl p 5 through the cell monolayer. With RBL assays and BATs, we demonstrated that this reduced allergen migration also has a decreasing effect on the activation and degranulation of basophils. Second, we proved the potential of bi-specific conjugates to prevent 16HBE14o- cells from infection by RV-B14, as representor of the major group of RVs, by performing experiments with the RTCA xCELLigence DP system.

Our results demonstrate that the topical non-invasive administration of a bi-specific antibody conjugate is a promising concept for the prevention of allergic immune responses as well as RV infection *in vitro*. Nevertheless, further investigations should be performed to clarify whether the ICAM-1-specific antibody is interfering with the physiological functions of human ICAM-1. Since the binding sites of RVs and LFA-1 are both located in domain 1 of human ICAM-1 (Staunton et al. 1990, Olson et al. 1993), the adhesion and migration of leukocytes could be affected by the interactions between anti-ICAM-1 IgG1 and ICAM-1. However, it was demonstrated that a monoclonal ICAM-1-specific antibody that was shown to bind to domain 1, did not alter the attachment of LFA-1 to human ICAM-1 *in vitro* (Traub et al. 2013). Furthermore, additional physiological tests need to assess the potential as well as possible consequences of internalization of human ICAM-1 together with the attached bi-specific conjugate. It was reported that nanoparticles, showing a molecular weight of

20 kDa - 90 kDa, were rapidly internalized after binding to human ICAM-1 on epithelial cells. In some cases, ICAM-1 mediated internalization of nanoparticles that are used as drug carrier is required to increase the efficacy of a drug by delivering it to the site of interest (Chittasupho et al. 2009). However, the bi-specific conjugate acts at the apical side of epithelial cells and therefore internalization would be contra-productive for preventing allergen uptake and RV infection. Since the bi-specific conjugate has a molecular weight of at least 300 kDa, it might not be internalized anyways. In addition, further investigations with *in vivo* models are needed to confirm the promising results of topical administered bi-specific conjugates as novel therapeutic tool for prevention of allergy and RV infection.

For allergy, the only causative treatment so far is AIT (reviewed in Larché et al. 2006), which was shown to be very effective also for grass pollen allergy (Jutel et al. 2005). Nevertheless, AIT carries some risks and is very time consuming and thus it may not be the therapy of choice for treating seasonal allergies. In contrast, a recent proof of concept study re-stimulated the idea that passive administration of monoclonal antibodies is also a convincing approach for allergy treatment (Orengo et al. 2018). These results demonstrated for the first time that monoclonal allergen-specific antibodies are useful for allergy treatment in patients. However, there are no approved therapeutic antibodies for the treatment of grass pollen allergy available so far (reviewed in Flicker et al. 2011). Other effective medications are antihistamines for treating symptoms, but not the disease itself (reviewed in Yamauchi and Ogasawara 2019). Therefore, the bi-specific conjugate applied via nasal spray or inhalator represents a convenient strategy to prevent allergic symptoms. First, it is feasible due to very low allergen concentrations in the air and thus it is realistic that the bi-specific conjugate can immobilize most of the allergens on the respiratory epithelium and hence prevent allergen migration and penetration. Second, the administration is easy and independent from a clinic.

For prevention of RV infection, several strategies including receptor-blocking by monoclonal antibodies or vaccines are under investigation (reviewed in Basnet et al. 2019). However, since no treatments are on the market to prevent infection by RVs so far, the bi-specific conjugate would be an elegant way to protect against RV infection as well as allergy. Especially for patients with allergic rhinoconjunctivitis it is important to prevent infection by RV because RVs can promote the progression to bronchial asthma (reviewed in Heymann et al. 2005). Moreover, RV infection leads to damage of the epithelial barrier

function and thus facilitate the allergen uptake (reviewed in Mattila et al. 2011). This synergism between RV and allergens should play an important role in the development of effective treatments for both diseases.

In addition, the bi-specific antibody conjugate could also be applied to reduce allergic symptoms caused by pollen-related food allergies. It was previously shown that antibody conjugates consisting of ICAM-1-specific and Phl p 2-specific antibodies reduce trans-epithelial migration of allergens and thus significantly decrease the activation of human basophils *in vitro* (Madritsch et al. 2015). This leads to the suggestion that antibody conjugates specific for ICAM-1 and Bet v 1, which is known to be highly cross-reactive with several dietary allergens (reviewed in Bohle 2007), may be usable for the prevention of pollen-related food allergies. Therefore, the concept of an antibody conjugate applied via nasal spray or inhalator could be used as prophylaxis in several seasonal allergies by targeting the respective major allergens and human ICAM-1 to prevent infection by the major group of RVs.

