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DISSERTATION

Prevention of IgE-Mediated Allergy by Molecular Chimerism and Costimulation Blockade in a Murine Model

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1 Abstract

IgE-mediated allergy is a hypersensitivity disorder whose prevalence is still increasing. IgE is produced by plasma cells after class switching which requires T cell help, costimulation and T_H2 cytokines. To date, immunotherapy is the only causative treatment but, next to limited effectiveness, it harbors risks such as anaphylaxis or additional sensitization. Therefore, development of strategies for tolerance induction towards allergens remains a desirable goal. Here we examined costimulation blockade and molecular chimerism as two potential solutions.

Costimulation blockade prolongs graft survival in transplantation but its potential in the treatment of allergy still has to be fully investigated. Recently, OX40L was suggested to play an important role in T_H2-mediated and memory T cell responses. We therefore analyzed the tolerizing potential of blocking OX40L alone or in addition to CD28 and CD40L blockade in a well-described model of IgE-mediated allergy using clinically relevant major allergens. Blocking OX40L alone had no therapeutic effect. Combination of anti-CD40L/CTLA4Ig significantly delayed production of allergen-specific antibodies. T cell response was suppressed even after established antibody production. Additional anti-OX40L treatment to anti-CD40L/CTLA4Ig showed no synergistic effect.

In the second part of my work we examined molecular chimerism as a promising concept in tolerance induction, which is brought about through the transplantation of autologous hematopoietic cells genetically modified *in vitro* to express a disease-causing antigen. Recently, our group has published a proof of principle study showing robust long-term tolerance towards the grass pollen allergen, Phl p 5, through molecular chimerism. Since allergens are structurally and functionally different, we investigated whether molecular chimerism establishes tolerance towards the major birch pollen allergen, Bet v 1. VSV-Bet

v 1-GFP transduced BMC were transplanted into preconditioned recipients. Engraftment of allergen-expressing stem cells was supported by inhibition of CD26 and tracked by the reportergene GFP. Molecular chimerism was detectable throughout long-term follow-up, preventing sensitization even after repeated challenges with Bet v 1. Production of Bet v 1-specific antibodies was completely abrogated throughout follow-up. Moreover, Bet v 1-specific T cell proliferation was suppressed.

Our findings show that combined costimulation blockade including OX40L blockade at the time of immunization with an allergen delays but does not prevent the humoral allergic response. In contrast, molecular chimerism induced robust long-term tolerance. Chimerism is a promising strategy to achieve life-long tolerance on the B-, T cell and effector cell levels towards a wide range of allergens.

2 Zusammenfassung

Die IgE–mediierte oder Soforttyp Allergie ist eine Erkrankung mit steigender Tendenz. Zurzeit ist die einzige kausale Therapieform die spezifische Immunotherapie. Allerdings bestehen auch Risiken, wie etwa Neusensibilisierungen oder Anaphylaxie. Stabile und lebenslange Toleranzinduktion wäre eine ideale Prophylaxe und Therapie. Um dieses Ziel zu erreichen wurden zwei verschiedene Ansätze untersucht, Kostimulationsblockade und molekularer Chimärismus.

Blockade von Kostimulationssignalen mittels spezifischer Antikörper hat in der Transplantation zu vielversprechenden Ergebnissen geführt. Diese Arbeit setzt sich mit der Kostimulationsblockade in der Allergieprävention und Therapie auseinander. Bei der T_H2 Immunantwort werden unter anderem die Zahl und Funktion der Gedächtniszellen durch das Kostimulationsmolekül OX40L reguliert. Welche Rolle OX40L im murinen Allergie-Modell spielt, wurde durch Blockierung dieser Interaktion analysiert. Es wurde auch eine mögliche synergistische Funktion von OX40L mit, CD28 (CTLA4Ig) und CD40L (anti-CD40L) untersucht.

Blockierung von OX40L hatte keinen Einfluss auf die allergen-spezifischen Antikörperproduktion oder die T-Zell Antwort. Wogegen die Kombination von anti-CD40L/CTLA4Ig zu einer supprimierten T Zell Antwort und signifikant verzögerter humorale allergische Sensibilisierung führte.

Eine andere Strategie, Toleranz zu induzieren ist der molekulare Chiärismus. Molekularer Chimärismus wurde schon erfolgreich in einem Allergie-Mausmodell mit dem Allergen Phl p 5 gezeigt. Da Allergene strukturell und funktionell unterschiedliche Proteine sind, wurde diese Methode mit Bet v 1, dem Hauptbirkenpollenallergen, umgesetzt. Ein Bet v 1-GFP Konstrukt wurde in hämatopoetische Stammzellen eingeschleust und anschließend in

vorbehandelte, syngene Mäuse transplantiert. Durch erfolgreiches Engraftment von allergen-exprimiernden Blutzellen im Empfänger wurde Langzeittoleranz gegen dieses Allergen erzielt. Die behandelten Mäuse waren tolerant gegen wiederholte Immunisierungen. Über den gesamten Verlauf entwickelte sich keine allergen-spezifische T-Zell Antwort und auch kein allergen-spezifisches IgE. Darüber hinaus konnte keine Effektorzellaktivität durch das Serum induziert werden.

Diese Ergebnisse zeigen, dass OX40L-Blockade keine Auswirkung auf die T_H2 Antwort in unserem Modell hat. Wogegen die Blockade von CD40L/CD28 zu einer stark verzögerten Immunantwort führt. Stabile Toleranz auf zellulärer und humoraler Ebene gegenüber einem Allergen konnte nur mit molekularem Chimärismus erreicht werden. Es konnte gezeigt werden, dass molekularer Chimärismus in der Allergie auch mit strukturell und funktional unterschiedlichen Allergenen erfolgreich angewendet werden kann.

3 Introduction

3.1 IgE-Mediated Allergy (Type I Allergy)

Hypersensitivity is a term which describes disorders caused by immune responses. Four different types of hypersensitivity are classified by Coombs and Gell according to the effector mechanisms of the immune response. The first type is the immediate type hypersensitivity, or IgE-mediated hypersensitivity. Its hallmark is the production of antigen-specific immunoglobulin E (1). IgE binds to the surface of mast cells and basophils, this leads to the release of a variety of pharmacoactive inflammatory mediators. These mediators cause the typical allergic symptoms which appear as local allergic rhinitis, allergic asthma, ocular allergy, allergic skin inflammation or, in worse cases, in systemic anaphylaxis (2). More than 20% of the population are abnormally responsive to allergens. Immediate type hypersensitivity is described in more detail later. The second type of hypersensitivity is antibody mediated (IgG, IgM) and results in complement activation and recruitment of inflammatory cells. The third type is caused by immune complexes of circulating antibodies (IgM, IgG), also recruiting the complement system. The fourth and last type is mediated by T cells. T lymphocytes kill target cells or activate effector mechanism of delayed type hypersensitivity (3).

The present thesis focuses on the immediate type hypersensitivity or IgE-mediated allergy.

3.1.1 Allergens

Type I allergy is provoked by a single antigen, an allergen. Allergens are common environmental proteins and chemicals. They do not stimulate the innate immune response, which would lead to a T_H1 response. There is as yet no known common structure or

function which is characteristic for allergens. Many allergens have low molecular weight, glycosylation and are highly soluble in body fluids. Some of them are enzymes like phospholipase A (bee venom) and the cysteine protease (house dust mite). Allergens provoke an immune response through repeated exposure. Healthy individuals can also produce allergen-specific antibodies but they develop IgM or IgG and only low levels of IgE. Therefore, they do not develop the typical allergic symptoms common in atopic patients (3).

3.1.2 Atopy

Atopy is the genetic predisposition to develop IgE-mediated allergy. The greatest susceptibility is given when hereditary and environmental factors come together. Susceptibility for the development of allergy and asthma were found together with genes triggering the immune system and directing to $CD4^+$ cell differentiation (e.g. TLR2, TLR4, STAT3) and regulation of T_H2-cell differentiation and effector function (e.g. GATA3, IL-4, IL-13, IL-5). Moreover, susceptibility to asthma was found in genes associated with mucosal immunity, epithelial biology and lung function (4). HLA genes might also play an important role in susceptibility to allergy. Increased IgE responses are associated with HLA-DR, -DQ and -DP polymorphism. Polymorphic residues are located within or next to the antigen-binding cleft, contributing to antigen presentation and T cell recognition. However, expression of the disease-associated HLA does not necessarily cause allergy because it is designed by several factors (4).

Interestingly, $CD4^+$ cells of healthy and atopic individuals recognize the same T-cell epitopes. Both develop allergen-specific T_H1 cells, T_H2 cells and IL-10 secreting T regulatory cells (T_R1). It is the balance of the different T cell subsets which causes the

allergic phenotype. An atopic individual would display a dominant T_H2 cell response, secretion of IL-4 and suppression of regulatory cells (5) and therefore develop allergy.

3.1.3 Sensitization Phase and Memory Induction

In order to provide a short overview of the development of IgE-mediated allergy, the allergic disease can be divided into sensitization phase and memory induction, immediate phase type 1 reaction and late phase allergic inflammation (6).

Sensitization to an allergen occurs after the first contact. The antigen enters through the epithelia. Dendritic cells capture and process the antigen and transport it to the draining lymph node. As the allergen is brought in from extracellular space it is presented by MHC class II molecule to naive T cells. Primed allergen-specific T_H2 cells release IL-4 and IL-13, leading to IgE class switch in B cells. B cells require the costimulatory signal of CD40 and IL-4 for heavy chain isotype switching. Moreover, the T_H2-produced cytokines IL-4, IL-5, IL-9 and IL-13 mediate development, survival and recruitment of eosinophils and mast cells, and lead to hypersecretion of mucus.

IgE circulates as a bivalent antibody and binds to the high affinity FcεRI receptor on mast cells and basophils. IgE also binds to a lower affinity FcεRII (CD23) at the surface of B cells. Binding of IgE to FcεRI/II amplify the process of allergen uptake by APC and presentation of peptides to $CD4^+$ cells. B cells undergo differentiation and secrete IgE as plasma cells (Fig. 1) (6).

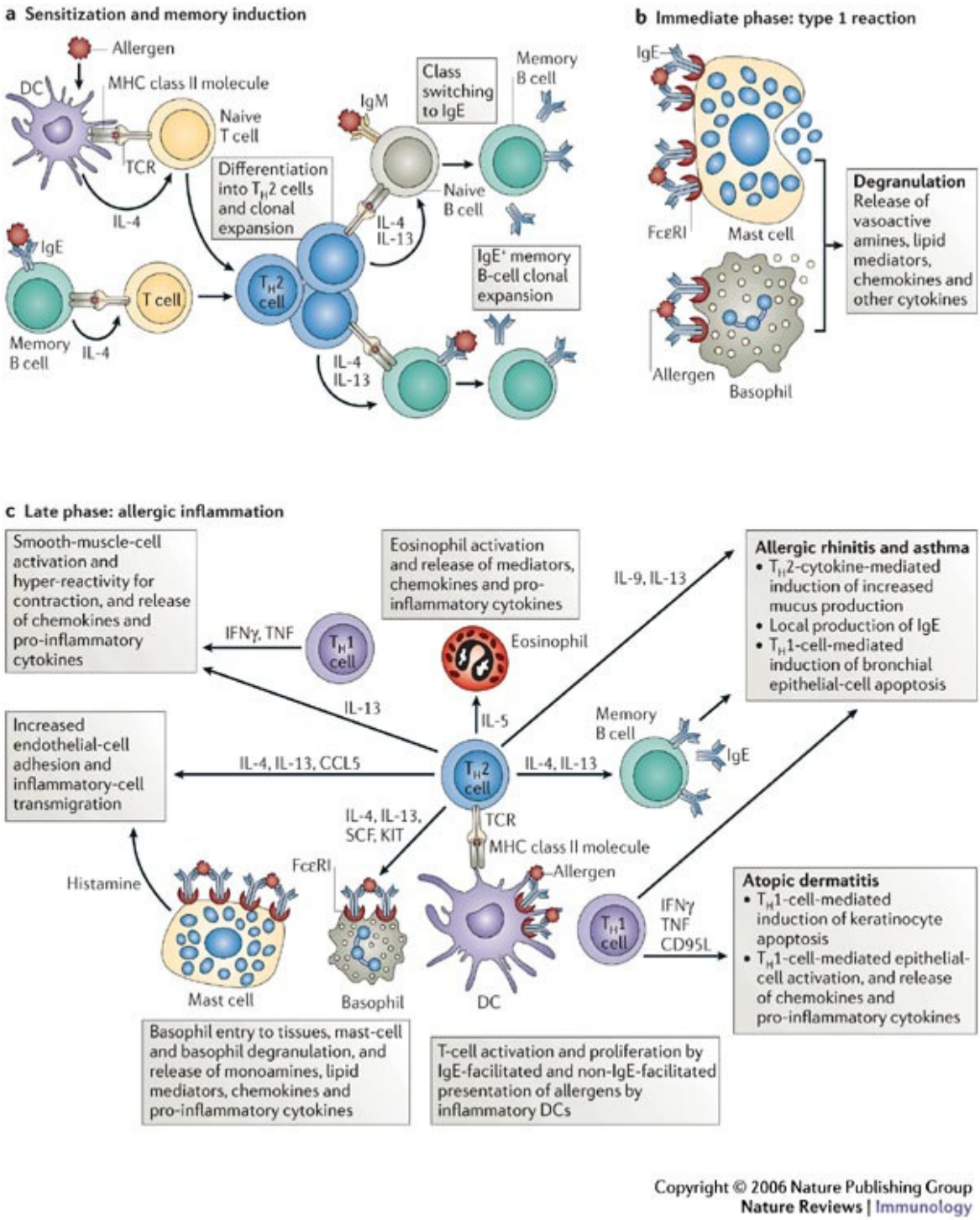

Figure 1: Mechanism of allergic reactions (6)

3.1.4 Immediate Phase Reaction

Allergen-specific IgE attaches to FcεRI/II on mast cell, eosinophils and basophils. Crosslinking of the bound IgE by an allergen leads to release of vasoactive substances, lipid mediators, chemokines and various cytokines.

Mast cells, basophils and eosinophils are essential elements of immediate hypersensitivity. These cells contain cytoplasmic granules (e.g. histamine, serine poteases), lipid mediators (e.g. prostaglandine D2, leukotriene) and cytokines (e.g. IL-4, IL-5, TNF-α) that induce inflammation (2).

Mast cells are derived from progenitors in the bone marrow. Immature mast cells migrate to the peripheral tissue and undergo differentiation. The typical T_H2 cytokines IL-4 and IL-5 upregulate expression of FcεRI and promote proliferation. Mast cells contribute to the immediate phase reaction as well as to late-phase reaction.

Basophils are circulating granulocytes; they represent a separate lineage. Basophils express FcεRI and FcεRII. They produce leukotriens and histamine like mast cells, but no prostaglandin or IL-5. Recruitment to inflammatory sites contributes to the immediate hypersensitivity by release of IL-4 and IL-13.

T_H2 cells also secrete IL-5, IL-9 and IL-13, increasing mucus production and activating eosinophils. IL-5 recruits eosinophils to allergic sites of inflammation. Differentiation and proliferation of eosinophils is also mediated by IL-5 (Fig. 1) (2).

3.1.5 Late Phase of the Allergic Reaction

The late phase reaction of immediate hypersensitivity starts about four hours after allergen exposure and lasts up to 48 hours. Late phase reaction is manifested by the production of IL-4, IL-5, IL-9 and IL-13 by allergen-specific T cells, survival and recruitment of eosinophils, differentiation of mast cells and hypersecretion of mucus. Production of IFNγ and tumor necrosis factor together with CD95 ligand (Fas ligand) on T_H1 cells lead to apoptosis of epithelial cells and impaired barrier function (Fig. 1) (6).

3.2 Current Therapies of IgE-Mediated Allergy

3.2.1 Treatment of Symptoms

The typical allergic symptoms induced by inhalation of allergens such as plant pollen and house dust mite are manifested in the upper respiratory tract. The symptoms appear as allergic rhinitis, coughing, sneezing, difficulty in breathing and conjunctivitis. They are most commonly medicated with anti-histamines. Ingested allergens lead to food allergies causing vomiting, diarrhoea and enhanced peristalsis. Food allergens such as peanuts and shellfish can trigger severe systemic reactions even in very small quantities. Urticaria, a symptom of the skin, is also often associated with food allergy. These symptoms are also medicated with anti-histamines.

In worse cases, a systemic response can lead to anaphylaxis. Life threatening systemic anaphylaxis is characterized by fall in blood pressure caused by vasodilatation and can be treated with epinephrine, glucocorticoids and antihistamines (3).

Repeated immediate hypersensitivity and late phase reactions can induce bronchial asthma. The bronchial inflammation is characterized by eosinophil infiltration, reversible airway obstruction and bronchoconstriction. The most important bronchoconstricting mediators are leukotriens C_4 and its breakdown products. The treatment aims to reverse inflammation, e.g. with corticosteroids and relaxation of airway smooth muscles. Leukotriene inhibitors block the bronchoconstricting effect.

3.2.2 Allergen-Specific Immunotherapy (SIT)

Allergen-specific immunotherapy is the only causative and antigen-specific approach currently available for the treatment of allergy. The curative characteristics of low dose allergen-application were discovered about a hundred years ago. SIT consists in the

repeated application of the sensitizing allergen in increasing doses, modulating the immune response towards the allergen. The ratio of T_H1 cells to T_H2 cells is renewed to favour T_H1 cytokines.

SIT alters the response of APC, T cells, B cells and the number and function of effector cells. APC, especially dendritic cells play a major role in tolerance induction. Immature dendritic cells mediate tolerance when they encounter and present an antigen in the absence of a danger signal. Repeated stimulation of T cells with immature DC lead to the generation of a regulatory cell population, the T_R1 cells. These cells secrete the immunodulatory cytokine IL-10. IL-10 production is also increased in APC, monocytes and macrophages. IL-10 mediate T cell anergy, increase regulatory cells and cytokines like TGFβ. IL-10 also modulates the function of effector cells by inhibiting further activation and production of IL-5 and IgE. Following SIT, production of allergen specific IgG increase. Induced IgG compete with IgE, if the same epitopes are recognized and therefore reduce IgE-mediated degranulation of mast cells and basophils. Moreover allergen presentation to T cells over IgE is decreased, so IgE producing B cells lose their signals for affinity maturation. In human patients SIT mainly results in production of IgG1 and IgG4. IgG4 does not induce the complement cascade and has anti-inflammatory characteristics (6).

Nowadays SIT is optimized by different protocols such as routes of administration. Routes of administration can be subcutaneous injection, sublingual, epicutaneous or, in recent protocols intralymphatic (7-8).

Furthermore, modifications of SIT, such as the replacement of allergen extracts with recombinant allergens are also under investigation. Currently, allergen extracts are widely used, although these extracts can cause various side effects. In contrast, recombinant allergens allow standardization of the amount and quality of the allergen. Thus, the

occurrence of other allergens, which harbour the risk of additional sensitization, can be avoided in the extracts. A number of studies exist which investigate modification of recombinant allergens. This technique would allow the reduction of allergenicity by changing IgE epitopes. Allergens can be optimized in the laboratory for optimal efficiency in SIT with minimal risk of side effects.

In a clinical study, hypoallergenic derivates of the major birch pollen allergen, Bet v 1, were used for treatment. The derivates were designed to exhibit reduced allergenic activity. Treatment led to enhanced allergen-specific IgG production in patients. Administration of the genetically engineered allergen diminished production of IgE towards the natural allergen during the birch pollen season (9).

Another strategy to reduce IgE-mediated side effects of SIT is the treatment with T cell peptides. T cell peptides represent only fragments of the native allergen and therefore reduced allergenicity. Treatment was shown to decrease systemic T_H1 and T_H2 cells, induce regulatory cells and production of IL-10.

First clinical trials with the cat allergen Fel d 1 were accompanied by frequently adverse effects caused by T cell recognition, however clinical efficacy was observed. The adverse effects were diminished through the use of a peptide mix with shorter peptides. Again a significant improvement of disease could be detected after challenge with cat dander allergen extracts. The protocol was optimized and a peptide vaccine was developed which consists of seven different peptides. The new vaccine was well tolerated and is under further investigation (10).

3.2.3 Future Perspectives for Treatment and Prevention

To investigate further potential strategies for causative treatment of allergen we translated two promising protocols from transplantation to IgE-mediated allergy, chimerism and costimulation blockade. The following studies examine mainly preventive approaches.

In transplantation and autoimmunity, successful therapies were achieved by blockade of costimulatory molecules, which contribute to full activation, B cell class switch and tolerance induction. Manipulation of these interactions has the potential to alter the immune response and induce non-responsiveness towards antigens. Costimulatory molecules, their families and how they contribute to a T_H2 response are briefly described below.

Induction of tolerance by chimerism has achieved promising results in transplantation and autoimmune diseases. This protocol is able to provide stable tolerance without the need of constant medication. An overview of chimerism is given in the next chapter where the potential of molecular chimerism in allergy is discussed.

3.3 Costimulatory Molecules in Allergy

Full T cell activation needs two independent signals, the T cell receptor stimulation by antigen-MHC complex and a second interaction by costimulatory molecules. Signal transduction needs the interaction of ligands to membrane bound molecules. Costimulatory molecules can provide activating and inhibitory signals. Recently, many new costimulatory molecules have been described and the two signal model has become more complex. Costimulatory molecules can be divided into groups according to their structure, like B7:CD28 superfamily and TNF/TNFR family.

3.3.1 CD28 Superfamily

The CD28 superfamily consists of seven structurally related ligands: CD80 (B7.1), CD86 (B7.2) – both binding to CD28 and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) furthermore, the programmed death-1/2 ligands (PD-L1, PD-L2) which bind to PD-1, the inducible costimulatory molecule ligand (ICOS-L) binding ICOS and lastly B7-H3 and B7-H4, where the receptors have not yet been identified. Binding mediates costimulatory (CD28, ICOS) or coinhibitory (CTLA4, PD-1) signals (11, 3).

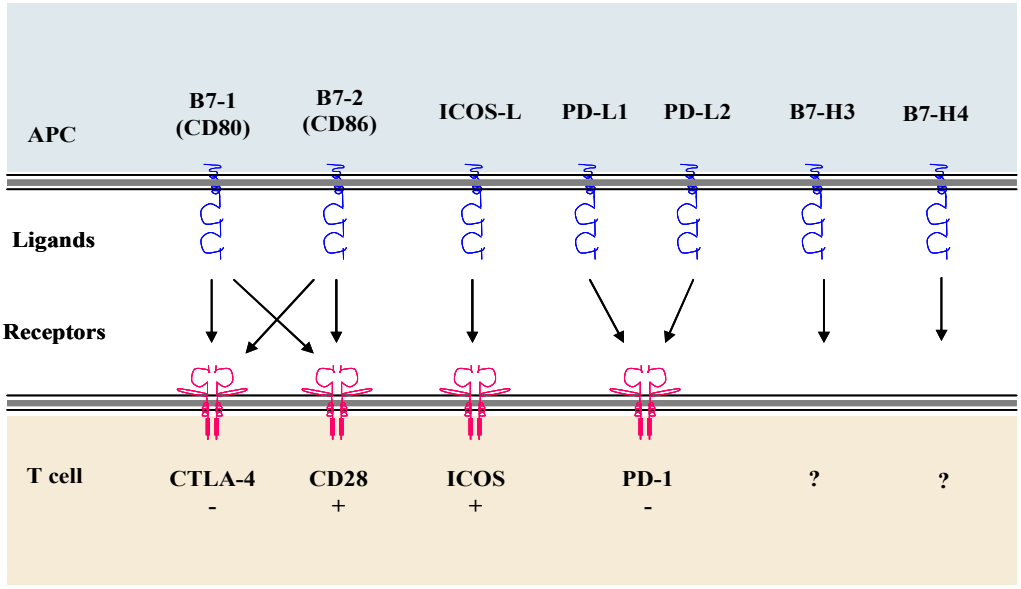

Figure 2: B7:CD28 family. Seven known B7 ligands are expressed on APCs. The ligands are transmembrane proteins, consisting of two Ig-like domains, V-like and C-like. Receptors are expressed on T cells. The transmembrane proteins harbour a single variable domain and a cytoplasmic tail.

CD80/CD86 is expressed on APC, DC, macrophages and B cells. Low levels of CD86 can be found on resting B cells, DC and macrophages. Activation of cells results in enhanced CD86 expression and *de novo* expression of CD80. CD80/CD86 deficient mice have profound deficits in humoral and cellular immune responses.

CD28 is constitutively expressed on T cells. CD28 mainly engages during initiation and amplification of the immune responses. Engagement enhances antigen-specific proliferation and cytokine production. TCR stimulation in the absence of CD28 signalling was initially shown by CD28 blockade *in vitro* and induces classical T cell anergy (11).

Binding of CD80/CD86 to CTLA4 (CD152) provides an inhibitory signal to T cells (12). CD80 binds with higher affinity to CTLA4 and CD86 to CD28. CTLA4 is a master switch for peripheral tolerance; it is upregulated upon activation. CTLA4 deficient mice develop multiple organ autoimmune pathologies and lymphoproliferative disease due to defects in immune regulation (12).

CD28-deficient mice are unable to express T_H2 cytokines or produce IgE and IgG1. The development of asthma was blocked and levels of IL-4, serum IgG1 and IgE were reduced. Interestingly, blockade of CTLA4 in CD28-deficient mice restores lung inflammation in a model of allergic airway inflammation. But the inflammation was qualitatively different as that in wild type mice, cell infiltrates consisted of lymphocytes and lack eosinophils; suggesting an inhibitory role of CTLA4 in this model (13).

3.3.2 TNF/TNFR – Family

The tumor necrosis factor family receptor (TNFR) consists of OX40 (CD134), CD40, 4-1BB (CD137), CD30 and CD27. These molecules are induced or upregulated on the T cell surface hours or days after antigen recognition. Signals seems to take part in controlling the ongoing immune response in respect of continued cell division, prevention of excessive cell death, number of effector T cells and proportion of memory cells.

3.3.2.1 OX40 - OX40 Ligand Pathway

OX40 (CD134) is transiently expressed after activation of naïve T cells starting after about 12 – 24 hours, peak of expression is reached after about 3 days. Memory and effector T cells are able to re-express OX40 4 hours after reactivation (14). OX40 Ligand (OX40L, gp34 or CD252) is expressed hours to days after activation on APC (dendritic cells, B cells, macrophages), endothelial cells, mast cells, activated NK cells and also on responding CD4 T cells themselves (15). OX40L expression can be triggered by CD40 stimulation (16) or thymic stromal lymphopoietin (TSLP) (17). The OX40 pathway plays a critical role in the late primary T cell immune response and also in the generation and survival of memory T cells. OX40 transmits an anti-apoptotic signal, Bcl-2, Bcl-xL and survivin, and prevents excessive T cell death (16).

OX40 deficient mice are severely impaired in their ability to generate a T_H2 response. In a model of OVA induced airway disease, OX40 deficient mice exhibited diminished lung inflammation, reduced mucus production and eosinophilia infiltration. Moreover the typical T_H2 cytokines IL-4, IL-5 were reduced in bronchoalveolar lavage, and IgE levels in serum were diminished (18).

Seshasayee et al. investigated the role of OX40 pathway in a mouse model by blocking OX40L or depleting OX40L positive cells. Lung inflammation was induced by intranasal TSLP and anti-OX40L antibody was administrated, DC activated by TSLP expressed OX40L. Treatment resulted in significantly reduced levels of T_H2 cytokines (IL-4, IL-5, IL-13), reduced numbers of lymphocytes in BAL and also IgE and IgG1 in serum. Furthermore, blockade of OX40L in allergic asthma in rhesus monkeys led to reduction of effector/memory CD4 T cells, IL-5 and IL-13 in the BALF (17).

The activating potential of OX40 has been described in several studies. OX40 antibody (OX86) has been observed to break peripheral tolerance when mice were immunized with

soluble peptide (19). Administration of OX86 can trigger an immune response towards tumor cells, which is possibly due to the abrogation of Treg function. OX86 treatment was even more effective than CD25 depletion in regard to anti-tumor response (20). Furthermore, breaking tolerance by LPS was shown to be dependent on OX40 interaction (21).

$FoxP3^+$ Tregs constitutively express OX40, OX40 has been shown to abrogate the suppressive effects (22). OX40 engagement inhibits TGFβ-mediated conversion of $CD4^+$ cells to FoxP3 regulatory cell (23).

3.3.2.2 CD40 – CD154 Pathway

CD40L (gp39, CD154) is expressed primarily on activated T cells but also on B cells and platelets. CD40 is constitutively expressed on B cells, macrophages and DCs and is upregulated upon activation (24). CD40/CD40L interaction is sufficient for T cell dependent immunoglobulin heavy chain class switching, development of memory B cells, formation of germinal centers, activation and maturation of DCs, upregulation of MHC molecules and costimulatory molecule and increased inflammatory cytokine production. The importance of CD40L in humoral immunity is highlighted in patients with hyper IgM X-linked syndrome. Patients have profound defects in the generation of T cell dependent antibodies and isotype class switching (25).

In allergy, CD40/CD40L interaction is fundamental for development of allergen-specific antibodies. The interaction is only necessary during allergic sensitization. The time from CD40L blockade to allergen challenge had no effect on the amount of allergen-specific antibodies. On the contrary, T cell response was diminished regardless of whether CD40L was blocked due to sensitization or challenge (26).

3.4 Tolerance Strategies in Allergy

3.4.1 Costimulation Blockade

As described above, many costimulatory molecules exist with different and partly overlapping functions. The molecules guide the specification, strength and duration of the immune response. More and more, costimulatory molecules offer the possibility of modulating the immune response.

3.4.1.1 Prominent costimulation blockades

OX40 is a promising target in the creation of new therapeutic strategies for allergic inflammation, autoimmune disease, graft-versus-host diseases and anti-tumor-treatment. OX40L blockade is currently being assessed in a clinical trial of allergen-induced airway obstruction in adults with mild allergic asthma (NCT00983658). Blockade of the OX40 pathway inhibits activated cells which would allow specific targeting of activated effector T cells. While some studies suggest that long-term application would be necessary in diseases such as EAE, this would entail an undesireable suppression towards other inflammatory processes. Triggering the OX40-OX40L interaction was shown to be successful in cancer treatment (27).

Also blocking CD40L is widely and effectively employed in animal models prolonging allograft survival and preventing acute rejection (28). In autoimmune disease, blockade of CD40 interaction can abrogate or suppress the disease, especially in diseases with fundamental preponderance of B cells like systemic lupus erythematosus and myasthenia gravis (29). In a murine allergy model, blockade of CD40L led to prevention of sensitization and suppressed T cell response (26).

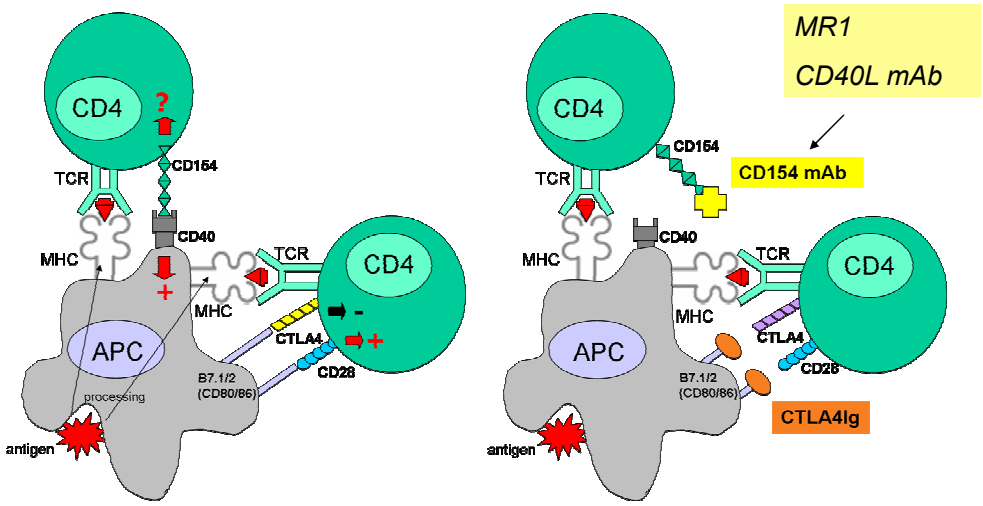

Figure 3: Schematic drawing of T cell–APC interaction with the costimulatory molecules CD40 and B7. Blockade of CD40-CD40L with anti-CD40L (MR1) prevents the activating signal. Blockade of B7 by CTLA4Ig prevents the interaction to CTLA4 (inhibitory signal) and CD28 (activating signal) (30-31).

Translation to human application is still an unmet goal. Human anti-CD154 antibodies were associated with severe thromboembolic complications in a clinical phase I trial, therefore, the clinical trial was put on hold, however, encouraging murine studies are ongoing (32). The adverse effect was caused by the function of CD40L in stabilizing thrombi. CD40L -/- mice exhibit unstable thrombi but exhibit a normal phenotype. Therefore investigations targeting CD40 are underway (24).

An outstanding way to block the CD28 pathway is through the chimeric protein CTLA4 immunoglobulin (CTLA4Ig). CTLA4Ig prevents the interaction of CD80/CD86 and CD28. Moreover, CTLA4Ig leads to a production of indolamine 2,3-dioxygenase (IDO) in APC which breaks down tryptophan, resulting in suppression of T cell proliferation and cell death.

In transplantation, blockade of CD28 signalling by CTLA4Ig was shown to prevent acute allograft rejection and induce donor-specific transplantation tolerance in certain strains (33). While the results in rodent models were very promising, the effect in non-human primates was moderate on allograft survival. CTLA4Ig, abatacept, was more effective in treatment of autoimmunity disease such as rheumatoid arthritis or psoriasis vulgaris. A second generation CTLA4Ig was developed (LEA29Y, belatacept) with a higher affinity to CD86 which has been successfully tested in a clinical phase III study (34).

The prominence of the CD28 pathway led to efforts in developing more specific treatment possibilities by targeting CD28 directly. However, a superagonistic CD28 antibody implementation had a fatal outcome in a clinical phase I trial. The antibody was considered for a possible treatment of chronic lymphocytic B cell leukemia. Six healthy volunteers experienced systemic inflammation following administration of the anti-CD28 antibody. Previously, the antibody had been successfully tested in non-human primates, the fatal consequences in humans could not have been predicted on the basis of this study (24, 35).

3.4.1.2 Synergy between CD40/CD28 and OX40

Blockade of a single costimulatory molecule may have a redundant function in the development of an immune response. Synergistic effects are often described. In the following section the synergy between CD40, CD28 and OX40 pathways are discussed, as it is these costimulatory molecules that are subject to the ensuing investigations.

In transplantation some models are resistant to the otherwise potent effect of CD28/CD40L blockade. Interestingly, OX40L was discovered to mediate CD28/CD40L independent rejection. In CD28/CD40L double knock out mice blockade of OX40L induced successful prolongation of skin graft survival (36).

Synergy of CD28 and CD40 has been reported in many studies. In a model of T_H2-mediated contact hypersensitivity (CHS), administration of CTLA4Ig led to inhibition of primary response, but secondary response was little affected. Interestingly, CTLA4Ig together with anti-CD40L during the primary response induced long-lasting unresponsiveness also towards secondary response in T_H2-mediated CHS. These results indicate a synergistic function of CD28 and CD40L pathway in a T_H2 response (37). In systemic lupus erythematosus, coadministration of anti-CD40L and CTLA4Ig led to significant improvement of the disease in contrast to treatment with only one molecule blocked (38).

In transplantation, the combined blocking of both costimulatory pathways has also led to long term survival of skin and cardiac allografts in fully MHC-mismatched model, while blockade of only one molecule, CD40L or CD28, has led to only slightly prolonged tolerance (39). Furthermore, costimulation blockade of CD28 and CD40 are able to induce mixed chimerism and long term allograft acceptance without cytoreductive treatment of the host (40).

CD28 deficient mice express OX40 on T cells after activation but expression is delayed and OX40L is minimally induced on APC. Costimulation of CD28 deficient T cells can be circumvented by OX40L expression on stimulated B cells which can provide CD28-independent costimulatory signals to T cells (41).

In an EAE model of CD28-deficient mice, antagonistic anti-OX40L therapy protects mice from EAE (induced by double immunization) but anti-OX40L treatment alone did not protect wild type mice. These data suggest that OX40 interaction can act as alternative costimulatory pathway and mediate CD28 independent immune responses (42). These results also support a redundant role of costimulatory molecules.

3.4.2 Chimerism

The following short overview of chimerism discusses cellular chimerism in the transplantation setting first, followed by molecular chimerism in transplantation, autoimmune disease and allergy.

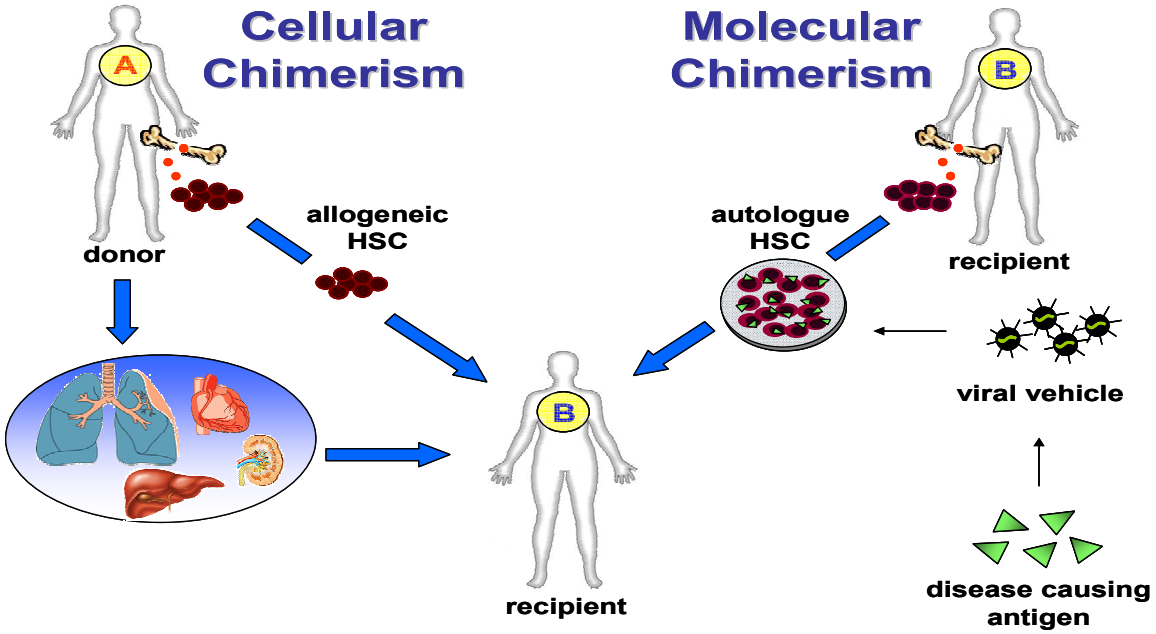

Figure 4: Difference between cellular and molecular chimerism. Cellular chimerism describes the coexistence of donor and recipient HSC, leading to tolerance towards donor organs. Molecular chimerism describes the coexistence of genetically modified recipient HSC and recipient HSC, tolerizing towards the new antigen.

3.4.2.1 Cellular Chimerism

Today, transplantation of organs is the therapy of choice for end-stage organ failure. However, long-term graft acceptance is hindered by immunological barriers and chronic rejection is a widespread complication. Patients are dependent on life-long immunosuppressive treatment. Besides the advantageous effects of immunosuppressive

drugs to prevent graft loss, unspecific suppression of the immune systems leads to increased morbidity and mortality and chronic graft rejection can still occur. A state of immunological tolerance without the loss of a functioning immune system is the major goal in transplantation medicine.

A promising approach is tolerance induction by a state of hematopoietic chimerism, also known as mixed cellular chimerism; this describes a state of coexistence of hematopoietic donor and recipient cells in the host. Once stable chimerism is induced, the immune system can also accept solid organ grafts from the BMC donor without further immunosuppressive treatment. To induce hematopoietic chimerism over MHC barriers, intense and cytotoxic treatment of the recipient previous to BMT is usually necessary.

Host conditioning in early experiments was based on myeloablation and T cell depletion. The development of costimulation blockers could obviate the need for myeloablation and global T cell depletion (43). Irradiation could only be avoided with the use of huge doses of BMC in combination with costimulatory blocking antibodies (anti-CD40L, CTLA4Ig), inducing long-term macrochimerism (40). However, these BMC doses are not feasible for clinical application.

Regulatory T cells were identified to play an important role in BMT. The cells are critical during the early phase but appear to be dispensable for maintenance of chimerism (44). Regulatory T cells are supported by costimulation blockers, like CD40L blockade, which was found to be dependent on $CD4^+CD25^+$ cells (45). Moreover, regulatory cells gained prominence as they allowed the development of protocols with less toxic host conditioning. Replacement of cytoreductive treatment was achieved with a clinically feasible BMC dose combined with anti-CD40L, CTLA4Ig, a short-course of rapamycin and cotransfer of regulatory T cells in a murine model (46-47).

Chimerism-induced tolerance includes both central and peripheral tolerance mechanisms. Central tolerance is established by donor bone-marrow derived dendritic cells, which migrate into the thymus and contribute to negative selection of newly developing thymocytes. Pre-existing donor reactive T cells undergo peripheral deletion early after BMT induced by costimulation blockade. Deletion was demonstrated to be thymus-independent, passive cell death as well as activation-induced cell death played an important role (30-31).

The chimerism induction protocol is still very toxic so that there are few studies on deliberate induction of chimerism in the clinical setting. Megan Sykes and colleagues published a study with six patients suffering from end-stage renal disease due to multiple myeloma. The patients simultaneously received kidney and BMT from HLA identical siblings. Chimerism was transient in four patients and turned into full chimerism in two patients, which also developed GVHD. Three patients remain operationally tolerant without any immunosuppression after a reported follow-up of up to 7 years. All in all, the study showed that renal allograft tolerance could be achieved with combined kidney and BMT (48).

In a subsequent study, David Sachs and colleagues showed successful transplantation of HLA single haplotype mismatched kidney transplants together with bone marrow. In four of five patients renal function remained stable after stopping immunosuppressive treatment. In one patient irreversible humoral rejection led to organ loss (49).

These results show that translation to the clinical setting is possible but still harbours many potential hurdles. A less toxic recipient treatment protocol would allow more clinical investigations and would also open the possibility to applying this concept to a broader range of diseases.

3.4.2.2 Molecular Chimerism

Not only in transplantation is chimerism a promising strategy towards establishing tolerance. Tolerance induction by chimerism would also be a causative treatment for autoimmunity and allergy. Molecular chimerism is induced by transplantation of *ex vivo* genetically-modified cells expressing the disease-causing antigen into a syngeneic recipient.

3.4.2.2.1 Molecular Chimerism in Transplantation

Nowadays, stable tolerance can be achieved by molecular chimerism in rodent and large animal models introducing MHC class I and MHC class II molecules. Long-term tolerance can only be achieved by persistent expression and presentation of the retroviral integrated gene on hematopoietic cells. Therefore, hurdles such as stable retroviral transduction, *in vitro* culture of HSC and engraftment of modified cells had to be overcome.

In a transplant model of molecular chimerism, lethally irradiated mice were transplanted with modified cells, expressing a disparate MHC class I. Chimeric mice developed chimerism and accepted skin grafts with the disparate MHC class I, where skin grafts represent the most stringent model in transplantation (50).

Iacomini and his team identified mature T cells as tolerizing cell population in a non-myeloablative pre-conditioning regime. Cells were originated from congenic mice, which were disparate in MHC class I ($K^k \rightarrow K^b$). Chimerism could be induced successfully and skin graft survival was followed over 100 days (51).

Presensitization is a major problem in transplantation, however, in all these models, chimerism was induced in mice which were not presensitized. Xenotransplantation could be a solution to organ shortage, but the human immune system harbours natural xenoantibodies towards gal carbohydrates. Recipients would thus be presensitized. As a

mouse model, αGT-/- knockout mice are used; they lack αGT and therefore the gal carbohydrate. These mice produce natural anti-αGal antibodies similar to humans. Chimerism was established successfully in αGT-/- knockout mice. B cells were successfully tolerized even in presensitized mice. Anti-αGal antibody production was stopped even after challenge with αGal-expressing cells. Furthermore, in a non-myeloablative murine model, heart acceptance over 100 days was followed after chimerism induction (52).

Although numerous studies on molecular chimerism have been carried out in the mouse model, there are few data regarding chimerism in large animal models.

Transplantation of autologous MHC class II transduced cells in a swine model led to chimerism and graft survival of subsequent transgene matched renal allograft (53).

In a non-human primate xenotransplantation model, molecular chimerism was established with autologous cells transduced with swine MHC class II genes in baboons. Expression of the transgene was transient and could be detected for 6 weeks, the proviral DNA was detected up to 123 weeks. Baboons were grafted with MHC class II matched renal and skin xenografts. Anti-Gal IgM and IgG were reduced at the day of transplantation; however, grafts were rejected after 8-22 days. Reduced production of non-anti-Gal IgG was observed (54).

Rhesus macaques also display a low serum level of preexisting anti-Gal IgM and IgG similar to human anti-Gal antibodies. In monkeys transplanted with autologous αGT transduced BMCs chimerism was still detectable after 5 months. Anti-Gal antibody production could be inhibited after immunization with porcine cells. IgM antibodies to other porcine xenoantigens were produced at similar levels in the chimeric monkeys (55).

3.4.2.2.2 Molecular Chimerism in Autoimmune Disease

Autoimmune diseases are an immune response towards self antigens resulting in pathology and clinical disease dependent on the culprit antigen. In some autoimmune disease models where a single defined antigen is known to cause disease, molecular chimerism can be applied. Induction of tolerance towards a specific antigen through chimerism has been achieved in autoimmune disease models such as type 1 diabetes, multiple sclerosis and autoimmune gastritis. Moreover, successful data were gained in the therapeutical approach.

NOD mice develop spontaneous type 1 diabetes. A successful preventive approach uses molecular chimerism to induce a protective MHC class II by transplantation of genetically modified HSC. Chimeric mice lose the susceptibility to develop diabetes (56).

The murine model of multiple sclerosis is experimental autoimmune encephalomyelitis (EAE). EAE is a T-cell mediated autoimmunity; mice develop a $CD4^+$ T cell response when immunized (with e.g. myelin basic protein, MBP). The disease manifests in ascending paralysis caused by demyelination and inflammation. Molecular chimerism was able to abrogate the induction of EAE even after repeated challenge in a preventive and therapeutic approach (57-58). These results were verified in different studies but there was also a study with disappointing results. Myelin basic protein-expressing BMC failed to induce tolerance or prevent susceptibility although the protein expression was verified (59).

3.4.2.2.3 Molecular Chimerism in Allergy

Since molecular chimerism has been shown to induce tolerance in autoimmune disease models in preventive as well as in curative approaches, we investigated the potential in

IgE-mediated allergy. Allergens are well characterized molecules and are therefore suitable candidates for molecular chimerism.

The first proof of principle experiment of molecular chimerism was done with Phl p 5, the major allergen of *Phleum pratense* (timothy grass). Phl p 5 is a highly immunogenic and clinically relevant respiratory allergen. For molecular chimerism, BMC were transduced with a retrovirus encoding the allergen Phl p 5 fused to a signal peptide and a transmembrane domain, thus, the allergen was membrane-anchored. Transduced BMCs were transplanted into syngeneic recipients preconditioned with a lethal dose of total body irradiation and T cell depleting antibodies one day prior BMT. The anti-CD40L-antibody was injected directly after BMT into recipients. Six weeks after BMT, the mice were repeatedly immunized with Phl p 5 adsorbed to aluminium hydroxide (and a second allergen as specificity control) within a time-span of three weeks. Expression of Phl p 5 within the peripheral blood of the recipients was assessed during the whole follow-up and was found to be detectable up to 40 weeks. Phl p 5-specific antibody levels in chimeric mice were not detectable throughout the whole follow-up in contrast to antibodies towards the control allergen. Moreover, T cell reactivity in response to the allergen Phl p 5 was prevented, thus, B-cell, T-cell and tolerance at the effector level could be established (60). In conclusion, a stable long-term tolerance towards the allergen Phl p 5 was induced through molecular chimerism.

Since allergens vary in their structure, function and source, we investigated whether molecular chimerism can be induced towards a Phl p 5-unrelated allergen. Therefore, we used the major birch pollen allergen, Bet v 1 (of the birch *Betula verrucosa*). More than 96% of tree pollen allergic individuals recognize Bet v 1, and over 60% exclusively display Bet v 1-specific IgE. Besides its frequent occurrence, Bet v 1 is a further cause of

symptoms because of the high cross-reactivity to food allergens like soy (61), cherry (62), celery (63) and others.

