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List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AIT	Allergen-specific immunotherapy
AKP	Alkaline phosphatase
BSA	Bovine serum albumin
CDR	Complementary determining region
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
Fab	antigen-binding fragment
FcεRI	High affinity receptor for IgE
HCAb	Heavy-chain antibodies
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
MHC	Major histocompatibility complex
Nb	Nanobody
OD	Optical density
PBS	Phosphor-buffered saline
PBST	Phosphor-buffered saline containing 0.05% Tween20
RBL	Rat basophilic leukemia
pNPP	para-Nitrophenylphosphate
ScFv	Single-chain variable fragment
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
T _H 2	T helper 2
VH	Variable region of the heavy chain in antibodies
VHH	Variable region of the heavy chain in heavy-chain antibodies
VL	Variable region of the light chain in antibodies

ABSTRACT

Type I allergy is an increasing world-wide health problem affecting almost 30% of the population. Especially in the northern hemisphere, birch pollen is a prevalent allergen source that triggers allergic symptoms such as hayfever and asthma in over 100 million patients, resulting in a decreased quality of life.

So far, two types of medication are on the market: symptomatic treatment (e.g. antihistamines) and allergen-specific immunotherapy (AIT). While the effect of antihistamines is temporary, AIT is the only treatment so far that offers a long-lasting protection by inducing blocking IgG antibodies with allergen extracts or, more recently, hypoallergenic derivatives. However, AIT is associated with time-consuming schedules over several years and if based on application of allergen extracts with side effect, e.g. possible allergic reactions to the given allergens.

Besides symptomatic medication and AIT a third option, passive immunization, has raised the interest of researchers. Recent studies have shown that administration of allergen-specific monoclonal IgG antibodies results in inhibition of IgE antibodies binding to the respective allergen, further in prevention of IgE-mediated effects on various immune cells and thus reduces allergic symptoms in patients. However, the generation and production of monoclonal IgG antibodies is laborious and expensive. A welcome alternative was found in nanobodies that derive from heavy-chain antibodies discovered in camelids such as camels and llamas. Those single-domain antibody fragments are chemically stable, easy to produce and recognize a variety of different epitopes due to their structural features while exhibiting high affinity and specificity.

The aim of this thesis was to characterize nanobodies specific for Bet v 1, the major birch pollen allergen, and evaluate if they are applicable for passive treatment of allergy. Nanobodies were raised in camels and expressed in *E. coli*. Specificity, cross-reactivity and epitope location were investigated by ELISAs. Affinity measurements were conducted using surface plasmon resonance (SPR). Finally, the ability to block polyclonal IgE from patients' serum and inhibit basophil activation was assessed by inhibition ELISAs and RBL cell assays.

We isolated three high-affine Bet v 1-specific nanobodies (Nb23, Nb24 and Nb32) that also recognize two important cross-reactive allergens, Aln g 1 from alder and Cor

a 1 from hazel. The binding site on Bet v 1 was mapped to the alpha helix on the C-terminus. Our results showed that the nanobodies were able to inhibit polyclonal IgE binding to Bet v 1, Aln g 1 and Cor a 1. Furthermore, Nb32, as a representative for all three nanobodies, reduced Bet v 1-induced activation of basophils.

In conclusion, the combined features of high-affinity, cross-reactivity and ability to inhibit IgE binding to Bet v 1 qualified Nb32 a potential candidate for a prospective passive treatment of allergy. We suggest Nb32 as one component for a nanobody cocktail that should be evaluated in a proof of concept testing in experimental animal models for efficacy to identify promising nanobody-based drugs to reduce birch pollen related allergic symptoms.

1. INTRODUCTION

1.1. Type I allergy

Allergy is a hypersensitivity disease which affects almost 30% of people worldwide (reviewed in Valenta et al. 2018). It is based on the reaction of the immune system to foreign proteins, called allergens. Normally, a response from the adaptive immune system is developed to effectively protect from these proteins, involving antibodies (mainly immunoglobulin (Ig) subclass IgG), B lymphocytes (i.e. B cells) and T lymphocytes (i.e. T cells). However, in patients suffering from type I allergy, a different Ig subclass, namely IgE, encounters the allergen and triggers a series of IgE-mediated symptoms which affect the respiratory tract, skin, cardiovascular system, and gastrointestinal tract. Therefore, the disease is also called IgE-mediated hypersensitivity (Abbas et al. 2005). Allergy represents an increasing health problem worldwide and is expected to affect 4 billion people in the 2050s (reviewed in Palomares et al. 2017).

The term “allergy” was coined by Clemens von Pirquet, a Viennese pediatrician, in 1906 to describe a ‘specifically altered reactivity of the organism’ when he investigated serum sickness in children (von Pirquet 1906). In 1921, pivotal experiments from Prausnitz and Küstner showed that it was possible to passively sensitize skin by serum transfer, concluding that a specific factor in the blood must be responsible for the allergic reaction (Prausnitz and Küstner 1921). Almost 50 years later this factor was identified and characterized as IgE (Ishizaka and Ishizaka 1967; Johansson et al. 1968; Bennich et al. 1968). To elicit an allergic response, Prausnitz and Küstner also postulated that a specific antigen (i.e. allergen) and a tissue component (i.e. mast cells) is necessary (Prausnitz and Küstner 1921).

1.2. Mechanism of type I allergy

The mechanism of type I allergy consists of two phases. First, an individual experiences a so-called sensitization, i.e., first exposure to a certain allergen. Second, a repeated exposure to the allergen leads to the IgE-mediated degranulation of effector cells, causing the typical symptoms of an allergic reaction, e.g., allergic rhinoconjunctivitis (i.e. hayfever) and asthma (reviewed in Shamji and Durham 2017).

1.2.1. Sensitization

The first step in developing an allergy is the sensitization of genetically predisposed (i.e. atopic) individuals by initial allergen confrontation, leading to an increased production of allergen-specific IgE antibodies (Figure 1). The exposure site for airborne allergens is the mucosa in the respiratory tract and eyes, while food allergens are absorbed in the gut (McGill et al. 1998; reviewed in Deckers et al. 2017; reviewed in Golebski et al. 2013; reviewed in Berin and Sampson 2013). Allergens entering the body are captured by antigen-presenting cells (APCs), e.g., dendritic cells (DCs) in the epithelium, which migrate to draining lymph nodes and process the allergen into small peptides. In the lymph nodes those peptides are presented to residing naïve CD4⁺ T cells via the class II major histocompatibility complex (MHCII) (Abbas et al. 2005). Three important signals are required for T cell activation and differentiation (reviewed in Deiftl and Bohle 2011). First, selected T helper (T_H) cells are binding with specific T cell receptors to the presented peptides. Second, costimulatory molecules on the surface of DCs (e.g., CD28 and B7-1/B7-2) ensure full T cell activation and proliferation. The third signal is provided by a certain cytokine milieu, which determines which subset naïve T cells are differentiating to e.g., T_H1 cells and T_H2 cells. T_H2 cells produce high amounts of the cytokines interleukin (IL)-4 and IL-13, while T_H1 cells synthesize INF- γ , an IL-4 antagonist. In atopic individuals the balance between T_H1 and T_H2 cells is inadequate due to the secretion of predominantly IL-4 (but also IL-5, IL-6, IL-10 and IL-11) during T cell priming, which drives T cells to differentiate to the T_H2 subset (reviewed in Deiftl and Bohle 2011). T_H2 cells then activate naïve allergen-specific B cells. The release of cytokines IL-4 and IL-13 by T_H2 cells promotes a heavy chain isotype switch in B cells towards IgE. IgE⁺ B cells develop into two subsets: Plasma cells and memory B cells. Plasma

cells produce high amounts of soluble IgE antibodies which are secreted in the blood and then bind to their high-affinity receptor FcεRI expressed on mast cells, basophils and eosinophils, where they operate as specific allergen receptors. Furthermore, IgE up-regulates the expression of FcεRI leading to an amplification of IgE-mediated reactions (Abbas et al. 2005). IgE⁺ memory B cells are immediately activated upon subsequent allergen encounter, which leads to a boost in allergen-specific IgE production (reviewed in Valenta et al. 2002, reviewed in Eckl-Dorna et al. 2019a). IgE⁺ memory B cells have been found in the blood of allergic patients, but also locally at allergen exposure sites such as the nasal and gut mucosa, as well as in lymphoid organs such as tonsils. However, the number of IgE⁺ B cells is scarce, and it is important to note that it is difficult to obtain certain tissue samples from patients such as bone marrow or spleen, as well as to develop sensitive methods to identify IgE producing cells in those samples. Therefore, IgE memory cells may also reside in sites that are not identified yet (reviewed in Eckl-Dorna et al. 2019a).

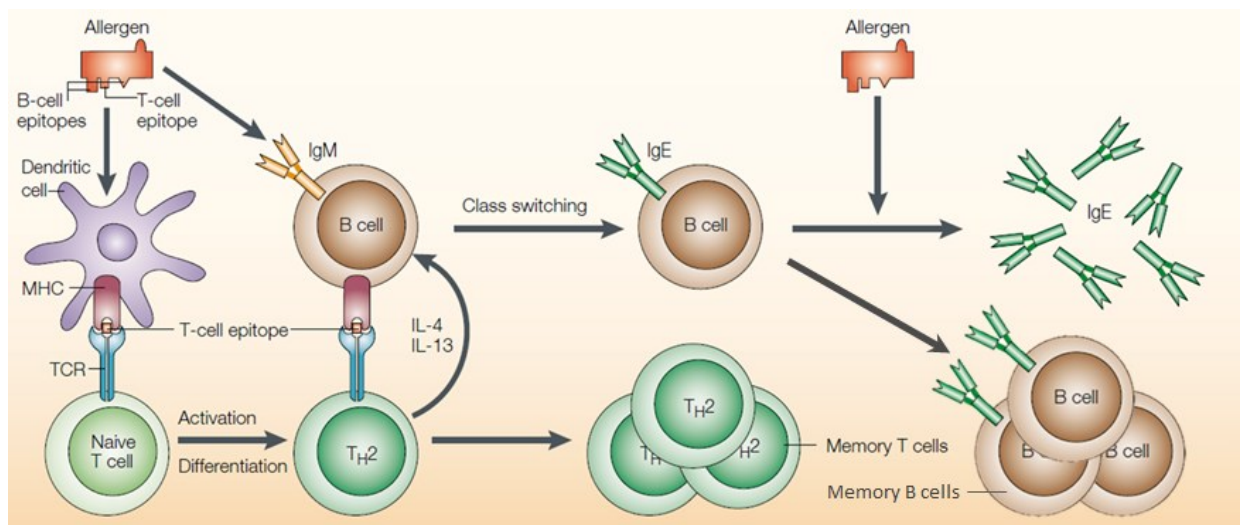


Figure 1 | The first step in developing an allergy: Sensitization and memory. (adapted from Valenta, Nat Rev Immunol 2002; 2:446-453)

1.2.2. Immediate-phase reaction

Sensitized effector cells, e.g., mast cells and basophils, are loaded with allergen-specific IgE on their surface. Thus, a repeated allergen exposure leads to an activation of effector cells by cross-linking FcεRI receptors (Figure 2). More precisely, two or more allergen-specific, surface-bound IgE antibodies must bind the same allergen molecule to achieve IgE-mediated activation (Abbas et al. 2005). Consequently, effector cells release inflammatory mediators like histamines and leukotrienes which trigger typical allergic symptoms like rhinitis and conjunctivitis for airborne allergy (reviewed in Valenta et al. 2002) or nausea and diarrhea in the case of food allergy (reviewed in Tordesillas et al. 2017) within minutes after allergen penetration.

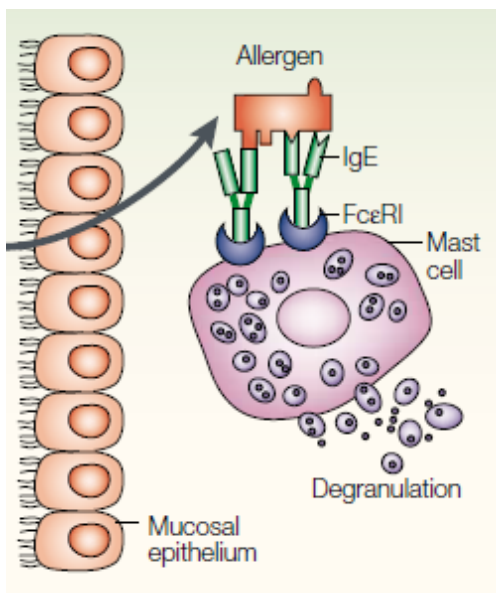


Figure 2 | Crosslinking of IgE- FcεRI complexes through allergens leads to degranulation. (from Valenta, Nat Rev Immunol 2002; 2:446-453)

1.2.3. Late phase reaction

The late phase reaction takes place several hours after encountering an allergen and is mainly driven by newly synthesized cytokines after mast cell activation. This leads to the recruitment of other immune cell such as eosinophils, neutrophils and T cells which infiltrate the inflamed tissue and secrete more inflammatory mediators. The reaction usually follows a clinically evident immediate phase and takes up to 2 days to fully resolve (reviewed in Galli et al. 2008).

1.2.4. Effector cells in type I allergy

The major effector cells in an immediate reaction are mast cells and basophils, while eosinophils are involved in the late-phase allergic reaction. They have in common that they express the high-affinity receptor Fc ϵ RI to capture IgE and they contain inflammatory mediators in cytoplasmic granules.

Mast cells derive from progenitors in the bone marrow and mature after relocation in peripheral tissue. They can be found in skin and mucosal epithelia, as well as around blood vessels and nerves (Abbas et al. 2005). Upon activation through cross-linking of IgE bound to Fc ϵ RI on their surface, they secrete preformed molecules such as biogenic amines (e.g. histamine) and granule macromolecules (e.g. tryptase) as well as newly synthesized lipid mediators (e.g. prostaglandin D₂ (PGD₂) and leukotrienes) and cytokines. Histamine and PGD₂ are vasoactive and increase vascular permeability resulting in swelling and redness of the tissue. Also, contraction of smooth muscle tissue (e.g. intestinal and bronchial) is induced, which results in bronchial spasms during an allergen-induced asthma attack. Secreted enzymes like tryptase cause tissue damage, while synthesized cytokines like IL-4, IL-5, IL-6 and IL-13 influence the proliferation of mast cells, differentiation of T cells to T_H2 cells and the class switch in B cells to IgE, enhancing and maintaining allergic responses (reviewed in Brown et al. 2008).

In contrast to mast cells, basophils already mature in the bone marrow and then circulate in the peripheral blood. Like mast cells, they contain IgE receptors and thus can be activated and subsequently release mediators (Abbas et al. 2005). Although they only make 1% of blood leukocytes, a small number infiltrating the exposure site is sufficient to act as initiators of inflammation, recruiting other inflammatory cells and releasing cytokines, mainly IL-4. Therefore, they also play a crucial role in allergic reactions (reviewed in Miyake and Karasuyama 2017).

Eosinophils mature in the bone marrow like basophils and circulate in the blood until they are recruited to inflamed tissue by certain cytokines and adhesion molecule interaction during the late phase reaction. Upon activation they secrete preformed acidic proteins which may damage the surrounding tissue. Furthermore, eosinophils release a range of different cytokines and lipid mediators, however the importance of

these mediators in allergic diseases is not completely understood yet (Abbas et al. 2005).

As already mentioned, to efficiently capture IgE, effector cells are equipped with a high affinity receptor. This receptor, FcεRI, consists of one α chain which is responsible for binding ligands, and one β and two γ chains, which are part of the signaling pathway. IgE binds strongly to the extracellular Ig-like domains with a dissociation constant (K_D) of approximately 1×10^{-10} M. The β and γ chains each contain one immunoreceptor tyrosine-based activation motif (ITAM) on the cytoplasmic side, which upon cross-linking is phosphorylated and initiates the signaling cascade to activate effector cells (Abbas et al. 2005).

1.3. Allergens

Allergens are usually environmental proteins that are *per se* harmless to humans but can induce hypersensitivity upon repeated exposure in atopic persons. The resulting symptoms from this disorder range from mild ones like rhinoconjunctivitis to severe ones such as asthma and anaphylactic shock. Typical allergen sources include plants (e.g. tree, grass and weed pollen), foods (e.g. vegetables, fruits), animals (e.g. cats, dogs, house dust mites), insects' venom (e.g. bees, wasps) and chemicals (e.g. penicillin).

While it is not completely understood why some proteins induce strong allergic reactions and others do not, several common features have been found among characterized allergens. Those include a low molecular weight and high solubility in body fluids (Abbas et al. 2005). Additionally, it has been found that allergens are spread among few protein families and display only restricted biological functions. Those include enzymes (proteases), lipid- and calcium-binding proteins, pathogenesis-related (PR) proteins, trypomycins, profilins, and storage proteins (reviewed in Chapman et al. 2007; reviewed in Radauer et al. 2008; reviewed in Deifl and Bohle 2011; reviewed in Scheurer et al. 2015). The most important prerequisite of an allergen is to exhibit multiple binding sites (i.e. epitopes) to IgE to trigger degranulation of effector cells. An antigen that contains only one epitope is not able to crosslink receptor bound IgE antibodies, and thus cannot activate the mediator cascade leading to immediate allergic symptoms. The more epitopes an allergen

contains, the stronger is the induced response to it (reviewed in Valenta et al. 2018). Furthermore, flexibility provided by two domains connected by a linker increases its potency (Göbl et al. 2017; reviewed in Valenta et al. 2018). It is worth mentioning that also the proximity of epitopes influences the allergenic activity (Gieras et al. 2016).

Since my project deals with nanobodies directed against pollen allergens and primarily against the major pollen allergen Bet v 1, I will focus on this allergen in the further course of this work.

1.3.1. Bet v 1 – the major birch pollen allergen

Pollen allergy is the most common allergy worldwide. In fact, it accounts for approximately 40% of all allergic patients (reviewed in Asam et al. 2015). It is associated with hay fever, but also asthma, which significantly decreases the life quality of people affected. (Kiotseridis et al. 2013).

One of the major sources for pollen is the European white birch (*Betula verrucosa*). It belongs to the tree family Fagales and is widely distributed in the northern hemisphere, particularly in Central and Northern Europe (Biedermann et al. 2019). Bet v 1 was characterized as the only major birch pollen allergen and elicits an allergic reaction in 95% of patients sensitized to birch (Jarolim et al. 1989; Niederberger et al. 1998). It is a 17 kDa protein belonging to the PR-10 protein family (WHO/IUIS Allergen Nomenclature Sub-Committee), which allows the proposal that it is involved in pathogen resistance of pollen (Breiteneder et al. 1989).

Bet v 1 displays a high homology to other allergens of trees of the Fagales order like alder and hazel, but also to allergens of fruits and vegetable families, mainly Rosaceae (e.g. apple) and Apiacea (e.g. celery, carrot) resulting in cross-reactivity. Patients sensitized against Bet v 1 also experience allergic symptoms when challenged with the mentioned allergen sources (reviewed in Bohle 2007; reviewed in Popescu 2015). Pollen-related food allergy which displays mostly mild symptoms such as swelling and itching of lips, tongue, and throat, but rarely also life-threatening anaphylaxis, is termed Oral Allergy Syndrome (OAS) (reviewed in Alessandri et al. 2020).

The cross-reactivity of related allergens leads back to similar IgE epitopes, which are recognized by IgE specific for the primary allergen, i.e. the allergen a patient has been sensitized to. Pollen allergens have been shown to be the primary elicitor of food allergy in patients experiencing OAS (Kazemi-Shirazi et al. 2000). In this regard, Bet v 1 is considered as one of the most important sensitizing agents, as over 70% of birch pollen-allergic patients develop allergic symptoms to Bet v 1-related food allergens (reviewed in Bohle 2007).

1.4. Therapeutic approaches for type I allergy

1.4.1. Symptomatic treatment

The first line approach for type I allergy is treating patients with antihistamines. Histamine is a key mediator in hypersensitivity disease and therefore an attractive target to stop the immediate reaction after allergen exposure (reviewed in Rotiroti et al. 2016). Histamine can bind to four receptors, termed H1-4. The most important one in regard to allergy is H1, which is present on epithelial cells, smooth muscle cells, vascular endothelial cells and sensory nerves. It has been found to be overexpressed in the nasal mucosa of patients suffering from allergic rhinitis. Thus, most antihistamines are H1-antihistamines which act as inverse agonists and keep the H1 receptor in an inactive state upon binding (reviewed in Rotiroti et al. 2016). Although they are quite successful in relieving symptoms of mild to modest allergic rhinitis and conjunctivitis, they are less effective in treating severe asthma and are only used as supplementary drugs besides leukotriene receptor antagonists (reviewed in Yamauchi and Ogasawara 2019). Furthermore, they were long associated with side effects like drowsiness and their effective period only lasts for several hours. Therefore, newer antihistamines (second and third generation) have been developed to avoid those side effects (reviewed in Rotiroti et al. 2016; reviewed in Kawauchi et al. 2019).

