universität wien

MASTERARBEIT

Titel der Masterarbeit

The Antigenicity of the Major Grass Pollen Allergen Phl p 5 Expressed in Balb/c Mice

Verfasser

Andreas Farkas, B.rer.nat.

angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 066 830
Studienrichtung lt. Studienblatt:	Masterstudium Molekulare Mikrobiologie und Immunbiologie
Betreuerin / Betreuer:	Univ.-Prof. Dr.med.univ. Rudolf Valenta

Zusammenfassung:

Die adaptive Immunität reagiert auf Pathogene indem sie Antigene spezifisch erkennt. Um auf die Vielzahl von Antigenen reagieren zu können, muss die Spezifizierung hochflexibel sein. Diese Flexibilität birgt jedoch auch die Gefahr eine Immunantwort gegen normalerweise harmlose Antigene (z.b. Allergene), mit zum Teil sehr negativen Nebenwirkungen, zu entwickeln. Um dies zu verhindern ist die Entwicklung neuer Ansätze, die eine solche Immunantwort verhindern, von sehr großer Bedeutung. Eine Herangehensweise ist der molekulare Chimärismus, bei welchem Toleranz gegen ein bestimmtes Antigen induziert werden soll. Dies geschieht durch Transplantation von autologen hämatopoetischen Stammzellen (HSC), die gentechnisch verändert wurden, um ein bestimmtes Antigen zu exprimieren. Nach der erfolgreichen Transplantation kommt es zu einer Koexistenz von modifizierten und unmodifizierten HSC in einem Individuum, was man als molekularen Chimärismus versteht. Unsere Forschungsgruppe hat ein Mausmodell entwickelt, bei welchem HSC einer Balb/c Maus retroviral transduziert werden, um das Graspollenhauptallergen Phl p 5 als Transmembranprotein zu exprimieren. Diese modifizierten HSC werden anschließend in naive Balb/c Mäuse transplantiert, der dadurch entstehende molekulare Phl p 5 Chimärismus führt zu einer spezifischen stabilen Langzeittoleranz gegen Phl p 5. Um die Toleranzinduktion dieses Ansatzes weiter erforschen zu können, wurde eine transgene Maus entwickelt welche ubiquitär Phl p 5 als Transmembranprotein, mit zytoplasmatischer GFP (i.e. grün fluoreszierendes Protein) Co-expression, exprimiert. In dieser Masterarbeit wurde die Phl p 5 Expression der Phl p 5 transgenen Maus durch Durchflusszytometrie, Konfokalmikroskopie und Immunoblotting charakterisiert.

Des Weiteren ist es wichtig, dass ein Antigen ausreichend immunogen ist um als Modellantigen für experimentelle Toleranzinduktion geeignet zu sein. Weshalb im zweiten Teil der Arbeit die Immunogenität des Phl p 5 Transgens bestimmt wurde. Hierfür wurde ein stringentes Toleranzmodell aus der Transplantationsimmunologie gewählt bei welchem Haut von einem Spender auf einen Empfänger transplantiert wird. Sollten sich in der Spenderhaut Antigene befinden, welche vom Immunsystem des Empfängers als ‚Nicht-Selbst' erkannt werden, wird die Haut abgestoßen. Da sich in unserem Ansatz die Haut der Phl p 5 transgenen Balb/c Maus von einer naiven Balb/c Maus nur durch Phl p 5 und GFP unterscheidet, erlaubte die

Geschwindigkeit der Abstoßung einen Rückschluss auf die Immunogenität der Antigene. In diesem Experiment zeigte sich, dass die Haut der Phl p 5 transgenen Maus unerwartet schnell abgestoßen wurde (Medianes Überleben: 9 Tage). Des Weiteren konnten wir zeigen, dass für diese schnelle Abstoßung $CD4^+$ T Zellen nötig sind. Nach der Abstoßung wurden die Phl p 5-spezifischen Antikörper im Serum der Mäuse mittels ELISA bestimmt. Die induzierten Antikörperspiegel waren mit einer subkutanen Immunisierung durch rekombinantes (r)Phl p 5 mit Aluminiumhydroxid vergleichbar. Außerdem wurde gezeigt, dass die induzierten Phl p 5-spezifischen IgE und IgG1 Antikörperspiegel mindestens 58 Wochen lang stabil waren. In einem *in vitro* Basophil Assay konnte gezeigt werden, dass die Phl p 5-spezifischen IgE Antikörper eine Degranulierung induzieren. In weiteren Versuchen wurden Splenozyten und Splenozytenproteinextrakte der Phl p 5 transgenen Maus subkutan injiziert und die Phl p 5 spezifischen Antikörperspiegel zu verschiedenen Zeitpunkten mittels ELISA bestimmt. Hierbei konnte gezeigt werden, dass nur Splenozyten eine stabile mindestens 14 Wochen anhaltende Immunantwort induzieren können. Nach Beendigung der Mausexperimente wurde erhöhte Phl p 5-spezifische T-Zellproliferation in Splenozyten von Mäusen, welche hauttransplantiert oder mit Splenozyten behandelt wurden, festgestellt.

Darüber hinaus konnte gezeigt werden, dass die Lokalisation von Phl p 5 an der Zellmembran nicht notwendig für die Induktion einer Immunantwort ist. Hierfür wurden murine Zellen transduziert, um entweder membrangebundenes oder zytoplasmatisches Phl p 5 zu exprimieren. Danach werden die Zellen subkutan in naive Balb/c Mäuse injiziert. Anschließend wurden die Phl p 5 spezifischen Antikörperspiegel mittels ELISA bestimmt.

Zusammenfassend ist dieses neuartige Mausmodell ideal zur Erforschung der Toleranzinduktion durch molekularen Chimärsmus geeignet. Zudem könnte dieses Modell sich zur Erforschung der humoralen Immunantworten gegen nicht-MHC Alloantigene eignen, da sich das in murinen Zellen exprimierte Transgen Phl p 5, ohne die Zugabe von Adjuvantien, als hoch immunogen erwiesen hat.

Abstract:

Adaptive immune responses against pathogens are specifically directed towards antigens. To achieve specific immune responses towards the wide variety of antigens, our adaptive immune system has to be highly versatile. This flexibility also holds the potential to react on antigens which are normally innocuous to the individual (e.g. allergens). But immune responses towards innocuous antigens have the potential to do tremendous damage to the individual. Therefore it is of great importance to find new strategies to avoid such immune responses. One approach to those immune responses is molecular chimerism. This approach strives for induction of long-term tolerance towards defined antigens by transplantation of autologous hematopoietic stem cells (HSCs) genetically modified to express the disease causing antigen. Our group has established a murine model whereby Balb/c HSCs are retrovirally transduced to express the major grass pollen allergen Phl p 5 as a membrane anchored protein. The modified HSCs are then transplanted into naïve Balb/c mice leading to a presence of both genetically modified and naïve HSCs (i.e. molecular chimerism). This molecular chimerism leads to specific, stable and long term tolerance towards Phl p 5. To facilitate the further development of tolerogenic cell therapies for allergy, a transgenic mouse expressing membrane-bound Phl p 5 ubiquitously with green fluorescent protein (GFP) co-expression, was generated. In this thesis the Phl p 5-transgenic Balb/c was characterised concerning expression of Phl p 5 in white blood cells by flow cytometry, localisation at the cell membrane by confocal microscopy, and full length expression via immunoblotting. Additionally it is crucial for experimental tolerance induction that the model antigen chosen is immunogenic. Therefore this thesis also strived for the determination of the immunogenicity of Phl p 5 expressed in Balb/c mice. In order to determine the immunogenicity a stringent tolerance model in allotransplantation, the skin graft approach was used. In this approach tail skin of a donor mouse is transplanted onto a recipient mouse. If foreign (i.e. non-self) antigens presented on the skin tissue are immunogenic the skin tissue is rejected by the recipient. The skin of the Phl p 5-transgenic mouse (whose only foreign antigens were Phl p 5 and GFP) was readily rejected in a $CD4^+$ T cell dependent manner after transplantation on naïve Balb/c mice (median graft survival: 9 days). Subsequently Phl p 5-specific antibody levels were determined in sera of mice via ELISA at several time points. Furthermore we

determined the immunogenicity of splenocytes and splenocyte cell extracts by subcutaneous injection into naïve Balb/c mice and subsequent Phl p 5-specific ELISAs. We showed that the rejection of tail skin of Phl p 5-transgenic Balb/c mice is capable of inducing a stable levels of anti-allergen antibodies for at least 58 weeks and was comparable to subcutaneous immunisation with recombinant (r)Phl p 5 + aluminium hydroxide (including high levels of Phl p 5-specific IgE). High levels of Phl p 5-specific effector cell mediator release were also found for skin grafted mice *in vitro*. Additionally splenocytes of the Phl p 5-transgenic Balb/c induced a stable Phl p 5-specific immune response for at least 14 weeks.

Furthermore we found elevated Phl p 5-specific T cell proliferation *in vitro* in splenocytes of skin grafted and splenocyte treated mice.

To assess whether localisation of Phl p 5 at the cell membrane is crucial for the Phl p 5-specific immune response, NIH-3T3 cells were transduced to express Phl p 5 either as transmembrane protein or cytoplasmically. The transduced cells were then injected subcutaneously and Phl p 5-specific antibody levels were determined via ELISA. Both membrane-bound and cytoplasmic Phl p 5 induced Phl p 5-specific antibodies.

Therefore we conclude that this novel transgenic mouse model is a suitable model for the investigation of tolerance induction via molecular chimerism. Since the transgene Phl p 5 expressed in murine cells appears to be highly immunogenic without adjuvants, it might also serve as a model for a better understanding of humoral immune responses towards non-MHC alloantigens antigens.

This master thesis was conducted under the supervision of:

Univ.-Prof. Dr.med.univ. Thomas Wekerle

Univ.-Prof. Dr.med.univ. Rudolf Valenta

Mag.Dr.rer.nat. Ulrike Baranyi

at the **Medical University of Vienna,**
Department of Surgery, Division of Transplantation

Introduction: 11

The immune response towards exogenous antigens: 11

Antigens: 11

Humoral immune responses towards antigens: 12

Sensitisation towards TD antigens: 12

Activation of Th cells: 13

Immunogenicity of protein antigens: 14

Adjuvants amplify immune responses towards poorly immunogenic antigens: 14

Importance of adjuvants in vaccination: 15

Aluminium hydroxide ($Al(OH)_3$): 16

Undesirable immune responses towards exogenous antigens: 16

Allergy: 16

Allergens: 17

IgE-mediated allergy: 17

Sensitisation towards allergens: 19

Allergen-specific immunotherapy (SIT): 19

Recognition of major and minor histocompatibility antigens in transplant immunology: 20

Major and minor histocompatibility antigens: 20

Haematopoietic chimerism, a cell therapy approach to prevent antigen specific immune responses: 22

Molecular chimerism approaches to prevent immune responses towards defined antigens: 22

Tolerance induction by molecular chimerism in IgE-mediated allergy: 23

Phl p 5-transgenic Balb/c mouse: 25

Aims of this study: 27

Characterisation of the Phl p 5-transgenic mouse: 27

Determination of the immunogenicity of Phl p 5 as a transgene in Balb/c mice: ... 27

Materials and Methods: 28

Materials: 28

Cell culture: 30

Methods: 31

Mouse Methods: 37

Statistical analysis: 39

Results: 40

Characterisation of the Phl p 5-transgenic mouse: 40

Ubiquitous Phl p 5 expression in leucocytes of transgenic Balb/c mice: 40

Localisation of membrane-bound Phl p 5 in confocal microscopy: 41

Full length expression of the transgene Phl p 5: 41

Determination of the immunogenicity of Phl p 5 as a transgene in Balb/c mice: ... 42

Transplanted skin of Phl p 5-transgenic Balb/c mice is highly immunogenic: 42

The rejection of Phl p 5-transgenic skin grafts is accompanied by a strong humoral response: 46

Splenocytes of Phl p 5-transgenic mice induce a high immune response: 50

Phl p 5-specific IgE induced by cells of transgenic mice leads to high effector cell release: 57

Skin grafted mice show high Phl p 5-specific T-cell proliferation: 58

A Phl p 5-specific humoral immune response can also be triggered by irradiated cells and cytoplasmatic Phl p 5: 59

Discussion: 65

Characterisation of the Phl p 5-transgenic mouse: 65

Determination of the immunogenicity of Phl p 5 as a transgene in Balb/c mice: ... 65

References: 69

Appendix: 75

Curriculum vitae: 75

Acknowledgments: 77

Introduction:

The immune response towards exogenous antigens:

Our immune system has to manage a wide variety of pathogens. In contrast to the innate immune system that recognises patterns that are typical for pathogens, the adaptive immune system has the capability to specifically identify antigens. Furthermore a specific immunological memory can be induced to react readily in subsequent encounters with the same antigen. This can only be achieved by a very flexible interplay of different cells. This gives the adaptive immunity and thereby the individual itself the possibility to respond and cope with evolving pathogens. Though the flexibility of recognising antigens appears to be a clear advantage, it might not always be for the benefit of the individual.

Antigens:

Antigens can be referred as substances (e.g. proteins) with the capability of inducing a specific immune response. There are two main ways for the adaptive immune system to recognise antigens. First, via B cells which identify antigens with their main antigen receptors (i.e. membrane-bound antibodies). Antigens that can be recognised by antibodies are substances that bind to the variable regions of antibodies and can be composed of a wide variety of biological molecules (e.g. carbonhydrates, lipids, sugars, proteins and nucleic acids). This binding occurs via noncovalent forces and the specificity is determined by hypervariable loops within the variable regions of an antibody.

T cells and their antigen receptors represent the second route of recognition. [1] In contrast to antibodies T cells recognise antigen peptides that are presented on the surface of the individual's own cells. Therefore antigens presented to T cells have to be processed within the cell and displayed at the surface via the major histocompatibility complex (MHC) class I or II. Those antigens can be derived from intracellular bacteria or viruses or they are exogenous proteins which have to be internalised via endocytosis by antigen presenting cells. [2] The sites that are recognised by B- or T-cells are referred as either B cell or T cell epitopes.

Humoral immune responses towards antigens:

The specific humoral immune response is extremely important for the protection of extracellular spaces within the body. Antibodies produced by B cells have the capability to suppress expansion of infections through the destruction of extracellular microorganisms. [2] An antigen has to be recognised by resting mature IgM^+ or IgD^+ mature B cells followed by activation and clonal expansion and differentiation leading to either immediate antibody secretion, isotype switching, affinity maturation or differentiation to memory B cells. B cells can be activated mainly by two distinct mechanisms; non-protein antigens can directly activate B cells or protein antigens that usually require helper T cell (Th cells) contribution. The antigens are then either denoted as T-dependent (TD) or T-independent (TI) antigens (e.g. nucleic acids, polysaccharides and glycolipids). This work will focus on TD antigens. [1]

Sensitisation towards TD antigens:

Once a B cell binds a TD antigen it can be internalised, processed to antigen peptides and presented via MHC class II to activated Th cells. If the Th cell recognises the antigen a co-signal between CD40 (B cell) and CD40 ligand (CD40L, on the T cell) is crucial and leads to B cell activation and proliferation. Additionally Th cells start to produce cytokines that amplify B cell proliferation (e.g. IL-2, IL-5, IL-4 and IL-21) and can promote heavy chain isotype switching (figure 1). Class switch depends on several factors including the nature of the Th response (Th1 or Th2) triggered by the antigen [3]. One of the first publications to describe Th1 and Th2 responses was published by Mosman and Coffman in 1989. Th cells were approved to be divided in two distinct subsets (i.e. Th1 and Th2) and can be distinguished by their cytokine production patterns upon activation. While viruses and bacteria preferably activate the Th1 subset, helminths most likely trigger a Th2 response. [4] A Th1 response is characterised by an elevation cytotoxic effects and high IFN-γ production leading to an antibody class switch to opsonising and complement fixing antibodies (e.g. in murine models IgG2a [5-6], IgG2b and IgG3[6]). Th2 cells mainly

produce IL-4 which leads to IgG1 and IgE production[6] additionally IL-5 which is also highly secreted, causes eosinophil recruitment. [4, 7]

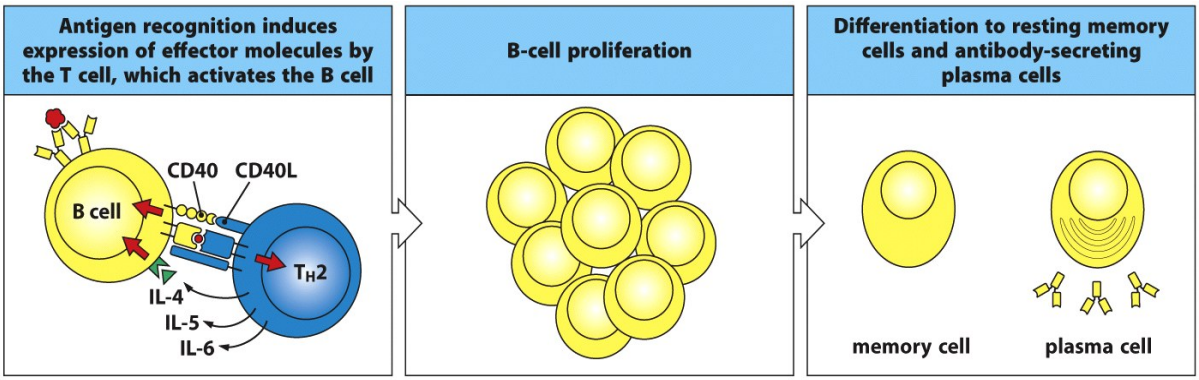

Figure 10.3 Janeway's Immunobiology, 8ed. (© Garland Science 2012)

Figure 1: B cell activation by TD antigens [8]

Activation of Th cells:

As mentioned before, activated antigen specific Th cells are required to activate B cells and subsequently promote a humoral immune response. Therefore naïve Th cells require three signals for activation. First, the interaction of the T cell receptor with MHC class II on antigen presenting cells (APCs) usually dendritic cells. After a successful binding of T cell receptor and MHC class II the cell requires a co-stimulator (e.g. B7-1/CD80 or B7-2/CD86) for its activation, this co-stimulation represents the second signal. To enable a high expression of co-stimulation molecules, dendritic cells have to be activated. Then the co-stimulation molecule interacts with CD28 on the naïve Th cell [9], thereby inducing a required survival signal for the Th cell, followed by IL-2 production. IL-2 in context with antigen binding causes Th cells to proliferate leading to an autocrine clonal expansion of antigen-specific Th cells. [1] The third signal is the polarisation by cytokines either produced by the APC itself or by a third cell (e.g. natural killer – or mast cells). The polarisation leads to the differentiation into a subset of Th cells (e.g. Th1 or Th2) enabling them to produce a wide variety of cytokines to stimulate and aid other immune cells (e.g. B cells as mentioned above). [9]

Immunogenicity of protein antigens:

The immunogenicity of proteins is determined by several factors, it can be due to structural features (e.g. glycosilation, sequence variation and frequency of potential B cell and T cell epitopes) [10]. But also the efficient antigen uptake and presentation by APCs is crucial for the Th cell activation. Once the antigen is internalised it has to be presented via the MHC complex to naïve Th cells, but as mentioned before for high expression of MHC molecules the APC has to become activated. This is mainly achieved by recognition of the protein via pattern recognition receptors (PRRs). [11] Due to this properties which play a role in the immunogenicity of protein antigens it can be assumed that not all proteins are equally immunogenic.

Adjuvants amplify immune responses towards poorly immunogenic antigens:

Protein antigens by themselves (i.e. exclusively the protein) are often poorly or not immunogenic due to lack of additional signals for immune cell activation (e.g. toll like receptor activation). In order to generate a proper adaptive immune response a mixture of protein antigens and adjuvants can be used. Adjuvants generally enhance the immune response in two different ways. First, soluble proteins (which are poorly taken up by most APCs) can be adsorbed on particles to be taken up more efficiently. Second, they mostly contain substances (e.g. bacterial components or ligands for PRRs like toll like receptors (TLR)) to render antigen presentation more efficient [2] and commonly activate innate immune responses. This is crucial for two applications, first amplification of the specific immune response to ensure long lasting and protective immunity towards pathogens in vaccination, which can only be achieved when memory B- and T cells are induced. The second application, especially used in basic science is to change the bias (e.g. Th1 or Th2) of the immune response towards an antigen if administered with a certain adjuvants. [12]

Importance of adjuvants in vaccination:

Rendering certain antigens immunogenic is important for a wide range of applications. In vaccination non-living vaccine, antigens are mostly poorly immunogenic. Therefore adjuvants are administered together with the antigen for a successful immunisation [12]. Adjuvants are also used to boost immune responses in individuals with impaired seroconversion [13], enable a lower dose of antigen used for immunisation [14], and allow fewer doses for vaccination[15]. Most of the adjuvants create a bias in the way the immune system responds to an antigen (i.e. Th1 or Th2 response), thereby guiding the adaptive immunity to respond in the most efficient way for each pathogen. [12] Additionally combination of different adjuvants can be used to increase differentiation of memory cells to certain effector cells. [16]

But adjuvants are also commonly used in basic science to induce and study immune responses towards poorly immunogenic antigens in animal models. [17]

In basic science adjuvants help to alter or induce an immune response in animals for simulation and studying of diseases or immunological disorders. To stimulate desired specific immune responses (e.g. antibodies, Th1 or Th2 responses) distinct adjuvants are used. [12] A list of the major adjuvants that are currently used can be found in figure 2, but this work will focus on aluminium hydroxide.