Molecular chimerism would be a promising tool to prevent type I allergy by induction of stable long-lasting tolerance (chapter 4.1.2).

4 Aim of the Thesis

IgE-mediated allergy is a steadily rising disease. To date, the only causative treatment is allergen-specific immune therapy, while other current treatments primarily focus on allergic symptoms. Further prevention of IgE-mediated allergy is still a challenging goal.

The specific aim of my thesis was to investigate two preventive approaches in a murine model, the blockade of costimulation molecules and induction of molecular chimerism.

One potent immunomodulating strategy is the blockade of costimulatory molecules. Non-responsiveness towards alloantigens can be induced by costimulation blockade e.g. in allo-transplantation. Effective approaches are not clinically applicable so far. Therefore we aimed to investigate the potential suppressive effect of blocking the costimulatory molecule OX40L. OX40L was recently shown to play an important role in the development of a full T_H2 response. Moreover, the combination of blockade of different molecules (CD28/CD40L) additional to OX40L blockade was tested to assess the potential bypass of OX40L.

We have recently shown that molecular chimerism induces stable tolerance towards one allergen in a preventive approach. Since allergens differ in their function and structure we investigated whether this approach can be extended to several allergens from different sources. Therefore we chose the well described clinically highly relevant major birch-pollen allergen Bet v 1 as a possible candidate for induction of tolerance.

5 Papers

5.1 Original Papers

5.1.1 Anti-OX40L Alone or in Combination with anti-CD40L and CTLA4Ig Fails to Inhibit the Humoral and Cellular Response to a Major Grass Pollen Allergen

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Keywords: Costimulation, suppression, allergy, costimulation blockade, OX40

Running title: Anti-OX40L does not prevent IgE-mediated allergy

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Abstract

IgE-mediated allergy is a common disease characterized by a harmful immune response towards an otherwise harmless antigen. Induction of specific immunological non-responsiveness towards allergens would be a desirable goal. Blockade of costimulatory pathways is a promising strategy to modulate the immune response in an antigen-specific manner. Recently OX40 (CD134) was identified as a costimulatory receptor important in Th2 mediated immune responses. Moreover, synergy between OX40 blockade and 'classical' costimulation blockade (anti-CD40L, CTLA4Ig) was observed in models of alloimmunity. Here we investigated the role of the OX40 pathway in an established murine model of IgE-mediated allergy in which BALB/c mice are immunized with the clinically relevant grass pollen allergen Phl p 5. Early or late blockade of OX40 (anti-OX40L mAb) did not modulate the allergic response on the T cell, humoral or effector cell levels, whereas early administration of a combination of anti-CD40L/CTLA4Ig delayed the allergic immune response. Antibody-production could not be inhibited after repeated immunization independent of long-term suppressed T cell response. Additional blockade of OX40 had no detectable supplementary effect. Delayed response was partly mediated by regulatory T cells as depletion of $CD25^+$ cells led to restored T cell proliferation. To conclude our results, the allergic immune response towards Phl p 5 was independent of OX40. No synergistic function of OX40 to CD40L/CD28 could be detected. According to the literature OX40 is more effective in T cell mediated disease like allergic lung inflammation. In contrary blockade of CD40L/CD28 led to non-responsiveness of T cells without prevention of antibody production.

Introduction

IgE-mediated allergy is a hypersensitivity disorder whose prevalence is still increasing (1). The main feature of type I allergy is the development of IgE which is bound on mast cells. Crosslinking by an allergen leads to the release of cytokines and vasoactive substances, causing the typical allergic symptoms (2). A desirable but still unmet goal would be the induction of antigen-specific immunological non-reactivity towards these antigens.

Blockade of costimulatory molecules is a promising approach to modulate the immune response. Induction of an immune response requires engagement of the T cell receptor simultaneously with costimulatory molecules. One of the first discovered costimulation molecules was CD28. Ligation of CD28 is important for activation of T cells. Missing CD28 signalling leads to anergy, what means functional deficiency of T cells (3). Another well-known costimulation molecule is CD40. Interaction of CD40 - CD40L is required for generation of high titers of isotype-switched antibody, survival, maturation and allograft rejection (4). Blocking CD40L is widely and effectively employed in animal models prolonging allograft survival and preventing acute rejection (5). More effective is the blockade of both, CD28 together with CD40 in diminishing allo-immunity (6).

In recent years, OX40 was shown to participate in Th2 mediated immune responses (7-8). OX40, a member of the TNF/TNF-R superfamily, has a critical role in the late primary T cell response and in the generation and survival of memory T cells. Stüber and Strober observed decreased production of IgG1, IgG2a, IgG2b and IgG3 when anti-OX40 antibodies were administered together with TNP-KHL immunization, provoking a T cell-dependent immune response. T cell independent immune responses were not influenced (9). In OVA induced lung inflammation blockade of OX40L was able to attenuate disease with suppressed production of IgE, IgG and decreased Th2 cytokines after anti-OX40L

treatment (10). Anti-OX40L was currently tested in a clinical trial for allergen-induced airway obstruction in adults with mild asthma (NCT00983658).

OX40 is also expressed on regulatory T cells (Tregs). Tregs of OX40-deficient mice are present in normal numbers and function whereas OX40 ligation on mature T cells abrogates Treg function and conversion. In some studies enhanced OX40 stimulation preferentially converts T cells to memory cells instead of Tregs (11-12). So OX40 is not only a costimulatory molecule to activated effector T cells but also negatively regulates $FoxP3^+$ Tregs.

Synergistic functions of OX40L to the costimulatory molecules CD28 and CD40L have been reported in the literature in various models (13-15). For example CD28 interaction was shown to be necessary for high and early expression of OX40 and OX40L (16-17). In transplantation, CD40L blockade-induced cardiac allograft acceptance can be overridden by agonistic OX40 antibodies (14). Another model describes that mice, resistant to CD28/CD40L blockade exhibit prolonged skin graft survival when OX40L is blocked (18).

In experimental autoimmune encephalomyelitis (EAE) OX40L was shown to be able to bypass CD28 independent development of disease. Blockade of OX40L in CD28 deficient mice prevented induction of EAE (19).

In allergy, blockade of CD28 and CD40L during allergic sensitization and ongoing disease was assessed. Blocking CD40L prevented allergic sensitization but had no influence when administrated to already sensitized mice. Blocking CD28 (CTLA4Ig) early or late had no influence on the humoral response but allergen-specific T cell proliferation was diminished (20).

In order to delineate the potential of costimulatory molecules in IgE-mediated allergy, we applied a well-defined mouse model of IgE-mediated allergy, focusing the humoral response (20).

Our results demonstrate that OX40 is negligible for an IgE-mediated allergic response towards Phl p 5. Surprisingly this effect was not caused by redundant function of OX40 to CD40 or CD28. Interestingly, blockade of the costimulatory molecules CD28 and CD40L led to delayed humoral response contrary to long-term extended T cell non-responsiveness. These findings together with published data of OX40 underline that maybe OX40 mainly takes part in T cell mediated responses like allergic lung inflammation. Moreover there is lots of potential in modulation of immune responses by costimulatory molecules in allergy but the effect mainly targets T cells as humoral immunity is only temporary suppressed.

Material and Methods

Animals

Female BALB/c mice were obtained from Charles River (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.

Recombinant Allergens and Immunization

Purified recombinant (r) timothy grass pollen allergen (rPhl p 5) and birch pollen allergen (rBet v 1) were obtained from Biomay (Vienna, Austria). Mice were immunized subcutaneously with 5 µg rPhl p 5 (major grass pollen allergen) and in selected groups with 5 µg rBet v 1 (major birch pollen allergen), adsorbed to $Al(OH)_3$ (Alu-Gel-S, Serva,

Ingelheim, Germany). Immunization started at day 0 and was repeated every three weeks (days 0, 21, 42, …) till the end of follow-up.

Costimulation Blockade

Treatment with anti-CD40L (MR1) and human CTLA4Ig (abatacept, 0.5 mg/mouse) was given by intraperitoneal (i.p.) injection early (days 0, 2, 4) or late (days 21, 23, 25) (20). OX40L was blocked by i.p. injection of RM134 (0.5 mg/mouse) early (days 0, 2, 4, 8) or late (days 21, 23, 25, 29) (18). For depletion of $CD25^+$ cells a cytotoxic anti-CD25 mAb (PC61) was given i.p. at days 0 (0.5 mg/mouse), 4 and 8 (0.25 mg/mouse) or days 21, 25, 29**.** In combination to anti-CD40L/CTLA4Ig/anti-OX40L treatment $CD25^+$ cells were depleted at day 16 (PC61, 1 mg/mouse). Anti-CD40L and anti-OX40L were purchased from BioXCell (West Lebanon, NH, USA), hCTLA4Ig (abatacept) was generously provided by Bristol-Myers, Squibb Pharmaceuticals (Princeton, NJ, USA).

Flow Cytometry and Antibodies

For analysis of Tregs mAbs with specificity against CD4 (RM4-4) and CD25 (7D4) were used. Antibodies were conjugated to FITC and PE and detected in FL1 and FL2. Surface staining was performed according to standard procedures and flow cytometric analysis was done on a Coulter Cytomics FC500. CXP software (Coulter, Austria) was used for acquisition and analysis.

ELISA

To measure antigen-specific antibodies in the sera of immunized mice ELISAs were performed as described previously (21). Blood samples were taken from the tail vein and serum was stored at -20°C until analysis. Plates were coated with rBet v 1 or rPhl p 5 (5

µg/ml), sera were diluted 1:20 for IgE, 1:100 for IgM, IgA, IgG2a, IgG3 and 1:500 for IgG1, bound antibodies were detected with monoclonal rat anti-mouse IgE, IgG1, IgG2a, IgG3, IgA and IgM antibodies (BD Pharmingen) diluted 1:1000 and a HRP-coupled goat anti-rat antiserum (Biosciences, UK) diluted 1:2000. The substrate for HRP was ABTS (60 mM/l citric acid, 77 mM/l Na_2HPO_4 x $2H_2O$, 1.7 mM/l ABTS (Sigma-Aldrich, MO), 3 mM/l H_2O_2). Absorbance was measured at 405 nm (0.1 s) and 490 nm (0.1 s). For calculation values obtained at 490 nm were abstracted from 405 nm values.

Lymphocyte Proliferation Assay

Spleens were removed under sterile conditions and homogenized. Single cell suspension was filtered through a 70 µm Nylon cell strainer to remove remaining tissue. Erythrocytes were removed by adding cold lysing buffer (Red Blood Cell Lysing Buffer, Sigma-Aldrich). Cells were diluted to a final concentration of 5 x 10^5 cells/well, 2 µg/well allergen-stimulant was added and 0.5 µg/well Concanavalin A (Sigma-Aldrich) as control. The 96 well-plates were incubated at 37°C, 5% CO_2. On day 4, 0.5 µCi H^3 thymidine ([methyl-3H] Thymidine, Amersham) per well were added. Sixteen hours later cells were harvested and thymidine uptake was measured in a beta counter (Beta scintillation liquid, Wallac) (21).

Rat Basophile Leukaemia (RBL) Cell Degranulation Assay

RBL-2H3 cell subline was cultured as described previously (22)**,** in RPMI 1640 medium (Biochrome AG, Berlin, Germany) containing 10 % fetal calf serum. 6 x 10^4 cells were plated in 96 well tissue culture plates (Greiner, Bio-One, Germany), loaded with 1:30 diluted mouse sera and incubated for 2 hours at 37°C and 5 % CO_2. Supernatants were removed and the cell layer was washed 2x with Tyrode´s buffer (137 mM NaCl, 2.7 mM

KCL, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM D-glucose, 12 mM $NaHCO_3$, 10 mM HEPES and 0.1% w/v BSA, pH 7.2). Preloaded cells were stimulated with rPhl p 5 or rBet v 1 (0.03 µg per well) for 30 min. at 37°C. The supernatants were analyzed for ß-hexosaminidase activity by incubation with the substrate 80 µM 4-methylumbelliferyl-N-acetyl-ß-D-glucosamide (Sigma-Aldrich) in citrate buffer (0.1 M, pH4.5) for 1 hour at 37°C. The reaction was stopped by addition of 100 µl glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and the fluorescence was measured at λ_{ex}: 360/λ_{em}: 465 nm using a fluorescence microplate reader (Wallac, Perkin Elmer, Vienna, Austria). Results are reported as percentage of total ß-hexosaminidase released after addition of 1 % Triton X-100. Determinations were done in triplicates.

Statistical analysis

Means, standard deviations (SD) and standard error of the mean (SEM) were calculated for statistical documentation. P values were calculated with an unpaired two tailed T-test. Values of $p < 0.05$ were considered as statistical significant.

Results

Blockade of OX40L has no detectable effect on the humoral and cellular response towards Phl p 5

To investigate the role of OX40, a well characterized model of IgE-mediated allergy was employed in which BALB/c mice are repeatedly immunized with recombinant Phl p 5 (days 0, 21) (table I: group A, untreated control group). Groups of mice (n=6/group) received anti-OX40L mAb early, at the time of first immunization (group B) or late, at the time of second immunization (group C). Consistent with previous reports (20), untreated immunized mice (control group) produced high levels of allergen-specific IgE, IgG1, IgG2a, IgA and IgM (Fig. 1a–1f). Treatment with anti-OX40L early or late had no detectable effect on the levels of allergen-specific antibody production (Fig. 1a–1f).

The impact of anti-OX40L treatment was further demonstrated using RBL cell degranulation assay. RBL cells were sensitized by transfer of serum, challenge with Phl p 5 led to a release of β-hexosaminidase in the control group. Anti-OX40L treatment did not affect mediator release in *in vitro* RBL assays (Fig. 1g).

We further investigated the T cell response to Phl p 5 by *in vitro* T cell proliferation assays. Splenocytes of immunized controls strongly proliferated in response to Phl p 5. Treatment with anti-OX40L early or late did not significantly reduce the proliferative response (Fig. 1h).

According to these data, blockade of OX40L does not significantly alter the primary or secondary immune response towards Phl p 5.

Blockade of OX40L has no additive effect when combined with anti-CD40L and CTLA4Ig

The OX40 pathway is described in the literature to act synergistically with the costimulatory molecules CD40 and CD28 (16, 18-19). To investigate the effect of

blocking OX40L in combination with CD40L (anti-CD40L) and CD28 (CTLA4Ig) blockade, groups of mice were treated early with anti-CD40L/CTLA4Ig (group D) with or without additional anti-OX40L (group E). Mice receiving anti-CD40L/CTLA4Ig showed significantly delayed allergen-specific antibody production compared to the control mice. IgE levels were increased after the third immunization (by day 63, Fig. 2a). Also the other Ig isotypes (IgG1, IgG2a, IgG3, IgA, IgM) were significantly suppressed for at least that long, in case of IgG2a and IgG3 even longer (day 84, Fig. 2b-2f). Unexpectedly, additional treatment with anti-OX40L had no detectable additive effect on the course or levels of allergen-specific antibody production.

IgE reactivity was assessed in RBL assays as described previously. β-hexosaminidase release was due to IgE levels (Fig. 2g). No significant difference was observed when mice were treated with anti-CD40L/CTLA4Ig or additional anti-OX40L.

T cell proliferation in response to Phl p 5 was significantly suppressed (Fig. 2h) in anti-CD40L/CTLA4Ig treated mice in comparison to the control group. Again no difference was detectable in mice treated additionally with anti-OX40L.

Late treatment with anti-CD40L/CTLA4Ig had no effect on an already ongoing allergic response (20). Since anti-OX40L, in contrast, was effective in modulating a primed response in models of allotransplantation (13), we tested the effect of anti-OX40L in addition to anti-CD40L/CTLA4Ig (late) in a primed allergic response (group F). Allergen-specific IgG1, IgG2a levels stayed constant after second immunization and late treatment. IgE and IgA were unaffected by late administration of anti-CD40L/CTLA4Ig/anti-OX40L (data not shown).

Early treatment with anti-OX40L had no additional effect on the prolonged non-responsiveness achieved with anti-CD40L/CTLA4Ig. No synergistic effect of blocking OX40L was discovered in this model focusing humoral allergy response. Also late

administration of anti-CD40L/CTLA4Ig/anti-OX40L had no effect on IgE, as levels staed constant.

CD25 positive cells partly mediate costimulation blockade induced suppression

Our results demonstrate that blocking CD40L, CD28 and OX40L results in non-responsiveness towards Phl p 5. Blocking CD40L acts in part through the induction of Tregs, as shown in a transplant model (23). To shed some light on the mechanism of this immune modulation we investigated the role of $CD25^+$ T cells, T regulatory cells (Tregs). Therefore, $CD25^+$ cells were depleted shortly before the second immunization (day 16) of anti-CD40L/CTLA4Ig/anti-OX40L early treated mice (group H). Delayed antibody production in response to the allergen was reversed by depletion of Tregs. The reversing effect was first detected at day 63 and still significant at day 84 (**Fig. 3a**). In T cell proliferation assays, mice treated with anti-CD40L/CTLA4Ig/anti-OX40L exhibited significantly suppressed T cell proliferation (week 15), whereas proliferation was completely restored by depletion of $CD25^+$ cells (Fig. 3b).

Influence of Tregs alone on Phl p 5 immunization was investigated by single $CD25^+$ depletion early or late (group I and J). Allergen-specific IgE levels were not influenced and comparable to those of the control group. No influence of $CD25^+$ depletion alone on antibody production was detected (Fig. 3c). Successful depletion of $CD25^+$ was verified in flow cytometric analysis (Fig. 3d).

Tregs are activated in an antigen-specific manner but their suppressive function is reported to be antigen non-specific (24). Therefore, we designed an experiment to test whether Tregs activated with one allergen suppress the immune response towards another, unrelated allergen, Bet v 1 (major birch pollen allergen). Mice were immunized with Phl p 5 and treated with anti-CD40L/CTLA4Ig +/- anti-OX40L early. Three weeks later mice

were immunized with Phl p 5 and additionally Bet v 1 (group L and M). Double immunized control mice (group K) developed Bet v 1-specific IgE after the first immunization. Noteable, IgE levels of immunized mice, treated with anti-CD40L/CTLA4Ig +/- anti-OX40L early exhibited a delayed immune response, also towards Bet v 1. Bet v 1-specific IgE levels were significantly suppressed till day 63 (Fig. 3e). Once again, no significant difference between mice receiving anti-OX40L or not was detected.

In Fig. 3f Bet v 1-specific β-hexosaminidase release of RBL cells (week 12) was assessed. Sera of mice treated with anti-CD40L/CTLA4Ig +/- anti-OX40L early led to diminished Bet v 1-specific release. Notable, depletion of $CD25^+$ cells (group N) reversed the effect partly, β-hexosaminidase release was comparable to the double immunized control group.

Also T cell proliferation in response to Bet v 1 was investigated. Proliferation was significantly lower in anti-CD40L/CTLA4Ig +/- anti-OX40L treated groups than in the double immunized control group. Depletion of $CD25^+$ cells was able to restore proliferation partly but significant (Fig. 3g).

These results demonstrate that regulatory cells, induced by anti-CD40L/CTLA4Ig modulate the allergic response towards Phl p 5 in an antigen non-specific manner, although the effect on the T cell level was more pronounced.

Persistent in vivo antibodies are capable to delay humoral response towards Phl p 5

Delayed immune response towards Bet v 1 is partly mediated by Tregs as shown in Fig. 3g. To exclude the possibility of immunosuppression caused by remaining anti-CD40L/CTLA4Ig/anti-OX40L in the sera, we investigated how long blocking antibodies stay therapeutically effective after *in vivo* administration. Anti-CD40L/CTLA4Ig/anti-OX40L were injected into naïve mice three weeks before the first immunization with Phl p

5 (group G) and compared with the control group (A) and mice receiving anti-CD40L/CTLA4Ig/anti-OX40L early (group E).

As previously observed control mice produced high levels of IgE after the first immunization and mice treated with anti-CD40L/CTLA4Ig/anti-OX40L early exhibited delayed development of IgE (day 63). Surprisingly mice receiving antibody treatment three weeks before the first immunization also showed delayed antibody production. In both cases allergen-specific antibodies developed upon the immunization 6 weeks after anti-CD40L/CTLA4Ig/anti-OX40L treatment, independent of the time of the first immunization (d 0) (Fig. 4a). Thus, a suppressive effect on antibody production was still detectable three weeks after costimulation blockade.

Interestingly, T cell proliferation was different. Proliferation was only suppressed when mice received anti-CD40L/CTLA4Ig/anti-OX40L early together with the first immunization. Delayed immunization led to normal T cell proliferation in response to the allergen Phl p 5 (Fig. 4b).

These results indicate that the remaining antibodies are still capable to delay the humoral immune response whereas for suppression of T cell response simultaneous treatment and immunization is obligatory.

Discussion

OX40L is currently discussed in the literature as a potent costimulatory molecule triggering Th2 responses. These findings are further investigated in a clinical trial for allergen-induced airway obstruction in adults with mild asthma (NCT00983658).

We investigated the effect of costimulation blockade, especially blockade of OX40:OX40L pathway in IgE-mediated allergy. Surprisingly, our results did not reveal any influence of OX40 pathway blockade on the allergic response towards Phl p 5. Early or late blockade of OX40L did not significantly influence humoral, T cell or effector cell response.

In T cell mediated diseases, like allergic lung inflammation, OX40 pathway was shown to play an important role in antibody and cytokine production as well as T cell responses. The present model focuses on the humoral response, which seems to be independent of OX40L signalling (Fig. 1).

Moreover, we wanted to investigate potential synergy of OX40 to CD28 and CD40L, as synerigstic effects are described in the literature (15) (18). Therefore the potency of a costimulation blockade based combination-therapy (anti-CD40L/CTLA4Ig/anti-OX40L) was tested in the humoral allergy model. Blockade of CD40L led to similar results as shown in the literature, prevention of allergic sensitization and suppressed T cell response (data not shown). We also investigated the effect of combined blockade of CD40:CD40L and CD28:B7 pathway by anti-CD40L/CTLA4Ig treatment. Consistent with published data, T cell response was suppressed. But contrary to the literature of the humoral response (20), antibody production was prevented for several weeks. But additional effect of blocking CD28 and CD40L was observed in many other models like in transplantation (6) and murine allergic contact dermatitis (25).

As we aimed to investigate the synergistic potential of OX40L, we analyzed the effect of anti-OX40L together with anti-CD40L/CTLA4Ig treatment. Nevertheless no effect due to additional anti-OX40L treatment was observed in our experiments (Fig. 2). These data suggest that OX40L plays a negligible role in emerging an allergic response towards Phl p 5. Moreover OX40L did not bypass delayed development of allergen-specific antibodies in anti-CD40L/CTLA4Ig treated mice. Notably, also IgE production was only delayed, despite the longterm suppressed T cell response induced by costimulation blockade. As IgE is the reason for mast cell degranulation and subsequent allergic symptoms, reduction IgE production would be desirable.

Combined treatment of mice with anti-CD40L/CTLA4Ig had a significant suppressive effect. We wanted to gain insight into the underlying mechanism. So we further investigated the role of regulatory cells in the combinational treatment with anti-CD40L/CTLA4Ig/anti-OX40L. The suppressive effect of anti-CD40L in allergy is partly caused by $CD4^+CD25^+$ cells, as depletion of $CD25^+$ cells reverse suppression to some extent (data not shown). Importance of $CD25^+$ cells was shown in our model of CD40L/CTLA4Ig/anti-OX40L treatment, where these cells maintained T cell unresponsiveness and depletion restored proliferation (Fig. 3b). Moreover, allergen-specific IgE was significantly higher in mice treated with $CD25^+$ depleting Ab.

As regulatory T cells can suppress non-specific, upon TCR-specific activation; we investigated suppression towards an additional administrated allergen, Bet v 1. Nonspecific suppression was shown by delayed antibody production and suppressed T cell proliferation. Bet v 1-specific IgE production was similar to Phl p 5-specific antibody production (Fig. 3g).

To test whether delayed antibody production is not caused by persistent serum levels of *in vivo* antibodies, mice were immunized three weeks after *in vivo* antibody treatment.

Interestingly antibody production was delayed, even when mice were treated three weeks before the immunization (Fig. 4a). Suggesting that unspecific suppression of antibody production was shown to result from persisting anti-CD40L and CTLA4Ig in the sera. In the literature, application of 3 x 250µg anti-CD40L (MR1) was demonstrated to be still biological active (5%) 3 weeks after administration (26). Furthermore, CTLA4Ig was found to be functionally active 5 weeks after treatment (6 x 200 µg). These data suggest that prolonged costimulation blockade-based suppression of allergic response was induced but no permanent tolerance (27). Interestingly, only antibody production was effected by unspecific immunosuppression. Persisting anti-CD40L/CLTA4Ig/anti-OX40L in the sera of mice did not affect T cell response. T cell response was suppressed only when mice were immunized at the day of *in vivo* antibody treatment.

In allergy, regulatory T cells were shown to inhibit allergic disease. Tregs secrete the immunoregulatory cytokine IL-10, capable to indirectly modulate allergen-specific B-cells. Also in peptide-immunotherapy T cells were shown to play a protective role. We have shown that antibodies were produced despite non-responsive T cells, whereas the effect of induced regulatory cells has to be further investigated *in vivo*.

All in all, costimulation blockade can only provide limited suppression of the allergic immune response, mainly effective during sensitization. Currently, the only causative therapy of allergy is specific immunotherapy, modulating the allergic immune response. The only approach to induce longterm robust tolerance is molecular chimerism (28-29). But this therapy is still in an early development state of proof-of-principle testing.

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Figures and Tables

Figure 1: Blockade of OX40L has no detectable effect on the allergen-specific response in an IgE-mediated allergy model.

Serum samples were collected at baseline (preimmune, d 0), three weeks after the first immunization (d 21) and three weeks after the second immunization (d 42). Allergen-specific antibody production was analyzed by ELISA (IgE, IgG1, IgG2a, IgG3, IgA, IgM). Three groups (described in table I) are shown on every diagram **a – f**. Immunized mice (group A, designated as control group), immunized mice with early anti-OX40L treatment (day 0, 2, 4, 8, group B) and immunized mice with late anti-OX40L treated mice (day 21, 23, 25, 29, group C). Antibody levels are displayed as OD values in box-and-whisker plots, 6 mice per group are shown. **(g)** To analyze the effector function of IgE in the sera, allergen-specific β-hexosaminidase release of serum coated RBL cells in response to Phl p 5 was assessed of the control group or groups with anit-OX40L early and anti-OX40L late treatment. Serum samples of day 0, 21, 42 were tested and are represented in a scatter plot (n=6). **(h)** T cell reactivity was tested in a proliferation assay, (week 7). Bars represents means of 6 mice per group with SEM, immunized mice without treatment or treated with anti-OX40L early or anti-OX40L late. Legends are shown in the figure**.**

Figure 2: OX40L blockade together with anti-CD40L/CTLA4Ig treatment has no detectable additional effect

Shown are three groups, control group (group A), immunized mice treated early with anti-CD40L/CTLA4Ig (group D) and mice treated early with anti-CD40L/CTLA4Ig/anti-OX40L (group E) (n=6). Serum samples were collected every three weeks for a follow-up of 15 weeks. Results of group D and E are representative for two and three independent experiments, respectively. Allergen-specific antibody levels of different isotypes were

analyzed over the whole follow-up. **a)** shows Phl p 5-specific IgE, **b)** IgG1, **c)** IgG2a, **d)** IgG3, **e)** IgA and **f)** IgM. OD values are represented in box-whiskers blots. Effector function of IgE was analyzed in RBL assays. β-hexosaminidase release in response to Phl p 5 of serum coated cells, collected to the different time points is shown in a box-and-whiskers plot (n=6). **Fig. 2g** presents the whole follow-up of the control group, of immunized mice treated early with anti-CD40L/CTLA4Ig and mice treated early with anti-CD40L/CTLA4Ig/anti-OX40L**.** To analyze the tolerizing effect on allergen-specific T cells, proliferation was analyzed at about week 15 and mean SI values of 6 mice per group are shown in a column bar graph with SEM **(h).**

Figure 3: Depletion of CD25 positive cells restores allergic T cell response

Effect of $CD25^+$ cells was assessed by depletion at day 16, 5 days before the second immunization. Shown are **a)**, control group (group A), anti-CD40L/CTLA4Ig/anti-OX40L (early) treated mice (group E) and same regime with $CD25^+$ cell depletion (group H). Serum samples were collected every three weeks. Allergen-specific IgE levels are depicted in OD values (n=6) and presented in box-and-whiskers plot. One of two independent experiments is shown. **b)** T cell proliferation was assessed in response to Phl p 5 at week 15, suppressed proliferation was restored when $CD25^+$ cells were depleted in the antibody treated group. Mean SI values of 6 mice per group are shown in a column bar graph with SEM. **c)** Influence of $CD25^+$ cell depletion in allergen-specific IgE production was tested. Groups of mice received anti-CD25 early (group I) or late (group J) and IgE levels were compared to the control group. Mean OD values are shown in a box-and-whiskers plot. Depletion of $CD25^+$ was verified in flow cytometric analysis **(d)**. To analyze Treg function mice were immunized with additional Bet v 1 starting at day 21 (group K, L, M, N, n=6). Serum samples of double immunized mice were collected (group K) and represent the

control group in this graph. Further groups shown are mice treated with anti-CD40L/CTLA4Ig (group L), anti-CD40L/CTLA4Ig/anti-OX40L (group M) and the last group with CD25 depletion on day 16 (group N). OD values of Bet v 1-specific IgE are shown in a box-and-whiskers blot, groups are described in the figure **e**. **f)** shows the β-hexosaminidase release of serum coated RBL cells in response to Bet v 1 at week 12. Groups are described in the figure. **g)** T cell proliferation was assessed at week 12 (n=3). Shown are mean Bet v 1-specific SI values with SEM in a column bar graph.

Figure 4: Potency of remaining anti-CD40L/CTLA4Ig/anti-OX40L three weeks before immunization

a) Remaining function of circulating anti-CD40L, CTLA4Ig and anti-OX40L was tested by administration 21 days prior the first immunization. Three different groups were followed, the control group (group A), mice treated with anti-CD40L/CTLA4Ig/anti-OX40L early (group E) and mice receiving the same treatment at day -21 (group G). Serum samples were collected as depicted in the diagram. Shown is a box-and-whisker plot with OD values (n=6). **b)** T cell response of mice receiving antibodies at day -21 was analyzed at week 12 and compared to the other groups in a T cell proliferation assay. Mean values of 3 mice with SEM are shown.

Table I: Immunization and treatment protocol

Groups	Immunization	Treatment				
	Phl p 5	Bet v 1	anti-OX40L	CTLA4Ig	anti-CD154	anti-CD25
A	+					
B	+		early			
C	+		late			
D	+			early	early	
E	+		early	early	early	
F	+		late	late	late	
G	start day 21		early	early	early	
H	+		early	early	early	16
I	+					0, 4, 8
J	+					21, 25, 29
K	+	start day 21				
L	+	start day 21		early	early	
M	+	start day 21	early	early	early	
N	+	start day 21	early	early	early	16

Groups of BALB/c mice (6/group) were immunized with the allergen rPhl p 5 and/or rBet v 1 adsorbed to aluminium hydroxide, in a span of three weeks (days 0, 21, 42, ..). Additional mice were treated with anti-CD40L (early, days 0, 2, 4 or late, days 21, 23, 25) and CTLA4Ig (early, days 0, 2, 4 or late, days 21, 23, 24) and/or anti-OX40L (early, days 0, 2, 4, 8 or late, days 21, 23, 25, 29) and/or anti-CD25, days of antibody administration are described in the table. Group A is used as control group.

Figure 1

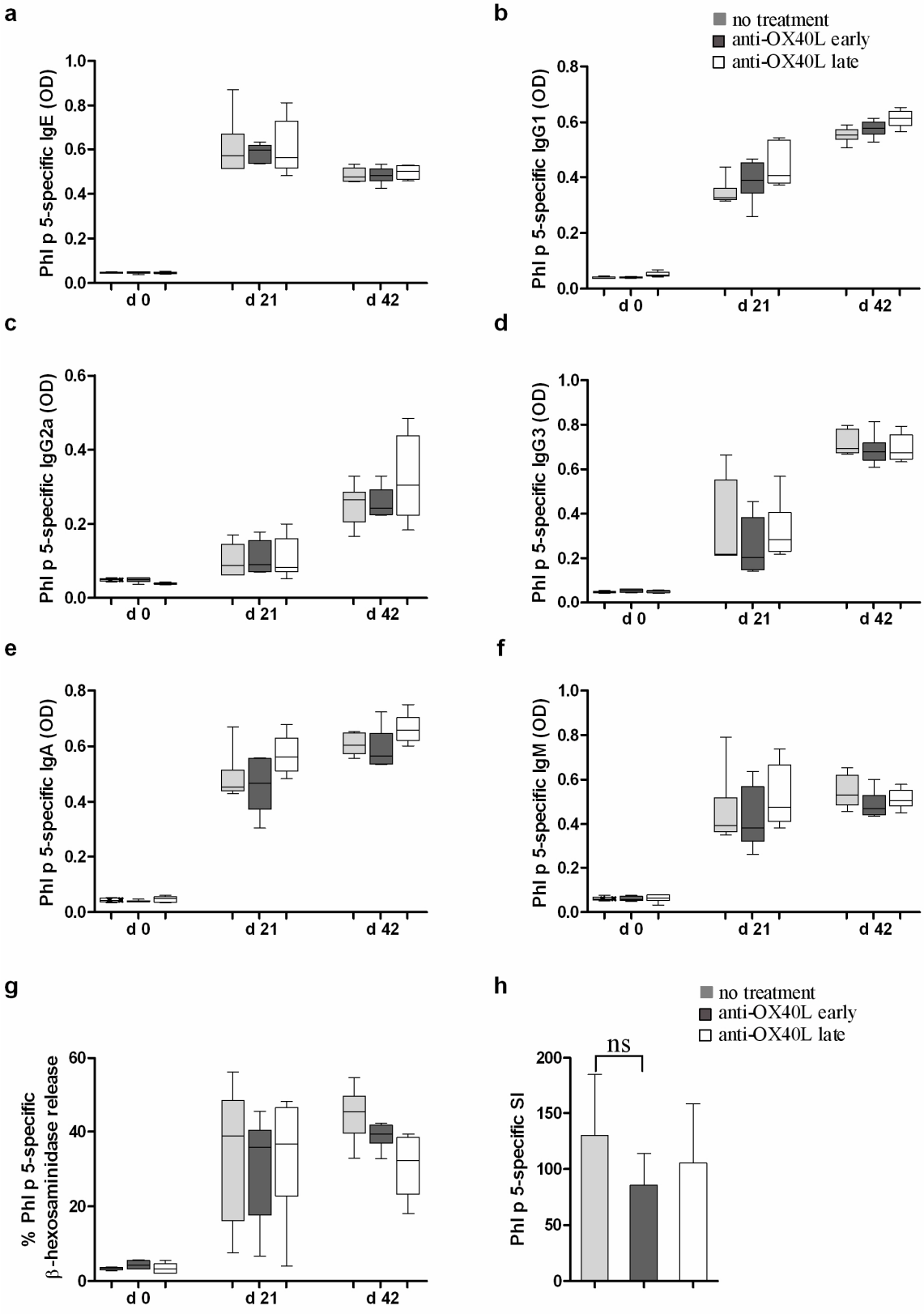

a
Phl p 5-specific IgE (OD)
1.0
0.8
0.6
0.4
0.2
0.0
d 0
d 21
d 42
b
no treatment
anti-OX40L early
anti-OX40L late
Phl p 5-specific IgG1 (OD)
0.8
0.6
0.4
0.2
0.0
d 0
d 21
d 42
c
Phl p 5-specific IgG2a (OD)
0.6
0.4
0.2
0.0
d 0
d 21
d 42
d
Phl p 5-specific IgG3 (OD)
1.0
0.8
0.6
0.4
0.2
0.0
d 0
d 21
d 42
e
Phl p 5-specific IgA (OD)
0.8
0.6
0.4
0.2
0.0
d 0
d 21
d 42
f
Phl p 5-specific IgM (OD)
1.0
0.8
0.6
0.4
0.2
0.0
d 0
d 21
d 42
g
% Phl p 5-specific β-hexosaminidase release
60
40
20
0
d 0
d 21
d 42
h
no treatment
anti-OX40L early
anti-OX40L late
ns
Phl p 5-specific SI
200
150
100
50
0

Figure 2

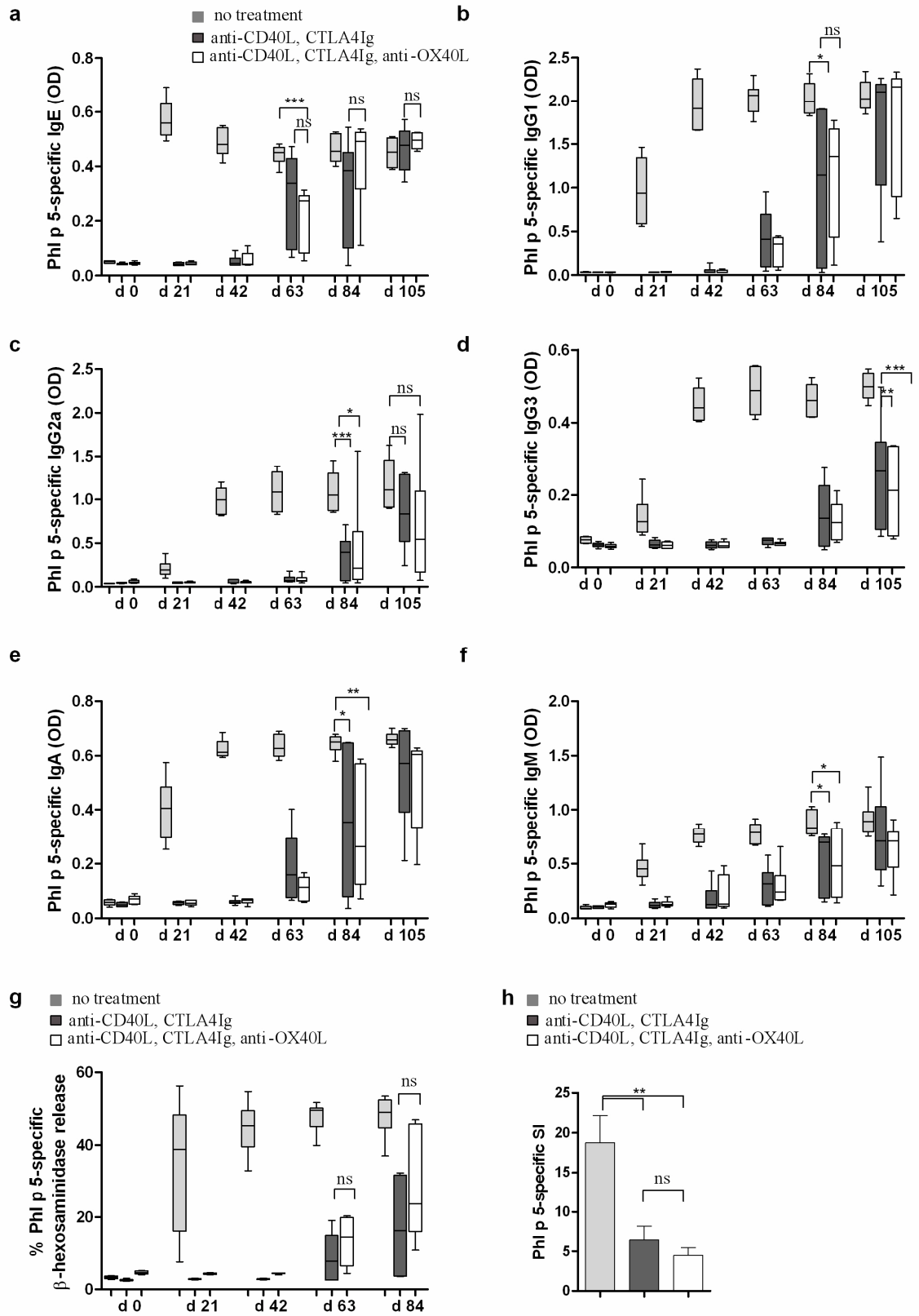

a
no treatment
anti-CD40L, CTLA4Ig
anti-CD40L, CTLA4Ig, anti-OX40L
Phl p 5-specific IgE (OD)
b
Phl p 5-specific IgG1 (OD)
c
Phl p 5-specific IgG2a (OD)
d
Phl p 5-specific IgG3 (OD)
e
Phl p 5-specific IgA (OD)
f
Phl p 5-specific IgM (OD)
d 0
d 21
d 42
d 63
d 84
d 105
ns
g
% Phl p 5-specific β-hexosaminidase release
h
Phl p 5-specific SI

Figure 3

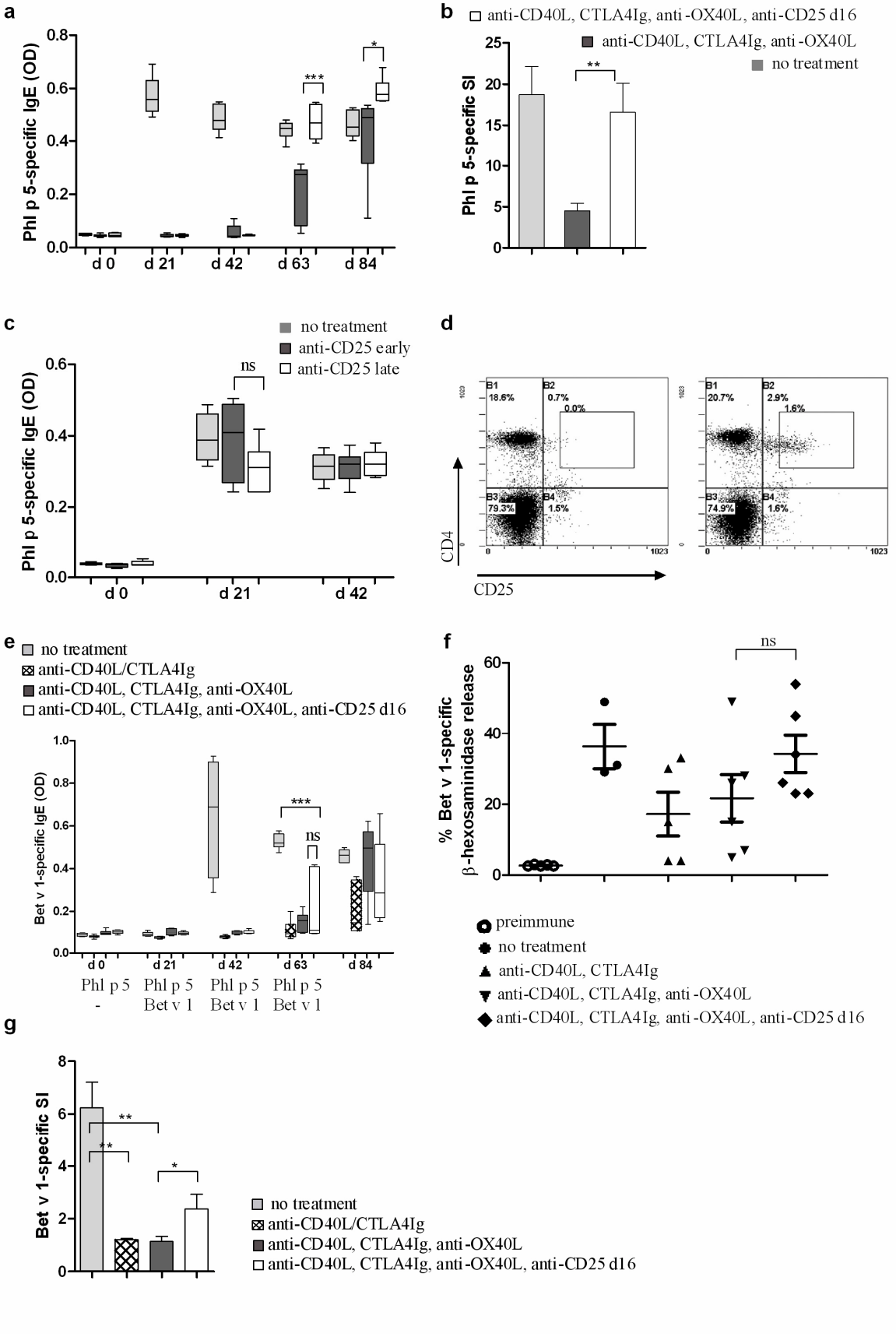
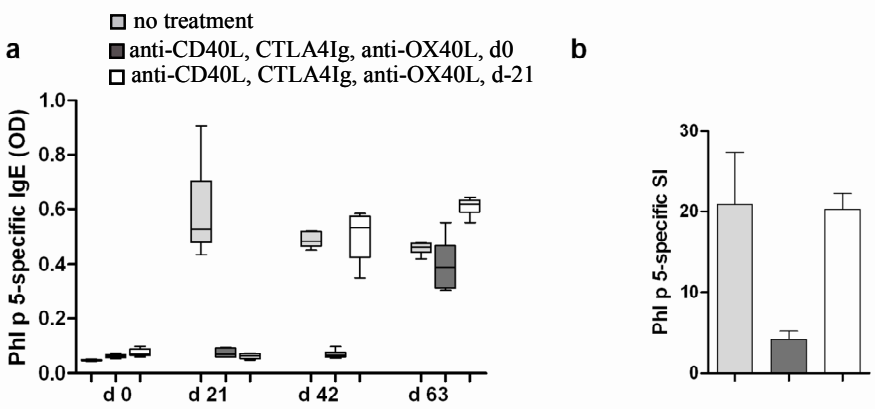

a
Phl p 5-specific IgE (OD)
d 0
d 21
d 42
d 63
d 84
b
anti-CD40L, CTLA4Ig, anti-OX40L, anti-CD25 d16
anti-CD40L, CTLA4Ig, anti-OX40L
no treatment
Phl p 5-specific SI
c
no treatment
anti-CD25 early
anti-CD25 late
ns
d
CD4
CD25
e
no treatment
anti-CD40L/CTLA4Ig
anti-CD40L, CTLA4Ig, anti-OX40L
anti-CD40L, CTLA4Ig, anti-OX40L, anti-CD25 d16
Bet v 1-specific IgE (OD)
Phl p 5 -
Phl p 5 Bet v 1
f
% Bet v 1-specific β-hexosaminidase release
preimmune
no treatment
anti-CD40L, CTLA4Ig
anti-CD40L, CTLA4Ig, anti-OX40L
anti-CD40L, CTLA4Ig, anti-OX40L, anti-CD25 d16
g
Bet v 1-specific SI
no treatment
anti-CD40L/CTLA4Ig
anti-CD40L, CTLA4Ig, anti-OX40L
anti-CD40L, CTLA4Ig, anti-OX40L, anti-CD25 d16

Figure 4


a
no treatment
anti-CD40L, CTLA4Ig, anti-OX40L, d0
anti-CD40L, CTLA4Ig, anti-OX40L, d-21
Phl p 5-specific IgE (OD)
1.0
0.8
0.6
0.4
0.2
0.0
d 0
d 21
d 42
d 63
b
Phl p 5-specific SI
30
20
10
0

5.1.2 Engraftment of Retrovirally-Transduced Bet v 1-GFP Expressing Bone Marrow Cells Leads to Allergen-Specific Tolerance

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Running title: Tolerance towards the allergen Bet v 1 by molecular chimerism

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Abstract

Background Molecular chimerism is a promising strategy to induce tolerance to disease-causing antigens expressed on genetically modified haematopoietic stem cells. The approach was employed successfully in models of autoimmunity and organ transplantation. Recently, we demonstrated that molecular chimerism induces robust and lasting tolerance towards the major grass pollen allergen Phl p 5.

Objective Since allergens are a group of antigens differing widely in their function, origin and structure we further examined the effectiveness of molecular chimerism using the Phl p 5-unrelated major birch pollen allergen Bet v 1, co-expressed with the reporter GFP. Besides, inhibition of CD26 was used to promote engraftment of modified stem cells.

Methods Retrovirus VSV-Betv1-GFP was generated to transduce 5-FU-mobilized BALB/c hematopoietic cells to express membrane-bound Bet v 1 (VSV-GFP virus was used as control). Myeloablated BALB/c mice received Betv1-GFP or GFP expressing bone marrow cells, pre-treated with a CD26 inhibitor. Chimerism was followed by flow cytometry. Tolerance was assessed by measuring allergen-specific isotype levels in sera, RBL assays and T-cell proliferation assays.

Results Mice transplanted with transduced BMC developed multi-lineage molecular chimerism which remained stable long-term (>8 months). After repeated immunizations with Bet v 1 and Phl p 5 serum levels of Bet v 1-specific antibodies (IgE, IgG1, IgG2a, IgG3 and IgA) remained undetectable in Betv1-GFP chimeras while high levels of Phl p 5-specific antibodies developed. Likewise, basophil degranulation was induced in response to Phl p 5 but not to Bet v 1 and specific non-responsiveness to Bet v 1 was observed in proliferation assays.