For treating severe allergic asthma and also chronic urticaria, another medication is available for several years on the market (https://www.accessdata.fda.gov/drugsatfda_docs/label/2015/103976s5224lbl.pdf).

Omalizumab which is the only anti-IgE antibody approved by the U.S. Food and Drug

Administration (FDA), acts by binding to the C3 domain in the constant region of circulating IgE molecules and thus hindering IgE to bind to its receptors (FcεRI and FcεRII) (Presta et al. 1993; Shields et al. 1995). Simultaneously, it does not interact with already bound IgE to effector cells, which therefore does not result in cross-linking and mediator-release. Since Omalizumab binds to the constant region of IgE, it inhibits all IgE interaction regardless of allergen-specificity. By reducing free IgE, the expression of FcεRI on effector cells is downregulated resulting in mitigation of severe symptoms (Holgate et al. 2009). Currently it is also investigated whether Omalizumab can be used for other forms of IgE-associated conditions such as food allergy, in combination with immunotherapy (reviewed in Lieberman and Chehade 2013; Langlois et al. 2020).

For the sake of completeness, it should be mentioned that beside Omalizumab, the most prominent monoclonal antibody that is applied to reduce heavy allergic symptoms, also several other monoclonal antibodies are currently used for treatment of severe asthma. Amongst them are Benralizumab, Dupilumab, Mepolizumab and Reslizumab which are all directed either against IL-4 receptor/IL-5 receptor or directly against IL-5 with the aim to prevent asthma exacerbations (reviewed in Agache et al. 2020).

1.4.2. Allergen-specific immunotherapy

So far, allergen-specific immunotherapy (AIT) remains the only disease-modifying therapy available for allergic patients and is based on the administration of the corresponding allergen in form of extracts (natural allergen) or modified hypoallergenic derivatives as well as allergen fragments (recombinant allergen) (reviewed in Zhernov et al. 2019). Throughout the course of the treatment, which usually lasts two to three years, patients are building up a tolerance against the disease-eliciting allergen by the induction of allergen-blocking IgG antibodies and an altered response of cells involved in the immunological reaction, which has a long-lasting effect even after discontinuation of the therapy (reviewed in Dorofeeva et al. 2021).

The first study that showed the effect of AIT was conducted by Leonard Noon in 1911 (Noon 1911). He demonstrated that symptoms of patients allergic to grass pollen

were eased after injection of grass pollen extract. At this time point, neither the mechanism of allergy nor of the induced immunity was understood. It was believed that 'toxins' contained in pollen were responsible for the exaggerated reactions and that 'anti-pollen toxins' were formed during the treatment. Since then, a lot of progress has been made towards understanding the underlying mechanism of the treatment, improving efficacy and reducing side effects (reviewed in Dorofeeva et al. 2021).

First, it is well established that during AIT IgG antibodies are induced, which compete for binding to the allergen (reviewed in Valenta 2002; reviewed in Shamji and Durham 2017). The most abundant subtypes that are formed are IgG1 and IgG4 (Jutel et al. 2005; Purohit et al. 2008; Eckl-Dorna et al. 2019b). Especially IgG4 is thought to play a major role in inhibiting binding of IgE to the allergen and hence preventing the release of mediators from effector cells, IgE-facilitated allergen presentation to T cells and IgE memory cell stimulation (reviewed in Wachholz and Durham 2004; James and Till 2016; Groh et al. 2017). Not only IgG formation, but also an altered cell reaction has a positive impact on the outcome of AIT. These exact mechanisms are, however, not fully understood yet. Nevertheless, it has been found that T cell differentiation is modulated by a distinct cytokine expression pattern. More precisely, the secretion of cytokines IL-12, IL-27 and IL-10 by tolerogenic regulatory dendritic cells (DCreg) drives the differentiation of T cells to T_H1 cells which, in contrast to T_H2 cells, produce interferon- γ (INF- γ) and IL-10 that promotes Ig class switching to IgG in allergen-specific B cells. Moreover, IL-10 induces maturation of regulatory T (Treg) and B (Breg) cells, which themselves produce more IL-10 to suppress T_H2 proliferation and IgE+ B cell activity. Additionally, a decrease in IL-4, IL-5 and IL-13 has been observed explained by the lower number of T_H2 cells (reviewed in Shamji and Durham 2017). Another positive outcome that has been discovered is that the numbers of effector cells involved in allergic reactions like mast cells, basophils and eosinophils are reduced (reviewed in Larché et al. 2006).

Second, efficacy and safety of AIT have been improved over the decades by using recombinant allergens and even short allergen fragments and allergen-derived peptides over allergen extracts. Extracts often vary in their composition, lacking important allergens and thereby inducing an incomplete immunity (reviewed in Zhernov et al. 2019). They can also be contaminated and may cause unwanted

sensitization (Dzoro et al. 2018). Purified recombinant allergens bypass these problems and induce similar immunity responses in patients (Pauli et al. 2008). Recombinant allergens have also been used in combination to improve efficacy. For example, in a double-blind, placebo-controlled study a vaccine for timothy grass pollen allergy compiled of five allergens had been very successful (Jutel et al. 2005). Since recombinant allergens may still elicit an unwanted allergic reaction by binding to IgE which might even result in anaphylactic shock, they have been modified by mutation or fragmentation to alter the structure and termed (recombinant) hypoallergens (Niederberger et al. 2004; Chen et al. 2012; Swoboda et al. 2013). Such fragments do not show IgE reactivity (mostly because they are not properly folded for IgE to recognize conformational epitopes) but contain T cell epitopes to induce blocking IgG antibodies (Vrtala et al. 1997; Focke et al. 2001; Focke et al. 2004, Twaroch et al. 2011; Focke-Tejkl et al. 2015). Interestingly, a study from Pree et al. showed that unfolded Bet v 1 fragments as well as a folded recombinant Bet v 1-trimer both lead to the formation of IgG1 and IgG4 against new sequential epitopes, which overlap with conformational IgE epitopes (Pree et al. 2007). In a following study, a Bet v 1-specific ScFv isolated from the blood of a non-allergic subject that was vaccinated with these fragments, showed the potential to inhibit Bet v 1-allergic patients' IgE binding to Bet v 1 and partially suppressed allergen-mediated basophil activation (Gadermaier et al. 2018). These findings underline the importance of epitope specificity of IgG antibodies, formed during AIT with hypoallergens, for a protective outcome.

The latest development in AIT are peptide carrier-based vaccines which contain B cell epitopes to bypass unwanted late phase reactions when using vaccines that contain T cell epitopes (reviewed in Curin et al. 2018). This concept uses viral carrier proteins that are already used in other types of vaccines, such as the hepatitis surface antigen PreS. Not only lack the non-folded allergen-derived peptides IgE-reactivity, but also reduce allergen-specific T cell reactions. Furthermore, the carrier protein is highly immunogenic which helps to raise an appropriate immunological response to less immunogenic allergen-derived peptides, while still targeting the IgE binding site (reviewed in Curin et al. 2018).

Overall, AIT has advanced much over the years and its unique disease-modifying effect is well documented. It can prevent the progression from mild to severe

symptoms (e.g. in asthma) and hence improve the situation of patients. Furthermore, AIT is envisioned to serve as a prophylactic vaccination to stop the worldwide increase of allergic patients (reviewed in Tulaeva et al. 2020). Still, there are a few drawbacks remaining, including time-consuming schedules for patients and possible side-effects which have to be monitored (reviewed in Zhernov et al. 2019).

1.4.3. Passive immunization by administration of allergen-specific blocking antibodies

Another method to alleviate symptoms is transferring blocking antibodies from an immunized individual to achieve passive immunization in non-treated patients. These allergen-specific blocking antibodies were initially discovered by Robert Cooke in 1935 during a study investigating the mechanism of AIT. More precisely, he found that an inhibiting substance was formed in the course of AIT, which prevents the activation of effector cells, and that this substance was transferable to other sensitized patients (Cooke et al. 1935). In 1940 Mary Hewitt Loveless could show that the inhibiting antibodies bind to the same allergen, thus protecting by competing with IgE (Loveless 1940). The next important step was the identification of the blocking substance as IgG, which was published by Lawrence M. Lichtenstein (Lichtenstein et al. 1968).

Nowadays it is well established that the induction of allergen-specific IgG antibodies during AIT is largely contributing to its disease modifying effect (reviewed in Flicker et al. 2003). Through modern biotechnological methods (combinatorial libraries, phage display, hybridoma technique, EBV transfection, recombinant protein expression) it is possible to isolate such induced blocking IgGs and produce them as monoclonal antibodies in larger quantities to assess their usefulness in allergy therapy. Since the half-life of IgGs is approximately 35 days, a single injection of allergen-specific IgG antibodies is envisioned as useful and convenient alternative treatment for seasonal allergies (e.g. pollen allergy) (reviewed in Flicker et al. 2011).

Blocking antibodies act at least in three modes (Figure 3). First, they can inhibit degranulation of effector cells by competing with IgE for the same epitope (Figure 3a). Second, they may also prevent IgE facilitated allergen-presentation to T cells by hindering binding of the allergen to IgE, thus decrease T cell activation and

proliferation (Figure 3b). Last, blocking antibodies may be able to capture allergens before they can induce IgE production in memory B cells (Figure 3c) (Flicker et al. 2011).

Looking at the listed mechanism it seems obvious that epitope specificity is probably the most important feature of a blocking antibody. If it does not occupy an IgE epitope (fully or partial) it cannot prevent the allergen from binding to IgE (Flicker et al. 2011). However, it has been demonstrated that binding to a different epitope than IgE, allergen-specific IgG can still prevent crosslinking of two FcεR1s and instead lead to crosslinking of FcεR1 and FcγRIIB (a receptor for IgG), which inhibits degranulation in mouse and human basophils (Daëron et al. 1995; Cassard et al. 2012).

Besides epitope specificity there are several other features which need to be fulfilled. A high affinity (i.e. strength of binding), preferably in the nanomolar range, is required for a blocking antibody to be able to compete with IgE. Affinity has also an influence on the half-life of antibodies, which affects dosing and frequency of administration. It is desired to use a low dose and frequency for the highest effect, which is achieved when the antibody displays high affinity (reviewed in Flicker et al. 2011). Furthermore, cross-reactivity to related allergens is an advantage, as many patients do not only react to the sensitizing allergen but also homologous ones (reviewed in Flicker et al. 2011).

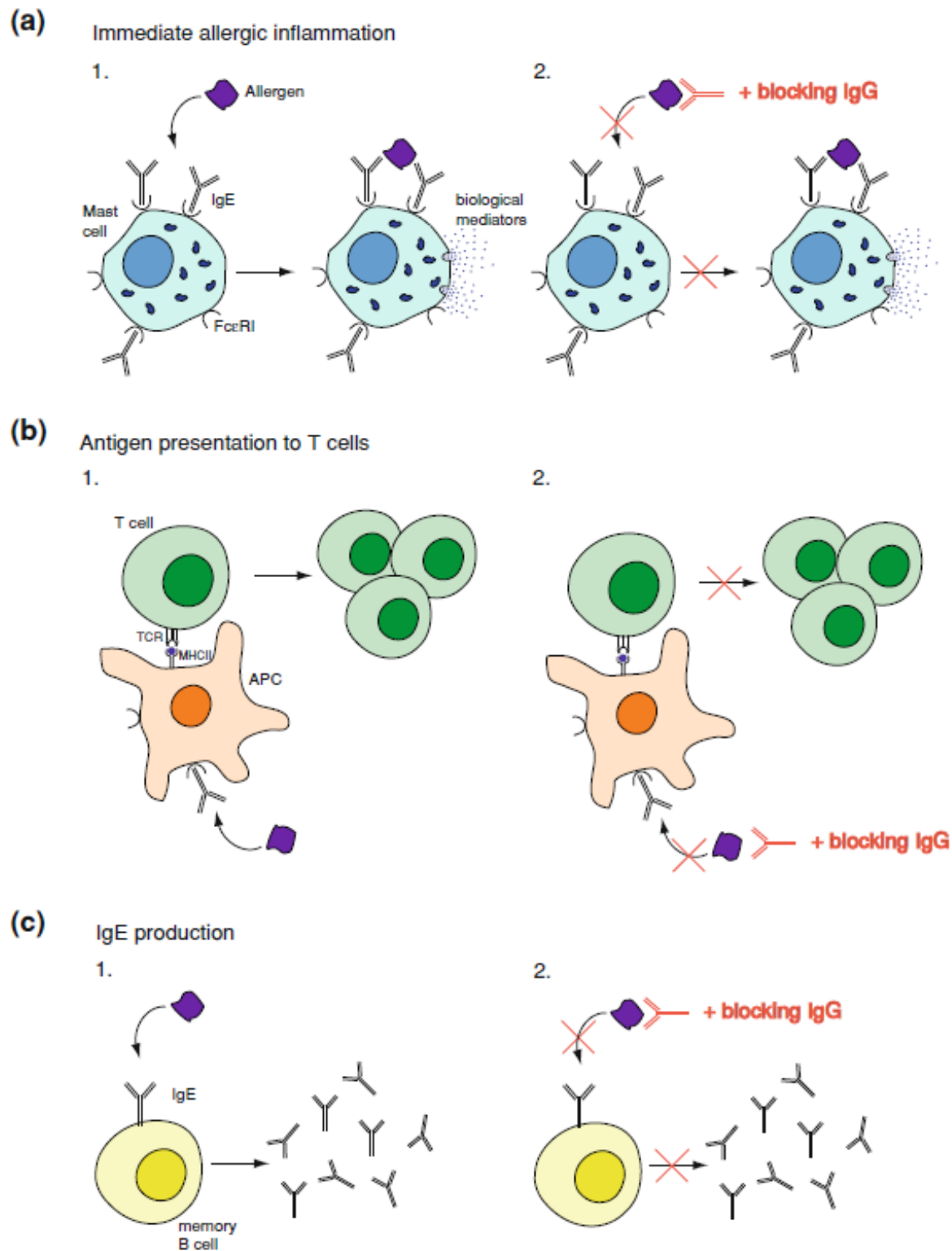


Figure 3 | Possible mechanisms with which blocking antibodies can decrease allergic symptoms upon allergen encounter. (from Flicker et al. Curr Top Microbiol Immunol 2011;352:141–159).

All these prerequisites have made it hard to find a suitable candidate which may be of use in a passive therapeutic approach. For example, Visco et al. isolated five Bet v 1-specific IgG antibodies from a patient receiving AIT, but only one (titled Bab 1) was able to inhibit binding of IgE to Bet v 1, while three had no influence on the binding and one IgG antibody even enhanced patients' IgE binding to Bet v 1 (Visco et al. 1996), which is clearly not desired for a successful therapy. So far only one group managed to generate two IgG antibodies (REGN1908 and REGN1909) which in combination block binding of IgE to the cat allergen Fel d 1 in mouse models and cat-allergic patients (Orengo et al. 2018). These recombinant antibodies bind two separate epitopes, have affinities in the subnanomolar range, and only a single subcutaneous injection was necessary to significantly reduce allergic symptoms over a period of three months (Durham et al. 2017; Orengo et al. 2018). Thus, they are fulfilling the requirements mentioned above. Recently, the therapeutics successfully finished the phase II clinical study (<https://clinicaltrials.gov/ct2/show/NCT03838731>).

Since generating and producing full monoclonal antibodies in sufficient amounts is cumbersome and expensive, researchers are also investigating whether smaller antibody fragments (antigen-binding fragment, Fab; single-chain variable fragment, ScFv; nanobody, Nbs), which are easier to produce, may display similar blocking abilities. Several allergen-specific Fabs and ScFvs have been isolated but only few could inhibit IgE binding (de Lalla et al. 1996; Flicker et al. 2002; Jakobsen et al. 2004; Jylhä et al. 2009; Padavattan et al. 2009; Gadermaier et al. 2014; Gadermaier et al. 2018). Nanobodies, however, have not been investigated so far in regards of an allergy treatment.

1.5. Antibodies

Throughout evolution our body has developed a complex immune system to fight of any kind of intruders. Antibodies play a major role in our defense system. They are highly specified for their cognate antigen and can recognize foreign structures, such as bacteria, virus particles, fungi or toxic molecules, and lead to their destruction.

1.5.1. Structure of antibodies

Antibodies, or immunoglobulins (Igs), are highly conserved among mammals. An Ig molecule is composed of two identical heavy (H) and two identical light (L) chains, which are about 55 to 70 kDa and 24 kDa, respectively (Figure 4A). Each L chain is covalently attached to an H chain by a disulfide bond, while the two H chains are also connected to each other via a disulfide bond. H and L chains can further be divided into a constant (C) and variable (V) region. Based on the constant region, Igs are separated into five classes or isotypes: IgA, IgD, IgE, IgG and IgM. The constant region is the same in the respective Ig class and is important for distinct effector functions for example recruiting other cells of the immune system (e.g. phagocytes, natural killer cells) by binding to their cell receptors. The (N-terminal) variable region determines the specificity of an antibody. The antibody repertoire, i.e. the total collection of antibodies with different specificities, of an individual can recognize up to 10^9 different structures. This high variability is achieved by somatic recombination, somatic hypermutation and combination of the variable H and L chain domains during the maturation of B cells, which are the sole cell type that produce antibodies (Abbas et al. 2005).

The diverse variable region contains three hypervariable regions, called complementarity-determining regions (CDRs). Three CDRs from the H chain and three CDRs from the L chain form the paratope, with which antibodies primarily bind to their antigen. The CDRs each contains around 10 amino acids in the form of loops that together form a three-dimensional surface, which, as the name suggests, is complementary to the binding site on the antigen (Figure 4B). The antibody-antigen affinity is dependent on the complimentary fit between paratope and epitope and involves several non-covalent bonds such as van-der-Waals force, ionic forces, hydrogen bonds and apolar bonding (Cruse and Lewis 1999).

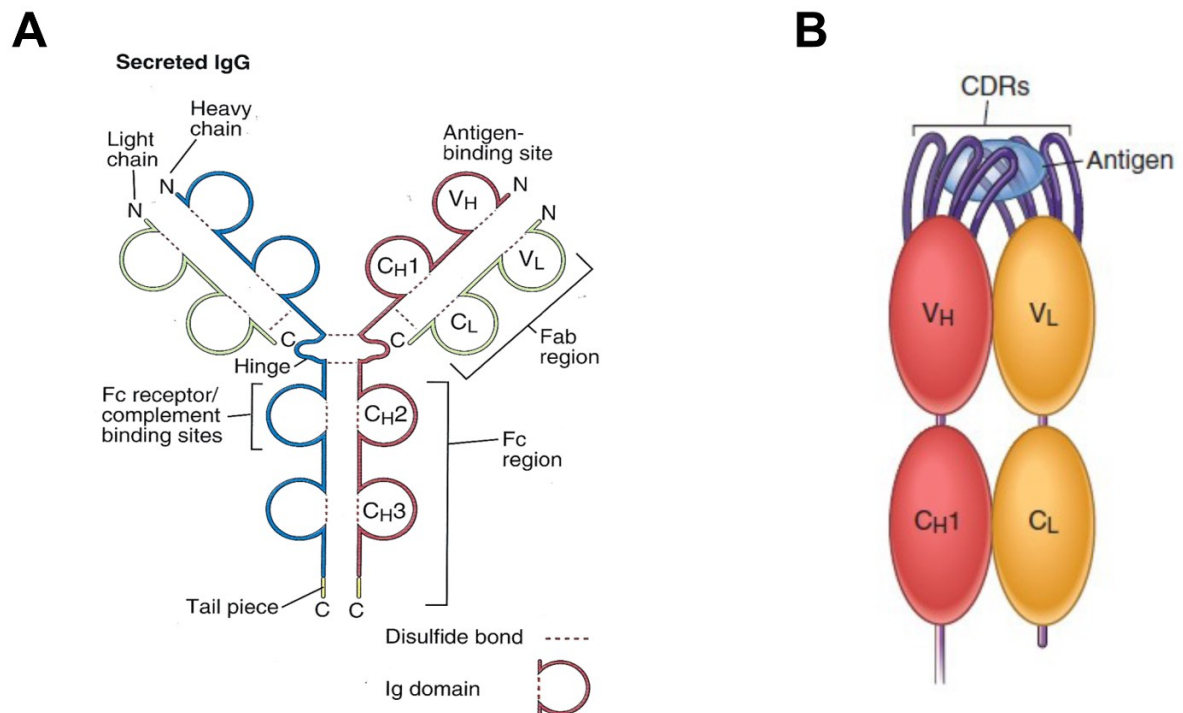


Figure 4 | A. Structural composition of IgG. Heavy chains (blue and red) and light chains (light green) are subdivided into Ig domains, with the N-terminal domain of each heavy and light chain forming the paratope (antigen-binding site). **B. Schematic view of the antigen binding site.** 3 CDRs from the V_H and V_L each bind the antigen. (adapted from Abbas et al. Cellular and molecular immunology 2005; 5. ed., Elsevier Saunders)

1.5.2. Antibody derivatives

Monoclonal antibodies i.e., antibodies produced from a cell line that derive from one single B cell clone, are exploited in numerous analytical tasks such as detection and quantification of proteins (Shire 2015), but also as therapeutics for diseases like cancer, viral infections, or asthma (reviewed in Berger et al. 2002; reviewed in Posner et al. 2019; reviewed in Agache et al. 2020). However, the production is often time-consuming and cumbersome. Furthermore, antibodies are large molecules (150kDa) and not suitable in some cases, e.g., when the penetration of tissue is favored (Nelson et al. 2010), thus smaller versions are preferable.