Adjuvant	Major Immunostimulatory Component(s)	Innate Receptors or Pathway Activated	Principal Immune Responses Stimulated
Licensed Adjuvants			
Alum	aluminum salts	NLRP3 inflammasome (?)	Ab, Th2 (+ Th1 in humans)
MF59 and AS03	squalene-in-water emulsions	tissue inflammation (no receptors defined)	Ab, Th1 + Th2
AS04	MPL plus alum	TLR4 and inflammasome (?)	Ab, Th1
Adjuvants in Widespread Experimental Use or in Late Stage Clinical Development			
Poly-IC (also Poly-ICLC)	synthetic derivatives of dsRNA	TLR3, MDA5	Ab, Th1, $CD8^+$ T cells
MPL and formulations (AS01, AS02)	MPL and QS-21	TLR4 (MPL), ? (QS21)	Ab, Th1
Flagellin, flagellin-Ag fusion proteins	Flagellin from *S. typhimurium*	TLR5	Ab, Th1 + Th2
Imiquimods	imidazoquinoline derivatives	TLR7, TLR8 or both	Ab, Th1, $CD8^+$ T cells (when conjugated)
CpG oligodeoxynuceotides and formulations (IC31, QB10)	synthetic phophorothioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, $CD8^+$ T cells (when conjugated)
CAF01	trehalose dimycolate (cord factor)	Mincle	Ab, Th1, Th17
ISCOMS and ISCOMATRIX	saponins	mechanism undefined	Ab, Th1+ Th2, $CD8^+$ T cells
IFA (and Montanide formulations)	mineral or paraffin oil + surfactant	mechanism undefined	Ab, Th1 + Th2
CFA	IFA + peptidoglycan, trehalose dimycolate	NLR, inflammasome, Mincle, TLR?	Ab, Th1, Th17

The principal immune response stimulated is based on results from human and mouse studies, although it may be limited to one species in some cases. Where indicated, conjugation of TLR ligand to antigen is necessary to obtain significant $CD8^+$ T cell responses.

Figure 2: Major adjuvants and the components of the immune system they activate. [12]

Aluminium hydroxide ($Al(OH)_3$):

Probably one of the oldest adjuvants used for immunisation is aluminium hydroxide ($Al(OH)_3$). It has been used for more than 80 years [18-19] and is still commonly used in basic science. Furthermore it is one of the most important adjuvants in clinical application since it is one of the few adjuvants approved for human application. [20] It is known to activate dendritic cells [21], monocytes and macrophages, thus leading to enhanced expression of adhesion molecules CD54 and CD86 and co-stimulatory molecules CD40 and CD86, molecules which are needed for T cell activation. Furthermore it is discussed that $Al(OH)_3$ is partially dependent on CD21 and CD35 (complement receptors) to induce affinity maturation, antibody production and memory cell differentiation of B cells [11]. $Al(OH)_3$ also induces molecules of the innate immune system via the nucleotide-binding domain and leucine-rich-repeat-containing gene family pyrin-domain-containing 3 (NLRP3) inflammasome to cleave and activate caspase-1. But if this is crucial for the adjuvanticity to induce the adaptive immune response is still being discussed. There is a controversy whether there is a NLRP3 dependence [22] [23] [24] [25] or not. [26]

Despite that the mechanisms leading to its adjuvanticity are still not fully understood $Al(OH)_3$ remains as one of the most important adjuvants.

Undesirable immune responses towards exogenous antigens:

Allergy:

The term “allergy” was first defined by the Viennese immunologist Clemens von Pirquet as “an altered capacity of the body to react to a foreign substance” [27], which was a very broad definition and can be defined today as “a disease following a response by the immune system to an otherwise innocuous antigen”. Therefore antigens that cause allergy are known as allergens. Allergy belongs to a certain class of hypersensitivity reactions of the body that cause tissue damage. This work will focus on IgE-mediated allergy (also known as type I hypersensitivity), but the features of the other hypersensitivity types are listed in figure 3. [2]

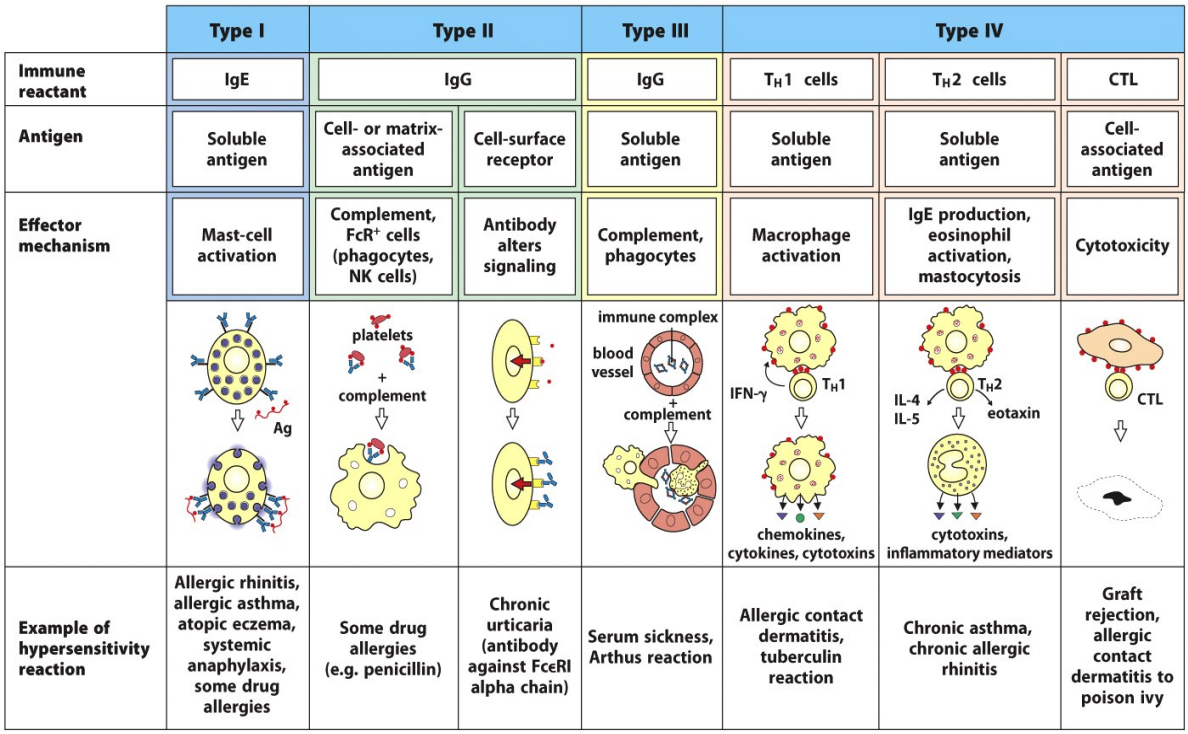

Figure 14.1 Janeway's Immunobiology, 8ed. (© Garland Science 2012)

Figure 3: An overview of the types of hypersensitivity [8]

Allergens:

Allergens are proteins that induce specific IgE in atopic individuals upon encounter. Therefore they have to contain both, B cell and T cell epitopes. Allergen sources can be e.g. pollen, food, insect venom, animal dander, or pharmaceuticals. [28] They also have a wide variety of molecular properties, and no common features have been unequivocally established which can be found in every allergen. Rather there are several factors that could enhance the allergenicity (i.e. the capability of an antigen to cause allergy) of some proteins. This can be for example biological activity (e.g. protease activity), glycosylation, resistance to proteolysis [29], or functional mimicry. [30]

IgE-mediated allergy:

The IgE-mediated allergy also known as immediate hypersensitivity occurs within 2-30 minutes after contact with the allergen and is caused by an allergen specific IgE mediated immune response. As a first step allergic individuals have to be sensitised upon allergen contact, in a way that allergen-specific IgE is produced by B cells (like described in the next section). This allergen-specific IgE binds to their high affinity

FcεRI receptor on effector cells (e.g. mast cells and basophils). In subsequent allergen contacts the allergen causes an immediate reaction, thereby it induces cross-linking of effector cell bound IgE and thus leading to a release of mediators (e.g. histamine) that cause the immediate allergic symptoms (e.g. itching and vasodilation) [1]. Especially in individuals suffering from asthma and chronic forms of allergy a late phase reaction 2-4 hours after the immediate reaction can be observed. In the late phase reaction activated allergen specific T cells undergo clonal expansion and release pro-inflammatory cytokines (e.g. IL-4, IL-5, IL-13). This is followed by upregulation of leukocyte adhesion molecules on endothelial cells leading to an accumulation of leukocytes in the tissue. Th1 cells that are accumulated secrete tumor necrosis factor (TNF) and interferon-γ (IFN-γ) which is accompanied by expression of CD95 ligand. These highly inflammatory molecules lead to apoptosis of endothelial cells thereby utterly compromising their barrier function. [31] An overview of the stages of type I hypersensitivity can be seen on figure 4.

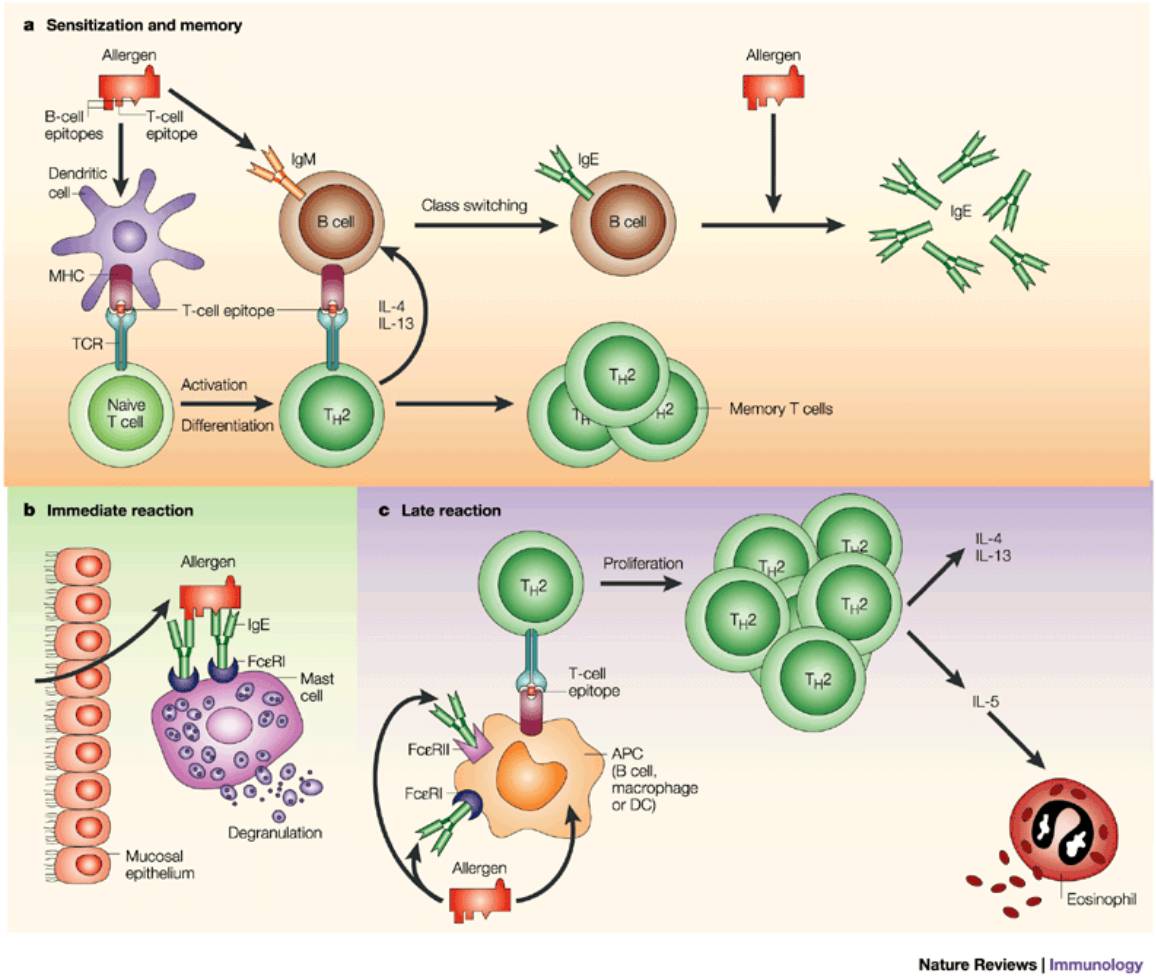

Figure 4: Sensitisation, immediate and late reaction towards allergen. [32]

Sensitisation towards allergens:

As mentioned above the class switch to IgE requires activated allergen specific Th cells that in turn depends on antigen presentation via MHC class II on APCs (e.g. dendritic cells). APCs constantly take up exogenous antigens and present them to Th cells. While non-allergic individuals do not react to allergens presented by APCs by IgE production, atopic allergic individuals are prone to an allergen-specific Th2 cell activation. Furthermore B cells can efficiently bind allergen and present it to activated Th cells, therefore thought to be one of the major antigen presenting cells in allergy. [33] The subsequent cytokine production of Th2 cells (e.g. IL-4 and IL-13) induces an IgE class switch. [34] [35] After sensitisation memory B and T cells develop and rapidly respond to further contacts with the allergen. After recognising the allergen, effector memory T cells that circulate in the periphery, start to secrete cytokines for rapid effector cell recruitment. After recognition of an allergen memory B cells differentiate into IgE-secreting plasma cells. Those cells appear to produce allergen-specific IgE in an IL-4 and IL-13 independent manner. [36] Additionally activation of naïve B cells can be inhibited by addition of co-stimulation blockade (i.e. anti-CD40L) to prevent Th cell aid. Contrary B cells that were preliminarily activated are not impaired by addition of anti-CD40L.[37]

Allergen-specific immunotherapy (SIT):

One therapy approach is the SIT. In SIT it is aimed for a desensitisation of allergic individuals. This can be achieved by repeated sublingual or subcutaneous administration of the sensitised allergen in repeated applications with increasing doses. The first publication to describe the application of SIT was released over 100 years ago by Noon [38]. Since the very beginning of SIT grass-pollen extracts are being used for SIT. But pollen extracts have several disadvantages since they are composed of several proteins making it hardly possible to define and purify single allergens from this extracts. Additionally it could lead to new sensitisations towards other allergens if they are administered with the pollen extract. Therefore in newer approaches cDNAs of allergens were generated and used for production of recombinant allergens. Recombinant allergens have the advantage that they can be produced with a very high yield. Additionally they can be quantified and purified. [39] In subsequent experiments the recombinant allergens were modified to mimic properties of the natural allergen but also with reduced allergenic activity to render

SIT more efficiently. [40] The desensitisation in SIT occurs by shifting the immune response from a Th2 to a Th1 driven response, to induce an allergen-specific T cell anergy, or by using adjuvant-bound allergens to induce a new allergen-specific response (e.g. a IgG driven antibody response) that competes and diminishes the IgE driven response. [41]

Recognition of major and minor histocompatibility antigens in transplant immunology:

Transplantation of organs, cells or tissues from one individual to another is called allo (i.e. non-identical)-transplantation.
Since our immune system distinguishes between self and non-self antigens (i.e. tolerance towards self), the recognition and response towards alloantigens leads to rejection of the transplant if no immunosuppressives are administered. [42] Therefore it is a critical obstacle for graft survival and of high clinical relevance since more than 30000 organ transplantations a year are performed in the United States alone. [1]

Major and minor histocompatibility antigens:
Major histocompatibility antigens (i.e. Ma antigens) are molecules that belong to the MHC. The MHC is a highly polymorphic group of genes even within the very same species. The MHC proteins are crucial for the immune system to distinguish between self and foreign [1]. Ma antigens tend to be highly immunogenic due to an exclusive mechanism of T cell recognition, the direct allorecognition. Ma antigens can be recognised by T cells on two different pathways (direct and indirect allorecognition). Direct allorecognition occurs when donor MHC molecules are presented to recipient T cells on donor stimulator cells. Indirect allorecognition occurs when donor MHC molecules are processed and presented on recipient APCs to recipient T cells (figure 5). [42] In early transplant immunology papers it was proposed that the direct allorecognition is predominant in graft rejection, but since the 1990s novel findings suggested that also indirect allorecognition is strongly impairing graft survival. [43] Allorecognition of Ma antigens can occur by $CD4^+$ and $CD8^+$ T cells but also allospecific B cells can be stimulated. [1]

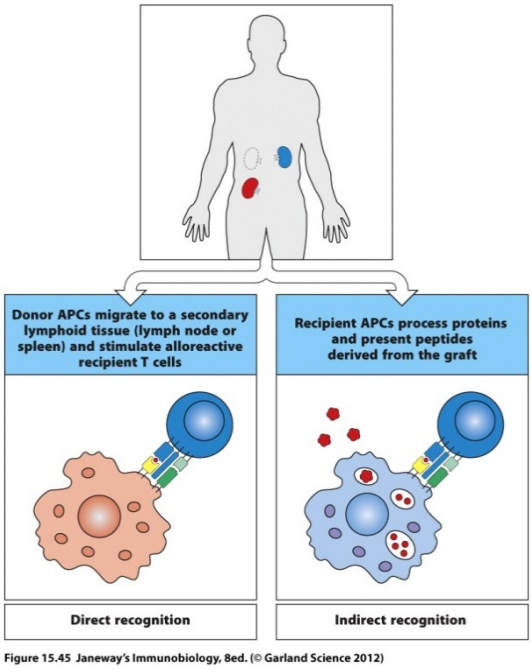

Figure 5: Direct and indirect allorecognition. [8]

In contrast to Ma antigens, minor histocompatibility antigens (i.e. Mi antigens) are non-MHC antigens that are immunogenic and have the capability to cause graft rejection. [44] One of the first reports of Mi antigens causing graft rejection was shown by skin transplantation within different inbred strains. [45] It should be noted that in this context the term major and minor does not necessarily refer to the immunogenicity of the antigen, since some Ma antigens have a lower immunogenicity than some minor antigens. [46] Mi antigens can only be recognised when they are internalised by recipient APCs and presented to T cells [47]. It was believed that graft rejection caused by Mi antigens is T cell mediated but newer findings reported that Mi antigens can also induce specific humoral responses. [48-49]

One example of Mi antigens are the MHC I chain related gene A (MICA) antigens. Encoded on chromosome 6 they are highly polymorphic with already over 68 different alleles known. This high variety of alleles makes MICA for an ideal target for alloresponses. Humoral immune responses towards MICA have already been related to impaired graft survival in renal, heart and pancreas transplantations. [48]

Haematopoietic chimerism, a cell therapy approach to prevent antigen specific immune responses:

Haematopoietic mixed chimerism denotes the presence of haematopoietic cells genetically differing from other cells existing in one organism. It was observed almost 70 years ago that dizygotic bovine twins sharing a common placenta (i.e. so called 'freemartin cattle') accept skin transplants of each other without triggering an allo-specific immune response even though they were disparate in their MHC molecules. It was achieved by a naturally occurring haematopoietic chimerism via placental circulation. [50] This was the basis of extensive research to prevent allo-specific immune responses and thereby the rejection of allo-transplants. In subsequent experiments it was sought to induce chimerism in adult animal models (i.e. mice) and as a final step, translate it to clinical application [51-52] [53]. Recently clinical trials for tolerance induction through mixed chimerism were already published with quite promising perspectives for the future. [54] [55]

Molecular chimerism approaches to prevent immune responses towards defined antigens:

In contrast to haematopoietic chimerism molecular chimerism is used to induce tolerance towards defined antigens. Therefore autologous haematopoietic stem cells (HSCs) are genetically modified to express defined antigens. Subsequently they are transplanted back into their donor and induce tolerance towards those defined antigens. [56] [57] This approach has several advantages since it allows tolerisation towards defined antigens. Additionally almost all current protocols for transplantation of allogeneic bone marrow cells (BMCs) hold at least some risks of causing graft versus host disease (GVHD) [52], a disease whereby mature donor T cells react to alloantigens of the host with the potential to cause tremendous damage to the host. [1] Therefore the danger of GVHD is avoided since autologous BMCs are used [58]. Molecular chimerism was already shown to be effective in transplantation models [59] several autoimmune disease models (e.g. multiple sclerosis models [60] and type I diabetes models [61])

Tolerance induction by molecular chimerism in IgE-mediated allergy:

Since molecular chimerism can prevent immune responses towards auto- and alloantigens, it was also considered to be capable to induce tolerance towards allergens. To develop a new immunotherapy approach, the highly immunogenic allergen Phl p 5 was chosen for a molecular chimerism approach.

The major grass pollen allergen Phl p 5 is one of the three major grass pollen allergens of *Phleum pratense* (i.e. timothy grass). Phl p 5 has a size of 32kDa. Furthermore 80% of patients with grass pollen allergy react to Phl p 5, therefore it is of high clinical relevance and further the molecule is well-studied. [62]

In this approach syngenic BMCs of Balb/c mice were isolated and subsequently genetically modified via retroviral transduction to express Phl p 5 as a membrane-anchored protein. The modified BMCs were then transplanted into naïve Balb/c mice leading to molecular chimerism. The percentage of Phl p 5-expressing cells (and thereby the percentage of chimerism) was determined via flow cytometry in different cell lineages at different time points (Fig. 6). To analyse the specific unresponsiveness of the chimeric recipient mice, the mice were challenged with recombinant (r)Phl p 5 and the unrelated allergen rBet v 1 (i.e. major birch pollen allergen) both adsorbed to $Al(OH)_3$ via subcutaneous injection. The allergen administration was carried out 4 times followed by Phl p 5-specific ELISAs to analyse Phl p 5-specific antibody levels in sera. Additionally T cell proliferation assays and *in vitro* and *in vivo* effector cell release assays were performed to determine T cell and effector cell responses. In this approach a Phl p 5-specific immune response on B cell, T cell and effector cell level was successfully prevented (figure 6). [63] This approach can be extended to a variety of allergens as shown for the unrelated allergen Bet v 1 (Gattringer et al., submitted).