Conclusions and Clinical Relevance These data demonstrate successful tolerization towards Bet v 1 by molecular chimerism. Stable long-term chimerism was achieved under

inhibition of CD26. These results provide evidence for the broad applicability of molecular chimerism as tolerance strategy in allergy.

Keywords: Molecular chimerism, tolerance, allergy

Introduction

The immune system protects the body from harmful events, but the immune response can become destructive in case of an overwhelming response to otherwise harmless antigens. Such exaggerated immune responses can result in autoimmune diseases or allergy. In case of transplantation, donor-specific immune responses can mediate graft loss. Specific non-responsiveness towards the disease-causing antigens in these settings can be achieved by the induction of chimerism by hematopoietic stem cell transplantation. Successful application of this approach has been shown in mouse models for allotransplantation and autoimmune diseases [1-2].

In case of cellular chimerism, allogeneic hematopoietic stem cells (HSC) are transplanted into an appropriately conditioned host. This strategy has been used to induce donor-specific tolerance and permanent acceptance of allogeneic grafts in transplantation in the mouse model as well as in the clinical setting [3-4]. The remaining toxicity of host preconditioning and the risk of graft versus host disease, however, currently still prevent this strategy from widespread clinical application. Molecular chimerism presents an alternative in which only the disease causing antigens are integrated into (autologous) hematopoietic cells. HSCs, genetically modified to express the antigen(s) of interest, are transplanted in a syngeneic setting. Transplantation of bone marrow cells (BMC) expressing an allogeneic MHC class I led to long-term skin graft acceptance of MHC class I disparate skin grafts [5-6]. Molecular chimerism was also successfully tested in autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), type I diabetes [7-8] and autoimmune gastritis [9]. Induction of disease was prevented and even remission of ongoing disease was achieved in some settings. In EAE, the mouse model for multiple sclerosis, different antigens were used for expression in syngeneic BMC [10-12]. Interestingly, not every protocol was able to induce tolerance in EAE [13].

Type I allergy is caused by IgE-mediated responses towards well defined antigens, making it a technically suitable candidate for molecular chimerism. Recently we successfully applied the concept of molecular chimerism in a mouse model for type I allergy using the grass pollen allergen Phl p 5 [14]. Allergens originate from a broad variety of sources like animal dander, tree pollen or dust mites. The common features of allergens are still largely unknown [15] and there is great diversity in structure and function. Therefore the effectiveness of the molecular chimerism strategy needs to be confirmed with an allergen unrelated to Phl p 5, ruling out that molecular chimerism's effectiveness is limited to certain protein families. Bet v 1 is such a Phl p 5-unrelated protein, belonging to pathogenesis-related protein family 10 [16]. It is the major allergen of the birch pollen of the birch *Betula verrucosa*, located in the cytoplasm of birch pollen grains [17]. It is of particular clinical relevance, as more than 96% of tree pollen allergic individuals recognize Bet v 1, and more than 60% exclusively display Bet v 1-specific IgE [18]. Bet v 1 is also causing symptoms because of the high cross-reactivity to food allergens in soy, cherry, celery and others [16, 19-21]. In regard of its clinical relevance we chose the Phl p 5-unrelated allergen Bet v 1 for our investigations.

In the present study engraftment of transduced cells was supported by inhibition of CD26 which was shown to promote HSC engraftment in other models. Optimized homing would allow transplantation of lower numbers of transduced cells. BMC were pretreated with the CD26 inhibitor (Diprotin A), shown to inhibit CD26 specifically. CD26 would otherwise cleave CXCL12 (SDF-1α) and prevent the interaction with CXCR4 which is critical for homing of BMC [22-23]. Moreover the integrated cDNA of the allergen Bet v 1 was fused to an IRES-eGFP construct to allow better tracking of the transplanted BMC.

Materials and Methods

Animals

Female BALB/c mice were obtained from Charles River (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.

Retroviral constructs and virus production

A retroviral construct was created for transduction of murine cells to express the allergen Bet v 1 in a membrane-anchored fashion. Three different plasmids were used for transfection, MD.G (envelope expression plasmid), encoding for vesicular stomatitis virus G (VSV)-G protein, MLV (murine leukemia virus) encoding for viral proteins gag pol and MMP-Bet v 1-TMD-IRES-eGFP. The plasmid pMMP-Bet v 1-TMD-IRES-eGFP was composed of the Bet v 1 cDNA (accession number X15877) [24], and an IRES-eGFP side flanked by long terminal repeats (LTR). Previously cDNA of Bet v 1 (pMW172 [25]) was fused to a murine Ig signal sequence (S) and a transmembrane domain (TM) (pDisplay, Invitrogen, Carlsbad, CA) by overlapping PCR technique [26]. Overlapping primers were used to fuse the sequences: NcoI restriction site and start ATG fused to S: 5′ GGC G CC ATG GAG ACA GAC ACA CTC CTG-3′; S fused to cDNA of Bet v 1: forward 5′-TCC ACT GGT GAC GGT GTT TTC AAT TAC-3′, reverse 3′-GTA ATT GAA AAC ACC GTC ACC AGT GGA-5′; cDNA of Bet v 1 fused to the TMD: forward 5′-GAT GCC TAC AAC GCT GTG GGC CAG-3′, reverse 3′-CTG GCC CAC AGC GTT GTA GGC ATC-5′; TMD fused to a XhoI restriction site and a stop codon. pMMP-IRES-eGFP was used as control. For virus production 293T (human embryonic kidney cell line) cells were

cotransfected with the three plasmids using calcium phosphate precipitation method [27]. Viral supernatant was concentrated by ultracentrifugation (33620 x g for 2h), replication deficient viruses are named VSV-Bet v 1-GFP and VSV-GFP (control virus). Titers were evaluated by infection of NIH 3T3 (murine fibroblast cell line) cells.

Bone marrow transduction and transplantation

Bone marrow transduction (BMT) was performed as described previously [14, 28]. Briefly, bone marrow of 5-FU-treated BALB/c mice [29] was collected and transduced with VSV-Bet v 1- GFP or control virus VSV-GFP three times with multiplicity of infection of 2.4 and 2.8. One day before BMT, recipients received 8 Gy total body irradiation and a depleting dose of anti-CD8 (2.43; 0.5mg/mouse) and anti-CD4 (GK1.5; 0.5mg/mouse) mAbs [30]. On the day of reconstitution mice were transplanted with $2.5x10^6$ transduced BM cells [31]. For induction of Bet v 1-GFP or GFP chimerism, BMC were additionally incubated with 5mM Diprotin A (Sigma-Aldrich) at 37°C for 15 min [22]. Immediately after BMT mice received anti-CD40L mAb (MR1; 0.5mg/mouse) [30]. All *in vivo* mAbs used *in vivo* were purchased from BioXCell (West Lebanon, NH, USA).

Recombinant allergens and immunizations

Purified recombinant (r) birch pollen allergen (rBet v 1) and timothy grass pollen allergen (rPhl p 5) were obtained from Biomay (Vienna, Austria). All groups of mice were immunized subcutaneously (s.c.) with 5µg rBet v 1 and 5µg rPhl p 5, adsorbed to $Al(OH)_3$ (Alu-Gel-S, Serva, Ingelheim, Germany) [32] at weeks 6, 9, 12, 22 and 37.

Flow cytometric analysis

To detect transduced cells among various leukocyte lineages, white blood cells were stained with PE-conjugated antibodies against CD3, B220, Mac-1, CD45.2 and isotype controls (Abs from BD Pharmingen) and analyzed by flow cytometry (FCM). Two-colour FCM was used to determine the percentage of GFP positive cells of particular lineages. The percentage of transduced cells was calculated as the net percentage of GFP positive cells among leukocyte lineages. To detect Bet v 1 expression on GFP-positive cells, Bet v 1 polyclonal antiserum against rBet v 1 was purified from rabbit serum by a protein G column (Pierce). Polyclonal anti-Bet v 1 IgG was biotinylated and developed with PE streptavidin (PEA, BD Pharmingen). A Beckman Coulter (Werfen, Austria) flow cytometer (FC500) was used for acquisition, and Beckman Coulter CXP software (for FC500) was used for analysis of flow cytometric data.

ELISA

To measure antigen-specific antibodies in the sera of immunized mice ELISAs were performed as described previously [33]. Plates were coated with rBet v 1 or rPhl p 5 (5µg/ml), sera were diluted in PBS/0.05% Tween 1:20 for IgE, 1:100 for IgM, IgA, IgG2a and IgG3 respectively and 1:500 for IgG1. Bound antibodies were detected with monoclonal rat anti-mouse IgM, IgG1, IgE, IgA, IgG2a and IgG3 antibodies (BD Pharmingen) diluted 1:1000 and a HRP-coupled goat anti-rat antiserum (Biosciences, UK) diluted 1:2000. The substrate for HRP was ABTS (60mM/l citric acid, 77mM/l Na_2HPO_4 x $2H_2O$, 1.7mM/l ABTS (Sigma-Aldrich, MO), 3mM/l H_2O_2).

Lymphocyte proliferation assay

Spleens were removed under aseptic conditions and homogenized. Single cell suspensions were filtered through a 70µm nylon cell strainer to remove remaining tissue. Erythrocytes were removed by adding cold lysing buffer (Red Blood Cell Lysing Buffer, Sigma-Aldrich). Cells were diluted to a final concentration of $2x10^6$ cells/ml and triplicates of 100µl/well were seeded in 96-well round-bottom plates. Stimulants were added at a concentration of 2µg/well allergen and 0.5µg/well concanavalin A (Sigma-Aldrich) used as proliferation control. The plates were incubated at 37°C, 5% CO_2, on day 4, cells were pulsed with 0.5µCi H^3 thymidine ([methyl-3H] thymidine, Amersham) per well. Sixteen hours later cells were harvested and thymidine uptake was measured in a beta counter (Beta scintillation liquid, Wallac).

Rat basophil leukaemia (RBL) cell degranulation assay

RBL-2H3 cell subline was cultured as described previously [14]. Aliquots of $4x10^4$ cells/well were plated in 96-well tissue culture plates (Greiner, Bio-One, Germany), loaded with 1:50 diluted mouse sera in completed RPMI medium and incubated for 2 hours at 37°C and 5% CO_2. Supernatants were removed and the cell layer was washed with 2x Tyrode´s buffer (137mM NaCl, 2.7mM KCL, 0.5mM $MgCl_2$, 1.8mM $CaCl_2$, 0.4mM NaH_2PO_4, 5.6mM D-glucose, 12mM $NaHCO_3$, 10mM HEPES and 0.1% w/v BSA, pH 7.2). Preloaded cells were stimulated with rPhl p 5 or rBet v 1 (0.03µg per well) for 30 min at 37°C. Supernatants were analyzed for ß-hexosaminidase activity by incubation with the substrate 80µM 4-methylumbelliferyl-N-acetyl-ß-D-glucosamide (Sigma-Aldrich) in citrate buffer (0.1M, pH4.5) for 1 hour at 37°C. The reaction was stopped by addition of 100µl glycine buffer (0.2M glycine, 0.2M NaCl, pH 10.7) and the fluorescence was measured at λ_{ex}:360/λ_{em}:465nm using a fluorescence microplate reader (Wallac, Perkin

Elmer, Vienna, Austria). Results are reported as percentage of total ß-hexosaminidase released after addition of 1% Triton X-100. Determinations were done in triplicates and are displayed as mean values with standard deviations (SD).

Immunofluorescence microscopy

Transduced NIH 3T3 cells were grown on gelatine coated glass slides. Cells were washed twice with PBS, fixed for 10 min with 3% paraformaldehyde and incubated with antiserum of rabbits immunized with rBet v 1 and rPhl p 5 were diluted 1:200 and stained with fluorochrome-conjugates as secondary antibodies (goat α-rabbit IgG Alexa-Fluor 546nm, Invitrogen, Oregon, USA). Samples were treated with ProLong Gold antifade reagent (Molecular probes, Invitrogen, Oregon, USA). Samples were analyzed with confocal laser scanning microscope LSM 510 Meta (Zeiss, Jena, Germany).

Statistical analysis

Mean, SD and standard error of the mean (SEM) were calculated for statistical documentation. P values were calculated with an unpaired two tailed T-test. Values of $p < 0.05$ were considered as statistical significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Surface expression of Bet v 1 in mammalian cells

For production of retroviral particles the fusion gene S-Bet v 1-TMD was cloned into a viral vector (pMMP-IRESeGFP) containing an IRESeGFP site flanked with long terminal repeats, resulting in pMMP-Bet v 1-TMD-IRESeGFP. pMMP-IRESeGFP without the transgene was used as control vector (Fig. 1a). Viral particles were produced by cotransfection with pMLV, pMD.G and pMMP-Bet v 1-TMD-IRESeGFP or control pMMP-IRESeGFP in 293T cells resulting in VSV-Bet v 1-GFP and VSV-GFP. Co-expression of Bet v 1 and GFP was confirmed after transduction of NIH 3T3 with VSV-Bet v 1-GFP by flow cytometry (data not shown). Moreover localization of Bet v 1 was analyzed by immunofluorescence microscopy and clearly showed surface-expression of Bet v 1 and cytoplasmic co-expression of GFP (Fig. 1b). As control NIH 3T3 cells were transduced with VSV-Phl p 5 and stained with a Phl p 5-specific rabbit antiserum (Fig. 1b) [14].

Induction of molecular chimerism in BMC chimeras

Based on our previous work that long-term molecular chimerism can be established with modified BMC [14], we examined the induction of chimerism by transplantation of VSV-Bet v 1-GFP transduced BMC. Since GFP is co-expressed with Bet v 1 (Fig. 1b), GFP was used as a surrogate marker for Bet v 1 expression in flow cytometry when analyzing levels of Bet v 1-chimerism. *In vitro* transduction of BMC with VSV-Bet v 1-GFP virus led to 4.5% GFP-positive cells (transduction with VSV-GFP control virus led to 25.6% positive cells) (Fig. 2a). Transduced cells were additionally treated with Diprotin A (a CD26 inhibitor) prior to transplantation which was described to enhance homing and engraftment of BMCs [22]. To avoid rejection of modified transplanted cells, recipients were irradiated

(8 Gy total body irradiation), T cell depleted (anti-CD4, anti-CD8, 0.5mg/mouse) and treated with the costimulation blocker anti-CD40L (0.5mg/mouse) (Fig. 2b). Preconditioned recipients received $2.5x10^6$ transduced BMCs [31].

Chimerism was successfully induced in all recipients (4/4 Bet v 1-GFP chimeras, 4/4 GFP chimeras). Mean leukocyte chimerism of Bet v 1-GFP (% GFP^+) was 3%, 15 weeks post BMT and remained relatively stable throughout the follow-up of 35 weeks (Fig. 2c). Moreover, chimerism was of multi-lineage nature and as it was detected in T cells ($CD3^+$), B cells ($B220^+$) and myeloid cells (Mac-1^+). Although the transduction efficiency was low in the Bet v 1-GFP chimeric group, the modified allergen-expressing cells were detectable in FCM through the whole follow-up (Fig. 2c). Chimerism was higher in the group that received GFP- transduced BMCs (e.g. leukocyte chimerism 19%, 15 weeks post BMT, Fig. 2d) correlating with the higher transduction efficiency (~25%). The experiment was terminated at week 40, at this time point cells in spleen ($B220^+$, $CD3^+$, $CD45.2^+$) and bone marrow ($B220^+$, Mac-1^+, $CD45.2^+$) were subjected to FCM analysis. GFP positive cells were found in every tested lineage in both spleen and BM in recipients of both groups (GFP positive leukocytes in spleens of Bet v 1-GFP chimeric mice: 1.4%, 1.8%, 19.5%, 4.2% and GFP chimeric mice: 33.6%, 26.6%, 31.2%, 27.4%).

These data provide evidence that transplantation of Diprotin A-treated Bet v 1-GFP-transduced BM leads to long-term molecular chimerism. Chimerism remained remarkably stable over time while chimerism levels declined in our previous study without CD26 inhibition [14].

Molecular chimerism prevents Bet v 1-specific antibody production

BALB/c mice were repeatedly immunized with $Al(OH)_3$ adsorbed rBet v 1 and rPhl p 5 as specificity control (weeks 6, 9, 12, 22 and 37 after BMT) **(Fig. 2b)**. To assess the humoral response to these two allergens, immunoglobulin isotypes IgE, IgA, IgM and IgG subtypes were measured in a Bet v 1-specific ELISA (Fig. 3 left column) or Phl p 5-specific ELISA (specificity control; Fig. 3 right column). Non-BMT immunized mice and GFP chimeric mice, produced Bet v 1 specific IgE and Phl p 5-specific IgE after immunization with the recombinant allergens (w9 to w40). In contrast, Bet v 1-GFP chimeras failed to produce IgE over the whole follow-up even after repeated immunizations. The specificity of unresponsiveness is shown by Phl p 5-specific ELISAs, in which Bet v 1-GFP chimeric mice were able to produce IgE in response to the control allergen Phl p 5. Likewise, no Bet v 1-specific IgG1 could be detected in Bet v 1-GFP chimeric mice in contrast to the control groups (Fig. 3b). Moreover IgG2a, IgG3 and IgA levels of Bet v 1-specific antibodies were not detectable in Bet v 1-GFP chimeric mice (Fig. 3c, d, e). Bet v 1-specific IgM was elevated in Bet v 1-GFP chimeric mice after BMT (in contrast to control group and GFP chimeras), however levels of IgM remained constant in spite of repeated allergen challenge (Fig. 3f). A similar pattern was also observed in our previous study transducing Phl p 5. In general control mice without BMT (Fig. 3, white bars) showed a more rapid allergen-specific humoral response than BMT controls, probably due to the toxicity of BMT protocol. These results demonstrate that long-term stable tolerance was established at the humoral level in molecular chimeras.

Basophils, loaded with sera of Bet v 1-GFP chimeric mice degranulate only in response to the control allergen Phl p 5 but not to Bet v 1

Tolerance on the effector cell level was assessed in rat basophilic leukaemia cell (RBL) assay. ß-Hexosaminidase release of basophils triggered by sera of Bet v 1-GFP chimeric mice was significantly lower in response to rBet v 1 compared to sera of GFP chimeric mice or immunized mice that did not receive BMT (control group; Fig. 4a). Sera of Bet v 1-GFP chimeric mice were still able to mount ß-hexosaminidase release in response to rPhl p 5 (Fig. 4b). On the contrary, sera of control mice and GFP chimeric mice were able to induce ß-hexosaminidase release in response to both allergens, rBet v 1 and rPhl p 5. Thus allergen-specific tolerance was induced also at the effector cell level

T cell tolerance in Bet v 1 chimeras

To test tolerance on the T cell level proliferation assays were performed at week 40 (Fig. 5). Unseparated splenocytes were stimulated with rBet v 1 and rPhl p 5. Splenocytes of Bet v 1-GFP chimeric mice (Fig. 5, black bars) proliferated in response to rPhl p 5 (Fig. 5b) but not in response to rBet v 1 (Fig. 5a). Splenocytes of GFP chimeric mice (Fig. 5, grey bars) showed proliferation in response to both allergens. Hence, Bet v 1-specific T cell tolerance is induced in Bet v 1-GFP chimeric mice.

Discussion

In a proof-of-principle study performed with the major timothy grass pollen allergen Phl p 5, we have previously shown that molecular chimerism is a possible strategy for prevention of IgE-mediated allergy through induction of durable tolerance [14]. Given the available literature that molecular chimerism is not universally successful but fails to induce tolerance towards some antigens [13], we felt it necessary to investigate whether molecular chimerism is capable of tolerizing an unrelated allergen. In the current study, tolerance towards the allergen Bet v 1 was established. Our results provide evidence that molecular chimerism is working also for different types of allergen families.

To promote engraftment of transduced cells – which only accounted for 4.5% of total BMCs in the current study – we used Diprotin A (CD26 inhibitor). Diprotin A is described to enhance homing and engraftment even of low numbers of transduced BMC [22]. The beneficial effect was demonstrated by transplanting limited numbers of HSC which were retrovirally transduced with an allogeneic MHC class I gene and transplanted into lethally treated mice. The results of the current study show that multilineage chimerism was successfully induced over a long-term follow-up despite low transduction efficiency, underlining the fact that retrovirally transduced stem cells engrafted [22]. Only 4.5% transduced cells of $2.5x10^6$ BMC were sufficient to induce stable chimerism when engraftment was enhanced by Diprotin A treatment. In fact, chimerism was more stable than in previous experiments without Diprotin A.

Investigation of levels of different immunoglobulin isotypes showed successful induction of tolerance on the B cell level. Production of IgE was successfully suppressed over the whole follow-up, likewise no Bet v 1-specific production of IgG1, IgG2a, IgG3 and IgA

was detected after BMT treatment or repeated allergen challenge. IgM levels, however, were elevated after transplantation of Bet v 1-transduced BMC, even before immunization with recombinant Bet v 1. These antibodies had no detectable untoward effect on the longterm outcome as chimerism remained stable. Interestingly, this phenomenon has also occurred in our previous experiments with Phl p 5-transduced BM [14], in which only limited time points were analyzed. Here, we show that IgM levels rise after BMT independent of immunization. Likely, IgM production is due to a T cell-independent response which is induced by BMT and surface expression of an allergen. Notably, elevated levels of allergen-specific IgM were not observed in chimerism studies investigating the cytoplasmatic (as opposed to cell surface) expression of an allergen (Baranyi et al, manuscript submitted). Notably, no class switch was induced after contact with the Bet v 1-positive BM or recombinant allergen indicating that T cell help was suppressed.

For clinical translation of this tolerizing protocol, several hurdles need to be overcome, including the toxicity of irradiation, the hazards currently associated with retroviral transduction and the risk of anaphylaxis. The development of minimally toxic conditioning regimens is a major focus of research in the area of cellular chimerism [1, 34]. Irradiation-free protocols have recently become feasible even for the transplantation of MHC-mismatched BMT and should thus be an attainable goal for allergen-transduced BM as well. Current concerns regarding the proto-oncogenic potential of retroviral transduction of HSCs could be alleviated in the future through the development of advanced transduction techniques [35]. Anaphylaxis can be avoided through the use of hypoallergenic derivatives for transduction [36].

While many problems still have to be solved, induction of tolerance is a much needed goal for the treatment of allergies [37]. Molecular chimerism is a promising approach in this context. Its potential has been shown in many different mouse models [38]. Moreover, clinical observations of tolerance induction through cellular chimerism in transplant patients underline the potential of chimerism as future treatment strategy [3, 39].

In conclusion, the present study supports the further development of the molecular chimerism approach for tolerance in IgE-mediated allergy.

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Figure Legends

Figure 1: Vector constructs and localization of Bet v 1 in the murine cell line, NIH 3T3.

(a) Schematic drawing of pMMP-based retroviral vector encoding Bet v 1 fused to a signal peptide (S) and a transmembrane domain (TMD), resulting in S-Bet v 1-TMD construct. The start codon was inserted with a NcoI site, the stop codon was inserted immediately after the XhoI site and fused to IRESeGFP, resulting in MMP-Bet v 1-TMD-IRESeGFP. The control vector, MMP-IRESeGFP is shown below. LTR, long terminal repeat, SD splicing donor, SA splicing acceptor. **(b)** Immunofluorescence of transduced NIH 3T3 cells, expressing Bet v 1 on the cell surface and GFP in the cytoplasm (upper image) or Phl p 5 on the surface of cells (lower image). Allergens are shown in red.

Figure 2: Transduction efficiency of BMCs and stable long-term chimerism.

(a) Transduction efficiency of infected BMC was analyzed by GFP expression in FCM. On the left side VSV-Bet v 1-GFP infected BMC are shown, exhibiting a transduction efficiency of 4.5% and on the right side a histogram of the control group is shown, where 25% of VSV-GFP infected cells were transduced. Black lines in the histograms represent non-transduced cells **(b)** Experimental design of the *in vivo* experiment. Mice were treated with anti-CD4 mAb (0.5mg, d -1), anti-CD8 mAb (0.5mg, d -1) and total body irradiation (8 Gy on d -1). Transduced BMCs were transplanted on day 0, BM recipients additionally received anti-CD40L (MR1, 0.5mg, d 0). Mice were immunized with rBet v 1 and rPhl p 5 (specificity control) starting 6 weeks after BM reconstitution and challenged 9, 12, 22 and 37 weeks after BMT**.** Stable multilineage chimerism was detected by GFP expression in FCM analysis of Bet v 1-GFP chimeric mice **(c)** and GFP chimeric mice **(d)** every 5 weeks over a follow-up of 40 weeks. Mean multilineage chimerism was analyzed in B cells

(B220), T cells (CD3), myeloid lineages (Mac1) and whole lymphocyte population (CD45.2). Mean values of 4 mice/group are shown.

Figure 3: Suppressed Bet v 1-specific antibody production in Bet v 1-GFP chimeric mice

Antibody isotypes were detected by ELISA, 6 different time points are shown. Left side columns show Bet v 1-specific antibody levels and right columns show Phl p 5-specific antibody levels. Three groups of mice were tested: immunized mice without BMT (n = 5, □), GFP chimeric mice (n = 4, ▨) and Bet v 1-GFP chimeric mice (n = 4, ■). Week 0 represents baseline antibody levels of mice previously to treatment; week 6 shows antibody levels after BMT and week 9, 12, 22, and 40 show the ongoing development. OD values corresponding to allergen-specific levels of **(a)** IgE, **(b)** IgG1, **(c)** IgG2a, **(d)** IgG3, **(e)** IgA and **(f)** IgM are shown (y-axes). Data shown represent mean values with SD obtained from 4 to 6 mice, significant statistical differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) are indicated.

Figure 4: Tolerance towards the allergen Bet v 1 on the B cell level

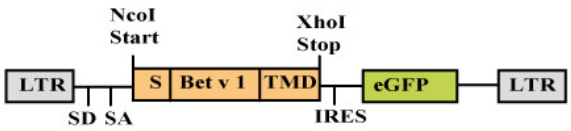
ß-Hexosaminidase release of rat basophilic leukaemia cells coated with serum of the different groups (n=4) in response to rBet v 1 **(a)** or rPhl p 5 **(b)** was analyzed. Three different groups were tested, immunized mice without BMT (n = 5, □), GFP chimeric mice (n = 4, ▨) and Bet v 1-GFP chimeric mice (n = 4, ■). Data shown are the mean values with SD obtained from 4 to 6 mice per group, significant statistical differences (* $p < 0.05$) are indicated.

Figure 5: Tolerance towards the allergen Bet v 1 on the T cell level

Splenocyte proliferation assays were performed 40 weeks after BMT, immunized mice without BMT mice were used as controls (n = 4, □). GFP chimeric mice (n = 4, ▢) are shown in grey and Bet v 1-GFP chimeric mice (n = 4, ■) in black. Cpm values in response to Bet v 1 are shown in **(a)** and to Phl p 5 in **(b)**. Data shown are the mean values with standard error of the mean (SEM) obtained from 4 mice per group.

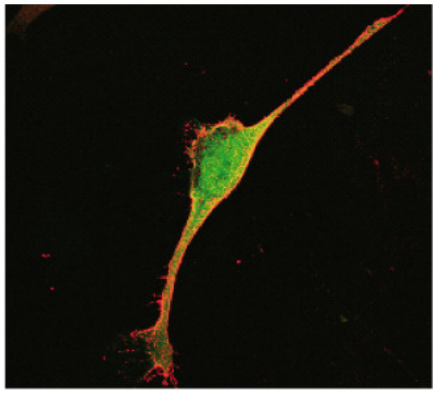
Fig. 1

(a) MMP-Bet v 1-TMD-IRESeGFP



MMP-IRESeGFP

(b) VSV-Bet v 1-GFP transduced cells



VSV-Phl p 5 transduced cells

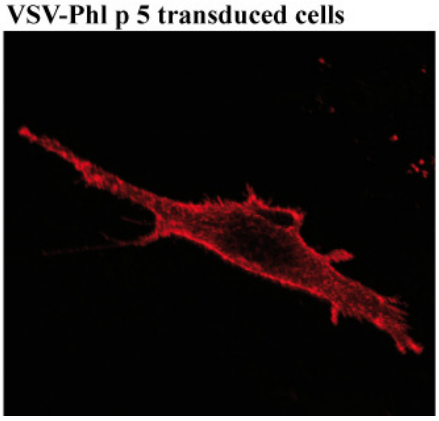
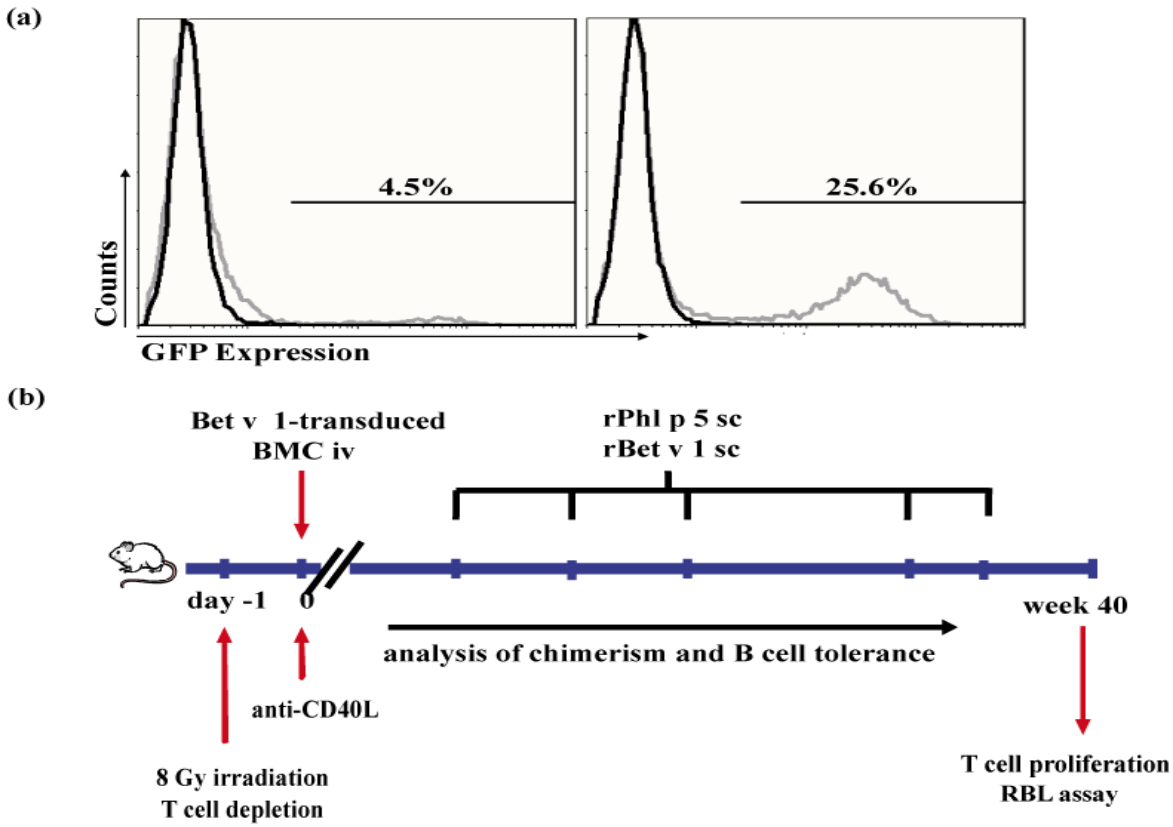
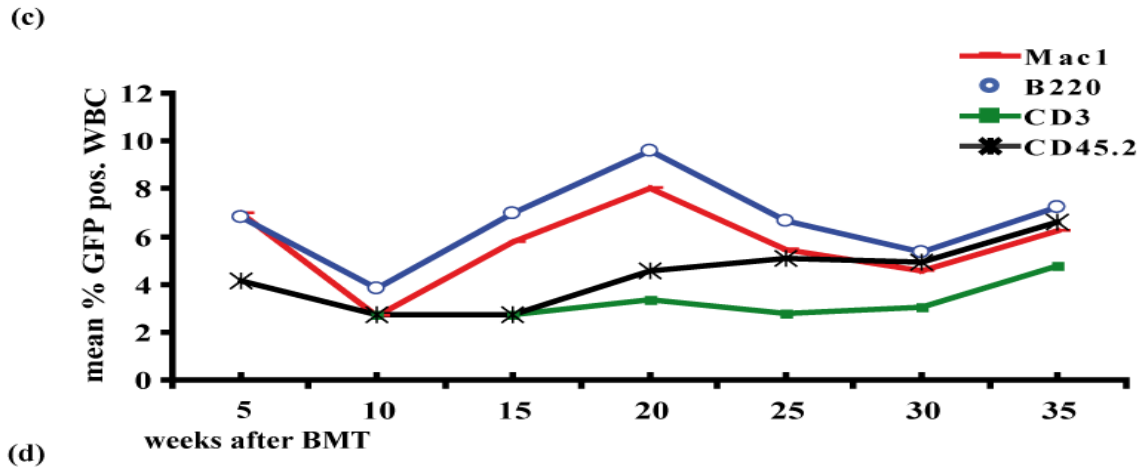


Fig. 2


(a)
4.5%
25.6%
Counts
GFP Expression
(b)
Bet v 1-transduced
BMC iv
rPhl p 5 sc
rBet v 1 sc
day -1
0
week 40
analysis of chimerism and B cell tolerance
anti-CD40L
8 Gy irradiation
T cell depletion
T cell proliferation
RBL assay


(c)
Mac1
B220
CD3
CD45.2
mean % GFP pos. WBC
weeks after BMT

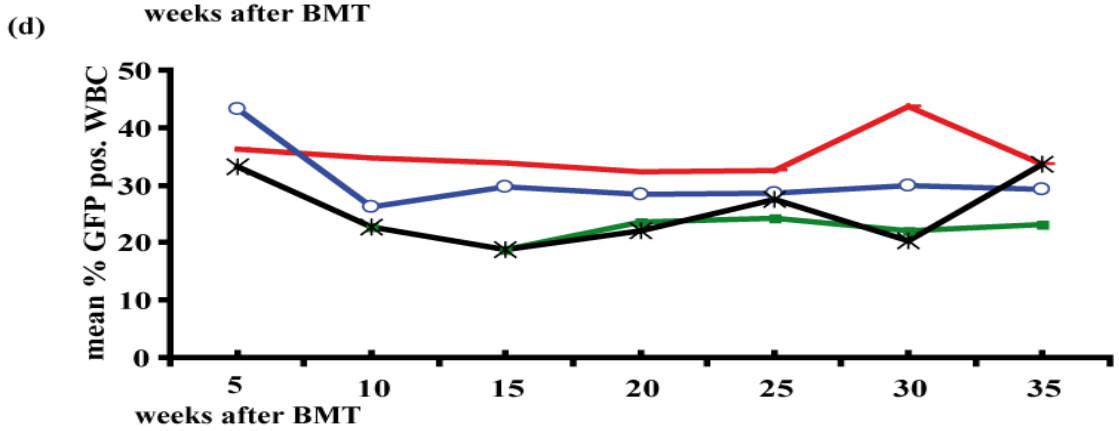

(d)
mean % GFP pos. WBC
weeks after BMT

Fig. 3

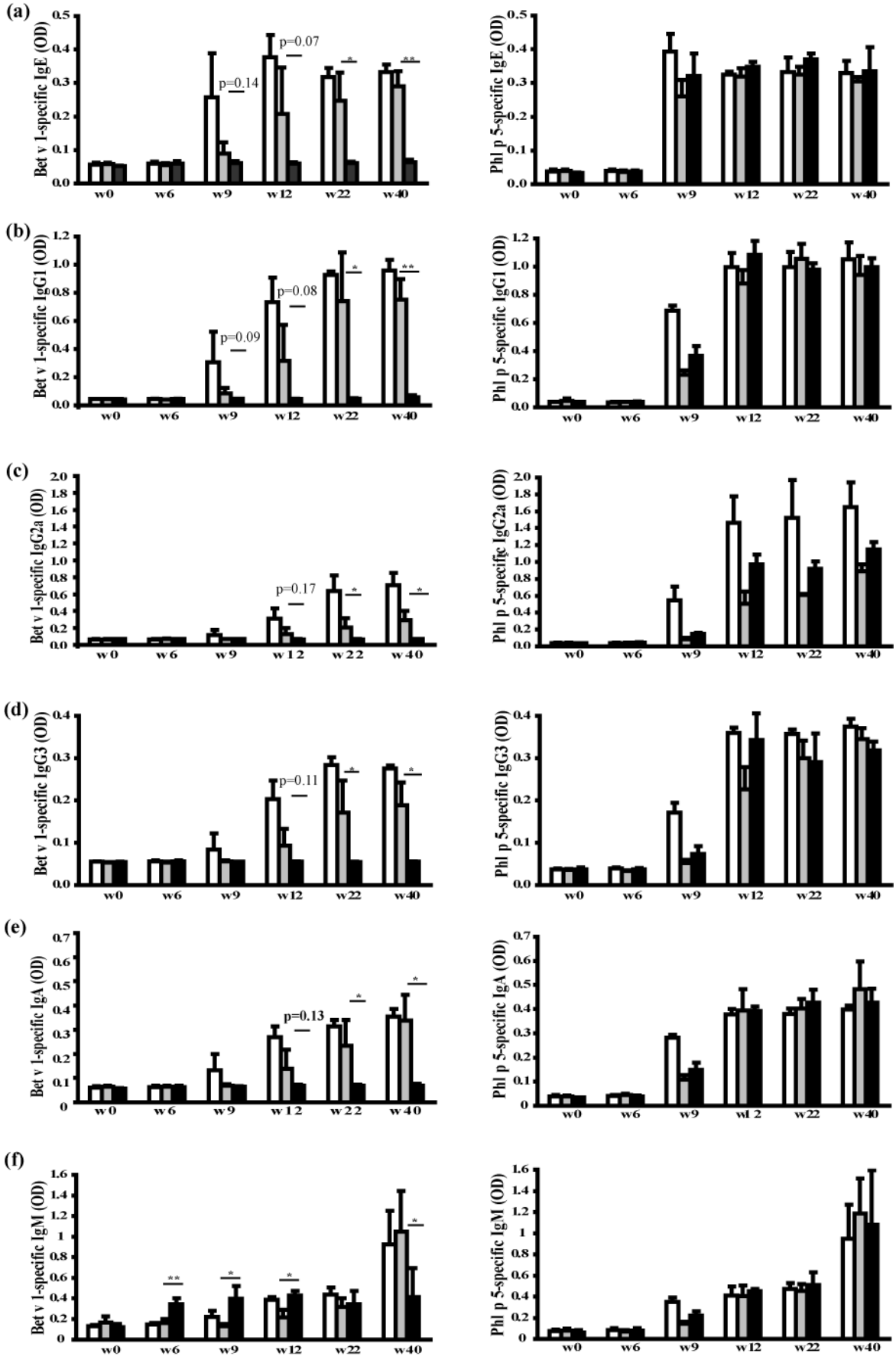

(a)
Bet v 1-specific IgE (OD)
p=0.14
p=0.07
*
**
Phl p 5-specific IgE (OD)
(b)
Bet v 1-specific IgG1 (OD)
p=0.09
p=0.08
*
**
Phl p 5-specific IgG1 (OD)
(c)
Bet v 1-specific IgG2a (OD)
p=0.17
*
*
Phl p 5-specific IgG2a (OD)
(d)
Bet v 1-specific IgG3 (OD)
p=0.11
*
*
Phl p 5-specific IgG3 (OD)
(e)
Bet v 1-specific IgA (OD)
p=0.13
*
*
Phl p 5-specific IgA (OD)
(f)
Bet v 1-specific IgM (OD)
**
*
*
*
Phl p 5-specific IgM (OD)
w0
w6
w9
w12
w22
w40

Fig. 4

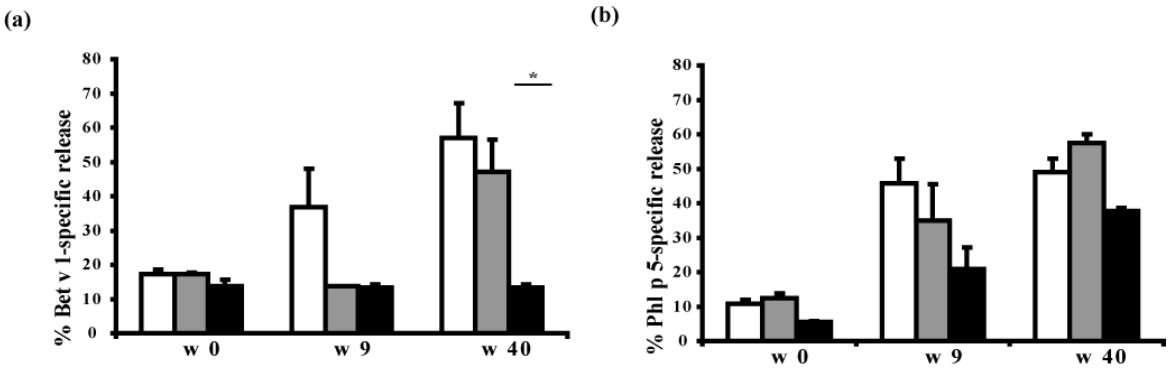
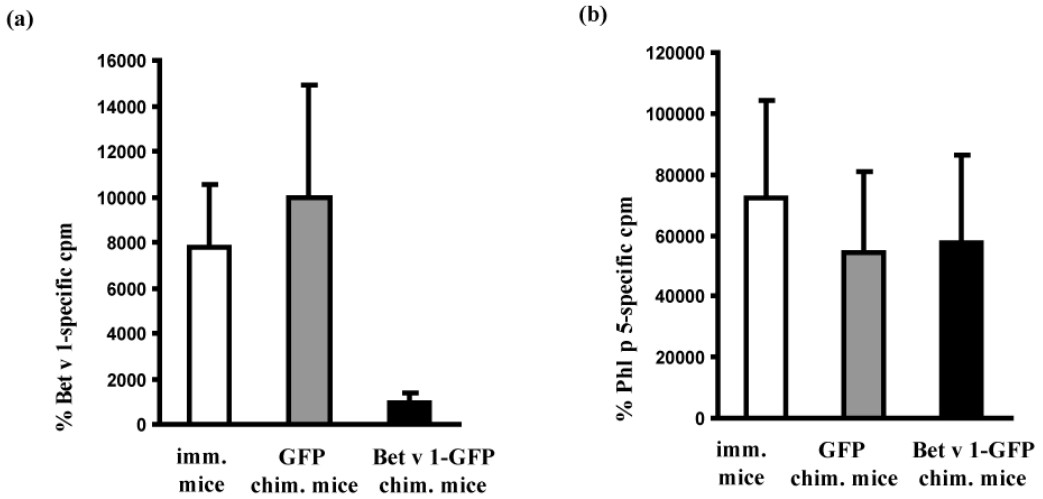

(a)
% Bet v 1-specific release
80
70
60
50
40
30
20
10
0
*
w 0
w 9
w 40
(b)
% Phl p 5-specific release
80
70
60
50
40
30
20
10
0
w 0
w 9
w 40

Fig. 5


(a)
% Bet v 1-specific cpm
16000
14000
12000
10000
8000
6000
4000
2000
0
imm. mice
GFP chim. mice
Bet v 1-GFP chim. mice
(b)
% Phl p 5-specific cpm
120000
100000
80000
60000
40000
20000
0
imm. mice
GFP chim. mice
Bet v 1-GFP chim. mice

5.1.3 Persistent Molecular Microchimerism Induces Long-Term Tolerance towards a Clinically Relevant Respiratory Allergen

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Running title: Persistent low level molecular chimerism induces tolerance in allergy

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Abstract

Back*ground* Development of antigen-specific preventive strategies is a challenging goal in IgE-mediated allergy. We have recently shown in proof-of-concept experiments that allergy can be successfully prevented by induction of durable tolerance via molecular chimerism. Transplantation of syngeneic hematopoietic stem cells genetically modified to express the clinically relevant grass pollen allergen Phl p 5 into myeloablated recipients led to high levels of chimerism (i.e. macrochimerism) and completely abrogated Phl p 5-specific immunity despite repeated immunizations with Phl p 5.

Objective It was unclear, however, whether microchimerism (drastically lower levels of chimerism) would be sufficient as well which would allow development of minimally toxic tolerance protocols.

Methods Bone marrow cells were transduced with recombinant viruses integrating Phl p 5 to be expressed in a membrane-anchored fashion. The syngeneic modified cells were transplanted into non-myeloablated recipients that were subsequently immunized repeatedly with Phl p 5 and Bet v 1 (control). Molecular chimerism was monitored by flow cytometry and PCR. T-cell, B-cell and effector-cell tolerance was assessed by allergen-specific proliferation assays, isotype levels in sera and RBL assays.

Results Here we demonstrate that transplantation of Phl p 5-expressing bone marrow cells into recipients having received non-myeloablative irradiation resulted in chimerism persisting for the length of follow-up. Chimerism levels, however, declined from transient macrochimerism levels to persistent levels of microchimerism (followed for 11 months). Notably, these chimerism levels were sufficient to induce B-cell tolerance as no Phl p 5-specific IgE and other high affinity isotypes were detectable in sera of chimeric mice. Furthermore T-cell and effector-cell tolerance were achieved.

Conclusion and Clinical Relevance Low levels of persistent molecular chimerism are

sufficient to induce long-term tolerance in IgE-mediated allergy. These results suggest that it will be possible to develop minimally toxic conditioning regimens sufficient for low level engraftment of genetically modified bone marrow.

Key words: Molecular chimerism, allergen Phl p 5, membrane-expression, T-cell tolerance, B-cell tolerance,

Introduction

IgE-mediated allergy is a hypersensitivity disease and a growing problem in developed countries. Development of antigen-specific therapeutic and preventive strategies implies fundamental knowledge of antigens causing allergy (i.e. allergens). In the last decades a variety of allergens has been cloned, characterized and three-dimensional structures have been decoded [1, 2]. Currently, the only antigen-specific, disease-modulating and long-lasting treatment can be provided by allergen-specific immunotherapy (SIT). An obvious risk of SIT using crude allergen-containing extracts is that of provoking a systemic allergic reaction which may lead to fatalities [3, 4]. Therefore *prevention* would be a desirable goal especially for individuals with high risk. It remains an unmet challenge to develop tolerance strategies that avoid the occurrence of pathogenic immunological responses towards allergens. Although some prophylactic approaches such as mucosal tolerance have been explored [5] additional strategies are necessary to avoid T cell priming and antibody production (mainly IgE).

Molecular chimerism is a strategy to induce tolerance by transplantation of autologous (or syngeneic in experimental models) hematopoietic stem cells (HSC) genetically modified to express the disease-causing antigen [6, 7]. This approach has been demonstrated in predominantly T_H1-dependent models of organ transplantation as well as in selected autoimmune disease models [8, 9]. Induction of T cell tolerance has been demonstrated in several studies in molecular chimerism suggesting central T cell tolerance and peripheral regulation [10-14]. Nevertheless, only few studies in molecular chimerism exist regarding humoral responses towards the introduced antigens [15, 16]. In a proof-of-concept study we showed for the first time that molecular chimerism is a promising tool for prevention of IgE-mediated allergy [17]. However, intense preconditioning of recipients [i.e.

myeloablative total body irradiation (TBI)] is a major hurdle for clinical application.

The degree of conditioning (e.g. the dose of irradiation) directly correlates with the ensuing chimerism levels. However, the minimum level of chimerism necessary to induce tolerance still remains unclear. Donor bone marrow transplantation (BMT) into a conditioned host establishing mixed cellular chimerism (denoting a state of macrochimerism with a donor fraction >1% <100 %) reliably leads to immunological tolerance towards a donor allograft in several settings including clinical pilot trials [18-23]. In contrast, the spontaneous persistence of "passenger" leukocytes after organ transplantation (i.e. microchimerism), seems to have no causal role in graft acceptance [24, 25]. Moreover, in experimental models microchimerism is not sufficient for tolerance induction towards MHC-mismatched grafts [26, 27]. Contrarily, microchimerism can lead to the tolerization of certain antigens (i.e. viral antigens) [28, 29].

In the study presented herein we investigated if both, B cell- and T cell-tolerance towards a clinically relevant major allergen can be achieved with a reduced-intensity BMT protocol which results only in low molecular chimerism.

Materials and Methods

Animals

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.