So far, three antibody fragments decreasing in size have emerged (Figure 5). The first derivate developed were antibody binding fragments (Fabs). They consist of the variable domain and the first constant domain of both heavy and light chain. The chains are only connected via a disulfide bond. Next, a smaller variant was generated

by getting rid of the constant region and connecting the two remaining variable regions (VH and VL) with a peptide linker. This form is called single-chain variable fragment (ScFv). While Fabs can be generated by enzymatic digestion of monoclonal antibodies, ScFvs are purely recombinant molecules that are expressed as one chain and thus considered easy to produce in *E. coli* or other expression systems (Zhao et al. 2009; Ahmad et al. 2012). The third group worth mentioning are nanobodies that derive from heavy-chain antibodies and are made up of one single, variable domain. Nanobodies will be discussed in detail in the next chapter.

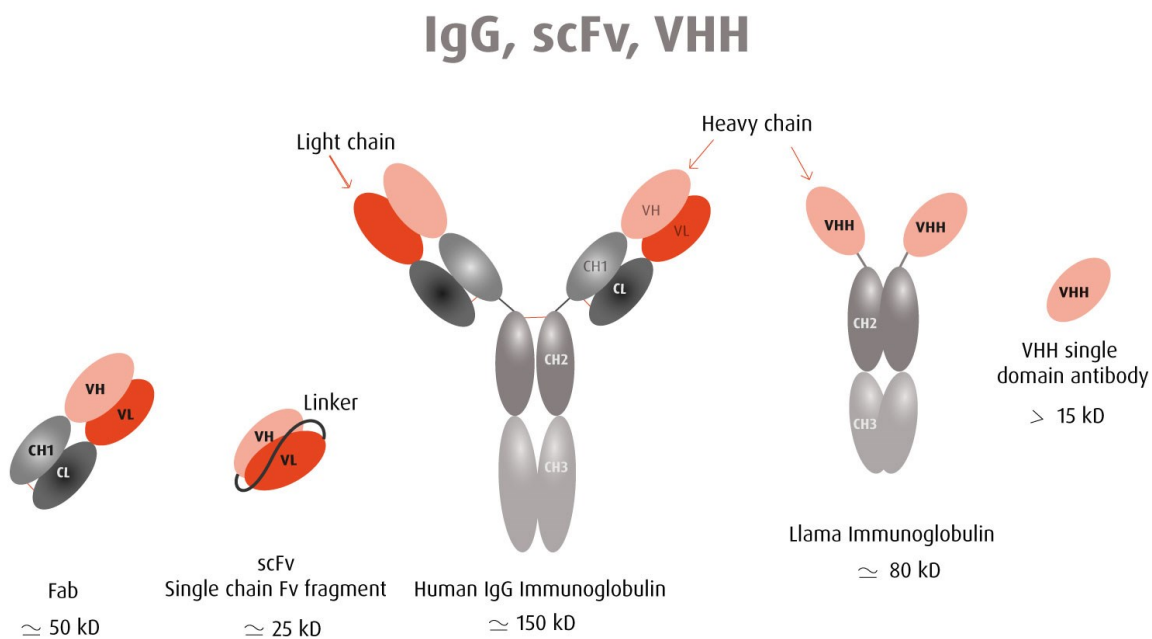


Figure 5 | Overview of antibodies and their derivatives. (from Hybribody: VHH Nanobody Properties, available at <https://www.hybribody.com/contenu/synthetic-vhh-library-menu/take-advantage-of-vhh-antibody-properties>, accessed 21.12.2020)

1.6. Nanobodies – small but powerful antibody fragments

1.6.1. History

In 1993, a new antibody class was reported that differs distinctly from the conventional model. When isolating the IgG fraction from camels' blood, the authors found that beside the familiar IgG1 subclass comprising of two heavy and two light chains two other subclasses (IgG2 and IgG3) exist, which are devoid of light chains and were therefore named heavy chain antibodies (HCAbs). Additionally, the first constant domain of the heavy chain (CH1) is missing and partly substituted by an extended hinge between the variable and the constant region (Hamers-Casterman et al. 1993). HCAbs are present in all other camelids like llamas or alpacas and were also surprisingly found in certain sharks (Greenberg et al. 1995; reviewed in Nguyen et al. 2001; Nuttall et al. 2001). Before this discovery, HCAbs were only known to occur in the so called heavy chain disease reported in human patients, where due to mutations non-functional antibodies lacking the light chain and CH1 are formed (reviewed in Wahner-Roedler and Kyle 2005). In contrast, HCAbs in camelids and sharks are fully functional and have evolved besides conventional antibodies (reviewed in Conrath et al. 2003). Depending on the camelid family, they make up to 75% of the IgG fraction (reviewed in Arbabi-Ghahroudi 2017).

1.6.2. Properties and generation

The variable domain of the heavy chain (VHH) serves as the single antigen binding domain and is the equivalent to Fabs in conventional antibodies. The isolated, cloned and recombinantly expressed VHH is called single-domain antibody (sdAb) or nanobody (Nb) because its dimensions are in the nanometer range. VHHs consist of three hypervariable regions (H1, H2, H3) forming the CDR, and four more conserved framework regions (FR1, FR2, FR3, FR4) (reviewed in Muyldermans 2013). Therefore, it displays a similar composition like the conventional VH. However, comparative studies revealed a few crucial differences between these two antigen-binding fragments regarding amino acid sequences and structure. Four important amino acid substitutions (positions 37, 44, 45, 47; Kabat numbering) in framework 2 have been noticed and are considered hallmarks to distinguish between VH and

VHH: V37F/Y, G44E/Q, L45R/C and W47G/S/L/F (Desmyter et al. 1996; Vu et al. 1997; reviewed in Muyldermans et al. 2001) (Figure 6). In conventional antibodies these non-polar amino acids cover the VH-VL interface to allow hydrophobic van der Waals interaction, but in VHHs this part is solvent exposed. The substitutions render the surface more hydrophilic, which improves solubility and represents the adaption to the loss of an associated VL (Desmyter et al. 1996). Indeed, exchanging those amino acids in a mouse or human VH (termed 'camelized' VH) led to better stability and removed their reported 'sticky' behavior (Davies and Riechmann 1994; Riechmann 1996, reviewed in Muyldermans 2013).

Nanobodies are ten times smaller than conventional antibodies (approximately 15 kDa compared to 150 kDa) and are missing the high antigen binding repertoire resulting from heavy and light chain pairing. Conclusively, other strategies must have been evolved to assure high variability, as they are binding with similar affinities and comparable specificity as conventional antibodies (reviewed in Mitchell and Colwell 2017). One noticeable feature of nanobodies is an extended CDR3 (compared to conventional antibodies), which is thought to partially make up for the missing VL domain to ensure a large binding surface (Figure 6). Furthermore, CDR3 is relatively flexible due to its length and can therefore insert itself into cavities of antigens, and thus bind to epitopes which are inaccessible for the larger conventional antibodies (reviewed in Muyldermans 2013). Additionally, it seems that not only the CDRs but also part of the framework (especially FR2) is involved into antigen binding (reviewed in Arbabi-Ghahroudi 2017). Along with a higher structural flexibility in CDR1 and CDR2 (reviewed in Mitchell and Colwell 2017) and a larger number of unique VHH genes for combining (reviewed in Arbabi-Ghahroudi 2017), these features are considered the strategies by which nanobodies retain high specificity regardless of the size.

Moreover, nanobodies convince with high thermal and chemical stability while retaining antigen binding ability (van der Linden et al. 1999), and also remain functional for several months when being stored at 4°C or -20°C, making them very suitable for various biochemical, diagnostic and therapeutic applications (reviewed in Muyldermans 2013).

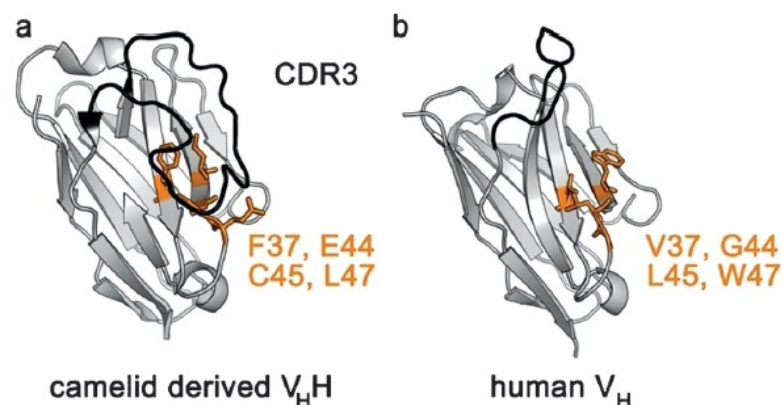


Figure 6 | Comparison of a camelid VHH and human VH. (from Schumacher et al. *Angew Chem Int Ed* 2018;57:2314 –2333)

The traditional method to generate nanobodies *in vivo* is to immunize a camelid several times with the respective antigen and, after a few weeks (e.g. six weeks for llamas), isolate B lymphocytes from the blood of the immunized animal. The mRNA coding for VHHs is extracted and amplified by PCR and subsequently reverse transcribed to DNA using RT-PCR. The DNA of VHHs is then cloned into a phage display vector using specific primers for the hinge region and the N-terminus to build an immune library. From this library, high affinity clones can be selected by 2-3 round of phage panning. The DNA representing high affinity clones can then be cloned into vectors suitable for different expression systems (e.g. *E. coli*, yeast) or modified to add tags for facilitated purification and/or detection purposes (e.g. His₆-tag, HA-tag) (Pardon et al. 2014).

More recently, other forms of libraries, such as naïve or (semi-) synthetic, have emerged to enable the production of nanobodies against antigens which are possibly toxic or incapable to raise a natural immune response (Monegal et al. 2009; Goldman et al. 2006; Moutel et al. 2016).

1.6.3. Applications and future visions

Due to the discussed properties nanobodies have emerged as useful tools in biomedical and biochemical fields. They represent meanwhile well accepted alternatives to conventional antibodies and have shown great potential for diagnosis as well as therapy (reviewed in Huang et al. 2010; reviewed in Bannas et al. 2017; reviewed in Jovčevska and Muyldermans 2020; reviewed in Sroga 2020).

Besides diagnostics, possible applications as therapeutics have been explored within the last fifteen years. An important point to consider regarding the development of drugs is safety. A crucial advantage of nanobodies is the ease of humanization to reduce immunogenicity. Due to a high similarity to the human VH, only a few amino acid substitutions are necessary are required to improve safety (reviewed in Vincke et al. 2009). Furthermore, nanobodies are well susceptible to engineering and can be modulated to fit their purpose. Consequently, bivalent, bispecific, or oligomeric constructs have been reported, as well as fusion to Fc domains to enable effector functions. The small size of nanobodies is considered a disadvantage as therapeutic because of the fast renal clearing, therefore strategies have evolved to elongate their half-life, for example the fusion to PEG or human serum albumin (HSA) or building larger constructs by oligomerization (reviewed in De Vlieger et al. 2018). The ease of manufacture of nanobodies in *e.g.*, *E. coli* resulting in low production costs also provides a positive aspect from an economical view in comparison to monoclonal antibodies (reviewed in Arbabi-Ghahroudi 2017).

The above mentioned and well-documented qualities make nanobodies attractive agents to be applied for a wide spectrum of human diseases. In general, nanobodies are highly researched in oncology. Different strategies are applied where nanobodies can function as receptors antagonists, target effector domains or target moieties of drug delivery systems (reviewed in Kijanka et al. 2015; reviewed in Jovčevska and Muyldermans 2020). For example, nanobodies binding to classical cancer receptors such as epidermal growth factor receptor (EGFR) or human epidermal growth factor (HER2) can inhibit signaling from their respective ligands, hence delay tumor growth (reviewed in Bannas et al. 2017). Another route to cancer therapy may be the use of nanobody-based immunotoxins, where nanobodies targeting a cancer marker protein (*e.g.* VEGFR2) are genetically fused with a toxin that is designed to kill cancer cells (reviewed in Bannas et al. 2017).

Furthermore, nanobodies are currently used in clinical trials for other diseases like rheumatoid arthritis, osteoarthritis and RSV-infection, as well as more have been developed against other viral infections like influenza and HIV (reviewed in De Vlieger et al. 2018; reviewed in Jovčevska and Muyldermans 2020; reviewed in Sroga et al. 2020). Recently, the first nanobody based drug, Caplacizumab, has been approved by the US Food and Drug Administration (FDA) and the European Medicinal Agency (EMA) for treating thrombotic thrombocytopenic purpura. Due to the mentioned advantages and characteristics, they have recently also been envisioned as a possible treatment for allergy (reviewed in Flicker et al. 2020).

2. PROLOGUE

Several treatment options are currently available for allergic patients, with antihistamines being the most common ones, while AIT promises the only causative treatment that modulates the immune response to allergens. However, time schedules for AIT can be inconvenient for patients and the therapy may elicit side effects, although much effort has been put into developing safer strategies over the past years. The passive administration of blocking antibodies seems to be a welcome alternative that is especially applicable for seasonal allergies such as pollen allergy. The first blocking antibodies with specificity for Bet v 1 are currently evaluated in phase I clinical trials (www.regeneron.com/pipeline) while Fel d 1-specific antibodies have shown promising results in phase II clinical trials by reducing allergic symptoms (Orengo et al. 2018). Since nanobodies can be described as the smaller variant of antibodies with high stability and low production cost, it is tempting to explore the therapeutic potential of pollen allergen-specific nanobodies. To our knowledge no one else has yet investigated the use of nanobodies as treatment of pollen allergy.

For this proof of principle study, we chose Bet v 1 as our allergen of interest, as it represents an important sensitizing allergen in the northern hemisphere. We hypothesize that Bet v 1-specific nanobodies, when binding to an IgE epitope, can inhibit IgE from interacting with Bet v 1 in a similar way as blocking antibodies. Thus, degranulation of effector cells resulting from crosslinking of IgE through Bet v 1 should be prevented.

3. AIM OF THE THESIS

The aim of this thesis is the expression, purification, and immunological characterization of nanobodies raised against the major birch pollen allergen Bet v 1. To investigate whether those nanobodies might be useful for treatment for birch allergic patients, we checked if they display cross-reactivity to Bet v 1-related allergens, determined the epitope specificity and measured affinity constants. Lastly, we investigated their potential to inhibit IgE-binding to Bet v 1 and basophil activation *in vitro*.

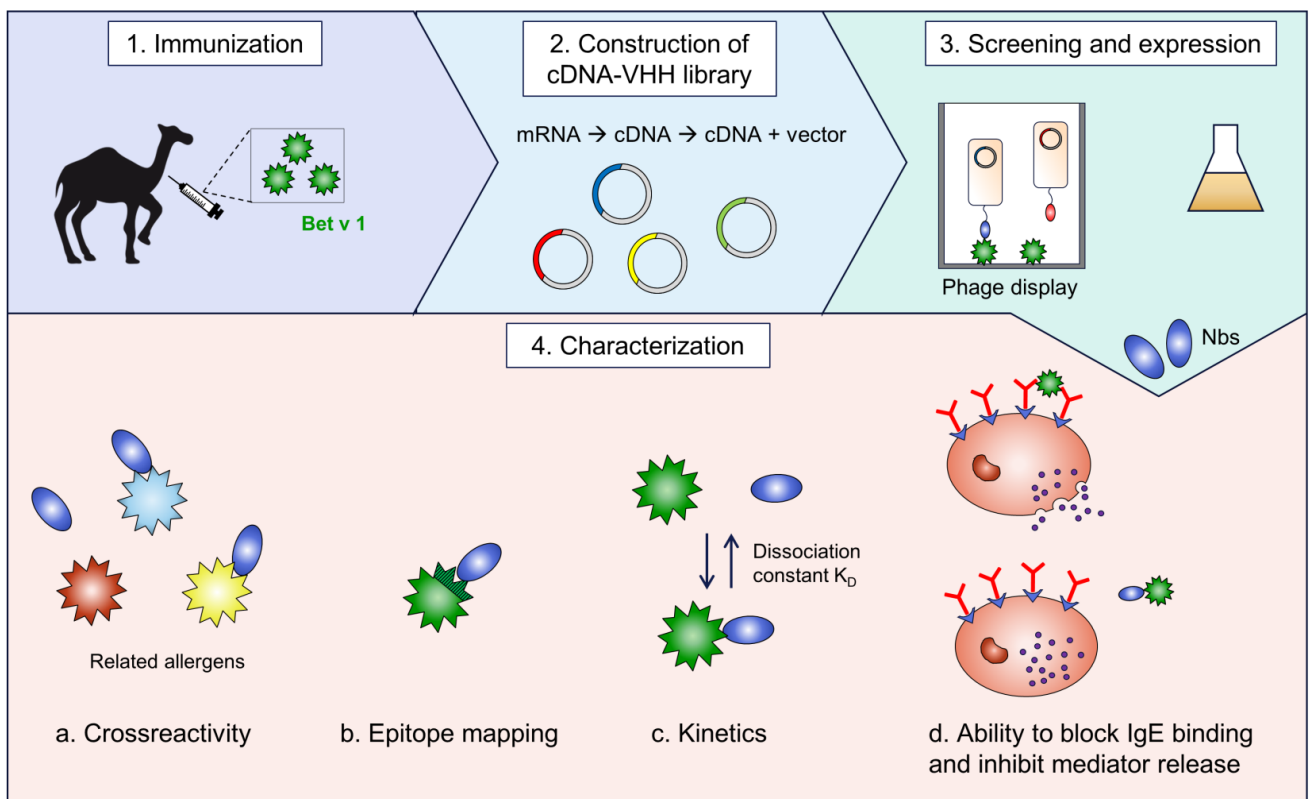


Figure 7 | Schematic workflow of the project.