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8. Appendix

Abstract (German)

Über 25% der westlichen Bevölkerung leiden unter IgE-vermittelter Hypersensitivität, auch Typ I Allergie genannt. Besonders im Frühjahr und Sommer sind Gräserpollen weit verbreitet und die darin enthaltenen Allergene führen bei bereits sensibilisierten Patienten zu den typischen allergischen Symptomen. Diese werden durch Fcε-Rezeptor gebundene IgE-Allergen-Komplexe, die zur Freisetzung von biologischen Mediatoren (z.B. Histamin) führen, ausgelöst. Zu einem der schwerwiegendsten Symptome von Typ I Allergie zählt Asthma bronchiale. Neben Allergie spielen auch Viren, die die Atemwege betreffen, wie z.B. humane Rhinoviren, eine wichtige Rolle in der Pathogenese von Asthma. Humane Rhinoviren gelten zusätzlich als Hauptursache von Erkältung, welche bei bestimmten Individuen zu lebensbedrohlichen Symptomen führen kann. Aufgrund einer möglichen synergistischen Wirkungsweise von inhalativen Allergenen und humanen Rhinoviren hinsichtlich Entzündungen der Atemwege ist es von großer Bedeutung, erfolgreiche Strategien zur Prävention von Typ I Allergie und Infektionen mit humanen Rhinoviren zu entwickeln. Zur Behandlung von Allergie gibt es bereits einige Möglichkeiten, wie z.B. Allergen-spezifische Immuntherapie, wohingegen noch keine Medikamente zur Prophylaxe von Infektionen mit humanen Rhinoviren auf dem Markt erwerblich sind.

Ziel dieser Arbeit ist es, eine topische, nicht invasive Applikation, z.B. in Form eines Nasensprays oder Inhalators, zu entwickeln, die einerseits zur Prävention von allergischen Reaktionen durch Gräserpollen eingesetzt werden kann und andererseits vor Infektionen mit humanen Rhinoviren schützt. Hierfür wurde ein Antikörper Komplex generiert, der bispezifisch für Phl p 5, ein Hauptallergen von Gräsern, und humanes ICAM-1, dem Rezeptor für die Hauptgruppe der humanen Rhinoviren, ist. Durch Streptavidin-Biotin-Konjugation von Phl p 5-spezifischen humanen IgG1 und ICAM-1-spezifischen murinen IgG1 Antikörpern wurden stabile bispezifische Antikörper Komplexe gebildet. Der ICAM-1-spezifische Antikörper verankert den bispezifischen Komplex durch die Bindung an ICAM-1 auf respiratorischen Epithelzellen, sodass der Phl p 5-spezifische Antikörper das Allergen auf der Zelloberfläche immobilisiert und dadurch dessen Migration durch die Mukosa verhindert wird. Gleichzeitig blockiert der ICAM-1-spezifische Antikörper durch seine Bindung an ICAM-1 die Interaktion zwischen dem Virus und seinem Rezeptor, wodurch

einer Infektion mit humanen Rhinoviren vorgebeugt wird. Um diese Hypothese zu testen, wurden zunächst *in vitro* Experimente mit Durchflusszytometrie und Immunfluoreszenz Mikroskopie durchgeführt, um herauszufinden, ob der bispezifische Antikörper Komplex ICAM-1 auf der Zelloberfläche der humanen bronchialen Epithelzelline 16HBE14o- erkennt und weiters Phl p 5 bindet. Transwell Migrationtests wurden zur Untersuchung des Potentials des Antikörper Komplexes, die Migration des Allergens durch eine Schicht von 16HBE14o- Zellen zu verhindern, eingesetzt. Mittels Basophilen Aktivierungstests sowie Experimenten mit humanisierten basophilen Rattenleukämiezellen wurden die Auswirkungen auf die Aktivierung und Degranulation von Effektorzellen analysiert. Des Weiteren wurden die präventiven Effekte des Antikörper Komplexes auf Infektionen mit humanen Rhinoviren von 16HBE14o- Zellen durch Impedanzmessungen des xCELLigence Real Time Cell Analysis Systems ermittelt.

Die *in vitro* Ergebnisse zeigen, dass der generierte bispezifische Antikörper Komplex Phl p 5 auf der Oberfläche von Epithelzellen immobilisiert, die Allergenmigration signifikant reduziert und dadurch die Allergen-induzierte Aktivierung und Degranulation von Basophilen gesenkt wird. Zusätzlich wurde demonstriert, dass der Antikörper Komplex eine Infektion der 16HBE14o- Zellen mit humanen Rhinoviren vorbeugt.

Zusammenfassend lässt sich sagen, dass der aus einem Allergen-spezifischen und einem ICAM-1-spezifischen Antikörper bestehende Komplex großes Potential hinsichtlich der Prävention von allergischen Symptomen sowie Infektionen mit humanen Rhinoviren aufweist. In Form von Nasensprays oder Inhalatoren könnten diese bei Atemwegserkrankungen, welche durch Allergie oder humane Rhinoviren induziert wurden, zur Behandlung eingesetzt werden.