Retroviral constructs and production of retroviruses

To generate membrane-anchored Phl p 5, full length Phl p 5 was fused to a signal sequence and a transmembrane domain (TMD) (both pDisplay, Invitrogen, Carlsbad, CA) by overlapping PCR technique as described in Baranyi et al [17] As control vector, the membrane-anchored GFP was fused to a signal sequence and a transmembrane domain by overlapping PCR technique as described above. Primer sequences are as follows: leader peptide: 5'-GGCGCCATGGAGACAGACACACTCCTG-3', 5'-CTTGCTCACGTCACCAGT-3' eGFP: 5'-ACTGGTGACGTGAGCAAG-3', 5'-GCCCACAGCTCTAGATCC-3'

TMD: 5'-GGATCTAGAGCTGTGGGC-3', 5'-CCGGCCTCGAGCTAACGTGGCTTCTTCTG-3'

PCR product was cloned into the retroviral vector pMMP NcoI and Xho I sites resulting in pMMP-GFP-TM. The start codon was inserted with the Nco I site, the stop codon was inserted with the Xho I site. For virus production plasmids pMMP-Phl p 5-TM or pMMP-eGFP-TM, VSV-G protein and pMLV, encoding for viral proteins gag and pol, were co-transfected using the calcium phosphate method into 293 T cells resulting in VSV-Phl p 5-TM or VSV-GFP-TM viruses [30]. Viral supernatants were concentrated by ultracentrifugation (16500 rpm 2h).

Retroviral transduction of bone marrow cells

BALB/c donor mice were injected i.p. with 5-fluorouracil (150mg/KG) 7 days before BM isolation. Mice were sacrificed and BM was harvested from tibiae, femurs, humeri and pelvis [31]. BM cells were cultured and transduced with VSV-Phl p 5-TM or transduced with VSV-GFP-TM as described in [17] with a multiplicity of infection (MOI) of 5 [32].

Bone marrow transplantation

One day before BMT, recipients received 6 Gy total body irradiation (TBI) and a depleting dose of anti-CD8 (2.43; 0.5mg/mouse) and anti-CD4 (GK1.5; 0.5mg/mouse) monoclonal antibodies (mAb). On the day of reconstitution mice were transplanted with $4x10^6$ transduced BM cells i.v. After BMT mice received anti-CD40L mAb (MR1; 0.5mg/mouse). Anti-CD4, anti-CD8 and anti-CD40L were used as they were shown to enhance engraftment of transduced BM. All mAb used *in vivo* were purchased from BioXCell (West Lebanon, NH).

Recombinant allergens and immunization of mice

Purified recombinant (r) timothy grass pollen and birch pollen allergens (rPhl p 5, rBet v 1) were obtained from Biomay (Vienna, Austria). All groups of mice were immunized s.c. with 5 µg rPhl p 5 and 5µg rBet v 1 adsorbed to aluminum hydroxid (Alu-Gel-S, Serva, Ingelheim, Germany) as described previously [33].

Flow cytometric analysis

Non-specific Fcγ receptor binding was blocked with mAb against mouse FcγII/III receptor (CD16/CD32). Phl p 5 polyclonal antiserum against full length rPhl p 5 was purified from rabbit serum (Charles River) by a protein G column (Pierce, Rockford, USA) according to

the manufacturer's instructions. Polyclonal anti-Phl p 5 IgG was biotinylated and developed by counterstaining with phycoerythrin streptavidin. To detect Phl p 5^+-expressing cells among various leukocyte lineages white blood cells were stained with FITC-conjugated antibodies against CD4, CD8, B220, Mac-1 and isotype controls (all antibodies from Pharmingen, San Diego, CA) and analyzed by flow cytometry. Propidium iodide staining was used to exclude dead cells. Two-color flow cytometric analysis was used to determine the percentage of Phl p 5-expressing cells of particular lineages. The percentage of Phl p 5^+ cells (i.e. molecular chimerism) was calculated by subtracting control staining from quadrants containing Phl p 5^+ and Phl p 5 negative cells expressing a particular lineage marker, and by dividing the net percentage of Phl p 5^+ cells by the total net percentage of Phl p 5^+ plus Phl p 5 negative cells of that lineage as described in [17]. An Cytomics FC500 flow cytometer (Coulter Werfen, Austria) was used for acquisition and the CXP software (Coulter Werfen, Austria) was used for analysis of flow cytometric data.

Isolation of genomic DNA and detection of Phl p 5-specific products

Genomic DNA of splenocytes was isolated as described in [29]. A 250-bp Phl p 5-specific product was amplified and sequenced (for confirmation) using primers: Phl p 5 fw: 5'-CTGCAGGTCATCGAGAAGGT-3', Phl p 5 rev: 5'-TTTCAGTGCGGTCTCAAAGA-3', ß-actin fw: 5'-TGGAAATCCTGTGGCATCCATGAAAC-3'. ß-actin specific primers: ß-actin rev. 5'-TAAAACGCAGCTCAGTTACAGTCCG-3'. PCR products were separated on a 5% Acrylamidgel (Bio-Rad) in 1xTBE and visualized by EthBr.

ELISA

To measure allergen-specific antibodies in the sera of immunized mice ELISAs were

performed as described previously [34]. Plates were coated with rPhl p 5 (5µg/ml), sera were diluted 1:20 for IgE, 1:100 for IgM, IgA, IgG2a and IgG3 respectively and 1:500 for IgG1. Bound antibodies were detected with monoclonal rat anti-mouse IgM, IgG_1, IgE, IgA, IgG_{2a} and IgG_3 antibodies (Pharmingen, San Diego, CA) diluted 1:1000 and a HRP-coupled goat anti-rat antiserum (Amersham, Biosciences, UK) diluted 1:2000. The substrate for HRP was ABTS (60mM/l citric acid, 77mM/l Na_2HPO_4 x $2H_2O$, 1.7mM/l ABTS [Sigma, St. Lois, MO.], 3mM/l H_2O_2).

Lymphocyte proliferation assay

Spleens were removed under aseptic conditions (week 44) homogenized and erythrocytes were lysed. Suspended splenocytes were plated into 96-well round-bottom plates at a concentration of $2x10^5$ cells / well in triplicates and stimulated with concanavalin A (Con A; 0.5µg/well, Sigma), rPhl p 5 (2µg/well) and rBet v 1 (2µg/well). On day 5 cultures were pulsed with 0.5µCi/well [^{3}H]thymidine (Amersham, Biosciences, UK) and harvested approximately 16 hours thereafter. The proliferative response was measured by scintillation counting. The stimulation index (SI) was calculated as the ratio of the mean proliferation after allergen stimulation and medium control values.

Rat basophil leukaemia (RBL) cell degranulation assay

RBL-2H3 cell subline was cultured as described previously, in RPMI 1640 medium (Biochrome AG, Berlin, Germany) containing 10 % fetal calf serum. Aliquots of $4x10^4$ cells were plated in 96 well tissue culture plates (Greiner, Bio-One, Stuttgart, Germany), loaded with 1:50 diluted mouse sera and incubated for 2 hours at 37°C and 5 % CO2. Supernatants were removed and the cell layer was washed with 2x Tyrode´s buffer (137 mM NaCl, 2.7 mM KCL, 0.5mM $MgCl_2$, 1.8mM $CaCl_2$, 0.4mM NaH_2PO_4, 5.6mM D-glucose, 12mM $NaHCO_3$, 10mM HEPES and 0.1 % w/v BSA, pH 7.2). Preloaded cells

were stimulated with optimal concentrations of rPhl p 5 or rBet v 1 (i.e., 0.03µg per well) for 30 min. at 37°C. The supernatants were analyzed for ß-hexosaminidase activity by incubation with the substrate 80µM 4-methylumbelliferyl-N-acetyl-ß-D-glucosamide (Sigma-Aldrich, Vienna, Austria) in citrate buffer (0.1M, pH4.5) for 1 hour at 37°C. The reaction was stopped by addition of 100µl glycine buffer (0.2M glycine, 0.2M NaCl, pH 10.7) and the fluorescence was measured at λ_{ex}: 360/λ_{em}: 465nm using a fluorescence microplate reader (Wallac, Perkin Elmer, Vienna, Austria). Results are reported as percentage of total ß-hexosaminidase released after addition of 1 % Triton X-100. Determinations were done in triplicates and are displayed as mean value + SD.

Statistical analysis

The reported p-values are results of two-sided Student's T tests. P-values < 0.05 were considered statistically significant. Error bars indicate standard deviations (SD).

Results

Transplantation of Phl p 5-transduced BM cells in non-myeloablatively conditioned syngeneic recipients

A previously established murine model was used to determine whether long-term tolerance in IgE-mediated allergy can be induced via molecular chimerism under non-myeloablative host conditioning [17]. The molecularly well-defined, clinically relevant major timothy grass pollen allergen Phl p 5 was retrovirally transduced into HSC to be expressed on the cell surface. BM cells (BMC) of BALB/c mice (treated with 5-FU seven days earlier) were transduced with recombinant virus VSV-Phl p 5-TM *in vitro*. As control the reporter gene GFP was fused to an signal peptide and a transmembrane domain and cloned into the retroviral vector pMMP and retroviruses VSV-GFP-TM were produced as described in [17, 35] (Fig. 1a). Transduction of VSV-Phl p 5-TM and VSV-GFP-TM showed an efficiency of 7% Phl p 5- and 6% GFP-expression, respectively on the surface of BMC as determined by flow cytometry (Fig. 1b).

BMT of Phl p 5- transduced cells leads to low level chimerism in an irradiation reducing protocol

BALB/c recipients, known to be a high IgE responder strain [analogous to the susceptible (atopic) human phenotype] [36] were preconditioned with non-myeloablative TBI of 6 Gy in addition to T cell-depleting antibodies and anti-CD40L mAb which were shown to enhance BM engraftment [37]. Levels of molecular Phl p 5-chimerism were determined by flow cytometry (FCM) at multiple time points (weeks 3, 6, 15 and 23) after BMT (Fig.2a). Although mean levels of Phl p 5-positive cells within the myeloid line were clearly *macro*chimeric at week 15 (1.51% ± 0.44%), blood cells expressing Phl p 5 declined over time to less than 1% in most (7 of 10) mice, the cut-off level commonly defined as

indicating *micro*chimerism [29]. Mean levels of Phl p 5 positive cells at week 23 post BMT were only 0.7 % ± 0.9% in myeloid subtypes. Similarly, within the B cells chimerism was 0.67% ± 0.5 % at week 23 (vs. 1.81% ± 1.11% 6 weeks after BMT) and within CD4+ T cells 0.67% ± 0.29% (n=10). These levels of expression reached the detection limit in FCM. Indeed, by FCM none of the chimeric mice showed any Phl p 5+ CD8 cells at week 23. Therefore *macro*chimerism was no longer detectable at 23 weeks post BMT and hence FCM analysis was not performed thereafter (Fig. 2a).

To distinguish between the possibilities that molecular chimerism either persisted at lower levels or disappeared (i.e. were of transient nature), Phl p 5-specific PCR was performed at the end of follow up (week 44; Fig. 2b). Chromosomal DNA of splenocytes, described to develop similar chimerism levels as BM, was isolated at the time of sacrifice [17]. In recipients transplanted with Phl p 5-transduced BMC (n=10), Phl p 5-specific products were detectable in 9/10 mice suggesting that Phl p 5-expressing cells were still available and chimerism was persistent. In chromosomal DNA of splenocytes of one Phl p 5-BM transplanted mouse (Fig. 2b, marked with *) no Phl p 5-specific PCR-product could be detected suggesting that chimerism in this mouse might have been lost. Thus, almost all recipients of Phl p 5-transduced BM developed persistent microchimerism.

B cell tolerance in low level Phl p 5-chimeric mice

Recipients of syngeneic Phl p 5-transduced BMC (n=10) were immunized with recombinant (r) Phl p 5 and the unrelated major birch pollen allergen Bet v 1 at weeks 6, 9 and 12 after BMT [17]. To assess if low level chimerism is sufficient to induce humoral tolerance, sera of chimeric mice were tested for Phl p 5-specific antibodies by ELISA (Fig. 3-5). Eight of 10 recipients developed no detectable amounts of Phl p 5-specific IgE, IgG_1

or IgA, while recipients of GFP-transduced BM and naïve controls developed high levels (Fig. 3a and 4a). Interestingly, in chromosomal DNA of one mouse developing low level Phl p 5-specific IgE and IgG_1 no Phl p 5-specific product was detectable suggesting that loss of chimerism in this mouse had led to loss of tolerance (Fig. 2b*). In contrast, mice of all groups developed high levels of Bet v 1-specific IgE, IgG_1 and IgA (Fig. 3a and b, 4a right panels). Interestingly recipients of Phl p 5-tranduced BMC developed high amounts of Phl p 5-specific IgM, a phenomenon also observed in recipients of Phl p 5-transduced BMC after myeloablative conditioning [17]. Bet v 1-specific IgM levels were similar in all groups of mice (Fig. 4b right panel). None of the 10 mice receiving Phl p 5-transduced BMC developed detectable Phl p 5-specific IgG_{2a} or IgG_3 (Fig. 5 a and b left panels) at late time points but high levels of Bet v 1-specific IgG antibody levels (Fig. 5a and b right panels). These results demonstrate that low-level persistent Phl p 5 molecular chimerism leads to long-lasting humoral tolerance.

Tolerance at the T cell level in low chimeric recipients

To assess if recipients of Phl p 5-transduced BM showed T-cell unresponsiveness towards Phl p 5, splenocytes of these mice were isolated at the end of follow up (week 44) and stimulated with r Phl p 5 in *in vitro* proliferation assays. Phl p 5-chimeric mice (n=10) showed a significantly reduced proliferation rate upon Phl p 5 stimulation compared to control groups (Fig. 6). These results demonstrate that Phl p 5-chimeric mice are tolerant at the T cell level.

Unresponsiveness at the effector cell level in sera of Phl p 5-chimeric mice

The rat basophilic leukaemia (RBL) cell degranulation assay allows evaluation of activation and mediator release of effector cells *in vitro* [38]. To determine if Phl p 5-

chimeric mice are tolerant at the effector cell level, RBL cells were loaded with sera of recipients of Phl p 5-transduced BMCs, or sera of control groups and stimulated with rPhl p 5 or rBet v 1. Phl p 5-specific release of ß-hexosaminidase was induced when cells were loaded with sera of control groups (Fig. 7a), whereas sera of Phl p 5-chimeric mice did not induce Phl p 5-specific release above the spontaneous release similar as when cells were loaded with pre-immune sera. Bet v 1-specific release was detectable in sera of all groups of mice (Fig. 7b). Thus, recipients of Phl p 5-transduced BMC showed tolerance towards Phl p 5 at the effector cell level.

Discussion

Here it is shown that low levels of molecular chimerism following non-myeloablative recipient conditioning are sufficient for tolerance induction in IgE-mediated allergy. These results may be considered as an important step for advancing allergen-specific gene therapy towards clinical application in allergy. In preclinical studies stable long-lasting macrochimerism is required for robust tolerance towards alloantigens but the minimum level of chimerism has not been defined. However, about 2 % of myeloid chimerism at late time points post BMT is sufficient to induce tolerance in some murine protocols [39, 40]. In molecular chimerism protocols levels of 1% - 5 % of persistent leukocyte chimerism were required to maintain tolerance towards skin grafts in an MHC I-congeneic and an experimental autoimmune encephalitis (EAE) model [9, 14, 26]. Therefore macrochimerism seems to be indispensable for induction of long-term tolerance in cellular and molecular chimerism protocols. Yet it was found that microchimerism was sufficient for maintaining peripheral tolerance towards a viral antigen [29], but not MHC antigens [26].

Transient mixed macrochimerism seems to be sufficient also for operational tolerance in clinical pilot trials. Following combined kidney and BMT taken from an HLA-mismatched donor, macro- and microchimerism became undetectable after some weeks [20, 22]. Mechanistically, it was suggested recently that regulatory T cells may play an early role during the lymphopenic period in tolerance induction, while deletion or anergy mechanisms might be responsible for long-term tolerance in clinical studies [41]. Similarly, in mice regulation contributes to tolerance induction early after BMT, while clonal deletion of donor-reactive T cells is the main mechanism maintaining tolerance long-term [40, 42]. These data are encouraging in that transient chimerism might be

sufficient (with further less toxic preconditioning protocols) for tolerance induction also for IgE-mediated allergy.

Molecular chimerism is a gene-therapeutic approach integrating transgenes into HSC with viral vectors. Gene therapeutic trials in infants suffering e.g. from SCID–X1 (X-linked severe combined immune deficiency) were successfully transferring modified viral transgene-integrated autologous HSCs but with the risk of severe immunological side effects by insertional mutagenesis in some patients resulting from the gene transfer vector used. Therefore ongoing research is focusing on improving safety and efficiency of different gene transfer systems [43]. An alternative to the gene-therapeutic approach integrating transgenes would be coupling of allergens to hematopoietic cells. In fact, in a recently published study prevention of food allergy was demonstrated by fixing proteins from whole peanut extracts to syngeneic splenocytes before transfer into recipient mice. After several oral challenges with these extracts mice were at least partially tolerant (no specific IgE but IgG_1) towards these extracts short term indicating that prevention of allergy using allergen-modified stem cells may be a feasible strategy [44].

In our approach we show that non-myeloablative recipient conditioning leads to robust B-cell tolerance by persistent low level chimerism avoiding the production of any high affinity Phl p 5-specific isotypes, mainly Phl p 5-specific IgE. Additionally no anaphylactic activity was triggered by this approach as determined in commonly accepted basophil *in vitro* assays testing for allergenic activity. Interestingly, Phl p 5-specific IgM was produced in detectable amounts but no class switch recombination seems to occur possibly due to the lack of T-cell help [45]. In mice, IgG_1 and IgE antibodies are both generated during T cell-dependent B cell responses mediated by Th2 lymphocytes, IgG_{2a} and IgG_3, in contrast, by Th1 lymphocytes [46-48]. Here we demonstrate tolerance at the B

cell level regarding Phl p 5-specific IgE and IgG_1 as well as complete avoidance of Phl p 5-specific IgG_{2a} and IgG_3 production, suggesting that both Th1 and Th2 cells were tolerized.

The allergen Phl p 5 is a highly immunogenic and allergenic protein even when expressed in mammalian cells and therefore a very stringent model for induction of tolerance via molecular chimerism [7]. This approach can be extended to a variety of different allergens. In fact we have data suggesting that tolerance can also be induced to the unrelated allergen Bet v 1 with this approach (Gattringer et al, submitted). Also tolerance induction against several clinical allergens should be possible because it has been demonstrated that functional or hypoallergenic hybrid allergens/allergen derivatives can be constructed by fusion of the DNAs coding for several different allergens [49-51]. It should therefore be possible to transduce stem cells with DNA constructs coding for several allergens or immobilize hybrid molecules consisting of several allergens on their surfaces for tolerance induction. Furthermore, intracellular expression of allergens or the use of hypoallergenic derivatives allows to targeting selectively either T cells alone or both, T and B cells and thus risks of allergic sensitization or anaphylaxis can be potentially minimized.

Our findings show a robust tolerance protocol that permanently prevents IgE-mediated allergy and may advance molecular chimerism strategies towards clinical application for the prevention of allergy.

Figure Legends

Figure 1: Expression levels of membrane anchored Phl p 5 and GFP in retrovirally transduced BM

(a) Schematic representation of pMMP-Phl p 5-TM retroviral construct and pMMP-eGFP-TM retroviral construct. Full length Phl p 5 and eGFP were fused to a leader peptide (S) and a transmembrane domain (T) and ligated into retroviral vector pMMP. LTR-long terminal repeats. **(b)** Expression levels of membrane-anchored Phl p 5 (left histogram) and membrane-anchored GFP as control (right histogram) in BM cells before BMT demonstrated in flow cytometric analysis. Dashed lines represent untransduced cells.

Figure 2: Phl p 5-chimerism is detectable in flow cytometry at early and PCR at late time points

(a) Phl p 5-specific expression within lineages of WBC as indicated was detected by flow cytometry at early time points. Data represent the mean % of Phl p 5 chimerism in recipients of Phl p 5-transduced BM (n=10). Arrows in grey indicate time points of FCM analysis, arrow in black indicates PCR analysis at 44 weeks post-BMT. *Note:* Phl p 5-expressing CD4+ and CD8+ cells became detectable only 6 to 15 weeks after BMT due to delayed recovery of T cells after administration of T-cell depleting antibodies **(b)** Upper gel: Phl p 5-specific PCR-products (lane 1-4) shown in genomic DNA of recipients transplanted with GFP-transduced BM (GFP-transduced) and recipients of Phl p 5-transduced BM (Phl p 5-transduced; lane 5 to 14). In chromosomal DNA of mouse 9 (lane 13) no Phl p 5-specific product was detectable (*). Lower gel: Lane 1-14 show ß-actin specific PCR-products in genomic DNA of splenocytes of recipients of GFP-transduced BM (lane 1-4) and Phl p 5-transduced BM (lane 5-14).

Figure 3: Lack of Phl p 5-specific IgE and IgG_1 in Phl p 5-chimeric mice

Allergen-specific IgE and IgG_1 levels in sera of recipients of Phl p 5-transduced BM (n=10), sera of recipients of GFP-transduced BM (n=4) and sera of non-transplanted immunized mice (n=5), were demonstrated before (pre imm), and after BMT (week 9,12,15,37 and 44) and analyzed by ELISA. Mean antibody levels (+SD) are shown for each group. **(a**) Left panel: Phl p 5-specific IgE levels. Right panel: Bet v 1-specific IgE levels. **(b)** Left panel: Phl p 5-specific IgG_1 levels. Right panel: Bet v 1-specific IgG_1 levels. P-values for Phl p 5-specific IgE and IgG_1 in sera of recipients of Phl p 5-transduced BM versus recipients of GFP-transduced BM are demonstrated.

Figure 4: Phl p 5-chimeric mice do not develop Phl p 5-specific IgA but IgM

Allergen-specific IgA and IgM levels in sera of recipients of GFP-transduced BM (n=4), recipients of Phl p 5-transduced BM (n=10) and non-BMT sensitized mice (n=10) were analyzed by ELISA at late time points (weeks 37 or 44, pooled data) compared to pre immune (pre imm) sera. Mean antibody levels (+SD) are shown for each group. **(a**) Right panel: Phl p 5-specific IgA levels. Right panel: Bet v 1-specific IgA levels. (**b)** Left panel: Phl p 5-specific IgM levels. Right panel: Bet v 1-specific IgM levels. P-values for Phl p 5-specific IgA and IgM in sera of recipients of Phl p 5-transduced BM versus recipients of GFP-transduced BM are demonstrated and recipients of Phl p 5-transduced BM versus non-transplanted immunized mice.

Figure 5: Phl p 5-chimeric mice do not develop Phl p 5-specific IgG_{2a} and IgG3

Allergen-specific antibody levels in sera of recipients of GFP-transduced BM (n=4), recipients of Phl p 5-transduced BM (n=10) and non-BMT sensitized mice (n=10) were analyzed by ELISA at late time points (weeks 37 or 44, pooled data) compared to pre

immune (pre imm) sera. Mean antibody levels (+SD) are shown for each group. **(a)** Left panel: Phl p 5-specific IgG_2 levels. Right panel: Bet v 1-specific IgG_{2a} levels. (**b)** Left panel: Phl p 5-specific IgG_3 levels. Right panel: Bet v 1-specific IgG_3 levels. P-values for Phl p 5-specific IgG_{2a} and IgG_3 in sera of recipients of Phl p 5-transduced BM versus recipients of GFP-transduced BM are demonstrated.

Figure 6: T-cell non-responsiveness of splenocytes of Phl p 5-chimeric mice.

Splenocytes of recipients of GFP-transduced BM (n=4), Phl p 5-transduced BM (n=10), non-transplanted sensitized mice (n=5) and naïve mice (n=3) were stimulated with r Phl p 5. Proliferation rates are shown for splenocytes of each group after H^3-labeled thymidine incorporation and demonstrated by Phl p 5-specific stimulation indices (SI). P-values for recipients of Phl p 5-transduced versus recipients of GFP-transduced BM are demonstrated.

Figure 7: Complete lack of Phl p 5-specific effector cell degranulation in Phl p 5-chimeric mice

RBL cells were loaded with sera of Phl p 5-chimeric mice (n=10) collected at time points indicated, sera of recipients of GFP-transduced mice (n=4) and sera of non-BMT immunized mice and the corresponding preimmune sera (pre imm). Subsequently cells were challenged with r allergens. **(a)** Phl p 5-specific release of ß-hexosaminidase. **(b)** Bet v 1-specific release of ß-hexosaminidase. The mean percentages of allergen-specific ß-hexosaminidase release (+ SD) are shown for each group. P-values for recipients of Phl p 5-transduced versus recipients of GFP-transduced BM are demonstrated.

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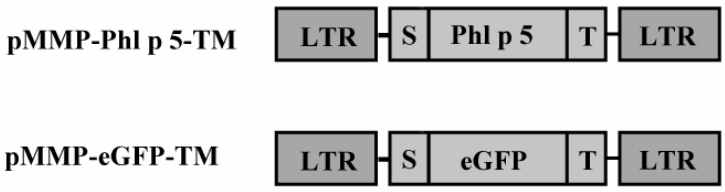
Fig. 1

(a)


pMMP-Phl p 5-TM
LTR
S
Phl p 5
T
LTR
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LTR
S
eGFP
T
LTR

(b)

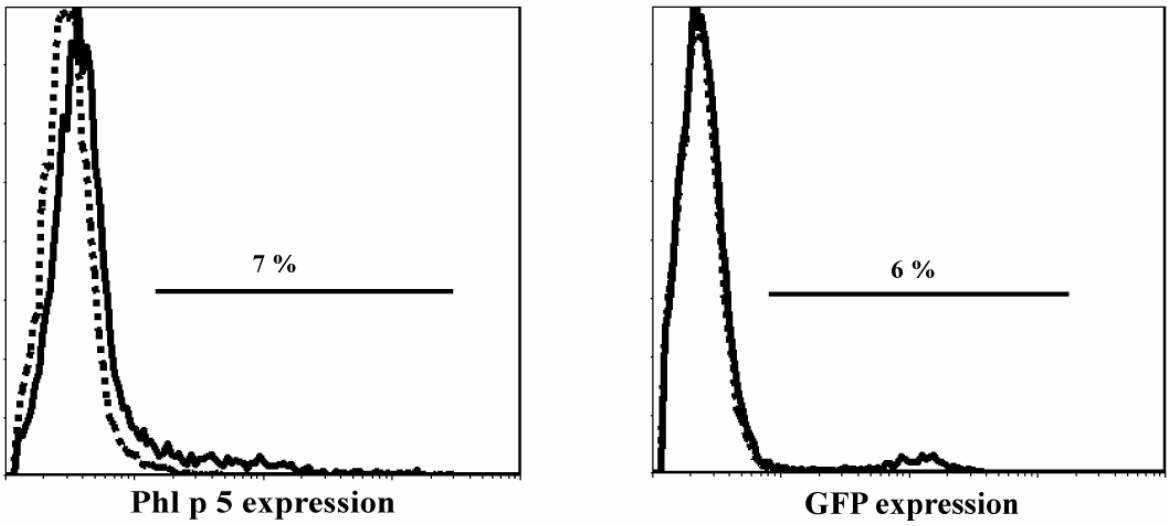
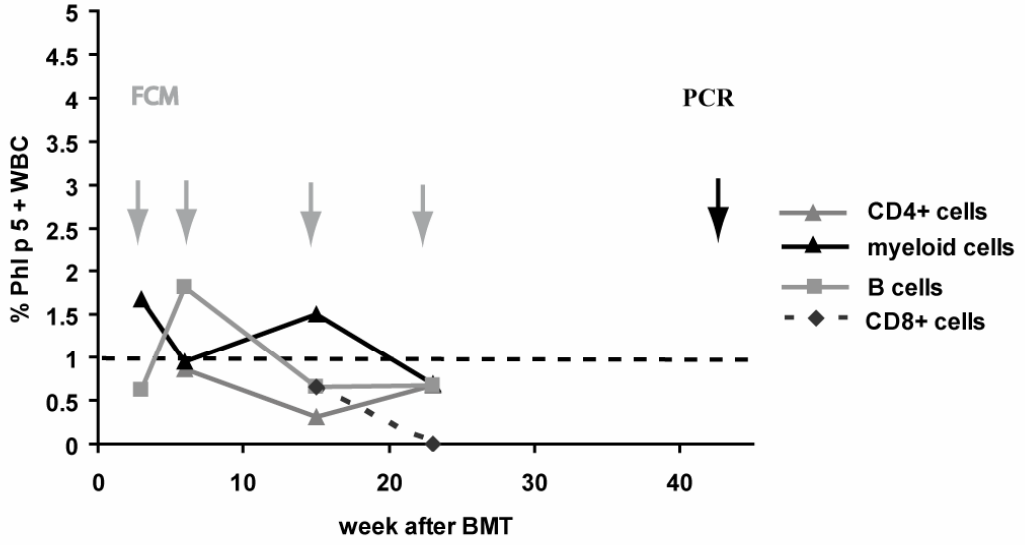

7 %
Phl p 5 expression
6 %
GFP expression

Fig. 2

(a)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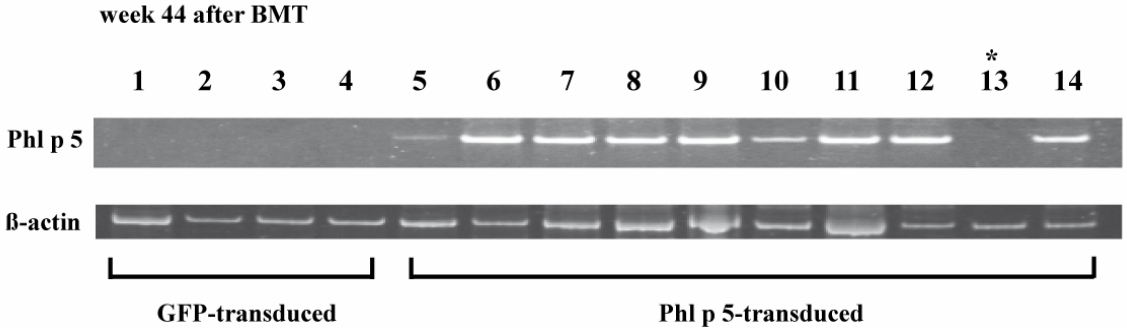
PCR
CD4+ cells
myeloid cells
B cells
CD8+ cells
week after BMT

(b)


week 44 after BMT
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*
Phl p 5
ß-actin
GFP-transduced
Phl p 5-transduced

Fig. 3

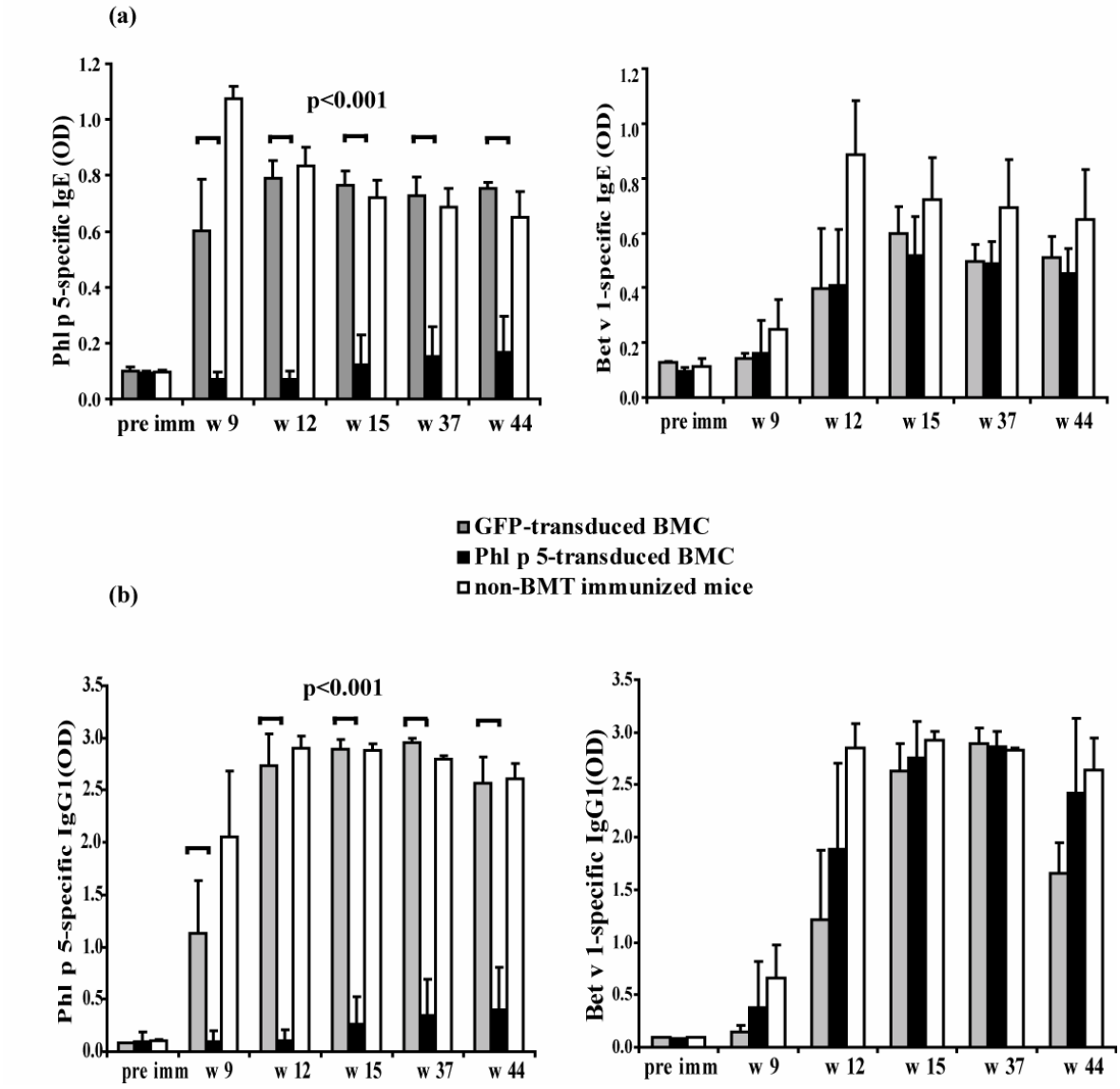

(a)
Phl p 5-specific IgE (OD)
p<0.001
Bet v 1-specific IgE (OD)
pre imm
w 9
w 12
w 15
w 37
w 44
GFP-transduced BMC
Phl p 5-transduced BMC
non-BMT immunized mice
(b)
Phl p 5-specific IgG1(OD)
p<0.001
Bet v 1-specific IgG1(OD)

Fig. 4

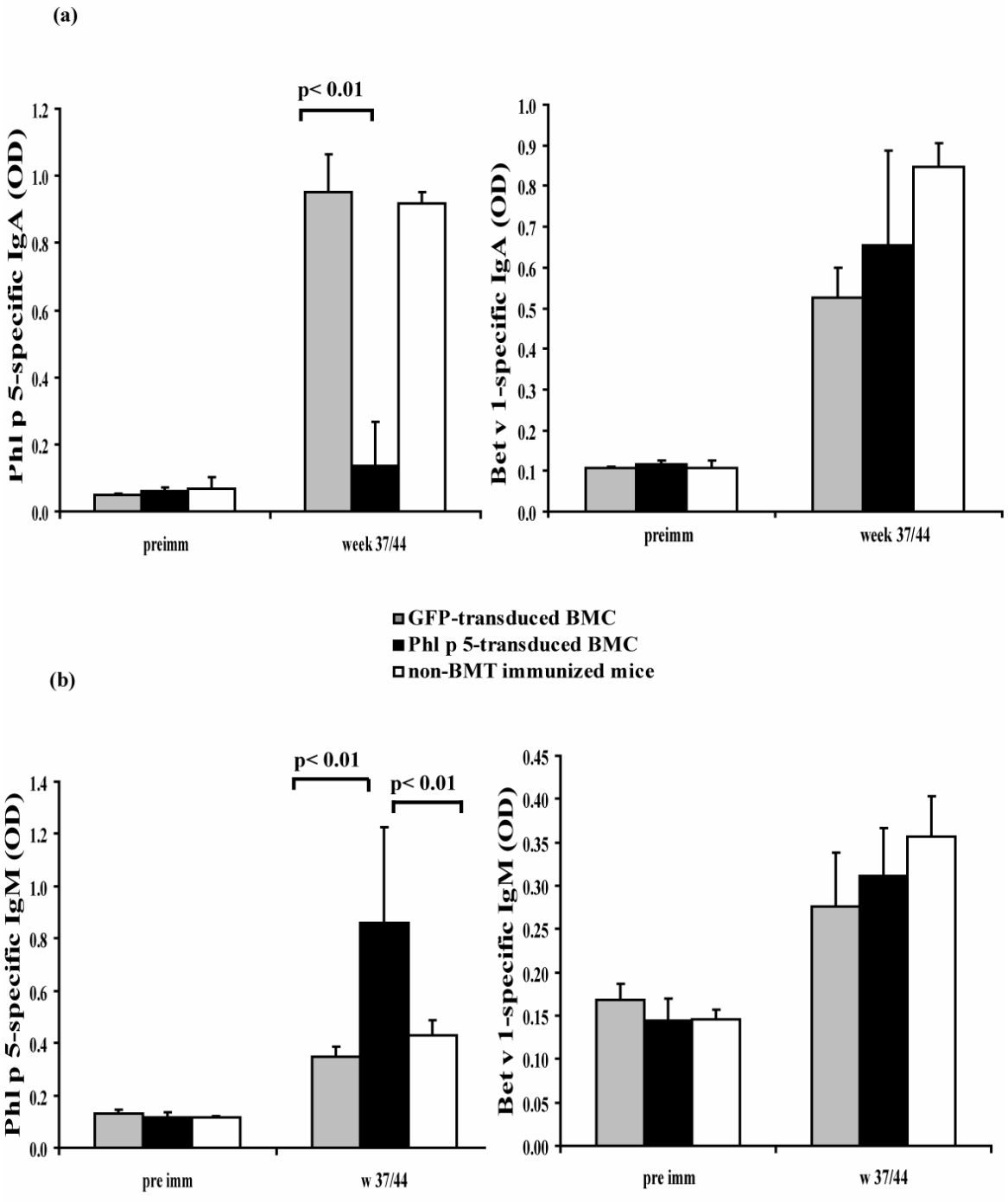

(a)
p< 0.01
Phl p 5-specific IgA (OD)
Bet v 1-specific IgA (OD)
preimm
week 37/44
GFP-transduced BMC
Phl p 5-transduced BMC
non-BMT immunized mice
(b)
p< 0.01
p< 0.01
Phl p 5-specific IgM (OD)
Bet v 1-specific IgM (OD)
pre imm
w 37/44

Fig. 5

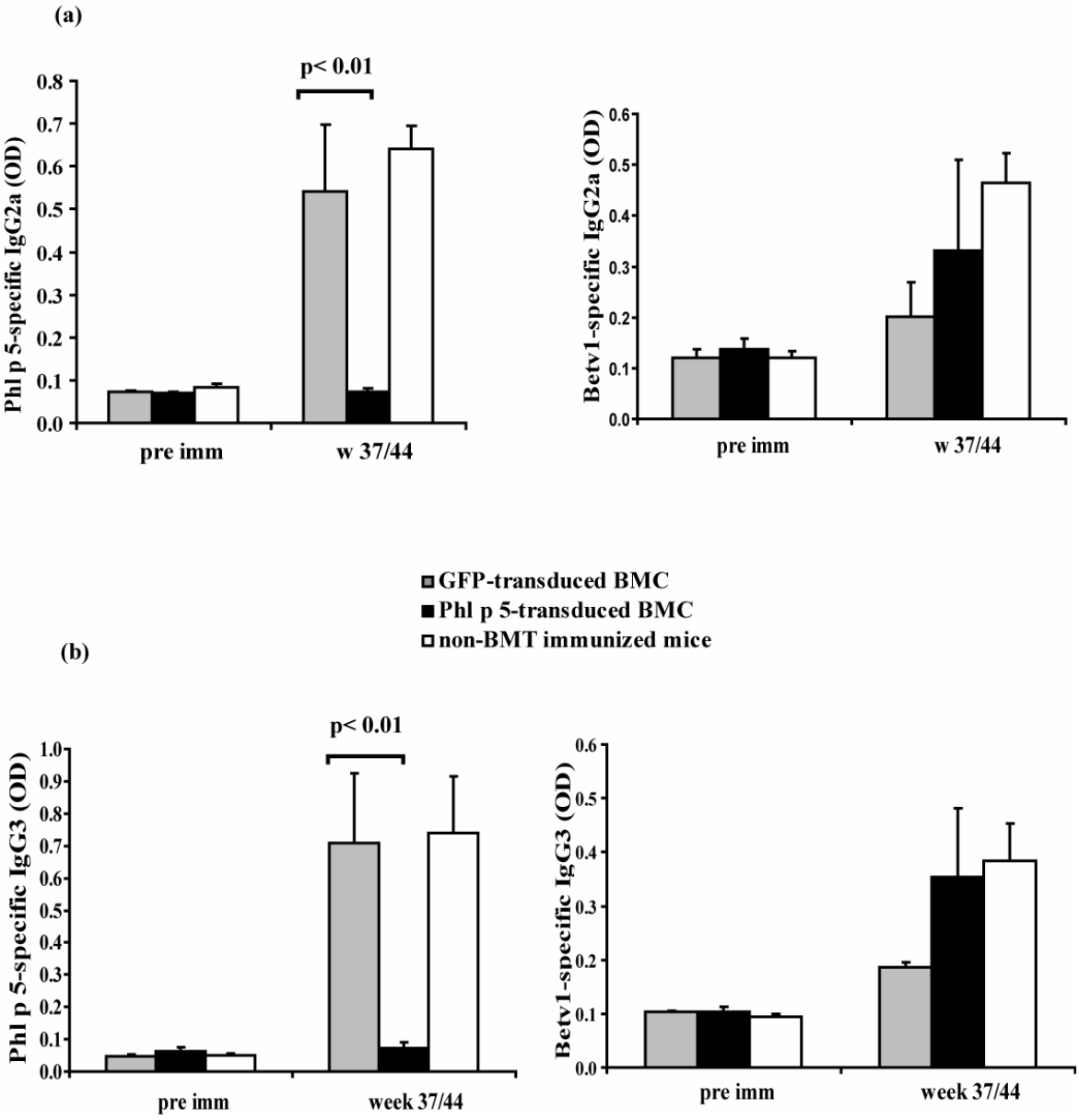

(a)
p< 0.01
Phl p 5-specific IgG2a (OD)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pre imm
w 37/44
Betv1-specific IgG2a (OD)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pre imm
w 37/44
GFP-transduced BMC
Phl p 5-transduced BMC
non-BMT immunized mice
(b)
p< 0.01
Phl p 5-specific IgG3 (OD)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pre imm
week 37/44
Betv1-specific IgG3 (OD)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pre imm
week 37/44

Fig. 6

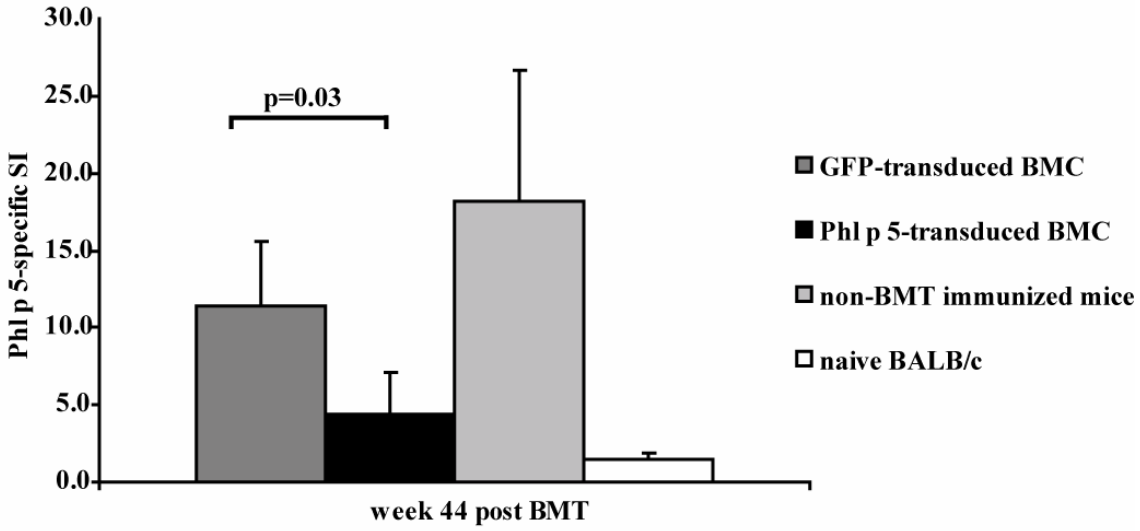
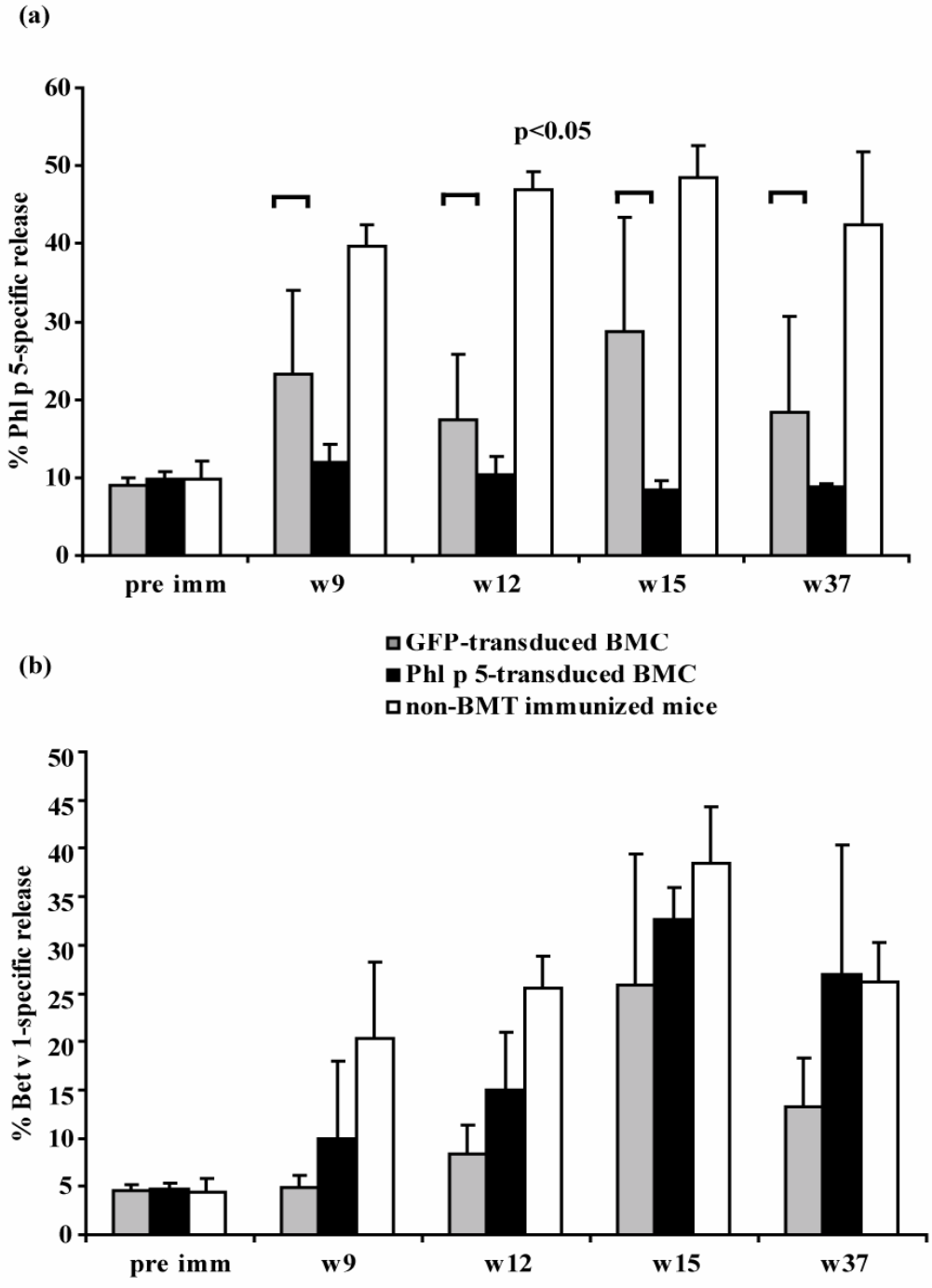

30.0
25.0
20.0
15.0
10.0
5.0
0.0
Phl p 5-specific SI
p=0.03
week 44 post BMT
GFP-transduced BMC
Phl p 5-transduced BMC
non-BMT immunized mice
naive BALB/c

Fig. 7


(a)
% Phl p 5-specific release
60
50
40
30
20
10
0
p<0.05
pre imm
w9
w12
w15
w37
GFP-transduced BMC
Phl p 5-transduced BMC
non-BMT immunized mice
(b)
% Bet v 1-specific release
50
45
40
35
30
25
20
15
10
5
0
pre imm
w9
w12
w15
w37

5.1.4 Tolerization of a Type I Allergic Immune Response through Transplantation of Genetically Modified Hematopoieitic Stem Cells

Tolerization of a Type I Allergic Immune Response through Transplantation of Genetically Modified Hematopoietic Stem Cells[1]

Ulrike Baranyi,* Birgit Linhart,† Nina Pilat,* Martina Gattringer,* Jessamyn Bagley,‡ Ferdinand Muehlbacher,* John Iacomini,‡ Rudolf Valenta,[2†] and Thomas Wekerle[2,3]*

Allergy represents a hypersensitivity disease that affects >25% of the population in industrialized countries. The underlying type I allergic immune reaction occurs in predisposed atopic individuals in response to otherwise harmless Ags (i.e., allergens) and is characterized by the production of allergen-specific IgE, an allergen-specific T cell response, and the release of biologically active mediators such as histamine from mast cells and basophils. Regimens permanently tolerizing an allergic immune response still need to be developed. We therefore retrovirally transduced murine hematopoietic stem cells to express the major grass pollen allergen Phl p 5 on their cell membrane. Transplantation of these genetically modified hematopoietic stem cells led to durable multilineage molecular chimerism and permanent immunological tolerance toward the introduced allergen at the B cell, T cell, and effector cell levels. Notably, Phl p 5-specific serum IgE and IgG remained undetectable, and T cell nonresponsiveness persisted throughout follow-up (40 wk). Besides, mediator release was specifically absent in in vitro and in vivo assays. B cell, T cell, and effector cell responses to an unrelated control allergen (Bet v 1) were unperturbed, demonstrating specificity of this tolerance protocol. We thus describe a novel cell-based strategy for the prevention of allergy. *The Journal of Immunology,* **2008, 180: 8168–8175.**

The pathophysiological hallmark of type I allergy is the Th2 cell-driven production of IgE against otherwise harmless Ags (i.e., allergens) in predisposed atopic individuals (1, 2). IgE-mediated allergy manifests itself clinically either locally (e.g., as hay fever, allergic asthma, or food allergy) or systemically, as is the case in anaphylaxis. Although infrequent, anaphylaxis is an acute life-threatening condition (e.g., induced by food allergens or insect venoms) (3). Allergy is mainly being treated symptomatically by various drugs that are associated with considerable side-effects and cost.

