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1 Abstract

Birch pollinosis is one of the most common allergic diseases in Central and Northern Europe as well as in North America. Several proteins in birch pollen have already been characterized as allergens causing sensitization and the development of allergic symptoms in atopic individuals. Recently, in a proteome profiling approach of birch pollen, high quantities of a 26 kDa protein of the Glutathione-S-transferase (GST)-family were identified. GSTs are a phylogenetically widespread enzyme family mainly involved in cellular detoxification processes and have already been described as allergens in house dust mite (Der p 8) and German cockroach (Bla g 5). However, no data about the role of birch-GST in birch pollen allergy were available so far. For this purpose we i) expressed and purified recombinant birch-GST (rGST) and ii) evaluated its immunological and biochemical features.

GST from birch pollen was expressed as a recombinant protein in *E. coli*. It was purified to homogeneity and protein-associated endotoxins were removed. Mice were immunized with recombinant birch-GST to generate specific antibodies (Abs). In parallel, another group of mice were immunized with recombinant Bet v 1 (rBet v 1), the major birch pollen allergen. Specific Ab and T cell responses were assessed. Both proteins induced specific IgG1 and IgE Abs and Th2-dominated T cell responses. Abs generated with rGST and rBet v 1 recognized the respective natural protein in birch pollen extract (BPE). RGST-specific Abs did not cross-react with house dust mite (HDM) extract.

Next, sera from 215 Austrian birch pollen-allergic individuals were tested for IgE-reactivity to birch-GST and the birch pollen minor allergens Bet v 3, Bet v 4 Bet v 6 and Bet v 7. Around 9% of the examined individuals displayed IgE-reactivity to birch-GST. Similar recognition frequencies were determined for the minor allergens.

In biochemical assays recombinant birch-GST was not enzymatically active, whereas BPE demonstrated weak enzymatic activity.

In summary, birch-GST was immunogenic in mice and induced specific Ab and T cell responses similar to the major allergen Bet v 1. However, we showed that it is still a minor allergen for humans. Whether this is due to its release from the pollen or intrinsic properties will be elucidated in future experiments.

2 List of abbreviations

Aa	amino acid
Ab	antibody
Amp	ampicillin
APC	antigen presenting cells
BCA	bicinchoninic acid assay
BLAST	basic local alignment search tool
BPE	birch pollen extract
BSA	bovine serum albumine
CBP	calcium-binding protein
CCD	carbohydrate-determinants
CD	cluster of differentiation
cpm	counts per minute
CTL	cytotoxic T lymphocytes
DC	dendritic cell
ddH_2O	double distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpm	delta counts per minute
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay

FCS	fetal calf serum
FcεRI	high-affinity receptor for IgE
GSH	glutathione
rGST	recombinant glutathione-S-transferase
HDM	house dust mite
HEK cells	human embryonic kidney cells
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IS	immune system
kDa	kilo Dalton
LB medium	lysogeny broth medium
LPS	lipopolysaccharide
mA	milliampere
MHC	major histocompatibility complex
OAS	oral allergy syndrome
O/N	over night
OD600	optical density at 600 nm
PAGE	polyacrylamid gel electrophoresis
PAMP	pathogen-associated molecular pattern

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
POX	peroxidase
PRR	pattern recognition receptor
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SIT	allergen-specific immunotherapy
SCIT	subcutaneous allergen-specific immunotherapy
SLIT	sublingual allergen-specific immunotherapy
TCR	T cell receptor
TF	transcription factor
TGF	transforming growth factor
Th	T helper cells
TLR	Toll-like receptor
TNF	tumor necrosis factor

3 Introduction

3.1 Immune system

The function of the immune system (IS) in multicellular organisms is to protect from pathogens. This involves two main features: the recognition of certain structures on pathogens and the induction of an immune response to eliminate harmful molecules or cells. Another very important property of the IS is to distinguish between foreign and the body's own patterns on cells (self-nonself discrimination). In consequence, the IS is not only able to eliminate invading pathogens but also to kill or neutralize altered host cells (tumour cells).

According to modern textbooks, we discriminate between the adaptive and the innate IS. Both systems show different characteristics but highly interact with each other. An efficient collaboration between both systems is required to combat pathogens and thus protect the organism.

3.2 Innate immune system

The innate IS is seen as a first line of defence and elicits a response within the first hours after the occurrence of an infection. Although the response mechanisms of this system are not highly specific, it poses the first barrier to pathogens. These first barriers include skin and mucosa (physical), the acidic milieu of the stomach and certain enzymes in body fluids (chemical). Moreover, specific cells belonging to the innate IS can carry out phagocytosis, a process in which harmful cells are taken up by specialized cells (monocytes, neutrophils or macrophages). Another important aspect of phagocytosis is the so-called receptor-mediated phagocytosis. In this event, pathogenic molecules bound to cellular receptors are internalized. Additionally, innate immunity also comprises the complement system, which releases serum

proteins that either kill or facilitate killing of invading microbial organisms. Cells of the innate IS (e.g. dendritic cells, macrophages) express germline-encoded pattern recognition receptors (PRRs) to detect certain structures that most pathogens have in common. PRRs bind to pattern-associated molecular patterns (PAMPs) and opsonize the invader. This event recruits cells that lyse or phagocyte the pathogen [1]. Most prominent member of PRRs are Toll-like receptors (TLRs). To date, 10 TLRs have been identified in humans and each receptor recognizes a specific structure or molecule expressed on bacteria, yeast or viruses [2]. Apart from TLRs various other PRRs such as lipopolysaccharide-binding protein (LBP), NOD (nucleotide-binding oligomerization domain) or scanvenger receptor exist. LBPs detect lipopolysaccharide derived from gram-negative bacteria, whereas NOD binds peptidoglycan present in the cell wall of gram-positive bacteria [2]. Cells encountering and recognizing PAMPs secrete various cytokines, which activate and recruit further cells enhancing the inflammatory response. Antigen presenting cells (APCs) function as a link between the innate and adaptive immune system. Pathogenic antigen is ingested by these cells, processed and displayed on the surface in the context of MHC (major histocompatibility complex) molecules. Activated APCs carrying processed antigen migrate into the lymphoid tissue and present it to cells of the adaptive IS (T lymphocytes) [3]. This process underlines the importance of the cooperation between both defence mechanisms to effectively protect the body from damage. Hence, the adaptive IS requires signals, elicited by cells belonging to the innate immunity, to become fully effective. Moreover, this event also demonstrates how both systems regulate each other [4].

3.3 Adaptive immune system

The adaptive IS is a highly variable defence strategy by means of antigen recognition. It recognizes unique differences among antigens and this feature leads to an extremely specific cellular and humoral immune response. To mount an adaptive immune response, different lymphocyte subpopulations

showing a diverse spectrum of functions are required. These cells mediate events that are pivotal for a fully effective humoral (B cell-mediated) and cellular (T cell-mediated) response. The diversity of lymphocytes is necessary to effectively combat a manifold range of antigens. Numerous gene rearrangements contribute to the vast specificity of the B cell and T cell receptor, which recognizes even very subtle differences in the antigen structure.

Apart from antigen specificity and cellular diversity, the memory function of specific lymphocytes plays a key role in the adaptive immune response. This process allows the IS to adapt efficiently and react more rapidly upon repeated (second) encounter with antigen [5, 6].

The activation of the adaptive IS involves the presentation of antigenic peptides in association with MHC molecules to naïve T lymphocytes. Before presentation, the antigens, which are either taken up in the periphery or originate from the cytosol (e.g. infected or tumour cells), are processed and converted to peptide fragments. These fragments are then loaded on MHC molecules and the peptide-MHC-complex is expressed on the cell surface. The physiological function of MHC molecules, also termed human leukocyte antigen (HLA) complex in the human system, is to display processed antigens (peptides) to T lymphocytes. MHC molecules are generally divided into two classes: MHC class I molecules are ubiquitously expressed on all nucleated cells in the human body, whereas MHC class II molecules are only found on APCs [7]. Depending on the origin of the antigen, peptides are presented by either class I (endogenous antigen) or class II (exogenous antigen) molecules.

Once professional APCs, which comprise dendritic cells (DCs), macrophages and B cells, have taken up and processed antigen, they migrate into regional lymph nodes and mature. Matured APCs mainly reside in the T cell zone of the lymph node where antigen-presentation to naïve T lymphocytes takes place. In addition, co-stimulatory signals provided by APCs, are required for the complete activation of naïve T cells. Activated T lymphocytes undergo a maturation process and differentiate into effector cells. Differentiation of

immature cells is accompanied with clonal expansion, which represents a key feature of adaptive immunity. During this event, lymphocytes divide rapidly in order to fight also increasing numbers of pathogen [5, 6].

DCs are the most effective APCs expressing a variety of co-stimulatory molecules [8]. Besides DCs and macrophages, B cells show antigen-presenting function. B cells stimulate T helper cells (Th), which again induce a humoral, B cell-associated immune response**.** Follicular dendritic cells (FDCs), a specialized cell subset not related to DCs, can also activate B cells [6].

3.3.1 B lymphocytes

B lymphocytes are mediators of the humoral immune response and their unique properties represent an integral part of the adaptive immunity. They reside as naïve B cells in the bone marrow, where they also partly mature. To become fully effective, B lymphocytes leave the bone marrow, circulate in lymphatic tissue and relocate to secondary lymphoid organs.

B cells recognize antigen by means of the B cell receptor. The B cell receptor consists of membrane-bound antibodies (Abs), which specifically bind and neutralize antigen. A unique feature of Abs is to recognize soluble antigen. Once B cells encounter antigen, they proliferate rapidly and turn into mature Ab-secreting plasma cells. Classically, plasma cells are grouped into two distinct subsets. Effector B cells, which show only little Ab bound on their surface release large quantities of Abs specific for a certain antigen. Memory B cells persist a prolonged period in the body and express certain types of Abs bound on the surface. Memory B cells are responsible for the induction of a much faster immune response (secondary response) and increased Ab secretion after repeated (secondary) antigen contact. In general, affinity maturation of B cells increases their specificity towards a particular antigen and enables the cells to clear infections more rapidly and efficiently [5, 6].

Abs represent the effector molecules of humoral immunity. They are either secreted by mature plasma cells or function as membrane-associated receptors. Five different Ab classes have been identified: IgM, IgD, IgG, IgE and IgA. All immunoglobulin (Ig) subtypes consist of the same core structure consisting of two heavy and two light chains. Igs are glycoproteins demonstrating an extremely diverse antigen-specificity caused by innumerable recombinations within Ig genes. During a humoral immune response IgM is the first Ab class to be produced. However, a certain cytokine environment provided by a specific T lymphocyte subset (T helper cells) cells can favour Ig-class switch to other Ab isotypes [5, 9, 10].

In healthy individuals IgE Abs are primarily produced after parasitic infections. However, atopic/allergic individuals classically demonstrate high IgE titers caused by an inadequate response to normally innocuous molecules (allergens) [11, 12]. In allergic diseases an Ig class switch to IgE takes place, which is promoted by a specific cytokine environment provided by T helper (Th) cells [9, 13, 14].

3.3.2 T lymphocytes

T lymphocytes represent the key mediators of cellular immunity. Various subsets of T lymphocytes are involved in the cellular immune response. CD4+ Th cells exert supportive effects on other immune cells, whereas CD8+ cells mediate cytotoxicity. The T cell receptor (TCR) complex expressed on these cells allows them to specifically recognize antigen, however, only in association with MHC molecules. T lymphocytes also show cellular memory although it is difficult to distinguish between effector and memory cells. The progeny of T lymphocytes arises in the bone marrow and migrates into the thymus where they mature.

3.3.3 T cell receptor complex

The TCR complex is a functional unit consisting of three molecules. All compounds are mandatory for T cell activation and, hence, to initiate a complete cellular immune response. The T cell receptor itself comprises two transmembrane polypeptides, which are covalently linked by disulfide bonds. In almost all T lymphocytes α and β polypeptide chains are expressed, while γ and δ T cell receptor heterodimers are only found in 5% of the T lymphocyte population. The polypeptide chains are closely associated with the membrane protein CD3, which is characteristically expressed on T cells. Furthermore, in a fully functional TCR complex another polypeptide, the ζ chain, is integrated. Similar to the B cell receptor, individual TCRs originating from a single clone exhibit the same specificity. However, the strength of antigen binding by the T cell receptor complex is lower compared to antibody-antigen binding.

The recognition of peptides incorporated in the MHC molecule entity by the TCR complex is required to activate T cells. However, self-MHC restriction allows T cells to recognize antigen presented by self-MHC molecules. Peptides (antigen) displayed on allogeneic (foreign) MHC molecules in combination with self-MHC do not induce a T cell response in healthy individuals. T cells are able to recognize even minor differences in MHC molecules and this feature is crucial for self/non-self discrimination. Additionally, certain T cells subsets are also restricted to different types of MHC molecules (MHC class I and MHC class II). However, T cells that express the γδ receptor are not MHC class restricted and, therefore, do not require antigen presentation by MHC [15]. In contrast to αβ T cells, this lymphocyte subtype can bind native antigen as well as lipids or phosphorylated molecules found on microbes [16, 17].

3.3.4 Cytotoxic T cells

Cytotoxic T cells (CTLs) are defined by the expression of CD8 on the cell surface. The main function of this lymphocyte subpopulation is to eliminate infected (by viruses or bacteria) or altered (tumour) cells. These cells contain allogeneic protein/antigen, which is consequently degraded by proteasome into peptides and presented in the context of MHC class I. CTLs are restricted to MHC class I-associated antigens. To clear degenerated cells, CTLs form close complexes with the target cell and release cytotoxic mediators such as perforins or granzymes, which induce apoptosis in the target cell.

For differentiation of naïve CTLs into effector CTLs, the presentation of peptide bound to MHC class II by professional APCs is required. Beyond that, complete activation is achieved by the interaction of co-stimulatory molecules (CD40–CD154) expressed on both cell types. Activating cytokines such as IL-12 released by CD4+ Th cells, foster this process [5, 6, 18].

3.3.5 T helper cells

This T lymphocyte subset was originally termed helper cells because they exhibit supportive effects on other cells of the immune system. Th cells show a restriction to antigen presented only in association with MHC class II and characteristically express the co-receptor CD4. Both features make these immune cells distinguishable from the CTL subset. Th cells residing in secondary lymphoid organs are activated by APCs displaying MHC-bound antigen. Additionally, complete activation requires the involvement of certain co-stimuli. Antigen-presenting DCs express high levels the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86), which both interact with the Th cell co-stimulus CD28 [19]. The absence of a co-stimulus leads to induced cell death (apoptosis) or promotes T cells anergy. Activated Th cells produce IL-2, which concomitantly upregulates the expression of IL-2 receptor. This autocrine-signalling loop promotes proliferation and clonal expansion of CD4+

T cells. To maintain homeostasis, most of the cells that have proliferated die by apoptosis after clearance of pathogen. However, some Th cells differentiate into either effector CD4+ Th cells or memory cells. Effector Th cells can support and stimulate B cells, macrophages or CTLs resulting in a fully-fledged immune response. The activation of various immune cells by Th cells is driven by a certain cytokine environment, which is representative for distinct Th subsets [5, 6].

In the 1980s *Mosmann et al.,* first discriminated between two functional distinct Th cell subtypes and established the Th1/Th2 paradigm [20, 21]. In this model the type of infection and, in particular, the cytokines secreted define the respective CD4+ Th subset. However, in autoimmune diseases contradictory observations were made and could not be completely explained by the Th1/Th2 model. The identification of new Th subset led to the adaption and expansion of the Th1/Th2 paradigm [22].

