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Research Paper

Cell Therapy for Prophylactic Tolerance in Immunoglobulin E-mediated Allergy

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ABSTRACT

Background: Therapeutic strategies for the prophylaxis of IgE-mediated allergy remain an unmet medical need. Cell therapy is an emerging approach with high potential for preventing and treating immunological diseases. We aimed to develop a cell-based therapy inducing permanent allergen-specific immunological tolerance for preventing IgE-mediated allergy.

Methods: Wild-type mice were treated with allergen-expressing bone marrow cells under a short course of tolerogenic immunosuppression (mTOR inhibition and costimulation blockade). Bone marrow was retrieved from a novel transgenic mouse ubiquitously expressing the major grass pollen allergen Phl p 5 as a membrane-anchored protein (BALB/c-Tg[Phlp5-GFP], here mPhl p 5). After transplantation recipients were IgE-sensitized at multiple time points with Phl p 5 and control allergen.

Results: Mice treated with mPhl p 5 bone marrow did not develop Phl p 5-specific IgE (or other isotypes) despite repeated administration of the allergen, while mounting and maintaining a strong humoral response towards the control allergen. Notably, Phl p 5-specific T cell responses and allergic airway inflammation were also completely prevented. Interestingly allergen-specific B cell tolerance was maintained independent of Treg functions indicating deletional tolerance as underlying mechanism.

Conclusion: This proof-of-concept study demonstrates that allergen-specific immunological tolerance preventing occurrence of allergy can be established through a cell-based therapy employing allergen-expressing leukocytes.

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1. Introduction

Cell-based therapy may have high potential to prevent or treat immunological diseases (Fischbach et al., 2013). T regulatory cells (Tregs) have received the most interest in recent years. Their use is currently explored in several clinical pilot trials for the treatment of autoimmune diseases, organ and bone marrow transplantation (Di Ianni et al., 2011; Brunstein et al., 2011; Trzonkowski et al., 2009; Marek-Trzonkowska et al., 2014; Wood et al., 2012). While of high interest, pharmacological efficacy remains to be established for such treatment strategies. Besides, conceptually their direct effects are of limited duration as most Treg subtypes gradually die off. Whether they can establish a lasting effect — through triggering a 'regulatory cascade', for instance, remains to be seen (Edozie et al., 2014). In IgE-mediated allergy the transfer of Tregs ameliorated allergic inflammation but the effects on IgE were less clear in mice (Kearley et al., 2005; Leech et al., 2007; Xu et al., 2012).

An alternative strategy is the transplantation of hematopoietic stem cells (Pilat and Wekerle, 2010). If stem cell engraftment is achieved, this approach has the advantage of establishing tolerizing mechanisms of unlimited duration (Sykes, 2001). Transplantation of allogeneic donor hematopoietic stem cells has been used in clinical pilot trials of renal transplantation successfully establishing donor-specific tolerance

Abbreviations: CB, costimulation blockade; BMT, bone marrow transplantation; BMC, bone marrow cells; ELISA, enzyme-linked immunosorbent assay; OD, optical density; FCM, flow cytometry; Rapa, rapamycin; WBC, white blood cells; AIT/SIT, allergen-specific immunotherapy; WBP, whole body plethysmography; s.c., subcutaneous; i.n., intranasal; AHR, airway hyperresponsiveness.

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(Kawai et al., 2008; Leventhal et al., 2012; Scandling et al., 2015). Transplantation of *autologous* hematopoietic stem cells modified to express the disease-causing antigen is employed for immunological disorders caused by defined antigens (Alderuccio et al., 2011). For instance, promising results have been achieved in a clinical trial of multiple sclerosis with this approach (Lutterotti et al., 2013).

Allergen-specific immunotherapy (AIT or SIT) is an established vaccination strategy in IgE-mediated allergy. The induction of allergen-specific IgG_4 to compete with allergen-specific IgE is among its main mechanisms (Niederberger et al., 2004; Larche et al., 2006), and also other mechanisms, such as induction of regulatory cells, including Tregs and Bregs, were described (Akdis and Akdis, 2015). Beside the well-established SIT, prophylactic approaches are an important unmet medical need (Valenta et al., 2012). Several studies performed in children found that oral immunotherapy was often effective but not always safe in peanut allergy (Jones et al., 2014). Interestingly a recently published clinical study showed that the prophylactic consumption of peanuts in early childhood led to peanut-specific IgG_4 induction and reduced the prevalence of peanut-specific IgE in children with a high risk to develop peanut allergy (Du Toit et al., 2015). Although oral tolerance might be effective in severe food allergy additional, widely applicable preventive strategies are needed.

Therefore we aimed to develop a cell therapy strategy for achieving a long-lasting prevention of IgE-mediated allergy by inducing robust allergen-specific tolerance.

2. Materials and Methods

2.1. Mice

Female BALB/c mice of SPF quality were purchased from Charles River Laboratories and housed in a barrier animal facility. Mice 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 approved by the Austrian Federal Ministry of Science, Research and Economy, BMWFW (GZ: BMWF-66.009/0295-1I/3b/2011) and were performed in accordance with national and international guidelines of laboratory animal care.

2.2. Sera of Allergic Patients

Sera of Phl p 5-allergic patients were used with the approval of the local ethics committee 235/05/2013 EK Nr. 565/2007 according to the Austrian Federal Ministry of Science, Research and Economy.

2.3. Generation of the mPhl p 5 Transgenic Mice

The Phl p 5 cDNA was fused to a signal peptide and a transmembrane domain as described (Baranyi et al., 2011). The vector pccall2-IRES-EGFP was transformed into an *E. coli* cre strain to excise the neomyocin-Lac Z cassette (both kindly provided by Prof. Maria Sibilia). The Phl p 5-fusion gene was cloned via XbaI and BglII into the recombined pccall2 vector-IRES-EGFP. The clone was confirmed by double-strand DNA sequencing. Before pronuclear injection the bacterial backbone was removed by restriction with ScaI and SfiI and elution from agarose gel. Briefly, the linearized construct was microinjected into a pronucleus of fertilized BALB/c inbred oocytes that were transferred afterward into the oviduct of pseudopregnant surrogate mothers according to standard protocols for generating transgenic mice (Rulicke, 2004). Transgene integrations were identified by PCR of tail DNA with Phl p 5-specific and GFP-specific primers. g Phl p 5 3 fw: 5′-CTGCAG GTCATCGAGAAGGT-3′, g Phl p 5 3 rev: 5′-TTTCAGTGCGGTCTCAAAGA-3′, PL EGFP-F fw: 5′-CGCACCATCTTCTTCAAGGACGAC-3′, PL EGFP-R rev: 5′-AACTCCAGCAGGACCATGTGATCG-3′. Of 6 identified transgenic founders we chose BALB/c-Tg (Phlp5-GFP) 304Biat expressing GFP and Phl p 5 at high level in white blood cells determined by flow cytometry.