Allergen-specific immunotherapy, whereby increasing doses of the sensitizing allergen are repeatedly administered in the form of crude extracts, is used in selected patients and is currently the only allergen-specific treatment of allergy (4). However, allergen-specific immunotherapy is associated with limited effectiveness and substantial risks, as exemplified by anaphylactic reactions or therapy-induced sensitization to additional allergens (5). Today, the molecular structure of the most common allergens has been revealed, and advanced experimental allergen-specific strategies have been developed (6). They include the use of allergen-derived T cell epitope-containing peptides (7), genetically engineered allergens (5), and DNA-based forms of treatment (8). Several experimental approaches for tolerance induction in allergy have also been explored but are characterized by limited robustness and relatively short-lived effects (9–11). So far no robust allergen-specific tolerance approach has been reported that permanently prevents allergy.

It is one of the main features of the immune system to be tolerant toward self (12). A major mechanism of self-tolerance is mediated by subpopulations of hematopoietic cells expressing self-Ags (13, 14). This principle has been emulated in organ transplantation by introducing donor hematopoietic stem cells (HSC)[4] into the recipient in a way to create a chimeric state in which recipient and donor bone marrow (BM) coexist, thereby inducing tolerance toward donor (allo)-Ags (15, 16). Alternatively, disease-associated Ag(s) can be introduced into an individual by transplanting autologous (i.e., in the experimental rodent setting syngeneic) HSC after they have been genetically modified in vitro to express the relevant Ag(s), leading to so-called molecular chimerism (17). Where successful, regimens relying on hematopoietic chimerism are characterized by a state of Ag-specific tolerance that is particularly robust and long-lasting.

Molecular chimerism models have been used experimentally to tolerize an allogeneic immune response (using single MHC Ags) (18, 19), a xenogeneic response (introducing the enzyme α-1,3-galactosyltransferase) (20), and selected autoimmune responses (21, 22). However, other studies have failed to achieve tolerance in

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[4] Abbreviations used in this paper: HSC, hematopoietic stem cell; BM, bone marrow; BMT, BM transplantation; r, recombinant; RBL, rat basophil leukemia; SI, stimulation index; VSV, vesicular stomatitis virus.

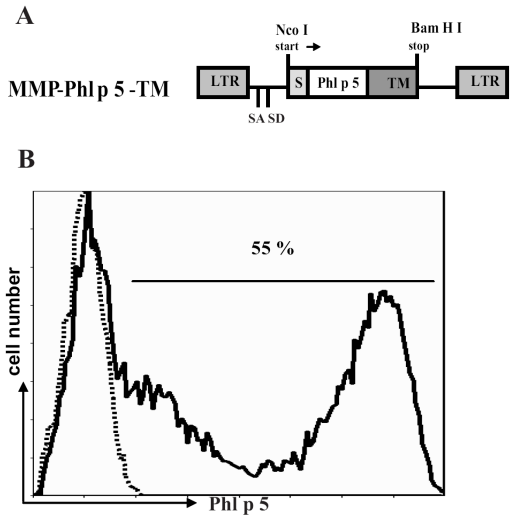

FIGURE 1. Efficient retroviral transduction of BM with membrane-bound allergen. *A*, Schematic representation of the MMP-Phl p 5-TM retroviral construct. Phl p 5 was fused to a signal peptide (S) and a transmembrane domain (TM). LTR represents long terminal repeats; SD and SA, splicing donor and splicing acceptor. The drawing is not to scale. *B*, Histogram depicting flow-cytometric analysis of Phl p 5 expression on the surface of VSV-Phl p 5-transduced BM immediately after transduction (solid line). Dashed line represents mock-transduced BM. One of two similar experiments is shown.

particular autoimmune disease models (23) and have even enhanced the susceptibility for disease development. No studies attempting tolerization of the distinct allergic immune response through molecular chimerism have been reported so far.

We wanted to investigate whether the immune response of IgE-mediated allergy can be tolerized by transplantation of syngeneic HSC expressing an allergen.

Materials and Methods

Animals

Female BALB/c mice were purchased from Charles River Laboratories. All mice were housed under specific pathogen-free conditions and were used between 6 and 12 wk 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.

Retroviral constructs and production of retroviruses

To generate membrane-anchored Phl p 5, full-length Phl p 5 (accession number X74735) was fused to a signal sequence and a transmembrane domain (both pDisplay, Invitrogen) by overlapping PCR technique (24). The original signal sequence of Phl p 5 was replaced by the murine Ig signal sequence pDisplay. Primer sequences are used as follows: leader peptide: 5′-GGCGCCATGGAGACAGACACACTCCTG-3′, 5′-GTAACCGAGATCGGCGTCACCAGTGGA-3′; Phl p 5: 5′-ACTGGTGACGCCGATCTCGGTTAC-3′, 5′-GCC CAC AGC GAC TTT GTA GCC ACC-3′; transmembrane domain: 5′-TACAAAGTCGCTGTGGGC-3′, 5′-GGCGGATCCTAACGTGGCTTCTTCTG-3′. PCR product was cloned into the retroviral vector pMMP *Nco*I and *Bam*HI sites, resulting in pMMP-Phl p 5-TM. The start codon was inserted with the *Nco*I site; the stop codon was inserted with the *Bam*HI site. For virus production plasmids, pMMP-Phl p 5-TM, pMD.G, encoding for vesicular stomatitis virus (VSV)-G protein, and pMLV, encoding for viral proteins gag and pol, were cotransfected using the calcium phosphate method (25) into 293 T cells, resulting in VSV-Phl p 5-TM viruses. Viral supernatants were concentrated by ultracentrifugation (33,620 × g for 2 h). Mock viruses were produced in the same manner using empty pMMP vector.

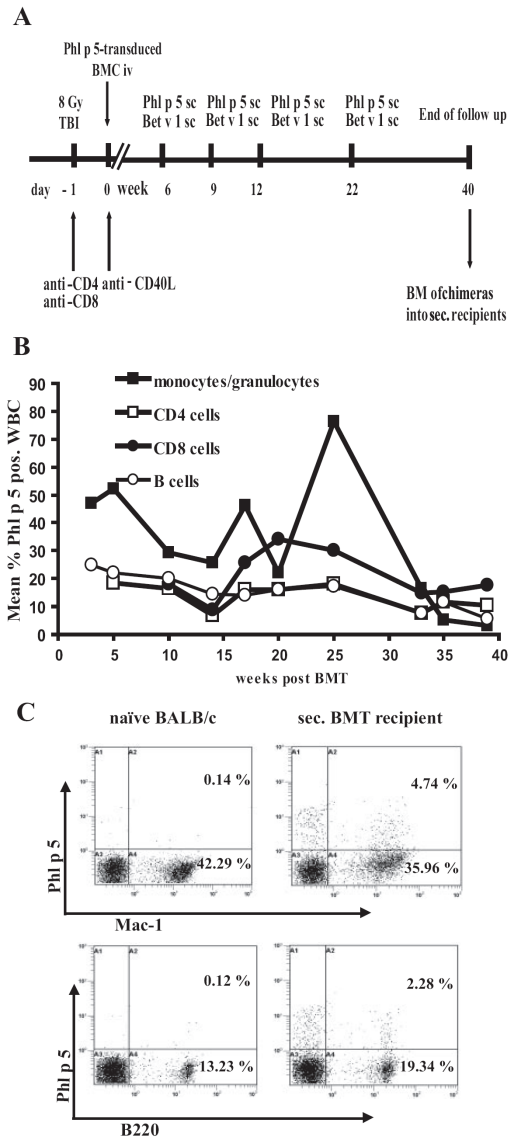

FIGURE 2. Transplantation of syngeneic HSC retrovirally transduced to express Phl p 5 leads to high levels of permanent multilineage molecular chimerism. *A*, Schematic drawing of the experimental in vivo protocol for the transplantation of Phl p 5-transduced BM. Preconditioned BALB/c mice received 2–4 × 10^6 transduced BM cells. Mice were repeatedly immunized s.c. with rPhl p 5 and Bet v 1 at weeks 6, 9, 12, and 22. At the end of follow-up, BM of chimeras was isolated and transplanted into secondary preconditioned recipients. *B*, Percentages of Phl p 5^+ cells among various leukocyte lineages were determined in blood by two-color flow cytometry in recipients of Phl p 5-transduced BM ($n = 3$) at multiple time points and are presented as means. Results from one of two independent similar experiments are shown. *C*, In secondary recipients the molecular chimerism within the Mac-1^+ and B220$^+$ populations was analyzed in peripheral blood 15 wk after secondary BMT (*right panels*). A representative plot of one secondary recipient is shown. *Left panels* show white blood cells of a naive BALB/c mouse as control.

Retroviral transduction of BM cells

BALB/c donors were injected i.p. with 5-fluorouracil (150 mg/kg) 7 days before BM isolation (26). Mice were sacrificed and BM was harvested

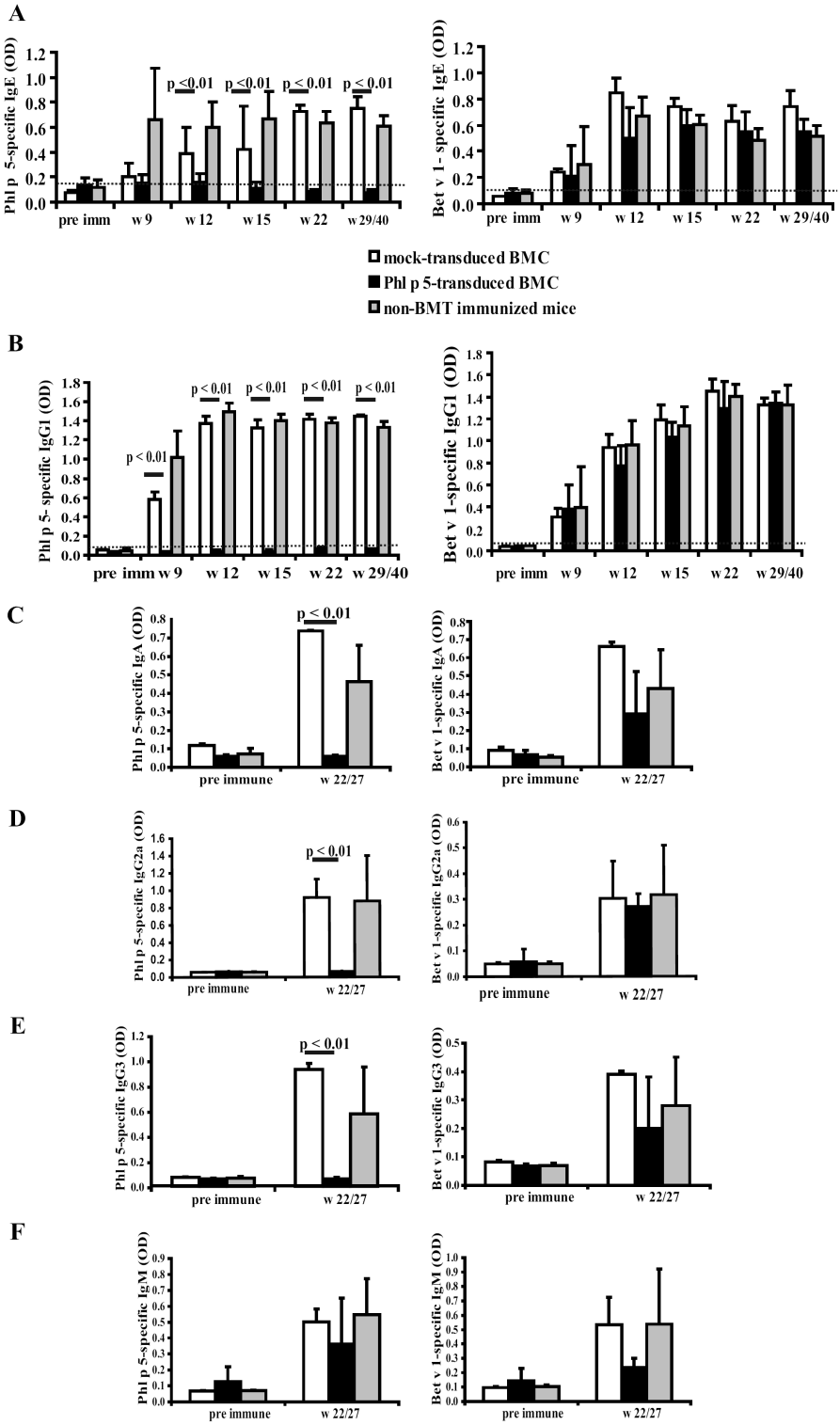

FIGURE 3. Recipients of Phl p 5-transduced BM are specifically tolerant toward Phl p 5 at the B cell level. Allergen-specific Ab levels in sera of recipients of Phl p 5-transduced BM ($n = 10$), mock-transduced BM ($n = 3$), and non-BMT immunized mice ($n = 10$) were analyzed by ELISA at the indicated time points. Mean Ab levels (+SD) are shown for each group. Dotted horizontal lines represent the baseline preimmune (pre imm) allergen-specific

from tibiae, femurs, humeri, and pelvis. BM cells were cultured and transduced with VSV-Phl p 5 or mock transduced as described by Bagley et al. (27) with a multiplicity of infection of 3–5.

BM transplantation (BMT)

One day before BMT, recipients received 8 Gy total body irradiation and a depleting dose of anti-CD8 (2.43; 0.5 mg/mouse) and anti-CD4 (GK1.5; 0.5 mg/mouse) mAbs. On the day of reconstitution mice were transplanted with 2–4 $\times$ 10^6 transduced BM cells i.v. After BMT mice received anti-CD40L mAb (MR1; 0.5 mg/mouse). Anti-CD4, anti-CD8, and anti-CD40L were used, as they were shown to enhance engraftment of transduced BM (28). All mAbs used in vivo were purchased from BioExpress.

Recombinant allergens and immunization of mice

Purified recombinant (r) timothy grass pollen and birch pollen allergens (rPhl p 5, rBet v 1) were obtained from BIOMAY. All groups of mice were immunized s.c. with 5 μg rPhl p 5 and 5 μg rBet v 1 adsorbed to aluminum hydroxide (Alu-Gel-S, Serva) as described previously (29).

Secondary BMT

Forty weeks after BMT, BM cells were harvested from primary recipients and transplanted into secondary BALB/c mice preconditioned like primary recipients (described above). Each secondary recipient received 3 $\times$ 10^7 BM cells harvested from one chimera.

Flow cytometric analysis

Nonspecific Fcγ receptor binding was blocked with mAb against mouse FcγII/III receptor (CD16/CD32). Phl p 5 polyclonal antiserum against full-length rPhl p 5 was purified from rabbit serum (Charles River Laboratories) by a protein G column (Pierce) according to the manufacturers' instructions. Polyclonal anti-Phl p 5 IgG was biotinylated and developed with PE streptavidin. To detect Phl p 5^+-expressing cells among various leukocyte lineages, white blood cells were stained with FITC-conjugated Abs against CD4, CD8, B220, Mac-1, and isotype controls (all Abs from BD Pharmingen) and analyzed by flow cytometry. Propidium iodide staining was used to exclude dead cells. Two-color flow cytometric analysis was used to determine the percentage of Phl p 5-expressing cells of particular lineages. The percentage of Phl p 5^+ cells (i.e., molecular chimerism) was calculated by subtracting control staining from quadrants containing Phl p 5^+ and Phl p 5^- cells expressing a particular lineage marker, and by dividing the net percentage of Phl p 5^+ cells by the total net percentage of Phl p 5^+ plus Phl p 5^- cells of that lineage as described (30). Mice were considered chimeric if they showed at least 1% Phl p 5^+ cells within the myeloid lineage and at least one lymphoid lineage. An EPICS XL-MCL flow cytometer (Beckman Coulter) was used for acquisition, and EXPO32 ADC software (Applied Cytometry Systems) was used for analysis of flow cytometric data.

ELISA

To measure Ag-specific Abs in the sera of immunized mice, ELISAs were performed as described previously (31). Plates were coated with rPhl p 5 (5 μg/ml), sera were diluted 1/20 for IgE, 1/100 for IgM, IgA, IgG2a, and IgG3, respectively, and 1/500 for IgG1, and bound Abs were detected with monoclonal rat anti-mouse IgM, IgG1, IgE, IgA, IgG2a, and IgG3 Abs (BD Pharmingen) diluted 1/1000 and a HRP-coupled goat anti-rat antiserum (Amersham Biosciences) diluted 1/2000. The substrate for HRP was ABTS (60 mM/L citric acid, 77 mM/L $Na_2HPO_4 \times 2H_2O$, 1.7 mM/L ABTS (Sigma-Aldrich), 3 mM/L H_2O_2).

Lymphocyte proliferation assay

Spleens were removed under aseptic conditions (weeks 29/40) and homogenized. Suspended splenocytes were plated into 96-well round-bottom plates at a concentration of 2 $\times$ 10^5 cells/well in triplicates and stimulated with Con A (0.5 μg/well, Sigma-Aldrich), rPhl p 5 (2 μg/well), and rBet v 1 (2 μg/well). On day 5 cultures were pulsed with 0.5 μCi/well [^{3}H]thymidine (Amersham Biosciences) and harvested ~16 h thereafter. The proliferative response was measured by scintillation counting. The stimulation index (SI) was calculated as the ratio of the mean proliferation after allergen stimulation and medium control values (32, 35).

Rat basophil leukemia (RBL) cell degranulation assay

RBL-2H3 cell subline (33) was cultured as described previously (34) in RPMI 1640 medium (Biochrom) containing 10% FCS. Cells (4 $\times$ 10^4) were plated in 96-well tissue culture plates (Greiner Bio-One), loaded with 1/50 diluted mouse sera, and incubated for 2 h at 37°C and 5% CO_2. Supernatants were removed and the cell layer was washed with 2$\times$ Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM D-glucose, 12 mM $NaHCO_3$, 10 mM HEPES, and 0.1% (w/v) BSA (pH 7.2)). Preloaded cells were stimulated with rPhl p 5 or rBet v 1 (0.03 μg/well) for 30 min at 37°C. The supernatants were analyzed for β-hexosaminidase activity by incubation with the substrate 80 μM 4-methylumbelliferyl-*N*-acetyl-β-D-glucosamide (Sigma-Aldrich) in citrate buffer (0.1 M (pH 4.5)) for 1 h at 37°C. The reaction was stopped by addition of 100 μl glycine buffer (0.2 M glycine, 0.2 M NaCl (pH 10.)7) and the fluorescence was measured at λ_{ex}: 360/λ_{em}: 465 nm using a fluorescence microplate reader (PerkinElmer Wallac). Results are reported as percentage of total β-hexosaminidase released after addition of 1% Triton X-100. Determinations were done in triplicates and are displayed as mean values + SD.

Cutaneous type I hypersensitivity reaction

Thirty weeks after BMT, mice were injected i.v. with 100 μl of 0.5% Evans blue (Sigma-Aldrich). Subsequently, 30 μl of rPhl p 5 and rBet v 1 (0.5 μg/ml each, diluted in PBS) were injected intradermally into the shaved abdominal skin as described previously (35). As positive control, the mast cell-degranulating compound 48/80 (20 μg/ml, Sigma-Aldrich) was injected intradermally whereas PBS was injected as a negative control. Twenty minutes after injection, mice were sacrificed and the blue color intensity of a positive skin reaction due to vascular permeability was compared with the individual positive and negative control on the inverted skin.

Statistical analysis

The reported p values are results of Wilcoxon-Mann-Whitney U test and exact significances. SPSS statistical software system 14.0 was used for calculations. Values of $p < 0.05$ were considered statistically significant. Error bars indicate SDs.

Results

Membrane-anchored expression of an allergen on murine BM after retroviral transduction

We generated a membrane-anchored fusion protein of full-length Phl p 5 (*Phleum pratense* 5, timothy grass), (36), one of the most relevant respiratory allergens. The fusion gene was cloned into retroviral backbone pMMP (37) (Fig. 1*A*). Transient cotransfection of plasmids carrying viral structural proteins and VSV-G protein envelope and pMMP-Phl p 5-TM into 293 T cells resulted in VSV-Phl p 5-TM pseudotyped recombinant retroviruses (27). BALB/c donors were treated with 5-fluorouracil, and BM was isolated 7 days later, cultured ex vivo, and transduced with VSV-Phl p 5-TM retrovirus. Following transduction, 35 and 55% of BM cells, respectively (in two independent experiments), expressed Phl p 5 on their membrane (Fig. 1*B*).

Long-term molecular chimerism after transplantation of allergen-transduced HSC

Transduced BM cells were transplanted into preconditioned BALB/c recipients (Fig. 2*A*). The percentage of cells expressing Phl p 5 among various leukocyte lineages (i.e., molecular chimerism) was determined in blood by flow cytometry at multiple time points after BMT. All mice transplanted with Phl p 5-transduced BM (n = 10) developed high levels of chimerism in all tested lineages (e.g., 11% Phl p 5^+ B cells and 22% Phl p 5^+ T cells, 25 wk post-BMT). Multilineage chimerism persisted throughout follow-up (>39 wk) (Fig. 2*B*). Comparable levels of chimerism in

Ab level (collected 6 wk after BMT). *A*, Phl p 5-specific and Bet v 1-specific IgE levels; *B*, Phl p 5-specific and Bet v 1-specific IgG1 level; *C–F*, Phl p and Bet v 1-specific Ab levels IgA, IgG2a, IgG3, and IgM at the indicated time points. Values of p for recipients of Phl p 5-transduced BM vs recipients of mock-transduced BM are shown. Results are from pooled data of two independent experiments.

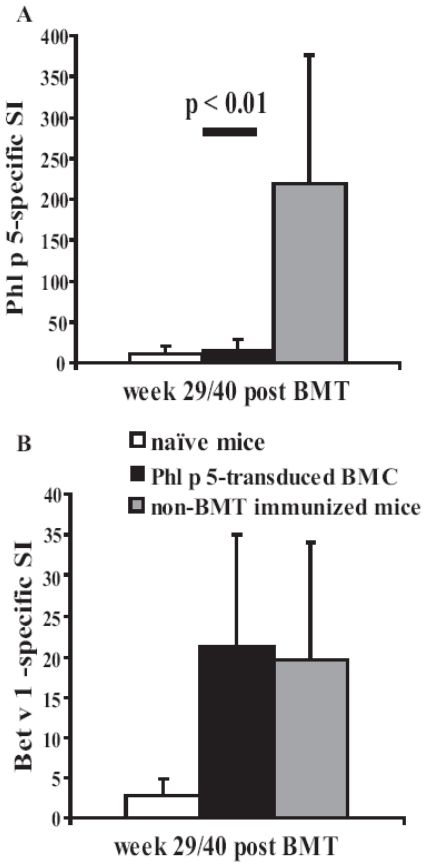

FIGURE 4. Recipients of Phl p 5-transduced BM are specifically tolerant toward the Phl p 5 at the T cell level. Results from in vitro allergen-specific proliferation assays performed at the time of sacrifice (weeks 29/40). Splenocytes of naive age-matched mice ($n = 5$), recipients of Phl p 5-transduced BM ($n = 4$), and non-BMT immunized mice ($n = 7$) were stimulated with rPhl p 5 (*A*) and rBet v 1 (*B*). Columns represent the mean stimulation indices (SI + SD) from two independent experiments.

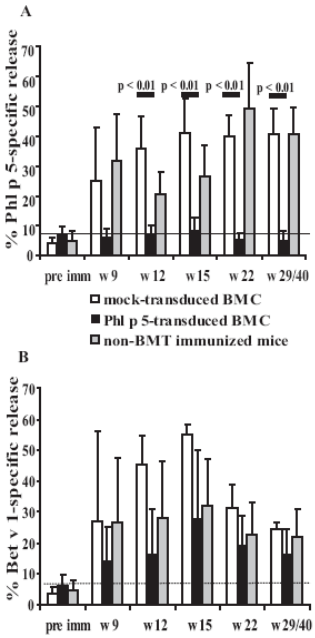

FIGURE 5. Recipients of Phl p 5-transduced BM are specifically tolerant toward Phl p 5 at the effector cell level as determined in vitro. Results are from in vitro mediator release assays. RBL cells were loaded with sera collected at the indicated time points from Phl p 5 chimeras ($n = 10$), recipients of mock-transduced BM ($n = 3$), and non-BMT immunized mice ($n = 10$). Loaded cells were incubated with rPhl p 5 (*A*) or rBet v 1 (*B*). The mean percentages of allergen-specific β-hexosaminidase release (+SD) are shown. p values for recipients of Phl p 5-transduced vs recipients of mock-transduced BM are shown. Pre imm, preimmune.

recipients of Phl p 5-transduced BM were also found in spleen and BM at the time of sacrifice (data not shown). Recipients of mock-transduced BM did not show any detectable Phl p 5 expression on leukocytes (data not shown). Persistent multilineage chimerism beyond 39 wk in recipients of Phl p 5-transduced BM is indicative of successful transduction and engraftment of HSC (38). To directly test whether HSC had been successfully transduced with Phl p 5-integrating recombinant retroviruses, we harvested BM cells from Phl p 5 chimeras 40 wk post-BMT and transplanted them into myeloablatively irradiated secondary BALB/c recipients ($n = 3$). Multilineage chimerism was again detectable and persisted for the length of follow-up (15 wk after secondary BMT, Fig. 2*C*), demonstrating that HSC had indeed been transduced to express Phl p 5 and had successfully engrafted and survived in the primary recipients.

Specific absence of Phl p 5-specific humoral responses in recipients of Phl p 5-transduced BM

To assess whether tolerance was induced, we used an established model in which Phl p 5-immunized BALB/c mice develop characteristics of clinical type I allergy such as production of allergen-specific IgE, other allergen-specific isotypes, and IgE-mediated effector cell degranulation (29, 39). Following this protocol we sensitized BMT recipients through repeated immunization with the recombinant allergens Phl p 5 and an unrelated control allergen, the major birch pollen allergen Bet v 1 (Fig. 2*A*). No Phl p 5-specific IgE was detectable in sera of any of the Phl p 5 chimeric mice throughout follow-up as determined by ELISA (Fig. 3*A*). In contrast, Phl p 5 chimeric mice developed high levels of Bet v 1-specific IgE postimmunization (Fig. 3*A*). Recipients of mock-transduced BM ($n = 3$) and non-BMT immunized mice (i.e., naive mice treated with the same immunization regimen but not receiving BMT, $n = 10$) developed high levels of IgE in response to both allergens. Likewise, Phl p 5 chimeras developed no Phl p 5-specific IgG1, whereas recipients of mock-transduced BM and non-BMT immunized mice showed high levels of Phl p 5-specific IgG1 upon repeated immunizations (Fig. 3*B*). Bet v 1-specific IgG1 levels were comparably high in all groups of mice. Similar results were obtained for allergen-specific IgA, IgG2a, and IgG3 (Fig. 3*C–E*). Phl p 5-specific low-affinity IgM could be detected in sera of Phl p 5 chimeras (Fig. 3*F*) reminiscent of natural autoantibodies directed against self-Ag (40). Thus, transplantation of Phl p 5-transduced BM led to specific tolerance toward the allergen at the B cell level, preventing the production of allergen-specific IgE and other high-affinity isotypes.

Specific T cell unresponsiveness in recipients of Phl p 5-transduced BM

In the course of an IgE-mediated allergic immune reaction, APCs induce activation and proliferation of allergen-specific T cells (1,

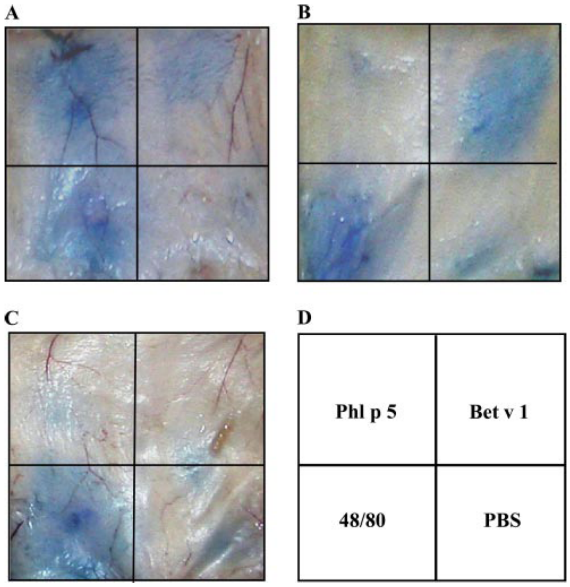

FIGURE 6. Recipients of Phl p 5-transduced BM are specifically tolerant toward the Phl p 5 at the effector cell level as determined by type I allergen-specific skin responses in vivo. Mice were injected i.v. with Evans blue dye and subsequently rPhl p 5 and rBet v 1 were injected intradermally into abdominal skin. Injection of the mast cell-degranulating compound 48/80 resulted in blue staining (positive control), whereas PBS resulted in no staining (negative control). The blue color intensity of the allergen-specific response was compared with the individual positive and negative controls. Reactions were assessed on the inverted abdominal skin. *D*, Scheme of intradermal injection. Representative skin sections of naive mice ($n = 4$) (*C*), non-BMT immunized mice ($n = 5$) (*A*), and Phl p 5 chimeras ($n = 7$) (*B*) are shown.

2). We therefore determined T cell responsiveness in in vitro proliferation assays by stimulating splenocytes isolated from recipients of Phl p 5-transduced BM with Phl p 5 and Bet v 1. The lymphocytes were isolated at the end of follow-up from chimeric mice of two independent experiments 40 wk and 29 wk after BMT, respectively (Fig. 4). Proliferation in response to Phl p 5 was reduced by 90% in recipients of Phl p 5-transduced BM cells compared with proliferation of splenocytes from non-BMT immunized mice (stimulation indices of 21 vs 219, $p = 0.006$) (Fig. 4*A*). In contrast, the proliferation response to Bet v 1 was high both in Phl p 5 chimeras and non-BMT immunized controls (Fig. 4*B*). Thus, recipients of Phl p 5-transduced BM showed allergen-specific T cell tolerance.

IgE-mediated degranulation of basophils and mast cells is specifically abolished in recipients of Phl p 5-transduced BM

Cross-linking of IgE on tissue mast cells and basophils by allergens results in local release of inflammatory mediators (including histamine) that cause many symptoms of the acute phase of an allergic reaction (41). We therefore analyzed effector cell function in vitro and in vivo. In an RBL degranulation assay, sera from Phl p 5 chimeras and control groups were loaded onto RBL cells, and mediator release (β-hexosaminidase as surrogate marker for histamine (33)) was measured after challenge with allergen. In recipients of Phl p 5-transduced BM no mediator release was detectable in response to Phl p 5 whereas release occurred upon challenge with Bet v 1 (Fig. 5). Non-BMT immunized mice and recipients of mock-transduced BM cells showed mediator release in response to both Phl p 5 and Bet v 1. To investigate anaphylactic activity of skin mast cells in vivo, we measured allergen-specific immediate-type hypersensitivity responses by intradermal allergen challenge and Evans blue staining. No positive skin reaction was detectable after intradermal challenge with Phl p 5 in 6 of 7 tested Phl p 5 chimeric mice, whereas a positive reaction was visible in all mice upon Bet v 1 challenge (Fig. 6*B*). Non-BMT immunized mice showed positive skin reactions to both allergens (Fig. 6*A*). In contrast, naive BALB/c mice did not show any mast cell skin reaction upon allergen challenge (Fig. 6*C*). The results from these in vitro and in vivo assays reveal that recipients of Phl p 5-transduced BM developed allergen-specific tolerance at the effector cell level.

Discussion

The data presented herein provide "proof-of-concept" that tolerance toward an allergen can be induced through transplantation of genetically modified BM. This novel approach for tolerizing a type I allergic immune response has two unique characteristics: permanence and robustness. Tolerance persisted for the length of follow-up (40 wk). As our data point to the successful engraftment of HSC transduced with the allergen, it appears safe to assume that molecular chimerism would persist for the physiological lifespan of the recipient and would continue to maintain tolerance. With this approach all relevant levels of a type I allergic immune response, namely T cells, B cells, and effector cells, were rendered specifically tolerant toward the immunogenic grass pollen allergen used in these experiments for BM transduction. Notably, allergen-specific IgE, IgG subtypes, and IgA remained undetectable throughout follow-up. Besides, T cell responses and effector cell responses toward the allergen could not be detected. A comparably complete degree of tolerance has, to the best of our knowledge, not been reported with other experimental or clinical approaches that have been used for allergy treatment or prevention.

Allergen-specific immunotherapy, the only causative treatment of allergy currently available in the clinical setting, was suggested to lead to immunomodulation of T cell responses in large part through the induction of a Th1 shift and the generation of regulatory T cells. Additionally, the humoral response is affected by the induction of high levels of allergen-specific IgG (and other isotypes), which is then competing with allergen-specific IgE (4). At the experimental level, dominant T cell epitope-containing polypeptides of three different allergens were administered intranasally in a murine mucosal tolerance approach. After subsequent sensitization with allergens, allergen-specific humoral and effector cell responses were merely reduced but not completely prevented (10). Another recently published approach relied on the blockade of the ICOS–ICOS ligand pathway, which induced regulatory T cells and inhibited OVA-induced airway hyperreactivity, but OVA-specific IgE was still detectable (9). A fusion protein consisting of an allergen and a truncated Fcγ1 portion that was designed for the purpose of immunomodulation was shown to inhibit allergen-induced basophil and mast cell degranulation by co-crosslinking of FcεRI and Fcγ receptors (follow-up ~6 wk), but it did not prevent Ab production (11). Overall, the causative approaches described in the literature so far, as exemplified above, led to immunomodulation and reduction of an allergic reaction, but not to the complete, permanent absence of all relevant levels of an allergen-specific immune response. Molecular chimerism, in contrast, establishes such a state of complete tolerance toward an allergen. Although detailed mechanistic studies are beyond the scope of this paper, we consider it likely that central tolerance plays a critical role in our model, as it does in all chimerism-based protocols (16). However, nondeletional mechanisms, in particular T

regulatory cells, might also be of importance, as we have recently shown in an allogeneic mixed chimerism model (42, 43).

Previously it had been shown that molecular chimerism can be used to tolerize allogeneic and xenogeneic immune responses, not only in rodents, but also in large animals (20, 27, 44). This concept failed, however, in a specific autoimmune model for unclear reasons (23). Thus, while molecular chimerism overall is a highly attractive tolerance approach, its effectiveness needs to be individually assessed for each particular immune response, and regimens potentially need to be adapted accordingly. To the best of our knowledge, none of the reported molecular chimerism studies has investigated IgE responses and none has used allergens.

Transplantation of retrovirally transduced BM has been able to correct life-threatening lymphoid and myeloid immunodeficiencies in the clinical setting (45, 46), but it was associated with serious side effects (47). However, substantial advances in vector design are continuously being achieved so that safe vectors may some day become available (47, 48). Molecular chimerism relies on the transplantation of autologous BM modified to differ in a single Ag (or at most a small, limited number of Ags), thereby avoiding graft-vs-host disease, one of the gravest risks associated with allogeneic BMT and cellular chimerism. Minimally toxic regimens for recipient conditioning have recently been developed for the experimental transplantation of allogeneic BM that could eventually also be used for the molecular chimerism approach (15, 30, 49, 50). Besides, autologous and allogeneic BMT have become a therapeutic option in selected clinical cases of autoimmune disease, and the range of indications for which BMT is a valid treatment is expected to increase substantially during the coming years (51, 52).

According to advances made in the field of molecular allergen characterization, a limited number (~30) of major and clinically relevant allergens can be defined that cover the most relevant allergen sources (6). Using the recently described hybrid technology it should be possible to engineer a few hybrid molecules (5–6) that may be sufficient to tolerize populations against the most common allergen sources in certain areas (53).

The concept presented herein provides a novel cell-based approach for tolerizing a type I allergic immune response through transplantation of genetically modified hematopoietic cells. As most relevant allergens have been cloned, this approach may theoretically be used for the prevention of many common forms of allergy.

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5.1.5 Expression of a Major Plant Allergen as Membrane-Anchored and Secreted Protein in Human Cells with Preserved T Cell and B Cell Epitopes

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Expression of a Major Plant Allergen as Membrane-Anchored and Secreted Protein in Human Cells with Preserved T Cell and B Cell Epitopes

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Key Words
IgE-mediated allergy · Recombinant allergen Phl p 5 · Cell-based therapy · Allergenic activity · Surface protein expression

Abstract
Background: Expression of allergens in human cells is a prerequisite for the development of antigen-specific cell therapy in IgE-mediated allergy. We developed a strategy how the clinically relevant major grass pollen allergen Phl p 5 can be efficiently secreted or expressed on the surface of human cells with preserved allergenic activity. ***Methods:*** The cDNA of Phl p 5 was fused to a leader peptide with or without a transmembrane domain and both constructs were ligated into a mammalian expression vector. Transfection of these plasmids into human cells resulted in a membrane-anchored or secreted version of Phl p 5, respectively, as determined by ELISA or flow cytometric analysis. ***Results:*** Both the secreted and membrane-anchored Phl p 5 proteins bound IgE from allergic patients in an immunoblot assay and induced specific histamine release and CD203c upregulation in basophils of grass pollen-allergic patients. Proliferation of peripheral blood mononuclear cells from Phl p 5-allergic individuals was induced upon stimulation with both variants of Phl p 5 expressed in human cells similar to recombinant Phl p 5. ***Conclusions:*** Secreted and membrane-anchored Phl p 5 expressed in human cells preserved B cell as well as T cell epitopes and may be used to develop and test various cell-based strategies for allergen-specific immunomodulation and to delineate the tolerance mechanisms involved therein.

Introduction

Allergen-specific immunotherapy is the only causative treatment in IgE-mediated allergy so far, but prevention of allergy is still an unmet goal. Transplantation of hematopoietic stem cells that have been genetically modified to express disease-causing antigens, such as auto- and allo-antigens, is a therapeutic strategy to induce tolerance towards these disease-causing antigens.

R.V. and T.W. are co-senior authors.

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Robust long-term B and T cell tolerance has been demonstrated with this molecular chimerism approach in experimental models including allergy [1, 2]. Therefore, expression of functional disease-causing antigens is a critical prerequisite for the exploration of a chimerism-based tolerance strategy for any specific disorder.

Allergens are highly immunogenic, exposing a number of IgE-binding sites (B cell epitopes) on the surface [3]. T cell epitopes, in contrast, are small peptides resulting from proteolytic processing of allergens and their presentation via MHC molecules. Allergens are antigens derived from a wide range of sources such as plants, mammals, moulds and hymenoptera. The immunologic properties and allergenic activities of allergens may vary dramatically depending on their fold, posttranslational modification, state (i.e. monomeric vs. oligomeric), dose and site of entry into the body [4, 5]. Therefore, allergens expressed in cellular systems must be compared with a reference allergen equaling the natural allergen in order to understand the mechanisms underlying strategies for cell-based allergen-specific immunomodulation and to further develop cell-based therapies.

Phl p 5 is one of the most potent and frequent environmental allergens affecting 80% of grass pollen-allergic individuals. Recombinant (r) Phl p 5 equals the immunological properties and allergenic activity of the corresponding natural Phl p 5 allergen [6].

We constructed fusions of Phl p 5 with a leader peptide for secretion and an additional transmembrane domain for surface anchoring of the allergen for expression in human cells. We studied the allergenic activity of Phl p 5 expressed in human cells determining IgE-binding, mediator release and T cell reactivity.

Materials and Methods

Constructs

cDNA sequence (aa 25–313) of Phl p 5 (accession number X74735) was fused to a signal sequence and a transmembrane domain (TMD) (both pDisplay, Invitrogen, Carlsbad, Calif., USA) by overlapping PCR technique [7]. Primer sequences were used as follows: leader peptide: 5′-GGCGCCATGGAGACAGACACACTCCTG-3′, 5′-GTAACCGAGATCGGCGTCACCAGTGGA-3′, Phl p 5: 5′-ACTGGTGACGCCGATCTCGGTTAC-3′, 5′-GCCCACAGCGACTTTGTAGCCACC-3′, TMD: 5′-TACAAAGTCGCTGTGGGC-3′, 5′-GGCGGATCCTAACGTGGCTTCTTCTG-3′. PCR products were cloned into the vector pcDNA3.1(–) (Invitrogen) under the control of a CMV promoter using EcoRI and BamHI sites resulting in p Phl p 5-TM or p Phl p 5. Positive clones were confirmed by DNA sequencing (VBC Biotech, Vienna).

Cell Culture and Transfection

HEK (human embryonic kidney) 293 T cells were grown in DMEM media including 10% FCS and antibiotics. 5×10^5 and 2×10^6 cells were grown in 6-well plates or 10-cm dishes (Falcon, Becton Dickinson, Franklin Lakes, N.J., USA) and transfected (1–10 μg) using the Ca_2PO_4 transfection method or transduced resulting in efficient surface expression of Phl p 5 as described previously [8, 9].

Recombinant Allergens, Antibodies

Purified recombinant timothy grass pollen allergen (rPhl p 5) was obtained from BIOMAY (Vienna, Austria). Phl p 5-specific polyclonal antibodies were used as described in [9].

Immunoblotting

Cell extracts, cell culture supernatants of transfected cells and rPhl p 5 were separated on a 16% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. Sera from timothy grass pollen-allergic individuals, non-Phl p 5-allergic donors and healthy donors were diluted 1:10 and used to probe nitrocellulose-blotted extracts and supernatants from Phl p 5-expressing 293 T cells. Extracts and supernatants of 293 T cells transfected with empty vector were used as a negative control (mock). Bound IgE was detected with ^{125}I-labelled anti-human IgE (Phadia, Uppsala, Sweden) [10].

ELISA

Amounts of secreted Phl p 5 in supernatants of p Phl p 5- and p Phl p 5-TM-transfected 293 T cells were quantified by Phl p 5-specific ELISA as described in Focke et al. [11].

Flow Cytometric Analysis

Biotinylated polyclonal Phl p 5-IgG was used as described in Baranyi et al. [9] and developed with phycoerythrin streptavidin. A Coulter Cytomics FC500 flow cytometer (Coulter, Austria) was used for acquisition and CXP software (Coulter) for analysis of flow cytometric data.

Histamine Release and CD203c Upregulation Experiments

Heparinized peripheral blood samples were obtained from individuals with grass pollen allergy known to react against Phl p 5, from individuals with different allergies (with known non-reactivity to Phl p 5) and healthy individuals. Basophils were enriched from blood of 2 allergic patients by Dextran sedimentation, washed and resuspended in histamine release buffer (HRB; Immunotech, Marseille, France) as described in Valent et al. [12]. Cells (3×10^6/ml) were incubated with various concentrations of rPhlp 5/transfected 293 T cells/supernatants of transfected cells in 96-well microtiter plates (TPP, Trasadingen, Switzerland) for 30 min (37°C). After incubation, cells were centrifuged at 4°C for 10 min. Cell-free supernatants were recovered, and histamine was measured by a commercial radioimmunoassay (RIA; Immunotech). Histamine release was calculated as percentage of total (extracellular and cellular) histamine measured in lysates of cells that were prepared by freeze-thawing. For investigation of CD203c upregulation, blood aliquots were incubated with rPhl p 5 (0.001 and 0.1 μg/ml), cells expressing membrane-anchored Phl p 5 and supernatants containing secreted Phl p 5 produced in 293 T cells. A monoclonal anti-IgE antibody E-124.2.8 (1 μg/ml; Immunotech) and PBS were used as control. Thereafter, cells

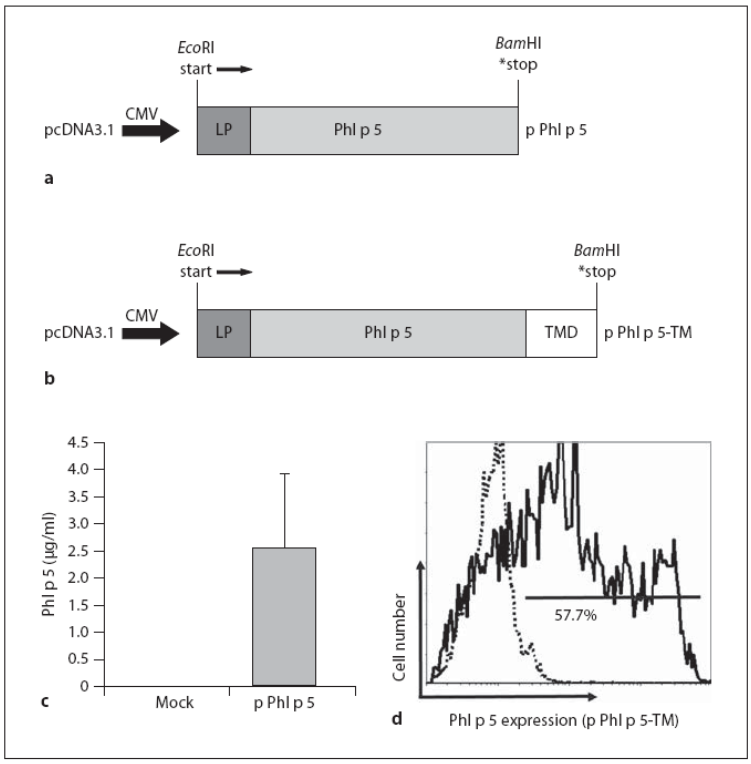

Fig. 1. High-level expression of secreted and membrane-anchored Phl p 5 in transfected human cells. **a** Construct for secretion of Phl p 5 (p Phl p 5). **b** Construct for membrane-anchored expression of Phl p 5 (p Phl p 5-TM). Both constructs were cloned into pcDNA3.1 vector under the control of a CMV promoter. LP denotes leader peptide and TM transmembrane domain. The drawing is not to scale. **c** Secretion of Phl p 5 was quantified by Phl p 5-specific ELISA in supernatants of 293T cells transfected with the construct shown in **a**. The mean (+SD) of 3 independent transient transfections is shown. As control, supernatant of 293T cells transfected with empty vector (mock) was quantified for soluble Phl p 5. **d** Efficient expression of Phl p 5 on the surface of 293T cells transfected with construct p Phl p 5-TM is shown as determined by flow cytometry. Dotted line indicates mock-transfected 293 T cells (empty vector).

were stained with phycoerythrin-conjugated CD203c mAb 97A6 (Immunotech). After erythrolysis cells were washed and resuspended in PBS and analyzed by 2-color flow cytometry. Upregulation of CD203c was calculated from mean florescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells and is expressed as stimulation index (SI) (MFI_{stim}:$MFI_{control}$).