3.3.6 T helper 1 cells

Th1 cells are mostly associated with immune responses directed against intracellular bacteria or viruses. This Th subset predominantly produces IL-2, Interferon (IFN)-γ, tumour necrosis factor (TNF)-α and favours a mainly IgG-driven humoral immune response, especially the IgG2a isotype in mice [23]. It was shown that Th1 cells releasing the above-mentioned cytokines are involved in delayed type hypersensitivity and chronic inflammation (autoimmune diseases). DCs and macrophages secreting IL-12, IFN-γ or IL-18 promote the differentiation of naïve Th cells into the Th1 subset [6]. However, these cells are not terminally committed since they can differentiate into other Th subsets in the respective cytokine milieu [24].

3.3.7 T helper 2 cells

IL-4, IL-5, IL-13 have been designated as Th2 signature cytokines. These cytokines induce the activation of certain cell subsets (eosinophils, mast cells) and support Ig class switch from IgM to IgE. Th2 cells characteristically express the transcription factor (TF) GATA-3, which is upregulated upon production of Th2-associated cytokines. In particular, IL-4 promotes the differentiation of naïve CD4+ Th cells into the Th2 subset [25]. This can be observed even in the presence of Th1-biased cytokine environment and highlights the importance of IL-4 for Th2 differentiation. The TF STAT6 is required for IL-4 and IL-5 production and downregulates the expression of IFN-γ [26]. However, the *in vivo* situation is not that clear since it was reported that Th2-associated IL-4 producing cells can still secrete IFN-γ [27].

Typically, Th2 cells are required upon infections with helminths but are also strongly involved in the pathogenesis of allergy. In most allergic individuals a Th2-biased cytokine milieu leads to IgE class switch and the development of allergic diseases.

3.3.8 T helper 17 cells

Recently, a new CD4+ Th subset has been identified and was termed Th17 cells. Th17 cells bear characteristic features distinct from Th1/Th2 cells and express the signature cytokines IL-17A and IL-17F as well as IL-22 [28]. *In vitro*, differentiation into Th17 requires the activation of STAT3 and is independent of any Th1 or Th2-related TFs. IFN-γ and IL-4 were shown to exert a rather suppressive effect on Th17 differentiation whereas IL-6 and TGF-β enhance the differentiation process [28, 29]. Additionally, RORγt and RORα were determined as a subset specific TF [30, 31].

3.3.9 T helper 22 cells

IL-22 producing cells were identified as a distinct Th cell subset within the Th17 lineage and were designated Th22 cells [32]. They express high levels of the signature cytokine IL-22 and were reported to be associated with atopic dermatitis and psoriasis [33, 34]. In contrast the involvement of IL-22 in inflammatory skin diseases, it facilitates tissue remodelling and to down-regulates the pro-inflammatory effects IFN-γ in asthmatic patients [35, 36]. IL-22 is part of the IL-10 cytokine family and is recognized by the IL-22 receptor (IL-22R) complex consisting of IL-22R1 and IL-10R2 subunits [37]. The receptor has not been detected on immune cells, whereas it is expressed in the tissue of several organs (liver, kidney, colon) and keratinocytes [38].

3.3.10 T regulatory cells

CD4+ regulatory T cells (Tregs) were shown to effectively suppress the proliferation of other T cell populations *in vitro*. This T cell subset classically expresses high levels of the lineage defining TF Foxp3 and the surface marker CD25 [39, 40]. The suppressive effect is also mediated by cell-to-cell contact and the release of various anti-inflammatory cytokines such as IL-10 [41]. Suppression of the immune response is antigen specific since the activation of the T cell receptor is necessary [40].

The induction of Tregs and their regulatory features is an interesting principle for the treatment of allergy and autoimmune disease and has already been intensively investigated [42-45].

3.4 Hypersensitivity

The clearance of an infectious agent by the IS is characterized by a localized inflammation. However, the inflammatory response can be triggered in an inadequate way leading to opposite effects. In this case, the initially beneficial reaction is rather harmful causing extensive tissue damage, disease or even death. Gell and Coombs introduced a classification system, which discriminates four different hypersensitivity reactions according to the type of immune response and effector molecules involved. Type 1-3 is characterized by a humoral, B cell-mediated immune response, type 4 is T cell-mediated.

Type 1 hypersensitivity reactions refer to immediate reactions (immediate hypersensitivity) upon encounter with antigen since the symptoms manifest within a few minutes or hours.

In type 2 reactions IgG and IgM Abs directed against cell surface or extracellular matrix antigens lead to opsonisation. This process activates the complement system and induces Fc receptor-mediated recruitment of neutrophils or macrophages.

Type 3 is also characterized by complement activation through formation of Ag-Ab complexes in various tissues. Additionally, infiltration of neutrophils enhances the inflammatory response.

In delayed type hypersensitivity (type 4) activated Th cell secrete various cytokines (mainly Th1-associated) and chemokines. These mediators attract macrophages, which contribute to inflammation. Clinical symptoms are observed after several days.

3.4.1 Type 1 hypersensitivity/Allergy

In 1906 the Austrian paediatrician Clemens von Pirquet first introduced the term “allergy” and described changes in reactivity to antigens after secondary or subsequent encounter [46]. Nowadays, “allergy” refers to type 1-immediate hypersensitivity reactions, which are characterized by an inadequate immune response directed against normally innocuous molecules (allergens) leading to the development of pathological symptoms. In predisposed individuals this involves the inappropriate (excessive) production of IgE, the key mediator of type 1 hypersensitivity. Specific IgE Abs bind to high affinity IgE (FcεRI) receptors of basophils and mast cells causing sensitization of the individual. Repeated contact with allergen cross-links IgE Abs on basophils and mast cells and results in the release of mediator substances. Within only a few minutes or hours, these pharmacologically active molecules lead to the clinical manifestation of allergic disease (immediate hypersensitivity reaction). Allergic symptoms include, for example, allergic rhinitis, allergic conjunctivitis, eczema/dermatitis, itching or swelling of the oropharynx and in the worst-case anaphylaxis.

In relationship with the expression “allergy” we often refer to “atopy”. Atopy (greek atopos; meaning out of place) is characterized by the extensive production of IgE Abs in genetically predisposed individuals upon repeated encounter with common environmental antigens. In westernized countries up to 25% of the population are estimated to be atopic and this percentage seems to rise in the future [47]. The so-called hygiene hypothesis tries to explain this phenomenon. It is believed that microbial and viral infections as well as a non-sterile environment in early childhood might have a beneficial effect on the IS and prevent the development of allergic diseases in adult life. A hereditary, genetic predisposition promotes the production of abnormally high quantities of IgE. For example, several genes have been mapped on chromosome 5 encoding disease-relevant cytokines (IL-4, IL-5, IL-9, IL-13) that might be affected in these individuals. However, in general the occurrence of allergic diseases is caused by a complex interplay between environmental factors and genes.

3.4.2 Molecular Mechanism of Type 1 hypersensitivity

Multiple factors are involved in the development of allergic diseases. Primarily, inhaled or ingested common environmental molecules (allergens) are recognized and taken up by APCs (Fig. 1 upper, pattern). Allergens mostly represent glycosylated proteins with a molecular mass of 5-80 kDa or molecules (e. g. pharmaceutical drugs) linked to proteins [47]. APCs intracellularly process antigens/allergens and present peptides in the context of MHC class II molecules to naïve T cells. In atopy-prone individuals this event induces differentiation of T cells into Th2 cells. This is a key step in allergic diseases, since activated Th2 cells create a cytokine microenvironment favouring the development of allergic reactions. IL-4 and IL-13 mediate Ig class switch and enhance IgE production. IL-5 together with IL-13 stimulate the activation of eosinophils; IL-3 and IL-4 induce the development of basophils; the maturation of mast cells is mediated by IL-4, IL-9 and IL-13 [48, 49].

Interaction of Th2 cells with activated B cells causes Ig class switch to IgE (Fig. 1, upper pattern). This process is supported by CD40/CD154 (CD40 ligand)-interaction as well as IL-4 secretion. Atopic individuals produce abnormal high quantities of IgE Abs, which enter circulation and bind to high affinity Fc receptors specific for ε heavy chains (FcεRI) predominantly expressed on basophils and mast cells. This process is called sensitization phase since mediator-releasing cells carry now allergen-specific IgE Abs [5, 6].

In already sensitized individuals repeated exposure to allergen causes cross-linking of specific IgE Abs and triggers mast and basophil activation. Eosinophils, which represent another important cell subset involved in allergic diseases, express only low levels of FcεRI. This cell type mostly is involved in the late phase of allergic reactions and is recruited to the site of inflammation by IL-5. In activated mast cells and basophils a complex signalling cascade is initiated, which leads to the secretion of various mediator substances (Fig 1, middle pattern). These include histamine, major basic protein, proteases or

carboxypeptidases, lipid mediators (leukotrienes, prostaglandin, heparin) and cytokines and cause vascular leakage, vasodilation, bronchoconstriction or mucus secretion. These allergic manifestations of type 1 hypersensitivity develop within several minutes and last up to one hour [5, 6].

In late phase reactions the presence of various cytokines and chemokines attracts allergen-specific Th2 cells to the site of allergen exposure secreting pro-inflammatory cytokines such as IL-5 (Fig.1, lower pattern). This cytokine recruits cells, neutrophils, macrophages, mast cells and, in particular eosinophils to the site of inflammation. Eosinophils, which are characteristic for late-phase or chronic allergic inflammations release cationic granule proteins leading to tissue damage and enhance inflammation. The recruitment and tissue infiltration by eosinophils is strongly dependent on the presence of IL-5 [50]. Symptoms of late-phase reactions classically occur 4 to 6 after antigen exposure and last 1 to 2 days [5, 6].

To treat immediate symptoms of type 1 hypersensitivity, pharmaceutical drugs (antihistamine, corticosteroids) are administered. Up to now, SIT is the only causative treatment for allergic diseases and achieves long-term tolerance to allergens. Repeated administration of allergen, either subcutaneously (SCIT) or sublingually (SLIT) induces immunological tolerance and improves the medical condition as well as the quality of life of allergic individuals. The immunological mechanisms of SIT are still a matter of controversy. It is believed that repeated exposure to allergens or hypoallergenic variants of them upregulate the production IgG Abs. In particular, IgG4 is supposed to compete with circulating IgE for allergen-binding and, thus might have a blocking effect [51-53]. Moreover, SIT promotes the induction of CD4+ CD25+ Foxp3+ Treg cells, which are associated with increased secretion of IL-10 and TGF-β. Both cytokines exhibit tolerogenic effects on immune cells such as reduced production of Th2-associated cytokines [54, 55]. Generally, SIT aims to shift the cytokine milieu from a Th2 towards a Th1 pattern. However, the induction of allergen-specific tolerance is very time-consuming and the risk of (severe) side effects cannot be completely excluded.

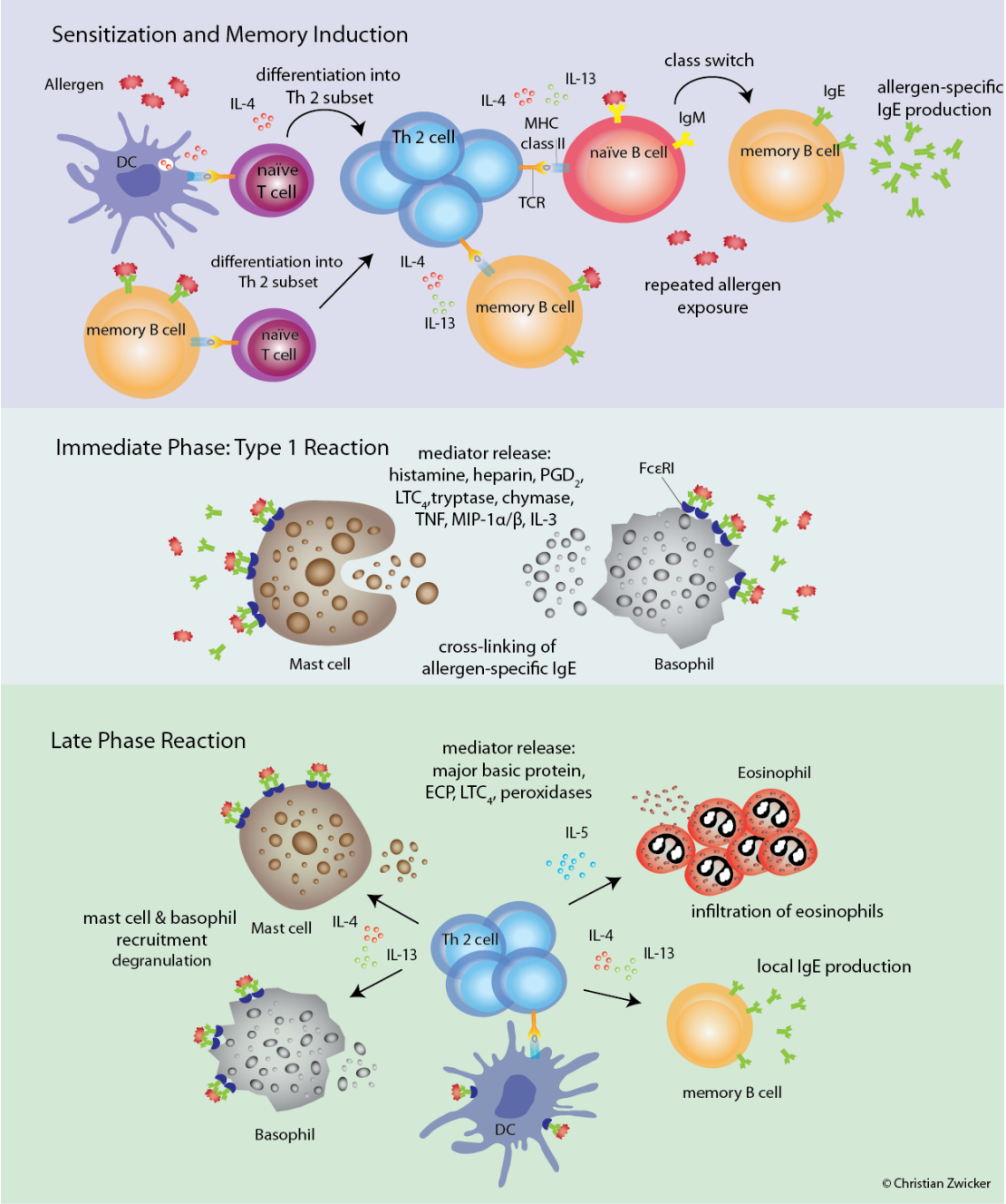

Figure 1 Stages of allergic reactions. During the sensitization phase allergen is taken up by APCs (e. g. DCs), processed and peptides are presented to naïve T cells (upper pattern). In atopy-prone individuals this process leads to differentiation of naïve T cells into Th2 cells providing a cytokine milieu (IL-4, IL-13) that facilitates Ig class switch to IgE and induces clonal expansion of memory B cells. Upon repeated encounter with allergen B cells produce high quantities of allergen-specific IgE. Cross-linking of IgE bound to FcεRI on mast cells or basophils by allergen triggers the release of vasoactive amines (histamine,..), lipid mediators (PGD2, LTC4,…), enzymes (proteases,…) and various cytokines (IL-3, TNF, MIP-1) contributing the development of allergic symptoms (immediate phase, middle pattern). In the late phase of allergic reactions Th2 cells are attracted to the site of inflammation by different cytokines and chemokines (lower pattern). IgE-facilitated antigen-presentation by DCs induces recruitment and clonal expansion of Th2 cells as well as local IgE production. IL-4 and IL-13 activate of mast cells and basophils, while IL-5 recruits eosinophils to the site of allergen exposure. Eosinophils infiltrate the tissue in high numbers and secrete cationic polypeptides causing tissue damage and promoting symptoms associated with late phase allergic reactions. PGD_2, prostaglanding-2; LTC_4, leukotriene C-4; ECP, eosinophilic cationic protein; MIP-1, monocyte inflammatory protein-1; TNF, tumor necrosis factor

3.5 Birch Pollen Allergy

In the Northern hemisphere, especially in Central and Northern Europe as well as in North America birch pollinosis is one the most common allergic diseases [56, 57]. Several proteins in birch pollen (see following sections) have been identified to cause sensitization in allergic individuals. Repeated exposure to these allergens leads to the development of allergic symptoms during the flowering season (spring until early summer) of birch trees (*Betula verrucosa)*. Birch pollen-mediated allergic reactions are characterized by hay fever (rhinoconjunctivits) and rarely allergic asthma. Moreover, in up to 60% of the pollinosis patients the consumption of certain fruits (eg, apples cherry, pea) or vegetables (eg, celery, carrots) leads to the occurrence of allergic symptoms confined to the oral cavity [58-61]. This reaction is commonly known as oral allergy syndrome (OAS) and can be explained by the high sequential and structural identity between proteins from plant-derived food and birch pollen. Although birch pollen allergy usually does not cause severe, life threatening reactions such as anaphylaxis, it strongly affects the health-related quality of life. Therefore, various therapeutic approaches have been made to improve diagnosis and treatment [62, 63].