2.4. Flow Cytometry

Phl p 5^+ cells were stained with Phl p 5 BG-6 mIgG1 (Petersen et al., 1994), incubated with rabbit anti-mouse Ig BIO and stained with PE or Cy5 streptavidin conjugates (Biolegend). B220-Bio CD25-Bio (clone 7D4) (stained with PE streptavidin conjugates) and CD4-APC Cy7 were obtained from Biolegend. Treg depletion-CD25 downregulation was assessed by staining with 7D4 Abs (anti CD25). Cells were measured in a FACS Canto II (BD) or FC 500 (Beckman Coulter) and analyzed using the FlowJo software (Miltenyi, Germany). Phl p 5 + cells were gated to CD45.2. Thymocytes are additionally gated to CD4/CD8 positive cells.

2.5. Immunofluorescence

Frozen sections of tail skin, hearts and spleens of mPhl p 5 transgenic mice or naïve BALB/c mice were blocked with 10% serum and incubated with rabbit anti-Phl p 5 antibodies (Focke-Tejkl et al., 2014) (1:500) and stained with a goat anti-rabbit Alexa fluor 633 antibody (Invitrogen). Nuclei were stained with propidiumiodid 1:200 from stock 1 mg/ml (Sigma, Germany). Specimens were analyzed by a confocal LSM510 Meta microscope (Zeiss, Germany) using the ZEN software.

2.6. Allergen-specific ELISA

Allergen-specific ELISAs were performed as described in Baranyi et al. (2008). For IgE sera were diluted 1:20, for IgG isotypes 1:500, for IgM and IgA 1:100. Plates were coated with 5 μg of r Phl p 5 or r Bet v 1 (Biomay, Austria) or with Phl p 5 peptides (5 μg/ml each) (Focke-Tejkl et al., 2014).

2.7. In Vivo Antibodies

Monoclonal antibodies, anti-CD25 (clone PC61) Ab (0.5 mg/mouse/dose), anti-IL-2 (clone S4B6) (0.3 mg/mouse/dose), anti CTLA4 (clone 9H10) (0.5 mg/mouse/dose) and anti PD-1 (clone J43) (0.25 mg/mouse/dose) were purchased from BioXcell (West Lebanon, NH). Mouse anti-mouse CD20 antibody (clone 5D2, isotype IgG2a) (250 μg per dose) was generously provided by Genentech (San Francisco, CA, USA). hCTLA4Ig (abatacept) (1 mg per mouse) was generously provided by Bristol-Myers, Squibb Pharmaceuticals (Princeton, NJ, USA).

2.8. Bone Marrow Transplantation

Bone marrow was isolated as described in Pree and Wekerle (2007). Aliquots of 15×10^6 BMCs were transplanted into the tail vein of preconditioned BALB/c recipients. Rapamycin was injected i.p. 150 mg/kg per dose for each mouse.

2.9. 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 sowed in 96-well round-bottom plates. Stimulants were added at a concentration of 2 μg/well allergen or Con A as control for proliferation at 0.5 μg/well (Sigma-Aldrich). The plates were incubated at 37 °C, 5% CO_2. On day 4, 0.5 μCi H3 thymidine ([methyl-3H], Amersham) per well was added. Sixteen hours later, cells were harvested and thymidine uptake measured in a beta counter (Beta scintillation

liquid, Wallac). The ability of splenocytes to proliferate was confirmed by unspecific stimulation with Con A.

2.10. Airway Hyperresponsiveness and Histology

Mice were treated with r Phl p 5 (10 μg per dose in 50 μl PBS) (Biomay, Austria) intranasally on day-3, -2 and -1 before whole body plethysmography (Buxco Research Systems, Wilmington, NC, USA). Mice were challenged with different concentrations of methacholine (8, 16, 32 and 64 mg/ml) (Sigma) and PBS as baseline. After methacholine provocation mice were killed and lungs were stored in 4.5% phosphate buffered formaldehyde. Lung sections were stained for hematoxylin and eosin (H&E) and periodic acid stain (PAS).

2.11. Statistical Analyses

The reported p-values are results of two-sided Mann–Whitney U tests and were calculated using Graph Pad Prism 5 and compared between groups. p-Values < 0.05 were considered statistically significant. Whiskers in box plots indicate the minimum to maximum range of the values and error-bars indicate the standard deviation (SD).

3. Results

3.1. Membrane-anchored Phl p 5 Is Ubiquitously Expressed in Cells and Tissues of a Newly Generated mPhl p 5-transgenic Mouse

Phl p 5, a major pollen allergen of timothy grass, was selected for our studies as it is highly allergenic (Westritschnig et al., 2008) and as it is relevant for more than 80% of grass pollen allergic patients from temperate regions (Gangl et al., 2013). To develop cell-based strategies for tolerance induction with autologous cells (i.e. syngeneic in animal models) we first generated a transgenic BALB/c mouse — a high IgE responder strain - expressing Phl p 5 on the cell surface (Dearman and Kimber, 2009). For ubiquitous expression of Phl p 5 a fusion gene of signal peptide (S) cDNA of Phl p 5 and transmembrane domain (TM) was cloned into vector pcall2-IRES eGFP, containing the chicken-β-actin promoter and CMV-enhancer (CAGGS-promoter) for ubiquitous high level expression and the reporter protein GFP as an IRES (internal ribosomal entry site) (Fig. 1A) (Baranyi et al., 2011; Novak et al., 2000; Rulicke and Hubscher, 2000; Ittner and Gotz, 2007). Virtually all WBC, bone marrow (BM), splenocytes and thymocytes expressed Phl p 5 and GFP as assessed by FCM (Fig. 1B). Additionally sections of cardiac, tail skin and splenic tissue showed widespread expression of mPhl p 5 (Fig. 1C). Specific expression of the transgene was further confirmed by immunoblot in splenocytes of mPhl p 5 transgenic mice (Fig. E1). These data demonstrate that mPhl p 5-transgenic mice express mPhl p 5 and GFP ubiquitously and at high level.