T Cell Proliferation Assay

Peripheral blood mononuclear cells (PBMC) were obtained from allergic and nonallergic donors by Ficoll-Hypaque density-gradient centrifugation (Pharmacia Diagnostics, Uppsala, Sweden). PBMC (2×10^5) were cultured for 6 days in triplicates in 96-well plates (Nunclone, Nunc, Denmark) in 200 µl of serum-free Ultra Culture Medium (Bio Whittaker, Walkersville, Md., USA) supplemented with 2 mM/l glutamine and 2×10^{-5} M 2-ME in the presence of rPhl p 5- (1–30 µg) and p Phl p 5-TM-transfected or transduced cells or supernatants of p Phl p 5-transfected cells like described above. During the last 16 h of culture [^{3}H]-thymidine (0.5 µCi per well) was added and the incorporated radioactivity was measured by scintillation counting in counts per minute (cpm). The stimulation index (SI) was calculated as ratio between cpm obtained in T cell cultures plus PBMC plus allergen and cpm obtained in cultures plus PBMC without allergen or as ratio between cpm obtained in T cell cultures plus PBMC and membrane-anchored or secreted Phl p 5 and cpm obtained in cultures plus PBMC and control cells or supernatants from control cells.

Results

Construction of Plasmids Coding for Secreted and Membrane-Anchored Phl p 5

For construction of secreted and membrane-anchored Phl p 5, we fused a murine immunoglobulin leader peptide to Phl p 5 [6] replacing the original plant signal peptide for efficient secretion. Additionally, to express Phl p 5 in a membrane-anchored fashion, the transmembrane domain of platelet-derived growth factor receptor, which has demonstrated efficacy in anchoring fusion proteins in human and murine cell lines, was added [9, 13]. The fusion genes were cloned under the control of a CMV promoter into vector pcDNA3.1 known to be a high level expression vector in mammalian cells (fig. 1a, b).

Membrane-Anchored Expression and Secretion of Phl p 5 in Human Cells

293T cells were transiently transfected with vector p Phl p 5 (fig. 1a) and Phl p 5 was quantified in the supernatant of transfected cells by Phl p 5-specific ELISA [11]. In all experiments, we used rPhl p 5, produced and purified from *Escherichia coli*, as a standard reference. Amounts of 1–3 μg/ml allergen secreted by p Phl p 5-transfected human cells were detected in the supernatant (fig. 1c). As a negative control, empty vector pcDNA3.1 was transfected (mock) in 293T cells and supernatants were analyzed by ELISA (fig. 1c). Transient transfection of 293T cells with p Phl p 5-TM (fig. 1b) led to cell surface expression of Phl p 5 (about 60% of cells were Phl p 5+) as quantified by flow cytometry with Phl p 5-specific antibodies [9] (fig. 1d). These cells also liberated Phl p 5 fused to the transmembrane domain (as evident from the supernatant; fig. 2) but at lower quantities than p Phl p 5-transfected cells (about 0.7 μg/ml Phl p 5, as determined in Phl p 5-specific ELISA, data not shown).

Phl p 5-Specific IgE Binding of Phl p 5 Expressed in Human Cells

The IgE-binding capability of Phl p 5 expressed in human cells was assessed with sera of Phl p 5-allergic patients. Secreted and membrane-anchored Phl p 5 of supernatants and extracts of transfected human 293T cells were loaded onto a denatured protein gel and blotted to a nitrocellulose membrane. Membranes were incubated with sera from allergic patients reacting to Phl p 5 (fig. 2a, b), sera from healthy donors as negative control and sera from donors with different allergic history (non-Phl p 5-allergic donors) and bound IgE was detected with labeled anti-IgE. rPhl p 5 was loaded as the positive control. Phl p 5-binding IgE was detected in supernatants and cell extracts of Phl p 5-expressing human 293T cells (fig. 2a, b). Extracts from cells transfected with p Phl p 5-TM showed a higher mobility shift due to the transmembrane domain fusion (additional 50 aa). The IgE reactivity of mammalian cell-expressed Phl p 5 in extracts and supernatants of secreted or membrane-anchored Phl p 5 was comparable to the IgE reactivity of rPhl p 5.

Both Surface-Expressed Phl p 5 and Phl p 5 Secreted by Human Cells Induce Mediator Release

Basophils store a number of vasoactive mediators like histamine in their granules. In allergic individuals, basophils bind IgE via high-affinity receptors (i.e. FcεRI) and release their mediators into the extracellular space upon allergen exposure [14]. The in vitro basophil degranulation assay has been shown to closely reflect the cascade of the allergic effector reaction as a model for the activity of natural and recombinant allergens similar to the in vivo situation [15]. Further, the ectoenzyme CD203c is expressed in basophils (not in other peripheral blood cells) and upregulated on IgE receptor cross-linkage with r allergens in basophils of sensitized individuals [12]. We examined the capacity of secreted and membrane-anchored Phl p 5 to induce degranulation and CD203c upregulation of basophils of Phl p 5-allergic individuals (fig. 3a, b). Basophils of two allergic individuals were incubated with serial dilutions of rPhl p 5 resulting in a typical Phl p 5-dependent bell-shaped peak in dose-response curves described to differ among allergens and allergic individuals [12]. The Phl p 5-specific release varied from about 60% Phl p 5-specific histamine release (fig. 3a1, patient 1) to almost 100% Phl p 5-specific histamine release in basophils of patient 2 (fig. 3a1). Escalating numbers of human cells expressing membrane-anchored Phl p 5 (or mock-transfected cells as control) were co-incubated with basophils of the same patients (fig. 3a2). Membrane-anchored Phl p 5 expressed on human cells efficiently triggered histamine release in a dose-dependent manner in basophils of patient 1 (50–60%). Patient 2-derived high responding basophils yielded a maximum histamine release of about 100% even with the lowest number of transfected cells. Secreted Phl p 5 in supernatants of transfected human cells led to a histamine release comparable to rPhl p 5 (fig. 3a3). Likewise, basophils of a Phl p 5-allergic patient showed upregulation of CD203c with cells expressing membrane-anchored Phl p 5 or secreted Phl p 5 comparable to upregulation with rPhl p 5 (fig. 3b1). On the contrary, basophils of non-Phl p 5-allergic patient and healthy donor showed no CD203c upregulation with rPhl p 5 and membrane-anchored Phl p 5 as well as soluble Phl p 5 incubation (fig. 3b2, b3). These results demonstrate Phl p 5-specific activation of basophils by cross-linking of membrane-anchored and secreted Phl p 5 expressed in human cells by two independent techniques.

T Cell Proliferation of PBMC of Allergic Donors Stimulated with Phl p 5-Expressing Human Cells

In atopic individuals allergen-specific memory T cells respond to repeated allergen contact leading, for example, to chronic asthma [16]. Stimulation of PBMC with either supernatant containing secreted Phl p 5 or with membrane-anchored Phl p 5 both led to responses similar to rPhl p 5, while nonallergic donors did not respond (table 1).

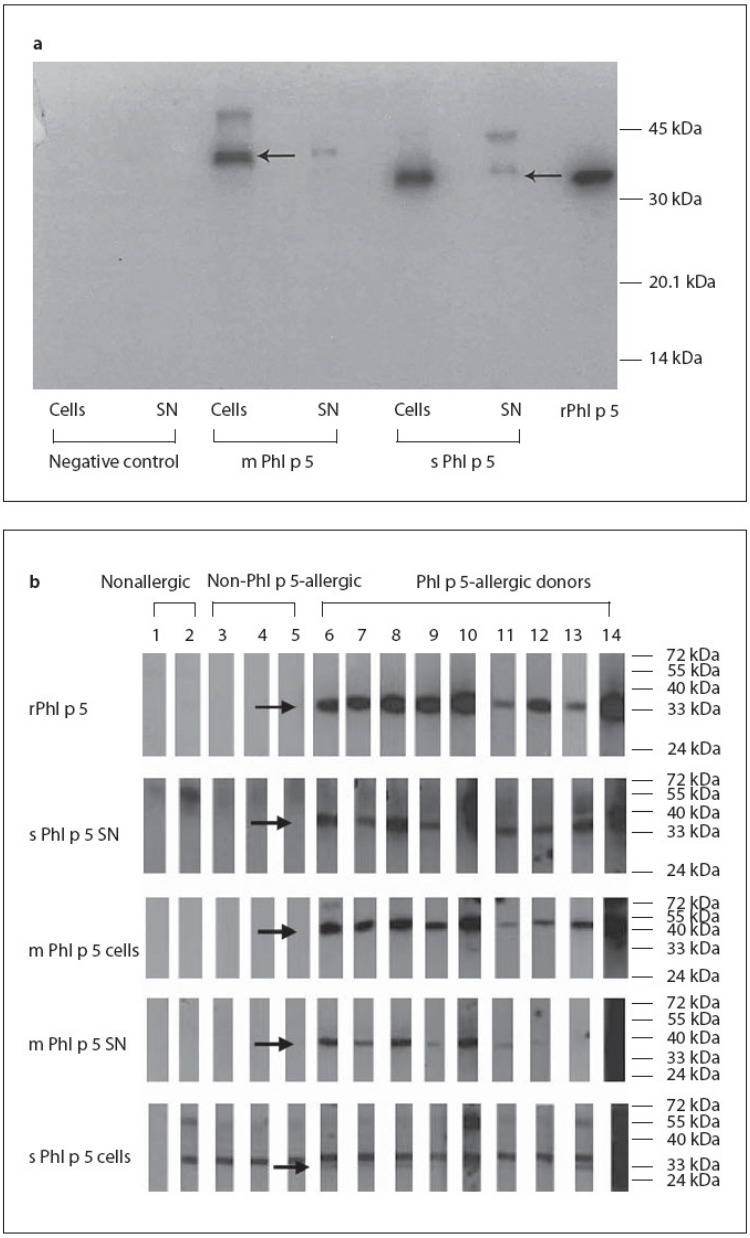

Fig. 2. Serum-IgE of allergic patients recognizes Phl p 5 expressed in human cells. **a**, **b** Nitrocellulose-blotted lysates (cells) and supernatants (SN) of 293T cells transfected with constructs for secreted Phl p 5 (s Phl p 5) or membrane-anchored Phl p 5 (m Phl p 5) were incubated with sera of Phl p 5-allergic donors, donors with a history of different allergic disease and healthy donors. Bound IgE was detected with ^{125}I-labeled anti-human IgE and visualized by autoradiography. rPhl p 5 served as positive control. Negative control denotes equal amounts of supernatants and cells from mock-transfected cells. Predominant proteins are indicated by arrows.

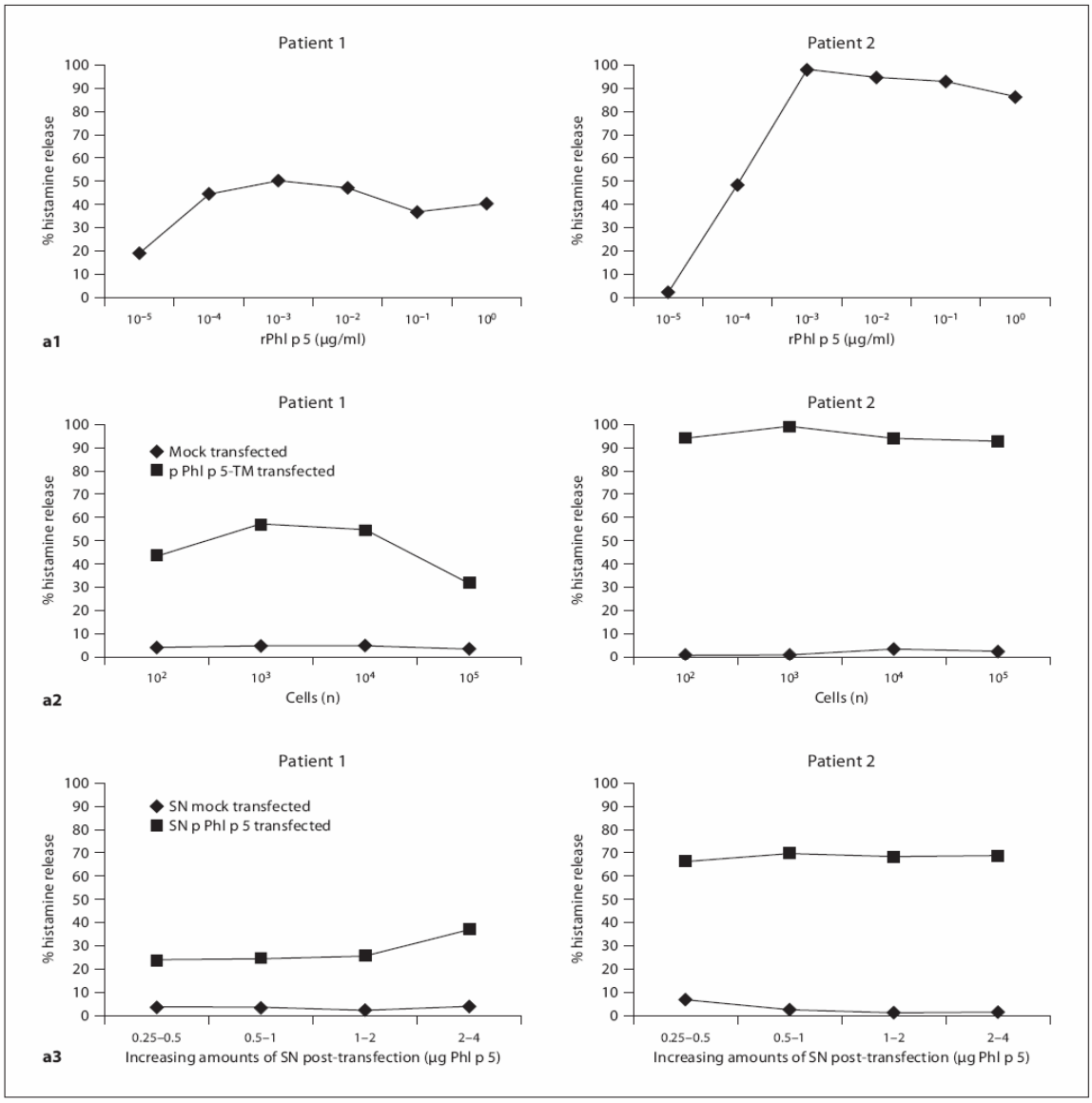

Fig. 3. Membrane-anchored and secreted Phl p 5 lead to histamine release and CD203c upregulation in basophils of allergic patients. Basophils of two allergic patients (patients 1 and 2) were incubated with different concentrations of rPhl p 5 (**a1**), increasing amounts of cells expressing membrane-anchored Phl p 5 or cells transfected with empty vector as control (**a2**), or different amounts of supernatants from cells secreting Phl p 5 (**a3**). Supernatants of mock-transfected cells were used as a negative control. CD203c upregulation was determined in blood of an allergic donor (**b1**) and a non-Phl p 5 allergic donor (**b2**) as well as in blood of a healthy donor (**b3**). Cells were incubated with rPhl p 5, membrane-anchored Phl p 5-expressing cells (m Phl p 5 cells) and supernatants of p Phl p 5-transfected cells (s Phl p 5). Anti-IgE was used as the positive control. Unstimulated cells (US) were used as the baseline. Mock-transfected cells and supernatants of mock-transfected cells were used as negative controls.

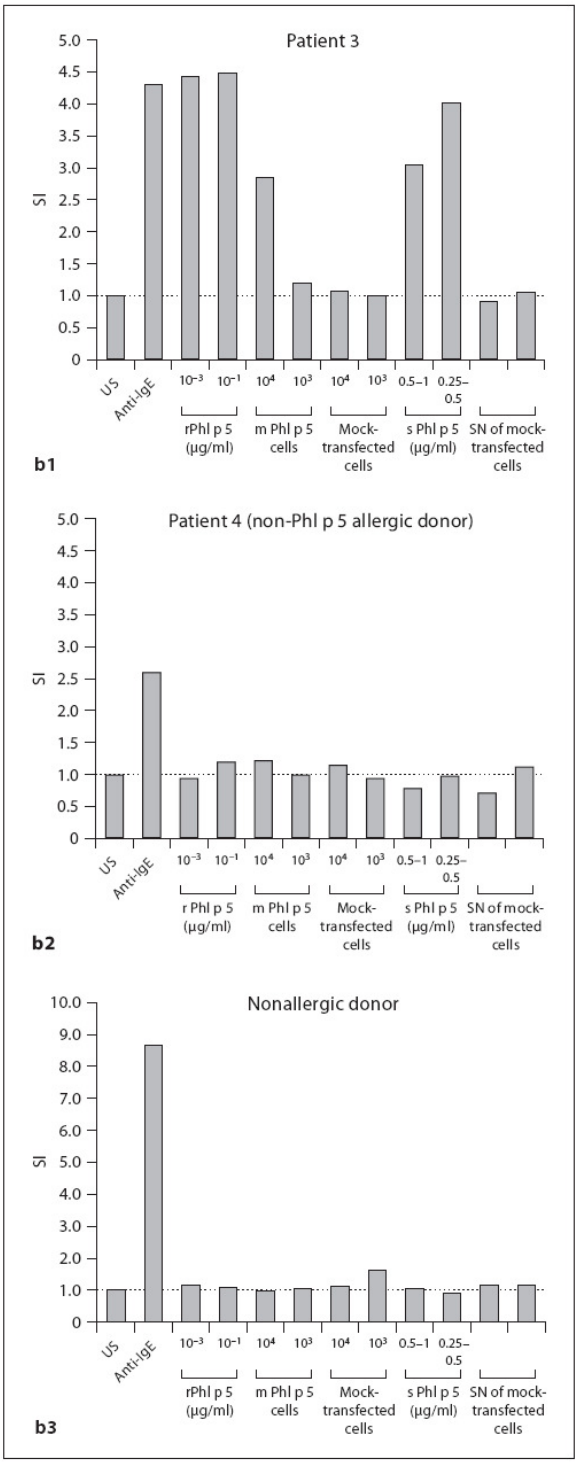

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Table 1. Lymphoproliferation (SI) of PBMC derived from three grass pollen-allergic patients (AD) and nonallergic donors (NAD) in response to stimulation with secreted (s) and membrane-bound (m) Phl p 5 derived from transfected human 293T cells

	Il-2	rPh1 p 5	s Phl p 5	m Ph1 p 5
AD 1	22.3	3.3	9.6	4.8
AD 2	59.4	4.3	3.4	1.1
AD 3	29.6	3.5	2.8	6.1
NAD 1	8.1	1.0	0.8	1.1
NAD 2	4.8	1.6	1.6	1.6

As negative controls, nontransfected 293T cells and their supernatants (control cells) were used. For positive control, PBMC were stimulated with rPhl p 5 and r Il-2. Two patients showed no proliferation to the positive control rPhl p 5 in this assay (not shown).

Discussion

Here we demonstrate efficient expression of secreted and membrane-anchored grass pollen allergen Phl p 5 in human cells. The fusion of a leader peptide and/or an additional transmembrane domain leads to the secretion or to the anchoring of Phl p 5 to the surface of human cells.

Saloga and colleagues [17] have previously reported the expression of the full-length allergen Phl p 1 in human dendritic cells by adenoviral transfer. Cytoplasmic expression of this allergen led to stimulation of T cell proliferation in vitro. This approach has been directed to induce T_H1 responses for potential immunotherapy by presentation of T cell peptides of the allergen Phl p 1 for CD8+ T cell proliferation. In contrast, our approach focuses on prophylactic tolerance induction at the T and B cell level and therefore displays allergens containing B and T cell epitopes on the cell surface or as secreted product. Both forms, membrane-anchored and secreted Phl p 5, recognize IgE obtained from a Phl p 5-allergic individual. Furthermore, basophil mediator release and T cell proliferation suggest correct expression of B cell epitopes and presentation of T cell epitopes of the released and membrane-anchored allergen Phl p 5 expressed by human cells.

Allergenic activity similar to the wild-type allergen is successfully retained in both instances.

In a murine model, we successfully induced long-term tolerance towards Phl p 5 by transplantation of hematopoietic stem cells expressing membrane-anchored Phl p

5 into syngeneic recipients. No Phl p 5-specific IgE, IgA and IgG subtypes were detectable in these mice after numerous immunizations with rPhl p 5 [9]. Similar results were observed after tolerization with the membrane-anchored major birch pollen allergen Bet v 1 expressed in hematopoietic stem cells [M.G. and T.W., unpubl. data]. Therefore, it appears likely that this approach can be extended to a variety of allergens. Although several impediments have to be overcome, molecular chimerism is a promising strategy for tolerization type I allergic immune responses [2].

Here we show the successful expression of two forms of Phl p 5 in human cells offering establishment of cell-based therapy approaches for prophylaxis and treatment of allergy.

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5.1.6 Therapeutic Efficacy of Polyclonal Tregs does not Require Rapamycin for the Induction of Tolerance in a Low-Dose Irradiation BMT Model

Therapeutic Efficacy of Polyclonal Tregs Does Not Require Rapamycin in a Low-Dose Irradiation Bone Marrow Transplantation Model

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Background. Mixed chimerism is an effective strategy for the induction of transplantation tolerance but the toxicity of recipient conditioning makes current bone marrow (BM) transplantation (BMT) protocols unsuitable for widespread clinical application. Therapies promoting BM engraftment under minimal conditioning would facilitate translation of this concept to the clinic. Recently, we have shown that regulatory T cell (Treg) therapy has potent engraftment-enhancing effects in an irradiation-free noncytotoxic BMT protocol, but only if it is combined with rapamycin treatment.

Methods. Here, we investigated whether polyclonal Treg therapy is effective in promoting chimerism and tolerance in an otherwise unsuccessful BMT protocol using low-dose total body irradiation (1 Gy) and costimulation blockade and determined whether Tregs do so on their own without rapamycin.

Results. The application of polyclonal FoxP3-transduced recipient Tregs led to durable multilineage chimerism and donor-specific skin graft tolerance whereas recipients receiving costimulation blockade alone or green flourescent protein (GFP)-transduced cells failed to develop chimerism. Infused Tregs had a limited life span as indicated by polymerase chain reaction analysis but rather contribute to de novo induction of subsequent Treg generations. Deletion of donor-reactive T cells was observed but progressed more slowly over time compared with recipients of a nonmyeloablative BMT protocol using 3 Gy total body irradiation.

Conclusions. In conclusion, Treg therapy promotes BM engraftment on its own in a low-dose irradiation BMT protocol, leading to chimerism and tolerance maintained through deletional and nondeletional mechanisms.

Keywords: Transplantation tolerance, Mixed chimerism, Regulatory T cells, Rapamycin.

(*Transplantation* 2011;92: 280–288)

Induction of donor-specific immunological tolerance would obviate the need for lifelong immunosuppressive therapy in organ transplant recipients. Numerous tolerance protocols have been developed in the rodent system but so far most of them failed when translated into large animal models. The mixed chimerism approach is a rare exception in this respect because it was shown to induce donor-specific tolerance in nonhuman primates (*1*) and notably also in clinical pilot trials (*2*, *3*). The toxicities of currently used bone marrow (BM) transplantation (BMT) protocols, however, preclude routine clinical application of this approach (*4*).

Therefore, strategies have been sought that allow allogeneic BM to be successfully transplanted with reduced recipient conditioning. "Mega" doses of BM (*5–7*), rapamycin (*8*), natural killer (NK) cell depletion (*9–11*), anti-CD8 and anti-CD4 monoclonal antibodies (mAbs) (*12–14*), and notably costimulation blockade (*5*, *15–17*) are among the identified engraftment-promoting strategies. Particularly, rapamycin positively affected BM engraftment in costimulation blockade-based models (*8*, *18*, *19*), allowing reduction of recipient preconditioning (*8*). However, using clinically feasible amounts of BM cells, rapamycin on its own was not sufficient to induce BM engraftment without low-dose total body irradiation (TBI) (*18*). More recently, regulatory T cells (Tregs) came into focus because they promote engraftment of allogeneic BM in various settings (*20–23*). However, to be successful, Treg therapy required additional (short term) treatment

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with rapamycin (*20*) or a substantial dose of TBI (5 Gy) (*24*). Several studies have demonstrated a potent effect of rapamycin on Treg survival and function (*25–28*). Although data on Treg expansion by rapamycin are somewhat controversial (*25, 29*), suppressive capacity remains preserved in all studies. Rapamycin and Tregs act synergistically in promoting BM engraftment (*20*); however, it is unclear whether this is due to the immunosuppressive effects of rapamycin or its beneficial effects on Treg generation/function. It thus remains undetermined whether polyclonal Tregs can promote BM engraftment on their own under low-dose recipient TBI.

We therefore investigated the potency of polyclonal recipient derived Tregs in a costimulation-blockade-based BMT model under low-dose irradiation. Such low-dose irradiation is commonly used in the clinical setting of "minitransplant" BMT protocols for hematological indications with reduced morbidity (*30*). FoxP3 Tregs have shown similar engraftment enhancing potency as nTregs in vivo (*20*) and comparable efficacy in vitro. As they can be generated efficiently in sufficient quantities, we chose to use FoxP3-transduced Tregs in the current study. The current study provides evidence that the therapeutic use of Tregs enhances BM engraftment on its own and thereby increases the reliability of clinically relevant minimal conditioning BMT protocol.

RESULTS

Generation and Characterization of FoxP3-Transduced Tregs In Vitro

Polyclonal FoxP3-Tregs were generated by retroviral transduction of wild-type B6 $CD4^+$ lymphocytes with a retroviral vector containing FoxP3 followed by an internal ribosomal entry site for direct green flourescent protein (GFP) translation (Fig. 1a) (*31*). Through FoxP3 transduction, large numbers of Tregs can be generated for experimental purposes whose properties resemble those of natural Tregs (*20*). After transduction with FoxP3-GFP virus, cells expressed high levels of FoxP3, CD25, CD62L, GITR, and CTLA4 (Fig. 1b), a phenotypic pattern characteristic of Tregs (*32*), whereas cells transduced with GFP control virus failed to upregulate Treg-associated markers (FoxP3, CD62L, GITR, and CTLA4) (Fig. 1b). In vitro co-culture assays revealed a regulatory function for FoxP3-Tregs as they suppressed proliferation of CD4 T cells in response to alloantigen in a dose-dependent manner (Fig. 1c). Moreover, FoxP3-Tregs seemed superior over freshly sorted $CD4^+CD25^+$ Tregs at lower doses, as suppression was still evident at a ratio of 10:1 (responder cells vs. Tregs) with FoxP3-Tregs but not $CD4^+CD25^+$ Tregs. Similar findings were obtained in several repeat experiments (data not shown). Cells transduced with GFP control virus did not suppress proliferation in response to alloantigen, indicating that suppressor function is due to forced FoxP3 expression (Fig. 1d).

FoxP3-Tregs Promote Engraftment of Allogeneic BM Under Low-Dose Irradiation

First, we investigated whether FoxP3-Tregs by themselves promote engraftment of allogeneic BM in a BMT model using low-dose TBI (1 Gy). Using a well-characterized nonmyeloablative BMT protocol (*8*), B6 recipients were conditioned with 1 Gy TBI and costimulation blockade (anti-CD154 and CTLA4Ig) and received a conventional dose of fully allogeneic Balb/c BM cells (2×10^7 cells/mouse). This protocol is insufficient to reliably induce chimerism and tolerance (*8*), but addition of rapamycin (*8*) or addition of NK depletion (*10*) and unpublished data) leads to chimerism and tolerance.

Treatment with FoxP3-Tregs, but not GFP transduced cells, led to permanent mixed chimerism in the majority of BMT recipients, whereas the low-dose irradiation BMT protocol without Tregs led to transient chimerism only in some recipients (7/9 chimeras with FoxP3-Tregs at 7 months post-BMT vs. 3/10 without Tregs, $P=0.0698$, pooled results from two independent experiments), with chimerism levels being significantly higher in FoxP3-Treg recipients (e.g., 32.96% vs. 3.81% $CD4^+$, $P=0.0132$; 23.54% vs. 4.91% B cell, $P=0.0085$ chimerism at 7 months post-BMT) (Fig. 2a). Chimerism levels in BM and spleen correlated with chimerism in peripheral blood (Table 1). Even though some BMT recipients without FoxP3-Treg treatment initially showed chimerism, it was mostly restricted to the B cell and myeloid lineages (Fig. 2b) and persisting donor T-cell chimerism was detectable only in one mouse. In sharp contrast, FoxP3-Treg-treated mice displayed increasing levels of donor T-cell chimerism, which has been empirically found to correlate with tolerance development (*33*).

Chimerism in Treg-treated recipients persisted for the length of follow-up (>5 months post-BMT, Fig. 2a) and was of multilineage nature (Fig. 2b), suggesting that hematopoietic stem cells had successfully engrafted and survived in the recipient mice. BMT recipients treated with control GFP virus-transduced cells did not develop chimerism or donor skin graft survival (0/3 chimeras, $P=0.0455$ vs. FoxP3-Tregs, data not shown), demonstrating that the observed effects are due to FoxP3 expression in transduced cells. Already 2 weeks after BMT (earliest time point analyzed) no GFP^+ cells were detectable in peripheral blood by FCM analysis (neither in FoxP3-Treg nor GFP-T-cell-treated mice), suggesting that FoxP3-Tregs did not persist long term in large quantities. Lymphoid organs (BM, spleen, thymus, and lymph nodes) were analyzed for the presence of GFP positive cells at the end of follow-up (28–37 weeks post-BMT). Polymerase chain reaction analysis did not detect GFP expression (data not shown), suggesting a limited life span for transferred cells.

Treg Treatment Leads to Long-Term Donor Skin Graft Survival and Protects From Chronic Rejection

BMT recipients treated with additional FoxP3-Tregs demonstrated donor-specific tolerance as tested by skin grafting 1 to 2 months post-BMT (7/9 long-term donor skin graft acceptors with FoxP3-Tregs vs. 0/10 without; $P=0.0033$; pooled results from two independent experiments; third-party C3H skin grafts were rapidly rejected in both groups) (Fig. 3a). Skin grafts appeared macroscopically intact for the length of follow-up (22–30 weeks after skin grafting), histopathological analysis showed preserved skin architecture and virtually no signs of tissue destruction, although lymphocyte infiltrates were evident in some grafts (Fig. 3b). Histological signs of chronic rejection were more prevalent in donor skin grafts of a previous nonmyeloablative BMT protocol using 3 Gy TBI and costimulation blockade (*8, 15*). Furthermore, a substantial increase in the frequency of mast cells in donor

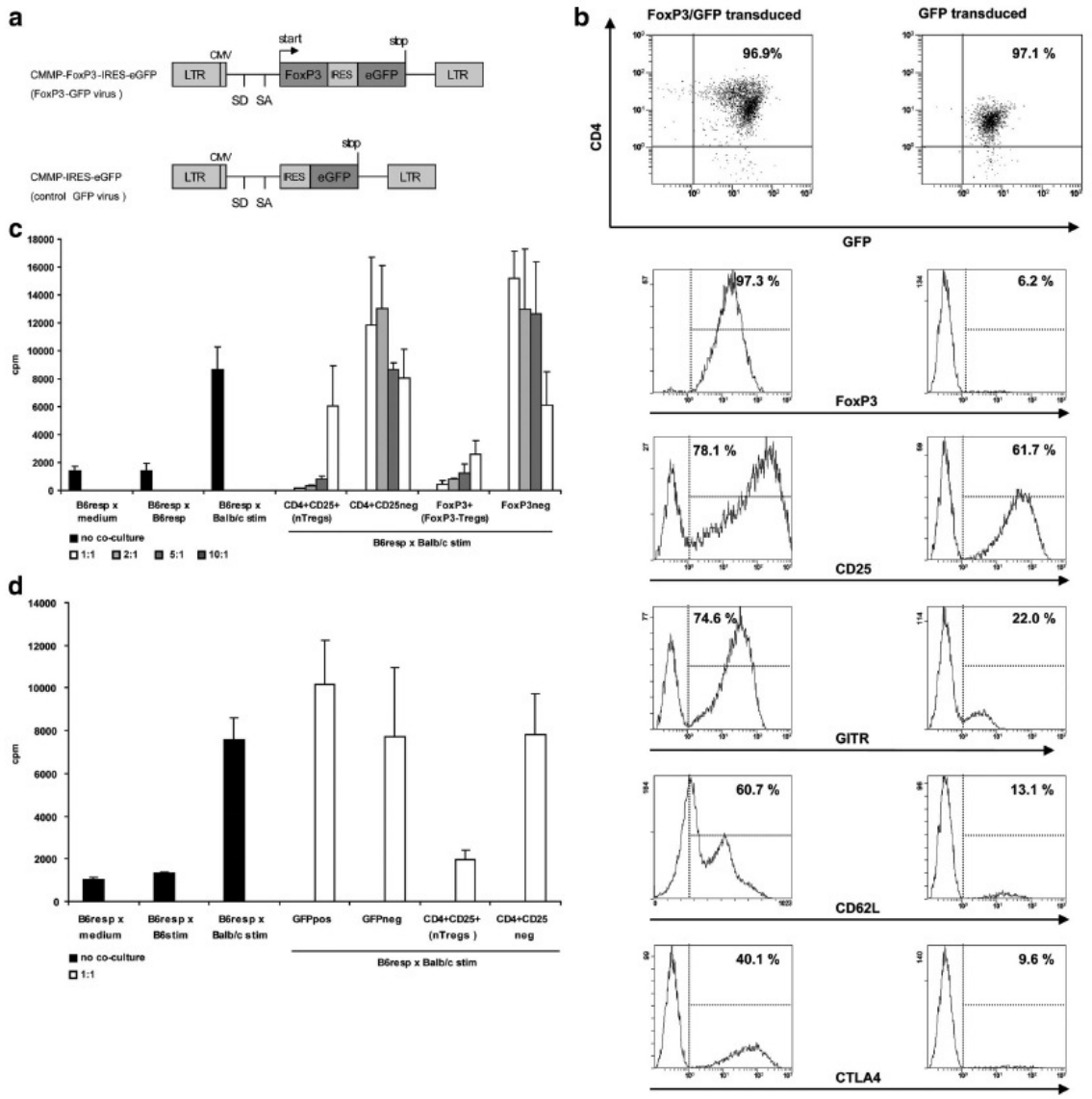

FIGURE 1. Phenotypical and functional characterization of FoxP3-Tregs. (a) Schematic representation of the CMMP-FoxP3-IRES-eGFP (FoxP3/green flourescent protein [GFP]) and CMMP- IRES-eGFP (GFP control) retroviral constructs. LTR represents long terminal repeats. SD and SA represent splicing donor and splicing acceptor, respectively. The drawing is not to scale. (b) B6 $CD4^+$ cells were transduced with FoxP3/GFP or GFP control virus and sorted for GFP expression. Phenotype of sorted cells was assessed by flow cytometric (FCM) analysis. (*Top*) Purity of sorted population. Typical histograms (gated on $CD4^+$ GFP^+ cells) for intracellular expression of FoxP3 and surface expression of Treg-associated markers are shown for FoxP3/GFP-transduced cells (*left*) and GFP-transduced cells (*right*). FoxP3/GFP-transduced cells are almost universally $FoxP3^+$ and express high levels of CD25, CD62L, GITR, and CTLA4. (c) FoxP3-Tregs suppress proliferation of B6 SPL in response to allogeneic stimulation (Balb/c SPL) with leastwise comparable potency as freshly sorted $CD4^+CD25^+$ Tregs, whereas co-culture with $FoxP3^{neg}$ cells $CD4^+CD25^-$ sorted cells results in enhanced proliferation (*black columns*: no co-culture; *white/gray columns*: co-culture; ratio Teff:Tregs 1:1 (*white*), 2:1 (*light gray*), 5:1 (*middle gray*), 10:1 (*dark gray*). Experiments were repeated at least three times with comparable results. Mean counts per minute + SD are shown. (d) Cells transduced with GFP control virus did not suppress proliferation, freshly sorted $CD4^+CD25^+$ Tregs were used as positive control (*black columns*: no co-culture; *white columns* co-culture: ratio Teff:Tregs 1:1).

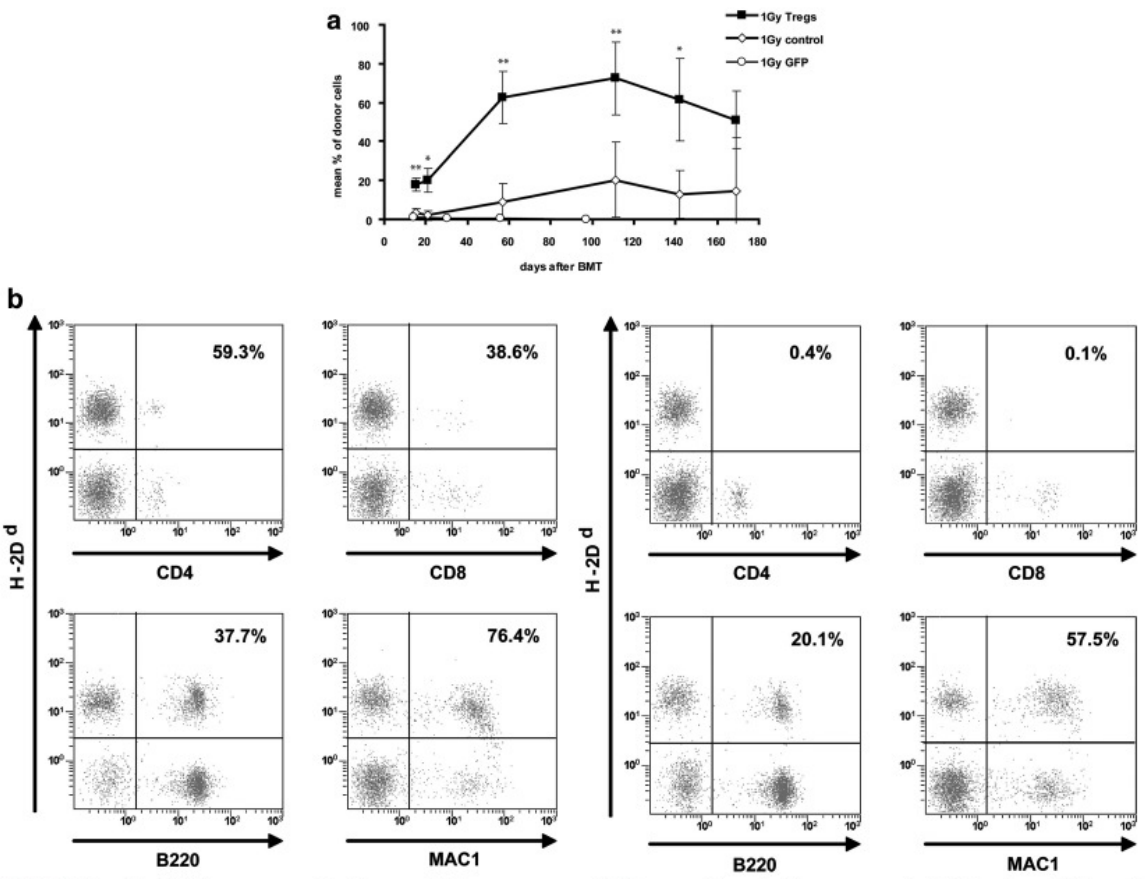

FIGURE 2. FoxP3-Tregs promote allogeneic bone marrow (BM) engraftment after nonmyeloablative irradiation. (a) Groups of B6 mice were grafted with Balb/c BM (2×10^7) after 1Gy total body irradiation (TBI) and costimulation blockade (anti-CD154, CTLA4Ig) and treated with (■, n=5) or without (◇, 4×10^6, n=5) recipient-derived FoxP3-Tregs or recipient derived GFP-transduced $CD4^+$ cells (○, 4×10^6, n=3). Long-term donor ($H\text{-}2D^d$) chimerism among leukocytes of the myeloid ($Mac1^+$) lineage was assessed by FCM analysis of peripheral blood at multiple time points and is shown as mean percent (error bars indicate standard deviation; representative data from 1 of 2 independent experiments) $^{**}P<0.0005$, $^{*}P<0.005$ (Student's *t* test). (b) Chimeras of the FoxP3-Treg group showed substantial levels of chimerism in all tested lineages, whereas in those few mice that developed chimerism without Tregs chimerism was limited to the B cell and myeloid lineages. FCM plots from representative BMT recipients treated with (*left*) or without FoxP3-Tregs (*right*) are shown (31 weeks post-BMT). Percentages indicate net chimerism in the depicted lineage.

skin was noted in Treg-treated recipients (Fig. 3c) compared with donor skin from BMT recipients prepared with 3 Gy TBI (Fig. 3d; chosen as control because donor grafts did not survive long-term in 1 Gy controls without Tregs), and also compared with syngeneic Balb/c grafts (not shown). Mean density of mast cells per high power field (HPF) was 47.1 (1 Gy Tregs) vs. 18.0 (3 Gy BMT recipients, $P=0.064$) vs. 16.2 (syngeneic grafts, $P=0.055$) (mast cells were counted in 5 HPF [high-power field]). Mast cells have been shown to be closely linked to Treg-mediated tolerance (*34*), suggesting a role for regulatory mechanisms for skin graft survival. These findings are substantiated by the intragraft presence of high numbers of FoxP3 positive cells (confirmed by immunohistochemical staining with FoxP3-specific mAb; Fig. 3c). Taken together, these results suggest that therapeutic application of Tregs protected grafts from chronic rejection.

Treg-Treated Chimeras Show Donor-Specific Hyporesponsiveness In Vitro and Preserved Immunocompetence Toward Third-Party Antigens

In vitro T-cell tolerance was evaluated by performing mixed lymphocyte reaction (MLR) assays at the end of follow-up (28–37 weeks post-BMT). Treg-treated chimeras showed specific hyporesponsiveness toward donor antigen in vitro, whereas alloreactivity toward third-party antigens was preserved (Fig. 4a). Donor responsiveness was reduced to the level of self-reactivity, in contrast to

TABLE 1. Multilineage chimerism levels in spleen and BM are significantly higher in FoxP3-Tregs treated mice at the end of follow-up (33 wk after BMT) compared with controls without Treg treatment

	Spleen			Bone marrow	
	$CD4^+$	$CD8^+$	$B220^+$	$B220^+$	$Mac1^+$
NL B6	0.07	0.30	0.53	0.05	0.26
Balb/c	100.00	100.00	100.00	100.00	100.00
1 Gy Tregs (n=5)					
AVG	13.95	14.24	50.85	55.06	60.89
STD	5.08	7.27	17.17	21.28	31.33
1 Gy control (n=4)					
AVG	1.91	2.29	16.02	16.64	13.65
STD	1.57	3.47	22.50	23.46	15.71
P	0.004	0.018	0.046	0.042	0.025

AVG, average; STD, standard deviation.

BMT recipients without Tregs whose response toward donor stimulators was preserved and comparable with naïve mice (reactivity toward donor antigen $P=0.048$ Treg chimeras vs. naïve B6; $P=0.022$ Treg chimeras vs. BMT recipients without Tregs).

Treg-Treated Chimeras Demonstrate Partial Deletion of Donor-Specific T Cells

Deletion of donor-reactive T cells is an important mechanism for the induction of tolerance after BMT (*15*) and can be assessed by following superantigen-reactive T-cell populations ($V\beta11^+$ and $V\beta5.1/2^+$ T cells in the strain combination used herein) (*5*, *15*). FoxP3-Treg-treated mice showed a significant and specific reduction in the percentages of $V\beta11^+$ and $V\beta5^+$ CD4 cells in comparison with naïve B6 mice, whereas no such reduction was seen in control groups without Tregs (Fig. 4b). Partial deletion of $V\beta5.1/2^+$ and $V\beta11^+$ CD4 T cells was evident early after BMT (week 6), indicating peripheral clonal deletion of donor-reactive T cells (*15*). Deletion progressed over time and was almost complete late after BMT (week 21; $P=0.0366$ for $V\beta11^+$, $P=0.0227$ for $V\beta5.1/2^+$ Treg group compared with control group without Treg treatment; $P<0.0001$ for $V\beta11^+$, $P=0.0037$ for $V\beta5.1/2^+$ compared with naïve B6 controls; no deletion of $V\beta8.1/2^+$ control populations was evident at any time point). In control mice preconditioned with 3 Gy TBI without Treg treatment, deletion progressed more rapidly and led to almost complete absence of donor-reactive T cells 12 weeks post-BMT.

Collectively, these results demonstrate that polyclonal FoxP3-transduced Tregs promote the engraftment of allogeneic BM after low-dose TBI, leading to donor-specific tolerance, which is induced both by deletional and nondeletional mechanisms.

DISCUSSION

The presented studies show that the therapeutic application of polyclonal recipient Tregs significantly promotes BM engraftment in a costimulation blockade-based mixed chimerism model. Using low-dose TBI, we demonstrate that Tregs affect immune responses after allogeneic BMT with high potency. This study indicates that polyclonal Tregs on their own are able to control costimulation blockade-resistant alloresponses.

The potency of Tregs in the prevention of allograft rejection was demonstrated by several groups, although success was mostly limited to models using lymphopenic (*35*, *36*) hosts or Tregs with a specific transgenic T cell receptor (*37*, *38*). Using more stringent, clinically relevant models, additional treatment with rapamycin was required to prevent rejection of allografts in several model systems (*20*, *37*, *39*). By contrast, rapamycin was not required in the present studies when Tregs were combined with a minimum-conditioning protocol. The combination of Tregs and costimulation blockade successfully induced long-term multilineage chimerism and donor-specific tolerance in a reduced intensity, low-dose irradiation BMT protocol. These results suggest that Tregs are not critically dependent on rapamycin to exert their suppressor function in vivo, but rather that rapamycin exerts additive effects on BM engraftment through any—yet to be determined— of its pleiotropic effects (*28*). Polyclonal Tregs are capable of promoting the engraftment of moderate doses of allogeneic BM if the immunological host-versus-graft barrier is lowered by a mild dose of irradiation, presumably by suppressing the remaining costimulation blockade-resistant immune response. The absence of a GFP signal in lymphoid organs and skin grafts (as revealed by polymerase chain reaction analysis) late after BMT indicates that initially transferred Tregs have a limited life span during which they promote BM engraftment but do not survive in large quantities. As high numbers of FoxP3 Tregs are present in accepted donor skin, transferred Tregs seem to induce subsequent Treg generations (*40*).

Although both rapamycin and Treg therapy (*8*) promote BM engraftment on their own, chimerism levels achieved were higher when Tregs were used than when rapamycin was used. Notably, chimerism levels in the T-cell lineage—which is associated with tolerance induction in several chimerism models (*5*, *41*, *42*)—were higher in Treg-treated mice compared with rapamycin-treated mice (13.8% vs. 2.9 mean% of CD4 chimerism, $P<0.02$; 4.1 vs. 1.2 mean % of CD8 chimerism, P=n.s., compared with historical controls [*8*]). As intrathymic alloresistance is critical in determining T-cell chimerism (*41*), Tregs seem particularly effective in breaking thymic alloresistance.