3.5.1 Bet v 1

Bet v 1 represents the birch pollen major allergen and is the main sensitizing protein in birch pollen allergy. It belongs to the pathogenesis-related protein family 10 (PR-10) and has been identified to be highly homologues to a pea disease resistance gene [64]. 95% of all allergic patients display IgE Abs specific for Bet v 1, whereof approximately 60% react exclusively to the birch pollen major allergen [65]. Bet v 1 is one of the best characterized allergens so far and has been demonstrated to share sequence homologies with several other allergens of the *Rosaceae* family (apple, Mal d 1; pear, Pyr c 1; cherry, Pru av 1) the *Apiaceae* family (celery, Api g 1; carrot, Dau c 1) as well as with hazelnut (Cor a 1), soybean (Gly m 4) or peanut (Ara h 8) [66-73]. As

a consequence, many birch pollen-allergic patients develop hypersensitivity reactions upon ingestion of the above-mentioned plant food, which manifest as irritation, itching, swelling of lips and oral mucosa. In the literature, this disease pattern is described as birch pollen-food syndrome [74-77]. In birch pollen usually several different isotypes of Bet v 1 can be identified and these variants display different IgE-binding as well as T lymphocyte activation capacity. Therefore, several attempts were made to apply low-IgE-binding isoforms as wells as genetically engineered, hypoallergenic Bet v 1-variants in immunotherapy [78-81].

Apart from the major allergen Bet v 1, several other allergenic molecules in birch pollen were identified and analyzed regarding their immunogenicity. They were designated as minor allergens since less than 50% of birch pollen-allergic patients are sensitized. Up to now, 5 minor allergens have been identified (Bet v 2, Bet v 3, Bet v 4, Bet v 6 and Bet v 7) and characterized. These molecules also contribute, however in most cases only partly, to the occurrence of allergic reactions in birch pollen-allergic individuals. All minor allergens undertake, in some cases still unknown, but important functions in the pollen and were shown to have several homologues in grass pollen, fruits, vegetables or nuts [60, 82]. Therefore, birch pollen-related food allergy can also be attributed to cross-reactivity between pollen minor allergens and its homologous proteins in plant food [61, 83-85].

3.5.2 Bet v 2

Bet v 2 belongs to the group of profilins, which are present in all eukaryotic cells and regulates actin polymerization [86]. Profilins are described as a very well-conserved protein family and various allergenic homologues have been detected in plants [87-89]. 10-20% of birch pollen-allergic individuals are sensitized to Bet v 2 [90]. Several lines of evidence proved that birch pollen profilin cross-reacts with several other plant profilins in apple, cherry, pear, celery, carrot, hazelnut, tomato and potato [61, 71, 85, 91, 92].

3.5.3 Bet v 3

This birch pollen minor allergen was identified as a calcium-binding protein (CBP) with a molecular mass of approximately 23 kDa [93]. Ca^{2+} is required for germination and therefore a balanced Ca^{2+}-household maintained by CBP is essential for plant growth [94, 95]. Unlike other CBPs such as calmodulin or certain phosphpolipids, Bet v 3 contains only three Ca^{2+}-binding motifs. *Seiberler et al*., demonstrated that IgE-binding of Bet v 3 heavily depends on the presence of Ca^{2+} since the experimental depletion of Ca^{2+} inhibited this process. Furthermore, this study showed that around 10% of birch pollen-allergic patients recognize Bet v 3 [93].

3.5.4 Bet v 4

Bet v 4 represents another member of the CBP-family and demonstrates high sequence homology with polcalcin proteins [96, 97]. It was reported that between 5 and 20% of the tested pollinosis patients display IgE Abs reacting with Bet v 4 [90, 98]. Bet v 4 possesses two EF-hand domains enabling the protein to regulate the pollen-specific Ca^{2+} metabolism. In contrast to Bet v 3, Ca^{2+} is not absolutely necessary for IgE-binding [96]. Bet v 4 is described as a highly cross-reactive allergen and shows sequence homology of around 65% with several other allergenic CBPs in Brassicaceae species (Timothy and Bermuda grass) and tree pollen (olive, alder) [99-106]. Since these allergenic polcalcins exist in numerous other plant species, these proteins are regarded as pan-allergens [82, 106, 107]. However, Bet v 4 homologues have not been identified in plant-derived food [106].

3.5.5 Bet v 6

Wellhausen et al., characterized first a 35 kDa protein in birch pollen that displayed IgE-binding capacity and cross-reacted with other comparable fruit and vegetable proteins [108]. It was shown to be homologous to isoflavone reductase-like proteins as well as to phenylcoumaran benzylic ether reductases, which both are believed to play a role in plant defence mechanisms [109, 110]. The protein was later designated as Bet v 6 and is recognized by 10% of birch pollinosis patients. Bet v 6 is associated with birch pollen-related food allergy since homologues were identified in apple, carrot, lychee fruit, orange, pear, strawberry and zucchini [90]. In particular, the pear allergen Pyr c 5 demonstrated high sequence identity and similar IgE binding properties [110].

3.5.6 Bet v 7

Bet v 7 belongs to the cyclophilin A family and is activated in response to plant-stress [111, 112]. However, the exact function of Bet v 7 in birch pollen remains unknown [113]. Allergenic cyclophilins from mould and yeast have been reported suggesting that these proteins might be another group of panallergens [114]. In the study of *Cadot et al.,* Bet v 7-specific monoclonal rat Abs recognized proteins in the same size range of alder and hazelnut but did not react with known allergenic mould cyclophilins [113].

3.6 Cross-reactivity

The phenomenon of cross-reactivity is constantly observed in many different types of allergy and is of important clinical relevance. According to the definition of allergen cross-reactivity, Abs originally raised against a specific allergen can recognize structurally related proteins from other allergen sources. Abs can not discriminate between different antigens (allergens) if they share the same structural determinant (epitope). Moreover, the affinity as well as the abundance of specific Abs is an important factor to allow cross-reactivity. This process also occurs at the T cell level, where the T cell receptor is unable to distinguish between similar epitopes. Cross-reactivity mostly has a clinical effect but can also be irrelevant for allergic patients. Generally, proteins require a structural identity of around 70% to cross-react, molecules showing lower homologies are normally not cross-reactive [115]. Evolutionary conserved epitopes on structurally related proteins classically enhance cross-reactivity. Besides similar folding and structure, glycosylation of proteins, so-called cross-reactive carbohydrate-determinants (CCDs), play an important role. Several studies provided evidence that IgE Abs recognize certain carbohydrate structures on allergens leading to cross-reactivity [90, 116-119]. The spectrum of cross-reactive proteins is very broad and *Ferreira et al*., grouped them into 28 different classes according to structural similarities [115]. Due to structural homologies some unexpected cross-reactions, e.g. between banana/mugwort, birch/apple or latex/banana/avocado, were observed [119]. A very prominent example for cross-reaction is the pollen-food syndrome. Here, birch or grass pollen-allergic individuals develop allergic reactions after the ingestion of fruits (apple, pear, cherry, kiwi), vegetables (celery, carrot, potato) or nuts (hazelnut). The occurrence of pollen-food syndrome can be attributed to the close structural identity of the birch pollen major allergen (Bet v 1) and birch pollen profilin (Bet v 2) with proteins in plant-derived food [61]. It was reported that about 70% of pollinosis patients show allergic reactions after the intake of the above-mentioned plant food [60, 90].

3.7 Glutathione-S-transferase (GST)

Originally, mammalian Glutathione-S-transferases (GST) have been first described in the 1960s and are nowadays regarded as a widely distributed enzyme superfamily. GSTs are mainly associated with the detoxification of endogenous and exogenous compounds (xenobiotics), however they also undertake several other functions in a cell [120-124]. GSTs were identified in virtually all phyla, reaching from bacteria and fungi to plants and vertebrates. The GST-family is grouped in several distinct classes: the alpha, mu, omega, pi, sigma, theta and zeta class were identified in mammals. Beta (bacterial), lambda, phi and tau were shown to be of non-mammalian origin. Apart from cytosolic variants, GSTs were also detected in mitochondria and were later summarized as the MAPEG (membrane-associated eiconsanoid and glutathione metabolism) class. The plant classification system for GSTs comprises four classes. The Phi and Tau class are plant-specific, theta and zeta class GST were also detected in other phyla [122, 124, 125]. Recently, dehydroascorbate reductase and tetrachlorohydroquinone dehalogenase (TCHQD) enzyme class were added to this category [126]. However, several other classes were also described in plants [124].

In plants, GSTs were identified shortly after their mammalian counterparts and are also involved in cellular detoxification processes [127]. In particular, it was demonstrated that certain plant GSTs play a role in herbicide resistance, can be activated by pathogens and participate in plant hormone metabolism [120, 128, 129]. Moreover, they were associated with the protection from reactive oxygen species and the biosynthesis of anthocyanins, which are responsible for the coloration of flowers [130]. Besides catalytic activity, plant GSTs also function as non-enzymatic carriers of cellular compounds [131].

Classically, GSTs form homo- or heterodimers consisting of 23-29 kDa subunits but monomers have also been reported [132]. The structure of the proteins displays two binding sites: a glutathione (G)-binding and substrate binding (H)-site bringing both molecules in close vicinity to each other to catalyze the glutathionylation [120]. Though all GSTs share a conserved

structure at the N-terminus, they do not display high overall amino acid sequence homology within different subclasses. The GSH-binding site is restricted to GSH or closely related molecules, whereas the substrate site recognizes and binds a broad spectrum of electrophilic compounds.

Phi and Tau class GSTs, which are most abundant among plants, were reported to be mainly involved in the detoxification of xenobiotics. GSTs of the theta class are responsible for the reduction of hydroperoxides formed during oxidative stress while zeta GSTs exhibit isomerase activity and play a role in tyrosine catabolism. Due to the altered structure of DHAR-associated GSTs, they demonstrate limited transferase activity but catalyze redox reactions. The function of the TCHQD-GST family has not been investigated so far [122, 126, 133].

Recently, a 27 kDa protein of GST-family was detected in a proteome profiling approach analyzing birch pollen extract. Compared to other proteins in the pollen, this protein was present in high amounts [134].

4 Aims of the thesis

The aims of this master thesis were

- to express and purify recombinant GST from birch pollen
- to characterize its biochemical properties
- to evaluate its immunogenicity/allergenicity

5 Material and Methods

5.1 Transformation of competent cells

Chemically competent *E.coli* BL21 DE3 cells were transformed with the expression vector (pHIS2) containing the sequence for birch-GST. The coding sequence was fused downstream of a T7 promotor and lac operon [135]. The expression vector also holds an ampicillin-resistance gene conferring clones carrying the plasmid antibiotic resistance. All centrifugation steps were performed with an Eppendorf 5714C tabletop centrifuge (Eppendorf, Austria).

- *E. coli* BL21 DE3 (Invitrogen, Austria) was thawed on ice for approx. 20 min
- 10 ng of plasmid DNA were added to the cells
- plasmid and cells were incubated on ice for 15 min
- the cells were heat-shocked for 30 sec at 42°C (heat block)
- cells were regenerated on ice for 2-5 min
- 1 mL of LB medium containing Ampicillin (LB-Amp, 100 µg*mL^{-1}) medium was added and the cell suspension was put on a heat block for 30 min at 42°C shaking
- the suspension was centrifuged for 1 min at 12.000 rpm and the cell-free supernatant was removed
- the cell pellet was resuspended in remaining LB-Amp medium
- cells were plated on LB-Amp plates
- Plates were incubated overnight (O/N) at 37°C

5.2 Protein expression

5.2.1 Set up of the pre and starter culture

- 50 mL preculture was inoculated with a single colony
- the preculture was incubated O/N at 37°C shaking (180 rpm)
- 1 L starter culture (LB-Amp) was prepared and approx. 25 mL of the preculture were added
- the starter culture was incubated at 37°C shaking (180 rpm) until the OD_{600} reached 0,6-1 units
- 1 mM IPTG was added to selectively induce protein production and the culture was incubated for 4 h shaking (180 rpm)
- the cell suspension was centrifuged in a Sorvall RC Plus centrifuge (Thermo Fisher Scientific, USA) at 10.000 rpm for 20 min (4°C)
- the cell pellet was resuspended in 25 mL lysis buffer (containing protease inhibitors)
- the cell suspension was frozen in liquid N_2 and thawed at 37°C in the water bath for 3 times
- DNAse was added (5 µg*mL^{-1}) and the cell lysate was incubated for another 30-60 min on RT
- the suspension was centrifuged in a Sorvall RC Plus centrifuge for 20 min at 10.000 rpm (4°C) and the supernatant was collected (including the recombinant protein)

5.2.2 Preparation of a bacterial glycerol stock

- 70 µL of sterile dimethylsulfoxide (DMSO) were added to 800 µL of the preculture under sterile conditions
- the sample was immediately stored at -80°C

5.3 Protein Purification

5.3.1 Nickel (Ni)-affinity chromatography

Due to the C-terminal 6x HIS-tag, the protein was purified by Ni-affinity chromatography. Ni ions are coupled to a resin that interacts and binds the histidine tag of a protein. In columns containing the Ni resin HIS-tagged proteins can be selectively retained in a protein mixture. Buffers containing imidazole (competes for the histidine residues) are used to elute the protein from the column [136].

5.3.1.1 Ni-NTA agarose (soluble resin)

- 50 mL of the supernatants including the protein were incubated with 3 mL of Ni-NTA agarose (Qiagen, Germany) for 60 min at 4°C on a shaker
- a column was loaded with the supernatant
- after 10 min of sedimentation the flowthrough was drained
- the resin was washed for 4 times with 5 mL wash buffer
- the protein was eluted with 0,5 mL elution buffer and protein fractions were collected

5.3.1.2 Fast liquid protein purification (FPLC)

The second purification step was performed using an Äkta Prime protein purification system (GE Healthcare, Germany).

- the system was washed with 40 mL of ethanol and wash buffer at a flow rate of 5 mL/min and a backpressure limit of 1 MPa. Subsequently, the system was equilibrated with 40 mL of lysis buffer
- the Ni agarose column (His-Trap FF crude 1 mL columns, GE Healthcare, Germany) was washed with 10 mL of ethanol (20% v/v) and ddH_2O. Equilibration of the column was done with binding buffer (10 mL). Washing and equilibration of the column was performed at a flow rate of 1 mL/min and backpressure limit of 0,3 MPa
- the sample was loaded onto the column and eluted with 30 mL lysis buffer by applying a linear imidazole gradient (100% lysis buffer – 0% elution buffer → 20% lysis buffer/ 80% elution buffer). Eluted protein was collected in fractions of 1 mL
- after elution, column and system were washed with ddH_2O and 20% ethanol. The column was stored in 20% ethanol at 4°C

5.3.2 Protein dialysis

The protein suspension was dialysed against sodium phosphate (Na_2HPO_4, 50 mM, pH=8.0) buffer to remove cell toxic imidazole

- the eluted protein samples were pooled and filled into a dialysis membrane with a cut-off of 6-8 kDa (Spectra/Por, Spectrum Labs, Netherlands)
- the protein sample was dialysed in approx. 4 L of dialysis buffer and the buffer was changed for 4 times

5.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Protein samples were analyzed by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For visualization gels were stained with Coomassie Brilliant Blue dye and subsequently exposed in a gel imager. All gels were prepared and run in a Mini Gel chamber (Biometra, Germany).