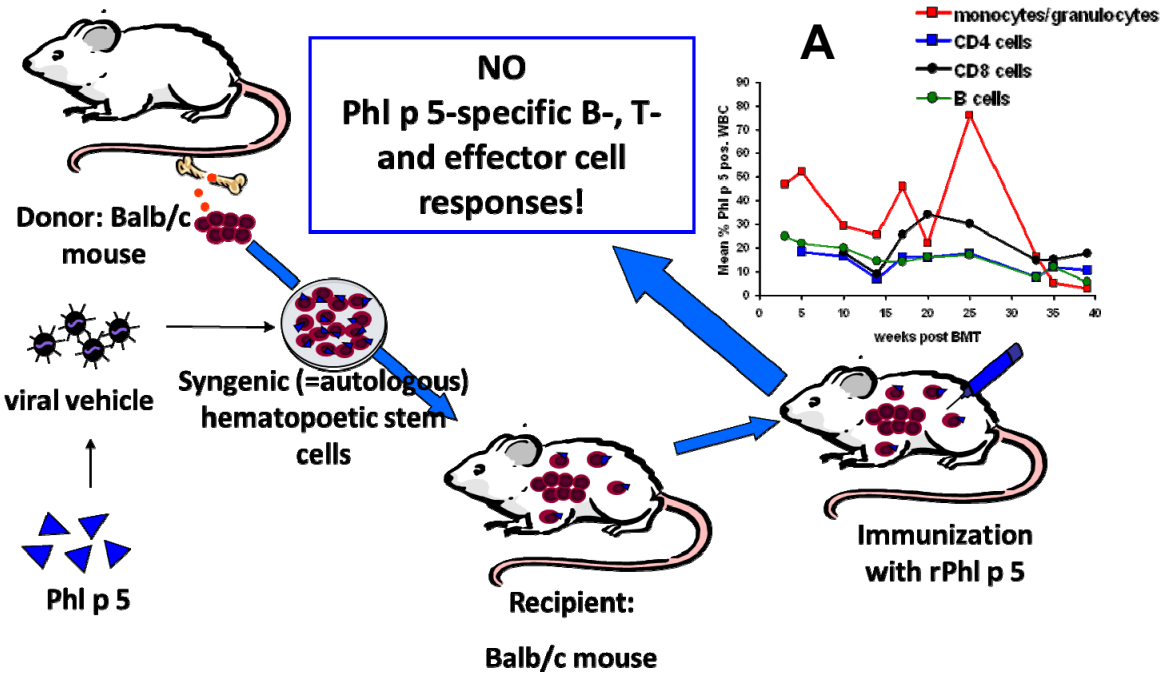

Figure 6: The Phl p 5 cell therapy approach. Balb/c BMCs are isolated and genetically modified to express Phl p 5. Subsequently the modified BMCs are transplanted into a recipient Balb/c mouse leading to molecular Phl p 5 chimerism (A, shows the mean percentage of Phl p 5 positive WBCs over a time course), leading to tolerance on the B-, T- and effector cell level.

Phl p 5-transgenic Balb/c mouse:

Retroviral transduction is accompanied by several disadvantages, since transduction efficiency varies between 5-55%. To obtain a higher and more constant amount of HSCs expressing Phl p 5 and to avoid the extensive retroviral transduction, a transgenic mouse, expressing Phl p 5 as membrane-anchored protein (figure 7), was generated by pro-nuclear injection as experimental 'tool'. For ubiquitous expression the construct was under the control of the strong chicken β-actin promotor and CMV-enhancer. Additionally eGFP is expressed as a reporter-gene via an internal ribosomal entry site (IRES). Preliminary to pro-nuclear injection of the vector, the bacterial backbone has been removed. (Baranyi et al., unpublished data) Pro-nuclear injection was carried out by Prof. Rülicke from Biomodels Austria and subsequent breeding of a homozygous line was carried out. The homozygous line was then used as BMC donors for tolerance induction towards Phl p 5 via molecular chimerism with the same protocol for induction of chimerism as with retrovirally transduced BMCs. Recipient mice showed high levels of chimerism nevertheless mice unexpectedly developed low levels of IgE and IgG1 specific for recombinant Phl p 5 although we were not able to detect Phl p 5-specific T cell proliferation (unpublished data). It therefore appeared that (subtle) differences between Phl p 5 expressed as transgene and Phl p 5 expressed through retroviral transduction exist. Studies aimed at delineating these differences are currently on going in our laboratory. In order to assess whether tolerance towards the transgene Phl p 5 has developed in recipients of Phl p 5-transgenic BM, tail skin of the Phl p 5- transgenic mouse was grafted unto chimeras. Skin is known as a highly immunogenic and

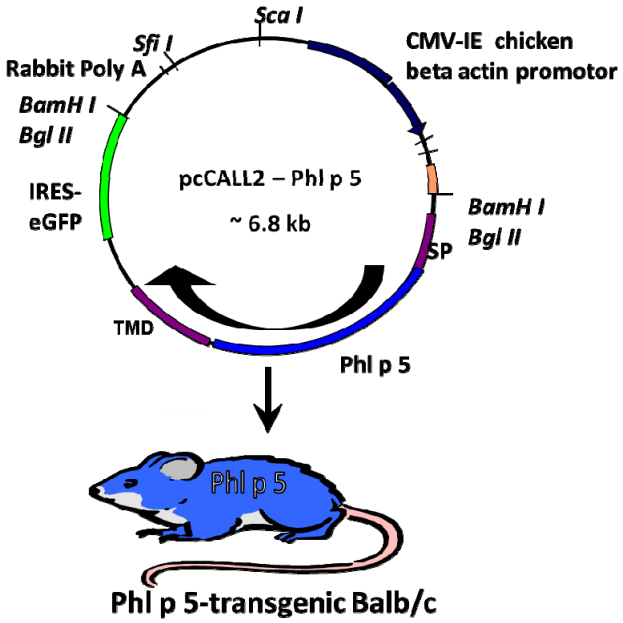

Figure 7: Generation of the Phl p 5-transgenic mouse. SP stands for leader peptide, TMD for transmembrane domain and eGFP is expressed via an IRES

readily rejected tissue and is therefore a stringent tolerance test in allo-transplantation. Phl p 5-chimeric permanently accepted the skin grafts of Phl p 5-transgenic mice (MST >25 weeks). This mouse model can be referred to a congenic strain that only differs from Balb/c mice by the antigens Phl p 5 and GFP. Since the grafts were not rejected it was suggested that the mice are either tolerant towards Phl p 5 on the T cell level or the mismatched antigens (i.e. Phl p 5 and GFP) are not sufficiently immunogenic in this tissue. Therefore, it was of importance to determine the immunogenicity of the Phl p 5 transgene. To this end skin of the Phl p 5-transgenic mouse was grafted onto naïve Balb/c mice. Surprisingly a prompt rejection of the skin grafts and high levels of Phl p 5-specific antibodies were observed (preliminary data, Baranyi et al., unpublished).

Aims of this study:

1) Characterisation of the Phl p 5-transgenic mouse:

To use the Phl p 5-transgenic mouse for mechanistic studies of tolerance induction via molecular chimerism, we aimed to determine whether Phl p 5 is expressed in full length and ubiquitously in all white blood cells (WBCs) and tissues.

2) Determination of the immunogenicity of Phl p 5 as a transgene in Balb/c mice:

Since our preliminary data suggested an unexpectedly high immunogenicity of the transgene Phl p 5 we aimed to define its immunogenicity in detail. Therefore the specific aims of this thesis regarding the immunogenicity of the transgene Phl p 5 were:

a) To further analyse the kinetics of the skin rejection, Phl p 5-specific antibody production and T cell mediated mechanisms after transplantation of tail skin of the Phl p 5-transgenic mouse onto naïve Balb/c mice.

b) We also aimed to address if the immunogenicity of the expressed transgene Phl p 5 is skin tissue specific.

c) Further we aimed to assess if the localisation (i.e. surface vs. cytoplasmic expression) of Phl p 5 expressed in murine cells is important for the immune response.

Materials and Methods:

Materials:

ABTS:

258mg citric acid

275.2mg Na_2HPO_4

20mg 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

2µl H_2O_2

→ ad 20ml ddH_2O

Blotting buffer 1x, 1.5l:

4.5g Tris

21.6g Glycine

1300ml Aqua dest.

200ml Methanol

Laemmli sample buffer (4X):

100 mM Tris/HCl ph 6.8 10ml

10% β-mercaptoethanol

4% SDS 1,96g

0.2% Bromphenol blue 100µl

20% Glycerine 11.5ml 87% Glycerine

→ ad 50ml ddH_2O

Running buffer 1x:

30.3g Tris (50mM) pH 8.5

71.3g Glycine (0.19 M)

50ml 10% SDS (0.1%)

→ ad 5l ddH_2O

FACS buffer:

1l PBS

1g NaN_3

1g BSA

210µl 1M NaOH

Coating buffer:

3.9g Na_2CO_3

5.3g $NAHCO_3$

→ ad 1000 ml ddH_2O

Tyrodes buffer:

9.5g tyrode salts: according to GIBCO (g/l)

$CaCl_2$ (anhyd.)	0.20	$CaCl_2$ x 2 H_2O	0.26
KCl	0.20		
$MgCl_2$ (anhyd)	0.0469	$MgCl_2$ x $6H_2O$	0.10
NaCl	8		
NaH_2PO_4 x H2O	0.05	NaH_2PO_4 x $2H_2O$	0.056
D-Glucose	1		

2,38 g HEPES (10 mM)

1 g $NaHCO_3$

Dissolved in bidest. pH adjusted to 7.2 with NaOH

0.1% BSA added before use

Glycine buffer:

15g Glycine

11.7g NaCl

dissolved in 1 l H_2O, pH adjusted to 10.7 with NaOH

Citrate buffer:

0.1 M citric acid (or sodium citrate) in bidest pH adjusted to 4.5 with NaOH

Assay solution:

5 ml citrate buffer + 80 ul 4-MUG (4-methyl umbelliferyl-N-acetyl-beta-D-glucosaminide, 10mM)

Antigen retrieval buffer:

2.94 g Tri-sodium citrate

dissolved in 1 l H_2O, pH adjusted to 6.0 with HCl

addition of 0.5 ml Tween 20 (Sigma-Aldrich)

Recombinant allergens:

recombinant Phl p 5 was obtained from Biomay.

***In vivo* antibodies:**

anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) monoclonal *in vivo* antibodies were obtained from Bio X Cell.

Cell culture:

RBL-Medium:

RPMI 1640 GIBCO with L-Glutamine

10% FCS

4 mM L-Glutamine

2 mM Sodium Pyruvate

10 mM HEPES

100 µM 2-Mercaptoethanol

1 % Penicillin / Streptomycin (Pen/strep)

T-cell proliferation medium:

RPMI 1640 GIBCO with L-Glutamine
10% FCS
0.1 mg/ml Gentamicin (50mg/ml Stock)

Methods:

SDS-Gel and Immunoblot:

- 2 Gels were prepared:

  Separation Gel 12% for 15ml
  - 3 ml Aqua dest.
  - 6 ml Acrylamide 30%
  - 5.7 ml Tris 1 M pH 8.8
  - 150 µl SDS (10% RT)
  - 150 µl APS 10%
  - 6 µl TEMED

  Stacking Gel 5ml
  - 3.4 ml Aqua dest.
  - 830 µl Acylamide 30%
  - 630 µl Tris 1M pH 6.8
  - 50 µl SDS (10%)
  - 50 µl APS
  - 5 µl TEMED
- Separation gels were poured and saturated butanol was added
- After polymerization butanol was decanted and the stacking gel was poured
- Samples were mixed with the reducing Laemmli buffer and subsequently boiled for 5 min at 95° C and put on ice afterwards
- 80 µl of each sample and 3µl of the prestained protein ladder by Fermentas, were loaded onto the gel
- The separation was carried out for 1.5 h at 100 V
- After electrophoresis the samples were blotted: 2 sheets of Whatman paper and a nitrocellulose membrane were wetted in blotting buffer
- 1 sheet of the Whatman paper was laid on the bottom followed by the gel, the nitrocellulose membrane and on the top another sheet of Whatman paper

- Blotting was carried out at an amperage of 250 mA for 1.5 h
- To block unspecific binding: 0.05% Tween/PBS plus 5% milk powder (1g in 20ml) was added and blot was shaken over night at 4°C
- wash 1x with PBS/Tween
- For detection of Phl p 5, Phl p 5 immunised rabbit serum 1:1000 diluted in 2% milk-powder/washing buffer was added and blot was shaken for 2.5 h at RT
- Blot was washed 3x for 5 min
- Second Ab (Pierce, goat-anti-rabbit-HRP) 1:2000 diluted in 2% milk-powder/washing buffer was added → shake for 1 h at room temperature
- Blot has been washed 4x for 5 min
- Bound Abs were detected with Pierce SuperSignal West Pico Kit

Allergen-specific ELISA:

- Allergen was diluted to a concentration of 5 µg/ml in coating buffer, 100 µl per well were added and incubated at 4° C over night, afterwards coating buffer was discarded
- Plates have been washed 2x with 250 µl of PBS/0.05% Tween and then saturated with 150 µl per well PBS/1% BSA/0.05% Tween for 2.5 h at RT
- The solution was decanted and sera were diluted in PBS/0.5% BSA/0.05% Tween as follows:

  IgE: 1:20

  IgA, IgM: 1:100

  IgG1, IgG2a, IgG2b and IgG3: 1:500

  100 µl per well were added and over night incubated at 4° C
- Plates were washed 5x with 250 µl of PBS/0.05% Tween
- 100 µl of rat-anti-mouse Ig's (PharMingen, 1:1000 diluted in PBS/0.5% BSA/0.05% Tween) per well were added followed by 2h incubated at RT and then discarded
- Plates were washed 5x with 250 µl of PBS/0.05% Tween
- 100 µl of goat-anti-rat HRP-labelled Abs (Amersham Biosciences, 1:2000 diluted in PBS/0.5% BSA/0.05% Tween) per well were added followed by two 30 min incubation steps at 37° C and 4° C afterwards the plates were decanted discarded
- Plates were washed 5x with 250 µl of PBS/0.05% Tween

- 100 µl of ABTS solution per well were added and incubated in a dark place for 30 min at RT
- OD was measured (405-490 nm)

Generation of cell extracts from spleens of Phl p 5-transgenic mice:

We used a modified version of the protocol published by Hughes et al [64]

- Two buffer solutions were prepared:
    - Buffer I:
        - 5mM Tris-HCl (pH 8.8)
        - 4mM KCl
        - 1mM EDTA
        - 1% Triton X100
    - Buffer II:
        - 20 mM Tris-HCl (pH8)
        - 100 mM NaCl
        - 1mM EDTA
        - 0.2% Triton X100
- Spleens were mashed through a 70µm cell strainer (BD Falcon) to generate a single cell suspension subsequently the cells were transferred into a 50 ml falcon tube by filtering them again through the cell strainer
- The cells were centrifuged at 20°C for 10 min with 1400 rpm
- Supernatant was discarded and cells were resuspended in 3 ml cold red blood cell lysing buffer afterwards the cells were incubated for 1 min on ice
- To stop lysis 10 ml of PBS were added and cells were centrifuged as before
- Supernatant was discarded again and the pellet was resuspended in 500 µl of Buffer I subsequently the cell suspension was incubated for 15 min at 37°C
- After incubation the cells were three times frozen with liquid nitrogen and then thawed at 37°C
- Afterwards the suspension was centrifuged for 5 min at 3000 x g
- The supernatant was dialysed against Buffer II for 1h at 4°C (Pierce, Slide-A-Lyzer Gamma Irradiated Dialysis cassette)
- The sample was then frozen at -20°C

Retroviral transduction:

- NIH-3T3-cells were seeded out 1 day before transduction. The cells have been harvested and centrifuged with 1640 rpm for 5 min at 20° C and then resuspended in 5 ml DMEM (10% FCS, 1% Pen./Strep., Gibco) medium. The calculated cell concentration of $4.93x10^6$ cells/ml was determined with Casy Cell Counter.
- Since $2x10^6$ cells per 10 cm dish were needed 4 dishes were prepared containing 410 µl of cell suspension and 4590 µl of DMEM medium
- On the next day cells were analysed via microscope and the media were removed
- The cells were transduced with the following virus concentrations:
  Dish 1 (neg. control): 5 ml DMEM medium + 5 µl polyprene
  Dish 2: 100 µl of virus + 4.9 ml DMEM medium + 5 µl polyprene
  Dish 3: 500 µl of virus + 4.7 ml DMEM medium + 5 µl polyprene
  Dish 4: 1000 µl of virus + 4.5 ml DMEM medium + 5 µl polyprene
  After addition of the virus the cells were put in the incubator for 4 hours.
- The medium was exchanged after incubation and transduction efficiency was checked via flow cytometry after 2 days

Flow cytometry-staining:

- 10^6 of the cells to be tested were transferred into FACS-tubes
- Cells were centrifuged for 5 min with 5000 rpm at 4° C
- Supernatant was discarded and tubes were resuspended
- Addition of 1 ml FACS medium and centrifugation as before
- Supernatant was discarded and tubes were resuspended
- Addition of 10 µl Abs (BioLegend)
- Incubation for 20 min at 4° C
- Addition of 1 ml FACS medium and centrifugation as before
- Biotinylated antibodies were incubated with 10 µl of streptavidin-PE (Phycoerythrin) or streptavidin-Cy5 (BioLegend) for 10 min at 4° C, followed by another washing and centrifugation step as before

- Addition of 100 µl FACS medium and measurement at the flow cytometer(EPICS XL-MCL Beckman Coulter was used for acquisition and EXPO32 ADC, Applied Cytometry Systems software was used for analysis)

Phl p 5 specific flow cytometry-staining:

- 10^6 of the cells to be tested were transferred into FACS-tubes
- Cells were centrifuged for 5 min with 5000 rpm at 4° C
- Supernatant was discarded and tubes were resuspended
- Addition of 1 ml FACS medium and centrifugation as before
- Supernatant was discarded and tubes were resuspended
- Addition of 10 µl BG-6 (previously described in [65] and kindly provided by Prof. Arndt Petersen, Borstel; 1:30 diluted) monoclonal Abs
- Incubation for 20 min at 4° C
- Addition of 1 ml FACS medium and centrifugation as before
- Supernatant was discarded and tubes were resuspended
- Addition of 10 µl rat anti mouse Biotin IgG1 Abs
- Incubation for 20 min at 4° C
- Addition of 1 ml FACS medium and centrifugation as before
- Supernatant was discarded and tubes were resuspended
- Addition of 10 µl Streptavidin-PE Abs
- Incubation for 10 min at 4° C
- Addition of 1 ml FACS medium and centrifugation as before
- Supernatant was discarded and tubes were resuspended
- Addition of 100 µl FACS medium and measurement at the flow cytometer(EPICS XL-MCL Beckman Coulter was used for acquisition and EXPO32 ADC, Applied Cytometry Systems software was used for analysis)

Rat basophil leukemia (RBL) cell degranulation assay:

- RBL-cells ($6x10^4$ cells/well, previously described in [66]) were seeded out in 96-well plates and incubated for 18 hours at 37° C, 5% CO_2
- 3.3 µl of mouse serum were added (1:30 diluted) per well and incubated for 2 hours at 37° C, 5% CO_2
- Medium was removed and cells were washed 2x with pre-warmed Tyrode Buffer

- Phl p 5 was diluted in Tyrode Buffer (0.3 µg rPhl p 5 / ml) and 100µl per well were added
- Cells were incubated for 30 min at 37° C, 5% CO_2
- For 100% release 10 µl of 10% Triton X-100 was added
- 50 µl of the supernatant was transferred into a new plate
- 50 µl of the assay solution was added to the supernatant and incubated for 1 hour at 37° C, 5% CO_2
- Reaction was stopped by addition of 100 µl glycine buffer and fluorescence was measured (PerkinElmer Wallac, ex. 360 nm and em. 465 nm)

Phl p 5-specific T-cell proliferation assay:

- Mice were sacrificed as described in animal methods section, spleens were isolated transferred into PBS and put on ice
- A single cell suspension was generated by using 70 µm cell strainers (BD Falcon) and subsequently centrifuged with 1400 rpm for 5 min at RT
- Supernatant was discarded, cells were resuspended in 3 ml cold red blood cell lysing buffer (Sigma) and subsequently incubated on ice for 1 min
- Lysis was stopped by addition of 15 ml PBS followed by centrifugation as before
- Supernatant was discarded and cells were resuspended in 5 ml proliferation medium
- Cells were counted with Casy® counter and diluted to an concentration of $2x10^6$ cells/ml
- 96-well round bottom plates were prepared with: 100 µl of medium (neg. control), medium + 2 µg rPhl p 5 or medium + 0.5 µg Concanavalin A (Biomay, pos. control)
- 100 µl/well of cell suspension ($2x10^5$ cells/well) were seeded out and triplicates were made of each mouse
- The plates were incubated for 5 days at 37° C, 5% CO_2
- ^3H ([methyl-^3H] Thymidine, Amersham) was added (0.5 µCi diluted in 50 µl medium) and subsequently incubated for 18 hours at 37° C, 5% CO_2
- The cells were harvested and measured in a beta counter. Before measurement the filter was transferred in a plastic bag, 5 ml of scintillator liquid enhancer (Wallac) was added and packed air tight.

Immunofluorescence:

- Mice were sacrificed as described in animal methods section, tissues were isolated transferred into 4.5% formaldehyde over night
- Samples were dehydrated, embedded in paraffin subsequently 5 µm sections were generated and transferred on microscope slides
- Slides were incubated over night at 58° C and deparaffinised (2x 10 min in xylene, 2x 5 min in 100% EtOH, 2x 5 min in 96% EtOH, 5 min in 80% EtOH, 5 min in 70% EtOH, 5 min in 50% EtOH, 5 min in ddH_2O)
- Antigens were retrieved by simmering the slides for 15 min in the antigen retrieval buffer and subsequently washed for 15 min in tap water and 2x 5 min in PBS
- Sections were bordered with a Pap Pen and transferred in a humidity chamber
- 2% BSA in PBS was added for 1 hour
- First antibody (Phl p 5 immunised rabbit serum 1:500 diluted in 1% BSA, PBS) was added for 1h 30 min
- Slides were washed 2x for 5 min in PBS
- Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, 1:1000 diluted in 1% BSA, PBS) and Hoechst33342 (1:1000) was added and incubated for 1hour
- Slides were washed 3x for 5 min in PBS and sections were covered with ProLong (Invtirogen) before cover slip was placed on the top and fixed with Eukit
- The slides were analysed at the Anna-Spiegel imaging core facility with a Zeiss LSM 700 microscope

Mouse Methods:

Animals:

Recipient female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All mice were housed under specific pathogen-free conditions and were used between 6 and 12 weeks of age. All experiments were approved by the local review board of the Medical University of Vienna, and were performed in accordance with national and international guidelines of laboratory animal care.