In most models of allergy mice are immunized in combination with alum as an adjuvant, known to activate the inflammasome and a type 2 innate response (Eisenbarth and Colegio, 2008; McKee et al., 2009). To develop a clinically more relevant model for allergen recognition we determined the immunogenicity of the mPhl p 5 transgene product by immunizing naïve BALB/c with splenocytes of mPhl p 5 transgenic mice ($n = 5$). Interestingly, using cell transfer high and constant recombinant Phl p 5 (r Phl p 5)-specific IgE levels were obtained (w 6 and w 23) during the whole follow up (Fig. E2B) as were Phl p 5-specific IgG_1, IgG_{2a}, IgG_3 and IgM (data not shown). In comparison, IgE epitope mapping using sera of grass pollen allergic patients ($n = 5$) revealed that IgE responses of allergic patients are directed to the intact Phl p 5 allergen molecule, with no detectable IgE reactivity to any of seven linear (sequential) peptides (P1–P7) (Focke-Tejkl et al., 2014) (Fig. E2A). In mice immunized subcutaneously with r Phl p 5 adsorbed to $Al(OH)_3$, IgE reactivity was again directed to the intact allergen but also towards some linear peptides (mainly P1, P4 and P6) of Phl p 5 (Fig. E2C). In contrast, in mice immunized with splenocytes of the mPhl p 5 transgenic mouse (mPhl p 5) no IgE-reactivity was detectable towards linear peptides but to the whole r Phl p 5 protein (Fig. E2C). Thus, the adjuvant-free immunization with mPhl p 5 splenocytes more closely reflects the IgE recognition of Phl p 5 of allergic patients than immunization with alum-adsorbed rPhl p 5. Thus, the Phl p 5-expressing transgenic BALB/c mouse may provide a suitable model for developing and investigating cell-based tolerance strategies.

3.2. Transplantation of mPhl p 5 Bone Marrow Cells With Short Course Rapamycin and CD40L or CTLA4Ig Leads to Permanent Tolerance

We injected mPhl p 5 BMCs (15×10^6) together with a short course of tolerogenic immunosuppression (the mTOR inhibitor rapamycin plus either anti-CD40L or CTLA4-Ig) into naïve BALB/c mice. Mice were subsequently immunized three times (w 4, 7, 10) with mPhl p 5-splenocytes and rBet v 1. Phl p 5-specific IgE remained undetectable for the duration of follow-up (20 weeks) (Fig. 2A), whereas high levels of Bet v 1-specific IgE developed quickly and persisted throughout follow-up (Fig. 2B). As CTLA4Ig and anti-CD40L were comparably effective in inducing tolerance, CTLA4Ig was selected for further development since it is clinically approved (termed hereafter 'CTLA4Ig/rapa cell therapy' protocol) (Fig. E3A). Additionally chimerism levels were determined at early time points (Fig. E3B). Beside the avoidance of Phl p 5-specific IgE, no Phl p 5-specific IgG1, IgG2a, IgG3, IgM and IgA isotypes were detectable in sera of CTLA4Ig/rapa cell therapy treated mice long term (Fig. 3), while Bet v 1-specific antibody levels were similar as in non-transplanted sensitized mice (Fig. E4). The combination of BMT with rapamycin and CTLA4Ig is necessary for full efficacy as anti-CD40L, CTLA4Ig or rapamycin alone did not completely prevent Phl p 5-specific IgE development (Fig. E5).

Moreover to investigate if T cell tolerance was achieved, splenocytes of mPhl p 5 BMC-treated mice were stimulated with rPhl p 5 in vitro. No proliferation towards Phl p 5 was detectable in mice receiving CTLA4Ig/rapa cell therapy, similar to splenocytes of naïve mice. On the contrary, splenocytes of sensitized mice showed high proliferation rates upon Phl p 5-stimulation (Fig. 2C). Thus, humoral and T cell responses were fully prevented with the transfer of Phl p 5-bearing BMCs under tolerogenic immunosuppression avoiding clinically not applicable irradiation protocols (Baranyi et al., 2008).

3.3. Airway Inflammation

Group 5 allergens are a major cause of allergic asthma (Suphioglu et al., 1992). Therefore we tested if airway inflammation can be avoided with the protocol described above. After additional intranasal application with rPhl p 5, mice were challenged with different doses of methacholine in whole body plethysmography (WBP). Sensitized non-BMT mice showed higher levels of enhanced pause (Penh) (Fig. 4A) than naïve mice. Mice receiving BMCs under rapa and CTLA4Ig responded weaker to methacholine provocation than sensitized mice and in a similar manner as naïve mice. Additionally in lung tissues no peribronchial chronic inflammatory infiltrations of lymphocytes were detectable in BMT recipients (Fig. 4B) and no mucus production was visible in tolerant mice in PAS staining (Fig. 4C). Thus, airway inflammation was avoided in mice receiving mPhl p 5 BMC under tolerogenic immunosuppression.

3.4. B Cells Are Not Required for Specific B Cell Tolerance Induction

B cells are involved in antigen presentation by recognizing conformational epitopes (Batista and Harwood, 2009). To assess whether B cells are important in tolerance induction with CTLA4Ig/rapa cell therapy we depleted them with an anti-CD20 mAb at the time of BMC transfer (d −7, 0, +7). Depletion of B cells was confirmed in the peripheral blood by flow cytometry (and was of a similar degree as in previous publications demonstrating a mechanistic effect of CD20 depletion