REFERENCES

- Abbas AK, Lichtman AH, Baker DL. (2005): Cellular and molecular immunology. 5. ed., updated ed. Philadelphia (PA): Elsevier Saunders; 2005.
- Agache I, Beltran J, Akdis C, Akdis M, Canelo-Aybar C, Canonica GW, et al. Efficacy and safety of treatment with biologicals (benralizumab, dupilumab, mepolizumab, omalizumab and reslizumab) for severe eosinophilic asthma. A systematic review for the EAACI Guidelines - recommendations on the use of biologicals in severe asthma. *Allergy*. 2020;75:1023-1042. doi: 10.1111/all.14221
- Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NB, Hamid M. scFv antibody: principles and clinical application. *Clin Dev Immunol*. 2012;2012:980250. doi: 10.1155/2012/980250
- Alessandri C, Ferrara R, Bernardi ML, Zennaro D, Tuppo L, Giangrieco I, et al. Molecular approach to a patient's tailored diagnosis of the oral allergy syndrome. *Clin Transl Allergy*. 2020;10:22. doi: 10.1186/s13601-020-00329-8
- Arbabi-Ghahroudi M. Camelid Single-Domain Antibodies: Historical Perspective and Future Outlook. *Front Immunol*. 2017;8:1589. doi: 10.3389/fimmu.2017.01589
- Asam C, Hofer H, Wolf M, Aglas L, Wallner M. Tree pollen allergens-an update from a molecular perspective. *Allergy*. 2015;70:1201–1211. doi: 10.1111/all.12696
- Bannas P, Hambach J, Koch-Nolte F. Nanobodies and Nanobody-Based Human Heavy Chain Antibodies As Antitumor Therapeutics. *Front Immunol*. 2017;8:1603. doi: 10.3389/fimmu.2017.01603
- Bennich HH, Ishizaka K, Johansson SG, Rowe DS, Stanworth DR, Terry WD. Immunoglobulin E: a new class of human immunoglobulin. *Immunology*. 1968;15:323–324.
- Berger M, Shankar V, Vafai A. Therapeutic applications of monoclonal antibodies. *Am J Med Sci*. 2002;324:14-30. doi: 10.1097/00000441-200207000-00004
- Berin MC, Sampson HA. Mucosal immunology of food allergy. *Curr Biol*. 2013;23:R389-400. doi: 10.1016/j.cub.2013.02.043
- Biedermann T, Winther L, Till SJ, Panzner P, Knulst A, Valovirta E. Birch pollen allergy in Europe. *Allergy*. 2019;74:1237–1248. doi: 10.1111/all.13758
- Bohle B. The impact of pollen-related food allergens on pollen allergy. *Allergy*. 2007;62:3–10. doi: 10.1111/j.1398-9995.2006.01258.x
- Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, et al. The gene coding for the major birch pollen allergen Betv1, is highly homologous to a pea disease resistance response gene. *EMBO J*. 1989;8:1935–1938.
- Brown JM, Wilson TM, Metcalfe DD. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. *Clin Exp Allergy*. 2008;38:4-18. doi: 10.1111/j.1365-2222.2007.02886.x

- Cassard L, Jönsson F, Arnaud S, Daëron M. Fcγ receptors inhibit mouse and human basophil activation. *J Immunol.* 2012;189:2995-3006. doi: 10.4049/jimmunol.1200968
- Chapman MD, Pomés A, Breiteneder H, Ferreira F. Nomenclature and structural biology of allergens. *J Allergy Clin Immunol.* 2007;119:414-20. doi: 10.1016/j.jaci.2006.11.001
- Chen KW, Blatt K, Thomas WR, Swoboda I, Valent P, Valenta R, et al. Hypoallergenic Der p 1/Der p 2 combination vaccines for immunotherapy of house dust mite allergy. *J Allergy Clin Immunol.* 2012;130(2):435-43.e4. doi: 10.1016/j.jaci.2012.05.035
- Conrath KE, Wernery U, Muyldermans S, Nguyen VK. Emergence and evolution of functional heavy-chain antibodies in Camelidae. *Dev Comp Immunol.* 2003;27:87-103. doi: 10.1016/s0145-305x(02)00071-x
- Cooke RA, Barnard JH, Hebal S, Stull A. SEROLOGICAL EVIDENCE OF IMMUNITY WITH COEXISTING SENSITIZATION IN A TYPE OF HUMAN ALLERGY (HAY FEVER). *J Exp Med.* 1935;62:733-50. doi: 10.1084/jem.62.6.733
- Cruse JM, Lewis RE. Antigen-Antibody Interaction. In: Atlas of Immunology. Springer, Berlin, Heidelberg. 1999. doi: 10.1007/978-3-662-11196-3_8
- Curin M, Khaitov M, Karaulov A, Namazova-Baranova L, Campana R, Garib V, et al. Next-Generation of Allergen-Specific Immunotherapies: Molecular Approaches. *Curr Allergy Asthma Rep.* 2018;18:39. doi: 10.1007/s11882-018-0790-x
- Daëron M, Latour S, Malbec O, Espinosa E, Pina P, Pasmans S, et al. The same tyrosine-based inhibition motif, in the intracytoplasmic domain of Fc gamma RIIb, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. *Immunity.* 1995;3:635-46. doi: 10.1016/1074-7613(95)90134-5
- Davies J, Riechmann L. 'Camelising' human antibody fragments: NMR studies on VH domains. *FEBS Lett.* 1994;339:285-90. doi: 10.1016/0014-5793(94)80432-x
- Deckers J, De Bosscher K, Lambrecht BN, Hammad H. Interplay between barrier epithelial cells and dendritic cells in allergic sensitization through the lung and the skin. *Immunol Rev.* 2017;278:131-144. doi: 10.1111/imr.12542
- Deifl S, Bohle B. Factors influencing the allergenicity and adjuvanticity of allergens. *Immunotherapy.* 2011;3:881-93. doi: 10.2217/imt.11.69
- de Lalla C, Tamborini E, Longhi R, Tresoldi E, Manoni M, Siccardi AG, et al. Human recombinant antibody fragments specific for a rye-grass pollen allergen: characterization and potential applications. *Mol Immunol.* 1996;33:1049-58. doi: 10.1016/s0161-5890(96)00061-2
- Desmyter A, Transue TR, Ghahroudi MA, Thi MH, Poortmans F, Hamers R, et al. Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. *Nat Struct Biol.* 1996;3:803-11. doi: 10.1038/nsb0996-803

- De Vlieger D, Ballegeer M, Rossey I, Schepens B, Saelens X. Single-Domain Antibodies and Their Formatting to Combat Viral Infections. *Antibodies (Basel)*. 2018;8:1. doi: 10.3390/antib8010001
- Dorofeeva Y, Shilovskiy I, Tulaeva I, Focke-Tejkl M, Flicker S, Kudlay D, et al. Past, present, and future of allergen immunotherapy vaccines. *Allergy*. 2021;76:131-149. doi: 10.1111/all.14300
- Durham S, Schwabe C, Robson R, Ahlström Emanuelsson C, Shamji M, Singh D, et al. A randomized clinical trial of passive immunotherapy with single-dose anti-Fel d 1 monoclonal antibodies REGN 1908-1909 in cat induced rhinoconjunctivitis: exploratory efficacy endpoints, safety, and pharmacokinetics. *Allergy*. 2017;72(S103):64–5.
- Dzoro S, Mittermann I, Resch-Marat Y, Vrtala S, Nehr M, Hirschl AM, et al. House dust mites as potential carriers for IgE sensitization to bacterial antigens. *Allergy*. 2018;73:115-124. doi: 10.1111/all.13260
- Eckl-Dorna J, Villazala-Merino S, Campion NJ, Byazrova M, Filatov A, Kudlay D, et al. (2019a): Tracing IgE-Producing Cells in Allergic Patients. *Cells*. 2019;8:994. doi: 10.3390/cells8090994
- Eckl-Dorna J, Weber M, Stanek V, Linhart B, Ristl R, Walzl EE, et al. (2019b): Two years of treatment with the recombinant grass pollen allergy vaccine BM32 induces a continuously increasing allergen-specific IgG4 response. *EBioMedicine*. 2019;50:421–432. doi: 10.1016/j.ebiom.2019.11.006
- Flicker S, Gadermaier E, Madritsch C, Valenta R. Passive immunization with allergen-specific antibodies. *Curr Top Microbiol Immunol*. 2011;352:141-59. doi: 10.1007/82_2011_143
- Flicker S, Steinberger P, Norderhaug L, Sperr WR, Majlesi Y, Valent P, et al. Conversion of grass pollen allergen-specific human IgE into a protective IgG(1) antibody. *Eur J Immunol*. 2002;32:2156-62. doi: 10.1002/1521-4141(200208)32:8<2156::AID-IMMU2156>3.0.CO;2-A
- Flicker S, Valenta R. Renaissance of the blocking antibody concept in type I allergy. *Int Arch Allergy Immunol*. 2003;132:13-24. doi: 10.1159/000073260
- Flicker S, Zettl I, Tillib SV. Nanobodies-Useful Tools for Allergy Treatment? *Front Immunol*. 2020;11:576255. doi: 10.3389/fimmu.2020.576255
- Focke M, Mahler V, Ball T, Sperr WR, Majlesi Y, Valent P, et al. Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. *FASEB J*. 2001;15:2042-4. doi: 10.1096/fj.01-0016fje
- Focke M, Linhart B, Hartl A, Wiedermann U, Sperr WR, Valent P, et al. Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. *Clin Exp Allergy*. 2004;34:1525-33. doi: 10.1111/j.1365-2222.2004.02081.x

- Focke-Tejkl M, Weber M, Niespodziana K, Neubauer A, Huber H, Henning R et al. Development and characterization of a recombinant, hypoallergenic, peptide-based vaccine for grass pollen allergy. *J Allergy Clin Immunol*. 2015;135(5):1207-7.e11. doi:10.1016/j.jaci.2014.09.012
- Gadermaier E, Flicker S, Blatt K, Valent P, Valenta R. Possible therapeutic potential of a recombinant group 2 grass pollen allergen-specific antibody fragment. *Allergy*. 2014;69:261-4. doi: 10.1111/all.12315
- Gadermaier E, Marth K, Lupinek C, Campana R, Hofer G, Blatt K, et al. Isolation of a high-affinity Bet v 1-specific IgG-derived ScFv from a subject vaccinated with hypoallergenic Bet v 1 fragments. *Allergy*. 2018;73:1425–1435. doi: 10.1111/all.13394
- Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. *Nature*. 2008;454:445-54. doi: 10.1038/nature07204
- Gieras A, Linhart B, Roux KH, Dutta M, Khodoun M, Zafred D, et al. IgE epitope proximity determines immune complex shape and effector cell activation capacity. *J Allergy Clin Immunol*. 2016;137:1557-65. doi: 10.1016/j.jaci.2015.08.055
- Göbl C, Focke-Tejkl M, Najafi N, Schrank E, Madl T, Kosol S, et al. Flexible IgE epitope-containing domains of Phl p 5 cause high allergenic activity. *J Allergy Clin Immunol*. 2017;140:1187-1191. doi: 10.1016/j.jaci.2017.05.005
- Goldman ER, Anderson GP, Liu JL, Delehanty JB, Sherwood LJ, Osborn LE, et al. Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library. *Anal Chem*. 2006;78:8245-55. doi: 10.1021/ac0610053.
- Golebski K, Röschmann KI, Toppila-Salmi S, Hammad H, Lambrecht BN, Renkonen R, et al. The multi-faceted role of allergen exposure to the local airway mucosa. *Allergy*. 2013;68:152-60. doi: 10.1111/all.12080
- Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. *Nature*. 1995;374:168-73. doi: 10.1038/374168a0
- Groh N, von Loetzen CS, Subbarayal B, Möbs C, Vogel L, Hoffmann A, et al. IgE and allergen-specific immunotherapy-induced IgG4 recognize similar epitopes of Bet v 1, the major allergen of birch pollen. *Clin Exp Allergy*. 2017;47:693-703. doi: 10.1111/cea.12835
- Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB et al. Naturally occurring antibodies devoid of light chains. *Nature*. 1993;363:446-8. doi: 10.1038/363446a0

- Holgate S, Smith N, Massanari M, Jimenez P. Effects of omalizumab on markers of inflammation in patients with allergic asthma. *Allergy*. 2009;64:1728–1736. doi: 10.1111/j.1398-9995.2009.02201.x
- Huang L, Muyldermans S, Saerens D. Nanobodies®: proficient tools in diagnostics. *Expert Rev Mol Diagn*. 2010;10:777-85. doi: 10.1586/erm.10.62
- Ishizaka K, Ishizaka T. Identification of gamma-E-antibodies as a carrier of reaginic activity. *J Immunol*. 1967;99:1187-98.
- Jakobsen CG, Bodtger U, Kristensen P, Poulsen LK, Roggen EL. Isolation of high-affinity human IgE and IgG antibodies recognising Bet v 1 and Humicola lanuginosa lipase from combinatorial phage libraries. *Mol Immunol*. 2004;41:941-53. doi: 10.1016/j.molimm.2004.05.009
- James LK, Till SJ. Potential Mechanisms for IgG4 Inhibition of Immediate Hypersensitivity Reactions. *Curr Allergy Asthma Rep*. 2016;16:23. doi: 10.1007/s11882-016-0600-2
- Jarolim E, Rumpold H, Endler AT, Ebner H, Breitenbach M, Scheiner O, et al. IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of Betula verrucosa. *Allergy*. 1989;44:385-95. doi: 10.1111/j.1398-9995.1989.tb04169.x.
- Johansson SGO, Bennich H, Wide L. A new class of immunoglobulins in human serum. *Immunology* 1968;14:265–272.
- Jovčevska I, Muyldermans S. The Therapeutic Potential of Nanobodies. *BioDrugs*. 2020;34:11-26. doi: 10.1007/s40259-019-00392-z
- Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol*. 2005;116:608-13. doi: 10.1016/j.jaci.2005.06.004
- Jylhä S, Mäkinen-Kiljunen S, Haahtela T, Söderlund H, Takkinen K, Laukkanen ML. Selection of recombinant IgE antibodies binding the beta-lactoglobulin allergen in a conformation-dependent manner. *J Immunol Methods*. 2009;350:63-70. doi: 10.1016/j.jim.2009.07.007
- Kawauchi H, Yanai K, Wang DY, Itahashi K, Okubo K. Antihistamines for Allergic Rhinitis Treatment from the Viewpoint of Nonsedative Properties. *Int J Mol Sci*. 2019;20:213. doi: 10.3390/ijms20010213
- Kazemi-Shirazi L, Pauli G, Purohit A, Spitzauer S, Fröschl R, Hoffmann-Sommergruber K, et al. Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy. *J Allergy Clin Immunol*. 2000;105:116-25. doi: 10.1016/s0091-6749(00)90186-6
- Kijanka M, Dorresteyn B, Oliveira S, van Bergen en Henegouwen PM. Nanobody-based cancer therapy of solid tumors. *Nanomedicine (Lond)*. 2015;10:161-74. doi: 10.2217/nnm.14.178

- Kiotseridis H, Cilio CM, Bjermer L, Aurivillius M, Jacobsson H, Dahl Å, et al. Quality of life in children and adolescents with respiratory allergy, assessed with a generic and disease-specific instrument. *Clin Respir J*. 2013;7:168-75. doi: 10.1111/j.1752-699X.2012.00298.x
- Langlois A, Lavergne MH, Leroux H, Killer K, Azzano P, Paradis L, et al. Protocol for a double-blind, randomized controlled trial on the dose-related efficacy of omalizumab in multi-food oral immunotherapy. *Allergy Asthma Clin Immunol*. 2020;16:25. doi: 10.1186/s13223-020-00419-z
- Larché M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol*. 2006;6:761-71. doi: 10.1038/nri1934
- Lichtenstein LM, Holtzman NA, Burnett LS. A quantitative in vitro study of the chromatographic distribution and immunoglobulin characteristics of human blocking antibody. *J Immunol*. 1968;101:317-24.
- Lieberman JA, Chehade M. Use of omalizumab in the treatment of food allergy and anaphylaxis. *Curr Allergy Asthma Rep*. 2013;13:78-84. doi: 10.1007/s11882-012-0316-x
- Loveless MH. Immunological Studies of Pollinosis: I. The Presence of Two Antibodies Related to the Same Pollen-Antigen in the Serum of Treated Hay-Fever Patients. *J Immunol*. 1940;38:25–50.
- McGill JI, Holgate ST, Church MK, Anderson DF, Bacon A. Allergic eye disease mechanisms. *Br J Ophthalmol*. 1998;82:1203-14. doi: 10.1136/bjo.82.10.1203
- Mitchell LS, Colwell LJ. Comparative analysis of nanobody sequence and structure data. *Proteins*. 2018;86:697-706. doi: 10.1002/prot.25497
- Miyake K, Karasuyama H. Emerging roles of basophils in allergic inflammation. *Allergol Int*. 2017;66:382-391. doi: 10.1016/j.alit.2017.04.007
- Monegal A, Ami D, Martinelli C, Huang H, Aliprandi M, Capasso P, et al. Immunological applications of single-domain llama recombinant antibodies isolated from a naïve library. *Protein Eng Des Sel*. 2009;22:273-80. doi: 10.1093/protein/gzp002
- Moutel S, Bery N, Bernard V, Keller L, Lemesre E, de Marco A, et al. NaLi-H1: A universal synthetic library of humanized nanobodies providing highly functional antibodies and intrabodies. *Elife*. 2016;5:e16228. doi: 10.7554/eLife.16228
- Muyldermans S, Cambillau C, Wyns L. Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. *Trends Biochem Sci*. 2001;26:230-5. doi: 10.1016/s0968-0004(01)01790-x
- Muyldermans S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem*. 2013;82:775-97. doi: 10.1146/annurev-biochem-063011-092449
- Nelson AL. Antibody fragments: hope and hype. *MAbs*. 2010;2:77-83. doi: 10.4161/mabs.2.1.10786.

- Nguyen VK, Desmyter A, Muyldermans S. Functional heavy-chain antibodies in Camelidae. *Adv Immunol*. 2001;79:261-96. doi: 10.1016/s0065-2776(01)79006-2
- Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, et al. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci U S A*. 2004;101 Suppl 2(Suppl 2):14677-82. doi: 10.1073/pnas.0404735101
- Niederberger V, Pauli G, Grönlund H, Fröschl R, Rumpold H, Kraft D, et al. Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. *J Allergy Clin Immunol*. 1998;102:579-91. doi: 10.1016/s0091-6749(98)70273-8.
- Noon, L. PROPHYLACTIC INOCULATION AGAINST HAY FEVER. *Lancet*. 1911;177:1572–1573. doi: 10.1016/S0140-6736(00)78276-6
- Nuttall SD, Krishnan UV, Hattarki M, De Gori R, Irving RA, Hudson PJ. Isolation of the new antigen receptor from wobbegong sharks, and use as a scaffold for the display of protein loop libraries. *Mol Immunol*. 2001;38:313-26. doi: 10.1016/s0161-5890(01)00057-8
- Orengo JM, Radin AR, Kamat V, Badithe A, Ben LH, Bennett BL, et al. Treating cat allergy with monoclonal IgG antibodies that bind allergen and prevent IgE engagement. *Nature communications* 2018;9:1421. doi: 10.1038/s41467-018-03636-8
- Padavattan S, Flicker S, Schirmer T, Madritsch C, Randow S, Reese G, et al. High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 as revealed by X-ray crystallography. *J Immunol*. 2009;182:2141-51. doi: 10.4049/jimmunol.0803018
- Palomares O, Akdis M, Martín-Fontecha M, Akdis CA. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. *Immunol Rev*. 2017;278:219-236. doi: 10.1111/imr.12555
- Pardon E, Laeremans T, Triest S, Rasmussen SG, Wohlkönig A, Ruf A, et al. A general protocol for the generation of Nanobodies for structural biology. *Nat Protoc*. 2014;9:674-93. doi: 10.1038/nprot.2014.039.
- Pauli G, Larsen TH, Rak S, Horak F, Pastorello E, Valenta R, et al. Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. *J Allergy Clin Immunol*. 2008;122:951-60. doi: 10.1016/j.jaci.2008.09.017
- Popescu FD. Cross-reactivity between aeroallergens and food allergens. *World J Methodol*. 2015;5:31-50. doi: 10.5662/wjm.v5.i2.31
- Posner J, Barrington P, Brier T, Datta-Mannan A. Monoclonal Antibodies: Past, Present and Future. *Handb Exp Pharmacol*. 2019;260:81-141. doi: 10.1007/164_2019_323