The Phl p 5-transgenic mice (male or female) with Balb/c background were generated via microinjection of the construct which can be seen on Figure 7. The pro-

nuclear injection was carried out by Prof. Rülicke of the University of Veterinary Medicine Vienna. Mice were bred under specific pathogen-free conditions in our animal facility at the Medical University of Vienna's Centre of Biomedical Research.

Ear tags for mice, collection of blood sera, anaesthesia, isolation of spleens:

- Were carried out according to our animal testing license.

Subcutaneous injections:

- Mice were injected with 5µg of rPhl p 5 diluted in 50µl of PBS + 50µl of aluminium hydroxide ($Al(OH)_3$, Serva)

Euthanasia of mice:

- Was carried out via cervical dislocation according to the guidelines of Current Protocols in Immunology [67].

Skin grafts:

- Anaesthetic solution was prepared
- The place for working was prepared by adding green textile bellow and fixed tape, two petri dishes were filled with PBS
- A Phl p 5-transgenic mouse was sacrificed the tail was cut with scalpel through long side then the skin was peeled off from the tail and the skin was laid in the petri dish filled with PBS
- Then the skin was cut in pieces of approximately 1cm
- each recipient mouse was injected with 0.25 ml of anaesthetics intraperitoneal
- Upon the onset of anesthesia, the fur was shaved around the place where the skin was grafted
- The spot was disinfected with 70% ethanol and the eyes were protected with eye ointment to prevent drying-out
- With forceps skin was pulled up and cut it with sterile scissors
- The skin of the Phl p 5-transgenic mice was added on the spot and fixed with 4 stitches subsequently a swab was added on the wound and fixed with band-aids
- After 7 days the band-aids were removed and grafts were monitored

- Skin grafts were considered as rejected if less than 10% of the grafted skin was intact

Statistical analysis:

The shown p-values were calculated with Man-Whitney U. P-values < 0.05 were considered statistically significant. Error bars indicate standard deviations (SD).

Results:

Characterisation of the Phl p 5-transgenic mouse:

Ubiquitous Phl p 5 expression in leucocytes of transgenic Balb/c mice:

We wanted to determine the expression of Phl p 5 in blood and tissues. Therefore cells of blood, thymus, spleen and bone marrow of the Phl p 5-transgenic Balb/c mouse were stained with the monoclonal Phl p 5-specific antibody BG-6 (kindly provided by Prof. Arnd Petersen, Borstel) then detected with biotinylated antimouse IgG antibodies and counterstained with streptavidin-Cy5. Subsequently the cells were analysed via flow cytometry (figure 8). In blood, spleen and bone marrow CD45.2$^+$ cells (a pan-leucocyte marker) were gated. The percentage of eGFP$^+$, Phl p 5+ double positive cells was 99.2% in blood (figure 8), 98% (figure 8) in the bone marrow and 98.9% (figure 8) in the spleen. In the thymus (figure 8) we determined 95.8% of eGFP$^+$, Phl p 5$^+$ cells gated on CD45.2$^+$, CD4$^+$ and CD8$^+$.

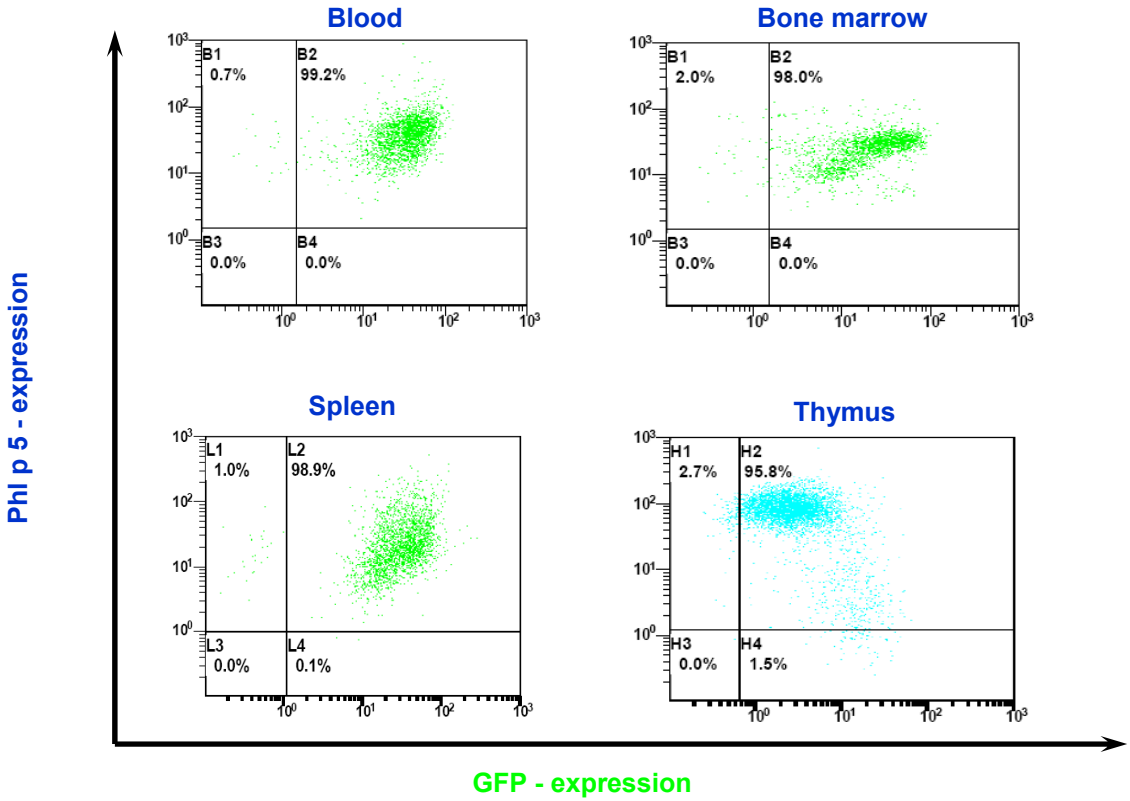

Figure 8: Representative FACS-Blot of one mouse for expression levels of Phl p 5 and GFP in different tissues, analysed via flow cytometry and gated on a pan-leukocyte marker (CD45.2, green), thymocytes were gated on CD4$^+$/CD8$^+$ (blue) double positive cells.

Localisation of membrane-bound Phl p 5 in confocal microscopy:

We also wanted to confirm the localisation of membrane-bound Phl p 5 via confocal microscopy in tissues (e.g. thymus). Therefore thymus sections of a Phl p 5-transgenic Balb/c were stained with polyclonal anti-Phl p 5 rabbit serum and detected bound antibodies with anti-rabbit Alexa-546 antibodies. Additionally nuclei were stained with Hoechst33342. As a negative control thymus sections of a naïve Balb/c were used. As shown on figure 9 there was a strong Phl p 5 specific signal on the cell membrane compared to the negative control.

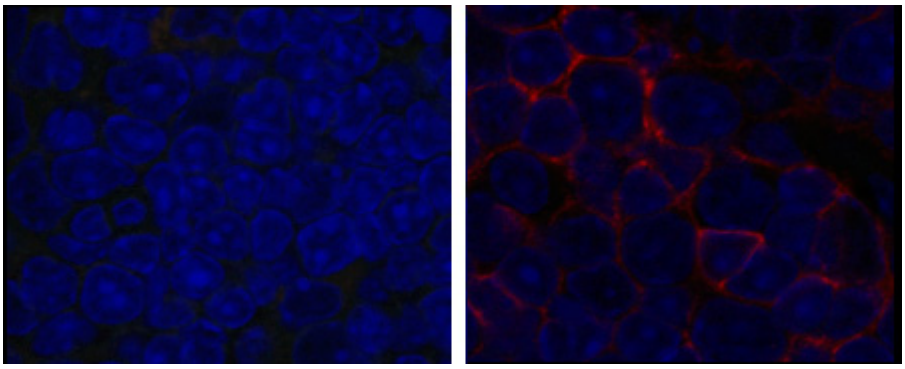

Figure 9: Thymus section (x400) of a naïve Balb/c mouse (left)
and a Phl p 5-transgenic Balb/c (right), nuclei visualised in blue, Phl p 5 in red

Full length expression of the transgene Phl p 5:

To assess whether Phl p 5 has a full length expression in transgenic mice, splenocyte lysates of a Phl p 5-transgenic mouse and 10ng rPhl p 5 as a control were used for immunoblotting. The lysates were separated on a SDS-gel and then transferred on a nitrocellulose membrane. Subsequently Phl p 5 was detected via chemiluminiscence.

As shown on figure 10 splenocyte lysates show a higher mobility shift than rPhl p 5 due to the transmembrane domain (consisting of 50 additional amino acids). Additionally a higher band Phl p 5-specific band was detected in splenocyte lysate at ~60kDa which might be a posttranslational modification (figure 12, black arrow).

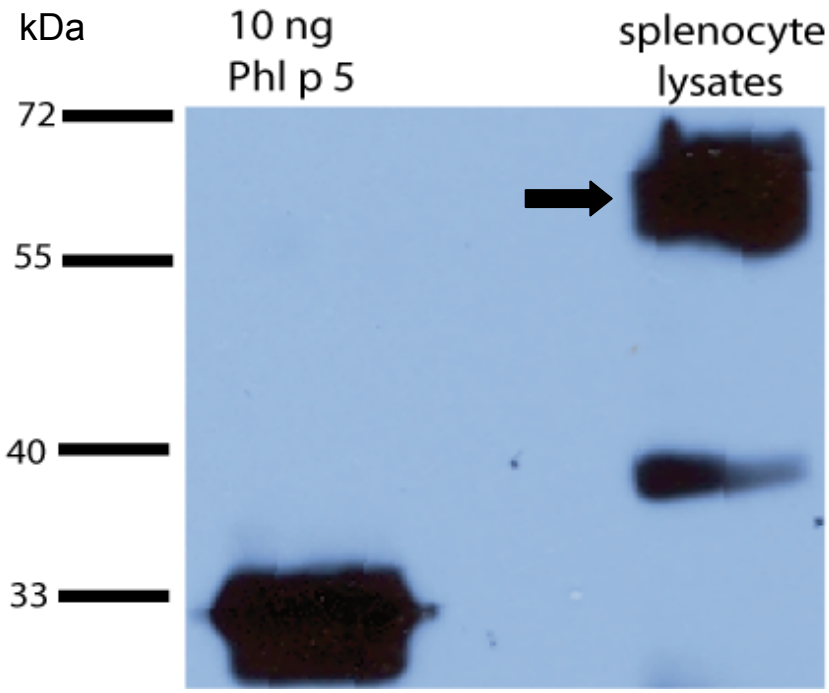

Figure 10: Nitrocellulose blotted rPhl p 5, and splenocyte lysates of a Phl p 5-transgenic mouse The blot was incubated with Phl p 5 immunised rabbit serum and detected as described in the methods section

Determination of the immunogenicity of Phl p 5 as a transgene in Balb/c mice:

Transplanted skin of Phl p 5-transgenic Balb/c mice is highly immunogenic:

To compare the rejection with another non-MHC mismatch, namely the male antigen H-Y, female Phl p 5 transgenic mice received skin grafts of male Phl p 5 transgenic mice (H-Y mismatch) known to lead to chronic rejection in skin grafts [68]. As controls, naïve Balb/c mice received skin grafts of Phl p 5 transgenic Balb/c mice. Furthermore we wanted to compare the rejection with an MHC mismatch. Therefore naïve C57BL/6 ($H2^b$) received skin grafts of B10.D2 ($H2^d$) mice, a congeneic strain sharing the same genetic background (B10/B6) but differing in the MHC. Afterwards mice receiving skin grafts of the Phl p 5-transgenic mice showed a surprisingly prompt rejection with a median graft survival of 9 days (n=18). This is comparable with an MHC-mismatched skin graft which also showed a mean graft survival of 9

days (n=2). Contrary H-Y mismatched skin led only to a chronic rejection (figure 11) ongoing till at least 150 days.

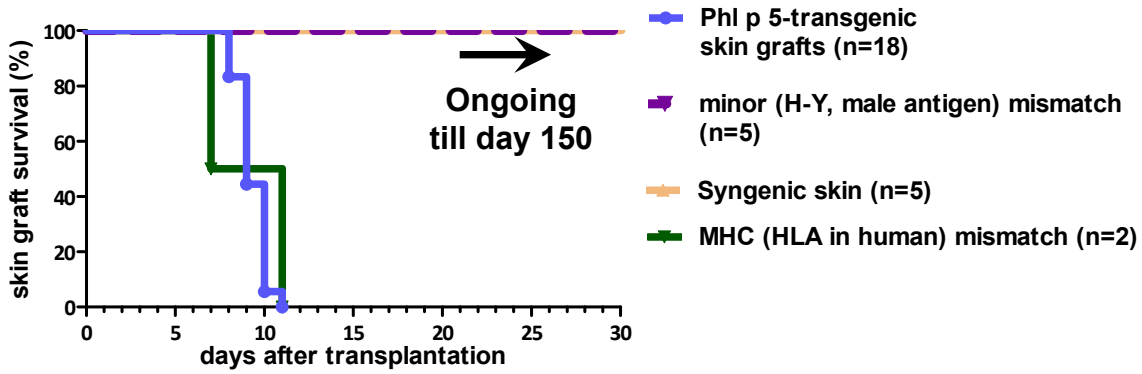

Figure 11: Skin graft survival of different donors visualized as Kaplan-Meier survival curve.

The chronic rejection H-Y mismatched skin was determined via hematoxylin and eosin (HE) staining by Prof. Wrba of the Institute of Pathology (Medical University of Vienna). Typical signs of the chronic rejection are shown on figure 12, thickening of the epidermis, elevated leukocyte infiltration (Fig. 12 A blue cells) and extinction of the epidermal appendages (Fig. 12 B marked by an arrow).

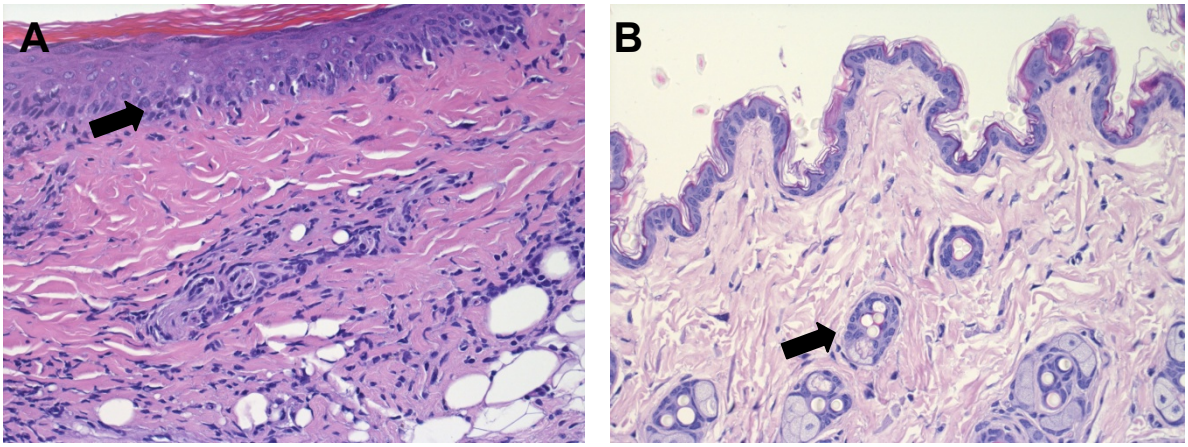

Figure 14: Tail skin section of a Balb/c mouse receiving a skin graft of a male Balb/c (A) or a syngenic graft (B) stained with HE x 400. The left arrow indicates the thickened epidermis; the right arrow shows the epidermal appendages that are extinct in A.

We also wanted to address which T-cell subset plays a role in this rapid rejection. In order to analyse this naïve Balb/c mice received either anti-CD4 or anti-CD8 depleting in vivo antibodies at several time points before skin grafting (figure 13). The CD4 or CD8 depletion was almost complete (~0.1% for each subset,) and assessed by flow cytometry at several time points (day 0, 7, 15 29) data not shown.

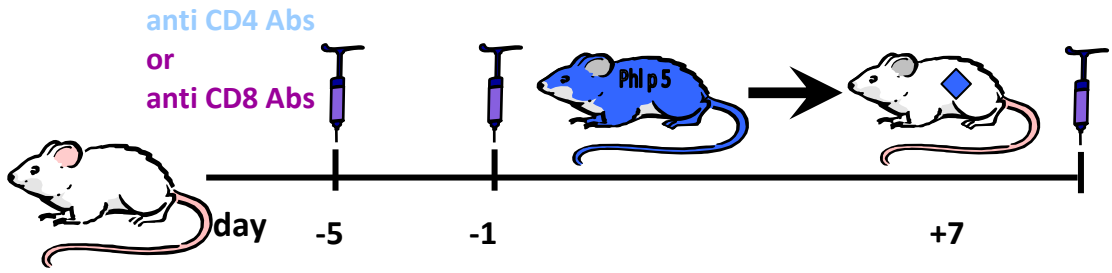

Figure 13: in vivo protocol for CD4 or CD8 depletion

For the CD8 depleted mice shown in figure 14 the median graft survival remained unchanged compared to untreated mice (9 days, n=5). In contrast CD4 depleted mice showed a prolonged graft survival with a median graft survival of 20 days which was clearly longer than in untreated or CD8 depleted mice (Figure 14).

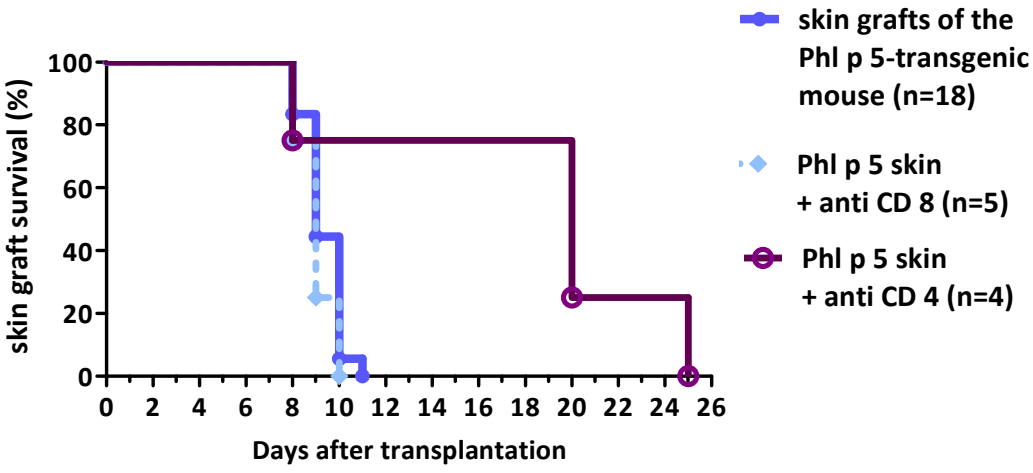

Figure 14: Skin graft survival of different donors visualized as Kaplan-Meier survival curve.

Since there was a clear atrophy in the skin grafts of the CD4 depleted mice, one mouse was sacrificed on day 25 post grafting to further analyse the graft via immunohistochemistry. According to Prof. Wrba there were signs of an acute rejection including massive leukocyte infiltration, thickening of the epidermis and extinction of the dermo-epidermal junction and epidermal appendages. Figure 15 shows the junction of its own skin tissue to the skin graft.

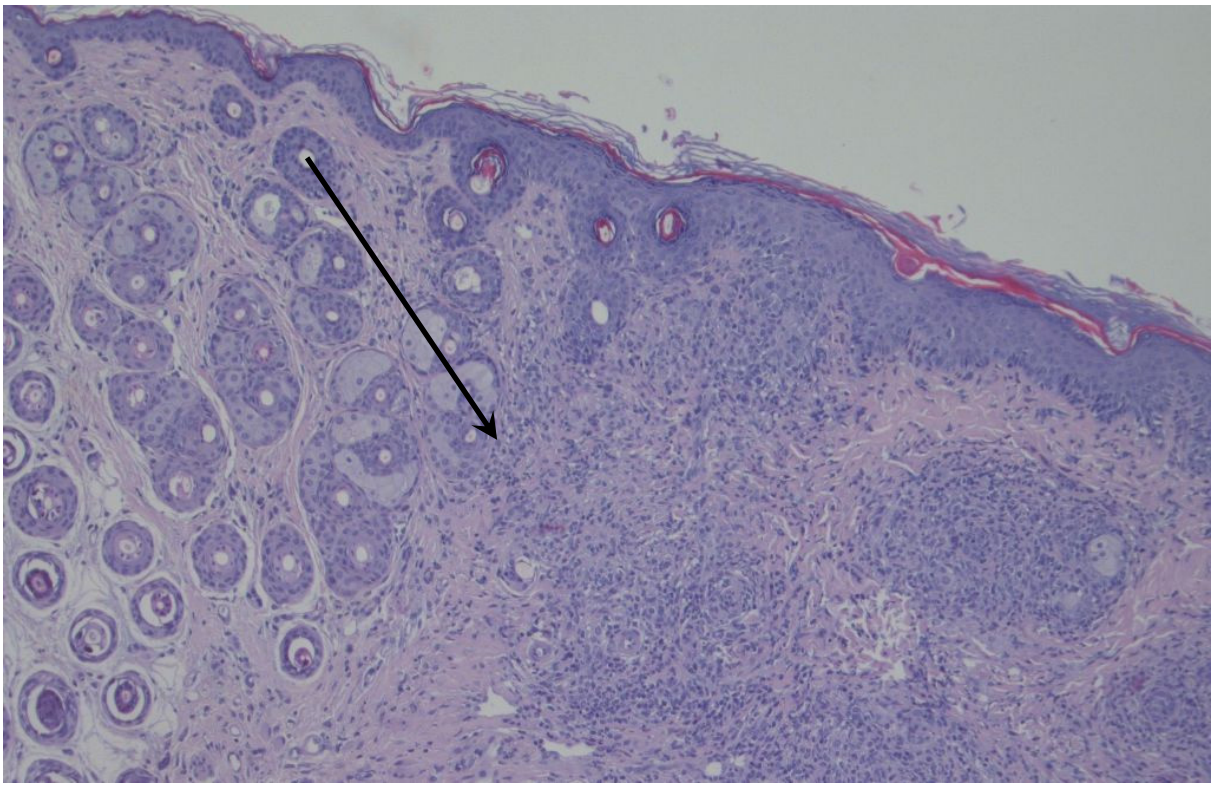

Figure 15: Skin section of the junction of the skin graft in a CD4 depleted Balb/c receiving a skin graft of a Phl p 5-transgenic Balb/c (A) or a syngenic graft stained with HE x 100.