	Separating Gel (15%)	Stacking Gel (15%)
Acryamide	6,6 mL	0,7 mL
Lower buffer	3,3 mL	1,1 mL
ddH_2O	3,3 mL	2,6 mL
Temed	13,2 µL	4,4 µL
APS	132 µL	44 µL

Table 1 reagents for 2-3 15% separating and stacking gels

- the reagents were mixed according to table 1
- the solution for the separating gel was poured in between two sealed glass plates. Shortly before, 10% APS was added to start polymerization
- isobutanol was added on top to create a completely even surface
- after polymerization the isobutanol was removed by rinsing with ddH_2O
- the stacking gel solution was added on top and a comb was inserted
- 15 µL of protein sample were mixed with 5 of µL 4x loading dye
- the protein sample were boiled at 95°C in heating block for 5 min
- after complete polymerization of the stacking gel, the glass plates were mounted in a gel chamber
- 3 µL of protein ladder (Page Ruler, Fermentas, Germany) and the samples were loaded
- an amperage of 0,025 A/gel at a constant voltage was applied (Power Pac, Bio-Rad Laboratories, USA) until the dye completely left the gel

5.3.3.1 Coomassie staining

Proteins were stained using a Coomassie Brilliant Blue G250 dye

- polyacrylamide gels were put in staining solution and incubated for 30 min at RT shaking.
- gels were destained using destaining solution until protein bands were clearly visible (at RT, shaking)
- destained gels were exposed in a gel imager (Gel Logic 2200, Carestream, USA)

5.3.4 Western Blot

Western Blots are used to specifically detect proteins. In a blotting chamber separated proteins are transferred onto a nitrocellulose membrane by applying an electric current. The membrane is then incubated with the primary Ab specifically recognizing the protein. The detection (secondary) Ab labeled with a reporter enzyme (e. g. horseradish peroxidase) is directed against the primary Ab. The reporter signal can be detected by chemiluminescence [137].

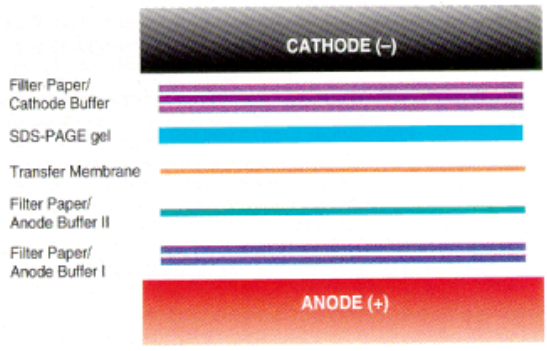

Figure 2 Western blot setup. Adapted from www.rndsystems.com

- a nitrocellulose membrane (Hybond ECL, GE Healthcare, Austria) was equilibrated in transfer buffer and the transfer unit was assembled according to Fig. 2
- the transfer unit was placed into the transfer chamber (TE 22 Mighty Small Transphor Tank, GE Healthcare, Austria) and an electric current was applied (Power Pac, Bio-Rad Laboratories, USA)
- the protein was blotted onto the membrane for 60 min and 240 mA at RT
- after blotting the membrane was blocked in blocking buffer (PBS, Tween 0,1% v/v, 5% w/v powder milk) for 60 min at RT (shaking) to avoid unspecific binding of proteins
- the primary Ab (mouse Penta-HIS IgG1, Qiagen, Germany) was diluted 1:6 000 in PBS Tween (PBST; 2,5% powder milk)
- the membrane was incubated with 10 mL of PBST 2,5% powder milk containing the primary Ab for 3 h at RT shaking
- the membrane was rinsed with 10 mL of blocking buffer
- the detection Ab (anti-mouse IgG1 HRP, Cell Signaling Technology, USA) was diluted 1:10 000 in PBST 2,5% powder milk
- the membrane was incubated with 10 mL of PBST 2,5% powder milk containing the detection Ab for 2 h at RT shaking
- the membrane was rinsed with 10 mL of blocking buffer 3 times and dried
- 2 mL of chemiluminescent reagent (Lumigen detection reagent, GE Healthcare, Austria) were put on the membrane
- in the dark room photo film (Amersham Hyperfilm, GE Healthcare, Austria) was exposed to the membrane for 5 sec – 5 min (depending on the strength of the signal)
- the photo film was developed in a developing unit (AGFA CP1000, Germany)

5.3.5 Bicinchoninic acid protein assay

Protein concentrations were determined by using a bicinchoninic acid (BCA) protein assay (BCA Protein Assay Kit, Pierce, USA). In alkaline medium proteins reduce Cu^{2+} to Cu^{+}, which subsequently can form a chelat-complex with BCA. A purple colored reaction-product develops upon complex formation, which adsorbs light at 562 nm. The measurement of absorption allows quantification of protein in a sample [138].

- standards were prepared according to manufacturer's instructions (working range: 25 – 2000 $\mu g*mL^{-1}$)
- the working reagent was prepared in a ratio of 50 parts of solution A and 1 part of solution B (1:50)
- 25 µL of protein sample or standard were added to a 96 well plate
- 200 µL of working reagent were mixed with sample or standard
- the plate was incubated for 30 min at 37°C
- the absorbance was measured at 562 nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA)

5.3.6 Limulus Amebocyte Lysate assay

Limulus amebocyte lysate (LAL) assays are used to quantitatively detect endotoxins derived from gram-negative bacteria. Endotoxins activate a pro-enzyme in LAL, which catalyzes a color reaction. The absorbance is then measured at 405-410 nm and correlates with the initial endotoxin concentration present in the sample [139]. The endotoxin concentrations in the protein sample were quantified with a LAL assay kit (Lonza, Switzerland).

- samples were diluted in endotoxin-free water
- the lyophilized endotoxin stock was reconstituted with 1 mL of endotoxin-free water and vortexed for 15 min

- an aliquot of the endotoxin standard stock was diluted to 1 EU*mL^{-1}, 0,5 EU*mL^{-1}, 0,25 EU*mL^{-1} and 0,1 EU*mL^{-1}
- chromogenic substrate was reconstituted with respective volume of endotoxin-free H_2O shown on the vial
- a 96 well plate was pre-warmed in a heating block at 37°C
- 50 µL of sample, standard or blank were added to the plate
- another 50 µL of LAL-reagent were added
- after 10 min the solution was mixed with 100 µL of chromogenic substrate
- after 6 min 100 µL of stop reagent were added
- the absorbance was measured at 405 nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA)

5.3.7 Endotoxin removal

To exclude any unwanted or adverse effects in cell culture assays and animal experiments, remaining endotoxins in the samples were removed. Therefore, two different methods were applied.

5.3.7.1 Endotoxin removal columns

Endotoxin removal columns are packed with a resin. This resin specifically binds and retains endotoxins while proteins pass through the column.

- prior to use all reagents were degased using a 0,22 µm sterile filter (Minisart sterile filter, Satorius Stedim, Germany)
- to activate the column (Endotrap Red, Hyglos, Germany), 3 mL of Regeneration Buffer were added and the column was drained completely
- 3 mL of Equilibration Buffer were applied to the column

- after complete draining of the column the sample was loaded and collected immediately
- 1 mL of Regeneration Buffer was added and collected again
- the column was washed with 3 mL of Equilibration Buffer
- all steps were repeated to allow efficient endotoxin removal
- the column was stored in 1 mL of Regeneration Buffer supplemented with 0,02% (v/v) sodium azide

5.3.7.2 Triton X-114 phase separation method

The detergent Triton X-114 forms micelles when the solution reaches a certain temperature level. Incubation of endotoxin-contaminated protein solutions with Triton X-114 promotes the incorporation of endotoxins into micelles by non-polar interactions. These micelles can than be subsequently removed by phase-separation [140].

- 100 µL of Triton X-114 (Sigma-Aldrich, USA) solution were added to 10 mL of the protein solution and incubated for 1 h at 4°C shaking
- the solution was centrifuged for 40 min at 7880 rpm
- the aqueous, micelle-poor supernatant was removed from the micelle-rich phase and transferred to a fresh tube
- these steps were repeated for at least 8 times to achieve efficient endotoxin removal
- remaining Triton X-114 was removed by incubating the supernatant for 1 h at 4°C
- the solution was centrifuged again for 40 min at 7880 rpm
- the supernatant was then transferred to a fresh tube
- these steps were repeated for at least 5 times

5.3.8 Triton X-114 removal columns

To eliminate any remaining traces of Triton X-114, the protein sample was loaded on detergent removal columns (Pierce, USA).

- the columns were placed in 50 mL tubes and storage solution was drained off by centrifugation at 1000 x g for 2 min using an Allegra X-12R centrifuge (Beckman Coulter, USA)
- afterwards 4 mL of wash buffer (50mM Na_2HPO_4) were added and the solution was centrifuged for 2 min at 1000 x g. This step was repeated for 3 times
- the detergent removal columns were placed in a fresh tube and up to 1 mL of the protein solution was loaded
- the samples were incubated for 2 min at RT and again centrifuged for 2 min at 1000 x g

5.3.9 Concentration and sterile filtration of the protein samples

All protein samples were concentrated in a vacuum centrifuge (Univapo 150 ECH, Uniequip, Germany) and filtrated through 0,22 µm sterile filters (Minisart sterile filter, Satorius Stedim, Germany).

5.4 Enzymatic activity assay

The enzymatic activity of rGST was determined in an enzymatic activity assay (Glutathione-S-transferase assay kit, Cayman Chemicals, USA) [141].

10 µl of GST positive control was diluted in 190 µl of ddH_2O and put on ice. 150 µL of assay buffer were added to flat bottom 96 well plates (Greiner, Germany). Another 20 µL of glutathione solution were pipetted to the blank and samples. To start the enzymatic reaction, 10 µL of the substrate (1-chloro-2, 4-dinitrobenze, CNDB) were added and the absorbance was

measured immediately at 340 nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA). The absorbance was determined every 5 min over a time period of 1 h.

5.5 Immunization of mice

Mice were immunized with rGST and rBet v 1 to induce specific Abs. 6 BALB/c mice (6 weeks old, female) were obtained from Charles River (Sulzfeld, Germany). The immunization protocol was started after a 2 weeks adaptation phase in the animal facility. Mice were immunized *intraperitoneally (i. p.)* with recombinant protein adsorbed to Alum ($Al(OH)_3$, Serva, Germany) according to the immunization protocol shown in fig. 3.

Two groups of mice were immunized

Group 1 (n=3): rGST + Alum *i. p.*

Group 2 (n=3) rBet v 1 (Biomay, Austria) + Alum *i. p.*

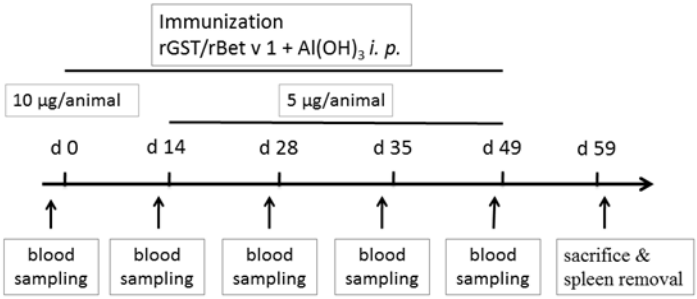

Figure 3 Mouse immunization protocol

In both groups mice were immunized on day (d) 0, 14, 28, 35 and 49. On d 59 the mice were sacrificed and spleens were removed. Mice were immunized *i. p.* with 10 µg (d 0) and 5 µg (d 14, 28, 35, 49), respectively, in a final volume

of 150 µL solution (Fig. 3). Equimolar amounts of rGST and rBet v1 were used. Alum (100 µL, Serva, Germany) was added to the respective amount of protein and the solution was filled up to 150 µL with PBS. To allow particle formation between Alum and protein, the solution was incubated for 30 min at RT before administration.

Prior to each immunization blood (200- 300 µL) was collected by means of tail bleeding. To prepare serum, blood samples were left at RT for approx. 30 min. The blood clot was removed and the sample was centrifuged for 5 min at 14.000 rpm in a tabletop centrifuge (5714C centrifuge, Eppendorf, Austria). The supernatants were transferred to a fresh tube and stored at -20°C until analysis.

5.5.1 Mouse ELISA

5.5.1.1 Mouse IgG subclass/IgE ELISA

The Ab levels in murine sera were determined by IgG-subclass (IgG1, IgG2a, IgG3) and IgE ELISA.

ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific, USA) were coated each with 100 µL of rGST (2 $\mu g*mL^{-1}$), rBet v 1 (1 $\mu g*mL^{-1}$) and BPE (50 $\mu g*mL^{-1}$) in carbonate buffer O/N at 4°C. Subsequently, the plates were washed twice with PBST (250 µL/well). To avoid unspecific binding, the plates were blocked with 150 µL/well of PBST (1% bovine serum albumin, BSA) for 6 h at RT. For detection of specific IgG1 and IgG2a, sera were diluted 1:1000 in PBST (0,5% BSA); for specific IgG3 1:400 and for specific IgE 1:20. Coated ELISA plates were then incubated with 100 µL/well of the respective pre-diluted mouse serum O/N at 4°C. PBST was used as a negative control. After washing the plates for 5 times, 100 µL/well of rat anti-mouse IgG1, IgG2a, IgG3 and IgE (1:1000 in PBST; BD Pharmingen, USA) were added and incubated for 90 min at 37°C followed by 90 min at 4°C. The plates were washed and 100 µL/well of goat anti-rat IgG HRP-linked (1:2000 in PBST; GE

Healthcare, Austria) were applied for 1 h at 37°C. The plates were washed again and 100 µL/well of ABTS solution ([1 mg*mL^{-1}]; 2,2'-azino-bis 3 ethylbenzolthiazonline-6-sulfate, Sigma Aldrich, USA) were added for color development. The absorbance was measured at 405 nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA).

5.5.1.2 Detection of cross-reactive mouse IgE Abs

A potential cross-reactivity between specific birch-GST Abs and house dust mite (HDM)-GST (Der p 8) was analyzed.

ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific, USA) were coated with 100 µL/well of BPE or HDM extract (each 50 µg*mL^{-1}) in carbonate buffer O/N at 4°C. Subsequently, the plates were washed twice with PBST and blocked with 150 µL/well of PBST (1% BSA) for 6 h at RT. Sera of rGST-immunized mice were diluted 1:20 (for IgE detection) in PBST (0,5% BSA). Pre-diluted mouse sera (100 µL/well) were applied to the plates and incubated O/N at 4°C. PBST was used as a negative control. After washing the plates for 5 times, 100 µL/well of rat anti-mouse IgE (1:1000 in PBST; BD Pharmingen, USA) were added and incubated for 90 min at 37°C followed by 90 min at 4°C. The plates were washed and 100 µL/well of goat anti-rat IgG HRP-linked (1:2000 in PBST; GE Healthcare, Austria) were applied for 1 h at 37°C. The plates were washed again and 100 µL/well of ABTS solution ([1 mg*mL^{-1}]; 2,2'-azino-bis 3 ethylbenzolthiazonline-6-sulfate, Sigma Aldrich, USA) were added for color development. The absorbance was measured at 405 nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA).

5.5.1.3 Inhibition ELISA

The folding of rGST was evaluated by inhibition. IgG-binding to rGST was inhibited by pre-incubation of the sera of rGST-immunized mice with rGST.