- Prausnitz C, Küstner H. Studien über die Überempfindlichkeit. *Centralbl Bakteriol.* 1921;86:160–169.
- Pree I, Reisinger J, Focke M, Vrtala S, Pauli G, van Hage M, et al. Analysis of epitope-specific immune responses induced by vaccination with structurally folded and unfolded recombinant Bet v 1 allergen derivatives in man. *J Immunol.* 2007;179:5309-16. doi: 10.4049/jimmunol.179.8.5309
- Presta LG, Lahr SJ, Shields RL, Porter JP, Gorman CM, Fendly BM, et al. Humanization of an antibody directed against IgE. *J Immunol.* 1993;151:2623-32
- Purohit A, Niederberger V, Kronqvist M, Horak F, Grönneberg R, Suck R, et al. Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. *Clin Exp Allergy.* 2008;38:1514-25. doi: 10.1111/j.1365-2222.2008.03042.x
- Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J Allergy Clin Immunol.* 2008;121:847-52.e7. doi: 10.1016/j.jaci.2008.01.025
- Riechmann L. Rearrangement of the former VL interface in the solution structure of a camelised, single antibody VH domain. *J Mol Biol.* 1996;259:957-69. doi: 10.1006/jmbi.1996.0373
- Rotiroti G, Dziadzio M, Radcliffe M. Antihistamines for the Treatment of Allergic Rhino-conjunctivitis. *Curr Treat Options Allergy.* 2016;3:1–17. doi: 10.1007/s40521-016-0073-5
- Scheurer S, Toda M, Vieths S. What makes an allergen? *Clin Exp Allergy.* 2015;45:1150-61. doi: 10.1111/cea.12571
- Schumacher D, Helma J, Schneider AFL, Leonhardt H, Hackenberger CPR. Nanobodies: Chemical Functionalization Strategies and Intracellular Applications. *Angew Chem Int Ed Engl.* 2018;57:2314-2333. doi: 10.1002/anie.201708459
- Shamji MH, Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. *J Allergy Clin Immunol.* 2017;140:1485-1498. doi: 10.1016/j.jaci.2017.10.010
- Shields RL, Whether WR, Zioncheck K, O'Connell L, Fendly B, Presta LG, et al. Inhibition of allergic reactions with antibodies to IgE. *Int Arch Allergy Immunol.* 1995;107:308-12. doi: 10.1159/000237010
- Shire S. (2015): Monoclonal Antibodies. 1. ed. Woodhead Publishing. ISBN: 9780081002964
- Sroga, P, Safronetz D, Stein DR. Nanobodies: a new approach for the diagnosis and treatment of viral infectious diseases. *Future Virol.* 2020;15. doi: 10.2217/fvl-2019-0167
- Swoboda I, Balic N, Klug C, Focke M, Weber M, Spitzauer S, et al. A general strategy for the generation of hypoallergenic molecules for the immunotherapy of

- fish allergy. *J Allergy Clin Immunol.* 2013;132:979-81.e1. doi: 10.1016/j.jaci.2013.04.027
- Tordesillas L, Berin MC, Sampson HA. Immunology of Food Allergy. *Immunity.* 2017;47:32-50. doi: 10.1016/j.immuni.2017.07.004
- Tulaeva I, Kratzer B, Campana R, Curin M, van Hage M, Karsonova A, et al. Preventive Allergen-Specific Vaccination Against Allergy: Mission Possible? *Front Immunol.* 2020;11:1368. doi: 10.3389/fimmu.2020.01368
- Twaroch TE, Focke M, Civaj V, Weber M, Balic N, Mari A, et al. Carrier-bound, nonallergenic Ole e 1 peptides for vaccination against olive pollen allergy. *J Allergy Clin Immunol.* 2011;128:178-184.e7. doi: 10.1016/j.jaci.2011.03.011
- Valenta R. The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol.* 2002;2:446-53. doi: 10.1038/nri824
- Valenta R, Karaulov A, Niederberger V, Gattlinger P, van Hage M, Flicker S, et al. Molecular Aspects of Allergens and Allergy. *Adv Immunol.* 2018;138:195-256. doi: 10.1016/bs.ai.2018.03.002
- van der Linden RH, Frenken LG, de Geus B, Harmsen MM, Ruuls RC, Stok W, et al. Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochim Biophys Acta.* 1999;1431:37-46. doi: 10.1016/s0167-4838(99)00030-8
- Vincke C, Loris R, Saerens D, Martinez-Rodriguez S, Muyldermans S, Conrath K. General strategy to humanize a camelid single-domain antibody and identification of a universal humanized nanobody scaffold. *J Biol Chem.* 2009;284:3273-84. doi: 10.1074/jbc.M806889200
- Visco V, Dolecek C, Denépoux S, Le Mao J, Guret C, Rousset F, et al. Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. *J Immunol.* 1996;157:956-62.
- von Pirquet C. Allergie. In *Munch Med Wochenschr.* 1906;30:1457–1461.
- Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest.* 1997;99:1673-81. doi: 10.1172/JCI119330
- Vu KB, Ghahroudi MA, Wyns L, Muyldermans S. Comparison of llama VH sequences from conventional and heavy chain antibodies. *Mol Immunol.* 1997;34:1121-31. doi: 10.1016/s0161-5890(97)00146-6
- Wachholz PA, Durham SR. Mechanisms of immunotherapy: IgG revisited. *Curr Opin Allergy Clin Immunol.* 2004;4:313-8. doi: 10.1097/01.all.0000136753.35948.c0
- Wahner-Roedler DL, Kyle RA. Heavy chain diseases. *Best Pract Res Clin Haematol.* 2005;18:729-46. doi: 10.1016/j.beha.2005.01.029

WHO/IUIS Allergen Nomenclature Sub-Committee: Allergen nomenclature. Available online at www.allergen.org, accessed on 7/16/2020.

Yamauchi K, Ogasawara M. The Role of Histamine in the Pathophysiology of Asthma and the Clinical Efficacy of Antihistamines in Asthma Therapy. *Int J Mol Sci*. 2019;20:1733. doi: 10.3390/ijms20071733

Zhao Y, Gutshall L, Jiang H, Baker A, Beil E, Obmolova G, et al. Two routes for production and purification of Fab fragments in biopharmaceutical discovery research: Papain digestion of mAb and transient expression in mammalian cells. *Protein Expr Purif*. 2009;67:182-9. doi: 10.1016/j.pep.2009.04.012

Zhernov Y, Curin M, Khaitov M, Karaulov A, Valenta R. Recombinant allergens for immunotherapy: state of the art. *Curr Opin Allergy Clin Immunol*. 2019;19:402-414. doi: 10.1097/ACI.0000000000000536

4. PAPER IN PREPARATION : Isolation of nanobodies with potential to reduce patients' IgE binding to Bet v 1, the major birch pollen allergen

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Abstract

Background: A recent proof of concept showed that a single injection of two human monoclonal allergen-specific IgG antibodies significantly reduced allergic symptoms in cat allergic patients. Since the production of full monoclonal antibodies in sufficient amounts is laborious and expensive, we sought to investigate if smaller recombinant allergen-specific antibody fragments, i.e., nanobodies deriving from heavy chain antibodies from camels have similar protective potential.

Objective: We generated nanobodies specific for Bet v 1, the major birch pollen allergen to evaluate their efficacy to inhibit IgE-mediated responses.

Methods: After an immune response against Bet v 1 was raised, a cDNA-VHH library was constructed and screened for Bet v 1 binding by phage display. Selected nanobodies were expressed, purified and analyzed in regards of epitope-specificity and affinity to Bet v 1, cross-reactivity to Bet v 1 homologous allergens, and their efficacy to inhibit IgE binding to ELISA-plate bound allergens and allergen-induced basophil activation.

Results: We isolated three nanobodies that recognize Bet v 1 with high specificity and affinity and cross-react with Aln g 1 (alder) and Cor a 1 (hazel), but not with Mal d 1 (apple). The epitope was mapped to the alpha-helix at the C-terminus of Bet v 1. All nanobodies inhibit allergic patients' polyclonal IgE binding to Bet v 1, Aln g 1 and Cor a 1 and partially suppress Bet v 1-induced basophil activation.

Conclusion: We isolated high affinity Bet v 1-specific nanobodies that reduce Bet v 1-induced basophil activation revealing the first proof that allergen-specific nanobodies comprise potential to be useful for future treatment of pollen allergy.

Key messages

- First-time isolation of Bet v 1-specific nanobodies from an immunized camel
- These Bet v 1-specific nanobodies bind Bet v 1 with high affinity in the nanomolar range and cross-react with Bet v 1 homologous allergens from alder (Aln g 1) and hazel (Cor a 1)
- These Bet v 1-specific nanobodies reduce polyclonal IgE binding to Bet v 1, Aln g 1 and Cor a 1 and decrease Bet v 1-induced IgE-mediated basophil activation

Capsule Summary

Nanobodies specific for Bet v 1, one of the most prominent and clinically potent pollen allergens were isolated and shown to have the capability to inhibit polyclonal IgE binding to Bet v 1.

Key words

allergy, Bet v 1, Nanobody, VHH, IgE, competitor

Abbreviations

AIT: allergen-specific immunotherapy

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

BSA: Bovine serum albumin

HCAb- Heavy Chain-only Antibody

HSA: human serum albumin

KLH: Keyhole limpet hemocyanin

PBMC: peripheral blood mononuclear cell

PBS: phosphate-buffered saline

PBST: phosphate-buffered saline + tween 20

RBL: Rat basophil leukemia

INTRODUCTION

Birch pollen allergy ranks among the most prevalent pollen allergies in Northern and Central Europe (Biedermann et al., 2019). Estimated 100 million individuals worldwide suffer from this IgE-mediated disease leading to clinical manifestations such as hayfever causing a major health and economic burden (Kiotseridis et al., 2013; Zuberbier et al., 2014). Bet v 1 was identified as the only major birch pollen allergen and is responsible for the sensitization of more than 90% of individuals allergic to birch pollen (Biedermann et al., 2019; Jarolim et al., 1989) and mainly contributes to pollen associated food allergy (Treudler & Simon, 2017). Representing such a clinically important allergen, a lot of effort was done in the last decades to understand its mode of action (Jensen-Jarolim, 2014). Based on this knowledge strategies have been elaborated to treat and ameliorate birch pollen allergy (Valenta et al., 2018, Moingeon et al. 2016; Linhart & Valenta, 2012; Mutschlechner et al., 2009). Many attempts have focused on the development of Bet v 1 hypoallergenic derivatives for vaccination of birch pollen allergic patients to induce protective IgG antibodies competing with the interaction between specific IgE antibodies and allergens causing a variety of effects on distinct immune cells (Niederberger et al., 2004; Gafvelin et al., 2005; Dorofeeva et al., 2021; Zhernov et al., 2019; Shamji & Durham, 2017).

Within this study we pursued a different approach to answer the question if recombinantly produced Bet v 1-specific antibody fragments, i.e. nanobodies are able to offer protection by inhibiting patients' IgE binding to Bet v 1. Support for this idea came from the recent finding that the mechanisms of action of passively administered monoclonal antibodies are comparable to those induced in the course of an active immunization if they are directed to or overlap with IgE epitopes (Orengo et al., 2018). The proof of principle study showed for the first time that passive immunization with allergen-specific IgG antibodies that inhibit patients' IgE binding to the culprit allergen is a rapid, effective and well-tolerated treatment to reduce allergic inflammation (Orengo et al., 2018).

Since the generation and identification of blocking monoclonal human or humanized antibodies is laborious and expensive, recombinant antibody fragments including nanobodies turned out to be attractive molecules extensively studied for diverse applications in the recent past. Thus, nanobodies deriving from naturally occurring

heavy-chain antibodies of camelids have been proven to be useful tools in imaging technology, diagnosis, prevention and therapy of several diseases as cancer, viral infection and blood disorders (Bannas et al., 2017; Jovceska et al., 2020; Sgora et al., 2020). They combine several advantageous qualities like unique biochemical characteristics and low immunogenicity (Muyldermans, 2020). Owing to their particular paratopes, i.e. finger like extensions that facilitate the recognition of a broad panel of various epitopes on antigens, and their reported antigen affinities comparable with affinities of monoclonal antibodies they recently aroused our interest to evaluate their potential to compete with IgE antibodies for allergen binding (Muyldermans, 2013; Bannas et al., 2017; Flicker et al., 2020).

We hypothesize that nanobodies raised against Bet v 1 may block IgE binding similar to protective whole IgG antibodies and thus prevent activation of effector cells. Therefore, we generated nanobodies specific for Bet v 1 to characterize their capacity to prevent binding of IgE antibodies of allergic patients to allergens with the long-term goal to identify nanobodies that are suitable tools for prospective passive allergy treatment.

METHODS

Materials, reagents, patients' sera

Recombinant allergens Bet v 1 (birch), Aln g 1 (alder), Cor a 1 (hazel) and Mal d 1 (apple) were obtained from Biomay AG (Vienna, Austria). Bet v 1 fragments 1 and 2 were kindly provided (Vrtala et al., 1997). Bet v 1-derived peptides 1-6 were freshly synthesized for the camel's immunization as previously described (Focke et al., 2004). Mouse anti-HA-tag antibody labelled with HRP was purchased from Sigma-Aldrich (St. Louis, MO, USA). Donkey anti-His₆-tag antibody labelled with HRP was purchased from Invitrogen (Carlsbad, CA, USA). Mouse anti-human-IgE labelled with AKP was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Sera were obtained from patients suffering from birch pollen allergy according to case history, skin prick testing and IgE serology after informed consent was obtained. Serum and blood samples were analyzed in an anonymized manner with permission from the Ethics committee of the Medical University of Vienna (EK641/2014).

Camel immunization with the major birch pollen allergen, Bet v 1 and Bet v 1-derived peptides

All animal work was undertaken in strict accordance with the recommendations of the National Standard of the Russian Federation GOST R 53434–2009. The camel immunization was carried out at the Scientific-Experimental Base "Chernogolovka" of the Severtsov Institute of Problems of Ecology and Evolution of the Russian Academy of Sciences (Chernogolovka, Russia) using a camel kept at the Center for Collective use "Live Collection of Wild Mammals". The work was approved on 11.02.18 (registration number 17) by the Commission on Bioethics formed on May 3, 2017 in the Severtsov Institute of Problems of Ecology and Evolution.

A young 1.5-year-old male camel (*Camelus bactrianus*) was immunized for the first time in its life. The immunization was carried out by 5-stage subcutaneous injections (each time the injection was carried out at 4-5 sites relatively more accessible for piercing and located at the upper part of the sides of the body or in the neck region). The second injection was carried out 3 weeks after the first, and the next 3 injections 8-14 days after the previous one. Complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) for the first stage and incomplete Freund's adjuvant (Difco Laboratories) for the subsequent stages were used as adjuvants for the immunization. The adjuvants were mixed in equal proportions with the antigen solution right before immunization (typically, it was a mix of 4 and 4 mL). We used both the full-length Bet v 1 protein and Bet v 1-derived peptides (25-32 aa long) corresponding to the surface-exposed regions of this protein (Focke et al., 2004). For immunization, peptides and full-size protein conjugated to the carrier protein were used in the following order: first, conjugates with KLH (Keyhole Limpet Hemocyanin, My Bio Sourc Inc., San Diego, CA, USA) for the first three stages of immunization, and conjugated with bovine serum albumin (BSA), Amresco, Solon, OH, USA) for the two final stages. For the first four steps, 500 µg of each peptide and a full-length protein conjugated with an equal weight of the carrier protein were taken for injection. For the last step, 200 µg of conjugates and 500 µg of full-length unconjugated Bet v 1 protein were taken. Peptides were conjugated to the carrier protein using the glutaraldehyde cross-linking procedure as described (Adrian, 2002). Full-size recombinant Bet v 1 protein was conjugated to the carrier protein using DSS (disuccinimidyl suberate) crosslinker according to the protocol of the manufacturing company (Thermo Fisher Scientific, Waltham, MA, USA).

The final blood sample (150 mL) was taken 6 days after the fifth injection to analyze the Bet v 1-specific IgG titer and to gain peripheral blood mononuclear cells (PBMCs) for the construction of a cDNA-VHH- library. To prevent clotting of the blood an equal volume of phosphate buffered saline (PBS) containing heparin (100 U/mL) and EDTA (3 mM) was added to the blood.

Monitoring of the anti-Bet v 1 –IgG titer available in the blood of the immunized camel was carried out using ELISA plates (MaxiSorp, Nunc, Roskilde, Denmark) immobilized with recombinant Bet v 1 (2 µg/ml in 1x PBS). After several washing steps (once in 1x PBS, twice in 1x PBS with 0.05% Tween 20 (1x PBST), or 1x PBT, and in 1x PBS once again), immune and pre-immune sera samples (diluted from 1:100 to 1:12800 in 1x PBS) were added for 2 hour incubation on a rotating platform at room temperature. After several washing steps, secondary anti-camel HCAb polyclonal affinity-purified rabbit antibodies conjugated to Horseradish Peroxidase (previously obtained by Tillib et al., unpublished) were added in concentration of 1 µg/ml. After 1 hour incubation and the final thorough washing step, the activity of the bound peroxidase was detected using ABTS (Sigma-Aldrich) as a chromogenic substrate. The optical density was measured at a wavelength of 405 nm using a planshet fluorometer. Results of the ELISA experiments are expressed as means of duplicate determinations with a deviation of less than 5%.

Construction of a cDNA-VHH library and selection of anti-Bet v 1 nanobody-coding sequences by phage display

The cloning of cDNA-sequences encoding the entire set of variable domains (VHHs) of specific heavy chain-only antibodies from PBMCs of immunized camel (RNA isolation, cDNA synthesis, two-stage PCR with specific primers, insert restriction and purification, and insertion into a phagemid vector) was performed as previously described (Hamers-Casterman et al., 1993; Tillib et al., 2010, 2013, 2014; Pardon et al., 2014). This procedure resulted in a new specific nucleotide sequence library of nanobody-coding sequences in the pHEN4 phagemid vector (Arbabi-Ghahroudi et al., 1997). This library consisted of two sub-libraries, which were different in the restriction sites used for insertion into the phagemid vector of sequences coding for antigen recognition domains, (i) NcoI + NotI and (ii) PstI + NotI. *Escherichia coli* cells (strain TG1) were transformed by electroporation with recombinant plasmid DNA

obtained by ligation of the two insert sub-libraries and two corresponding vector sequences. Standard cloning, bacterial and bacteriophage M13 procedures were performed following the techniques described (Sambrook et al., 1989; Barbas et al., 2001). The generated VHH-library satisfied the criteria of 10^7 clones and about 80% of inserts of the expected size.

The selection of nanobody-coding sequences by phage display, production of nanobodies in the bacterial periplasm, and the analysis of the nanobodies' ability to recognize a given antigen were performed using the previously described techniques (Tillib et al., 2010, 2013, 2014). Recombinant Bet v 1 (10 μ g in 100 μ L of 1x PBS) was immobilized on ELISA plates (MaxiSorp) overnight at 4°C. Casein blocking buffer (Sigma-Aldrich) and 1% BSA (Sigma-Aldrich) in 1x PBS were used as alternating blocking agents. On average, approximately 10^{11} phage particles from the original library were introduced into a well with the immobilized Bet v 1. After incubation and multiple washing steps (in 1x PBS and in 1x PBST), bound phages were eluted in 1.4% triethylamine and neutralized adding equal volume of 1 M Tris-HCl, pH 7.2. The eluted phages were used to infect growing bacterial E. coli K12 / TG1 cells with a purpose to amplify the eluted phages for the next round of selection. M13KO7 bacteriophage (New England Biolabs, Ipswich, MA, USA) was used as a helper phage. The phage display selection procedure was performed with alternating blocking proteins for 3 cycles. After the final round of selection eluted phages were used to infect E. coli K12/WK6 growing cells and after 30 minutes of incubation infected cells were plated in different dilutions on LB agar (with Ampicillin 100 μ g/ml and 1% glucose) in Petri dishes. Forty-eight most enriched clones of nanobody-coding sequences were analyzed in terms of DNA primary structures (fingerprint-like analysis (Tillib et al., 2010) or/and sequencing and of Bet v 1 binding activities (using ELISA with the corresponding bacterial periplasmic protein extracts) of expressing nanobodies (with the C-terminal HA-tag). Three major groups (variants) of cloned and selected nanobody-coding sequences were identified representing 52% (including clone 24), 35% (including clone 32) and 4% (including clone 23) of all selected clones correspondingly. The selected cDNA sequences (clones 23, 24 and 32) encoding the three selected major nanobody groups/variants were subcloned (by conventional or PCR cloning) into the pHEN6 expression plasmid (Conrath et al., 2001) together with the pelB leader sequence (for periplasmic production), camel upper hinge (the longest hinge variant) and two short tag sequences (HA-tag and

(His)₆-tag) at the C-terminus coding region to obtain the formatted nanobodies (Tillib et al., 2013, 2014) that are easy to purify (using Ni-NTA-agarose) and to detect (with antibodies against HA-tag).