Taken together skin grafts of Phl p 5-transgenic Balb/c mice transplanted onto naïve Balb/c are as promptly rejected as MHC-mismatched grafts. Additionally this prompt rejection does not require $CD8^+$ T-cells. Notably $CD8^+$ T-cells alone are also able to cause graft rejection of the minor antigen mismatched skin, but the graft survival is significantly prolonged compared to untreated mice.

The rejection of Phl p 5-transgenic skin grafts is accompanied by a strong humoral response:

Since the preliminary data also showed high levels of Phl p 5-specific IgE and IgG1 after graft rejection (Baranyi et.al., unpublished data), we wanted to further analyse the immunogenicity of skin grafts regarding the humoral response, especially Phl p 5-specific IgE. Further we wanted to assess if the rejection elicits a humoral response. Therefore sera of mice were taken at several time points (week 2, 7 and week 13/14) and subsequently analysed via Phl p 5-specific ELISA. Since some of the mice were sacrificed for T cell proliferation assays the lower mouse numbers at the last time point are indicated in the figure legends. Subcutaneous immunisation with rPhl p 5 and Al(OH)3 was shown to lead to a high immune response in Balb/c mice [37]. Therefore we compared the levels of Phl p 5-specific IgG1 and IgE with mice immunised subcutaneously with either rPhl p 5 (n=8) or rPhl p 5 plus $Al(OH)_3$. As shown on figure 16 and figure 17 levels of Phl p 5-specific IgE and IgG1 in sera were comparable at all indicated time points in mice receiving skin grafts or rPhl p 5 plus $Al(OH)_3$. However comparing the mice receiving skin grafts with the mice immunised with rPhl p 5 in absence of the adjuvant Phl p 5-specific IgE and IgG1 levels were significantly higher at week 2 and 7 in sera of skin grafted mice. In mice receiving only rPhl p 5 the Phl p 5-specific IgE and IgG1 levels in sera appeared to be less homogenous at all time points and some mice did not develop any Phl p 5-specific antibodies.

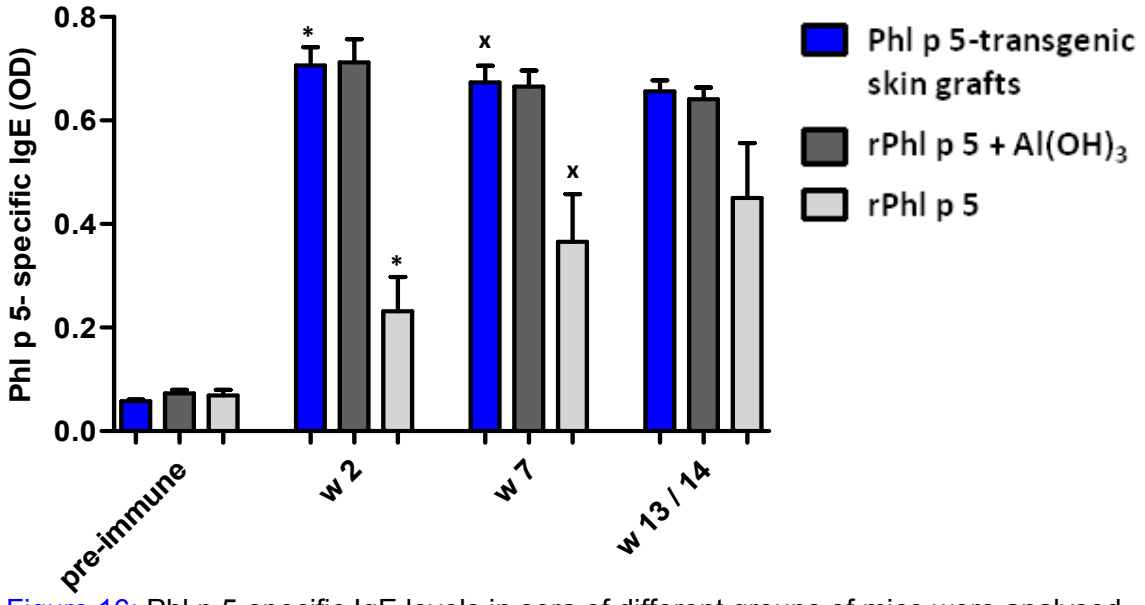

Figure 16: Phl p 5-specific IgE levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. $*/^{x}$ represents significant differences.

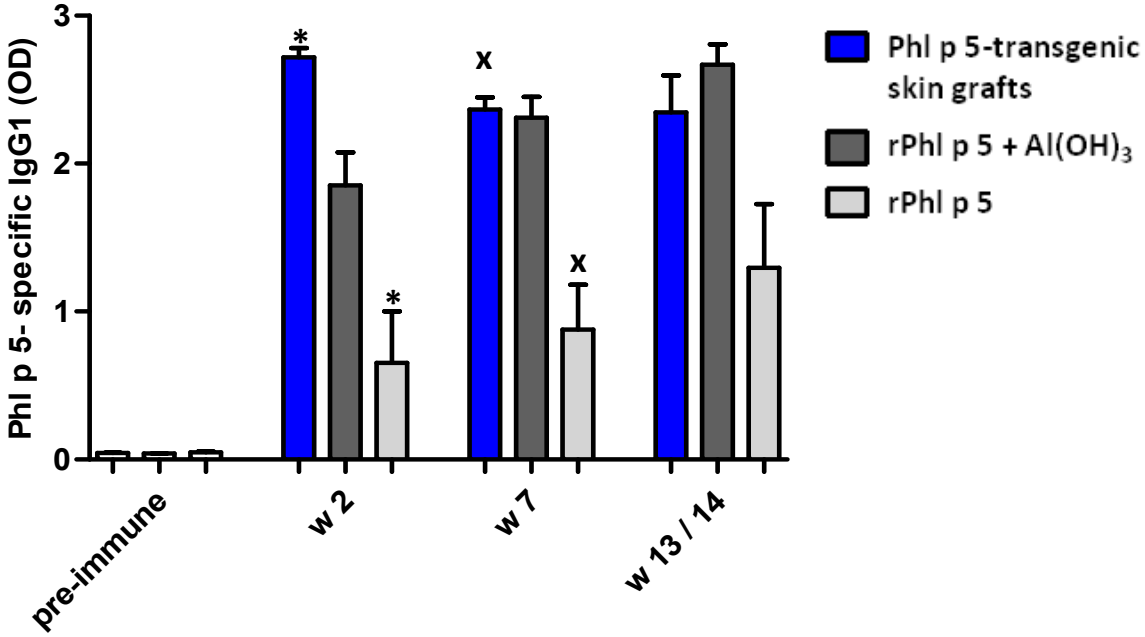

Figure 17: Phl p 5-specific IgG1 levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. $*/^{x}$ represents significant differences.

It was also of interest to determine the induction time point of the sensitisation. We were able to narrow the time frame of the induction of Phl p 5-specific IgE and IgG1. As shown on figure 18 Phl p 5-specific ELISA did not detect IgG1 and IgE at day 7 (n=8) but both isotypes were detectable by day 10 (n=5).

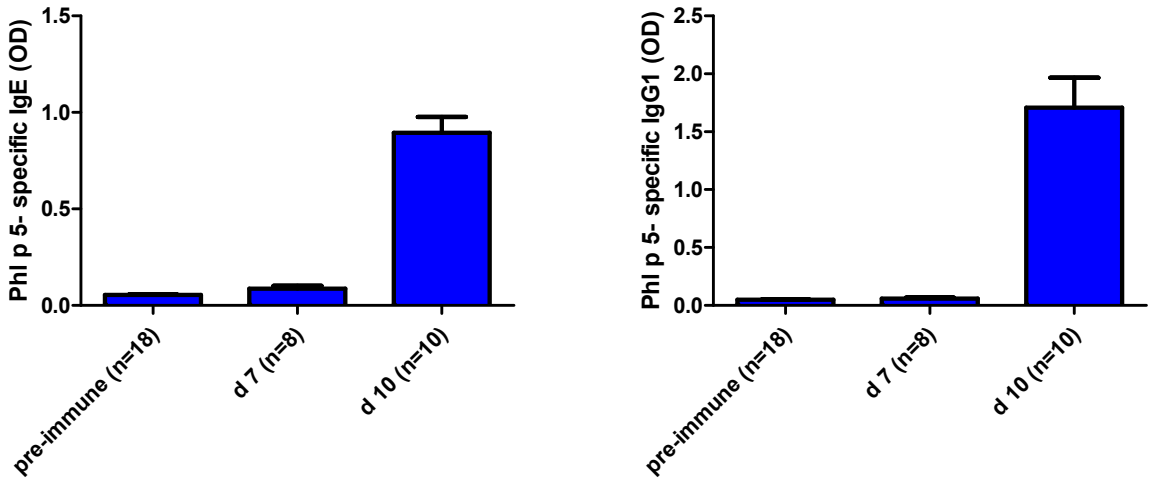

Figure 18: Time point of IgE / IgG1 induction. Phl p 5-specific IgE (left) IgG1 (right) levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group.

Additionally a long time course of Phl p 5-specific IgE and IgG1 in skin grafted mice was assessed. As shown on figures 19 and 20 both isotypes were stable and still detectable after 58 weeks (n=5). Time points are indicated in figure 19 and figure 20, unfortunately one mouse died before the blood withdrawal in week 40 and another mouse in week 57.

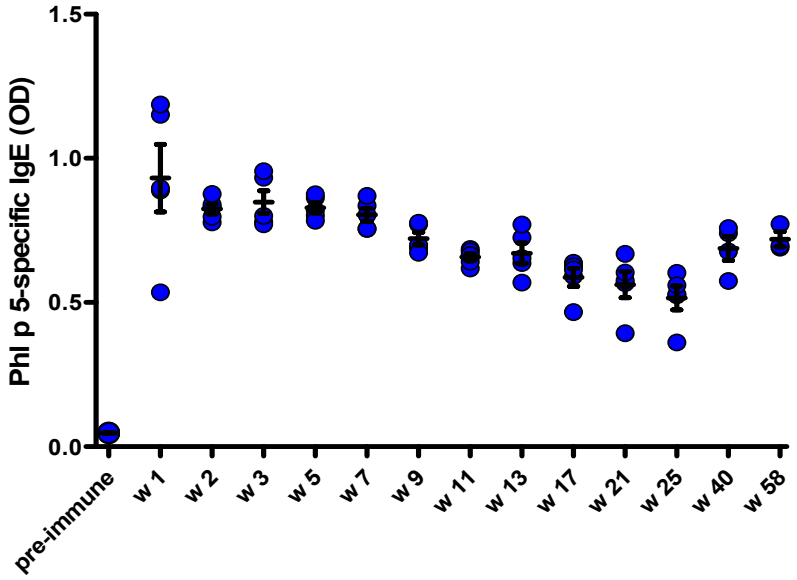

Figure 19: Time course of Phl p 5-specific IgE. Phl p 5-specific IgE levels in sera of different skin grafted mice were analysed by ELISA at indicated time points. Each circle represents an individual mouse + standard deviation for all mice.

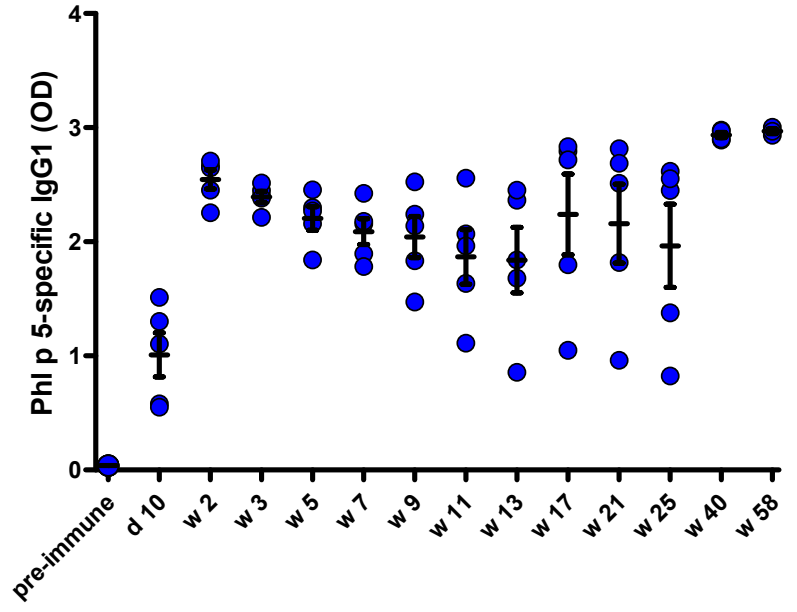

Figure 20: Time course of Phl p 5-specific IgG1. Phl p 5-specific IgG1 levels in sera of different skin grafted mice were analysed by ELISA at indicated time points. Each circle represents an individual mouse + standard deviation for all mice.

To recapitulate there was a strong induction of Phl p 5-specific IgE and IgG1 after skin graft rejection already detectable by day 10 up to week 58. Additionally the levels of antibodies in skin grafted mice were significantly higher in week 2 and 7 than in mice receiving rPhl p 5 without adjuvant.

Splenocytes of Phl p 5-transgenic mice induce a high immune response:

Skin grafts of Phl p 5-transgenic mice showed to be a very potent way to induce a Phl p 5-specific immune response. To assess if this is skin tissue specific we wanted to determine whether splenocyte single cell-suspensions or splenocyte cell extracts (with and without $Al(OH)_3$) of Phl p 5-transgenic mice are capable of eliciting a comparable response. For the generation of splenocyte cell extracts we used a slightly modified protocol, isolating mainly membrane proteins published by Hughes et.al. The content of Phl p 5 after Phl p 5 extraction was semi-quantified via western blotting. Therefore a serial dilution of rPhl p 5 and different cell numbers or different volumes of splenocyte cell extracts (gained from 5 spleens of female Phl p 5-transgenic mice) were loaded on a 30% polyacrylamide-gel and subsequently blotted on a nitrocellulose membrane and detected via chemiluminiscence (figure 21 and figure 22).

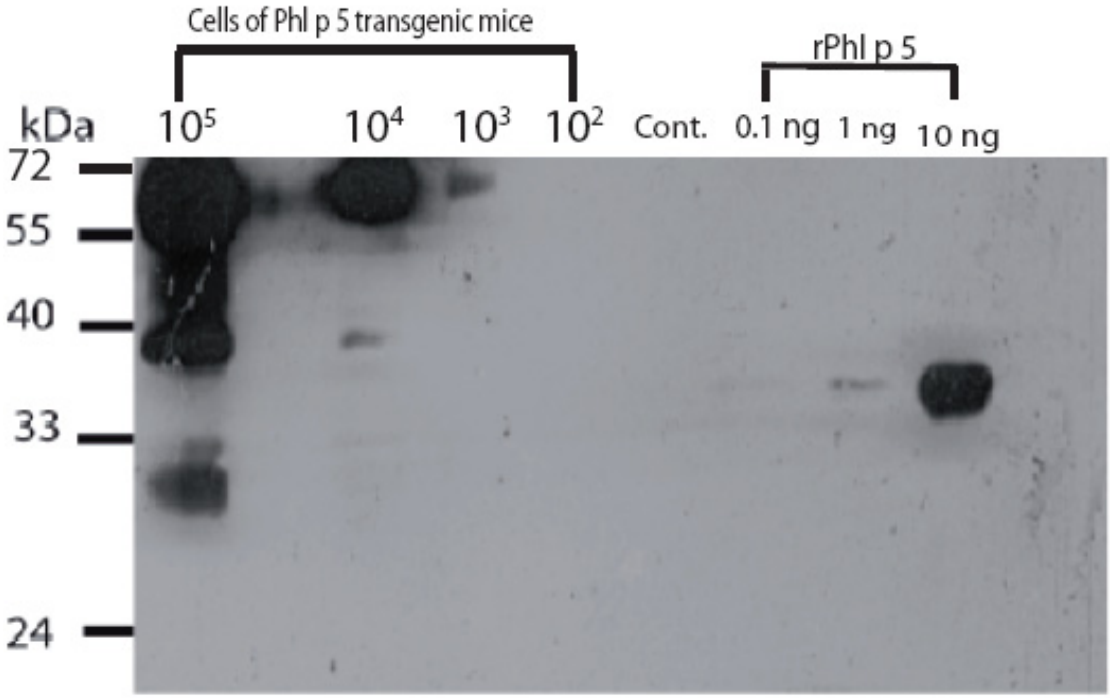

Figure 21: Semi-quantification of Phl p 5 content of Phl p 5-transgenic mice splenocytes. Nitrocellulose blotted serial dilution of lysates of Phl p 5-transgenic mice splenocytes, a serial dilution of rPhl p 5, and as a negative control splenocytes without Phl p 5 expression. The blot was incubated with Phl p 5 immunised rabbit serum and detected as described in the methods section.

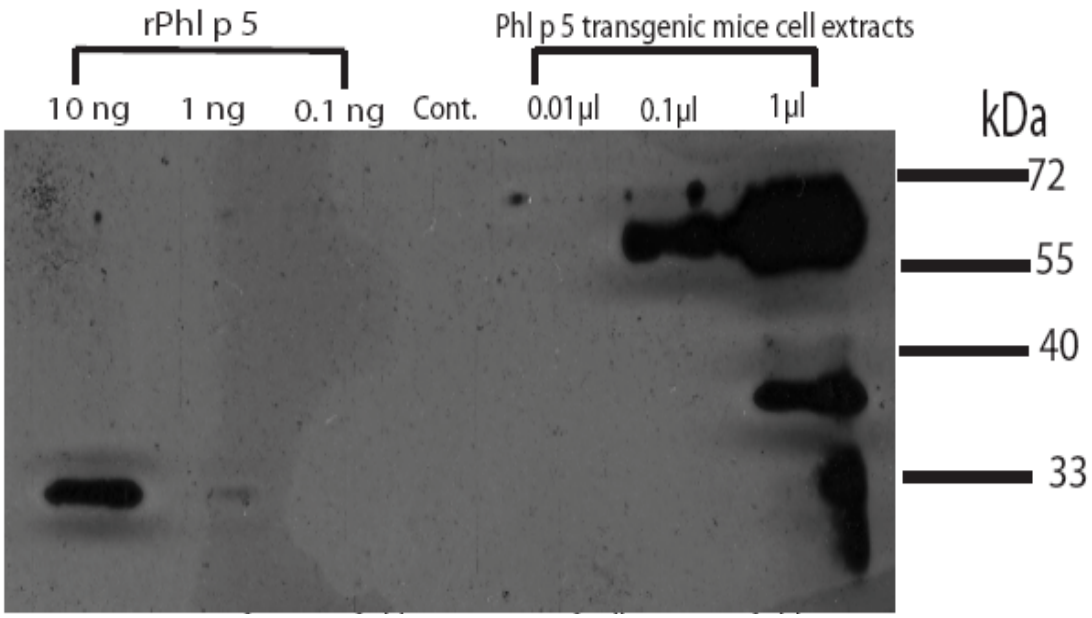

Figure 22: Semi-quantification of Phl p 5 content of cell extracts of Phl p 5-transgenic mice splenocytes. Nitrocellulose blotted serial dilution of cell extracts, a serial dilution of rPhl p 5, and as a negative control splenocytes without Phl p 5 expression. The blot was incubated with Phl p 5 immunised rabbit serum and detected as Described in the methods section.

By using the blot analysis software Quantity One® the intensity and area was calculated resulting in an approximate volume of the sample. From this measurement we estimated that 10^3 splenocytes contain approximately 1.95 ng of Phl p 5 and 1 µl of splenocyte cell extracts contains roughly 167 ng of Phl p 5. Naïve female Balb/c mice immunized with 5 µg or rPhl p 5 with or without $Al(OH)_3$ and naïve female Balb/c mice receiving skin grafts of Phl p 5-transgenic mice served as control groups. After semi-quantification we injected subcutaneously 2.5 x 10^6 splenocytes per mouse and assessed the levels of Phl p 5-specific antibodies in sera via ELISA at different time points (week 2, 7 and month 4). Since some of the mice were sacrificed for T cell proliferation assays the lower mouse numbers at the last time point are indicated in the figure legends.

There was an induction of Phl p 5-specific IgE in all groups at week 2. Although mice receiving skin grafts showed significantly higher levels of Phl p 5-specific IgE and IgG1 at week 2 and 7 than mice immunised with splenocytes. The splenocyte treated mice also showed a clear and quite homogenous induction of both isotypes (figure 23 and figure 24).