Although histopathological signs of chronic rejection were observed in some donor skin grafts in mice treated with a well-established BMT protocol using nonmyeloablative TBI (3 Gy) and costimulation blockade, chimeras treated with reduced TBI (1 Gy) and additional Treg therapy demonstrated no signs of chronic rejection. Interestingly, the frequency of graft-infiltrating mast cells was markedly increased in such Treg-treated mice over tolerant chimeras induced with the nonmyeloablative regimen (*43*). Mast cells were shown to favor the induction of Tregs through TGFβ release (*44*). Moreover, peripheral clonal deletion progressed over time in Treg-treated chimeras and was almost complete at the end of follow-up, suggesting an important role for deletional and nondeletional tolerance mechanisms in this model. Deletion progressed more slowly than in mixed chimeras conditioned with higher nonmyeloablative TBI (3 Gy) without Treg therapy (*15*), but it was more profound than in Treg-

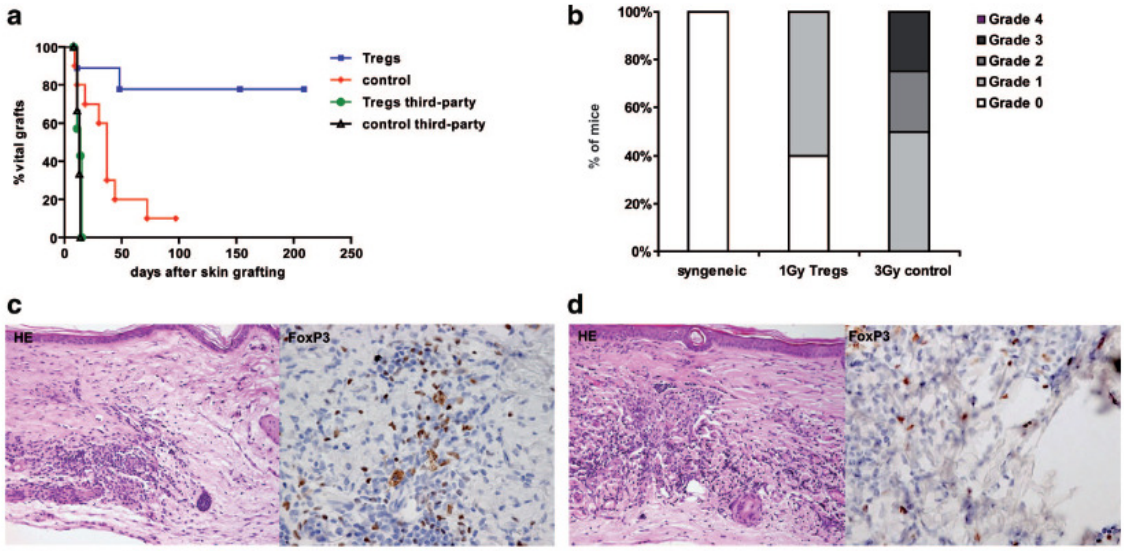

FIGURE 3. Treg treatment induces donor-specific skin graft tolerance in bone marrow (BM) transplantation (BMT) chimeras and protect grafts from chronic rejection. (a) Donor-specific tolerance was assessed by grafting donor and third party skin 4 to 6 weeks post-BMT. Donor skin graft survival is significantly prolonged in BMT recipients treated with (■, n=9) compared with those treated without FoxP3-Tregs (♦, n=10) (log-rank P=0.003). Third-party grafts are promptly rejected in both groups (Treg-treated, ●; control group, △). Pooled data from 2 independent experiments are shown. (b) Classification of skin allograft pathology for syngeneic grafts (n=2), Treg-treated group (n=5), and 3 Gy total body irradiation (TBI) group (n=4) are shown (*47*) (Grade 0: no or rare inflammatory infiltrates, skin architecture intact; Grade 1: mild inflammatory infiltration, no involvement of overlying epidermis; Grade 2: moderate perivascular inflammation with mild epidermal/adnexal involvement; Grade 3: severe inflammation, atrophy of epidermis, dyskeratosis and keratinolysis). (c, d) Representative histology of skin grafts from Treg-treated chimeras (c) and BMT recipients prepared with 3 Gy TBI (d; control because donor skin did not survive long-term in 1 Gy controls without Tregs) are shown. HE staining (*left*, hematoxylin-eosin stain, magnification 20×) and Foxp3 expression (*right*, immunohistochemistry with specific FoxP3 antibody, magnification 40×) are analyzed (30 weeks after grafting).

treated chimeras without cytoreductive conditioning (*20*). Taken together, these results support the conclusion that regulatory mechanisms are more effective than deletional mechanisms in preventing chronic rejection of skin allografts in the maintenance phase of tolerance. Thus, reduced-intensity conditioning combined with Treg treatment actually improves tolerance outcome over previous nonmyeloablative regimens.

Collectively, these data demonstrate that Tregs exert potent engraftment-enhancing effects that are not dependent on rapamycin. The therapeutic application of Tregs in the context of reduced intensity BMT leads to chimerism and robust tolerance through favoring nondeletional mechanisms in addition to clonal deletion.

MATERIALS AND METHODS

Animals

Female C57BL/6 (B6, recipient, H-2^b), Balb/c (donor, H-2^d), and C3H/N (third party, H-2^k) or SJL (third party, H-2^s) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). This donor-recipient strain combination (Balb/c → B6) crosses MHC and minor histocompatibility antigen barriers and B6 recipients are rather resistant to costimulation blockade, ensuring a stringent model (*12*, *45*, *46*). Mice were housed under specific pathogen-free conditions and 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.

Retroviral Transduction of CD4$^+$ T Cells

Retroviral vectors pCMMP-FoxP3-IRESeGFP (FoxP3/GFP) or control vector pCMMP-IRESeGFP (GFP), (Fig. 1a) were used for production of VSV-G pseudotyped retroviruses (*20*, *31*). Retroviral transduction of CD4$^+$ cells was performed as described previously (*20*, *31*). Transduction efficiencies approximately 15% were achieved, cells were sorted for GFP expression thereafter and purity of sorted populations was more than 95%.

Suppression Assay and MLR

A total of 4×10^5 B6 splenocytes (responder) were co-cultured with decreasing numbers of FoxP3-transduced Tregs (or GFP transduced cells), or freshly sorted CD4$^+$CD25^{high} Tregs (nTregs), respectively (4×10^5, 2×10^5, 8×10^4, 4×10^4 for a ratio of 1:1, 2:1, 5:1, 10:1 [responder cells vs. Tregs]), in the presence of 4×10^5 irradiated (30 Gy) Balb/c splenocytes (stimulator). FoxP3 negative cells (or GFP negative cells) or freshly isolated CD4$^+$CD25^{neg} cells were used as controls. MLRs were performed as described previously (*5*). Briefly, 4×10^5 responder splenocytes were incubated with 4×10^5 irradiated (30 Gy) stimulator cells of B6 (recipient), Balb/c (donor), or SJL (third party) origin or with medium only. Cells were pulsed with [3H]-thymidine (Amersham, Biosciences, UK) for 18 hr after 72 hr of incubation. Incorporated radioactivity was measured using scintillation fluid in a β-counter. For MLR analysis, stimulation indices (SI) were calculated in relation to medium controls.

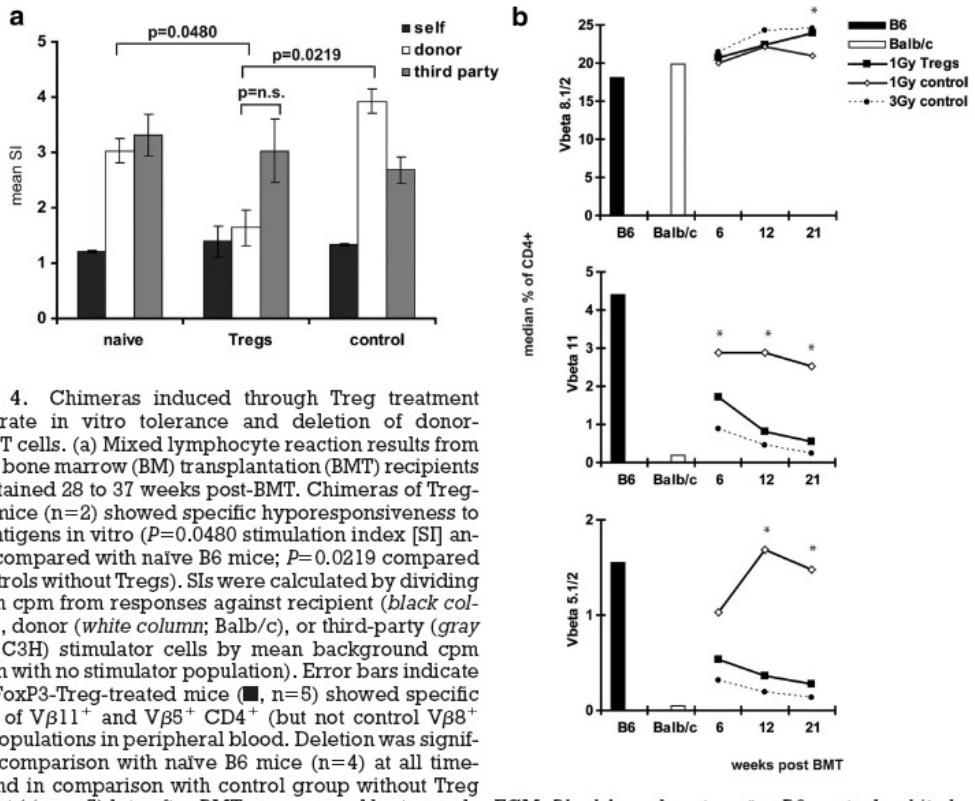

FIGURE 4. Chimeras induced through Treg treatment demonstrate in vitro tolerance and deletion of donor-reactive T cells. (a) Mixed lymphocyte reaction results from selected bone marrow (BM) transplantation (BMT) recipients were obtained 28 to 37 weeks post-BMT. Chimeras of Treg-treated mice (n=2) showed specific hyporesponsiveness to donor antigens in vitro ($P=0.0480$ stimulation index [SI] antidonor compared with naïve B6 mice; $P=0.0219$ compared with controls without Tregs). SIs were calculated by dividing the mean cpm from responses against recipient (*black column*; B6), donor (*white column*; Balb/c), or third-party (*gray column*; C3H) stimulator cells by mean background cpm (i.e., cpm with no stimulator population). Error bars indicate SD. (b) FoxP3-Treg-treated mice (■, n=5) showed specific deletion of $V\beta11^+$ and $V\beta5^+$ $CD4^+$ (but not control $V\beta8^+$ $CD4^+$) populations in peripheral blood. Deletion was significant in comparison with naïve B6 mice (n=4) at all time-points and in comparison with control group without Treg treatment (◇, n=5) late after BMT, as assessed by two color FCM. *Black bars* denote naïve B6 control, *white bars* denote naïve Balb/c control, mice preconditioned with 3Gy total body irradiation (TBI) are shown as additional control (● *dotted line*, n=4). Treg treatment promotes specific deletion progressing over time whereas no such deletion is seen in control mice without Treg treatment. *$P<0.005$ (Treg treatment vs. control group at time points indicated, Student's *t* test).

BMT Protocol

Groups of age-matched B6 recipients received 1 Gy nonmyeloablative TBI (day 1), costimulation blockade consisting of anti-CD40L (CD154) mAb (MR1, 1 mg, day 0) and CTLA4Ig (0.5 mg, day 2) (*8*, *15*) and approximately 2×10^7 unseparated BM cells harvested from Balb/c donors (day 0, intravenously) with or without additional FoxP3-transduced B6 Tregs (FoxP3-Tregs, 4×10^6 cells in two divided doses [2×10^6 cells, day 0 and day 6]) or control GFP-transduced cells. Mice receiving 3 Gy nonmyeloablative TBI (day 1), costimulation blockade and approximately 2×10^7 unseparated BM were used as additional controls as indicated. Anti-CD154 mAb was purchased from BioXCell (West Lebanon, NH), hCTLA4Ig (abatacept) was generously provided by Bristol-Myers, Squibb Pharmaceuticals (Princeton, NJ).

Skin Grafting

Full thickness tail skin from Balb/c mice and fully mismatched C3H (third party) were grafted 4 to 6 weeks after BMT and visually inspected thereafter at short intervals. Grafts were considered to be rejected when less than 10% remained viable.

Flow Cytometric Analysis of Treg Phenotype, Chimerism, and Deletion

Multicolor flow cytometric analysis of Treg phenotype, multilineage chimerism, and Vβ-subunit expression were performed as described previously (*15*, *20*). Chimerism was calculated as net percentages of donor MHC class I^+ ($H\text{-}2D^d$, 34-2-12) cells among leukocyte lineages (*15*). Mice were considered chimeric if donor cells were detectable by flow cytometry within both the myeloid lineage and at least one lymphoid lineage. Expression of Vβ-subunits was used as surrogate parameter for deletion of donor-reactive T cells. $CD4^+$ cells were counterstained with specific markers for Vβ-subunits (Vβ8.1/2, Vβ11, Vβ5.1/2, or irrelevant isotype control). For analysis and sorting of Tregs mAbs with specificity against CD4 (RM4-4), CD25 (7D4), CD62L (L-selectin, Mel-14), CTLA4 (UC10-4F10-11), and GITR (DTA-1) were used. For intracellular staining, FoxP3 (FJK-16s) staining Kit (eBioscience) was used according to the manufacture's protocol. Flow cytometric analysis was done on a Coulter Cytomics FC500. CXP software (Coulter, Austria) was used for acquisition and analysis. Cell sorting was performed on a FACS Aria (Becton Dickinson), purity of sorted populations was more than 95%.

Histological Analysis

Four micrometer sections were cut from paraffin-embedded tissue fixed in 4.5% formalin (pH of 7.5), stained with hematoxylin-eosin and Giemsa according to standard protocols, and analyzed by an experienced pathologist in blinded fashion. Mast cells were counted in 5 HPF (magnification ×400), and mean density per HPF was calculated. Immunohistochemistry was performed as described previously (*20*), mAb with specificity against Foxp3 (clone FJK-16S, eBioscience) was used.

Statistical Analysis

A two-sided Student's *t* test was used to compare percentages of Vβ-family positive cells and chimerism levels between groups, Fisher's exact test was used to compare chimerism rates between groups. Skin graft survival was calculated according to the Kaplan-Meier product limit method and compared between groups using the log-rank test. A *P* value less than 0.05 was considered to be statistically significant.

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5.2 Reviews

5.2.1 A Chimerism-Based Approach to Induce Tolerance in IgE-Mediated Allergy

Critical Reviews™ in Immunology, 29(5):379–397 (2009)

A Chimerism-Based Approach to Induce Tolerance in IgE-Mediated Allergy

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ABSTRACT: Immunoglobulin-E-mediated allergy (type I allergy) is a T-helper-2-mediated disease with increasing prevalence in industrialized countries. Immunotherapy is available as causative treatment, but an effective preventive strategy is still an unmet need. Molecular chimerism is an attractive experimental approach that induces tolerance through transplantation of autologous hematopoietic stem cells that are genetically modified to express the disease-causing antigen(s). Molecular chimerism leads to permanent and robust tolerance in experimental models of autoimmune diseases and organ transplantation. Recently, proof-of-principle studies demonstrated that a type I allergic immune response can be durably tolerized by transplantation of allergen-expressing syngeneic bone marrow. We review the concept of tolerance induction through chimerism and discuss the potential of this strategy in immunoglobulin-E-mediated allergy.

KEY WORDS: molecular chimerism, immunological tolerance, IgE-mediated allergy

I. INTRODUCTION

In susceptible individuals, the allergic immune response leads to a wide range of diseases, ranging from allergic rhinitis to life-threatening anaphylaxis.[1] The hallmark of type I allergy is the formation of immunoglobulin E (IgE) antibodies against environmental, otherwise harmless proteins known as allergens. A major characteristic of the immune system is that it is self-tolerant.[2] Self-tolerance is mediated by mechanisms that rely on the expression of self antigens by various subpopulations of hematopoietic cells. This physiological state is emulated in the concept of tolerance induction through hematopoietic chimerism.

In the case of cellular chimerism, allogeneic hematopoietic stem cells (HSC) are transplanted into an appropriately conditioned recipient in such a way that they engraft and persist. Cellular chimerism has been most extensively studied for the induction of donor-specific transplantation tolerance through transplantation of donor bone marrow (Fig. 1). Mixed chimerism is induced (i.e., a state of co-existence of both donor and recipient hematopoiesis) because it has several advantages over full donor chimerism.[3] The mixed chimerism concept has already been translated into the clinical setting in highly selected renal transplant patients.[4] Cellular chimerism can also prevent and treat autoimmune diseases.[5-7] While results

ABBREVIATIONS

IgE, immunoglobulin E; **HSC**, hematopoietic stem cells; **MHC**, major histocompatibility complex; **GVHD**, graft-versus-host disease; **BMC**, bone marrow cells; **BMT**, bone marrow transplantation; **HLA**, human lymphocyte antigen; **αGT**, α1,3-galactosyltransferase; **GT⁰**, αGT knockout; **αgal**, Gal α-1,3-Gal; **EAE**, experimental autoimmune encephalomyelitis

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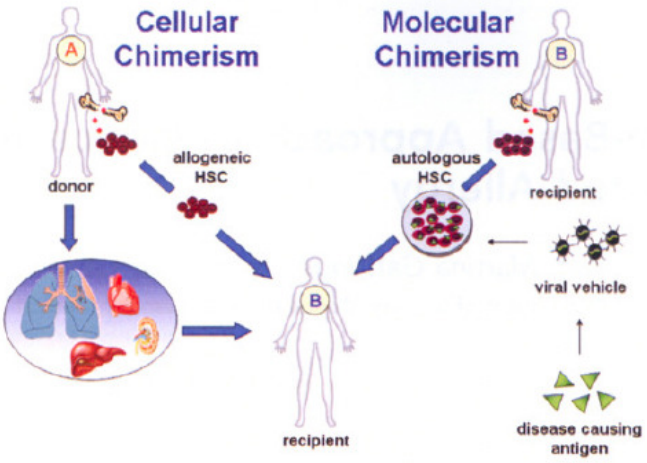

FIGURE 1. Cellular chimerism versus molecular chimerism. Hematopoietic stem cells from the donor are transplanted into an appropriately conditioned allogeneic recipient (A→B) to induce mixed chimerism and tolerance towards an organ graft from the donor (A). In molecular chimerism, autologous hematopoietic cells (B→B) are transduced in vitro to express the disease-causing antigen(s). The modified cells are injected back into the same recipient (B), inducing tolerance to the newly introduced antigen.

in experimental studies are promising, the clinical experience with HSC transplantation for treatment of autoimmune disorders remains limited.[8,9] In the case of molecular chimerism, autologous (or syngeneic in experimental models) HSC are genetically modified in vitro to express a disease-causing antigen before being transplanted back into the same individual (Fig. 1). This strategy has been explored mostly in experimental models of (allo- and xeno-) transplantation and autoimmune disorders. Chimerism-based models are among the most robust tolerance protocols reported to date and have therefore attracted a lot of attention. In this review we discuss tolerance induction via hematopoietic chimerism and suggest this strategy as a possible approach for tolerance induction in IgE-mediated allergy.

II. TOLERANCE INDUCTION THROUGH HEMATOPOIETIC CHIMERISM

A. Cellular Chimerism

1. Historical Perspective

The origins of the concept of tolerance through hematopoietic chimerism can be tracked back to the groundbreaking work of Owen published in 1945.[10] He described a naturally occurring case of hematopoietic chimerism in bovine dizygotic twins sharing a common placental circulation. These so-called "free-martin cattle" were tolerant to tissue antigens from each other, as demonstrated by the successful transplantation of major histocompatibility complex (MHC)-mismatched skin. In 1953, Billingham et al. induced "actively acquired tolerance" by transplanting a mixture of allogeneic cell types, including splenocytes, into fetal and neonatal mice.[11,12] Since 1955, allogeneic HSC transplantation has been used in the clinical setting as treatment for hematologic cancer and fatal immunodeficiencies.[13] However, the risk of graft-versus-host disease (GVHD) and the toxicity of host preconditioning remain substantial clinical problems after allogeneic HSC transplantation, precluding its use for a wider range of indications to the present day. Therefore, the development of HSC transplantation protocols sufficiently safe to be acceptable for the purpose of tolerance induction has been a major research goal.

2. Cellular Chimerism in Experimental Protocols

The first studies inducing hematopoietic chimerism in adult mice employed lethal (myeloabla-

tive) total body irradiation leading to complete elimination of the host hematopoietic system. Reconstitution with donor bone marrow cells (BMC) led to full (nearly 100%) chimerism and donor-specific tolerance. Full chimerism is associated with a higher risk of GVHD and reduced immunocompetence[14,15] than mixed chimerism, which is therefore preferred for the purpose of tolerance induction. Mixed chimerism describes a balanced state of co-existence of recipient and donor hematopoietic cells in the host (donor chimerism greater than 1% and less than 100%). The first experimental mixed chimerism protocols also required myeloablative recipient preconditioning.[3] Successful establishment of mixed chimerism with less drastic non-myeloablative recipient conditioning was achieved shortly thereafter.[16]

The use of costimulation blockade as part of bone marrow transplantation (BMT) regimens allowed a further reduction of conditioning toxicity. Non-myeloablative host conditioning with costimulation blockade (anti-CD40L [CD154] alone or together with the fusion protein CTLA4Ig) led to engraftment of clinically relevant doses of BMC in the presence of an intact recipient T-cell repertoire.[17-20] Irradiation-free protocols also became feasible with costimulation blockers, but require unrealistic mega-doses of bone marrow.[21,22]

3. Mechanisms of Tolerance Induction in Mixed Chimerism

Allogeneic mixed chimerism induces a particularly robust form of donor-specific tolerance across MHC barriers. Mixed chimeras permanently accept donor skin (while promptly rejecting third-party skin) in vivo, and demonstrate donor-specific unresponsiveness in mixed lymphocyte reaction and cell-mediated-lympholysis assays in vitro.

One hurdle to be overcome for the establishment of mixed chimerism is to achieve engraftment of allogeneic HSC. It has been long noted that myelosuppression enhances engraftment of HSC; however, the underlying mechanisms of this effect remain incompletely understood. Myelosuppression may open physical "niches" within the bone marrow, it may up-regulate cytokines that promote homing of HSC into the bone marrow, or its immunosuppressive effect may simply be preventing rejection of the allogeneic donor bone marrow. It is also possible that any combination of these modes of action might be at work in specific settings. Interestingly, the need for myelosuppression can be overcome by transplantation of very high doses of HSC.[21,22] Unfortunately, these doses are not available in the clinical setting. Several factors, including regulatory T cells, have been identified that promote HSC engraftment[23] and seem particularly promising.[24,25] Non-myelosuppressive protocols employing clinically feasible doses of HSC remain an important goal that would substantially advance the field.

In experimental models achieving stable mixed chimerism, central deletion is the main mechanism maintaining tolerance. Thymic dendritic cells of donor origin mediate intra-thymic clonal deletion of newly developing donor-reactive T cells by negative selection, resulting in a T-cell repertoire tolerant to self and to donor antigens.[26] Central tolerance is the unique characteristic distinguishing chimerism-based tolerance from virtually all other tolerance approaches. It assures that newly developing donor-reactive T cells are permanently tolerized and do not eventually overcome the capacity of peripheral mechanisms to maintain tolerance. At the time of BMT, additional mechanisms are required to render preexisting mature alloreactive T cells tolerant. In large animal models and humans, T-cell depletion using depleting antibodies and additional thymic irradiation are employed to globally destroy mature recipient T cells. Due to the slow T-cell recovery in adults, substantial risks are associated with this state of immuno-incompetence. In contrast, BMT with costimulation blockade achieves tolerization of the pre-existing mature T-cell repertoire without the need for global T-cell destruction. This peripheral tolerance is achieved by progressive clonal deletion of mature alloreactive T cells[17] and by non-deletional mechanisms.[27]

4. Costimulation Blockers in the Clinic

Costimulation blockers are a critical component of advanced mixed chimerism protocols, and thus are of great relevance for the clinical future of this tolerance approach. Blockade of the CD154:CD40 pathway through anti-CD40L mAb is a highly successful approach in rodents. Unexpectedly, all

clones of anti-CD40L mAb tested led to severe thromboembolic side effects in large animal models and in human trials,[28,29] and therefore further clinical development was put on hold. The use of anti-CD40 mAb would be an alternative approach for blocking the CD40 pathway, which showed promising results while avoiding thromboembolic complications in non-human primate studies, but its clinical future is currently unclear.[30] Thus, replacement of compounds targeting the CD154:CD40 pathway with clinically available drugs is still an unmet goal.

The fusion protein CTLA4Ig (abatacept) was designed to block CD28 signaling, but might also work through additional mechanisms.[31] CTLA4Ig has potent immunomodulating effects in autoimmune and transplant models,[32-34] and was recently approved for clinical use in rheumatoid arthritis. Since the immunosuppressive efficacy of CTLA4Ig was unsatisfactory in a non-human primate kidney transplant model, a modified CTLA4Ig with higher affinity for the B7 target molecules was developed and named LEA29Y or belatacept.[35] Belatacept was shown to be safe and effective as a primary immunosuppressant in renal transplant recipients in a phase II trial[36] and is currently under investigation in ongoing phase III trials.

5. Clinical Translation of Mixed Chimerism Protocols

The induction of tolerance via the mixed chimerism approach has been successfully tested in two pilot clinical trials. In one study, patients suffering from both end-stage renal failure and multiple myeloma simultaneously received a bone marrow and kidney graft from a human lymphocyte antigen (HLA)-identical sibling. All of the patients developed at least transient chimerism and operational tolerance towards the transplanted kidney. Two patients developed GVHD, and both of these exhibited full chimerism. Renal function remained stable after withdrawal from immunosuppressive drugs with follow-up of up to 7 years.[37] While persistent chimerism is important for the maintenance of skin graft tolerance in rodent models, transient chimerism was found to be sufficient for long-lasting tolerance in monkeys receiving combined kidney and bone marrow transplantation, which is consistent with the observed clinical outcome.[38] Recently, the researchers reported successful induction of tolerance in an HLA-mismatched setting with non-myeloablative pre-conditioning in patients suffering from chronic renal insufficiency without concomitant malignancy. Transient chimerism (persisting for 1–4 weeks post transplant) led to successful induction of tolerance in four of five treated patients without the development of GVHD, and stable renal function for up to 5 years after withdrawal of immunosuppressants. Because treatment-refractory humoral rejection occurred in the third patient, the protocol was modified by adding rituximab (anti-CD20 mAb). Unexpectedly, de novo anti-donor antibodies and auto-antibodies developed in three patients.[39,40] The donor-specific humoral response occurred despite in vitro T-cell hyporesponsiveness. Despite the presence of these antibodies, graft function was preserved long-term.[39] This is in contrast to the HLA-matched patients in the first clinical trial described above, in which no donor-directed B-cell immunity was detected. In the experimental setting, it was shown that mixed chimerism and molecular chimerism can induce B-cell tolerance.[41,42] Thus, it appears likely that anti-donor B-cell immunity is a potential concern in the MHC-mismatched clinical setting but not in molecular chimerism, in which modified autologous bone marrow is transplanted.

Overall, these two clinical series[4] highlight the potential of hematopoietic chimerism in the clinical setting during organ transplantation. Although pre-conditioning led to serious post-transplant side effects (e.g., severe leucopenia and capillary leak syndrome), withdrawal of immunosuppressive therapy revealed successful induction of tolerance towards allografts in these patients. Molecular chimerism employs modified autologous HSC transplantation (allergen-expressing HSC in the case of allergy), so a substantially lower antigen barrier needs to be crossed than in MHC-mismatched BMT. Therefore, markedly milder preconditioning is expected to be sufficient compared with allogeneic HSC transplantation.

Little is known about the relationship between BMT and allergy in the clinical setting. Isolated cases have been reported in which allergies were transferred with HSC transplantation in patients receiving HSC transplantation for leukemia. However, a few cases have also been documented

in which allergies were ameliorated after HSC transplantation, suggesting that BMT can, in principle, be an effective treatment for allergies under certain circumstances.[43]

The main impediments to using BMT for treatment of severe autoimmune disorders are treatment-related morbidity and mortality. Autologous HSC transplantation has a lower rate of mortality, in contrast to allogeneic HSC transplantation, but is associated with the risk of development of secondary autoimmune disorders.[44] Furthermore, allogeneic HSC transplantation aims at induction of tolerance through transfer of healthy HSC. Furthermore, allogeneic HSC transplantation causes an effect called graft-versus-autoimmuinity, in which donor T cells might eliminate remaining autoreactive host lymphocytes.[6] Although some case reports have revealed complete remission of autoimmune disease with full donor chimerism, mixed chimerism might be capable of inducing long-term remissions and also allows milder irradiation protocols, thus lowering the risk of GVHD.[45] In a retrospective study of patients with severe treatment-refractory autoimmune disease, 75% responded to allogeneic HSC transplantation; unfortunately, the mortality rate was approximately 20%.[46] In some reports, stable chimerism was associated with remission of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and psoriasis.[47,48] Although these data are encouraging, protocols and autoimmune disorders are heterogenous and further experience and studies are necessary.

In summary, several hurdles, in particular safety issues, still need to be overcome for mixed chimerism to become a routinely used clinical therapy for tolerance induction in transplantation and autoimmune disorders.[23]

B. Molecular Chimerism

Molecular chimerism is induced by the transplantation of autologous (syngeneic) HSC modified ex vivo to express the antigen(s) of interest. Molecular chimerism has major advantages over cellular chimerism: i) the risk of GVHD is avoided because autologous HSC are transplanted, and ii) the lower immunological barrier facilitates engraftment and therefore permits milder conditioning.[49] On the other hand, retroviral transduction of HSC with the gene of interest needs to be performed and the disease-causing antigen(s) needs to be known and of limited number. To date, gene transfer by retroviral or lentiviral gene integration is associated with severe side effects.[50] Notably, in several children suffering from severe combined immunodeficiency, correction of disease through transplantation of retrovirally transduced HSC resulted in T-cell leukemia due to insertion of the transgene near protooncogenes.[51,52] In two gene therapy studies published more recently, gene delivery appeared safer.[53,54] Thus, gene therapy approaches acceptable for widespread use might be developed in the future.

1. Molecular Chimerism for Tolerance Induction in Allo- and Xenotransplantation

Transplantation of syngeneic bone marrow expressing donor MHC is being explored for tolerance toward donor allografts. Molecular chimerism approaches in allo- and xenotransplantation are summarized in Table 1.[55,56] Introduction of either donor-MHC class I or MHC class II genes into recipient cells prolonged survival of cardiac allografts in a murine model.[57] The congenic mouse strains B10.AKM (H-2K^k) and B10.MBR (H-2K^b), which differ only in the MHC class one H-2K allele, are commonly employed in models of allogeneic molecular chimerism. Transplantation of B10.AKM BMC transduced with the H-2K^b gene resulted in prolongation of B10.MBR skin, while third-party grafts were rapidly rejected.[55,58,59] This approach has been successfully translated to a large animal model, in which autologous porcine bone marrow transduced with an allogeneic MHC II molecule was transplanted. Pigs transplanted with the transduced bone marrow became operationally tolerant to fully mismatched renal allografts (matched to the introduced class II molecule).[60,61]

Mechanistically, the lack of T-cell help played a role in graft prolongation of MHC I-disparate skin grafts in early, less-efficient models of molecular chimerism.[58,59,62] In more recent and efficient models, the main mechanism of T-cell tolerance was central thymic deletion, although deletion seems to be incomplete.[63,64] Induced CD4$^+$CD25$^+$ T-regulatory cells also contribute

TABLE 1. Tolerance Induction by Molecular Chimerism in Allo- and Xenotransplantation

Integrated Antigen	Model*	Outcome	P/T	Reference
H-2D^b/2K^b/2IA^b	Transplanted L cells in C3H mice	Prolonged survival of cardiac allograft	P	57
HLA-A2.1	Transplanted CD34$^+$ BMC in B6	Inhibition of antibody production; partially cell-mediated immunity	P	144
H-2K^b	Transplanted BMC in B10.AKM	Prolonged skin graft survival	P	55, 58
H-2K^b	CBK BMC in CBA	Dose dependence of BMC, heart graft survival >100 days	P	145
H-2K^b	Transplanted BMC in B10.AKM	Hyporesponsiveness	P	59
Secreted or cytoplasmic H-2K^b	Transplanted BMC in B10.AKM (CD4 or CD8 depleted, thymectomized before skin grafting)	Prolonged skin graft survival >50 days (cytoplasmic expression of antigen) >25 days (secretion of antigen)	P	62
H-2K^b	Transplanted BMC in B10.AKM	T-cell tolerance, skin graft survival prolonged	P	64
H-2K^b	Transplanted BMC + BMC of BM3.3 (T-cell depleted) in B10.AKM	Central deletional T cell tolerance, skin graft survival >90 days	P	63
H-2K^b	Transplanted BMC in B10.AKM	Improved protocol with co-stimulation blockade, skin graft survival prolonged	P	146
H-2K^b	Transplanted BMC + B10.AKM BMC in R^0 mice	Mature lymphocytes required for tolerance	P	68
H-2K^b	BMC (T-cell depleted) + mature CBK T or B cells in CBA	Mature T cells required for tolerance, skin graft survival >100 days	P	69
H-2K^b	Transplanted BMC in B10.AKM	Successful non-myeloablative protocol, skin graft survival >100 days	P	135
H-2K^b	Transplanted B10.AKM BMC and Tg361 BMC (CD4/CD8 depleted) in B10.AKM	CD4 T cells specific for K^b detectable, CD4$^+$CD25$^+$ are able to transfer tolerance, skin graft survival >200 days	T	65
H-2K^b transient	Transplanted BMC in B10.AKM	Persistent presentation of antigen required, skin graft survival slightly prolonged	P	67
H-2K^b	BMC + CBK CD4 or CD8 cells in CBA	Homeostatic proliferation necessary for T cells to re-enter thymus and induce tolerance, skin graft survival >100 days	P	71

H-2K^b	CBK T cells in CBA	Tolerance induced by T-cell transfer with non-myeloablative conditioning, skin graft survival >100 days	P	70
αGT	Transplanted BMC in GT^0 mice	Inhibition of antibody production	P	73
αGT	Transplanted BMC in GT^0 mice	Stable αGT expression on blood cells, B-cell tolerance	P	74
αGT	Transplanted BMC in GT^0 mice	B-cell tolerance, prolonged wt heart graft survival (MST 29 days)	P	75
αGT	Transplanted BMC in immunized GT^0 mice	Long-term αGal expression with high dose of BMC (1.5×10^7), no antibody production	T	76
αGT	Transplanted BMC in GT^0 mice	Gene transfer by lentivector, tolerance achieved, heart graft survival >50 days	P	77
αGT	Transplanted BMC in GT^0 mice	Gene transfer by lentivector, non-myeloablative conditions, heart graft survival >100 days	P	78
αGT	BMC and BM derived CD34$^+$ cells in Rhesus macaque	Gene transfer by lentivector, long-term chimerism achieved	P	79
Swine MHC class II	Transplanted CD34$^{+/-}$ BMC in baboon	Transient transgene expression, xenografts (kidney and skin) rejection after 8–22 days	P	147
DRB/DQA	Transplanted BMC in miniature swine	Prolonged kidney graft survival	P	61

* C3H mice (H-2^k), B10.AKM mice (K^k I^k D^q), CBA (H2^k), CBK (H2^k + K^b): CBA transgenic strain, express K^b as a transgene; GT^0 mice: lack functional glucosyltransferase uridine 5′-diphosphate galactosyl-1,4-acetyl-D-glucosaminide α(1-3)galactosyltransferase; BM3.3 mice: CBA background, express transgenic CD8 TCR specific for K^b-derived peptides; R^0 mice: Rag1$^{-/-}$ on B10.AKM background; Tg361 mice: CBA background, express transgenic CD4 TCR specific for K^b derived peptides.

BM, bone marrow; BMC, bone marrow cells; MST, median survival time; P, prophylactic; T, therapeutic.

to tolerance induction in such chimeras.[65] In cellular chimerism models, persistent chimerism is required to maintain allo-skin graft tolerance.[66] Similarly, persistence of molecular chimerism to an extent of at least 1% was required to maintain skin graft tolerance.[67] Antigen expression on mature lymphocytes has also been shown to be required for tolerance induction in molecular chimeras.[68] In an interesting and novel approach, induction of central tolerance via molecular chimerism was demonstrated by transplantation of mature T cells (instead of bone marrow) from congeneic mice differing in one MHC class I molecule (H2-K^b).[69] Re-entry of mature T cells into the thymus and deletion of alloreactive T cells have been demonstrated in this model.[70] Homeostatic expansion of mature T cells in the periphery is important for re-entering the thymus and delivering H2-K^b in a tolerogenic fashion.[71]

In the setting of discordant xenotransplantation, natural antibodies against the carbohydrate epitope Gal α-1,3-Gal (αgal), produced by the molecule α1,3-galactosyltransferase (αGT) is an additional major hurdle, requiring αgal-reactive B cells and T cells to be tolerized.[72] The αGT knockout mouse (GT^0), which develops IgM and IgG antibodies that bind αgal, is used as a model system to study natural antibodies.[72] Introduction of αGT into syngeneic BMC and transplantation into preconditioned recipients resulted in long-term molecular chimerism and B-cell tolerance even in presensitized recipients.[73-78] The mechanisms in B-cell tolerance in molecular chimerism models of xenotransplantation remain to be defined in detail. Furthermore, molecular chimerism led to acceptance of heart grafts and prevention of antibody-mediated rejection in this model.[75] Similarly, BMC transduced with αGT by lentiviral gene transfer and transplantation into GT^0 mice led to tolerance to cardiac grafts of GT+ mice with myeloablative and non-myeloablative regimens.[77,78] Transplantation of autologous BMC lentivirally transduced with αGT into sublethally irradiated rhesus monkeys resulted in engraftment of BMC and strong reduction of xenoantibodies after immunization with porcine hepatocytes, but induction of αgal-specific IgG1.[79]

Thus, molecular chimerism successfully induces T-cell and B-cell tolerance in experimental allo- and xenotolerance models.

2. Molecular Chimerism in Autoimmune Disorders

About 5% of the population in Western countries is affected by autoimmune diseases. Non-specific inhibition of immune inflammatory activity is partially effective, but is associated with several side effects. Therefore antigen-specific therapies for the treatment of autoimmune diseases are needed.[80] Both T-cell- and B-cell-mediated immunity contribute in various degrees to the pathogenesis of autoimmune diseases. For selected autoimmune diseases with defined disease-causing antigens, preventive and/or therapeutic experimental models inducing tolerance via molecular chimerism have been reported. Table 2 presents an overview of the published literature on tolerance via molecular chimerism in autoimmune models.[81] The most extensively studied models of molecular chimerism in autoimmune disorders are murine models of experimental autoimmune encephalomyelitis (EAE) and type 1 diabetes.

a. Molecular Chimerism in Experimental Autoimmune Encephalomyelitis

EAE—a rodent model to study the human disease multiple sclerosis (MS)—is a model for T cell-mediated autoimmunity. $CD4^+$ T cells respond upon immunization with MBP (Myelin Basic Protein), mediating central nervous system demyelination and inflammation, which results in ascending paralysis.[82] In one study, introduction of full-length MBP into HSC of MBP-susceptible mice failed to induce tolerance, as mice developed an even higher EAE incidence than control groups.[83] This failure is possibly related to an insufficient expression level of the transduced antigen and underscores the fact that specific protocols of molecular chimerism need to be tailor-made for each indication to ensure efficacy. In subsequent studies, MBP, MOG (Myelin Oligodendrocyte Glycoprotein), and phospholipid protein, all antigens inducing EAE, were introduced into HSC by retroviral transduction. EAE was abolished through transplantation of BMC transduced in this way.[84,85] In another successful therapeutic and preventive approach, MOG was transduced into HSC and subsequently transplanted into mice. Central T-cell tolerance was suggested as a main mechanism for tolerance induction in this model.[86] Recently, a preventive and a therapeutic approach transplanting BMC expressing the MOG_{40-55} linked to a murine MHC class II-associated invariant chain, replacing CLIP (CLass II-associated Invariant chain Peptide) by the MOG epitope,[87] showed that MOG_{40-55} expression was targeted to the MHC class II pathway.[88] In the preventive approach, T-cell tolerance was observed but B-cell tolerance failed (although humoral responses were diminished).

b. Molecular Chimerism in Models of Type 1 Diabetes

Most approaches for the induction of molecular chimerism in type 1 diabetes employ NOD (non-

TABLE 2. Tolerance Induction by Molecular Chimerism in Autoimmune Disease

Disease	Integrated Antigen	Model	Outcome	P/T	Reference
EAE	MBP	Transplanted BMC in B10.PL or SJL	No tolerance observed	P	83
EAE	PLP	Transplanted BMC in SJL	Tolerance in naïve recipients, primed animals show reduced disease scores	T	85
EAE	MOG	Transplanted BMC in B6	Tolerance in naïve and remission in primed recipients	T	86
EAE	MOG_{40-55}	Transplanted BMC (B6 CD45.1) in B6 (CD45.2)	Robust protection in naïve mice, reduction of disease in mice with established EAE	P	87
Diabetes	Pro-insulin II	NOD-PI BMC in NOD	Prevention of diabetes	P	92
Diabetes	MHC class II I-A β-chain	Transplanted BMC in NOD	Prevention of disease induction by restoration of protective MHC class II	P	90
Diabetes	Pro-insulin II	Transplanted BMC in NOD	Reduced degree of insulitis	P	93
Diabetes	MHC class II I-A β-chain	Transplanted BMC in NOD or in diabetic NOD mice after islet transplantation	Robust self-tolerance in naïve mice, prevention of recurrence in diabetic mice after islet transplantation	T	91
Autoimmune gastritis	β subunit of H/K-ATPase	IE-H/Kβ BMC in PC-GMCSF mice	T-cell tolerance, marked drop in H/K ATPase reactivity	P	148

BMC, bone marrow cells; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PLP, phosphlipid protein; MOG, myelin oligodendrocyte glycoprotein; NOD mice, nonobese diabetic mice; NOD-PI mice, transgenic for mouse proinsulin II; IE-H/Kβ mice, express the β subunit of the H/K ATPase; PC-GMCSF mice, express the pro-inflammatory cytokine granulocyte macrophage colony stimulating factor in the stomach; P, prophylactic; T, therapeutic.

obese diabetic) mice, which is commonly regarded as the murine model most closely resembling human type 1 diabetes.[89] In NOD mice, the MHC class II region encodes a single molecule, $I\text{-}A^{g7}$ (the murine homolog of HLA-DQB1). Transplantation of syngeneic bone marrow expressing an MHC class II I-Aß chain molecule that confers resistance to diabetes prevented type 1 diabetes in NOD mice.[90] In this model, tolerance was a result of central thymic deletion of autoreactive T cells. Molecular chimerism and expression of diabetes-resistant MHC class II alleles in NOD mice was also sufficient to restore normoglycemia in pre-existing type 1 diabetes after islet transplantation.[91]

Transduction of NOD bone marrow to express pro-insulin II dramatically reduced spontaneous insulitis after transfer into young NOD mice.[92,93] In a transgenic mouse model expressing the lymphocytic choriomeningitis virus gp peptide (LCMV-gp) on islet cells under the rat insulin promoter, diabetes is triggered upon LCMV infection.[94] Transplantation of LCMV-gp-transduced BMC led to absence of hyperglycemia in chimeric mice after LCMV infection.[95]

Thus, tolerance can be (re-)established via molecular chimerism in autoimmunity models.

III. IgE-MEDIATED ALLERGY

A. IgE-mediated Allergy, a B- and T-cell Disease

Atopic individuals are predisposed to developing allergy.[96,97] Allergic sensitization (the primary response) probably occurs within the first years of childhood in these patients.[98] Soluble allergens are released in small amounts from allergen-bearing particles (e.g., pollen) and are absorbed through the mucosa of the respiratory or gastrointestinal tract, promoting the differentiation of $CD4^+$ T cells to Th2 (T-helper 2) cells. Th2 cells produce cytokines such as IL (interleukin)-4 and IL-13, which induce immunoglobulin class switching to IgE, and then allergen-specific memory T and B cells develop. The immediate-phase reaction occurs when IgE bound to high-affinity Fc receptors on the surface of mast cells and basophils is cross-linked by allergen. Pro-inflammatory mediators, including histamine, are released, resulting in typical allergic symptoms such as rhinitis, conjunctivitis, or even anaphylaxis. While IgE-mediated symptoms predominate in the immediate phase, late-phase reactions (such as asthma) are mainly T-cell mediated.[1,99-101]

The prevalence of allergy in industrialized countries has risen to over 20%, with more than 700 million people currently affected worldwide. Although the mortality caused by allergic disease is relatively low, quality of life is significantly affected by asthma, rhinitis, and eczema.[43] The most dramatic clinical presentation of allergy is anaphylaxis, a relatively rare but life-threatening event affecting both pediatric and adult patients (0.05%–2%).[102] Studies of multi-generation families clearly indicate a strong genetic component of atopic diseases, with data from one study revealing that 51% of children with a family history of atopy developed allergic disease within the first 5 years of life.[103] Therefore, children whose parents both suffer from severe allergic diseases such as food and hymenoptera allergies (known to be potentially lethal) are at a substantial risk of developing severe allergies themselves.[104] For such individuals, preventive allergen-specific strategies would be potentially beneficial.[99]

B. Recombinant Allergens for Immunotherapy

To date, the only causal treatment in IgE-mediated allergy is allergen-specific immunotherapy. Immunotherapy has been in use for about 100 years, even before allergens were identified. In this method, crude extracts containing the sensitizing allergens are repeatedly administered in increasing doses in the presence of adjuvants. Several immunological mechanisms to modulate the immune system during immunotherapy have been suggested.[99] Induction of allergen-specific IgG-blocking antibodies is one of the most characteristic modulation events in immunotherapy, as is a shift of Th2- to a Th1-like response.[105] Recently, IL-10 and TGFß (Transforming Growth Factor beta)-dependent regulatory T cells have also been suggested to play a role.[106]

Crude extracts used for specific immunotherapy contain additional allergenic and non-allergenic material. Therefore, allergen-specific immunotherapy is associated with limited effectiveness and substantial risks, as exemplified by anaphylactic reactions or therapy-induced sensitization to additional allergens.[1,107] Recombinant allergens are alternative candidates for immunotherapy. In the last 20 years, most of the common allergen-encoding cDNAs have been isolated and recombinant allergens can thus be produced with high purity.[108] Recombinant allergens do not contain unknown components and can therefore be precisely "tailored" to the sensitization profile of one individual.[109,110] Moreover several modified recombinant allergens have been constructed with the goal of deliberately improving their properties.[111] For example, a trimer of the birch pollen allergen Bet v 1,[112] a multimer of different major grass pollen allergens,[113] or two homologous venom allergens,[114] have been constructed. Hypoallergenic allergen derivatives are modified allergens with reduced allergenicity, leading to a reduction of IgE-mediated effects, but preserved T-cell-mediated immunogenicity.[115] These recombinant hybrid and hypoallergenic allergen derivatives are attractive candidates for immunotherapy.

Recently, the efficacy of recombinant allergens has been evaluated in clinical studies.[116,117] A modified recombinant allergen (trimer) of the major birch pollen allergen significantly reduced allergic disease upon vaccination.[118] Additionally,

a modified allergen conjugated to CpG showed immunostimulatory activity to Th1 responses, ameliorating allergic disease.[119] Short linear peptides are not able to cross-link adjacent IgE molecules on mast cells and basophils and therefore lack the risk to induce anaphylaxis. Therefore T-cell peptide-based therapeutic vaccines for allergic and autoimmune disease have been suggested.[120] Unfortunately, intradermal administration of three dominant T-cell epitope peptides of the cat allergen Fel d induced late asthmatic reactions in some patients.[121,122]

C. Experimental Approaches for Preventive Tolerance in Allergy

Mucosal tolerance approaches have been assessed in various experimental models of allergy and autoimmune disease, but data from clinical trials in humans were overall disappointing.[123] Ovalbumin often serves as model antigen in IgE-mediated allergy, although it is not a relevant allergen in human allergy. The concept of inducing T-cell tolerance by administration of immunodominant T-cell epitopes has been successfully demonstrated by intranasal treatment of mice with high doses of house dust mite allergen (Der p 1) peptides. Lymph node T cells from tolerized mice were unable to provide cognate help in stimulating specific antibody production in vitro.[124] Although only one epitope was used for treatment, T-cell responses to the other epitopes of the antigen were subsequently down-regulated, suggesting a mechanism of "infectious" tolerance.[125]

Dominant T-cell epitope-containing polypeptides of three different allergens were administered intranasally in a similar approach. After subsequent challenge with allergens, allergen-specific humoral and effector cell responses were reduced but not completely prevented.[126] In other models, airway inflammation was reduced by intranasal and subcutaneous administration.[127] A recombinant allergen chimeric molecule consisting of T-cell epitopes of two different grass pollen allergens and full-length birch pollen allergen revealed reduction of IgE and IgG allergen-specific responses after intranasal application and evidence for induction of regulatory T cells upon application of two fragments of Bet v 1.[128,129] The intranasal application of the dominant T-cell epitope of olive pollen (Ole 1) induced an allergen-specific shift from Th2 to Th1 response.[130] Most of these experimental approaches for tolerance induction and prevention of allergy showed only partial T-cell and B-cell tolerance and included short follow-up times.

D. Molecular Chimerism in Allergy

Given the evidence that molecular chimerism is capable of inducing tolerance in transplantation and autoimmunity (as described above), we recently set out to investigate if this strategy can be used to establish genuine tolerance in IgE-mediated allergy.