Expression and purification of nanobodies

Phagemid vector pHEN6 containing the DNA of nanobodies specific for Bet v 1 were transformed into *E. coli* XL1-Blue competent cells (Agilent Technologies, Santa Clara, CA, USA) and plated on LB-plates containing 100 µg/mL ampicillin and 1% glucose. Single colonies were picked and incubated in 4 mL 2x YT medium containing 100 µg/mL ampicillin and 0.1% glucose overnight at 37°C. Cultures were transferred into 200 mL fresh YT-medium each, and after an OD₆₀₀ of 0.5 – 0.7 was reached, the temperature was reduced to 30°C and expression was induced by adding 0.2 mM IPTG. After incubation at 30°C overnight, cultures were centrifuged (3,500 rpm, 4°C, 15 min) and nanobodies were extracted from the periplasm by incubation of the pellet with 4 mL TES buffer (50mM Tris pH = 8.0, 0.5mM EDTA, 20% saccharose) containing 100 mg/mL PMSF and 0.04% (v/v) β-mercaptoethanol, and subsequently with 6 mL 50 mM Tris buffer (pH = 8.0) containing 5 mM MgCl₂ for 30 min on ice each. The suspension was centrifuged again (14,000 rpm, 4°C, 30 min) and the supernatant containing nanobodies was purified by affinity chromatography using HIS-Select® Nickel Affinity Gel (Sigma-Aldrich) according to protocol of Qiagen (The QIAexpressionist™, fifth edition, 2003). Purified nanobodies were dialyzed against DPBS (Gibco, Life Technologies, Carlsbad, CA, USA) containing 10 mM imidazole using 3K Amicon Ultra centrifugal filter tubes (Millipore, Billerica, MA, USA). Concentrations were determined by absorption at 280 nm (DeNovix DS-11 FX+ spectrophotometer, Wilmington, DE, USA) and Bradford-based colorimetric protein assay (Bio-Rad, Hercules, CA, USA). Extinction coefficients, theoretical isoelectric points and molecular masses for all three nanobodies, Nb23, Nb24 and Nb32, were calculated using the ExPASy tool ProtParam.

A successful purification was checked by SDS-PAGE (14%) under reducing conditions following Coomassie Brilliant Blue staining. Nanobodies were mixed with 4x sample buffer (50 mM Tris/HCl, 50% SDS, 8.5% glycerol, 0.005% bromophenolblue, 5% (v/v) beta-mercaptoethanol) and boiled at 95°C for 5 min.

Nanobodies were separated and blotted onto nitrocellulose membrane (0.2 µm, Amersham Protran, GE Healthcare). The membrane was blocked with buffer A (40 mM Na₂HPO₄, 0.6 mM NaH₂PO₄, 0.5% BSA, 0.5% Tween 20, 0.05% NaN₃) for 30 minutes and incubated with mouse anti HA-tag (1:5000 in buffer A) or donkey anti His-tag antibodies (1:2000 in buffer A) coupled with HRP for two hours at room temperature. Bound antibodies were traced with HRP-substrate DAB (0.6 mg/mL, Roth, Karlsruhe, Germany) in 50 mM Tris/HCl (pH = 7.3).

Reactivity of nanobodies to Bet v 1 and to cross-reactive allergens Aln g 1, Cor a 1 and Mal d 1

4 µg/mL of recombinant Bet v 1, Aln g 1, Cor a 1 and Mal d 1 (Biomay) were coated onto ELISA plates (MaxiSorp) at 4°C overnight. Wells were washed three times with 1x PBS and blocked with 1x PBS containing 1% (w/v) BSA (PBS/1%BSA) for three hours at room temperature. Plates were washed three times and purified Nb23, Nb24 and Nb32, diluted in 1x PBS containing 0.1% (w/v) BSA (PBS/0.1%BSA) to a final concentration of 1 µg/mL, were incubated overnight at 4°C. Bound nanobodies were traced with HRP-labelled mouse anti HA-tag antibody (1:4000 in PBS/0.1%BSA) and HRP substrate ABTS (1mg/ml, Sigma-Aldrich). ODs were measured at 405 nm and a reference wavelength of 495 nm with a TECAN Infinite F50 microplate reader (Männedorf, Switzerland). Results are shown as means of triplicates and error bars indicate standard deviation (SD).

Identification of the binding sites of the Bet v 1-specific nanobodies

For epitope mapping, ELISA plates were coated with 4 µg/mL Bet v 1, Bet v 1 fragment 1 (aa 1- 74) and 2 (aa 75-160) (Vrtala et al., 1997) or 5µg/mL Bet v 1 peptides 1-6 encompassing the entire Bet v 1 sequence (Focke et al., 2004) and saturated as described. Nanobodies diluted 1:10 in PBS/0.1% BSA were incubated overnight at 4°C and detected as detailed before. Results are shown as means of triplicates and error bars indicate standard deviation (SD).

Affinity studies of Nb32 by plasmon surface resonance

Affinity studies by plasmon surface resonance were performed on a Biacore 3000 (GE Healthcare, Uppsala, Sweden) at 25°C. The surface of a CM5-chip was activated by injecting a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) for 7 minutes at a flow rate of 5 µL/min. Bet v 1, diluted in 10 mM acetate buffer pH 4.5 according to pH scout, was immobilized on the surface to a level of 150 resonance units (RU) resulting in a theoretical R_{max} of 176 RUs for the nanobody. The surface was deactivated with 35 µL 1M ethanolamine at a flow rate of 5 µL/min. The reference cell was activated and deactivated as described above.

Multicycle kinetics were repeatedly conducted to determine binding affinities for Nb32. Nb32 diluted in HBS-EP (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% vol/vol surfactant P20 (pH 7.4)) was injected in two-fold increasing concentrations (0.98 nM – 500 nM) into the flow cells with a flow rate of 30 µL/min. Dissociation was investigated by injecting HBS-EP for 30min at 30 µL/min. Regeneration of the chip surface was established by injecting 10 mM glycine-HCl (pH 1.5) three times for 30 seconds at 30 µL/min. Dissociation constants and rate constants (on-rate, off-rate) were calculated with BIAevaluation software 3.2 (GE Healthcare) using a 1:1 (Langmuir) binding model.

Inhibition of allergic patients' IgE binding to ELISA plate bound Bet v 1, Aln g 1 and Cor a 1 by nanobodies

ELISA plates were coated with 1 µg/mL Bet v 1 overnight at 4°C. Wells were washed three times with 1x PBST and saturated with 2% (w/v) BSA in 1x PBST. In a pilot experiment, all three nanobodies applied individually and in combination (Nb23+24+32) were used at a predefined concentration of 10 µg/mL first to evaluate their specific potential to inhibit patients' IgE binding to Bet v 1. In a separate setting, Nb 32 was applied in 2-fold decreasing dilutions ranging from 20 µg/mL to 0.15 µg/mL in 1x PBST containing 0.5% (w/v) BSA (PBST/0.5%BSA) to determine the optimal concentration of Nb 32 for patients' IgE inhibition. Based on the results of these pilot experiments, for the following study Nb 32 was used in a concentration of 10 µg/mL to ensure excess of nanobodies.

ELISA plates coated with 1 µg/mL Bet v 1, Aln g 1 and Cor a 1, washed and saturated as described above were incubated with nanobodies overnight at 4°C. After washing with 1x PBST sera from patients allergic to Bet v 1, diluted 1:10 in PBST/0.5%BSA were added overnight at 4°C. Bound Bet v 1-specific IgE was traced with AKP-coupled mouse anti-human-IgE (BD Pharmingen) diluted 1:1000 in PBST/0.5%BSA and pNPP (1 mg/mL, Sigma-Aldrich). ODs were measured at 405 nm with a reference wavelength at 550 nm and are shown as means of triplicates. Percentage inhibition of patients' IgE binding after pre-incubation with Nb32 compared to buffer was calculated as follows: $100 - (\text{OD after pre-incubation with Nb32} \times 100 / \text{OD after pre-incubation with buffer})$.

Influence of nanobodies on Bet v 1-induced basophil degranulation tested by rat basophil leukemia cell-based mediator-release assay

Rat basophil leukemia (RBL) cells (RS-ATL8, Nakamura et al., 2010) transfected with the human FcεRI were cultivated in Minimal Essential Medium (Gibco) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (Gibco), 2 mM L-Glutamine (Gibco), 100 U/mL Penicillin-Streptomycin (Gibco), 0.2 mg/mL Geneticin (Life Technologies) and 0.2 mg/mL Hygromycin B (Life Technologies). 1.5×10^5 cells/well were seeded in transparent, flat-bottomed, sterile 96-well cell culture plates (Costar, Corning Incorporated, Corning, New York, USA) and incubated with allergic patients' sera diluted 1:10 in medium or medium alone overnight at 37°C. Cells were washed thrice with Tyrode's buffer (Tyrode's salt, Sigma-Aldrich; 24 mM NaHCO₃) containing 0.1% BSA and then exposed for one hour at 37°C to different concentrations of Bet v 1 or Bet v 1 preincubated with Nb32. In detail, Bet v 1 (three-fold dilutions, 0.01 nM - 0.00037 nM) had been preincubated with 20 µg/mL Nb32 diluted in buffer B (Tyrode's buffer with 50% D₂O and 0.1% BSA) or with buffer B only for two hours in flat-bottomed, transparent 96-well microplates (Greiner, Kremsmünster, Austria) before being added to the cells. For control purposes, cells loaded with IgE were incubated with buffer B only and cells not loaded with IgE were incubated with the highest concentration of Bet v 1 (0.01 nM) with or without Nb 32 (20 µg/mL) to determine the background release. After one hour, six wells with cells only were lysed using Triton-X100 with a final concentration of 1% to determine the total β-hexosaminidase content (100%). All cell supernatants were transferred to a fresh 96-

well microplate (Greiner) and mixed 1:1 with 0.16 mM 4-Methylumbelliferyl N-acetyl- β -D-galactosaminide (Sigma-Aldrich) in 0.1 M citric acid buffer (pH = 4.2). β -hexosaminidase amounts were measured one hour after adding 4-MUC by fluorescence (excitation wavelength: 360nm; emission wavelength: 465nm) with a TECAN infinite M200pro plate reader (TECAN). Allergen-specific releases are given as percentages of the total mediator content (Vogel et al., 2005). All results are shown as means of triplicates and error bars indicate standard deviation (SD).

RESULTS

Construction of a phage- displayed cDNA-VHH-library from a camel immunized with rBet v 1 and Bet v 1-derived peptides and isolation of Bet v 1-specific nanobodies

The camel had received five subcutaneous immunizations with Bet v 1 and Bet v 1-derived peptides according to a defined scheme (Fig 1, A). PBMCs to isolate RNA for the construction of the cDNA-VHH library were obtained 6 days after the fifth vaccination. At this time point, the camel had developed IgG antibodies specific for Bet v 1 as a result of the immunization (Fig 1, B). The cloning of cDNA-sequences encoding the entire set of VHHs of specific heavy chain-only antibodies led to a final library size of 1×10^7 independent clones. A restriction enzyme analysis of randomly picked clones from the original library revealed that about 80% of picked clones comprise inserts of the expected size displaying high divergence (data not shown).

Three rounds of panning were conducted against Bet v 1 resulting in a strong enrichment of phages displaying Bet v 1-specific nanobodies. Forty-eight clones were picked after the third round of panning and their nanobody-coding sequences were PCR amplified and investigated using fingerprint-like restriction analysis (Tillib et al., 2010). This analysis allowed us to identify three major groups (variants) of VHH sequences in a ratio of 25 clones to 17 clones to 2 clones.

Two to three representatives from each identified group of clones as well as all unique variants (about 4 variants out of 48 were left ungrouped) were further used to produce the corresponding soluble nanobody (in the format of VHH with C-terminal HA-tag and His-tag) in bacterial periplasmic extract. These extracts were analyzed

for their reactivity to Bet v 1 and for control purposes to Casein/BSA by ELISA. All tested clones were confirmed to recognize Bet v 1 but not Casein/BSA. After verified binding to Bet v 1, detailed sequence analysis of the binders revealed typical VHH sequences having hallmark amino acid substitutions (hydrophobic to hydrophilic) in the region corresponding to the framework 3 of VH sequence of a classical antibody, which represents the site of the interface between VH and VL. Furthermore, all three VHH variants show characteristically for VHH sequences two Cystein residues reflecting possible bond formation between CDR1 and CDR3 hypervariable regions stabilizing the nanobody paratope conformation. For further analysis, we took one representative clone from all three VHH variants designated clone 23, clone 24 and clone 32. Comparing their sequences in detail it turned out that they share highly homologous framework and CDR 1 hypervariable region but differ slightly in their CDR 2 and CDR 3 hypervariable regions resulting in a few amino acid residue substitutions (data not shown because they are currently evaluated by the Technology Transfer Office of the Medical University of Vienna for patent application).

Expression and purification of Bet v-1 specific nanobodies

Nanobodies (Nb23, Nb24 and Nb32) were expressed as C-terminally HA- and His-tagged proteins in *E. coli* XL1-Blue and purified via affinity chromatography. Theoretical isoelectric points of 7.00 (Nb23), 8.62 (Nb24) and 8.62 (Nb32) and molecular masses of 19.37 kDa, 18.92 kDa and 19.30 kDa, respectively were determined for each nanobody (13-15kDa) including linker, HA-tag and His-tag (~5 kDa). The analysis by SDS-PAGE under reducing conditions and Coomassie staining (Fig 2, A) as well as Western blot (Fig 2, B) revealed purified nanobodies of a size of approximately 20 kDa corresponding to the estimated molecular masses due to the C-terminally added tags.

Cross-reactivity and epitope mapping of Bet v 1-specific nanobodies

To further investigate the reactivity of Bet v 1-specific Nb23, Nb24 and Nb32 to Bet v 1-homologous allergens Aln g 1 (alder), Cor a 1 (hazel) and Mal d 1 (apple) ELISA

experiments were performed (Fig 3, A). Besides Bet v 1, all three nanobodies further recognized Aln g 1 and Cor a 1 in a similar intensity but did not bind to Mal d 1 at all. By increasing the avidity of nanobodies by formatting nanobody trimers, Mal d 1 was specifically bound but with a lower intensity compared to Aln g 1 and Cor a 1 (data not shown). Incubation of allergens with detection antibodies only gave no signal (Fig 3, A: buffer).

Next, we intended to map the binding sites of all three nanobodies on Bet v 1 by using ELISA-plated recombinant Bet v 1 fragments (F1 and F2) (Fig 3, B) and, for a more detailed analysis, six smaller synthetic Bet v 1-derived peptides (P1-6), containing each 25 to 32 amino acids covering almost the whole allergen sequence (Fig 3, C). It revealed that Nb23, Nb24 and Nb32 bound specifically to the C-terminal fragment 2 (F2; aa 75-160) (Fig 3, B) and to the C-terminal peptide 5 (P5; aa 130-160) (Fig 3, C). The fact that all three nanobodies bind to the C-terminus of Bet v 1 offers a possible explanation for their marginal binding to Mal d 1. Earlier sequence alignment of peptide 5 from Bet v 1 with the corresponding peptide sequences from alder, hazel and apple showed only 55 % sequence identity between Mal d 1 and Bet v 1 in this distinct region (Gadermaier et al., 2018).

Nb32 binds to Bet v 1 with high affinity and has a slow dissociation rate

Interaction between Nb32 as representative for all three Bet v 1-specific nanobodies and Bet v 1 was assessed using surface plasmon resonance (SPR). Nb 32 binds to Bet v 1 with high affinity exhibiting a slow dissociation rate ($k_d = 2 \cdot 10^{-4}/s$). Fig 4 displays recorded and calculated curves of Nb32 binding to immobilized Bet v 1 when Nb32 was applied in lower concentrations (i.e., 0.98 nM - 15.63 nM). As could be seen recorded and calculated curves are superimposable using the 1:1 binding model indicating a monomeric binding between the nanobody and the Bet v 1. When Nb32 was administered in high concentrations (i.e., > 30 nM) it revealed that the best overlay of curves was achieved applying the bivalent analyte model suggesting a slight tendency of Nb32 to also form dimers when available above a certain threshold in solution (data not shown).

Bet v 1-specific nanobodies compete with polyclonal IgE binding for Bet v 1 and for cross-reactive Aln g 1 and Cor a 1

To check whether the isolated nanobodies can inhibit polyclonal IgE from binding to Bet v 1, plate-bound allergen was incubated with a 10-fold excess of purified nanobodies and then exposed to birch allergic patients' sera containing specific IgE. Figure 5, A shows a similar blocking capability for all nanobodies for three representative patients (Patient 1, 2 and 3). Combining all three nanobodies (i.e., each still 10-fold excess) did not lead to a stronger inhibition of IgE binding to Bet v 1. Due to the fact that all three nanobodies yielded comparable IgE binding inhibition, Nb32 served as representative nanobody for further experiments. To determine the critical nanobody titer to compete with polyclonal IgE binding for ELISA-plate immobilized Bet v 1, increasing concentrations of Nb32 were applied. Exemplified again for three patients (Patient 1, 3 and 5) it could be proven that a nanobody concentration of at least 5 µg/mL is efficient to reach the best possible inhibitory effect (Fig 5, B). Testing the cross-protectivity of Nb32, the effect of 10 µg/mL Nb32 (to ensure 10-fold molecular excess) on IgE binding to plate-bound allergens Bet v 1, Aln g 1 and Cor a 1 was investigated for a larger cohort of birch pollen allergic patients (n=19). Table I summarizes results of patients' specific IgE binding to Bet v 1, Aln g 1 and Cor a 1 pre-incubated with Nb 32 or buffer displayed as OD_{405nm} values (Table I, columns 2, 3, 5, 6 and 8, 9, respectively). Nb32 caused a mean inhibition of IgE binding to Bet v 1 of 56.9% ranging from 74% to 40.6%. The IgE binding to Bet v 1-related allergens was reduced to a lesser degree with mean values of 46.2% for Aln g 1 (ranging from 71% to 0%) and 13.4% for Cor a 1 (ranging from 45.7% to 0%) (Table I, column 7 and 10).

Bet v 1-specific nanobodies reduce Bet v 1-induced basophil activation

To further analyze the capacity of the Bet v 1-specific nanobodies to block Bet v 1-triggered IgE-crosslinking on basophils and hence IgE-mediated effects, basophil activation assays using RBL cells and Nb32 as representative were performed. For this purpose, RBL cells were loaded with IgE from patients' sera (n=11) and the mediator release induced by Bet v 1 compared to Bet v 1 complexed with Nb32 was measured. Figure 6, A - K shows that Nb32 is able to reduce basophil activation in all

tested patients but to a varying degree. While the reduction of β -hexosaminidase release is more pronounced for 4 of 11 patients (i.e., patient 3, 4, 9 and 11) ranging between 30-fold (i.e., patient 11) to three-fold (i.e., patient 3), Nb32 has limited effect for all other 7 tested representative patients. These results revealed that Nb32 is a potential competitor for polyclonal IgE binding for certain but not all birch pollen allergic patients. These data further confirmed earlier observations (Focke et al., 2004) that beside this prominent IgE epitope on the C-terminus of Bet v 1, several additional major IgE epitopes on Bet v 1 must be blocked to achieve efficient prevention of mediator release. Figure 6, L summarizes the levels of β -hexosaminidase release of RBL cells not loaded with IgE antibodies but induced by Bet v 1 or Bet v 1 pre-incubated with Nb32 for each single assay.

DISCUSSION

Our study is the first to report the immunological characterization of Bet v 1-specific nanobodies. Three nanobodies (Nb23, Nb24, Nb32) were isolated from an immune library derived from a camel that was immunized with recombinant Bet v 1 and synthetic Bet v 1-derived peptides. The immunization induced high affinity nanobodies that not only recognize Bet v 1 but also homologous pollen allergens from alder and hazel. Since Bet v 1 exhibits a broad cross-reactivity (Biedermann et al., 2019), the ability of an antibody to bind those cross-reactive allergens is crucial for developing a protective treatment to reduce birch pollen allergic patients' symptoms (Kleine-Tebbe et al., 2020). Surprisingly, the generated nanobodies were not able to bind the important related food allergen Mal d 1. This may be explained by the lower sequence identity between Mal d 1 and Bet v 1 in the mapped epitope compared to the other Bet v 1-homologous allergens tested (Gadermaier et al., 2018). The nanobodies' binding site was located in the C-terminal alpha-helix of Bet v 1, which was identified as an important IgE epitope (Focke et al., 2004). Consequently, patients' IgE antibodies can be inhibited from binding to Bet v 1 by occupying this area, as shown in our inhibition assays.