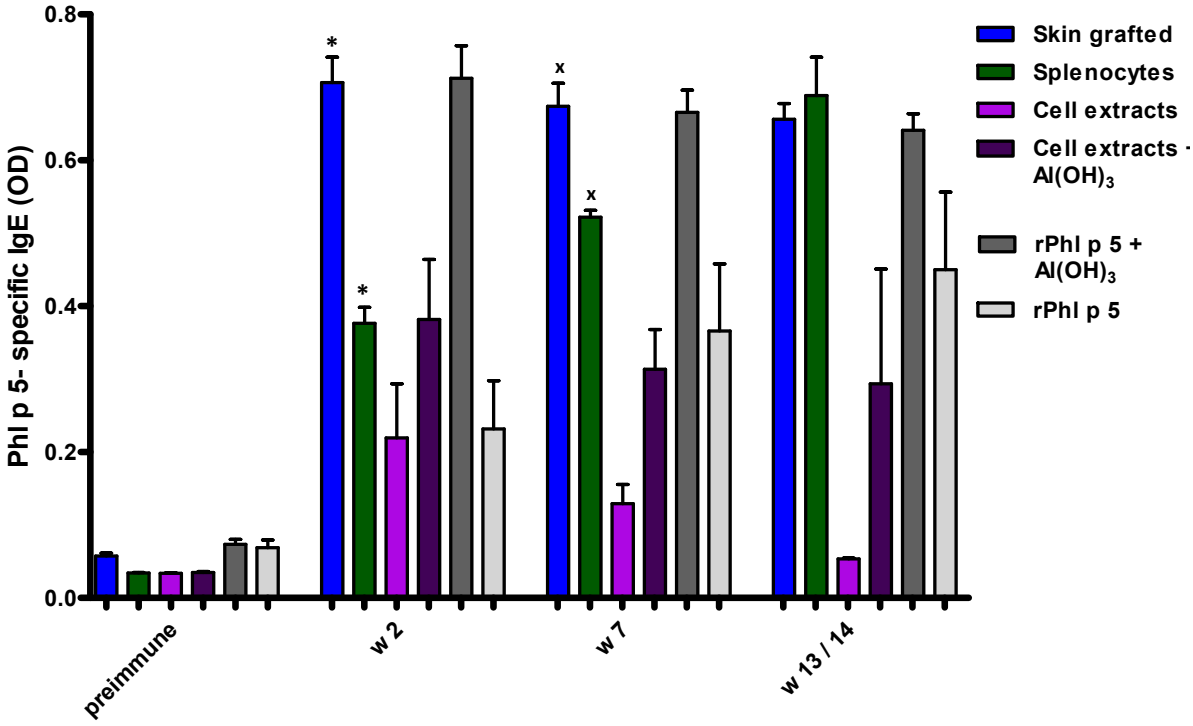

Figure 23: Phl p 5-specific IgE levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), splenocytes n=8, cell extracts n=8 (w 13 / 14 n=3), cell extracts + $Al(OH)_3$ n=8 (w 13 / 14 n=3), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. $*/^x$ represents significant differences.

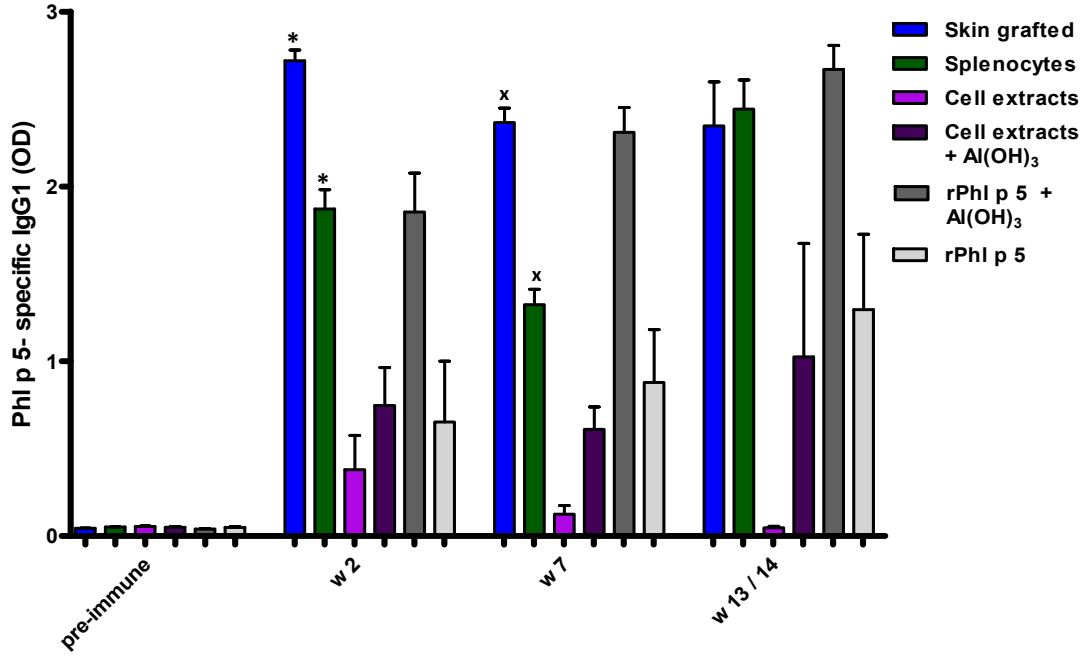

Figure 24: Phl p 5-specific IgG1 levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), splenocytes n=8, cell extracts n=8 (w 13 / 14 n=3), cell extracts + $Al(OH)_3$ n=8 (w 13 / 14 n=3), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. $*/^x$ represents significant differences.

Additionally Phl p 5-specific IgG2a, IgG2b, IgG3 and IgA levels were determined. For IgG2b, skin grafted mice showed Phl p 5-specific IgG2b levels significantly higher in week 7 and month 4 (figure 25). It was also assessed that Phl p 5-specific IgA was only significantly higher in week 2 for the skin grafted group (figure 26).

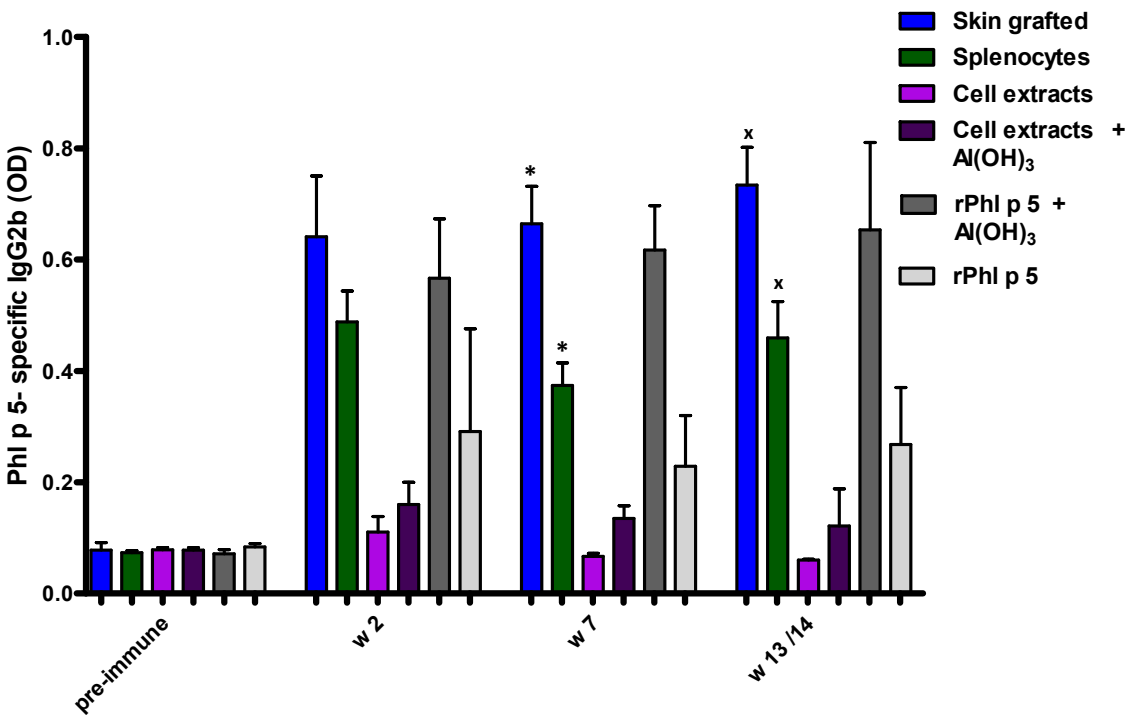

Figure 25: Phl p 5-specific IgG2b levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), splenocytes n=8, cell extracts n=8 (w 13 / 14 n=3), cell extracts + $Al(OH)_3$ n=8 (w 13 / 14 n=3), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. */^{x} represents significant differences.

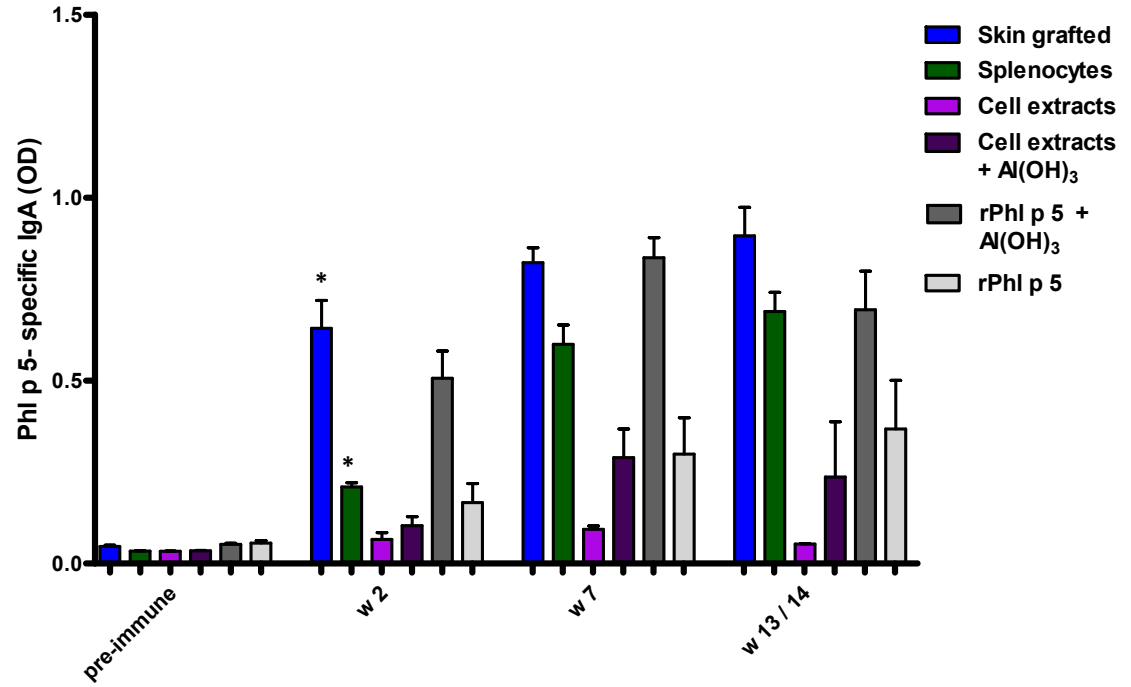

Figure 26: Phl p 5-specific IgA levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), splenocytes n=8, cell extracts n=8 (w 13 / 14 n=3), cell extracts + $Al(OH)_3$ n=8 (w 13 / 14 n=3), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. * represents significant differences.

In contrast immunisation with splenocytes induced significantly higher levels of Phl p 5-specific IgG2a, than in all other groups at all time points (figure 27). Additionally Phl p 5-specific IgG3 was significantly higher in week 2 for mice receiving splenocytes (figure 28).

Splenocyte cell extracts induced only very inconsistent levels of Phl p 5-specific antibodies (figures 23-28). For some mice we were not able to detect any Phl p 5-specific antibodies in sera. When the cell extracts were administered without adjuvants elevated levels of Phl p 5-specific IgE (figure 23) and IgG1 (figure 24), that peaked at week 2 and dropped again until week 14, were detected in sera of some mice. In contrast when the splenocyte cell extracts were injected with $Al(OH)_3$ the levels of Phl p 5-specific IgG1(figure 24) and IgE (figure 23) in sera were elevated and stable but quite inhomogeneous within the group. We were not able to detect Phl p 5-specific IgA (figure 26), IgG2a (figure 27), IgG2b (figure 25) and IgG3 (figure 28) in the serum of any mouse after injection of splenocyte cell extracts without adjuvants.

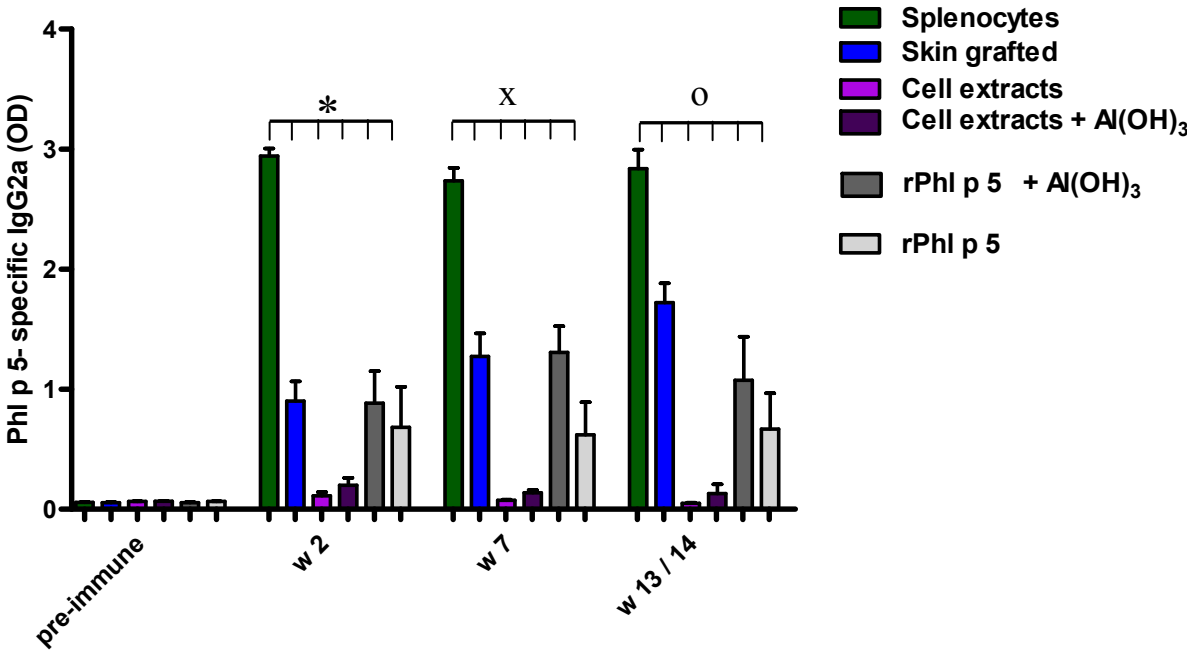

Figure 27: Phl p 5-specific IgG2a levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), splenocytes n=8, cell extracts n=8 (w 13 / 14 n=3), cell extracts + $Al(OH)_3$ n=8 (w 13 / 14 n=3), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. $*/^{x}/^{o}$ represents significant differences.

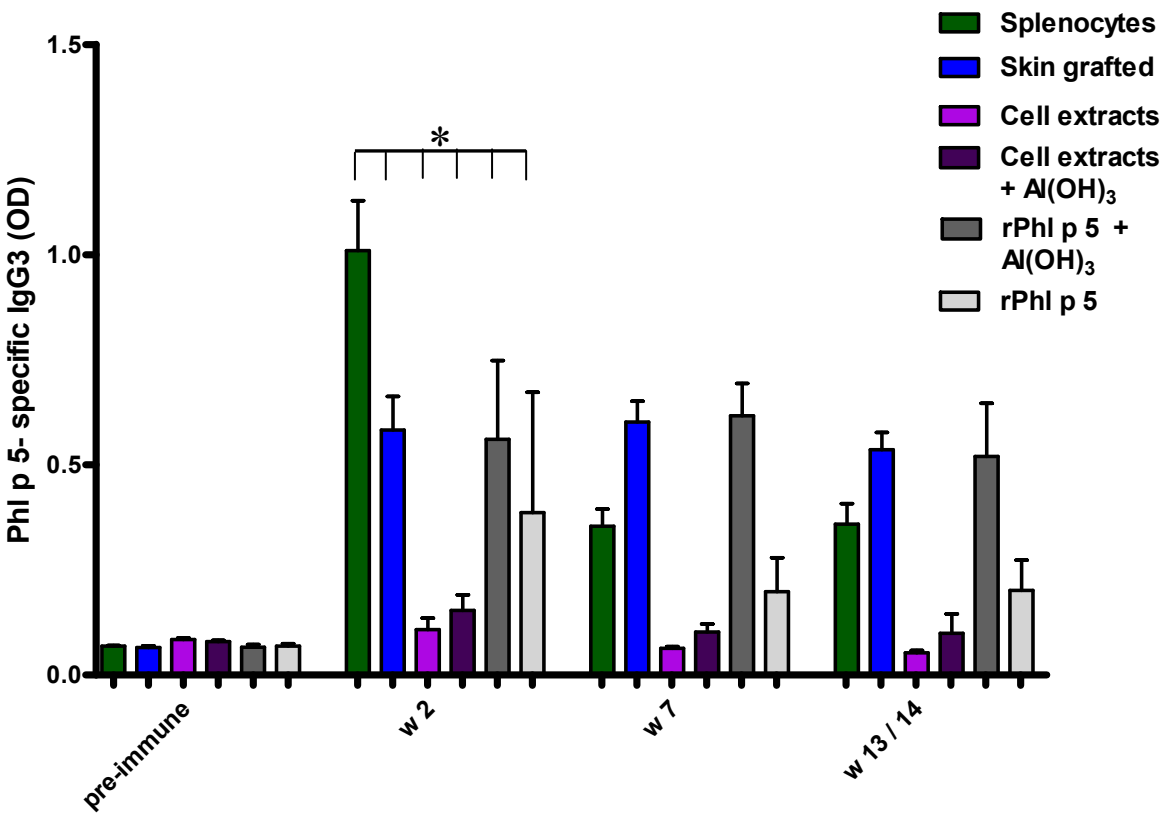

Figure 28: Phl p 5-specific IgG3 levels in sera of different groups of mice were analysed by ELISA at indicated time points. Mean Ab levels + standard deviation are shown for each group. Pooled data of three independent experiments with group size: Phl p 5-transgenic skin grafts n=13 (w 13 / 14 n=9), splenocytes n=8, cell extracts n=8 (w 13 / 14 n=3), cell extracts + $Al(OH)_3$ n=8 (w 13 / 14 n=3), rPhl p 5 + $Al(OH)_3$ n=13 (w 13 / 14 n=9), rPhl p 5 n=8. * represents significant differences.

To sum up splenocytes were capable of inducing a stable Phl p 5-specific humoral response in all recipient mice, additionally compared to all other groups splenocyte treated mice showed significantly elevated Phl p 5-specific IgG2a levels at all time points and Phl p 5-specific IgG3 levels at week 2.

Contrary to mice receiving cell extracts with and without $Al(OH)_3$ that appeared to develop only very inconsistent levels of Phl p 5-specific antibodies.

Phl p 5-specific IgE induced by cells of transgenic mice leads to high effector cell release:

We wanted to assess if induced Phl p 5-specific IgE, regarding mice that were immunised with cells or cell extracts of the Phl p 5-transgenic mouse, is able to induce effector cell release upon stimulation with rPhl p 5. Therefore the rat basophil leukemia (RBL) degranulation assay to observe whether induced Phl p 5-specific IgE is also capable to induce effector cell degranulation in vitro upon stimulation with rPhl p 5 was used. RBL-cells were loaded with sera taken at different time points (week 2, 7, 14) after mice were either immunised via skin grafts (n=5), splenocytes (n=5), cell extracts (n=3, + $Al(OH)_3$ n=3) of Phl p 5-transgenic mice or rPhl p 5 (n=5, + $Al(OH)_3$ n=5). β-hexasominidase release after stimulation with rPhl p 5 was measured as a surrogate for histamine. [66]

As shown in figure 28 skin grafted and mice immunised with rPhl p 5 + $Al(OH)_3$ elicited a comparable β-hexasominidase release at all time points. For the skin grafted the highest β-hexasominidase release was at week 7 with a mean of 59.5%. In sera of mice that were immunised with splenocyte cell extracts only in combination with $Al(OH)_3$ a clear β-hexasominidase release after allergen challenge was detectable but exclusively in week 2 (figure 29). (Due to technical problems no valid β-hexasominidase release data are available in splenocyte or rPhl p 5 treated mice.)

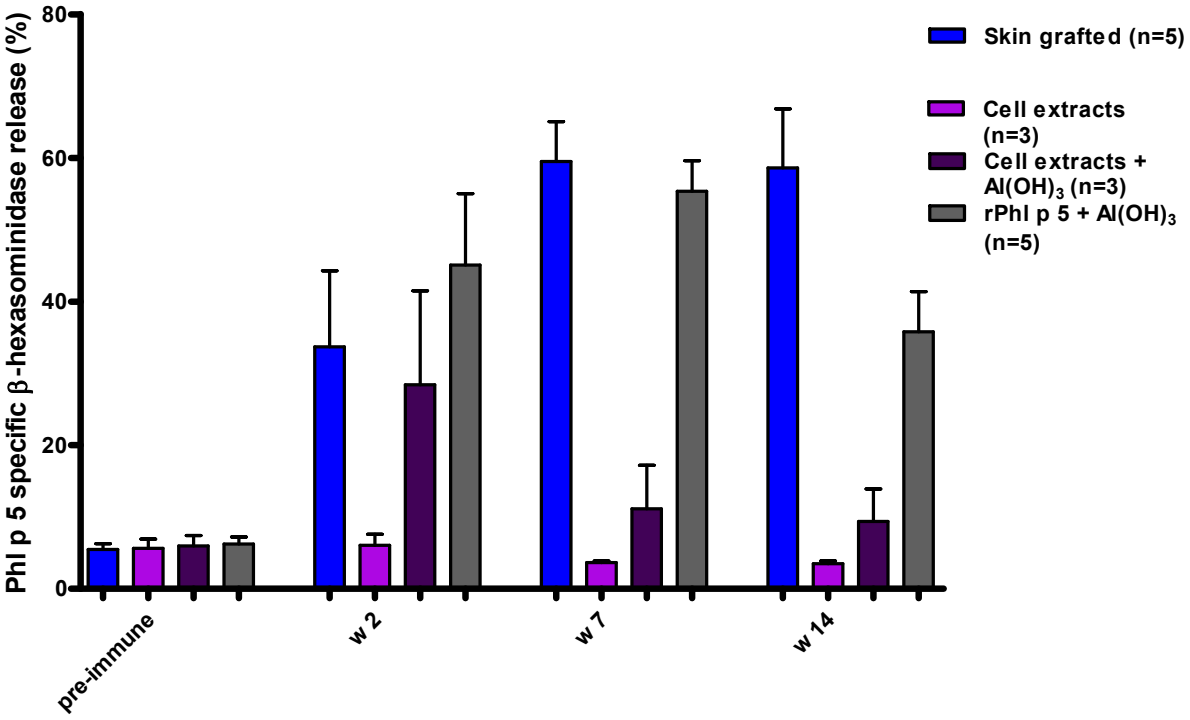

Figure 29: in vitro β-hexasominidase; the mean percentage of β-hexasominidase for each group + standard deviation at indicated time points are shown. Group size is indicated in the figure legend.

