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

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Abstract

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.

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.

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.

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.

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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

Allergy; Molecular chimerism; Tolerance

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 (Alderuccio et al. 2009; Pilat and Wekerle 2010).

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 (Kawai et al. 2008; Wekerle et al. 2000). 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 (Forman et al. 2005; Kang and Iacomini 2002). Molecular chimerism was also successfully tested in autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), type I diabetes (Chan et al. 2006; Tian et al. 2007) and autoimmune gastritis (Murphy et al. 2003). 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 (Chan et al. 2008; Eixarch et al. 2009; Xu et al. 2006). Interestingly, not every protocol was able to induce tolerance in EAE (Peters et al. 2000).

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 (Baranyi et al. 2008). 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 (Radauer et al. 2008) 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 (Bohle et al. 2003). It is the major allergen of the birch pollen of the birch *Betula verrucosa*, located in the cytoplasm of birch pollen grains (Grote 1999). 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 (Swoboda et al. 1995). Bet v 1 is also causing symptoms because of the high cross-reactivity to food allergens in soy, cherry, celery and others (Bohle et al. 2003; Fuchs et al. 2006; Kleine-Tebbe et al. 2002; Vieths et al. 2002). 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-1 α) and prevent the interaction with CXCR4 which is critical for homing of BMC (Christopherson et al. 2004; Tian et al. 2006). 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) [8] (Breiteneder et al. 1989), and an IRES-eGFP side flanked by long terminal repeats (LTR). Previously cDNA of Bet v 1 (pMW172 (Hoffmann-Sommergruber et al. 1997)) was fused to a murine Ig signal sequence (S) and a transmembrane domain (TMD) (pDisplay, Invitrogen, Carlsbad, CA) by overlapping PCR technique (Ho et al. 1989). Overlapping primers were used to fuse the sequences: NcoI restriction site and start ATG fused to S: 5' GGC GCC 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 (Pear et al. 1993). Viral supernatant was concentrated by ultra-centrifugation ($33,620 \times g$ for 2 h), 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 (Baranyi et al. 2008; Bracy and Iacomini 2000). Briefly, bone marrow of 5-FU-treated BALB/c mice (Bodine et al. 1991) 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; 0.5 mg/mouse) and anti-CD4 (GK1.5; 0.5 mg/mouse) mAbs (Bagley et al. 2002). On the day of reconstitution mice were transplanted with 2.5×10^6 transduced BM cells (Bracy and Iacomini 2002). For induction of Bet v 1-GFP or GFP chimerism, BMC were additionally incubated with 5 mM Diprotin A (Sigma–Aldrich) at 37 °C for 15 min (Tian et al. 2006). Immediately after BMT mice received anti-CD40L mAb (MR1; 0.5 mg/mouse) (Bagley et al. 2002). 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 with 5 µg rBet v 1 and 5 µg rPhl p 5, adsorbed to Al(OH)₃ (Alu-Gel-S, Serva, Ingelheim, Germany) (Linhart et al. 2007) 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 (Vrtala et al. 1998). Plates were coated with rBet v 1 or rPhl p 5 (5 µg/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 (60 mM/l citric acid, 77 mM/l $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, 1.7 mM/l ABTS (Sigma–Aldrich, MO, 3 mM/l H_2O_2).

Lymphocyte proliferation assay

Spleens were removed under aseptic conditions and homogenized. Single cell suspensions were 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 2×10^6 cells/ml and triplicates of 100 μl /well were seeded in 96-well round-bottom plates. Stimulants were added at a concentration of 2 μg /well allergen and 0.5 μg /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 0.5 μCi H^3 thymidine ([methyl-3 H] 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 (Baranyi et al. 2008). Aliquots of 4×10^4 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 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 per well) for 30 min at 37 °C. 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, 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 β -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 diluted 1:200 and stained with fluorochrome-conjugates as secondary antibodies (goat α -rabbit IgG Alexa-Fluor 546 nm, 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 statistically 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) (Baranyi et al. 2008).

Induction of molecular chimerism in BMC chimeras

Based on our previous work that long-term molecular chimerism can be established with modified BMC (Baranyi et al. 2008), 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 (Tian et al. 2006). To avoid rejection of modified transplanted cells, recipients were irradiated (8 Gy total body irradiation), T cell depleted (anti-CD4, anti-CD8, 0.5 mg/mouse) and treated with the costimulation blocker anti-CD40L (0.5 mg/mouse) (Fig. 2B). Preconditioned recipients received 2.5×10^6 transduced BMCs (Bracy and Iacomini 2002).

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 (Baranyi et al. 2008).

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–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. 3 C, 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 the 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 assay. β -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 β -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 β -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 (Baranyi et al. 2008). Given the available literature that molecular chimerism is not universally successful but fails to induce tolerance towards some antigens (Peters et al. 2000), 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 (Tian et al. 2006). 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 (Tian et al. 2006). Only 4.5% transduced cells of 2.5×10^6 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 (Baranyi et al. 2008), 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 (Pilat et al. 2010; Pilat and Wekerle 2010). Irradiation-free protocols have recently become feasible even for 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 (Mitsuhashi et al. 2006). Anaphylaxis can be avoided through the use of hypoallergenic derivatives for transduction (Larche et al. 2006).

While many problems still have to be solved, induction of tolerance is a much needed goal for the treatment of allergies (Baranyi et al. 2011). Molecular chimerism is a promising approach in this context. Its potential has been shown in many different mouse models (Baranyi et al. 2009). Moreover, clinical observations of tolerance induction through cellular chimerism in transplant patients underline the potential of chimerism as future treatment strategy (Fudaba et al. 2006; Kawai et al. 2008).

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

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Abbreviations

HSC	hematopoietic stem cells
MHC	major histocompatibility complex
VSV	vesicular stomatitis virus
LTR	long terminal repeat
5-FU	5-fluoruracil
RBL	rat basophile leukaemia
BMC	bone marrow cells
EAE	experimental autoimmune encephalomyelitis
S	signal sequence
TMD	transmembrane domain
BMT	bone marrow transplantation
mAb	monoclonal antibodies

FCM	flow cytometry
SEM	standard error of the mean
SD	standard error

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A MMP-Bet v 1-TMD-IRES-eGFP **MMP-IRES-eGFP** **B VSV-Bet v 1-GFP transduced cells** **VSV-Phl p 5 transduced cells** **Fig. 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 IRES-eGFP, resulting in MMP-Bet v 1-TMD-IRES-eGFP. The control vector, MMP-IRES-eGFP 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 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 (0.5 mg, d-1), anti-CD8 mAb (0.5 mg, 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, 0.5 mg, 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.

Fig. 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 ($n=5$, $\square$), GFP chimeric mice ($n=4$, $\square$) and Bet v 1-GFP chimeric mice ($n=4$, $\blacksquare$). Week 0 represents baseline antibody levels of mice previous 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.

Fig. 4.

Tolerance towards the allergen Bet v 1 on the B cell level β -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.

Fig. 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.