The sera of rGST-immunized mice were diluted 1:1000 in PBST (0,5% BSA). 50 µL/well of serum were added to 96 well plates (Greiner, Germany) and incubated with different amounts of double-concentrated protein (0,01; 0,05; 0,1; 0,5; 1; 5; 10 $\mu g*mL^{-1}$) O/N at 4°C. PBST served as a negative control. ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific, USA) were coated with 100 µL/well of rGST (0,5 $\mu g*mL^{-1}$) in carbonate buffer O/N at 4°C. Subsequently, the plates were washed twice with PBST. Pre-incubated sera were transferred to coated plates and incubated O/N at 4°C. After washing the plates for 5 times PBST, 100 µL/well of rat anti-mouse IgG1 (1:1000 in PBST; BD Pharmingen, USA) were added and incubated for 90 min at 37°C followed by 90 min at 4°C. The plates were washed and 100 µL/well of goat anti-rat IgG HRP-linked (1:2000 in PBST; GE Healthcare, Austria) were applied for 1 h at 37°C. The plates were washed again and 100 µL/well of ABTS solution ([1 $mg*mL^{-1}$]; 2,2'-azino-bis 3 ethylbenzolthiazonline-6-sulfate, Sigma Aldrich, USA) were added for color development. The absorbance was measured at 405 nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA) until OD reached 1-1.5.

5.5.2 Proliferation assay

Splenocytes were isolated from mice immunized with rGST and rBet v 1 and were stimulated with rGST, rBet v 1 and BPE. Antigen-specificity was tested upon stimulation with the respective protein and BPE. All centrifugation steps were performed with an Allegra X-12R centrifuge (Beckman Coulter, USA).

10 d after the last immunization mice were sacrificed and spleens were removed aseptically (done by Dr. Thomas Kolbe, Veterinarian University Vienna). Spleens were put in a sterile petri dish containing 10 mL of complete

medium. Spleen cells were separated using a 0,70 µm nylon filter (BD Falcon, USA) and the filter was washed with 5-10 mL of sterile PBS. Splenocytes were resuspended, centrifuged for 7 min at 1400 rpm and the supernatant was discarded. The cell pellet was resuspended in 3 mL of pre-chilled haemolysis buffer. Resuspended cells were put on ice for exactly 1 min and cell lysis was stopped with 8 mL of wash medium. The cell suspension was centrifuged for 5 min at 1400 rpm and the supernatant was discarded. The white cell pellet was resuspended in complete medium (4 mL). Cells were counted in a haemocytometer (Bürker-Türk, LO Labor Optik, Germany) and adjusted to 4 x 10^6 cells/mL with complete medium.

Proliferative responses were assessed in triplicates. Splenocytes isolated from GST-immunized mice were stimulated with different concentrations of rGST ranging from 0,39-2,5 µg*mL^{-1}. Cells of rBet v 1-immunized mice were stimulated with rBet v 1 (5 and 10 µg*mL^{-1}). As a positive control, cells were incubated with the mitogen conconavalin A (2,5 µg*mL^{-1}; Con A, Sigma Aldrich, USA). As a negative control, cells were either stimulated with rBet v 1 or GST. Additionally, cells were incubated with medium alone. 100 µL/well of stimulants or medium were transferred in a round bottom 96 well plate (Nunc, Thermo Fisher Scientific, USA).

Cells were added (2 x 10^5/well) and incubated for 5 days at 37°C. After 4 days ^{3}[H]-labeled thymidine (1 µCi*mL^{-1}; ^{3}[H]-dT, Perkin Elmer, USA) was added to a final concentration of 0,2 µCi*mL^{-1}. After 12-16 h of incubation cells were harvested onto a fiberglass filter (Filter Mat, Perkin Elmer, USA). Scintillation liquid (Betaplate Scint, Perkin Elmer, USA) was added on the filter and the proliferative response was determined by scintillation counting in a β-counter (MicroBeta TriLux, Perkin Elmer, USA)

5.5.3 Determination of the cytokine production

To determine the cytokine production, splenocytes of rGST-immunized and rBet v 1-immunized mice were incubated with optimal concentrations of rGST and Bet v 1.

The cell suspension was adjusted to 1,25 x 10^7/mL. Splenocytes (5 x 10^6/well in 400 µL) were transferred to 48 well plates (Costar, Incorning, USA) and stimulated with 100 µL/well of optimal concentrations of rGST (1,25 µg*mL^{-1}), rBet v 1 (5 µg*mL^{-1}), Con A (2, 5 µg*mL^{-1}) or left in medium alone. Cells were incubated for 40 h at 37°C. Supernatants were harvested and immediately stored at -20°C.

5.5.4 Cytokine detection

Cytokine production (IL-2, IL-4, IL-5, IL-10, IL-13, IL-17 and IFN-γ) was analyzed by using a Multiplex cytokine assay. This flow cytometry-based system can detect several different analytes (e. g. cytokines) in a single sample. The Multiplex assay uses polystyrene microbeads, also called microspheres, containing an internal dye. Each bead set has individual properties and can be coupled with a certain detection reagent (e. g. antibody) that allows capturing of the analyte. In the analyzing device a red lasers excites light emission of the internal bead dye and thus identifies a particular bead set linked with the detection reagent. A green laser detects the reporter dye, which is associated with the analyte-detection reagent-reporter complex. Light emission of the reporter dye enables quantification of the analyte.

Before transferring 200 µL of the bead solution (2,5 x 10^6 beads) to a Protein LoBind tube (Eppendorf, Germany), the bead microsphere stock solution was resuspended by vortexing and sonication. The bead solution was centrifuged for 2 min at 8.000 x g and the supernatant was removed. The beads were washed with ddH_2O (100 µL) and again resuspended by vortexing and sonication. After centrifugation (2 min, 8.000 x g) the supernatant was

removed and pelleted beads were resuspended in activation buffer (80 µL). Sulfo-NHS and EDC solution (each 10 µL) were added and the bead solution was vortexed. After 20 min of incubation at RT, the beads were centrifuged for 2 min at 8000 x g and the supernatant was removed (if no precipitate is detectable, the solution will be centrifuged at 12000 x g). Pelleted beads were washed twice in coupling buffer (200 µL) and finally collected in 100 µL of coupling buffer. After vortexing and sonication 100 µg of the respective coating antibody (IL-2, -4, -5, -10, -13, -17, INF-γ; Flow CytoMix, Ebioscience, USA) were added. The antibody-bead solution was filled up to 500 µL with coupling buffer and incubated on a rotating wheel for 2 h at RT in the dark. Thereafter, antibody-coupled microsphere beads were centrifuged (2 min, 8000 x g) and the supernatant was discarded. The beads were resuspended in 500 µL of Luminex buffer, vortexed and sonicated and incubated for 30 min at RT on a rotating wheel. After incubation the beads were washed with 1 mL of Luminex buffer. The wash step was repeated twice and the beads were finally resuspended in 500 µL of Luminex buffer. The bead concentration was measured in haemocytometer and the bead solution was stored at 4°C.

To prepare a bead working solution, the individual antibody-coupled bead stocks were combined in a single reaction tube. Therefore, the stocks were diluted to 60 beads/µL of each individual antibody-bead set in Luminex buffer. Next, a 1,2 µm 96 well filter plate was incubated with PBS 1% BSA (100 µL/well) to pre-wet the filter. The solution was aspirated and 30 µL/well of working bead solution were added. Standards and samples were diluted in Luminex buffer and analyzed in duplicates. As a control, supernatants of cells, incubated with medium alone, were used. 30 µL/well of the standard or sample were transferred to the bead solution and the plate was incubated at 4°C O/N shaking. Thereafter, the supernatants were removed, the plate was washed with PBS (100 µL/well) and the beads were finally collected in PBS (50 µL/well). The respective biotinylated detection Abs were diluted 1:200 in Luminex buffer and combined in one reaction tube. 25 µL/well of the detection Ab-solution were added and incubated for 1 h at RT shaking. After incubation the plates were washed twice with PBS (100 µL/well) and the antibody-coupled beads were resuspended in 50 µL of PBS. 30 µL of a streptavidin-R-

phycoerythrin reporter Ab (2 µg*mL^{-1}) were transferred to the plate and the solution was incubated for 30 min at RT shaking. Subsequently, the wells were washed twice with PBS (100 µL/well) and the beads were finally collected in 70 µL of PBS. The samples were analyzed by using a Luminex 100 analyzer (Luminex, USA).

5.6 Stimulation of TLR4-transfected HEK cells

Human embryonic kidney cells (HEK 293) transfected with the human TLR4 were stimulated with rGST to functionally assess the efficacy of endotoxin as well as Triton X-114 removal. TLR4 binds lipopolysaccharids derived from gram-negative bacteria inducing IL-8 release from the cells [142]. IL-8 can subsequently be detected in the supernatant by ELISA. For efficient TLR-signaling HEK 293 cells were co-transfected with the LPS-receptor (CD14) as well as MD2, a soluble molecule binding LPS.

5.6.1 Seeding of TLR4-transfected HEK 293 cells

Medium of cultured HEK 293 *hTLR4 CD14 MD2* (Imgenex, USA) cells was aspirated and the cells were detached from the flask. The cells were counted in a counting chamber (Bürker-Türk, LO Labor Optik, Germany), adjusted with HEK-TLR4 medium and seeded (40.000/well) in a flat bottom 96 well plate (Costar, Incorning, USA). After resting the cells for 20 h at 37°C, the supernatants were aspirated and 100 µL of the respective stimuli were added. Cells were stimulated with different concentrations of rGST ranging from 0,78-100 µg*mL^{-1}. As positive control, cells were stimulated with LPS (100 ng*mL^{-1}). Medium alone served as negative control. IL-8 production and cell proliferation was analyzed in triplicates.

5.6.2 Proliferative response of TLR4-transfected HEK 293 cells

To assess the viability of the cells, cell proliferation of HEK 293 *hTLR4 CD14 MD2* after incubation with rGST and LPS was analyzed.

On day 4 ^{3}[H]-dT was added (final concentration 0,2 µCi*mL^{-1}) to the cells. After 12-16h of incubation, cells were harvested onto a fiberglass filter (Filter Mat, Perkin Elmer, USA). Scintillation liquid (Betaplate Scint, Perkin Elmer, USA) was added on the filter and proliferative responses were determined by scintillation counting in a β-counter (MicroBeta TriLux, Perkin Elmer, USA).

5.6.3 Interleukin-8 ELISA

The supernatants of HEK 293 *hTLR4 CD14 MD2* were harvested and IL-8 production was determined.

ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific, USA) were coated with 100 µL/well of IL-8 capture Ab (2 µg*mL^{-1}) diluted in carbonate buffer. Plates were incubated O/N at RT and subsequently blocked with 150 µL/well of PBST (4% BSA) for 1 h at RT. Cell supernatants (75 µL) were collected and diluted 1:10 in PBS. After washing for 3 times with PBST (250 µL/well), 50 µL/well of standard solution or sample (diluted supernatants) were added to the plates and incubated for 1 at RT. Thereafter, 50 µL/well of a biotinylated Ab (1:2500 in PBST) were added and incubated for 1 h at RT. The plates were washed again for 3 times and incubated with 100 µL/well of HRP-conjugated Ab (1:2500 in PBST) for 30 min at RT. The plates were washed, 100 µL/well of TMB solution (TMB/E, Millipore, USA) were applied and incubated for 30 min in the dark. The color reaction was stopped with 100 µL of 0.18 M H_2SO_4. The absorbance was measured at 450/630nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA).

5.7 Birch pollen-allergic and non-allergic individuals

Sera of 215 birch pollen-allergic individuals were collected in Allergy Clinics in Graz, Innsbruck, Vienna and at the Medical University of Vienna. Diagnosis was made upon clinical history, positive skin prick test response to BPE and detection of specific IgE to BPE (≥ 0,35 kU_A/L [kilo units/L]). Non-allergic individuals did not show a clinical history as well as no specific IgE to BPE.

5.8 Human IgE ELISA

The sera of 215 birch pollen-allergic individuals were tested for specific IgE to rGST and the birch pollen minor allergens Bet v 3, Bet v 4, Bet v 6 and Bet v 7.

ELISA plates (Nunc Maxisorp, Thermo Fisher Scientific, USA) were coated with 100 µL of rGST or the minor allergens (2 $\mu g*mL^{-1}$, diluted in carbonate buffer) O/N at 4°C. After washing twice times with PBST (250µL/well), the plates were blocked with PBST 1% BSA (150 µL/well) for 6 h at RT. For allergen-specific IgE detection, sera were diluted 1:4 and 1:8 with PBST (0,5% BSA). As a control, buffer (PBST) and sera of non-allergic individuals were used. 100 µL/well of serum or buffer were added and incubated O/N at 4°C. Thereafter, the plates were washed for 5 times and 100 µL of alkaline-phosphatase conjugated anti-human IgE Ab (1:2000 in PBST; BD Pharmingen, USA) were applied. The Ab was incubated for 60 min at 37°C followed by 60 min at 4°C. The plates were washed again and 100 µL of paranitrophenylphosphate (pNPP; 1 tablet/5 mL diethanolamine buffer) were added for color development. The pnPP-solution was incubated in the dark and the absorbance was measured photometrically at 405 nm in a plate reader (Spectra Max Plus 384, Molecular Devices, USA). The mean value of all non-allergic controls plus 10 times standard deviation was set for the cut-off for positive IgE-reactivity.

5.9 Medium used

LB Amp medium:

5 g NaCl

10 g peptone

5 g yeast extract

Fill up to 1000 mL with ddH_2O

Autoclave and when cooled down (handwarm) add 1 mL Amp (stock: 100 $mg*mL^{-1}$) under laminar flow/sterile conditions and keep in fridge

LB Amp plates:

5 g NaCL

10 g peptone

5 g yeast extract

15 g Agar

Fill up to 100 mL with ddH_2O

Autoclave and when cooled down (handwarm) add 1 mL Amp (stock: 100 $mg*mL^{-1}$) and pour plates under laminar flow/sterile conditions

Transfer buffer

6.05 g Tris

28.82 g glycin

400 mL methanol

adjust to pH=8.3 with Tris or glycin

10x PBS:

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

pH = 7.4 (HCl/NaOH)

Fill up to 1000 mL with ddH_2O

Coomassie Brilliant Blue:

1 g Coomassie brilliant blue G-250

500 mL methyl alcohol

100 mL acetic acid

400 mL ddH_2O

Dissolve Brilliant Blue in methyl alcohol first O/N

Destaining solution:

100 mL methyl alcohol

100 mL acetic acid

800 mL ddH_2O

Na-Phosphate buffer (50 mM NaH_2PO_4 pH = 8.0)

6.90 g NaH_2PO_4 x H_2O

Adjust to pH = 8.0 with NaOH

Fill up to 1000 mL with ddH_2O

Lysis buffer (50 mM NaH_2PO_4 pH = 8.0, 300 mM NaCl, 10 mM imidazole):

6.90 g NaH_2PO_4 x H_2O

17.54 g NaCl

0.68 g imidazole

Adjust to pH = 8.0 with NaOH

Fill up to 1000 mL with ddH_2O

Wash buffer (50 mM NaH_2PO_4 pH = 8.0, 300 mM NaCl, 20 mM imidazole):

6.90 g NaH_2PO_4 x H_2O

17.54 g NaCl

1.36 g imidazole

Adjust to pH = 8.0 with NaOH

Fill up to 1000 mL with ddH_2O

Elution buffer (50 mM NaH_2PO_4 pH = 8.0, 300 mM NaCl, 500 mM imidazole):

6.90 g NaH_2PO_4 x H_2O

17.54 g NaCl

34.04 g imidazole

Adjust to pH = 8.0 with NaOH

Fill up to 1000 mL with ddH_2O

1 mg/mL DNase stock solution:

Buffer: 10 mM Tris/HCl pH = 7.5, 150 mM NaCl, 1 mM MgCl

For 25 mL of buffer:

0.03 g Tris

0.219 g NaCl

0.00508 g MgCl

Dissolve 2 mg of DNase in 1 mL of buffer and when DNase has dissolved completely add 1 mL of glycerol.