Skin grafted mice show high Phl p 5-specific T-cell proliferation:

To address Phl p 5-specific T-cell responses, T-cell proliferation assays were performed. The splenocytes were isolated at the end of the follow-up in three different experiments (week 14, week 17 and week 58). Phl p 5-specific T-cell proliferation was comparable in skin grafted and mice immunised with rPhl p 5 plus adjuvant (stimulation indices 25.03 vs. 23.38) (figure 30).

Splenocyte and cell extract treated mice also showed elevated Phl p 5-specific T-cell proliferation (stimulation indices 14.18 and 8.78), while the proliferation was comparable to naïve mice when cell extracts were used in combination with adjuvant (which was possibly due to a technical failure) (figure 30).

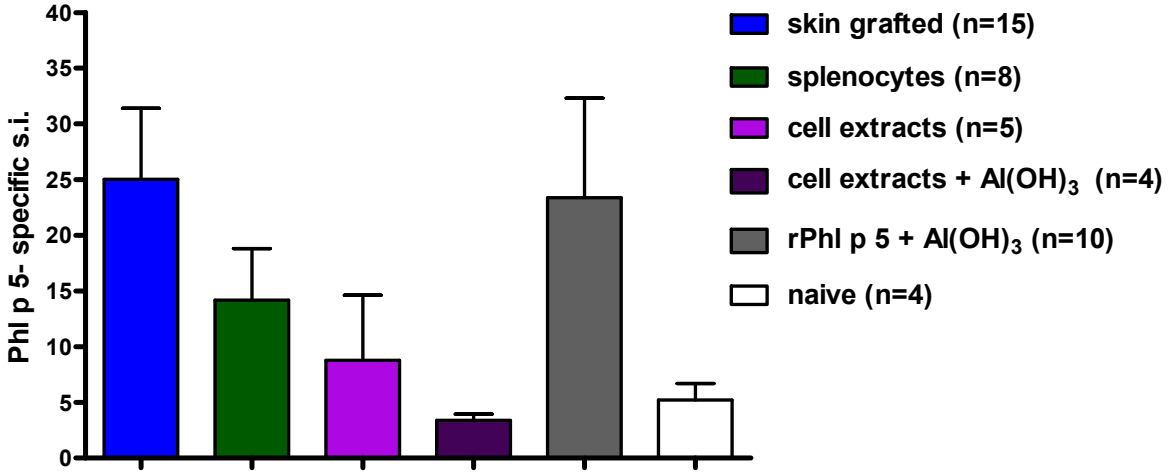

Figure 30: in vitro Phl p 5-specific T-cell proliferation; the mean percentage of stimulation indices for each group + standard deviation are shown. Group size is indicated in the figure legend.

A Phl p 5-specific humoral immune response can also be triggered by irradiated cells and cytoplasmatic Phl p 5:

Since preliminary experiments showed incomplete tolerance towards Phl p 5 via molecular chimerism when BMCs were transduced to express Phl p 5 cytoplasmatically, we wanted to assess if the localisation of Phl p 5 on the cell membrane is of importance for the antigen presentation. Therefore NIH-3T3 (murine fibroblast cell line) cells were transduced with recombinant viruses to express Phl p 5 either as a transmembrane protein (Phl p 5-TM) like described in [63] or as a cytoplasmic protein (Phl p 5-cyt, (Baranyi et al. unpublished data)). Additionally we wanted to determine if viable cells are needed to trigger a Phl p 5-specific response. This was done by irradiation of the cells. Each group of female Balb/c mice (n=3) received either 2.5 x 10^6 splenocytes of the Phl p 5-transgenic mouse, Phl p 5-TM cells or Phl p 5-cyt. Additionally three groups (n=3) received cells that were irradiated with 30 Gy before injection.

Prior to the mouse experiment we analysed the Phl p 5 expression of the transduced cells via flow cytometry. The Phl p 5-TM cells were stained with Phl p 5-specific antibodies and subsequently analysed. Since the cytoplasmic Phl p 5 construct also

contains GFP, expressed via an IRES the cytoplasmic Phl p 5 expression was determined indirectly via the GFP signal. Transduction efficiency was 78.1% for the Phl p 5-TM cells and 86.7% for the Phl p 5-cyt cells (figure 31).

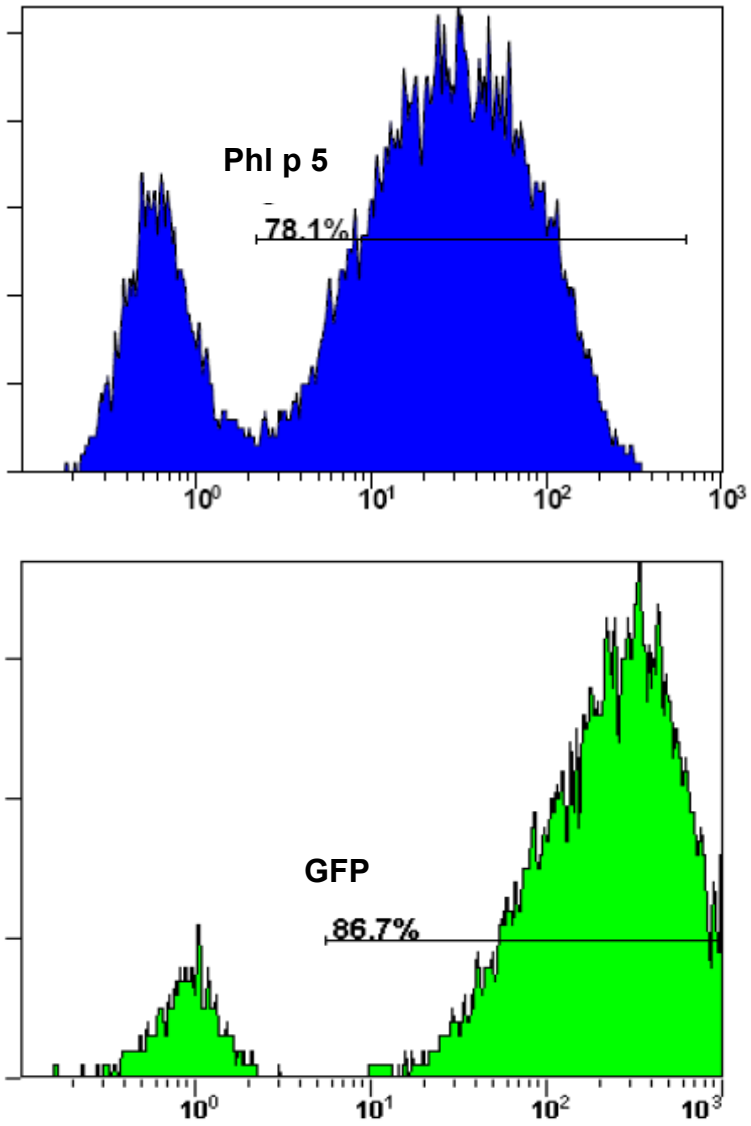

Figure 33: analysis of retroviral transduction efficiency; flow cytometric analysis of transduced 3T3 cells before injection in recipient mice. The upper histogram represents the percentage of surface stained Phl p 5, the lower histogram the GFP expression in the cytoplasm which is co-expressed with cytoplasmic Phl p 5.

After subcutaneous injection of the cells Phl p 5-specific antibody levels (IgG1, IgE, IgG2a and IgG3) in sera were determined via ELISA at week 2, 7 and 16.

As shown in figure 32 for irradiated splenocytes no Phl p 5-specific IgG1 was detectable in sera of the mice at week 2. After that time point Phl p 5-specific IgG1 levels were slightly elevated in week 7 and were still detectable in week 16 in sera of mice that were injected with irradiated splenocytes of Phl p 5-transgenic mice. All other groups showed elevated levels of Phl p 5-specific IgG1 at all time points compared with the pre-immune sera.

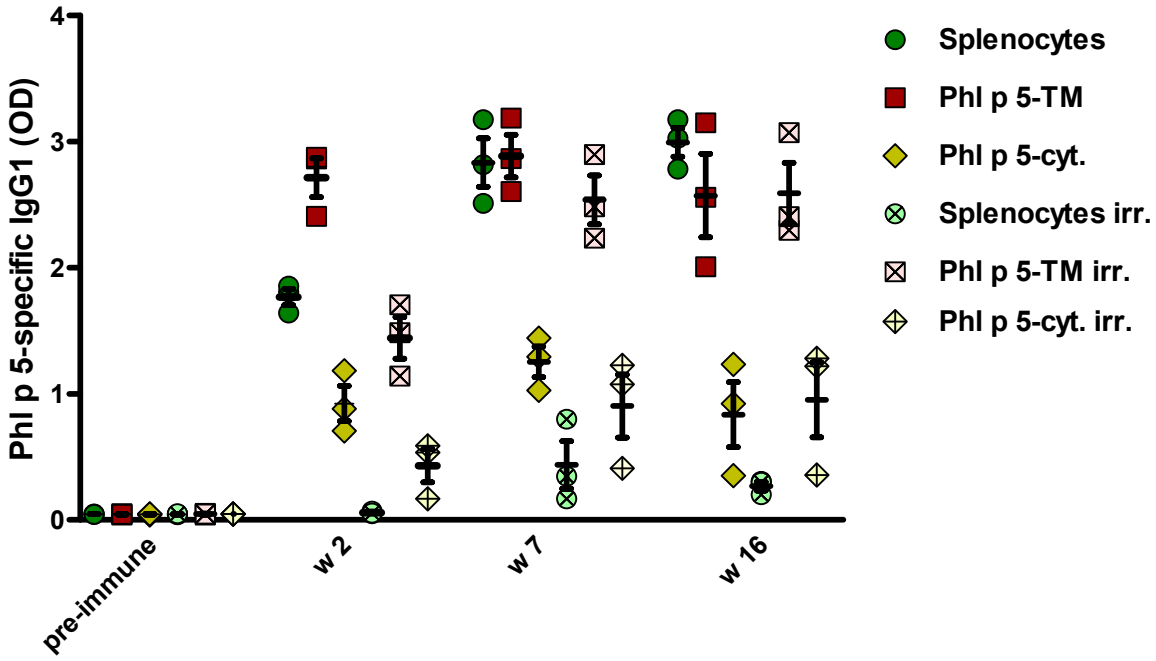

Figure 32: Phl p 5-specific IgG1 levels in sera of different groups of mice were analysed by ELISA at indicated time points. Each symbol represents an individual mouse + standard deviation for all mice.

Phl p 5-specific IgE levels were also induced in sera of all mouse groups, although in sera of mice receiving irradiated splenocytes only one mouse showed detectable Phl p 5-specific IgE at the first time point (figure 33).

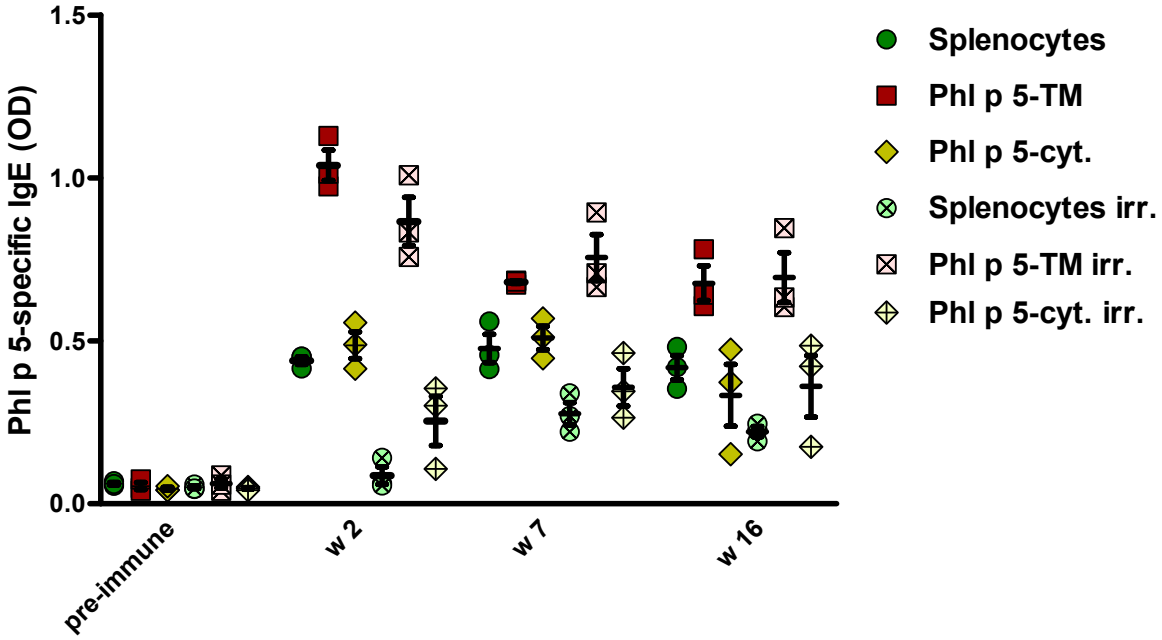

Figure 33: Phl p 5-specific IgE levels in sera of different groups of mice were analysed by ELISA at indicated time points. Each symbol represents an individual mouse + standard deviation for all mice.

Additionally we were interested in IgG2a and IgG3 levels since splenocyte treated mice showed clearly elevated levels of Phl p 5-specific IgG2a and IgG3 compared to the other groups in our prior experiments.

Consistent with the preliminary results, IgG2a levels were strongly increased at all time points in splenocyte-treated mice while the other groups showed only elevated levels of IgG2a in sera (figure 34).

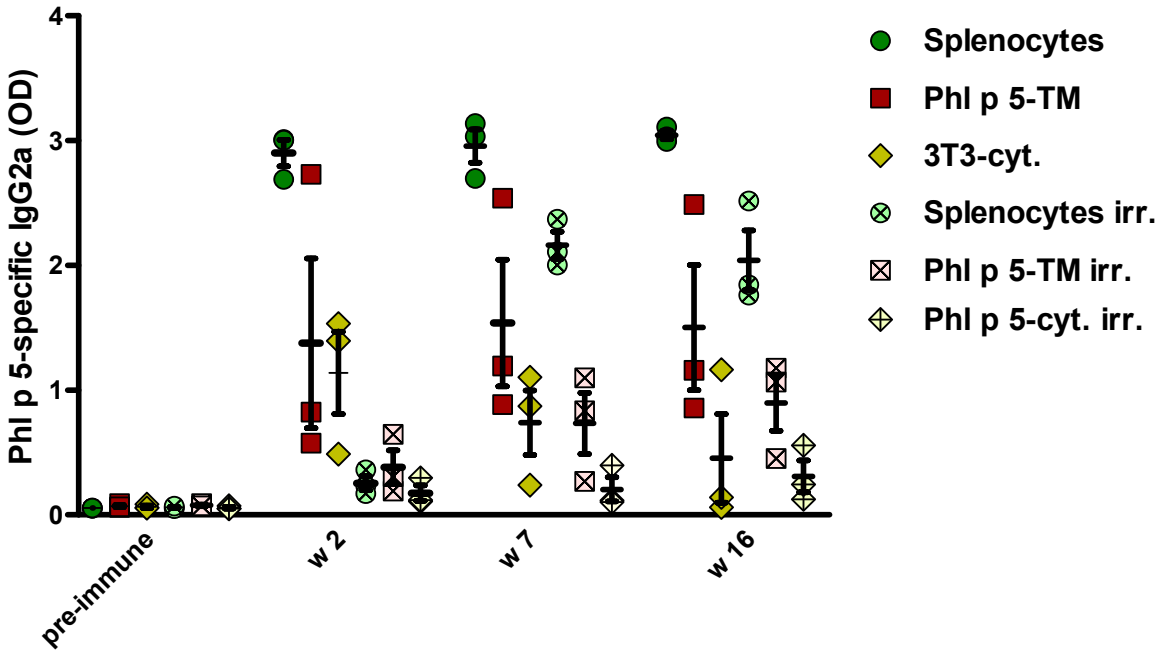

Figure 34: Phl p 5-specific IgG2a levels in sera of different groups of mice were analysed by ELISA at indicated time points. Each symbol represents an individual mouse + standard deviation for all mice.

Concerning Phl p 5-specific IgG3 splenocytes induced very high levels of specific antibodies in sera at week 2 that were also detectable in week 7 in this experiment and dropped until week 16. All the other groups showed only slightly increased levels of Phl p 5-specific IgG3 (figure 35).

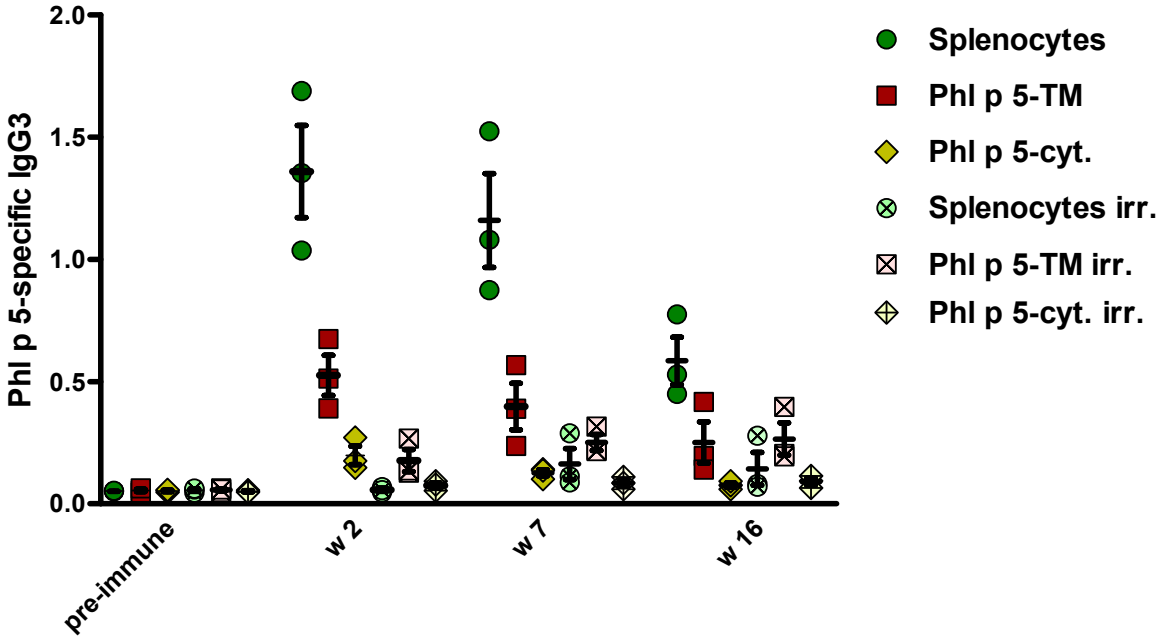

Figure 35: Phl p 5-specific IgG3 levels in sera of different groups of mice were analysed by ELISA at indicated time points. Each symbol represents an individual mouse + standard deviation for all mice.

Summarising, localisation of Phl p 5 on the cell membrane appears to be not essential for efficient antigen presentation and subsequent induction of Phl p 5-specific humoral responses. Furthermore irradiated cells can also be used for injection.

Discussion:

The mechanisms of molecular chimerism on tolerance induction in IgE-mediated allergy are still not completely understood. Therefore our new transgenic mouse model offers a vast pool of hematopoietic stem cells for a broad variety of approaches within the Balb/c strain. This can be done without the need for preliminary retroviral transduction. Furthermore the high immunogenicity of the transgene Phl p 5 in naïve recipients offers a novel approach to study immune responses towards highly immunogenic Mi antigens (i.e. non-MHC antigens).

Characterisation of the Phl p 5-transgenic mouse:

In this thesis we introduced a new transgenic mouse model, expressing an allergen ubiquitously as a transmembrane protein with GFP co-expression via an IRES. This mouse model can be used to study antigen-specific immune responses. Thereby the major grass pollen allergen Phl p 5 was fused to a leader peptide and a transmembrane domain and subsequently integrated into a vector for pro-nuclear injection. The ubiquitous expression of Phl p 5 in WBCs was shown via flow cytometry in several immunological relevant tissues. Furthermore confocal microscopy was used to visualise the membrane localisation in the thymus. Since there was no permeabilisation of the cells in the flow-cytometry protocol we can assume that Phl p 5 was located on the membrane in all positively tested cells. We also verified full length expression of Phl p 5 in splenocytes via immunoblotting.

Determination of the immunogenicity of Phl p 5 as a transgene in Balb/c mice:

As shown by the skin graft experiments the transgene Phl p 5 appears to be highly immunogenic. A surprisingly prompt rejection was elicited by transplantation of tail-skin of Phl p 5-transgenic mice onto naïve Balb/c mice. The median graft survival was 9 days which is comparable with an MHC mismatch. In contrast the H-Y antigen only led to a chronic rejection with a graft survival of more than 150 days. Furthermore it was determined that $CD4^+$ cells can elicit this prompt rejection when

$CD8^+$ cells are depleted with *in vivo* antibodies. Although there were also signs of an acute rejection, depletion of $CD4^+$ cells significantly prolonged the graft survival. Compared with another mouse model expressing ovalbumin (OVA) as transmembrane protein, the immunogenicity of Phl p 5 as a transgene appears to be clearly higher (median graft survival 33.9 vs. 9 days). Consistent with the findings of Ehst et.al. we observed a decrease of graft size when depleting $CD4^+$ cells. [69]. It was quite surprising that a non-MHC antigen can be rejected by $CD8^+$ cells without aid of $CD4^+$ cells since it is thought that non-MHC antigens can only be recognised by the indirect pathway of MHC-II presentation. Therefore it can be suggested that in this model Phl p 5 is presented via both MHC-I and MHC-II although MHC-II presentation appears to be more important for a prompt Phl p 5-specific response. Furthermore it would be interesting to determine the role of the B cells in this model. In order to analyse this B cell depleting antibodies could be used to assess the rejection kinetics of skin grafts of the Phl p 5-transgenic mouse grafted onto B cell depleted Balb/c mice.