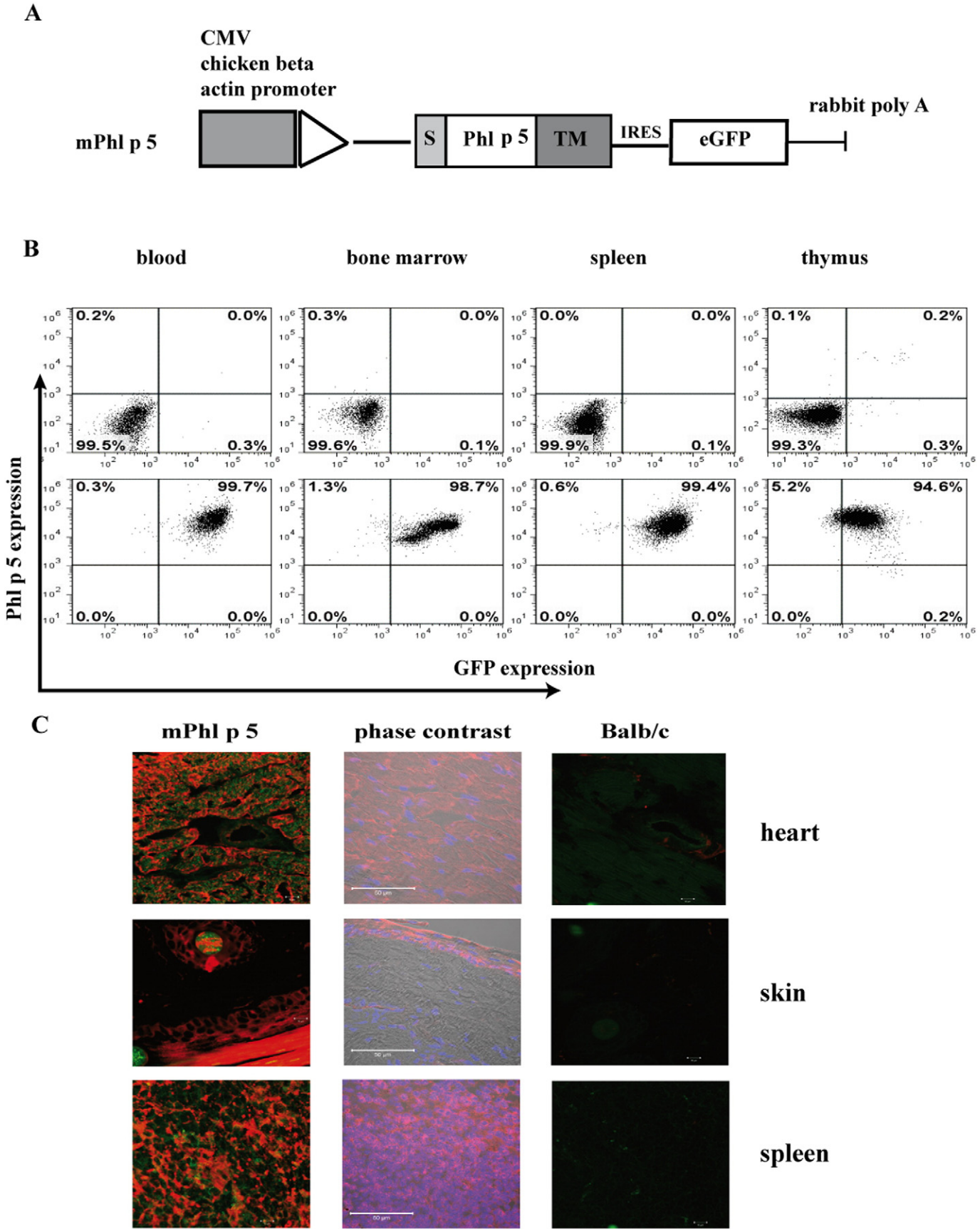

Fig. 1. Generation and characterization of mPhl p 5-transgenic mouse. (A) Construct of mPhl p 5 construct in pcall2 vector containing the CMV-chicken beta actin enhancer/promoter and a rabbit poly A tail. (B) Expression levels of Phl p 5 and GFP in white blood cells (blood), bone marrow, spleen and thymus. (C) Immunofluorescence of tissues of mPhl p 5 transgenic mice stained for Phl p 5 (red) (left panel and middle panels), nuclei are stained in blue (middle panels) GFP is also visible in green (left panels), and BALB/c mice (right panels).

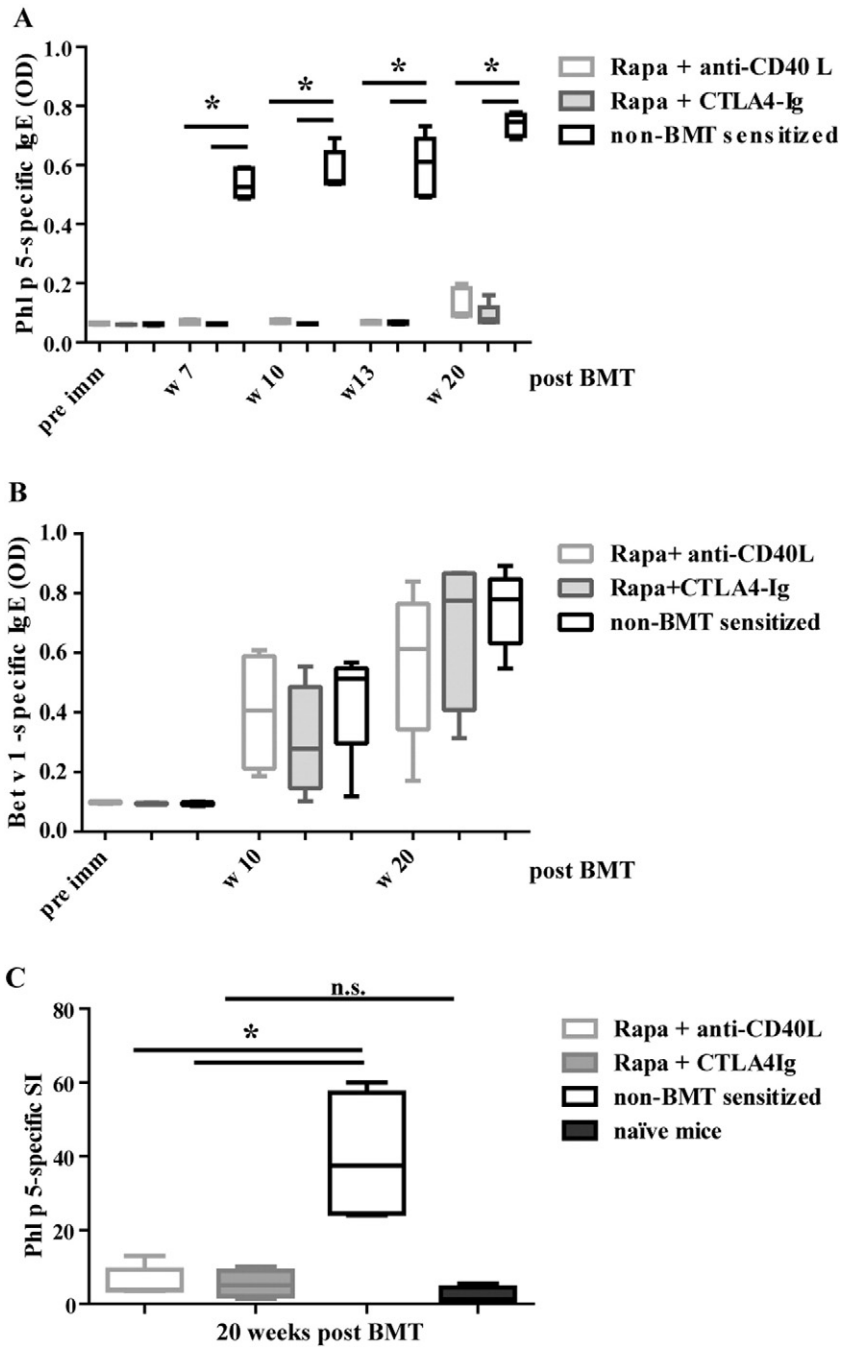

Fig. 2. mPhl p 5 cell therapy combined with rapamycin and CTLA4Ig leads to specific B cell and T cell tolerance (A) Phl p 5-specific IgE levels in sera of mice treated with 15×10^6 mPhl p 5 BMC and either rapamycin plus anti-CD40L (Rapa + anti-CD40L, n = 5), or rapamycin plus CTLA4-Ig (Rapa + CTLA4-Ig, n = 5) and non-transplanted immunized mice (non-BMT sensitized) n = 5 at different time points. (B) Bet v 1-specific IgE in groups of mice described in A (n = 5 each group). (C) T-cell proliferation assays of splenocytes of mice described in (A). Naive mice were used as control (n = 4). Results are demonstrated as box blots and medians are shown. * $p < 0.05$. n.s.—not significant.