Carbonate buffer (pH = 9.6):

1.965 g Na_2CO_3

2.645 g $NaHCO_3$

Ad 500mL of ddH_2O

ABTS buffer

6,76 g citric acid x H_2O

6, 88 g Na_2HPO_4

fill up with 500 mL of ddH_2O

Diethanolamine buffer

97 mL diethanolamine

200 mg NaN_3

750 mL ddH_2O

adjust with conc. acetic acid to pH = 9.8

fill up to 1000 mL with ddH_2O

PBS Tween 0,05% (PBST):

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

pH = 7.4 (HCl/NaOH)

add 500 µL Tween

Fill up to 1000 mL with ddH_2O

HEK TLR 4 medium

500 mL DMEM

10% FCS

1 mL gentamycin

550 µL blasticidin (stock: 10 $mg*mL^{-1}$)

275 µL Hydro Gold (stock 100 $mg*mL^{-1}$)

Complete medium (mouse splenocytes)

RPMI 1640 (Gibco, UK)

0.2% v/v gentamycin,

10% FCS

1% v/v glutamin;

0.5% v/v β-mercaptoethanol

Haemolysis buffer

1 g $KHCO_3$,

8,3 g NH_4Cl

0.37 mg*mL^{-1} EDTA,

fill up to 1000 mL with endotoxin free ddH_2O

Wash medium (for mouse splenocytes)

RPMI 1640 (Gibco, UK)

0,2% v/v gentamycin

Activation buffer (Luminex)

3 g/250 mL $NaHPO_4$, pH=6,2 (100 mM)

50 mg*mL^{-1} *N*-hydroxysulfosuccinimide (Sulfo-NHS)

50 mg*mL^{-1} 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)

sterile filtrate

Coupling buffer (Luminex)

4.88 g 2-(*N*-morpholino)ethanesulfonic acid (mM 50, MES)

adjust to pH=5.0 with NaOH

fill up to 500 mL with ddH_2O and sterile filtrate

Luminex Buffer

250 µL Tween

0,25 g NaN_3

5 g BSA

adjust to pH=7,4 with NaOH

fill up to 500 mL with PBS and sterile filtrate

6 Results

6.1 Production of recombinant GST from birch pollen

6.1.1 Sequence of birch pollen Glutathione-S-transferase

MADASVKEHLPTPLDATSNPPPIFDGTTRLYTCYTCPFAQRVWITRNYKGLQEKIKL
VPINLQNRPAWYKEKVYPENKVPALEHNGKVIGESLDLIKYVDINFEGPSLLPNDPA
KKAFAEELVAYSDTFNKTVFTSFKGDPVKEAGPAFDHLEKALHKFDDGPFFLGQFSA
VDIVYIPFVERFQIFLLDALKYDITAGRPKLAKWIEELNKIDAYKPTKTDPKELVEF
YKARFAAQQ LEHHHHHH

Figure 4 Aa sequence of birch-GST. The poly-(6x)-HIS-Tag introduced at the C-terminus of the sequence is highlighted in red.

The Aa sequence of birch-GST (kindly provided by the research group of Fatima Ferreira, University of Salzburg) consists of 245 amino acids and is flanked by a C-terminal poly-(6x) HIS-tag (Figure 4, shown in red). The computed molecular weight of the sequence is 28 kDa and the protein exhibits a theoretical isoelectric point (pI) of 6.56 (ExPasy, ProtParam). The sequence was cloned into a pHIS parallel 2 vector and fused with a C-terminal polyHIS-tag (6x) for protein expression and purification. Competent *E. coli* BL21 (DE3) cells were transformed with the expression vector and plated on agar containing Ampicillin to positively select successfully transformed cells.

6.2 Protein expression and purification

After incubating the plates overnight, colonies were picked and a 50 mL pre-culture was set up. For protein expression, a large-scale protein expression culture (1 L) was prepared and inoculated with the pre-culture. Protein production was induced by adding IPTG to the medium. After ~4.5 h cells

were harvested and lysed by several freeze/thaw cycles. Preliminary test expressions showed that recombinant birch-GST is expressed as a soluble protein and can be purified from the soluble fraction. Hence, the soluble cell lysate was incubated with nickel (Ni) agarose beads, which selectively bind polyhistidine (6x) tagged proteins. Protein fractions obtained after purification were loaded on a SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 5).

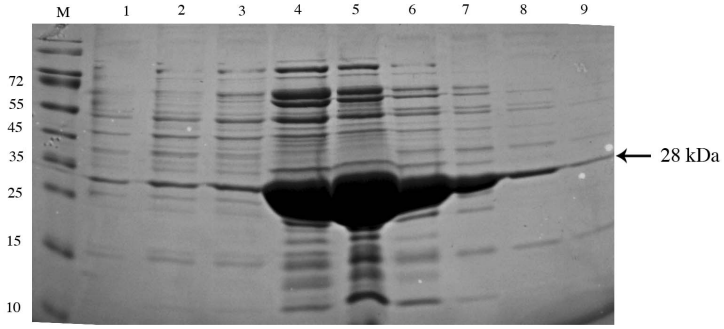

Figure 5 Batch purification of rGST by Ni-agarose beads. Different fractions (1-9) obtained after purification with Ni-agarose beads were analyzed by SDS-PAGE (15%) and stained with Coomassie. M, protein ladder (Fermentas page ruler). Black arrow indicates putative protein band of rGST running at the calculated size of 28 kDa.

Stained protein bands at a size of 28 kDa represent rGST produced in satisfactory yields. However, several other protein bands indicated that this purification method only roughly purified the protein. To reduce these impurities, an additional purification step was performed by FPLC. The pooled pre-purified protein sample was loaded onto a Ni-affinity column and protein was eluted by increasing imidazole concentrations. Collected protein fractions were analyzed by SDS-PAGE.

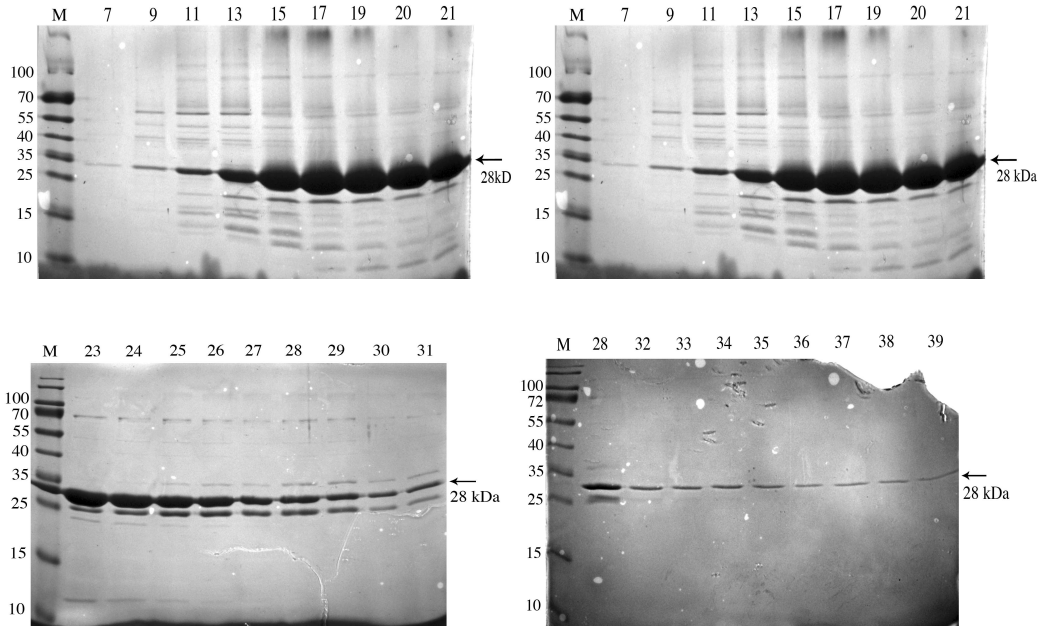

Figure 6 Purification of rGST by FPLC. Protein fractions collected after FPLC (1-39) were loaded onto a SDS-PAGE (15%) and Coomassie-stained. Black arrows show rGST at size of 28 kDa. Subsequently, the fractions were pooled in pool A (7-11, 32-42) and pool B (12-31). M, protein marker; sol, soluble protein after lysis; FT flowthrough of FLPC; W1-4, protein wash

After the second purification step, pure rGST was detected as a protein band at 28 kDa in SDS-PAGE (Fig. 6). The protein was available in sufficient amounts and perturbing impurities had been reduced to acceptable limits. Collected samples were pooled and separated into two aliquots (Pool A and B). Both aliquots were dialysed against sodium phosphate buffer to remove imidazole.

6.2.1 Detection of His-tagged birch-GST by Western blotting

Different protein fractions obtained from affinity chromatography were used for specific detection of rGST by western blotting. The proteins were transferred onto a nitrocellulose membrane and incubated with an anti-HIS-tag Ab.

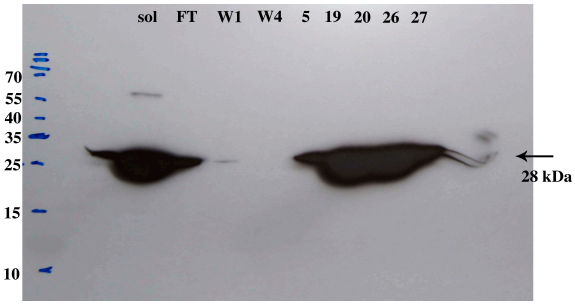

Figure 7 Detection of HIS-tagged rGST. Different protein samples (3, 19, 20, 26, 27) were separated by SDS-PAGE. Subsequently, proteins were transferred onto a nitrocellulose membrane and detected with a murine anti-HIS Ab. For visualization an anti-mouse POX-labeled IgG1 Ab was applied. rGST is indicated by a black arrow. M, protein marker; Sol, soluble protein after cell lysis; FT, flowthrough of FLPC; W1, W4, protein wash

His-tagged rGST was detected at a size of 28 kDa in all samples after purification (lane 3, 19, 20, 26, 27; Fig. 7). The correct size of the protein and the recognition of the C-terminal HIS-tag suggest that rGST was expressed as full-length protein. Additionally, this result demonstrates that the protein was present in the crude cell extract (sol; soluble protein fraction) before purification.

6.2.2 Proof of identity of recombinant birch-GST

The identity of recombinant birch-GST was confirmed by mass spectrometry (MS) at the University of Salzburg (Prof. Peter Briza, proteomics research group). The analysis showed the correct molecular mass of the protein and 90% of the peptides in the sequence were identified (Fig. 8).

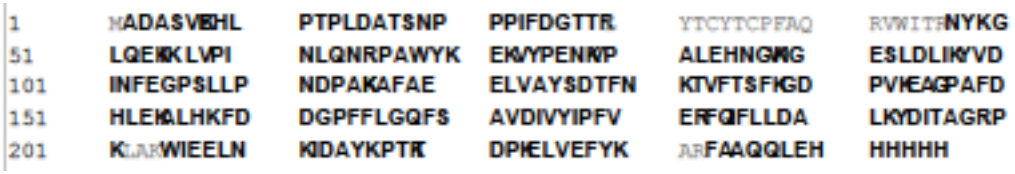

Figure 8 Peptide analysis of recombinant birch-GST by mass spectrometry (MS). The complete Aa sequence of rGST is shown. Bold characters indicate Aa/peptides found in MS analysis.

6.2.3 Endotoxin removal

Gram-negative bacteria, such as *E.coli*, are known to contain large quantities of endotoxins linked to the cell wall. Endotoxins (e. g. lipopolysaccharides; LPS) are released into the cell or media during the expression and purification cycle in recombinant protein production [143]. LPS triggers pro-inflammatory pathways via the PRRs TLR2 and TLR4 and can lead to tissue injury, septic shock or even death when it enters the blood stream in excessive amounts [140, 144]. To eliminate any remaining LPS contaminations in rGST, which might interfere with cell culture based assays or cause adverse reactions in animal experiments, two different endotoxin removal methods were applied [145].

One aliquot of the protein sample (Pool B) was loaded onto an endotoxin removal column containing a resin that specifically binds protein-associated endotoxins. The second aliquot (Pool A) was purified by applying the detergent Triton X-114. This purification procedure takes advantage of the property of Triton X-114 to form micelles. Micelles can incorporate LPS by

non-ionic interaction. The micelle rich hydrophobic phase including LPS can then be separated by centrifugation from the micelle poor, aqueous phase containing the protein [140].

Subsequently, protein and endotoxin concentrations in the samples were determined by using a bicinchinonic acid (BCA) protein assay and a chromogenic limulus amobecyte lysate (LAL) assay, respectively. In all samples endotoxin levels were reduced to satisfactory limits and the protein concentration was adjusted to a suitable working range as listed in table 2. Additionally, the detergent-treated rGST sample was applied to detergent removal columns to remove any residual Triton X-114 traces, since this group of compounds was reported to be toxic on cells [146]. All samples were stored in sodium phosphate buffer at -20°C.

Removal method	Protein Conc. [$mg*mL^{-1}$]	Endotoxin/mg Protein [$ng*mg^{-1}$]
rGST before Endotoxin removal	0,82	32
rGST Pool A (column method)	0,85	7
rGST Pool B (Triton method)	0,9	0,3

Table 2 Protein and endotoxin concentrations in different samples before and after endotoxin removal.

Possible remaining contaminations of LPS and Triton X-114 in Pool A and B were analyzed in a functional cell based assay using HEK 293 cells transfected with human TLR4. The activation of TLRs triggers the induction of a pro-inflammatory immune response and the release of antimicrobial agents. TLR4 binds gram-negative-derived LPS causing the release of inflammatory cytokines such as IL-8 [5, 144, 147].

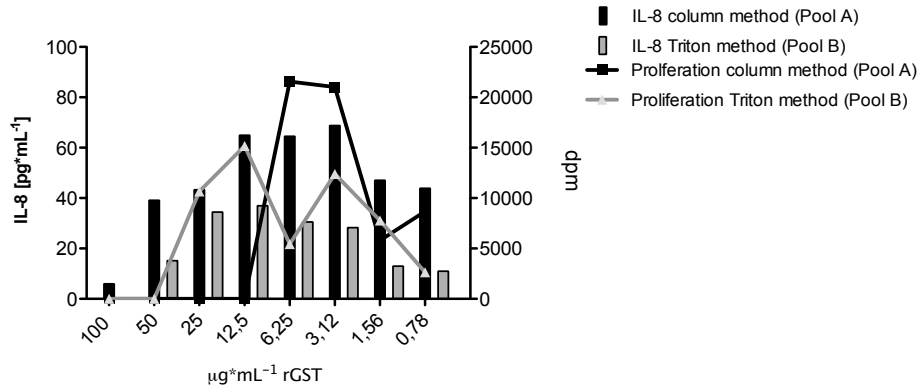

Figure 9 Proliferative and IL-8 responses of HEK-TLR4 cells to rGST. 2*10^5 cells/mL were incubated with various concentrations of rGST (derived from pool A or pool B) for 5 days. After 4 days supernatants were collected and IL-8 release was assessed. Proliferation was determined by ^{3}[H]-thymidin incorporation on day 6. Bars represent IL-8 in the supernatants, lines cell proliferation.

Concentrations higher than 50 µg*mL^{-1} of rGST were toxic on HEK-TLR4 cells independently of the protein pool used. At lower protein concentrations differences were observed for the individual endotoxin removal procedures. Cells stimulated with the Triton X-114-treated protein sample (Pool A) showed lower IL-8 release compared to cells incubated with rGST after column application. This finding was consistent with the results in previous experiments since pool B (Triton X-114-treated) exhibited low levels of endotoxin.