However, since Bet v 1 contains multiple important IgE epitopes (Gieras et al., 2011), one single antibody or antibody fragment appears not to be sufficient to completely block IgE binding and basophil activation. In fact, several studies have already shown

that two or more antibodies are needed to fully inhibit allergen-induced basophil activation (Lebecque et al., 1997; Orengo et al., 2018; Liao et al., 2019). Nevertheless, an observed mean inhibition of 56.9% of IgE binding exhibited by Nb32 is in accordance to that of other published monoclonal Bet v 1-specific antibodies or fragments using similar in vitro assays (ranging from 20% to 77%), indicating that this Bet v 1-specific nanobody comprises great potential to represent one component of a future mixture of protective nanobodies (Visco et al., 1996; Mirza et al., 2000; Gieras et al., 2011; Gadermaier et al., 2018; Brier et al., 2018; Köhler et al., 2020).

Furthermore, the dissociation rate of Nb32 ($k_d = 2 \cdot 10^{-4}/s$) is comparable to other high affinity allergen-specific antibodies, suggesting its dissociation constant is likely to be in the nanomolar range (James et al., 2012; Madritsch et al., 2015; Gadermaier et al., 2018; Brier et al., 2018). However, this has yet to be proven experimentally.

Allergy is a new field of application for nanobodies, despite being researched for almost 30 years. To our knowledge, only one other allergen-specific nanobody has been described yet (Chen et al., 2019). This nanobody with specificity to the major peanut allergen Ara h 3 has, however, not been explored in regards to IgE blocking capacity. Hence, it is not envisioned as a potential allergy treatment, but rather as diagnostic tool for peanut allergy detection. Therefore, we are the first to evaluate isolated allergen-specific nanobodies for their qualities to be useful tools for passive treatment of allergy. The finding that all three isolated nanobodies bind to the same epitope is certainly a limitation of our study due to the fact that several important epitopes are present on Bet v 1. The investigation and adjustment of half-life times have also to be considered before taking further steps towards pre-clinical trials, since nanobodies are known to be rapidly cleared from the body as a result of their size (Jovceska & Muyldermans, 2020). Nevertheless, our preliminary results point to Nb32 being a suitable first candidate that may be used in a nanobody cocktail to ameliorate symptoms of birch pollen allergic patients.

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REFERENCES

1. Biedermann T, Winther L, Till SJ, Panzner P, Knulst A, Valovirta E. Birch pollen allergy in Europe. *Allergy* 2019;74:1237-1248. doi: 10.1111/all.13758
2. Kiotseridis H, Cilio CM, Bjermer L, Aurivillius M, Jacobsson H, Dahl Å, et al. Quality of life in children and adolescents with respiratory allergy, assessed with a generic and disease-specific instrument. *Clin Respir J* 2013;7:168-75. doi: 10.1111/j.1752-699X.2012.00298.x
3. Zuberbier T, Lötvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2)LEN review. *Allergy* 2014;69:1275-9. doi: 10.1111/all.12470
4. Jarolim E, Rumpold H, Endler AT, Ebner H, Breitenbach M, Scheiner O, et al. IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. *Allergy* 1989;44:385-95. doi: 10.1111/j.1398-9995.1989.tb04169.x
5. Treudler R, Simon JC. Pollen-related food allergy: an update. *Allergo J Int* 2017;26:273–282. doi: 10.1007/s40629-017-0022-2
6. Jensen-Jarolim E. Happy 25th birthday, Bet v 1! *World Allergy Organ J* 2014;7:14. doi: 10.1186/1939-4551-7-14
7. Valenta R, Karaulov A, Niederberger V, Gattinger P, van Hage M, Flicker S, et al. Molecular Aspects of Allergens and Allergy. *Adv Immunol* 2018;138:195-256. doi: 10.1016/bs.ai.2018.03.002
8. Moingeon P, Floch VB, Airouche S, Baron-Bodo V, Nony E, Mascarell L. Allergen immunotherapy for birch pollen-allergic patients: recent advances. *Immunotherapy* 2016;8:555-67. doi: 10.2217/imt-2015-0027

9. Linhart B, Valenta R. Mechanisms underlying allergy vaccination with recombinant hypoallergenic allergen derivatives. *Vaccine* 2012;30:4328-35. doi: 10.1016/j.vaccine.2011.11.011
10. Mutschlechner S, Deifl S, Bohle B. Genetic allergen modification in the development of novel approaches to specific immunotherapy. *Clin Exp Allergy* 2009;39:1635-42. doi: 10.1111/j.1365-2222.2009.03317.x
- Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, et al. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci U S A* 2004;101 Suppl 2(Suppl 2):14677-82. doi: 10.1073/pnas.0404735101
12. Gafvelin G, Thunberg S, Kronqvist M, Grönlund H, Grönneberg R, Troye-Blomberg M, et al. Cytokine and antibody responses in birch-pollen-allergic patients treated with genetically modified derivatives of the major birch pollen allergen Bet v 1. *Int Arch Allergy Immunol* 2005;138:59-66. doi: 10.1159/000087358
13. Dorofeeva Y, Shilovskiy I, Tulaeva I, Focke-Tejkl M, Flicker S, Kudlay D, Khaitov M, Karsonova A, Riabova K, Karaulov A, Khanferyan R, Pickl WF, Wekerle T, Valenta R. Past, present, and future of allergen immunotherapy vaccines. *Allergy* 2021;76:131-149. doi: 10.1111/all.14300
14. Zhernov Y, Curin M, Khaitov M, Karaulov A, Valenta R. Recombinant allergens for immunotherapy: state of the art. *Curr Opin Allergy Clin Immunol*. 2019;19:402-414. doi: 10.1097/ACI.0000000000000536
15. Shamji MH, Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. *J Allergy Clin Immunol*. 2017 Dec;140(6):1485-1498. doi: 10.1016/j.jaci.2017.10.010
16. Orengo JM, Radin AR, Kamat V, Badithe A, Ben LH, Bennett BL, et al. Treating cat allergy with monoclonal IgG antibodies that bind allergen and prevent IgE engagement. *Nat commun* 2018;9:1421. doi: 10.1038/s41467-018-03636-8
17. Bannas P, Hambach J, Koch-Nolte F. Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. *Front Immunol* 2017;8:1603. doi: 10.3389/fimmu.2017.01603427

18. Jovceska I, Muyldermans S. The therapeutic potential of nanobodies. *BioDrugs* 2020;34:11–26. doi: 10.1007/s40259-019-00392-z
19. Sroga P, Safronetz D, Stein DR. Nanobodies: a new approach for the diagnosis and treatment of viral infectious diseases. *Future Virol* 2020;15(3). doi: 10.2217/fvl-2019-0167
20. Muyldermans S. A guide to: generation and design of nanobodies. *FEBS J.* 2020. doi: 10.1111/febs.15515. Epub ahead of print
21. Muyldermans S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem.* 2013;82:775-97. doi: 10.1146/annurev-biochem-063011-092449
22. Flicker S, Zettl I, Tillib SV. Nanobodies - Useful Tools for Allergy Treatment? *Front Immunol.* 2020;11:576255. doi: 10.3389/fimmu.2020.576255
23. Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest* 1997;99:1673-81. doi: 10.1172/JCI119330
24. Focke M, Linhart B, Hartl A, Wiedermann U, Sperr WR, Valent P, et al. Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. *Clin Exp Allergy* 2004;34:1525-33. doi: 10.1111/j.1365-2222.2004.02081.x
25. Adrian TE. Production of antisera using peptide conjugates. *The Protein Protocols Handbook*, ed. Walker JM, Humana Press Inc., 2002. P. 953-961. doi: 10.1385/1-59259-169-8:953
26. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. *Nature* 1993; 363:446–448. doi: 10.1038/363446a0
27. Tillib SV, Ivanova TI, Vasilev LA. Fingerprint-like analysis of "nanoantibody" selection by phage display using two helper phage variants. *Acta Naturae* 2010;2:85–93

28. Tillib S, Ivanova TI, Vasilev LA, Rutovskaya MV, Saakyan SA, Gribova IY, et al. Formatted single-domain antibodies can protect mice against infection with influenza virus (H5N2). *Antiviral Res* 2013;97:245-54. doi: 10.1016/j.antiviral.2012.12.014
29. Tillib SV, Privezentseva ME, Ivanova TI, Vasilev LF, Efimov GA, Gursky YG, et al. Single-domain antibody-based ligands for immunoaffinity separation of recombinant human lactoferrin from the goat lactoferrin of transgenic goat milk. *J Chromatogr B Analyt Technol Biomed Life Sci* 2014;949-950:48-57. doi: 10.1016/j.jchromb.2013.12.034
30. Pardon E, Laeremans T, Triest S, Rasmussen SG, Wohlkönig A, Ruf A, et al. A general protocol for the generation of Nanobodies for structural biology. *Nat Protoc* 2014;9:674-693. doi: 10.1038/nprot.2014.039
31. Arbabi Ghahroudi M, Desmyter A, Wyns L, Hamers R, Muyldermans S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett.* 1997;414:521-6. doi: 10.1016/s0014-5793(97)01062-4
32. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Edn. 2. Cold Spring Harbor, N.Y.; Cold Spring Harbor Laboratory Press, 1989. ISBN 0-87969-136-0
33. Conrath KE, Lauwereys M, Galleni M, Matagne A, Frere JM, Kinne J, et al. Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. *Antimicrob Agents Chemother* 2001;45:2807-2812. doi: 10.1128/AAC.45.10.2807-2812.2001.
34. Barbas III CF, Burton DR, Scott JK, Silverman GJ. *Phage Display: A Laboratory Manual*. Cold Spring Harbor, N.Y.; Cold Spring Harbor Laboratory Press, 2001. ISBN 978-087969740-2
35. Nakamura R, Uchida Y, Huguchi M, Nakamura R, Tsuge I, Urisu A, et al. A convenient and sensitive allergy test: IgE crosslinking-induced luciferase expression in cultured mast cells. *Allergy* 2010;65:1266-1273. doi: 10.1111/j.1398-9995.2010.02363.x.

36. Vogel L, Lüttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005;60:1021-8. doi: 10.1111/j.1398-9995.2005.00803.x.
37. Kleine-Tebbe J, Zuberbier T, Werfel T, Krüll M, Wagenmann M, Johansen N, et al. Is allergy immunotherapy with birch sufficient to treat patients allergic to pollen of tree species of the birch homologous group? *Allergy* 2020;75:1327-1336. doi: 10.1111/all.14130
38. Gadermaier E, Marth K, Lupinek C, Campana R, Hofer G, Blatt K, et al., Isolation of a high-affinity Bet v 1-specific IgG-derived ScFv from a subject vaccinated with hypoallergenic Bet v 1 fragments. *Allergy* 2018;73:1425-1435. doi: 10.1111/all.13394
39. Gieras A, Cejka P, Blatt K, Focke-Tejkl M, Linhart B, Flicker S, Stoecklinger A, et al. Mapping of conformational IgE epitopes with peptide-specific monoclonal antibodies reveals simultaneous binding of different IgE antibodies to a surface patch on the major birch pollen allergen, Bet v 1. *J Immunol* 2011;186:5333-44. doi: 10.4049/jimmunol.1000804
40. Lebecque S, Dolecek C, Laffer S, Visco V, Denépoux S, Pin JJ, et al. Immunologic characterization of monoclonal antibodies that modulate human IgE binding to the major birch pollen allergen Bet v 1. *J Allergy Clin Immunol* 1997;99:374-84. doi: 10.1016/s0091-6749(97)70056-3
41. Liao S, Patil SU, Shreffler WG, Dreskin SC, Chen X. Human monoclonal antibodies to Ara h 2 inhibit allergen-induced, IgE-mediated cell activation. *Clin Exp Allergy* 2019;49:1154-1157. doi: 10.1111/cea.13442
42. Visco V, Dolecek C, Denépoux S, Le Mao J, Guret C, Rousset F, et al. Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. *J Immunol* 1996;157:956-62.
43. Mirza O, Henriksen A, Ipsen H, Larsen JN, Wissenbach M, Spangfort MD, et al. Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J Immunol* 2000;165:331-8. doi: 10.4049/jimmunol.165.1.331

44. Brier S, Le Mignon M, Jain K, Lebrun C, Peurois F, Kellenberger C, et al. Characterization of epitope specificities of reference antibodies used for the quantification of the birch pollen allergen Bet v 1. *Allergy* 2018;73:1032-1040. doi: 10.1111/all.13364
45. Köhler VK, Crescioli S, Fazekas-Singer J, Bax HJ, Hofer G, Pranger CL, et al. Filling the Antibody Pipeline in Allergy: PIPE Cloning of IgE, IgG1 and IgG4 against the Major Birch Pollen Allergen Bet v 1. *Int J Mol Sci* 2020;21:5693. doi: 10.3390/ijms21165693
46. James LK, Bowen H, Calvert RA, Dodev TS, Shamji MH, Beavil AJ, McDonnell JM, Durham SR, Gould HJ. Allergen specificity of IgG(4)-expressing B cells in patients with grass pollen allergy undergoing immunotherapy. *J Allergy Clin Immunol* 2012;130:663-670.e3. doi: 10.1016/j.jaci.2012.04.006
47. Madritsch C, Gadermaier E, Roder UW, Lupinek C, Valenta R, Flicker S. High-density IgE recognition of the major grass pollen allergen Phl p 1 revealed with single-chain IgE antibody fragments obtained by combinatorial cloning. *J Immunol*. 2015;194:2069-78. doi: 10.4049/jimmunol.1402185
48. Chen F, Ma H, Li Y, Wang H, Samad A, Zhou J, et al. Screening of nanobody specific for peanut major allergen Ara h 3 by phage display. *J Agric Food Chem* 2019;67:11219-711 11229. doi: 10.1021/acs.jafc.9b02388

Figures and Tables

FIG 1. The camel immunization for the generation of anti-Bet v 1 antibodies. **(A)** A time course of the camel's immunization and of taking the pre-immune and immune blood for sera preparations are indicated. The time point of the isolation of mononuclear blood cells for the construction of the cDNA-VHH-library is marked. **(B)** Development of Bet v 1- specific IgG responses in the serum of the immunized camel after five immunizations with Bet v 1 and Bet v 1-derived peptides. OD values (y-axis) correspond to the amount of bound IgG antibodies and are shown as mean of duplicates with a variation of less than 5%.

FIG 2. **(A)** Coomassie brilliant blue-stained SDS-PAGE of Bet v 1-specific nanobodies under reducing conditions and **(B)** nitrocellulose-blotted Bet v 1-specific nanobodies detected with HRP-labeled mouse anti-HA-tag antibody. Lane 1: protein molecular mass marker; lane 2: purified Nb23, 0.5 µg; lane 3: purified Nb24, 1 µg; lane 4: purified Nb32, 1 µg. Molecular masses (in kDa) are indicated on the left margin.

FIG 3. **(A)** Reactivity of Nb23, Nb24 and Nb32 to plate-bound Bet v 1 and related allergens from alder (Aln g 1), hazel (Cor a 1) and apple (Mal d 1). **(B)** Reactivity of Nb23, Nb24 and Nb32 to two Bet v 1 fragments (F1 and F2) and **(C)** to six Bet v 1-derived peptides (P1-6). OD values (y-axis) correspond to the amount of bound nanobodies and are shown as mean of triplicates ± SDs.

FIG 4. Sensor chip-based study of the interaction between Bet v 1 and Bet v 1-specific nanobody Nb32. Bet v 1 was immobilized on the chip and Nb32 was injected in two-fold increasing concentrations from 0.98 nM to 15.63 nM. Recorded (colored lines) and calculated (black lines) curves, which represent fittings of the data to a 1:1 binding model, were superimposed. Signal intensities (RU) are displayed (y-axis) versus time (in seconds; x-axis). The dissociation rate constant (k_d) is indicated.

FIG 5. Pilot experiments for inhibition ELISAs. **(A)** The capacity of Nb23, Nb24, Nb32 and of their combination (Nb23+24+32) to inhibit patients' IgE binding (patient 1-3) to ELISA-bound Bet v 1 was determined and compared to IgE binding without hindrance (buffer). OD values (y-axis) corresponding to the amount of bound IgE are displayed as means of duplicates. **(B)** Titration of Nb32 (x-axis: two-fold increasing concentrations starting from 0.156 µg/mL to 20 µg/mL) to determine the critical nanobody concentration for prevention of IgE binding exemplified for three patients (Patient 1, 3 and 5). Shown OD values correspond to bound IgE and are displayed as means of triplicates ± SDs.

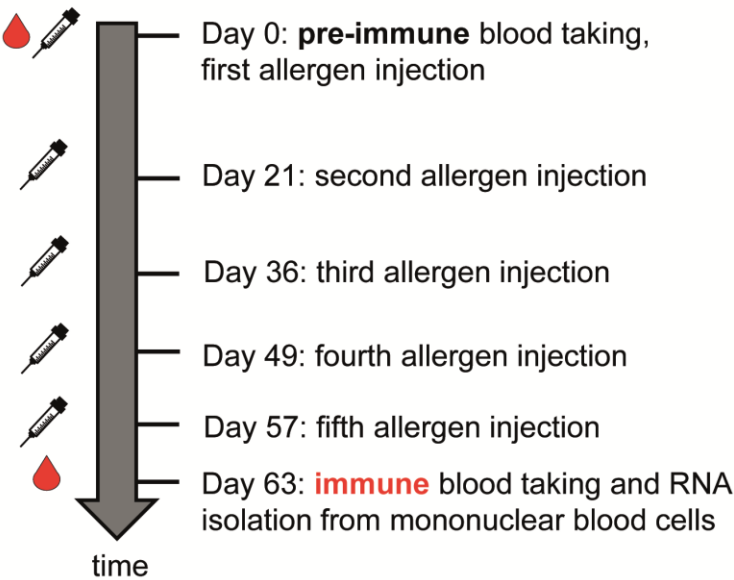
TABLE I: Inhibition of birch allergic patients' IgE binding to Bet v 1, Aln g 1 and Cor a 1 which had been pre-incubated with Nb32 or buffer. ELISA-plate bound allergens were pre-incubated with Nb32 or buffer and subsequently exposed to patients' sera. Displayed OD values correspond to allergen-bound IgE antibodies and are shown as mean of triplicates. Percentage of reduction of IgE-reactivity to Bet v 1, Aln g 1 and Cor a 1 pre-incubated with Nb32 in comparison to buffer are indicated in columns 4, 7 and 10, respectively.

FIG 6. Rat basophil leukemia (RBL) cell assay to determine the potential of Nb32 to suppress Bet v 1-induced IgE-mediated degranulation of basophils. RBL cells transfected with human FcεRI were loaded with sera from 11 birch pollen allergic patients (A-K). Decreasing concentrations of Bet v 1 (x-axes: 0.01 nM, 0.0033 nM, 0.0011 nM, 0.00037 nM) were preincubated with Nb32 (green lines) or buffer (black lines) for two hours at room temperature and then added to the IgE-loaded cells. The percentage of β-hexosaminidase release induced by Bet v 1 is displayed on the y-axes in relation to total β-hexosaminidase amount of lysed cells. Control experiments were performed for each assay (x-axis: graph A-K) with cells that have not been loaded with IgE, being exposed to 0.01 nM Bet v 1 preincubated with Nb32 (black line) or buffer (grey line) to evaluate background release (L). Values are shown as means of triplicates ± SDs.