Considering the humoral response towards Phl p 5 after graft rejection, high levels of Phl p 5-specific IgE were detectable by day 10. This was quite impressive since it is believed that the class switch from IgM to IgE has to occur via an intermediate IgG1 state within the germinal centre of lymph nodes followed by a subsequent class switch to IgE outside the germinal centre. [70] Furthermore Phl p 5-specific IgE levels were quite stable until week 58 after only one administration with Phl p 5. This can be considered as life-long sensitisation in mice without any further addition of adjuvants.

Compared to immunisation with rPhl p 5 only the addition of adjuvant resulted in a comparable humoral response after subcutaneous injection. When rPhl p 5 alone was administered Phl p 5-specific IgE and IgG1 levels were clearly lower (significantly in week 2 and 7) and less constant than in skin grafted mice, suggesting an adjuvant like effect when Phl p 5 is expressed in murine cells.

Additionally there was a strong and stable induction of Phl p 5-specific IgG2a, IgG2b, IgG3 and IgA for at least 14 weeks.

If the high levels of Phl p 5-specific antibodies induced by graft rejection play a role in the prompt rejection is planned to be analysed in further experiments. The role of the Phl p 5-specific antibodies could be assessed by transferring serum of pre-sensitised mice in to Balb/c scid mice (i.e. Balb/c background but lacking T and B cells) with subsequent grafting of skin of Phl p 5-transgenic mice.

It would be of great interest if the IgE induction observed in this approach is a general phenomenon of alloresponses or specific for this model. To our knowledge antigen-specific IgE upon transplantation was only briefly described once after liver transplantation. [71]

It was furthermore shown that not only skin tissue but also single cell suspensions of splenocytes are capable of inducing a Phl p 5-specific immune response when injected subcutaneously. Elevated levels of Phl p 5-specific IgE, IgG1, IgG2a, IgG2b, IgG3 and IgA were detectable for at least 14 weeks. Contrary to skin grafts of Phl p 5-transgenic mice the isotype IgG2a was strongly induced and IgE and IgG1 were elevated in mice receiving splenocytes. Since IgG2a is a Th1 isotype, and IgE and IgG1 Th2 isotypes [6], it could be suggested that a distinct Th bias might be induced by splenocytes of Phl p 5-transgenic mice. But cytokine profiles are not available yet. If a distinct Th bias is induced it would be of great interest to transplant other tissues or organs (e.g. cardiac grafts of Phl p 5-transgenic mice) and analyse if a Phl p 5-specific immune response is induced and if so, to assess whether the transplanted tissue has some influence on the Th bias.

Further it was already reported that dendritic cells have the capability to migrate into lymph nodes [72]. Therefore it could be suggested that injected APCs (e.g. dendritic cells) migrate into lymph nodes and serve there for antigen presentation. This antigen presentation of injected APCs could play a role in the strong immune response upon subcutaneous injection of splenocytes of the Phl p 5-transgenic mouse. Furthermore it should be added that the Phl p 5 specific band with a higher mobility shift (~60kDa) in splenocyte lysates might be a posttranslational modification (e.g. a glycosylation). This might elevate antigen uptake by dendritic cells and thereby the immunogenicity of the protein as shown for a glycosylated food allergen. [73] In order to assess possible modifications it is important to purify the protein expressed by the transgene Phl p 5 for further analysis.

Concerning the localisation of Phl p 5 we were able to show that the localisation of Phl p 5 on the membrane appears to be not crucial for a Phl p 5-specific humoral response. However it appears to be important for the prompt response that the transferred cells are still viable. Irradiated cells appear to induce only a delayed immune response. This could be caused by the diminished ability to proliferate and newly express Phl p 5, since irradiation is known to destroy RNA and chromosomal DNA thereby inhibiting the expression of Phl p 5.

When effector cell release was assessed, mice with high Phl p 5-specific IgE levels showed also high β-hexasominidase release in RBL-assays.
Additionally we analysed Phl p 5-specific T cell proliferation in *in vitro* assays. For skin grafted mice Phl p 5-specific T cell proliferation was significantly elevated compared to naïve mice. Splenocyte treated mice showed also increased T cell proliferation towards Phl p 5 *in vitro.* Due to a technical failure the T cell *in vitro* proliferation of mice immunised with rPhl p 5 alone could not be determined.
Regarding the splenocyte cell extracts of Phl p 5-transgenic mice we were not able to induce a stable immune response with splenocyte cell extracts therefore it could be assumed that their immunogenicity is impaired even when facilitated with adjuvants. Since some of the tested mice developed Phl p 5-specific antibodies and showed slightly elevated Phl p 5-specific T cell proliferation it can be considered that the immunogenicity is not completely lost.

In summary this novel transgenic mouse model is an ideal model for tolerance induction via molecular chimerism. Since the transgene Phl p 5 expressed in murine cells appears to be highly immunogenic without addition of adjuvants, it might also serve as a model for a better understanding of humoral immune responses towards antigens (e.g. non-MHC alloantigen).

This work was supported by the Austrian Science Fund FWF (project nr. P 21989-B11 to Ulrike Baranyi and Doctoral Programme W1212 to T.W.).

References:

1. Abbas, A.K., A.H. Lichtman, and S. Pillai, *Cellular and molecular immunology*. 2007, Philadelphia: Saunders Elsevier.
2. Janeway, C., *Immunobiology : the immune system in health and disease*. 2005, New York: Garland Science.
3. Elgueta, R., et al., *Molecular mechanism and function of CD40/CD40L engagement in the immune system.* Immunol Rev, 2009. 229(1): p. 152-72.
4. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. 7: p. 145-73.
5. Finkelman, F.D., et al., *Lymphokine Control of in Vivo Immunoglobulin Isotype Selection.* Annual Review of Immunology, 1990. 8(1): p. 303-333.
6. Nimmerjahn, F. and J.V. Ravetch, *Divergent Immunoglobulin G Subclass Activity Through Selective Fc Receptor Binding.* Science, 2005. 310(5753): p. 1510-1512.
7. Kurup, V.P., et al., *Anti-interleukin (IL)-4 and -IL-5 antibodies downregulate IgE and eosinophilia in mice exposed to Aspergillus antigens.* Allergy, 1997. 52(12): p. 1215-21.
8. Murphy, K., et al., *Janeway's immunobiology*. 2012, New York: Garland Science.
9. Corthay, A., *A three-cell model for activation of naive T helper cells.* Scand J Immunol, 2006. 64(2): p. 93-6.
10. Schellekens, H., *Factors influencing the immunogenicity of therapeutic proteins.* Nephrology Dialysis Transplantation, 2005. 20(suppl 6): p. vi3-vi9.
11. Aimanianda, V., et al., *Novel cellular and molecular mechanisms of induction of immune responses by aluminum adjuvants.* Trends Pharmacol Sci, 2009. 30(6): p. 287-95.
12. Coffman, R.L., A. Sher, and R.A. Seder, *Vaccine Adjuvants: Putting Innate Immunity to Work.* Immunity, 2010. 33(4): p. 492-503.
13. Beran, J., *Safety and immunogenicity of a new hepatitis B vaccine for the protection of patients with renal insufficiency including pre-haemodialysis and haemodialysis patients.* Expert Opinion on Biological Therapy, 2008. 8(2): p. 235-247.

14. Boyle, J., et al., *The utility of ISCOMATRIX™ adjuvant for dose reduction of antigen for vaccines requiring antibody responses.* Vaccine, 2007. 25(14): p. 2541-2544.
15. Schwarz, T.F., et al., *Single dose vaccination with AS03-adjuvanted H5N1 vaccines in a randomized trial induces strong and broad immune responsiveness to booster vaccination in adults.* Vaccine, 2009. 27(45): p. 6284-6290.
16. MacLeod, M.K.L., et al., *Vaccine adjuvants aluminum and monophosphoryl lipid A provide distinct signals to generate protective cytotoxic memory CD8 T cells.* Proceedings of the National Academy of Sciences, 2011. 108(19): p. 7914-7919.
17. Dearman, R.J. and I. Kimber, *Animal models of protein allergenicity: potential benefits, pitfalls and challenges.* Clinical & Experimental Allergy, 2009. 39(4): p. 458-468.
18. Rhoads, C.P., *Immunization with Aluminium Hydroxide Mixtures of Poliomyelitis Virus.* Science, 1930. 72(1876): p. 608.
19. Glenny, A., *The antigenic value of toxoid precipitated by potassium alum.* J. Pathol. Bacteriol., 1926. 29: p. 38-45.
20. Calabro, S., et al., *Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes.* Vaccine, 2011. 29(9): p. 1812-23.
21. Flach, T.L., et al., *Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity.* Nat Med, 2011. 17(4): p. 479-87.
22. Franchi, L. and G. Nunez, *The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity.* Eur J Immunol, 2008. 38(8): p. 2085-9.
23. Kool, M., et al., *Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome.* J Immunol, 2008. 181(6): p. 3755-9.
24. Eisenbarth, S.C., et al., *Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants.* Nature, 2008. 453(7198): p. 1122-6.
25. Li, H., et al., *Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3.* J Immunol, 2008. 181(1): p. 17-21.

26. McKee, A.S., et al., *Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity.* J Immunol, 2009. 183(7): p. 4403-14.
27. von Pirquet, C., *Allergie.* Münchner Medizinische Wochenschrift, 1906. 53: p. 1457-1458.
28. Valenta, R., et al., *From allergen genes to allergy vaccines.* Annu Rev Immunol, 2010. 28: p. 211-41.
29. Huby, R.D.J., R.J. Dearman, and I. Kimber, *Why Are Some Proteins Allergens?* Toxicological Sciences, 2000. 55(2): p. 235-246.
30. Trompette, A., et al., *Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein.* Nature, 2009. 457(7229): p. 585-8.
31. Larche, M., C.A. Akdis, and R. Valenta, *Immunological mechanisms of allergen-specific immunotherapy.* Nat Rev Immunol, 2006. 6(10): p. 761-771.
32. Valenta, R., *The future of antigen-specific immunotherapy of allergy.* Nat Rev Immunol, 2002. 2(6): p. 446-53.
33. Lanzavecchia, A., *Antigen uptake and accumulation in antigen-specific B cells.* Immunol Rev, 1987. 99: p. 39-51.
34. Romagnani, S., *Human TH1 and TH2 subsets: doubt no more.* Immunol Today, 1991. 12(8): p. 256-7.
35. Vercelli, D. and R.S. Geha, *Regulation of isotype switching.* Curr Opin Immunol, 1992. 4(6): p. 794-7.
36. Dolecek, C., et al., *Effects of IL-4 and IL-13 on total and allergen specific IgE production by cultured PBMC from allergic patients determined with recombinant pollen allergens.* Clinical & Experimental Allergy, 1995. 25(9): p. 879-889.
37. Linhart, B., et al., *Costimulation blockade inhibits allergic sensitization but does not affect established allergy in a murine model of grass pollen allergy.* J Immunol, 2007. 178(6): p. 3924-31.
38. Noon, L., *PROPHYLACTIC INOCULATION AGAINST HAY FEVER.* The Lancet, 1911. 177(4580): p. 1572-1573.
39. Valenta, R. and V. Niederberger, *Recombinant allergens for immunotherapy.* Journal of Allergy and Clinical Immunology, 2007. 119(4): p. 826-830.

40. Niederberger, V., et al., *Vaccination with genetically engineered allergens prevents progression of allergic disease.* Proc Natl Acad Sci U S A, 2004. 101 Suppl 2: p. 14677-82.
41. Valenta, R., et al., *Synthetic and genetically engineered allergen derivatives for specific immunotherapy of type I allergy.* Clin Allergy Immunol, 2002. 16: p. 495-517.
42. Game, D.S. and R.I. Lechler, *Pathways of allorecognition: implications for transplantation tolerance.* Transplant Immunology, 2002. 10(2–3): p. 101-108.
43. Gould, D.S. and H. Auchincloss Jr, *Direct and indirect recognition: the role of MHC antigens in graft rejection.* Immunology Today, 1999. 20(2): p. 77-82.
44. Simpson, E. and D. Roopenian, *Minor histocompatibility antigens.* Current Opinion in Immunology, 1997. 9(5): p. 655-661.
45. Snell, G., *Methods for the study of histocompatibility genes.* Journal of Genetics, 1948. 49(2): p. 87-108.
46. Perreault, C., et al., *Minor histocompatibility antigens.* Blood, 1990. 76(7): p. 1269-80.
47. Spencer, C.T., et al., *Minor histocompatibility antigens: presentation principles, recognition logic and the potential for a healing hand.* Curr Opin Organ Transplant, 2010. 15(4): p. 512-525 10.1097/MOT.0b013e32833c1552.
48. Zhang, Q. and E.F. Reed, *Non-MHC antigenic targets of the humoral immune response in transplantation.* Current Opinion in Immunology, 2010. 22(5): p. 682-688.
49. Montgomery, R.A., et al., *Humoral immunity and antibody-mediated rejection in solid organ transplantation.* Seminars in Immunology, 2011. 23(4): p. 224-234.
50. Owen, R.D., *Immunogenetic consequences of vascular anastomoses between bovine twins.* Science, 1945. 102: p. 400-401.
51. Wekerle, T., et al., *Mechanisms of tolerance induction through the transplantation of donor hematopoietic stem cells: central versus peripheral tolerance.* Transplantation, 2003. 75(9 Suppl): p. 21S-25S.
52. Pilat, N. and T. Wekerle, *Transplantation tolerance through mixed chimerism.* Nat Rev Nephrol, 2010. 6(10): p. 594-605.

53. Kawai, T., et al., *Long-term outcome and alloantibody production in a non-myeloablative regimen for induction of renal allograft tolerance.* Transplantation, 1999. 68(11): p. 1767-75.
54. Kawai, T., et al., *HLA-mismatched renal transplantation without maintenance immunosuppression.* N Engl J Med, 2008. 358(4): p. 353-61.
55. Leventhal, J., et al., *Chimerism and Tolerance Without GVHD or Engraftment Syndrome in HLA-Mismatched Combined Kidney and Hematopoietic Stem Cell Transplantation.* Sci Transl Med, 2012. 4(124): p. 124ra28.
56. Baranyi, U., et al., *Cell-based therapy in allergy.* Curr Top Microbiol Immunol, 2011. 820: p. 161-179.
57. Baranyi, U., et al., *A chimerism-based approach to induce tolerance in IgE-mediated allergy.* Crit Rev Immunol, 2009. 29(5): p. 379-97.
58. Copelan, E.A., *Hematopoietic stem-cell transplantation.* N Engl J Med, 2006. 345(17): p. 1813-1826.
59. Bagley, J., et al., *Induction of T-cell tolerance to an MHC class I alloantigen by gene therapy.* Blood, 2002. 99(12): p. 4394-9.
60. Eixarch, H., et al., *Tolerance induction in experimental autoimmune encephalomyelitis using non-myeloablative hematopoietic gene therapy with autoantigen.* Mol Ther, 2009. 17(5): p. 897-905.
61. Tian, C., et al., *Prevention of type 1 diabetes by gene therapy.* J Clin Invest, 2004. 114(7): p. 969-78.
62. Vrtala, S., et al., *Immunologic characterization of purified recombinant timothy grass pollen (Phleum pratense) allergens (Phl p 1, Phl p 2, Phl p 5).* Journal of Allergy and Clinical Immunology, 1996. 97(3): p. 781-787.
63. Baranyi, U., et al., *Tolerization of a type I allergic immune response through transplantation of genetically modified hematopoietic stem cells.* J Immunol, 2008. 180(12): p. 8168-75.
64. Hughes, E.N. and J.T. August, *Characterization of plasma membrane proteins identified by monoclonal antibodies.* J Biol Chem, 1981. 256(2): p. 664-71.
65. Petersen, A., W.M. Becker, and M. Schlaak, *Epitope analysis of isoforms of the major allergen Phl p V by fingerprinting and microsequencing.* Clinical & Experimental Allergy, 1994. 24(3): p. 250-256.
66. Hoffmann, A., et al., *Determination of the allergenic activity of birch pollen and apple prick test solutions by measurement of beta-hexosaminidase release*

from RBL-2H3 cells. Comparison with classical methods in allergen standardization. Allergy, 1999. 54(5): p. 446-54.

67. Donovan, J.C. and P. Brown, *Care and Handling of Laboratory Animals*, in *Current Protocols in Immunology*. 2001, John Wiley & Sons, Inc.
68. Hurme, M., et al., *Cytotoxic T-cell responses to H-Y: correlation with the rejection of syngeneic male skin grafts.* J Exp Med, 1978. 147(3): p. 768-75.
69. Ehst, B.D., E. Ingulli, and M.K. Jenkins, *Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection.* Am J Transplant, 2003. 3(11): p. 1355-62.
70. Erazo, A., et al., *Unique maturation program of the IgE response in vivo.* Immunity, 2007. 26(2): p. 191-203.
71. Farges, O., et al., *Anti-donor antibody class switching after liver transplantation.* Transplantation, 1995. 60(3): p. 296-300.
72. Lappin, M.B., et al., *Analysis of mouse dendritic cell migration in vivo upon subcutaneous and intravenous injection.* Immunology, 1999. 98(2): p. 181-8.
73. Shreffler, W.G., et al., *The Major Glycoprotein Allergen from Arachis hypogaea, Ara h 1, Is a Ligand of Dendritic Cell-Specific ICAM-Grabbing Nonintegrin and Acts as a Th2 Adjuvant In Vitro.* The Journal of Immunology, 2006. 177(6): p. 3677-3685.

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihr Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

Appendix:

Curriculum vitae:

Curriculum vitae

Farkas, Andreas

Personal data:

Date of birth: March 25th, 1987
Place of birth: Salzburg, Austria
Nationality: Austria

Professional experience:

2010 – present	Tutor Vienna Open Lab, dialog<>gentechnik Campus Vienna Biocenter 5 Ebene 1, 1030 Vienna, Austria

Education/University:

2001 – 2005	Borg-Nonntal, Salzburg
June, 2005	A-level degree final examination “Matura”
2005 – 2006	Studies of Information Technology and Systems Management at the University of Applied Sciences Salzburg
2006 – 2009	Bachelors course in Genetics at the University of Salzburg **Graduation:** 28.9.2009
2008 – 2011	Bachelors course in Japanese Studies at the University of Vienna
2009 – present	Masters course in Molecular Microbiology and Immunology at the University of Vienna
2010 – 2012	Master student at the Department of Surgery, Medical University of Vienna, group of Prof. Thomas Wekerle, MD

Memberships:
Member of the Austrian Society for Allergology and Immunology (ÖGAI)
Member of the Austrian Society for Transplantation

Abstracts:

Baranyi, U., M. Gattringer, A. Farkas, K. Hock, N. Pilat, J. Iacomini, R. Valenta and T. Wekerle. Introduction of cytoplasmically expressed Phl p 5 into hematopoietic stem cells induces molecular chimerism in a murine model in IgE mediated allergy. EAACI, Istanbul, Turkey, 2011. *Poster presentation*

Baranyi, U., M. Gattringer, A. Farkas, K. Hock, N. Pilat, B. Linhart, J. Iacomini, R. Valenta and T. Wekerle. The influence of expression site and chimerism level on B cell tolerance towards an allergen induced through molecular chimerism. Annual Meeting of the Austrian Society for Allergology and Immunology, Graz 2011. Oral presentation

Baranyi, U., M. Gattringer, A. Farkas, K. Hock, N. Pilat, B. Linhart, J. Iacomini, R. Valenta and T. Wekerle. The degree of tolerance towards an allergen induced through molecular chimerism depends on the site of antigen expression. 2nd International Conference on Immune Tolerance, Amsterdam, Netherlands, 2011. Poster presentation

Baranyi, U., A. Farkas, R. Valenta and T. Wekerle. Transplantation of a minor-mismatched skin graft induces a rapid humoral response including the production of antigen-specific IgE. 2nd International Conference on Immune Tolerance Amsterdam, Netherlands, 2011. Poster presentation

Farkas, A., U. Baranyi, R. Valenta and T. Wekerle. The major allergen Phl p 5 shows an unexpectedly high immunogenicity as membrane-bound transgene. Annual Meeting of the Austrian Society for Allergology and Immunology, Graz 2011. *Poster presentation*

Farkas, A., U. Baranyi, R. Valenta and T. Wekerle. Transplantation of a minor-mismatched skin graft elicits a rapid humoral response including the induction of antigen-specific IgE. Austrotransplant, Graz, Austria 2011. *Nominated for the Young Investigators Award*

Farkas, A., U. Baranyi, R. Valenta and T. Wekerle. High immunogenicity of the allergen Phl p 5 expressed in transgenic mice as a membrane-anchored protein. World Allergy Congress 2011, Cancun, Mexiko. *Oral presentation*

Acknowledgments:

I would like to express my deepest gratitude to all those who gave me the opportunity to work on this thesis. Especially I would like to thank Prof. Thomas Wekerle for giving the chance to join his group and for all his scientific support. I am also deeply grateful for the support I received by Dr. Ulrike Baranyi and my colleagues.

This project was done in collaboration with Prof. Rudolf Valenta, and of course I would like to thank him for his advices and the scientific support.

Furthermore I would like to thank Karl Emory Bauer and Verena Hameter for proofreading this thesis.

Besides the professional support, I would like to thank my family especially my parents for their extraordinary backing regarding my education. I would also like to thank all my friends for aiding me in a non-scientific way.