(Mollov et al., 2010)) and persisted for several weeks (Fig. 5A). After recovery of B cells (determined by FCM data, not shown) mice were immunized with mPhl p 5 splenocytes (weeks 7 and 10 after BMT). No Phl p 5-specific IgE was detectable after two immunizations with mPhl p 5 splenocytes in sera of mice treated with anti-CD20 antibody (Fig. 5B), suggesting that B cells are not the main regulator in establishment of B cell tolerance.

3.5. Regulation Is Not a Substantial Mechanism for Tolerance Induction

Rapamycin, anti CD40L and CTLA4Ig were all described to induce/promote regulatory T cells (Tregs) in certain experimental settings (Ahmadi et al., 2014; Muller et al., 2010; Linsley and Nadler, 2009). To assess if Tregs are important for tolerance induction we depleted CD25 positive cells (with anti-CD25 [PC61] mAb d-6, d-1) before cell therapy (mPhl p 5 BMC + Rapa + CTLA4Ig). Depletion was confirmed by FCM (Fig. E6). Treg depletion did not prevent development of B-cell tolerance, since again no Phl p 5-specific IgE was detectable just as without CD25 Ab treatment (Fig. 6A). Likewise CD25 depletion in already tolerant mice and subsequent immunization did not break B cell tolerance (data not shown). Additionally we determined if also Th1 dependent isotypes were detectable after CD25 depletion. Similarly, Phl p 5-specific IgG_{2a} was not detectable (Fig. E8). Anti-IL2 was described to reduce Treg numbers in different models, presumably through IL2

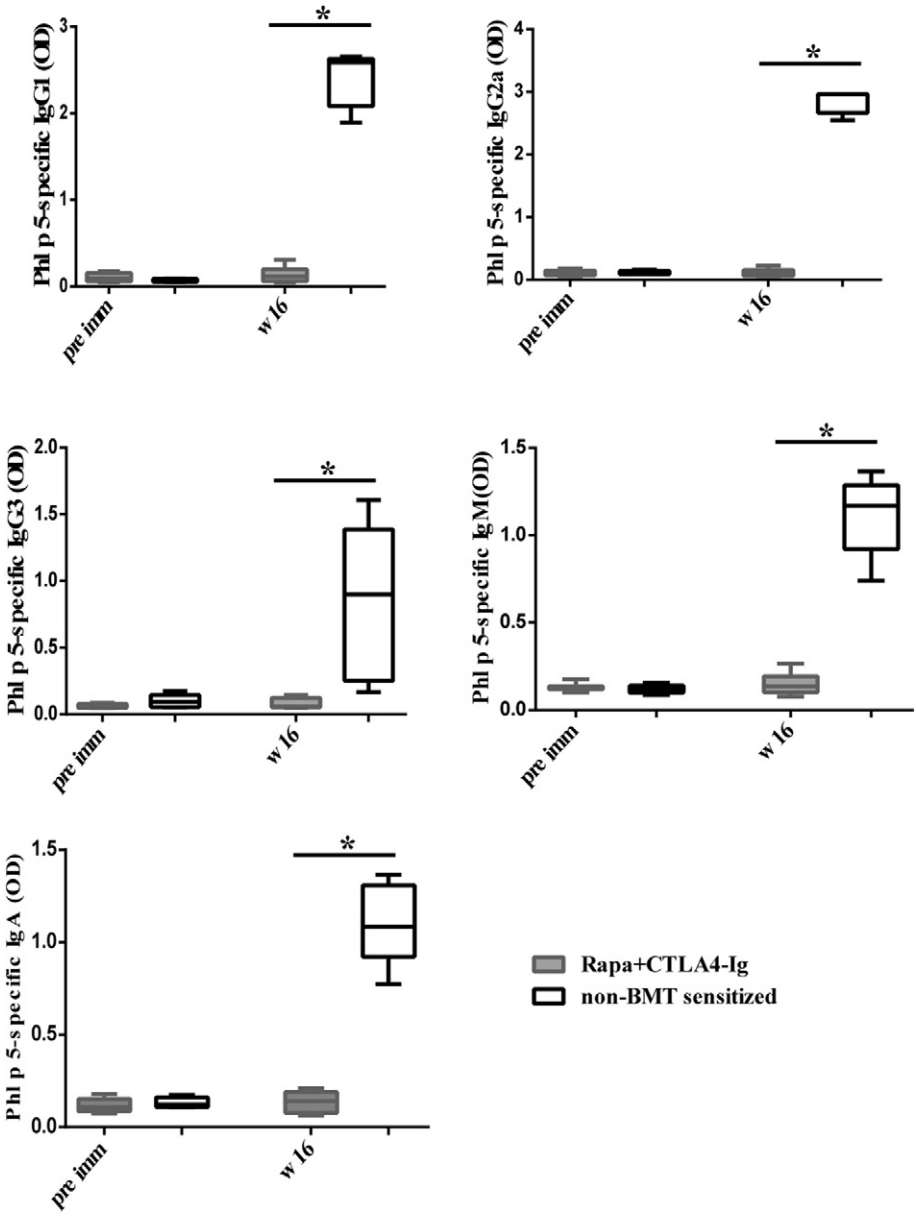

Fig. 3. CTLA4Ig/rapa cell therapy leads to B cell tolerance in several isotypes. Phl p 5-specific IgG_1, IgG_{2a}, IgG_3, IgM and IgA levels in sera of mice treated with 15×10^6 mPhl p 5 BMC, rapamycin and CTLA4-Ig (Rapa + CTLA4-Ig, n = 8) and non-transplanted immunized mice (non-BMT sensitized) n = 5 at week 16 after BMT. Results are demonstrated as box blots and medians are shown. * $p < 0.05$.

starvation, but more recently it was also shown to boost $CD8^+$ cells through stimulation of the low-affinity IL2 receptor (Bigenzahn et al., 2005; Phelan et al., 2008). We treated mice before and after BMT with anti-IL2 mAbs. Again, we could not break B cell tolerance in this setting (Fig. E7).

The co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA4) is critically involved in the suppressive function of Tregs, presumably by down-regulating CD80 and CD86 expression on antigen-presenting cells (Sakaguchi et al., 2008). Further, autoreactive B cells have to express PD-1 to be suppressed by Tregs (Gotot et al., 2012). Additionally Tregs themselves express PD-1 (Francisco et al., 2010; Sage et al., 2013). Neither a PD-1 (w 21) blocking antibody nor anti-CTLA4 blocking antibody (w 21) broke tolerance (Fig. 6B). Taken together our data suggest that Tregs are not critically required for B cell tolerance induced through the cell-based tolerance protocol.