FIG. 1

A

Timeline of immunization



B

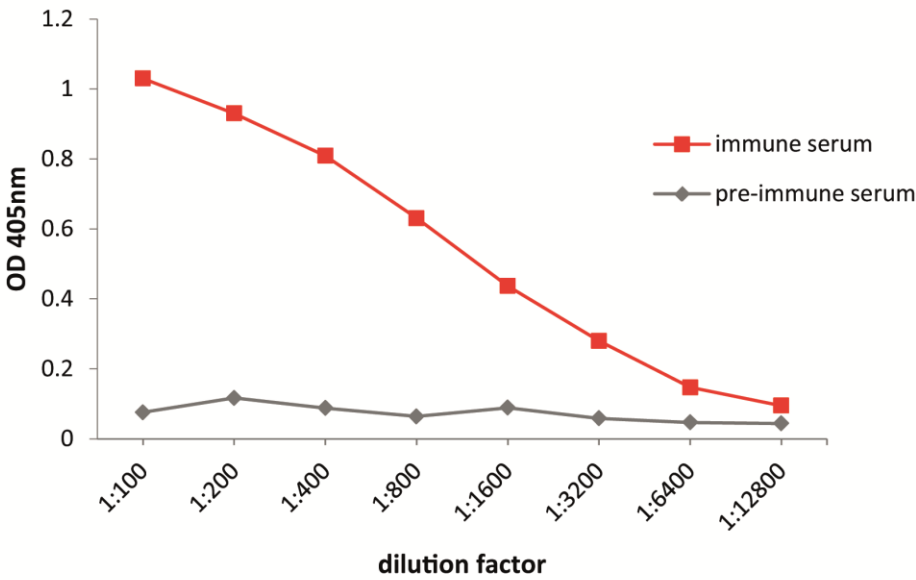
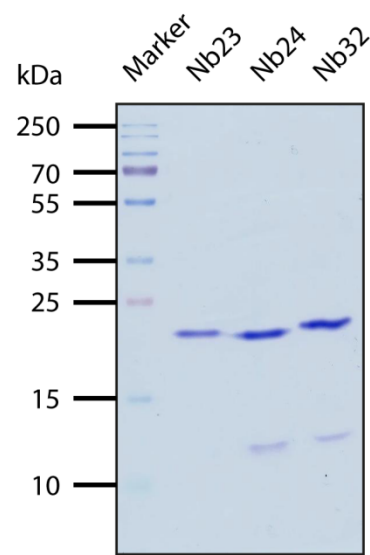


FIG. 2

A



B

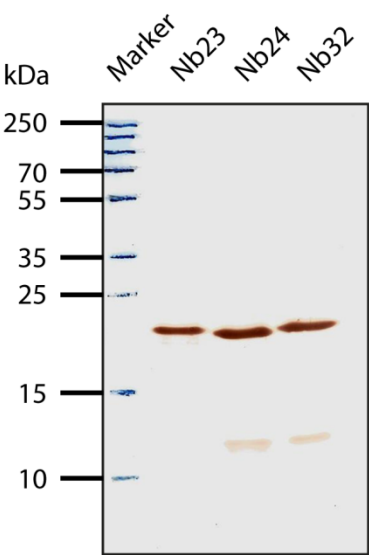
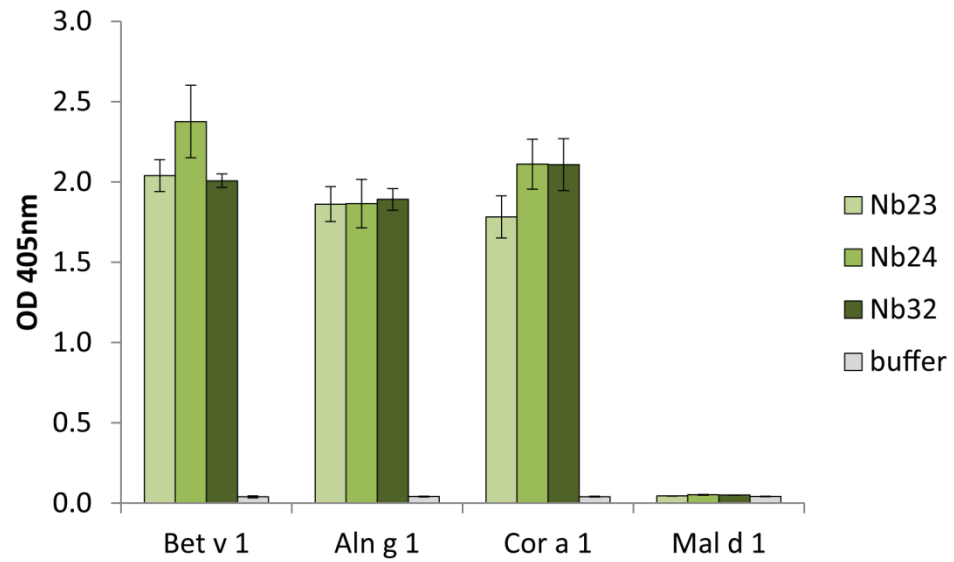
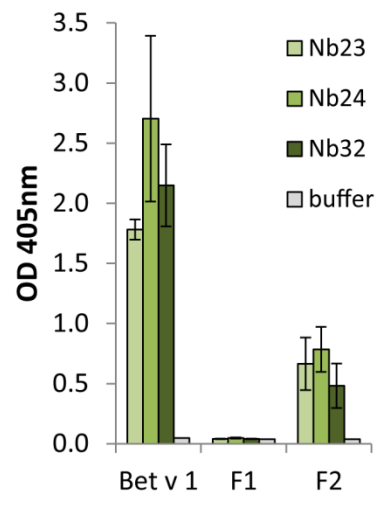


FIG. 3

A



B



C

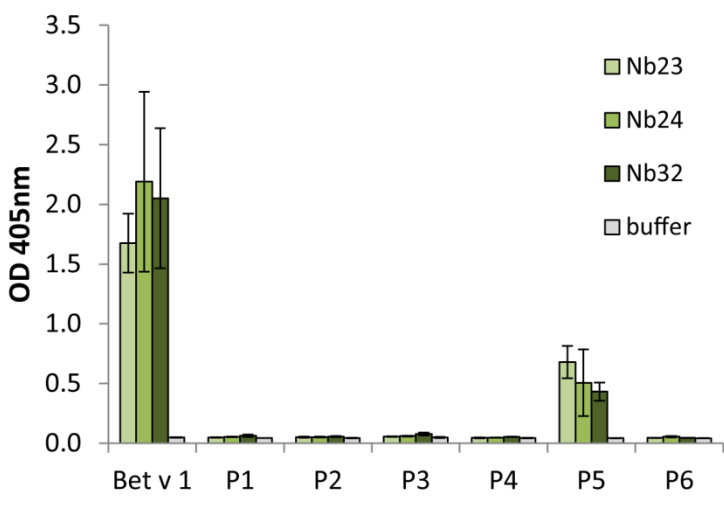


FIG. 4

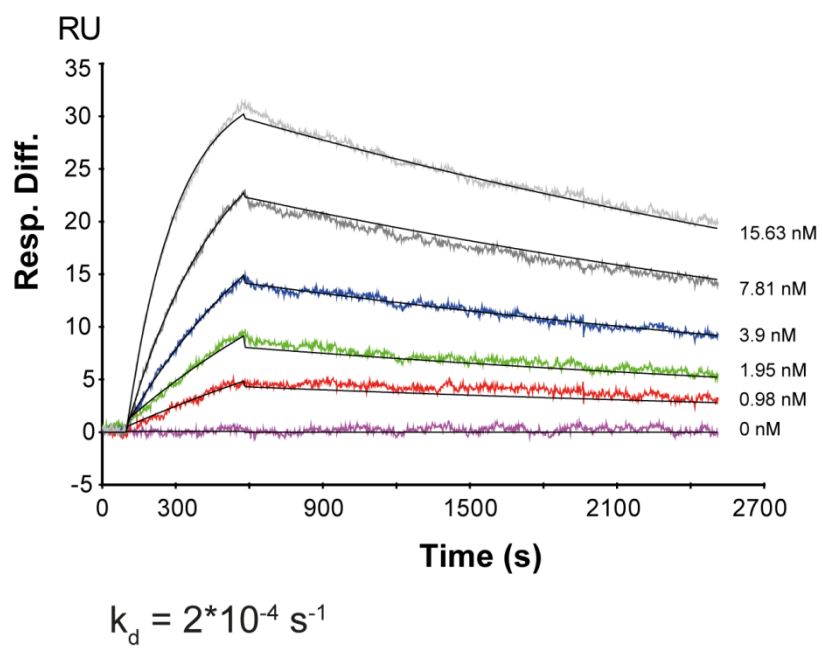
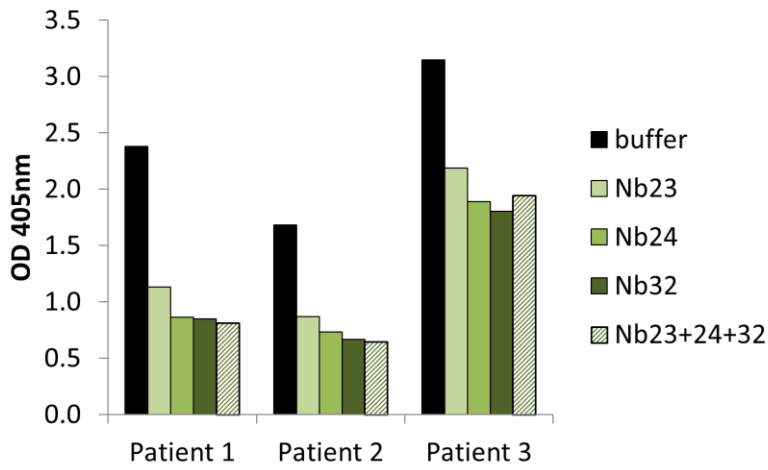


FIG. 5

A



B

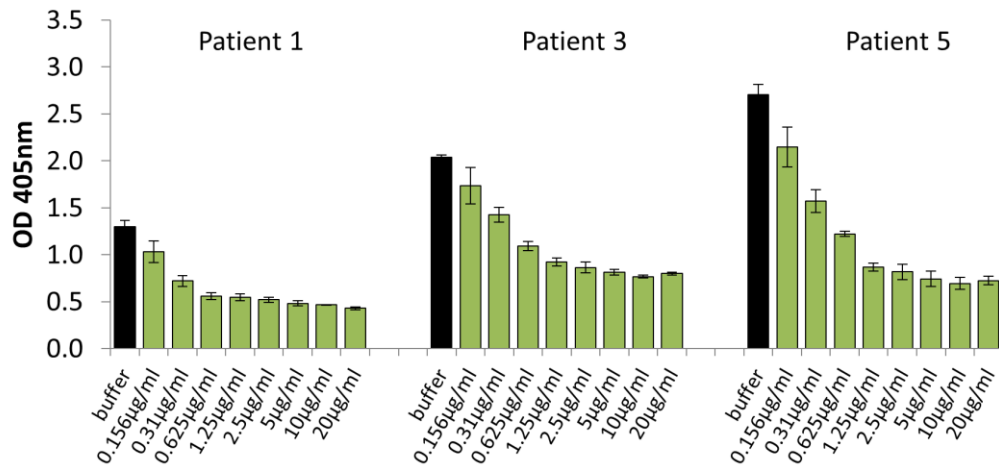
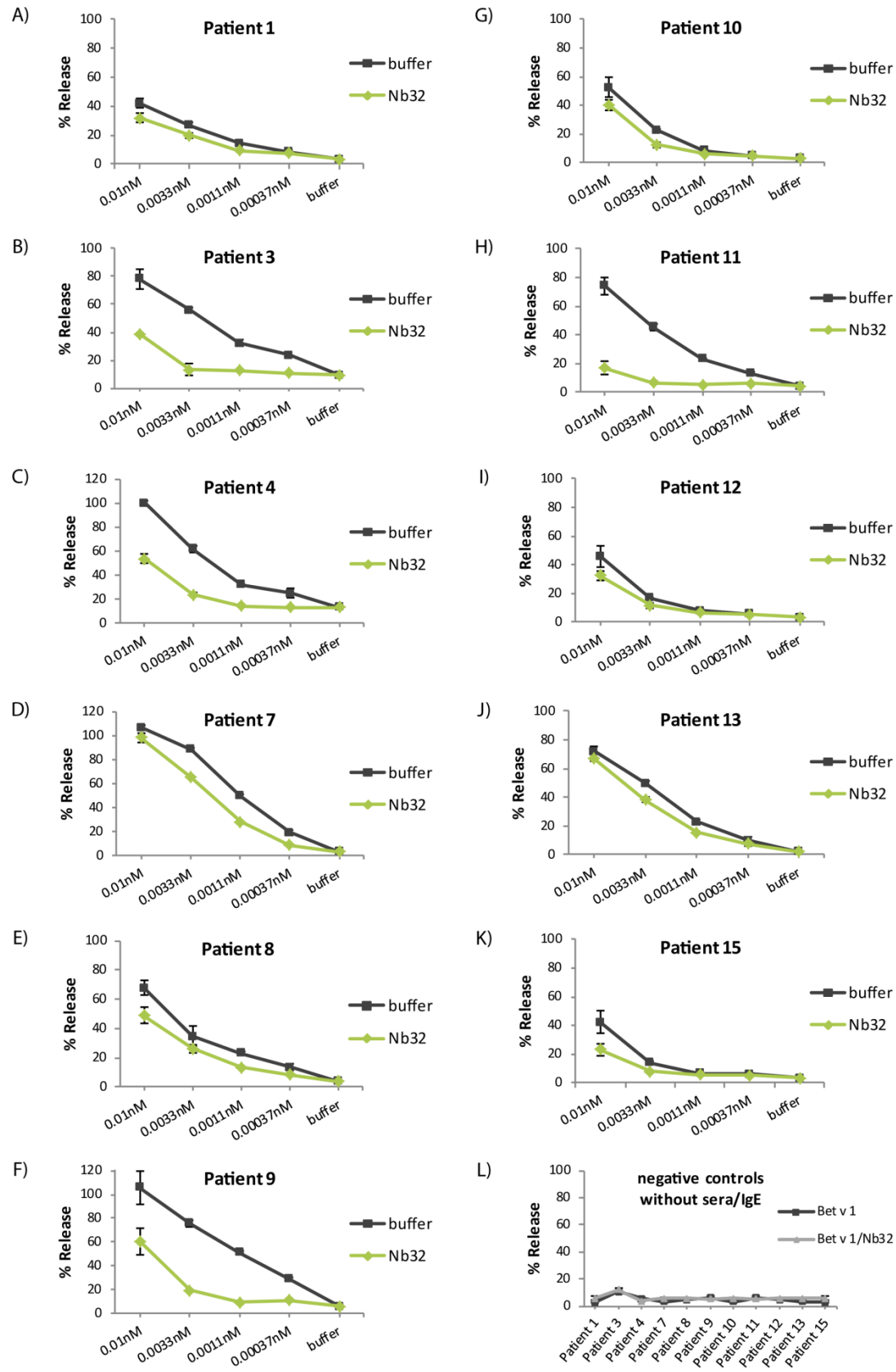


TABLE I

	% Inhibition			% Inhibition			% Inhibition		
	Nb32	buffer	to Bet v 1	Nb32	buffer	to A l n g 1	Nb32	buffer	to Cor a 1
Patient 1	0.662	2.129	68.9	0.699	1.342	47.9	0.743	0.957	22.4
Patient 2	0.492	1.300	62.1	0.162	0.307	47.1	0.301	0.426	29.4
Patient 3	0.852	2.240	62.0	0.390	0.481	18.9	0.705	0.578	0.0
Patient 4	0.948	1.748	45.7	0.464	0.766	39.4	1.194	1.309	8.8
Patient 5	0.899	2.562	64.9	0.365	0.928	60.7	0.972	1.093	11.1
Patient 6	1.050	3.555	70.5	0.753	2.150	65.0	0.959	1.767	45.7
Patient 7	0.693	2.702	74.3	0.453	0.747	39.3	0.443	0.408	0.0
Patient 8	0.521	1.942	73.2	0.114	0.395	71.0	1.108	1.476	24.9
Patient 9	1.087	2.723	60.1	0.400	1.050	61.9	1.405	1.644	14.5
Patient 10	0.319	0.658	51.5	0.255	0.478	46.7	0.371	0.420	11.7
Patient 11	0.630	1.096	42.5	0.117	0.304	61.4	0.412	0.422	2.3
Patient 12	0.589	1.007	41.5	0.185	0.253	26.8	0.411	0.480	14.4
Patient 13	0.867	1.458	40.6	0.123	0.383	67.8	0.772	0.816	5.4
Patient 14	1.963	3.750	47.7	0.449	1.284	65.0	2.211	2.529	12.6
Patient 15	0.711	1.292	45.0	0.309	0.218	0.0	0.322	0.223	0.0
Patient 16	0.275	0.541	49.2	0.084	0.117	28.0	0.292	0.280	0.0
Patient 17	0.394	0.795	50.4	0.089	0.093	4.3	0.198	0.170	0.0
Patient 18	1.637	3.850	57.5	0.668	1.939	65.6	0.818	1.073	23.8
Patient 19	0.696	2.680	74.0	0.456	1.176	61.2	0.879	1.212	27.5
Nonallergic	0.083	0.047		0.069	0.056		0.062	0.055	
Mean			56.9			46.2			13.4

FIG. 6



5. APPENDIX

Zusammenfassung

Typ I Allergie ist ein immer größer werdendes Gesundheitsproblem, das fast 30% der Menschen auf der ganzen Welt betrifft. Vor allem in der nördlichen Hemisphäre sind Birkenpollen eine weit verbreitete Allergenquelle, die allergische Symptome wie Heuschnupfen oder Asthma in über 100 Mio. Patienten auslöst und dadurch die Lebensqualität verringert.

Zurzeit werden zwei Behandlungsformen angeboten: symptomatische Medikamente (z.B. Antihistaminika) und allergen-spezifische Immuntherapie (AIT). Während Antihistaminika nur einen temporären Effekt haben, bietet AIT einen lang anhaltenden Schutz durch blockierende IgG Antikörpern, die nach der Injektion von Allergenextrakten oder, seit kurzem, Hypoallergenen gebildet werden. AIT wird allerdings mit einem aufwändigen Zeitplan für die Injektionen über mehrere Jahre sowie möglichen allergische Reaktionen, wenn Allergenextrakte verwendet werden, in Verbindung gebracht.

Neben symptomatischer Medikation und AIT gibt es noch eine dritte Option, nämlich passive Immunisierung, die das Interesse von Forschern geweckt hat. Erst kürzlich wurde gezeigt, dass die Verabreichung von allergen-spezifischen monoklonalen IgG Antikörpern die Bindung von IgE Antikörpern an das entsprechende Allergen inhibiert, weiters IgE-vermittelte Effekte an verschiedensten Immunzellen verhindert und somit allergische Symptome reduziert. Da die Herstellung von monoklonalen IgG Antikörpern aufwändig und teuer ist, wurde eine willkommene Alternative in Form von Nanobodies gefunden. Nanobodies stammen von „heavy-chain antibodies“ (Antikörper, die nur aus schweren Ketten bestehen) ab, die im Blut von Kameliden (z.B. Kamele und Lamas) entdeckt wurden. Diese Einzeldomänen-Antikörperfragmente sind chemisch stabil, ohne großem Aufwand zu produzieren und erkennen eine Vielzahl an verschiedenen Epitopen, was auf ihre strukturellen Eigenschaften zurückzuführen ist. Des Weiteren sind sie hochspezifisch und hochaffin.

Das Ziel dieser Arbeit war die Charakterisierung von Nanobodies, die spezifisch für das Hauptbirkenpollenallergen Bet v 1 sind, um ihre Anwendung als passive Behandlungsmethode von Allergie zu evaluieren. Nanobodies wurden durch die Immunisierung eines Kamels gebildet, isoliert und in *E. coli* exprimiert. Die Spezifität, Kreuzreaktivität und die Position des Epitops wurden durch ELISAs ermittelt. Die Affinität wurde mittels Oberflächenplasmonenresonance (surface plasmon resonance, SPR) gemessen. Schließlich wurden Inhibitions-ELISAs und RBL cell assays durchgeführt, um zu untersuchen, ob die Nanobodies die Bindung von polyklonalen IgE Antikörpern aus Patientenseren an Bet v 1 inhibieren und Bet v 1-induzierte Aktivierung von Basophilen verhindern können.

Es wurden drei hochaffine Bet v 1-spezifische Nanobodies (Nb23, Nb24 und Nb32) isoliert, die außerdem zwei wichtige kreuzreaktive Allergene erkennen: Aln g 1 aus der Erle und Cor a 1 aus der Hasel. Als Bindungsstelle für die Nanobodies auf Bet v 1 wurde die C-terminale α -Helix identifiziert. Alle drei Nanobodies können die Bindung von polyklonalem IgE an Bet v 1, Aln g 1 und Cor a 1 inhibieren. Des Weiteren reduziert Nb32, repräsentativ für alle drei Nanobodies, die Aktivierung von Basophilen durch Bet v 1.

Zusammenfassend lässt sich sagen, dass sich Nb32 aufgrund seiner hoher Affinität, Kreuzreaktivität und die Fähigkeit IgE-Bindungen an Bet v 1 zu inhibieren als potenzieller Kandidat für eine zukünftige Behandlungsmethode von Allergie qualifiziert hat. Wir schlagen Nb32 als Komponente eines Nanobody-Cocktails vor, welcher als „proof of concept“ in einem experimentellen Tiermodell untersucht werden sollte, um vielversprechende, auf Nanobodies basierende Medikamente zu identifizieren, die durch Birkenpollen hervorgerufene allergische Symptome mildern können.