6.3 Sequence alignment of birch-GST

Table 3 shows protein sequence homologies of birch-GST with plant and human GST proteins. Highest sequence homology was found with GST from *Jatropha curcas* (Purge nut). This protein was identified as an omega class GST. Alignment of birch-GST with allergenic GSTs from house dust mite (*Dermatophagoides pteronyssinus,* Der p 8; mu class) and German cockroach (*Blattella germanica,* Bla g 5; sigma/delta class) reveals sequence homology of 24% (Der p 8) and 22% (Bla g 5), respectively (Fig. 10 and table 3).

Aa sequence identity	GST family	Species
79%	Omega	*Jatropha curcas*
77%	Omega	*Ricinus comunis*
76%	Lambda	*Populus trichocarpa*
71%	Lambda	*Arabidopsis thaliana*
70%	n.d.	*Pisum sativum*
39%*	Mu	*Homo sapiens*
28%	Omega	*Homo sapiens*
27%	Sigma	*Homo sapiens*
24%**	Mu	*D. pteronyssinus*
22%***	Sigma/delta	*Blattella germanica*

Table 3 Sequence alignment of birch-GST with plant and human GST sequences. Alignments were performed with BLASTP [148]. Sequence identity within *21 Aa, **38 Aa and ***153 Aa; n. d., not determined

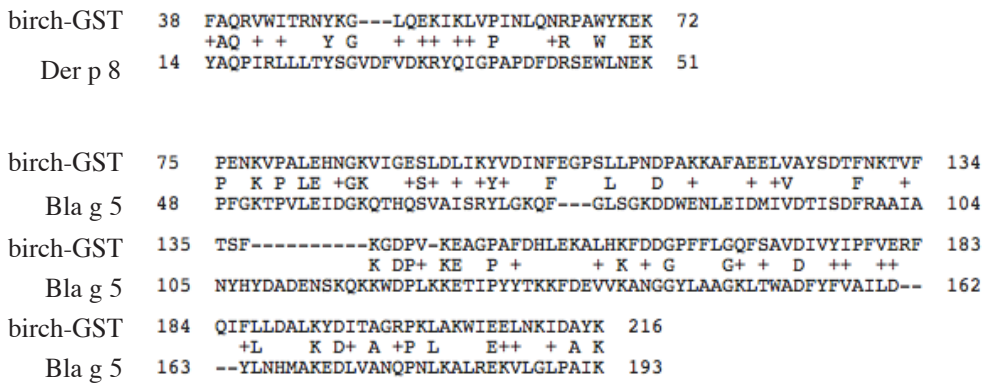

Figure 10 Sequence alignment of birch-GST with HDM-GST (Der p 8) and German cockroach-GST (Bla g 5). Sequence alignments were done with BLASTP.

6.4 Enzymatic activity of recombinant and natural GST

The enzymatic activity of rGST and BPE was investigated in a GST activity assay kit. The results showed that rGST was not enzymatically active, whereas BPE displayed weak enzymatic activity.

Sample	ΔA_{340}/min	GST Activity (U/mL)	GST Activity/μg Protein (U/μg)
rGST	0,71	1,42	0,002
nGST	4,70	9,35	n.d.
positive ctrl	26,72	53,12	n.d.

Table 4 Enzymatic activity of rGST and BPE. Enzymatic activity was measured in a GST activity assay kit and expressed in U/mL and U/mg protein, respectively. n. d.; not determined

6.5 Generation of murine anti-birch-GST Abs

BALB/C mice were immunized *intraperitoneally* (i. p.) with either rGST or rBet v 1 adsorbed to Alum to generate specific Abs. In total, 5 immunizations were carried out and before each injection blood was collected. Mice were sacrificed 10 days after the last immunization and spleen cells were isolated (Fig. 11).

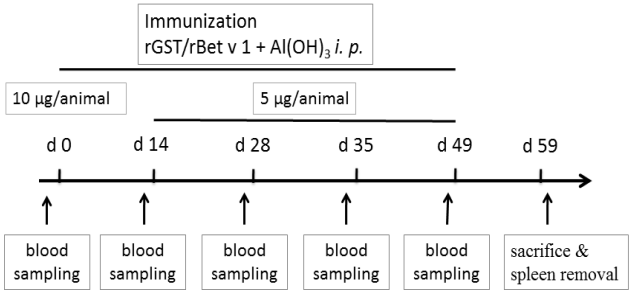

Figure 11 Immunization protocol. BALB/c mice were immunized with either rGST or rBet v 1 absorbed to $Al(OH)_3$ in biweekly intervals (except between d 28 and d 35).

6.5.1 Detection of Ab responses to birch-GST and Bet v1

Murine sera obtained from blood sampling were analyzed for specific IgG1, IgG2a, IgG3 and IgE responses to rGST and rBet v 1.

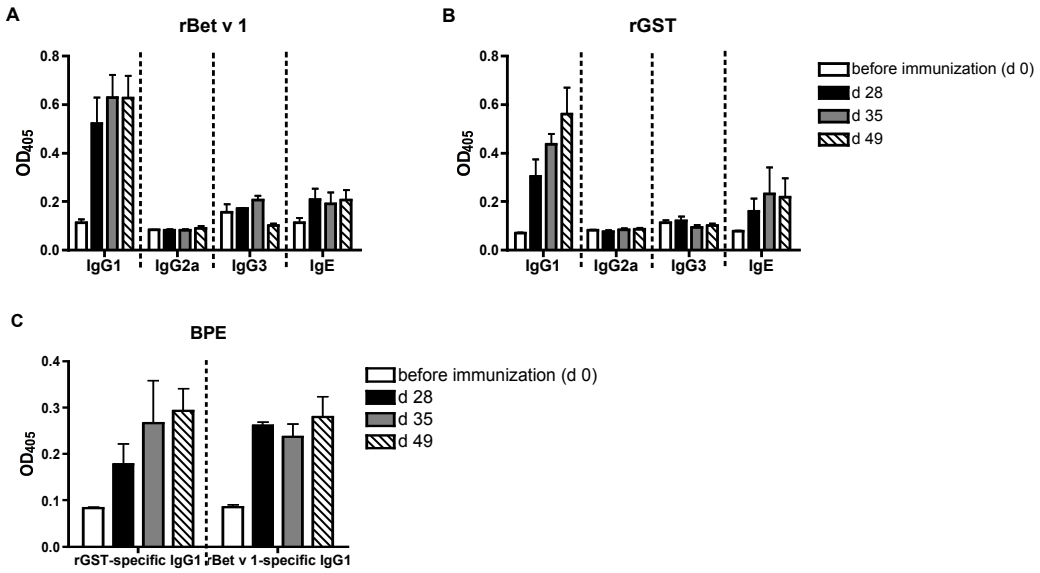

Figure 12 Ab levels determined after immunization with either rGST or rBet v 1. Plates were coated with (A) rBet v 1 (1 µg*mL^{-1}), (B) rGST (2 µg*mL^{-1}) or (C) birch pollen extract (50 µg*mL^{-1}) and incubated with sera from immunized mice collected at indicated time points. IgG1, IgG2a, IgG3 and IgE levels were assessed. O. D., optical density.

BALB/C mice immunized with either rGST or rBet v 1 showed a specific IgG1 response and Ab titers increased after every immunization (Fig. 12 A-C). IgG2a and IgG3 Ab levels did not rise after immunization neither in the GST nor in the Bet v 1 group. A moderate increase of IgE Ab titers was seen in both groups (Fig. 12 A, B). Specific IgG1 Abs raised against the recombinant proteins also recognized natural Bet v 1 and natural GST (nGST) in BPE (Fig. 12 C).

In summary, these findings demonstrated that rGST was immunogenic and induced rGST-specific Abs in mice. In addition to IgG1 Abs, slightly upregulated IgE levels were detected. The same applied to the major birch

pollen allergen rBet v 1. It was shown that Abs generated with rGST reacted with nGST in BPE. This fact suggests that rGST shows similar folding and structure compared to the natural occurring protein.

6.5.2 Cross-reactivity of recombinant birch-GST and Der p 8

Previously, proteins of GST-family were identified as allergens in HDM (Der p 8) and German cockroach (Bla g 5) [149, 150]. We investigated a potential cross-reactivity between rGST from birch and Der p 8. Therefore, ELISA-plates were coated with HDM extract and incubated with sera collected from rGST-immunized mice. BPE served as a positive control.

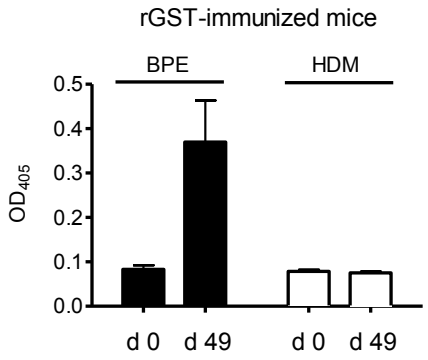

Figure 13. Detection of rGST-specific Abs cross-reacting with nGST in BPE and Der p 8 in HDM extract by ELISA. Plates were coated with HDM or birch pollen extract (each 50 µg/mL) and incubated with sera collected on d 0 and d 49. Bound IgG1 Abs were detected. O. D., optical density

No cross-reactivity between rGST-specific Abs and HDM-GST was observed (Fig. 13, white bars). In contrast, rGST-specific IgG1 clearly recognized nGST in BPE (Fig. 13, black bars).

6.5.3 Cross-reaction of recombinant and natural birch-GST

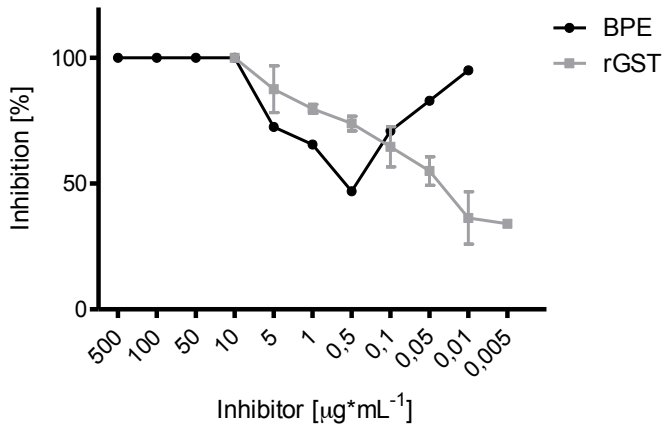

Figure 14 Binding of rGST-specific Abs to rGST was inhibited by BPE and rGST. Mouse sera obtained from rGST-immunized mice were pre-incubated with different concentrations of rGST and BPE. Bound rGST-specific Abs were detected by ELISA.

To evaluate whether Abs specific for rGST cross-react with BPE, inhibition studies were performed. Sera of rGST-immunized mice were pre-incubated with different concentrations of BPE and rGST. Pre-incubated sera were subsequently applied on ELISA plates coated with rGST. Inhibition of IgG1-binding to immobilized rGST by BPE and rGST was determined and expressed as percentage of inhibition (Fig. 14). Pre-incubation with rGST served as a control.

Similar inhibition curves for BPE and rGST were obtained showing that BPE could inhibit Abs specific for the recombinant protein. This finding suggests that rGST is correctly folded and rGST-specific Abs can be used to detect natural GST in BPE.

6.6 T cell response to birch-GST and Bet v 1

Splenocytes of rGST and rBet v 1-immunized mice were stimulated with different concentrations of rGST, rBet v 1 or BPE and proliferative responses were assessed.

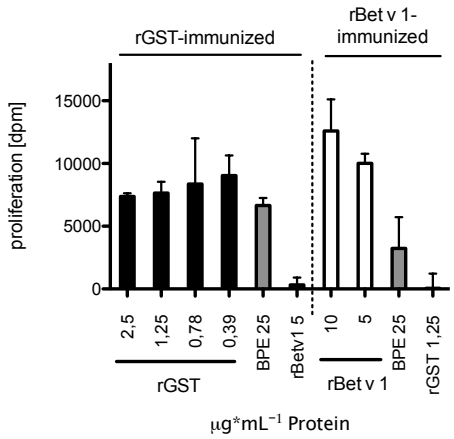

Figure 15 Proliferative responses of splenocytes isolated from mice immunized with rGST or rBet v 1. 2*10^5 cells/mL were incubated with rGST (black bars), rBet v 1 (white bars) and BPE (grey bars), respectively. Proliferation was measured by ^{3}[H]-labeled thymidin incorporation after 5 days. Results are shown in delta counts per minute (dpm). Medium values are subtracted.

Splenocytes obtained from rGST-immunized mice proliferated upon stimulation with rGST. Strongest proliferation was detected at low concentrations of antigen (0,39 µg*mL^{-1}; Fig. 15, black bars). Cells also proliferated upon stimulation with BPE (Fig. 15 left pattern, grey bar) and did not react to rBet v 1 (Fig. 15 left pattern).

Splenocytes isolated from rBet v 1-immunized mice showed a dose-dependent proliferation after stimulation with rBet v 1 (Fig. 15, white bars). Strongest proliferation was observed at 10 µg*mL^{-1} of rBet v 1. Cells also responded to BPE (Fig. 15 right pattern, grey bar) and did not proliferate upon incubation with rGST.

Taken together, T cells from mice immunized with either rGST or rBet v 1 proliferated upon stimulation with the recombinant as well as the natural protein.

To evaluate cytokine responses, spleen cells were incubated with rGST or rBet v 1 and IL-2, IL-4, IL-5, IL-10, IL-13, IL-17 and IFN-γ concentrations in the supernatants were determined by using a Multiplex bead system.

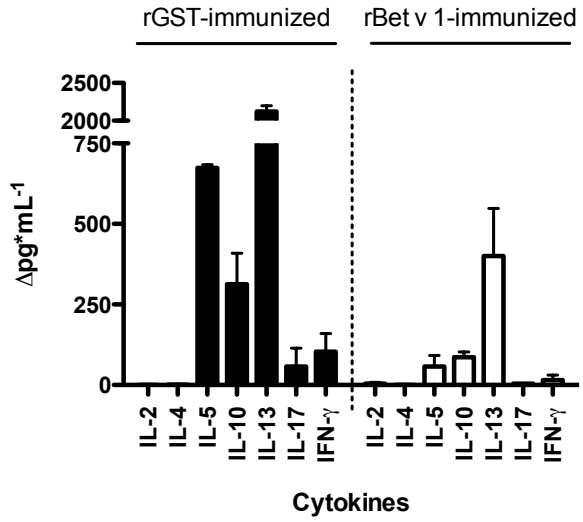

Figure 16 Cytokine responses of splenocytes after stimulation with rGST or rBet v1. Spleen cells isolated from rGST- and rBet v 1-immunized mice were incubated with optimal concentrations of rGST (1,25 µg*mL^{-1}, black bars) or Bet v 1 (5 µg*mL^{-1}, white bars) for 40 h. Thereafter, the supernatants were collected and analyzed for the indicated cytokines. Results are shown in pg*mL^{-1}. Δ; Medium values are subtracted.

Spleen cells obtained from mice immunized with rGST produced high amounts of the Th2-associated cytokines IL-5 and IL-13 after stimulation with rGST, whereas IL-2 and IL-4 were not detectable in the supernatants. Stimulation with rGST also induced the production of IL-10 and IFN-γ.

Splenocytes of rBet v 1-immunized mice cultured in the presence of rBet v 1 mainly released IL-13. Low levels of IL-5 and IL-10 were observed. No IL-2 and IL-4 were detectable. In contrast to rGST, cells stimulated with rBet v 1 did not produce IFN-γ.

6.7 Recognition of birch-GST by human IgE

IgE-reactivity to birch-GST and the birch pollen minor allergens Bet v 3, Bet v 4, Bet v 6 and Bet v 7 was assessed in the sera 215 Austrian birch pollen-allergic individuals [151]. All patients were diagnosed with birch pollen allergy on the basis of clinical history, positive skin prick test to BPE and the detection birch pollen-specific IgE (≥0,35 kU_A/L). The sera were provided by different Allergy Clinics (Graz, Innsbruck, Vienna) and the Medical University of Vienna (MUW). Plates were coated with the respective allergen and incubated with patient's sera O/N. Bound IgE Abs were detected by using a murine anti-human IgE Ab and plates were color-developed. Sera of non-allergic individuals were used as a control. The mean O. D. of all non-allergic controls plus 10 times standard deviation was defined as the cut-off for positive IgE-reactivity.