4. Discussion

In this murine proof-of-concept study we present a cell therapeutic approach for prophylaxis of IgE-mediated allergy. Robust allergen-specific tolerance towards a clinically relevant aero-allergen is induced permanently by treatment with allergen-bearing bone marrow cells and short-course immunosuppression. The cell-based prophylactic tolerance regimen is uniquely potent in completely preventing an allergen-specific T and B cell response, including all tested isotypes and does not require irradiation any more. Thus the described protocol may represent a significant step forward towards the clinical application of cell-based prevention for allergy. Since we did not obtain evidence that tTregs or pTregs are involved in the establishment of tolerance we assume that deletion through possibly central tolerance induction is the main mechanism in our model.

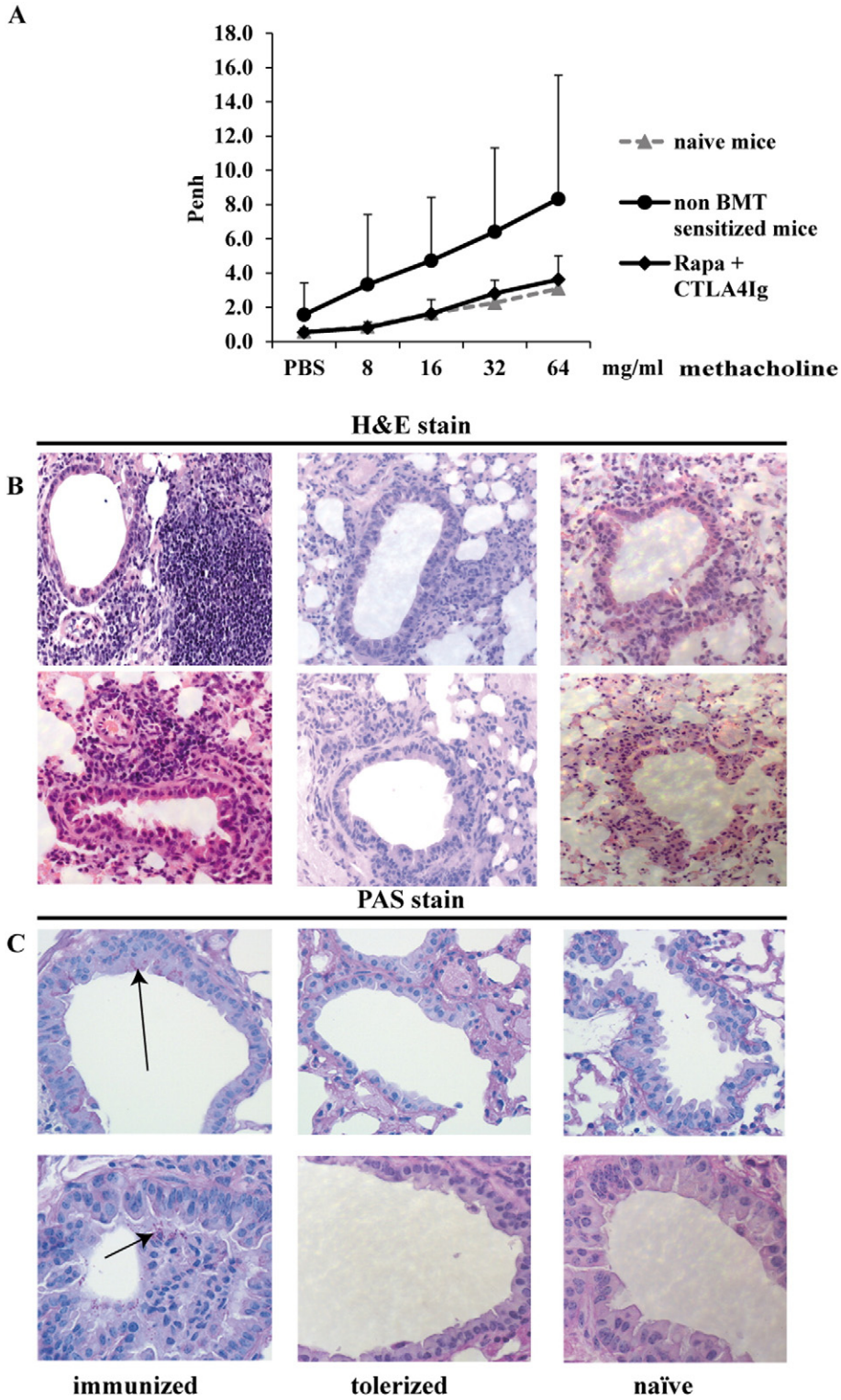

Fig. 4. CTLA4Ig/rapa cell therapy prevents allergic airway inflammation. (A) Penh (enhanced pause) after different concentrations of methacholine in WBP (whole body plethysmography) is shown in mice treated with mPhl p 5 BMCs, rapamycin and CTLA4Ig (n = 5). Non-BM transplanted immunized mice (n = 5) (non-BMT sensitized mice) and naïve mice (n = 4) were used as control. (B + C) Lung sections of mice described in (A) stained with H&E or PAS staining for mucus production. Two representative sections are shown for each staining and group. Pooled data of 2 independent experiments are demonstrated.

The prophylactic tolerance protocol entails the administration of immunosuppressive drugs. Rapamycin is an mTOR inhibitor approved for immunosuppression in organ transplantation (and is commonly used in children (Ganschow et al., 2013)) and has potent tolerogenic properties (Fischer et al., 2009; Battaglia et al., 2005). CTLA4Ig (abatacept) and its derivative belatacept are approved for use in rheumatoid arthritis and organ transplantation, respectively (Bonelli et al., 2013; Wekerle and Grinyo, 2012), and have tolerogenic effects as well. However, neither mTOR inhibition, nor CTLA4Ig are effective in establishing robust tolerance on their own, (Linhart et al., 2007; Pilat et al., 2011). The toxicity of