1. Proof-of-Principle Studies

As first step, we developed an experimental protocol for tolerance in IgE-mediated allergy as a preventive approach.[131] We employed the clinically relevant timothy grass pollen allergen *Phleum pratense* (Phl p 5),[132] which is highly immunogenic upon subcutaneous sensitization with adjuvans (aluminum hydroxide) in BALB/c mice.[133] Fusing full-length Phl p 5 cDNA to a signal peptide and a transmembrane domain, we generated a construct for membrane-bound expression of Phl p 5 on hematopoietic cells. Using this construct, BALB/c bone marrow was transduced to express high levels of Phl p 5. Myeloablated recipients were reconstituted with transduced bone marrow, resulting in stable and long-term molecular multi-lineage chimerism (follow-up time was approximately 9 mo). Persistent multi-lineage chimerism for this period of time is indicative of successful transduction and engraftment of true HSC, which was also confirmed by the presence of chimerism upon secondary BMT (Fig. 2).[134]

Complete absence of Phl p 5-specific high-affinity isotypes (no IgG isotypes, IgE and IgA) was observed in chimeras throughout follow-up, despite repeated immunization post-BMT with Phl p 5 and the unrelated birch pollen allergen Bet v 1. Likewise, allergen-specific T-cell unresponsiveness developed. Phl p 5-chimeras showed effective cellular and humoral immunity toward the unrelated control allergen Bet v 1, demonstrating the specificity of tolerance. Effector cell tolerance

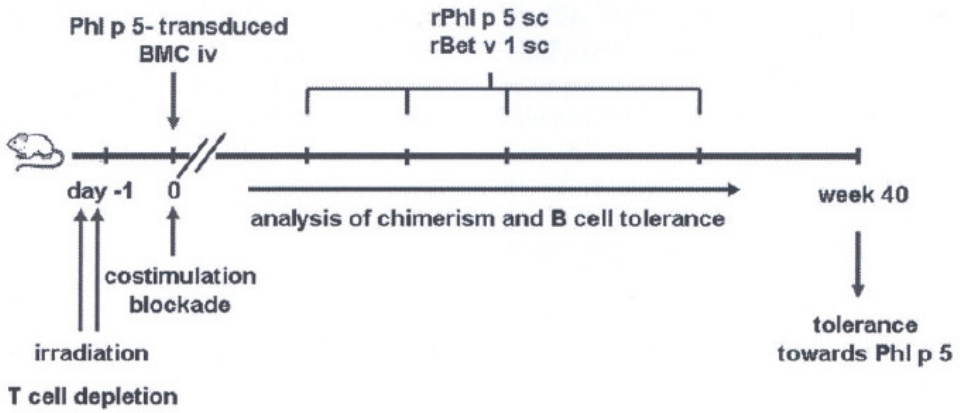

FIGURE 2. Experimental procedure for tolerance through molecular chimerism in allergy. The figure depicts the in vivo protocol of the proof-of-principle studies for tolerance induction towards the allergen Phl p 5 via molecular chimerism. Mice conditioned with T-cell-depleting antibodies, costimulation blockade, and lethal irradiation are transplanted with Phl p 5-transduced syngeneic bone marrow cells. Mice are subsequently repeatedly challenged with recombinant allergens (Phl p 5 and the unrelated control allergen Bet v 1). Chimerism and in vivo and in vitro tolerance are assessed throughout follow-up.

was shown in in vitro basophil assays (no release) and in vivo by type I skin testing (no mast cell response in the skin).

Thus, robust and permanent immunological tolerance toward the introduced allergen was established in this preventive approach at the T cell, B cell, and effector cell levels.

2. Limitations and Future Directions

While the proof-of-principle studies demonstrate that a complete state of tolerance toward an allergen can indeed be achieved through molecular chimerism, several impediments currently limit the applicability of this approach.

a. Reduction of Cytotoxic Recipient Conditioning

To allow engraftment of sufficient numbers of transplanted bone marrow cells, cytotoxic recipient conditioning is usually required. However, irradiation, cytotoxic drugs, or cytotoxic antibodies would not be clinically acceptable in the setting of allergy. Data from other molecular chimerism models and recent development in the mixed chimerism area demonstrate that toxicity of recipient conditioning can be substantially reduced and non-myeloablative and even non-cytotoxic regimens are possible.[22,70,87,135,136] Thus, it appears likely that molecular chimerism protocols in allergy with non-toxic conditioning will become feasible in the future.

b. Replacing Retroviral Gene Transfer with Safer Methods

Gamma-retro and lentiviral gene transfer are currently the only methods used to establish stable integration of a transgene in HSC, and they are associated with severe side effects in clinical trials. Therefore, non-viral gene transfer systems would be desirable for safety reasons. Recently, the transposase hyperactive *Sleeping Beauty* (SB) mutant (SB100X) was shown to be successful in stable gene transfer in $CD34^+$ HSC, offering the advantage of integrating more randomly rather than preferentially targeting actively transcribed genes as retro-and lentiviral systems do. These cells have the capacity to repopulate and differentiate into both lymphoid and myeloid lineages in vivo, as demonstrated in mice.[137,138] Integration into the genome would be necessary if persistent chimerism is required for long-lasting tolerance. Unlike in murine models, transient mixed chimerism is sufficient for lasting tolerance in non-human primate models and in

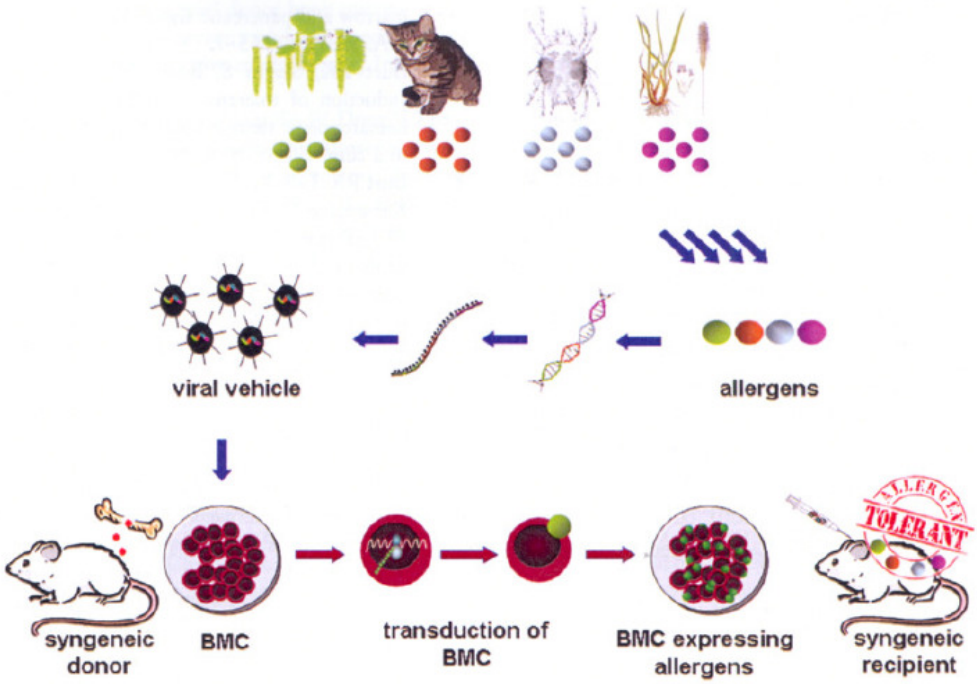

FIGURE 3. Molecular chimerism in allergy. Most clinically important allergens have been identified and are available as recombinant proteins. A combination of genes encoding the relevant allergens is stably integrated into the genome of hematopoietic stem cells (through retroviral transduction), which are then transplanted back into the same individual. Bone marrow, mobilized peripheral stem cells, or cord blood are conceivable sources of hematopoietic stem cells. Molecular chimerism and tolerance to the introduced allergens are established.

the clinical setting.[39,139] For transient chimerism it would be sufficient to only temporarily anchor antigens directly to the membrane or to deliver antigens or DNA extrachromosomally.[140,141]

c. Avoiding the Risk of Anaphylaxis

In our experimental model, full-length Phl p 5 was expressed on the surface of HSC, leading to B-cell tolerance toward Phl p 5. However, full-length expression of allergens increases the risk of anaphylaxis, especially in the therapeutic setting. It was demonstrated previously that expression of GFP (Green Fluorescent Protein) in the cytoplasm was effective in inducing T-cell tolerance, but it is unclear whether B-cell tolerance was also achieved.[142] Another possibility to avoid the risk of anaphylaxis would be to express only T-cell epitopes instead of the intact protein. This is conceivably sufficient to tolerize T cells, which in turn can lead to B-cell tolerance through a lack of T-cell help.[143] Employing peptides would also facilitate fusion of several allergen-fragments and would thus allow tolerance induction towards numerous allergens (Fig. 3).

d. Developing a Therapeutic Strategy

Our initial experimental studies used a preventive approach. While strategies for allergy prevention are urgently needed,[99] establishing allergen-specific tolerance in already sensitized patients is also an important goal. Chimerism-based protocols are indeed capable of inducing tolerance in sensitized recipients, as shown for allo-sensitized mice and mixed chimerism and autoimmunity and molecular chimerism.[41,76,86] Thus, it is likely that regimens can be developed that induce tolerance in mice sensitized to an allergen. However, such protocols are expected to require more intense recipient conditioning.

IV. CONCLUSIONS

Hematopoietic chimerism modulates the immune repertoire in such a way that extrinsic antigens are recognized like "self" antigens. Molecular chimerism offers the possibility to induce robust and long-lasting tolerance toward disease-causing antigens by genetic modification of autologous cells and subsequent reinfusion of these cells. Proof-of -principle studies have shown that this concept is applicable for tolerization of a type I allergic immune response. Molecular chimerism is a promising strategy, but is currently at an early stage of development. Development of less-toxic protocols and delivery systems is expected to contribute to the establishment of a cell-based protocol for preventing IgE-mediated allergy.

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Cell-Based Therapy in Allergy

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Abstract IgE-mediated allergy is an immunological disorder occurring in response to otherwise harmless environmental antigens (i.e., allergens). Development of effective therapeutic or preventive approaches inducing robust tolerance toward allergens remains an unmet goal. Several experimental tolerance approaches have been described. The therapeutic use of regulatory T cells (Tregs) and the establishment of molecular chimerism are two cell-based strategies that are of particular interest. Treg therapy is close to clinical application, but its efficacy remains to be fully defined. Recent proof-of-concept studies demonstrated that transplantation of syngeneic hematopoietic stem cells modified in vitro to express a major allergen leads to molecular chimerism and robust allergen-specific tolerance. Here we review cell-based tolerance strategies in allergy, discussing their potentials and limitations.

Contents

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1 Introduction

IgE-mediated allergic disorders are immunological hypersensitivity reactions. Allergic immune responses occur against environmental, otherwise innocuous antigens, known as allergens. Genetic and environmental factors influence susceptibility to allergic hypersensitivity (Akdis 2006; Vercelli 2008).

The only causative treatment available so far is allergen-specific immunotherapy (SIT), a vaccination strategy (Valenta 2002). Increasing doses of allergens (mostly in the form of crude extracts) are administered to allergic individuals with the goal to induce "desensitization". In this context, immunological tolerance in allergy was suggested to be defined as persistence of efficacy after treatment (Akdis and Akdis 2009). Numerous mechanisms have been suggested to show how SIT alters B cell and T cell responses. Mechanisms may vary depending on treatment protocol, route of administration and allergen preparation (Larche et al. 2006). SIT induces de novo humoral responses dominated by the production of protective allergen-specific IgG antibodies, mainly IgG4 (Nouri-Aria et al. 2004; Wachholz and Durham 2003). A Th2 to Th1 shift and induction of Tregs also contribute to effectiveness (Bohle 2008). SIT is, however, associated with limited effectiveness and considerable risks, as exemplified by anaphylactic reactions or therapy-induced sensitization to additional allergens when crude extracts are used (Valenta 2002). In addition to the need for more effective allergen-specific therapeutic strategies, prevention of allergy by prophylactic induction of tolerance is a major unmet need (Hamelmann et al. 2007).

Several experimental tolerance strategies have been described, with cell-based approaches having gained particular attention in recent years. This review discusses induction of tolerance through cell therapies as a possible approach to prevent and treat allergy.

2 Prevention of Allergy by Induction of Peripheral Tolerance

Peripheral, i.e., extrathymic, T cell tolerance is a critical mechanism of self-tolerance and can either occur by peripheral clonal deletion (Webb et al. 1990), anergy (Fathman and Lineberry 2007) or suppression (Sakaguchi 2005).

Potent tolerance models relying on peripheral tolerance have been developed. Conceptually, they suffer, however, from the drawback to have no mechanism in place to tolerize newly developing T cells and thus their effects are usually limited in duration (Wekerle et al. 2003).

2.1 Induction of Tolerance via the Mucosal Route

Specific non-responsiveness to inhaled and ingested allergens is a major goal in IgE-mediated allergy. Mucosal tolerance is induced through administration of antigens via the oral or nasal route (Faria and Weiner 2005). This phenomenon was reported very early as protection of systemic anaphylaxis was observed upon injection of hen′s egg protein in guinea pigs having been fed with the antigen (Wells 1911). Two primary mechanisms of mucosal tolerance have been described. Low-dose antigen- feeding induces active suppression by generation of Tregs, high-dose feeding regimens induce anergy (Friedman and Weiner 1994). TGFß (transforming growth factor beta)-producing T helper type 3 (Th3) cells are a Treg subtype specifically induced upon antigen-feeding (reviewed in Faria and Weiner 2005). Beside T cell-mediated immunoregulation, induction of allergen-specific IgA antibodies is a prominent mechanism of oral tolerance (Challacombe and Tomasi 1980).

Mucosal tolerance via the respiratory tract is more relevant to inhalant allergens. High-dose intranasal exposure of one single dominant T cell epitope of the house dust mite allergen Der p 1 was sufficient to induce T cell anergy toward the whole Der p 1 allergen by linked suppression, a phenomenon prominently described in transplantation (Hoyne et al. 1993, 1999; Qin et al. 1993). Dominant T cell epitopes of different allergens and in different combinations have been investigated for intranasal application, resulting in reduction of allergen-specific IgE and IgG responses. However, allergen-specific isotypes are not completely eliminated and only short-term follow up was reported in these models (Hufnagl et al. 2005, 2008; Marazuela et al. 2008; Wild et al. 2007). Involvement of Tregs and a Th2 to Th1 shift are important mechanisms in these protocols (Hufnagl et al. 2008; Marazuela et al. 2008; Winkler et al. 2006).

2.2 Induction of Tolerance by Intradermal Peptide Application

Systemic subcutaneous administration of T cell peptides of the major cat allergen Fel d 1 (without adjuvant) successfully led to peripheral T cell tolerance in experimental models. Translation to the clinical setting employing intradermal administration of T cell peptides of Fel d 1, however, induced late asthmatic reactions in some patients (Briner et al. 1993; Haselden et al. 1999; Oldfield et al. 2002). T cell responses were diminished, but humoral responses toward the full

length allergen persisted (Briner et al. 1993). In a novel transgenic mouse model of asthma mimicking the human situation systemic intradermal application of one Fel d 1 immunodominant peptide in pre-sensitized mice resulted in resolution of airway pathology through linked suppression, possibly through generation of Tregs (Campbell et al. 2009). To allow full assessment of the potential of T cell peptides as therapeutic vaccines, several parameters still need to be determined, such as optimum length and dose of peptides (Larche and Wraith 2005).

2.3 Tregs as Cell-Based Therapy in Allergy

Regulatory T cells play a critical role in maintaining self-tolerance (Sakaguchi and Powrie 2007). Numerous, incompletely defined, subpopulations of Tregs have been described, with CD4+CD25+FoxP3+ natural Tregs arising from the thymus, and adaptive/induced Tregs generated in the periphery playing major roles (Feuerer et al. 2009). Tregs regulate T cell and B cell responses and also innate immunity. Whether memory T cells are amenable to regulation by Tregs remains controversial (Levings et al. 2001; Yang et al. 2007). The therapeutic exploitation of Tregs has attracted enormous interest in recent years, fueled by hopes of developing antigen-specific treatments for transplant recipients, autoimmune diseases and allergies (Akl et al. 2008; Hutchinson et al. 2008; Riley et al. 2009). So far, however, the physiology of Tregs remains incompletely understood and their therapeutic potential largely unexplored (Schiopu and Wood 2008).

Several lines of evidence reveal a prominent role of Tregs in regulating, or preventing, respectively allergic immune responses. A role for T regulatory type 1 (Tr1) cells in modulating allergic immune responses has been found by comparing immune responses upon allergen exposure between healthy individuals and allergic patients (Akdis et al. 2004). Natural high exposure of venom allergen (phospholipase A2) induces a switch of specific Th1 and Th2 cells to Tr1 cells (Meiler et al. 2008). Tr1 cells are of special therapeutic interest, as they are currently under clinical investigation in bone marrow (BM) transplant recipients, as are natural Tregs (nTregs) (reviewed in Roncarolo and Battaglia 2007; Roncarolo et al. 2006; Schiopu and Wood 2008; Riley et al. 2009). First clinical trials using freshly isolated or expanded donor nTregs show encouraging results in the prevention of lethal graft versus host disease (GVHD) (Trzonkowski et al. 2009; Brunstein et al. 2011; Di Ianni et al. 2011) nTregs express the transcription factor forkhead box P3 (FoxP3) (Miyara et al. 2009) and play an indispensable role in maintaining self-tolerance (Sakaguchi et al. 2008). FoxP3-deficient patients suffering from immunodysregulation, polyendocrinopathy and enteropathy X-linked syndrome (IPEX) are affected by atopic disease, resulting in increased serum IgE levels, eosinophilia, eczema-like skin lesions and enhanced Th2 responses. This provides clear evidence that Fox P3-expressing Tregs play an essential role in suppression of Th2-driven immune responses in humans (Chatila 2005).

In experimental models in allergy, the therapeutic application of nTregs abolished allergic airway inflammation (Kearley et al. 2005, 2008; Leech et al. 2007). Transfer of nTregs resulted in reduction of allergen-specific IgE and a boost of IgG1 immunomodulation after nasal administration of Der p 1 in an IL-10 independent manner (Leech et al. 2007). In another model, ova-specific CD4+CD25+ T cells suppressed T cell responses in the lung but IgE responses were not altered in a preventive approach (Kearley et al. 2005). Features of chronic allergen-induced inflammation were resolved but established remodeling in the lung was not reversed and humoral responses to allergen were not altered (Kearley et al. 2008). Thus, the use of natural Tregs has a suppressive effect on allergic airway inflammation, but seems to be less effective in preventing IgE responses. Application of FoxP3-transduced polyclonal CD4+ cells is effective in models of autoimmunity and allo-transplantation (Chai et al. 2005; Jaeckel et al. 2005; Pilat et al. 2010). Using polyclonal transduced Tregs together with the application of a recombinant grass pollen allergen in preventive and therapeutic murine models, we observed that allergen-specific IgE was not significantly reduced and T cell responses were not suppressed (Baranyi, Pilat, Wekerle et al., unpublished data).

In summary, Treg therapy is of considerable interest for treating allergic diseases. Numerous basic biological questions still need to be resolved in this new field of investigation. Only then can the clinical potential of Treg therapy be adequately assessed.

3 Tolerance Induction by Hematopoietic Stem Cell Transplantation (HSCT)

Self-tolerance is mediated mostly by various subpopulations of hematopoietic cells (von Boehmer and Kisielow 2006). Chimerism-based tolerance strategies emulate this fundamental characteristic of physiologic self-tolerance with the aim of inducing tolerance toward introduced, disease-causing antigens (Fehr and Sykes 2008; Wekerle and Sykes 2001). Such chimerism protocols are the only strategies to reliably establish central tolerance, i.e., the intrathymic clonal deletion of antigen-specific thymocytes. Thereby they provide mechanisms not only for tolerizing pre-existing mature T cells through peripheral mechanisms (Bigenzahn et al. 2005; Wekerle et al. 2002), but also for continuously tolerizing the newly developing T cells constantly arising in the thymus (Wekerle et al. 1998; Wekerle and Sykes 2001). Moreover, chimerism is capable of inducing T cell, B cell and NK cell tolerance (Sykes et al. 1998), and also tolerance in pre-sensitized recipients (Colson et al. 2000). Tolerance induced by chimerism is thus of particular robustness and durability.

Two types of hematopoietic chimerism can be distinguished: cellular and molecular, with mixed chimerism being the most commonly used subtype of cellular chimerism. Allogeneic donor BM (containing HSC) is transplanted to establish mixed chimerism. In the case of molecular chimerism autologous (i.e., syngeneic in

the rodent setting) HSC are transplanted after genetic modification ex vivo to express the disease-causing antigen(s) (Bagley et al. 2002a).

Advanced experimental protocols of cellular hematopoietic chimerism have been systematically developed for several decades. Translation to large animal models and the clinical setting have been accomplished for transplantation tolerance (Fehr and Sykes 2008). Thus, chimerism-based tolerance is conceptually and empirically an attractive tolerance strategy with demonstrated clinical potential.

3.1 Mixed Chimerism

Following transplantation of donor BM into appropriately conditioned recipients, donor and recipient hematopoiesis co-exists (>1 < 100% donor cells), leading to donor-specific tolerance (reviewed in Sykes 2001; Wekerle and Sykes 2001).

Subsequent to the early observation of chimerism as a natural phenomenon in cattle, the concept has been translated to numerous experimental models (Billingham et al. 1953; Owen 1945). At first myeloablative recipient conditioning was used (Ildstad and Sachs 1984), which has been extensively modified so that substantially milder regimens have since become available (Pree et al. 2007). Mixed chimerism was found to be preferable over full chimerism as it reduces the risk of graft versus host disease (GVHD) and is associated with superior immunocompetence (Singer et al. 1981; Sykes et al. 1988). The use of co-stimulation blockers (mostly CTLA4Ig and anti-CD40L) as part of BM transplantation (BMT) protocols allowed chimerism and tolerance to be induced with the mildest, least toxic conditioning protocols developed to date (Durham et al. 2000; Pree and Wekerle 2006; Snanoudj et al. 2006; Wekerle et al. 2000). Permanent engraftment of conventional BM doses without recipient irradiation, remained however, an important goal. Either unrealistic mega doses of BM (Blaha et al. 2005; Durham et al. 2000; Wekerle et al. 2000), or recipient myelosuppression (by irradiation or cytotoxic drugs) have been necessary (Adams et al. 2001; Blaha et al. 2003; Koporc et al. 2008; Takeuchi et al. 2004). Co-transplantation of recipient Tregs allowed for the first time the engraftment of conventional doses of fully allogeneic BM without any myelosuppressive recipient conditioning (Pilat et al. 2010; Pilat and Wekerle 2010).

The clinical potential of the mixed chimerism strategy has recently been emphasized by two pilot trials. In the first series, patients suffering from chronic renal insufficiency plus concomitant myeloma were simultaneously grafted with a kidney and with BM from an HLA-identical sibling (Bühler et al. 2002; Fudaba et al. 2006). Patients accepted the renal grafts without maintenance immunosuppression. In a subsequent trial, HLA-mismatched BM plus a kidney were transplanted into recipients with chronic renal failure (without concomitant malignancy) (Kawai et al. 2008; LoCascio et al. 2010). Four out of five patients became tolerant, with long-term preserved kidney graft function (reported follow up of up to 5 years; one patient lost his graft due to humeral rejection). These

results provide clinical proof-of-concept that transplantation tolerance can be achieved through mixed chimerism (Pilat et al. 2009). Widespread application of this regimen is, however, prevented by the extensive myelosuppressive host conditioning which is required to allow even transient engraftment of HLA-mismatched BM and which is associated with substantial risks and toxicities. Therefore, more advanced, milder protocols that already exist in the rodent setting need to be translated to clinical use for routine application of the mixed chimerism strategy to become realistic.

3.2 Molecular Chimerism

Transplantation of syngeneic HSC that have been genetically modified in vitro to express disease-causing antigen(s) back into the same individual induces tolerance toward the introduced antigen(s). This gene therapy has been successfully employed in experimental models of allo- or xeno-transplantation and some selected autoimmune diseases. Autologous HSCT has the advantage compared to allogeneic HSCT that the risk of GVHD is avoided (Copelan 2006), and that the lower immunological barrier would allow milder host conditioning (Bagley and Iacomini 2003).

The safety issues associated with gene therapy, however, are a major drawback of molecular chimerism. Retro- or lentiviral vectors currently used for gene introduction into HSC cause substantial toxicity due to immune reactions or oncogenesis (Baum et al. 2003; Hacein-Bey-Abina et al. 2008). Recently, progress toward safer gene delivery has been reported (Aiuti et al. 2009; Mitsuyasu et al. 2009) and it is anticipated that acceptably safe vector systems will become available some time in the future.

3.2.1 Introduction of Allo- and Xeno- Genes to Establish Tolerance

Major histocompatibility complex (MHC) molecules are the primary target of immune responses in allotransplantation (Davidson and Diamond 2001; LeGuern 2007). Expression of single donor MHC class I and class II antigens can be sufficient for inducing tolerance toward fully allogeneic donors under certain circumstances, due to linked suppression and "infectious tolerance" (Frasca et al. 1997; Qin et al. 1993). Besides, certain alleles of MHC class I and II are strongly associated with the risk of developing autoimmune disease.

Donor MHC class I or class II genes transduced into recipient cells in vitro and transferred into recipients led to prolonged cardiac allografts survival (Madsen et al. 1988). Similarly, swine MHC class II DRB was integrated by retroviral transfer into murine and swine BM cells (BMC) in vitro (Emery et al. 1993; Shafer et al. 1991). By translating this method to a porcine in vivo model, prolonged renal allograft survival was achieved by transducing a single allogeneic MHC class II gene into recipient BMC (Emery et al. 1997; Sonntag et al. 2001).

In contrast, MHC class I gene transfer seems to be more difficult due to poor peptide presentation of MHC class I peptides (LeGuern 2007). The congeneic mouse strains B10.AKM (H-2K^k)-B10.MBR (H-2K^b)—differing in only one H-2K allele—serve as a model of allogeneic molecular chimerism. Several studies introducing the MHC class I (K^b) into murine BMC (K^k) showed hyporesponsiveness and prolongation of skin allografts (K^b). In these protocols low transduction efficiency at first resulted only in prolongation of skin grafts but not durable tolerance (Bagley et al. 2000; Fraser et al. 1995; Mayfield et al. 1997). By improving transduction efficiency of BMC by a different retroviral gene transfer system, stable multilineage molecular chimerism and long-term skin graft tolerance was achieved (Bagley et al. 2002b). Mechanistically, central thymic deletion plus peripheral mechanisms, including regulation by CD4+CD25+ Tregs, were shown to play a role in this model (Forman et al. 2005, 2006; Kang and Iacomini 2002) and existence of molecular blood chimerism is required for durable tolerance (Tian et al. 2006).

In xenotransplantation (pig to human), natural xenoantibodies against the carbohydrate epitope Gal α-1, 3-Gal (αgal) are an additional barrier. Thus both B cell and T cell tolerance are required for long-lasting acceptance of xenografts. Humans, Old World monkeys and Apes possess a diminished function of α1, 3-galactosyltransferase (αGT), the enzyme producing αgal (Galili et al. 1988). The αGT knockout mouse (GT^0) model (having IgG and IgM antibodies toward αgal) is used to study natural antibodies in xenotransplantation. By introduction of αGT into syngeneic BMC B cell tolerance through molecular chimerism can be studied. Long-term molecular chimerism and B cell tolerance were achieved even when mice were pre-sensitized with pig cells and cardiac grafts from αGT expressing mice were accepted in GT^0 mice (Bracy and Iacomini 2000, 2002; Bracy et al. 1998, 2001; Kearns-Jonker et al. 2004; Mitsuhashi et al. 2006). Autologous transduced BMC expressing αGT was also transplanted into rhesus monkeys and xenoantibody production was strongly reduced after sensitization with porcine cells (Fischer-Lougheed et al. 2006).

3.2.2 Introduction of Auto-Antigens to Establish Tolerance

Molecular chimerism has also been studied in selected autoimmune models (Alderuccio et al. 2003). In experimental autoimmune encephalomyelitis (EAE), a rodent model to study multiple sclerosis (Zamvil and Steinman 1990), genes encoding for myelin oligodendrocyte glycoprotein (MOG), phospholipid protein (PLP) , myelin basic protein (MBP) and antigens inducing EAE, were introduced into BMC and established tolerance in EAE (Chan et al. 2008; Xu and Scott 2004; Xu et al. 2006). In one study introduction of MBP resulted in worsening of the disease, possibly due to insufficient MBP expression (Peters et al. 2000). Introduction of one peptide epitope of MOG ($MOG_{40–55}$) fused to a murine MHC II-associated invariant chain into syngeneic BMC resulted in tolerance induction in preventive and therapeutic models reported recently (Eixarch et al. 2009).

In the NOD type I diabetes model autoreactive T cells respond to islet antigens (insulin, glutamic acid decarboxylase (GAD) and others). The MHC class II region in NOD mice encodes a single MHC class II molecule I-A^{g7} (homologue to human HLA-DQB1 lacking a charged amino acid at position 57 associated with susceptibility to develop type 1 diabetes) which confers susceptibility to spontaneous type I diabetes (Atkinson and Leiter 1999). Induction of molecular chimerism through transplantation of BM expressing a 'disease-resistant' MHC class II molecule prevented type I diabetes in NOD mice and was sufficient to restore normoglycemia after islet transplantation and led to central thymic deletion of autoreactive T cells (Tian et al. 2004a, 2007a).

3.3 Hematopoietic Stem Cell Transplantation and Allergy

Cases in which BM transplanted from allergic donors induced allergies in the recipient have been reported. In reverse, allergies have been described to be cured in allergic recipients receiving a BMT from a healthy donor, suggesting that BMT is an effective treatment for allergies (though of course not indicated for this disease). No systematic, prospective evaluation of these issues, however, is available (Hourinhane et al. 2005; Khan et al. 2009; Storek et al. 2011).

4 Induction of Tolerance via Molecular Chimerism in Allergy

Molecular chimerism is an effective strategy for tolerance toward allo- and autoantigens. We recently started to investigate this strategy for tolerance in allergy. As most clinically relevant allergens have been identified and are available as cDNAs (Valenta and Kraft 2002), and as only a limited number of allergens is relevant in any given patient (and even geographical) region, allergies seem particularly suited for the molecular chimerism approach.

4.1 Prevention of Allergy by Induction of Tolerance via Molecular Chimerism

In proof-of-concept studies, we developed a preventive experimental model for induction of tolerance via molecular chimerism (Baranyi et al. 2008). A clinically relevant grass pollen allergen, Phl p 5 (Vrtala et al. 1993), was employed as model allergen. Phl p 5 is highly immunogenic upon subcutaneous immunization with the adjuvant aluminiumhydroxide in wild type mice (BALB/c) (Linhart et al. 2007). For cell surface expression on HSC Phl p 5 was fused to a signal peptide and a

transmembrane domain and ligated into a retroviral expression vector. Syngeneic BMC were transduced with recombinant retroviruses integrating Phl p 5 and thereafter Phl p 5-expressing BMC were transplanted into myeloablated syngeneic recipients. Mice transplanted with Phl p 5-transduced BM and mice of control groups were repeatedly immunized with recombinant (r) Phl p 5 and an unrelated allergen (rBet v 1) post BMT. BMT of Phl p 5-transduced cells resulted in stable long-term multilineage chimerism (follow-up approximately 9 months). Transduction of true HSC, already suggested by long-term multi-lineage chimerism, was confirmed through successful transplantation of BM taken from chimeras into secondary recipients (Down and Ploemacher 1993).

In this model durable tolerance was demonstrated by the complete absence of Phl p 5-specific IgE, IgA and IgG antibodies throughout follow up while high antibody responses to Bet v 1 were preserved. Tolerance at the effector cell levels in vitro and in vivo toward Phl p 5 was also achieved. T cell tolerance toward the introduced allergen was demonstrated by unresponsivess in proliferation assays. These results have been confirmed by transplanting BM transduced with a distinct, unrelated allergen (Bet v 1) (Gattringer, Wekerle et al., unpublished). Again lasting molecular chimerism and allergen-specific tolerance were observed. Therefore molecular chimerism is uniquely potent in establishing complete and permanent tolerance at the T cell, B cell and effector cell levels in experimental type I allergy (Baranyi et al. 2008, 2009).

4.2 Anticipated Impediments to Translate Molecular Chimerism to the Clinical Setting and Possible Solutions

Safety aspects have high priority in prophylactic approaches in allergy (Kulig et al. 1999). Therefore non-toxic protocols are a pre-requisite for translation. The experimental protocol employed in our proof-of-principle study entails several components that cannot be applied clinically, such as retroviral gene transfer, irradiation and others. We propose that solutions to these problems can be envisioned (Fig. 1).

4.2.1 Moving to Safer Protocols

Retro- or lentiviral vector-based delivery to integrate transgenes is efficient but associated with oncogenicity and mutagenesis (Thomas et al. 2003). The hyperactive *Sleeping Beauty* DNA transposon was shown to integrate transgenes in a robust and stable manner into CD34+ HSC reconstituted in mice and was even successful to modify T cells before transfer in humans in a gene therapy approach (VandenDriessche et al. 2009). Therefore DNA transposons are a potential safer alternative for integrating genes into HSC.

In constrast to murine models, in which chimerism needs to persist in order to maintain robust tolerance (Tian et al. 2006), transient mixed chimerism was

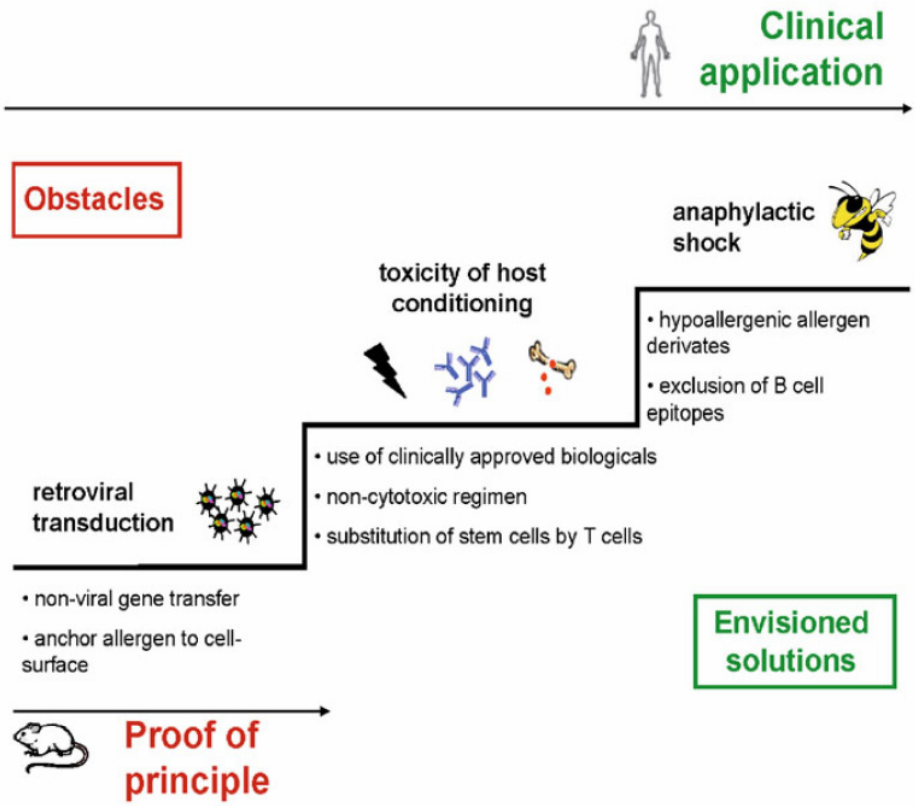

Fig. 1 Obstacles and envisioned solutions of cell based therapy by molecular chimerism, describing the hurdles on the way to clinical application starting with a proof-of-principle experiment in the mouse model

sufficient for tolerance in monkey models and clinical trials (Kawai et al. 2008; Ochiai et al. 2007). Thus, if transient chimerism suffices episomal, non-integrating delivery can be used, or antigens can be anchored directly to the cell surface (Belting and Wittrup 2009; Thomas et al. 2003; Yolcu et al. 2002).

In our proof-of-principle study, mice received a myeloablative dose of irradiation. Reduction of irradiation resulted in lower chimerism levels, but mice were still tolerant long-term (Baranyi, Wekerle et al., unpublished). In other models of molecular and mixed chimerism very mild experimental protocols have already been developed and could be translated to the allergy system in the future (Blaha et al. 2003; Eixarch et al. 2009; Forman et al. 2005; Tian et al. 2008; Wekerle et al. 2000; Pilat et al. 2010).

Application of the co-stimulation blocker anti-CD40L (anti-CD154) turned out to be very efficient in mixed chimerism rodent models (Wekerle et al. 1998). In a molecular chimerism model anti-CD40L was reported to enhance BM engraftment (Bagley et al. 2002c). Anti-CD40L is also effective in preventing sensitization to an allergen (Linhart et al. 2007). Unfortunately, this antibody induces severe

thromboembolic side effects in large animals and humans, and is therefore not used in the clinical setting (Kirk et al. 1999; Sidiropoulos and Boumpas 2004). Studies recently published show successful substitution of anti-CD40L by blockade of CD40 in murine models in allo-transplantation (Gilson et al. 2009), but clinical applicability remains uncertain. Blocking alternative co-stimulation pathways may also allow avoidance of anti-CD40L.

HSC engraftment requires—at least some—recipient conditioning. The transplantation of mature T cells instead of HSC in a murine model of molecular chimerism (crossing a MHC I barrier) led to long-lasting central tolerance by re-entry of mature T cells into the thymus (Tian et al. 2004b, 2007b, 2008). Thus, the use of allergen-expressing mature T cells, or other hematopoietic cell populations (McCurry et al. 2006), is an attractive alternative to be explored as a substitute for HSC.

4.2.2 Avoiding Anaphylaxis

Expression of full length allergens on transplanted BM—as in our proof-of-concept studies—implies the risk of anaphylaxis, mostly in the therapeutic but also in the preventive setting. Intracellular expression of an antigen was sufficient to induce T cell tolerance in mice (Tian et al. 2003). Expressing dominant T cell epitopes of allergens on the surface of HSC would conceivably avoid the risk of anaphylaxis, provided that T cell tolerance is sufficient to induce B cell tolerance, as it has been described (Li et al. 2008). Use of small peptides would also facilitate fusion of several dominant T cell epitopes of various allergens to achieve protection toward several allergens at the same time.

5 Conclusion

Preventive and therapeutic tolerance strategies are an important goal for improved medical management of allergies. Cell-based therapies are attractive candidates for development and substantial progress has been achieved in this field at the experimental level. Evaluation of Treg therapy has begun for certain indications and will allow a better appreciation of its usefulness in the treatment of allergy. Molecular chimerism is a powerful experimental approach leading to particularly robust allergen-specific tolerance. Its development is currently at an early proof-of-concept stage, but further progress toward clinical application in the long-term future can be envisioned.

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5.3 Contributions to Original Papers and Reviews

5.1.1 Anti-OX40L Alone or in Combination with anti-CD40L and CTLA4Ig Fails to Inhibit the Humoral and Cellular Response to a Major Grass Pollen Allergen

Manuscript in preparation

Developed experimental design, performed research (*in vivo* experiments, RBL cell degranulation assays, ELISAs, proliferation assays, flow cytometry), analyzed and interpreted data, wrote the manuscript

5.1.2 Engraftment of retrovirally-tranduced Bet v 1-GFP expressing bone marrow cells leads to allergen-specific tolerance

Manuscript submitted to Clinical and Experimental Allergy

Developed experimental design, performed research (cloned retroviral construct, production of retroviruses, transduction of BMC, *in vivo* experiments, RBL cell degranulation assays, ELISA, proliferation assays, flow cytometry and immunofluorescence microscopy), analyzed and interpreted data, wrote the manuscript

5.1.3 Persistent molecular microchimerism induces long-term tolerance towards a clinically relevant respiratory allergen

Manuscript submitted to Clinical and Experimental

performed research (supported ELISAs, proliferation assays and *in vivo* work), participated in analyzing data

5.1.4 Tolerization of a Type I Allergic Immune Response through Transplantation of Genetically Modified Hematopoieitic Stem Cells

J Immunol. 2008 Jun 15;180(12):8168-75

performed research (supported *in vivo* experiments, cell culture work and ELISAs), participated in analyzing data

5.1.5 Expression of a Major Plant Allergen as Membrane-Anchored and Secreted Protein in Human Cells with Preserved T Cell and B Cell Epitopes

Int Arch Allergy and Immunol, 2011 Jun 156(3):259-66

performed research (transfection of 293 T cells), analyzed data

5.1.6 Therapeutic Efficacy of Polyclonal Tregs does not Require Rapamycin for the Induction of Tolerance in a Low-Dose Irradiation BMT Model

Transplantation, 2011 Aug, 92(3):280-8

performed research (supported *in vivo* experiments and cell culture work)

5.2.1 A Chimerism-Based Approach to Induce Tolerance in IgE-Mediated Allergy

Crit Rev Immunol. 2009;29(5):379-97

Contributed to scientific writing and figures

5.2.2 Cell-Based Therapy in Allergy

Curr Top Mircrobiol Immunol. 2011 820:161-179

Contributed to scientific writing and figures

6 Conclusion

Currently, the risks of immunotherapy are under intensive investigation and some adverse effects could be reduced, e.g. by the exchange of allergen extracts to recombinant allergens or peptides. However, prevention of allergies would be a desirable alternative to symptomatic treatment and SIT.

Modification of the immune response by blockade of costimulatory molecules would be a strategy to renew the allergic response. Models of allergic inflammation revealed an important role of the costimulatory molecules OX40-OX40L, where blocking OX40L lead to diminished cell infiltration and antibody production. Therefore we investigated the role of the OX40 pathway. In our studies OX40L was negligible in the development of a T_H2 response towards an allergen. Furthermore, combined treatment of blocking CD28/CD40L and OX40L did not lead to an additional effect (chapter 4.1.1).

A successful, preventive strategy for allergy was achieved in a pilot project by induction of life-long tolerance towards Phl p 5 through molecular chimerism (chapter 4.1.3, 4.1.4, 4.2.1). In order to extend this approach of molecular chimerism to different clinically relevant allergens, tolerance towards Bet v 1 was investigated. Bet v 1 is structurally and functionally completely unrelated to Phl p 5. We were able to induce tolerance towards Bet v 1 in T-cell, B-cell and effector cell responses, even after repeated challenges of allergens, suggesting that this strategy is robust, specific and translatable for several allergens (chapter 4.1.2).

Although cellular and molecular chimerism are strategies with a high potential for inducing tolerance towards specific antigens, the development of low-toxicity protocols is still a major aim. In allo-transplantation, a strong immunological barrier, which is caused by pre-existing donor-reactive cells, has to be overcome. Recently, we published a

protocol for the transplantation of allogeneic BMC with only a low dose irradiation together with costimulation blockade and regulatory T cells with a clinically feasible dose of bone marrow cells (chapter 4.1.5). Not long ago, regulatory T cells in combination with rapamycin were shown to be able to fully replace irradiation (46). In allergy, identification of costimulatory molecules which are able to specifically block the immune response towards the allergen would possibly allow engraftment of allergen-expressing HSC. In this work, the potential of OX40L was tested yet this costimulatory molecule seems to play a negligible role in the development of an allergic immune response. It is noticeable that the immune response induced by allergen-expressing HSC is not necessarily identical with the T_H2 response induced by injection of alumn-adsorbed allergen. Therefore the role of costimulatory molecules is possibly not comparable and has to be tested separately.

Other hurdles such as the toxicity of retroviral transduction of BMC, the necessity for clinically unfeasible costimulation blockers, and the risk of anaphylaxis still exist (chapter 4.2.2). However, all these aspects are currently under investigation and promising strategies such as the use of modified recombinant allergens are being painstakingly developed. Successful translation to the clinical setting would allow molecular chimerism as a preventive and therapeutic strategy not only in allergy but also in autoimmunity. This protocol has the potential to induce stable and long-term tolerance, thus making ongoing medication dispensable.

7 Appendix

7.1 Abbreviations

APC	antigen presenting cell
Bet v 1	betula verrucosa allergen number 1
BMT	bone marrow transplantation
BMC	bone marrow cells
CHS	contact hypersensitivity
DC	dendritic cells
DNA	desoxyribonucleinacid
EAE	experimental autoimmune encephalomyelitis
Fel d 1	feline domesticus allergen number 1
GFP	green fluorescent protein
α GT	α-1,3 galactosyltransferase (αGT)
GVHD	graft versus host disease
HLA	human leucocyte antigen
HSC	hematopoietic stem cell
ICOS	inducible costimulatory ligand
IDO	indolamine 2,3-dioxygenase
IL	interleukin
IFN	interferon
MHC	major histocompatibility complex
NK	natural killer cells
OVA	ovalbumin
PD	programmed death
Phl p 5	phleum pratense allergen number 5
SIT	specific immunotherapy
TBI	total body irradiation
TIM	T cell immunoglobulin and mucin-domain containing molecule

7.2 References

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7.3 Acknowledgements

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7.4 Curriculum Vitae

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Education

2007 - 2011	Graduate student at the Medical University of Vienna General Hospital Vienna, Department of Surgery, Division of Transplantation
Doctoral Thesis:	Prevention of IgE–mediated allergy by molecular chimerism and costimulation blockade in a murine model

2000 - 2007	Studies of Biology with emphasis on Genetics and Microbiology at the University of Vienna, Institute of Microbiology and Genetics (Vienna Biocenter)
Diploma Thesis:	Induction of tolerance in type I allergy by transplantation of Bet v 1 transduced bone marrow
1995 - 2000	HBLA Auhof Linz

Work Experience

2005 – 2010	General Hospital Vienna, Department of Surgery, Division of Transplantation
Patent:	Co-Inventor of patent application no. WO 2008/148831 (Molecular chimerism for tolerance in allergy)

Additional Trainings

September 2008	"Labortierkundekurs", classified as category B by GV-SOLAS (Veterinärmedizinische Universität Wien)
October 2008	Intensive course in scientific writing (MUW)

Additional Skills

Languages	fluent English
	French (spoken and written)

7.5 Papers and Publications

7.5.1 Original Papers

1. Gattringer M, Baranyi U, Pilat N, Klaus C, Buchberger E, Ramsey H, Hock K, Valenta R, Wekerle T. Engraftment of retrovirally-transduced Bet v 1-GFP expressing bone marrow cells lead to allergen-specific tolerance. (Manuscript submitted)

2. Gattringer M, Baranyi U, Pilat-Michalek N, Hock K, Klaus C, Ramsey H, Hock K, Valenta R, Wekerle T. OX40L has no detectable effect on humoral and cellular response in IgE-mediated allergy not even in combination with anti-CD40L and CTLA4Ig. (Manuscript in preparation)

3. Baranyi U, Gattringer M, Boehm A, Marth K, Focke-Tejkl M, Bohle B, Valent P, Muehlbacher F, Valenta R, Wekerle T. Expression of a major plant allergen as membrane-anchored and secreted protein in human cells with preserved T cell and B cell epitopes. Int Arch Allergy and Immunol, 2011 Jun 156(3):259-66.

4. Nina Pilat, Christoph Klaus, Martina Gattringer, Elmar Jaeckel, Fritz Wrba, Dela Golshayan, Ulrike Baranyi, Thomas Wekerle. Therapeutic efficacy of polyclonal Tregs does not require rapamycin for the induction of tolerance in a low-dose irradiation BMT model. Transplantation, 2011 Aug, 92(3):280-8.

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6. Baranyi U, Pilat N, Gattringer M, Linhart B, Klaus C, Schwaiger E, Iacomini J, Valenta R, Wekerle T. Persistent molecular microchimerism induces long-term tolerance towards a clinically relevant respiratory allergen. (Manuscript submitted)

7.5.2 Review Articles

1. Baranyi U, Pilat N, Gattringer M, Wekerle T. A chimerism-based approach to induce tolerance in IgE-mediated allergy. Crit Rev Immunol. 2009;29(5):379-97.

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7.5.3 Posters and Presentations

1. Gattringer, M., Baranyi, U., Pilat, N., Klaus, C., Ramsey, H., Hock, K., Mühlbacher, F., Valenta, R., and Wekerle, T. Transplantation of Bet v 1 expressing bone marrow cells induces robust tolerance in a murine model. Proc. ÖGAI 2009 . 2009. Notes: Karl Landsteiner Meeting; Jahrestagung der Österreichischen Gesellschaft für Allergologie und Immunologie (ÖGAI); Salzburg, Austria, 6-7 September 2009. Poster Prize

2. Gattringer, M., Baranyi, U., Pilat, N., Klaus, C., Ramsey, H., Hock, K., Valenta, R., and Wekerle, T. Long-term tolerance in a murine model of type I allergy through molecular chimerism. 2009. Notes: World Allergy Congress; Buenos Aires, Argentinia; 6-10 December 2009.

3. Gattringer, M., Baranyi, U., Klaus, C., Pilat, N., Schwaiger, E., Muehlbacher, F., Valenta, R., and Wekerle, T. Influence of $CD4^{+}CD25^{+}$ cells during the sensitization phase or later allergen challenge in a mouse model. Proc. ÖGAI 2008, Wiener Klinische Wochenschrift 120, 138. 2008. Notes: Jahrestagung der Österreichischen Gesellschaft für Allergologie und Immunologie; Vienna, 3-6 September 2008.