The prevalence of IgE-reactivity to birch-GST was 5-20% in sera obtained from Innsbruck, Graz and Vienna while in the sera collected at the MUW only specific IgE for Bet v 4 was detectable (table 5). Bet v 3 and Bet v 7 showed relatively low IgE-recognition frequencies (3-11% and 5-7%, respectively), whereas IgE-reactivity to Bet v 4 was more pronounced (8-23%). IgE specific for Bet v 6 was found in 10-18% of the tested sera.

	n	rGST	rBet v 3	rBet v 4	rBet v 6	rBet v 7
Innsbruck	61	20	11	23	10	7
Graz	91	5	9	11	14	5
Vienna	39	5	3	8	18	5
MUW	24	0	0	13	0	0
Total	215	9*	7	14	12	5

Table 5 Percentage of birch pollen-allergic patients showing IgE-reactivity to rGST and rBet v 3-7 determined for each group. *Percentage of patients with IgE-reactivity are shown

In Fig. 17 the prevalence of IgE-reactivity to birch-GST and all known birch pollen allergens (Bet v 1-7) in the entire study population (215 individuals) is summarized. 9% (19/215) of the tested sera displayed IgE-reactivity to birch-GST. IgE specific for Bet v 3 and Bet v 4 was detected in 7% and 14% of the sera, respectively. 12% of the sera were IgE-positive for Bet v 6, 5% contained specific IgE for Bet v 7. 88% of the patients were sensitized to the major allergen Bet v 1 and 22% showed positive IgE-reactivity to birch pollen profilin (Bet v 2).

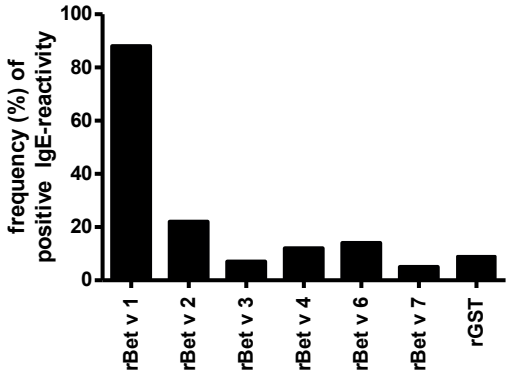

Figure 17 Percentage of patients showing positive IgE reactivity to birch-GST and all known birch pollen allergens.

7 Discussion

GSTs belong to a well-conserved enzyme superfamily which are, besides several other functions, mainly responsible for the detoxification of potential harmful substrates [122]. Members of the GST-protein-family have already been identified as allergens in HDM (Der p 8) and German cockroach (Bla g 5) [150, 152]. Recently, a GST-like protein has been found in birch pollen and was present in high amounts [153]. However, biochemical and immunological properties of birch-GST have not been investigated by now. Most interesting, to date, no data about the allergenicity of GST and its potential role in the development of birch pollen allergy has been gathered.

The aims of this thesis were to characterize the biochemical and immunological features of birch-GST. For this purpose, birch-GST was produced as recombinant protein and, for the first time, the recognition of recombinant birch-GST by IgE Abs from a high number of birch pollen-allergic individuals was analyzed. We also studied humoral and cellular responses in mice immunized with rGST to further evaluate the immunogenicity of this protein.

Since several attempts to purify natural GST from birch pollen by different chromatography methods were not successful, the sequence of the protein was deduced and cloned into an expression vector (kindly provided by Prof. Fatima Ferreira, University of Salzburg). Birch-GST was expressed as recombinant protein, purified to homogeneity and endotoxins were removed to negligible amounts. The identity of recombinant birch-GST was confirmed by mass spectrometry.

Sequence alignments of birch-GST with several known GSTs revealed a homology of 79% with a protein belonging to the GST omega class. Omega class GSTs have been identified in vertebrates (Pig) nematodes (*Caenorhabditis elegans*) or trematodes (*Schistosoma mansoni*) [154-156].

Despite of the low sequence homology (20%) with other GST subclasses (e.g. theta class), omega GSTs belong the to GST superfamily and exhibit similar characteristics. They are also located in the cytosol and are responsible for the detoxification of various endogenous and exogenous compounds [157]. Concerning the allergenicity of certain subclasses, GSTs of the mu (Der p 8, HDM) as well as sigma and delta (Bla g 5, German cockroach) class were reported to show IgE-reactivity [158].

Next, we investigated the enzymatic activity of recombinant birch-GST and BPE. It was reported that recombinant GSTs from *Alternaria alternata* or German cockroach were enzymatically active [159, 160]. In our study, recombinant birch-GST did not display any catalytic activity. In general, most GSTs are enzymatically active as hetero- or homodimers [120]. The formation of GST-dimers is facilitated by a dimerization interface located at the highly conserved N-terminal domain of the proteins. A potential Aa substitution or mutation might hamper or prevent dimerization of recombinant birch-GSTs and, thus leads to the lack enzymatic activity. In contrast, nGST in BPE seems to be still able to dimerize and shows at least weak enzymatic activity. A possible explanation for the weak activity might be that omega GSTs show only low affinity to the substrate CDNB, as shown for other subclasses, or too low concentrations of nGST were applied in the assay [124].

BALB/C mice were immunized with pure rGST to generate specific Abs. To compare the immune response to birch-GST with a major allergen, another group of mice were immunized with rBet v 1 in parallel. As described in several publications, immunization of atopy-prone mice with rBet v 1 led to a predominant production of IgG1 and IgE Abs [161-163]. Similar results were obtained with birch-GST. The induction of a rather Th2-like response is merely attributed to use of Alum as adjuvant, which is known to promote a Th2-associated phenotype, and not to the intrinsic properties of birch-GST [161, 164].

Spleen cells stimulated with birch-GST or Bet v 1 showed comparable proliferative responses for both antigens. However, on the cytokine level, birch-GST induced even stronger immune responses than Bet v 1. Cells

released considerably higher levels of IL-5 and IL-13 as well as IL-10 after stimulation with birch-GST compared to stimulation with Bet v 1. IL-5 and IL-13 are characteristic signature cytokines of Th2-associated responses and correlate with increased IgG1 and IgE titers, whereas IL-10 is preferentially produced by regulatory T cells favoring immune tolerance. Of note, that birch-GST also promoted the production of the Th1-related cytokine IFN-γ. In contrast, Bet v 1-stimulated splenocytes secreted only very low amounts of IFN-γ, which was consistent with other studies using a similar immunization protocol [161, 162]. Taken together, birch-GST induced comparable or even stronger Ab and T cell responses than Bet v 1 suggesting that, in a Th2-biased mouse strain, it shows similar immunogenicity to the major birch pollen allergen.

Sequence comparison of birch-GST and the known allergens Der p 8 (HDM-GST) and Bla g 5 revealed only little homology among these proteins. We analyzed whether birch-GST-specific Abs cross-react with Der p 8 and confirmed on experimental level that these GSTs do not share similar structure. The recognition of natural GST by Abs specific for recombinant birch-GST, however, shows that the recombinant protein is correctly folded and resembles the structure of its natural counterpart. In consequence, Abs directed against recombinant birch-GST can be used to detect nGST in BPE in future experiments.

Up to now, no studies have evaluated the prevalence of sensitization to birch-GST in birch pollen-allergic individuals. Hence, we investigated the recognition of rGST by IgE antibodies in the sera of 215 birch-pollinosis patients by ELISA. In parallel, IgE-reactivity to four recombinant birch pollen minor allergens (Bet v 3-7) was determined.

Around 9% of 215 pollen-allergic patients showed IgE-reactivity to birch-GST. 12 out of 19 (64%) individuals who were IgE-positive for birch-GST also recognized one or more minor allergens. Only 3,3% were exclusively sensitized to birch-GST indicating that probably primarily highly atopic individuals who react to several birch pollen allergens also develop IgE Abs

specific this protein. In summary, these findings lead to the conclusion that birch-GST is another minor allergen birch pollen.

Bet v 3 and Bet v 4 were recognized by IgE Abs in 7% and 14% of the tested patients, respectively. These data match previous studies demonstrating similar recognition frequencies in allergic individuals [93, 96, 97, 165]. Both minor allergens were identified as calcium-binding proteins (CBPs), which comprise a widespread protein family with several homologues found in alder (Aln n 4), timothy (Phl p 7) and bermuda grass (Cyn d 7) [101, 102, 104]. Due to the widespread distribution among various organisms, CPBs were characterized as panallergens [82]. Moreover, it was described that Bet v 4 and Phl p 7 show similar three-dimensional structures, which might be responsible for cross-reactivity between these proteins [166, 167]. However, up to now, it has not been investigated which allergen source causes primary sensitization in birch and grass pollen allergy.

In our study population 12% of the tested sera contained IgE specific for Bet v 6. This protein has been described to share sequential and structural homology to phenylcoumaran ether and isoflavonoid reductase with a molecular size of ≈35 kDa and similar IgE-recognition frequency (10%) [90, 108]. It has been shown that Bet v 6-specific IgE Abs reacted with homologous proteins in fruit such as pear, apple, banana, carrot or lychee fruit [168]. Especially Pry c 5, an isoflavonon reductase-like protein in pear displayed strong inhibition of IgE-binding to Bet v 6 [110]. However, it seems to be unlikely that birch pollinosis patients in our study were primarily sensitized to certain fruits and therefore developed cross-reactive IgE Abs to Bet v 6. It was demonstrated that homologous plant food allergens in celery, carrot or apple share common three-dimensional structures with Bet v 1 and Bet v 2 [61, 70, 169, 170]. Though, primary sensitization to pollen allergens is the main cause for the development of an OAS after the consumption of plant-derived food [171].

We also analyzed IgE-reactivity to Bet v 7. In 5% of the tested sera IgE Abs against Bet v 7 were detected but none of the patients exclusively recognized this allergen. This finding is in contrast to another study, in which

approximately 21% of the birch pollen-allergic individuals showed IgE-binding to Bet v 7 [112]. Cadot et al., identified Bet v 7 as a member of the cyclophilin protein family. Since allergenic cyclophilins were found in several yeast and fungi, Bet v 7 is also considered to be a potential panallergen [114].

In this thesis we assessed IgE-reactivity to birch-GST and the birch pollen minor allergens Bet v 3-7 in a study population of 215 birch pollen-allergic patients. In studies including a similar number of individuals comparable recognition frequencies were determined. This suggests that the prevalence for IgE-reactivity obtained in our study population is representative among birch pollinosis patients [56, 98].

In summary, we could demonstrate that birch-GST was immunogenic in mice and induced Ab and T cell responses similar to the major allergen Bet v 1. However, we found that it is still a minor allergen for humans. Although GST is abundant in birch pollen it does not show strong allergenicity. We speculate that the quantity and the release from pollen are detrimental for the allergenic properties of a protein. However, the quantity of GST in birch pollen is not yet well-defined. It has been shown that Bet v 1 accounts for 22% of the total water-soluble protein in birch pollen and is released immediately upon rehydration [172]. In contrast, the minor allergen Bet v 2, to which a much smaller percentage of allergic individuals are sensitized, is rather retained in birch pollen [173]. Whether GST also shows a limited release from birch pollen will be studied in the near future.

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11 Zusammenfassung

Birkenpollenallergie zählt zu den häufigsten allergischen Erkrankungen in Zentral- und Nordeuropa sowie in Nordamerika. Bis heute wurden mehrere Proteine des Birkenpollen, die zur Sensibilisierung und in weiterer Folge zum Auftreten von allergischen Symptomen in Birkenpollenallergikern beitragen, als Allergene identifiziert. In Analysen des gesamten Birkenpollen Proteoms wurde ein bisher unbekanntes, aber in großen Mengen vorkommendes Protein der Familie der Glutathione-S-Transferasen (GST), gefunden. GST sind eine phylogenetisch weit verbreitete Enzymfamilie und wurden bereits in der Hausstaubmilbe (*Dermatophagoides pteronyssinus*; Der p 8) und der Deutschen Schabe (*Blattella germanica*; Bla g 5) als Allergene beschrieben. Jedoch ist bis dato nichts über die Rolle der GST aus der Birke in Birkenpollenallergie bekannt.

Ziel dieser Masterarbeit war es i) GST aus Birkenpollen als rekombinantes Protein herzustellen und zu reinigen sowie seine ii) immunologischen und biochemischen Eigenschaften genauer zu charakterisieren.

GST wurde als rekombinantes Protein (rGST) in *E. coli* hergestellt, gereinigt und etwaige Endotoxinverunreinigungen entfernt. Mäuse wurden mit rekombinantem Birken-GST immunisiert um spezifische Antikörper herzustellen. Um die Immunantwort mit einem Hauptallergen zu vergleichen, wurde weiteren Mäusen Bet v 1 gespritzt. Die, durch Immunisierung mit rekombinantem Birken-GST hervorgerufene Immunantwort, war vergleichbar mit jener von Bet v 1. Beide Proteine lösten die Produktion von spezifischen IgG1 und IgE Antiköper sowie eine Th2-assozierte T Zell-Antwort aus. Antiköper, gerichtet gegen Birken-GST und Bet v 1, erkannten das jeweilig, natürlich vorkommende Protein in Birkenpollenextrakt. Kreuzreaktivität zwischen Birken-GST und Hausstaubmilben-GST wurde nicht festgestellt.

Anschließend wurden die Seren von 215 Birkenpollen Allergikern auf spezifische IgE Antikörper gegen Birken-GST und die Birkenpollen-Nebenallergene Bet v 3, Bet v 4, Bet v 6 und Bet v 7 getestet. In rund 9% der

untersuchten Seren wurde Birken-GST-spezifisches IgE nachgewiesen. IgE Antiköper gegen die Nebenallergene waren in ähnlicher Häufigkeit in den Patientenseren feststellbar.

In biochemische Analysen wurde nachgewiesen, dass rekombinantes Birken-GST enzymatisch nicht aktiv war. Birkenpollenextrakt hingegen zeigte geringe enzymatische Aktivität.

Im Rahmen dieser Masterarbeit wurde gezeigt, dass Birken-GST in Mäusen immunogen war und eine Immunantwort, vergleichbar mit jener des Hauptallergen Bet v 1, auslöste. Für den Menschen hingegen zeigt es die Charakteristika eines Nebenallergens. In zukünftigen Studien soll untersucht werden, ob dies auf die Freisetzung des Proteins aus dem Birkenpollen oder seinen intrinsischen Eigenschaften zurückzuführen ist.

12 Curriculum Vitae

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Education

1993-97	Elementary School Mondweg, Vienna
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Congresses

Christian Zwicker, Stephan Deifl, Claudia Kitzmüller, Gabriele Gadermeier, Fatima Ferreira and Barbara Bohle; *Immunological Characterization of Glutathione-S-Transferase in Birch Pollen*; Poster presentation at the Retreat of the Center of Infectiology, Immunology and Pathopysiology, Medical University of Vienna

Christian Zwicker, Stephan Deifl, Claudia Kitzmüller, Gabriele Gadermaier, Eva Vejvar, Fatima Ferreira and Barbara Bohle; *Immunological Characterization of Glutathione-S-Transferase in Birch Pollen*; Poster presentation at the annual Meeting of the Austrian Society for Allergology and Immunology, Klosterneuburg, Austria

Christian Zwicker, Stephan Deifl, Claudia Kitzmüller, Gabriele Gadermaier, Eva Vejvar, Fatima Ferreira and Barbara Bohle; *Immunological Characterization of Glutathione-S-Transferase in Birch Pollen*; Poster presentation at the EAACI Winterschool, Pichl, Austria