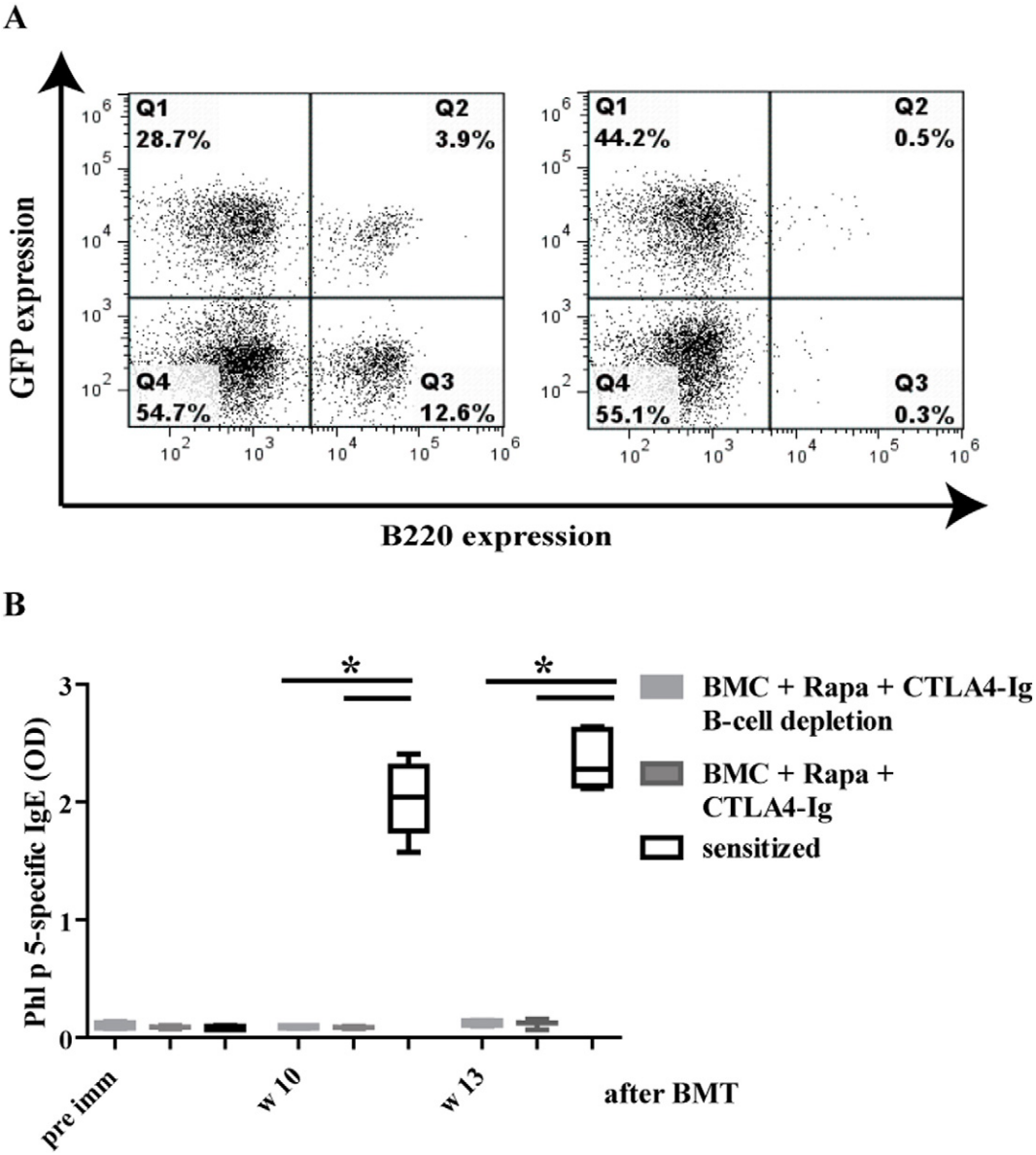

Fig. 5. B cells are not required for tolerance induction through CTLA4Ig/rapa cell therapy. Mice received 15×10^6 mPhl p 5 BMC plus rapamycin and CTLA4-Ig. (A) Dot blots of peripheral blood of one representative mouse without B cell depletion (left panel) and a B cell depleted mouse (right panel). GFP expression of donor cells is demonstrated (B) Phl p 5-specific IgE in sera of mice treated with anti-CD20 mAbs (d −7, 0, +7) (n = 4). Control mice were treated in the same manner without B-cell depletion or untreated but sensitized. Results are demonstrated as box blots and medians are shown. * $p < 0.05$.

these immunosuppressive drugs is expected to be low when given for a short-course treatment in the period of allergic sensitization (Westman et al., 2015). Specifically, the combination of rapamycin and belatacept has been tested in renal transplantation and was found to be safe (Ferguson et al., 2011). No anti-CD40L mAb is clinically available yet, but next generation anti-CD40L domain antibodies avoiding the thromboembolic side effects observed with conventional anti-CD40L mAbs are under pre-clinical development (Suchard et al., 2013; Xie et al., 2014), and anti-CD40 mAbs could soon offer an alternative as well (Goldwater et al., 2013). The presented cell therapy regimen does not require any cytotoxic or myelosuppressive preconditioning which has been necessary for BM engraftment in various previous BMT regimens (Baranyi et al., 2008; Baranyi et al., 2012), further limiting potential safety risks.

While cells from a transgenic mouse were used in the present experiments, expression of allergens on the surface of autologous cells might be feasible through various means. Retroviral transduction is currently insufficiently safe for non-vital indications (Hacein-Bey-Abina et al., 2010), but site-specific integration of genes into HSC of cord blood became recently possible and might eventually provide a viable option (Genovese et al., 2014). Chemical coupling of allergens might provide an attractive alternative (Jenkins and Schwartz, 2009; Getts et al., 2011). While not directly tested in the present study, antigen expression is likely required for a limited period only, as transient chimerism is effective in permanently tolerizing an alloresponse in experimental models (Tian et al., 2002; Tian et al., 2006) and the clinical setting (Kawai et al., 2008). The use of hypoallergenic allergen derivatives (Valenta et al., 2010), or allergen-derived peptides containing the relevant T cell epitopes (Focke-Tejkl et al., 2014), might further enhance the safety aspects of this approach, avoiding the risk of triggering anaphylaxis. Mobilized peripheral blood stem cells have been successfully used instead of BM cells in the setting of transplantation tolerance (Koporc et al., 2008; Scandling et al., 2008), and would be a clinically more acceptable cell source as they can be obtained non-invasively.

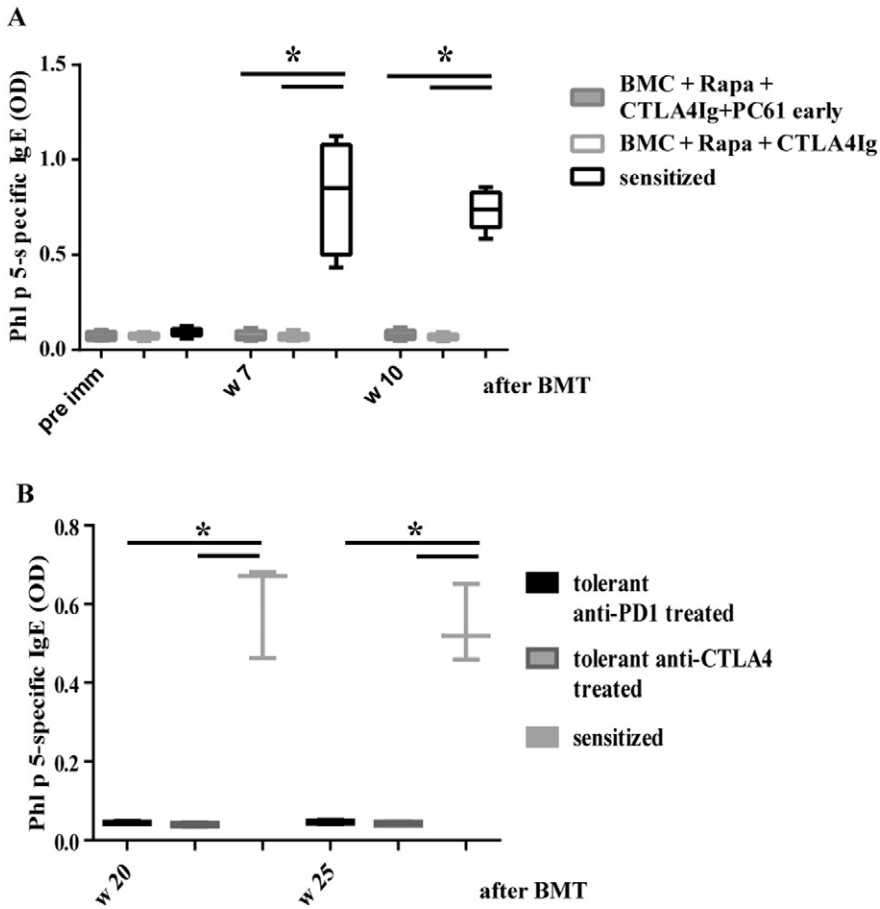

Fig. 6. Regulation is not a critical mechanism for tolerance induction and maintenance through CTLA4Ig/rapa cell therapy. (A) Half of recipients (BMC + Rapa + CTLA4-Ig) (n = 8) were treated with anti-CD25 mAb (PC61) before BMT (BMT + Rapa + CTLA4-Ig PC61 early) (n = 4) or left untreated (sensitized) (n = 5). Phl p 5-specific IgE is demonstrated after 1st and 2nd immunization (B) Phl p 5-specific IgE levels before (w20) and after treatment with anti PD1-mAb (tolerant anti-PD1 treated n = 4) or anti CTLA4 mAb (tolerant anti-CTLA4 treated) (n = 4) (w25) or sensitized only (n = 3). Results are demonstrated as box blots and medians are shown. * $p < 0.05$.

Eventually, cord-blood could serve as the ideal hematopoietic cells source (Pineault and Abu-Khader, 2015).

In conclusion, the results presented herein provide evidence that allergen-specific tolerance can be established prophylactically through cell therapy with allergen-expressing BM cells under tolerogenic immunosuppression without irradiation. They also offer a long-term vision of how step-wise clinical translation might eventually be realized to prevent IgE-mediated allergy by cell-based approaches.

Authors' Contributions

U.B., R.V. and T.W. performed experimental design and conception, interpreted data and wrote manuscript. U.B., A.F., K.H., B.M., and M.G., performed research, M. F.-T and A.P. contributed unique reagents, and T.R. constructed transgenic mouse.

Disclosure

The authors declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.03.028.

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Title: **Cell therapy for prophylactic tolerance in Immunglobulin E-mediated allergy**

Online Repository Materials

Additional Online Repository Materials include:

Figure E1: Expression of Phl p 5 in mPhl p 5 splenocytes

Figure E2: Transgenic Phl p 5-specific IgE recognizes only conformational epitopes comparable to IgE of Phl p 5-allergic patients

Figure E3: Timeline of the tolerogenic protocol and chimerism

Figure E4: Bet v 1-specific isotypes in Phl p 5-tolerized recipients

Figure E5: Combination of BMCs with CTLA4Ig and rapamycin is necessary to induce long-term tolerance

Figure E6: Depletion of CD4+CD25+ cells after treatment with PC61

Figure E7: Anti-IL-2 does not inhibit tolerance induction before and after BMT

Figure E8: Depletion of Tregs before BMT does not inhibit tolerance in Th1-mediated isotype

Figure E1

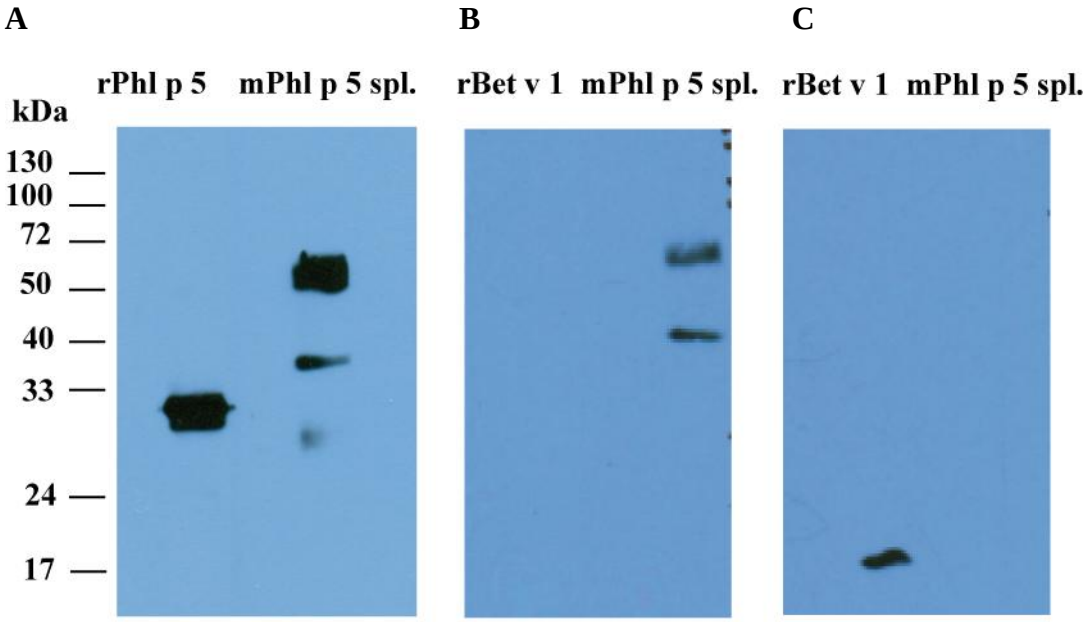

Fig. E1: Expression of mPhl p 5 in splenocytes (spl.) of mPhl p 5 transgenic mice demonstrated in immunoblot. rPhl p 5 and mPhl p 5 splenocytes or rBet v 1 and mPhl p 5 splenocytes were separated in SDS PAGE and blotted onto nylon membrane, incubated with rabbit anti-Phl p 5 antibodies (A and B) or with rabbit anti-Bet v 1 antibodies (C) and HRP-labeled anti-rabbit secondary antibodies.

Figure E2

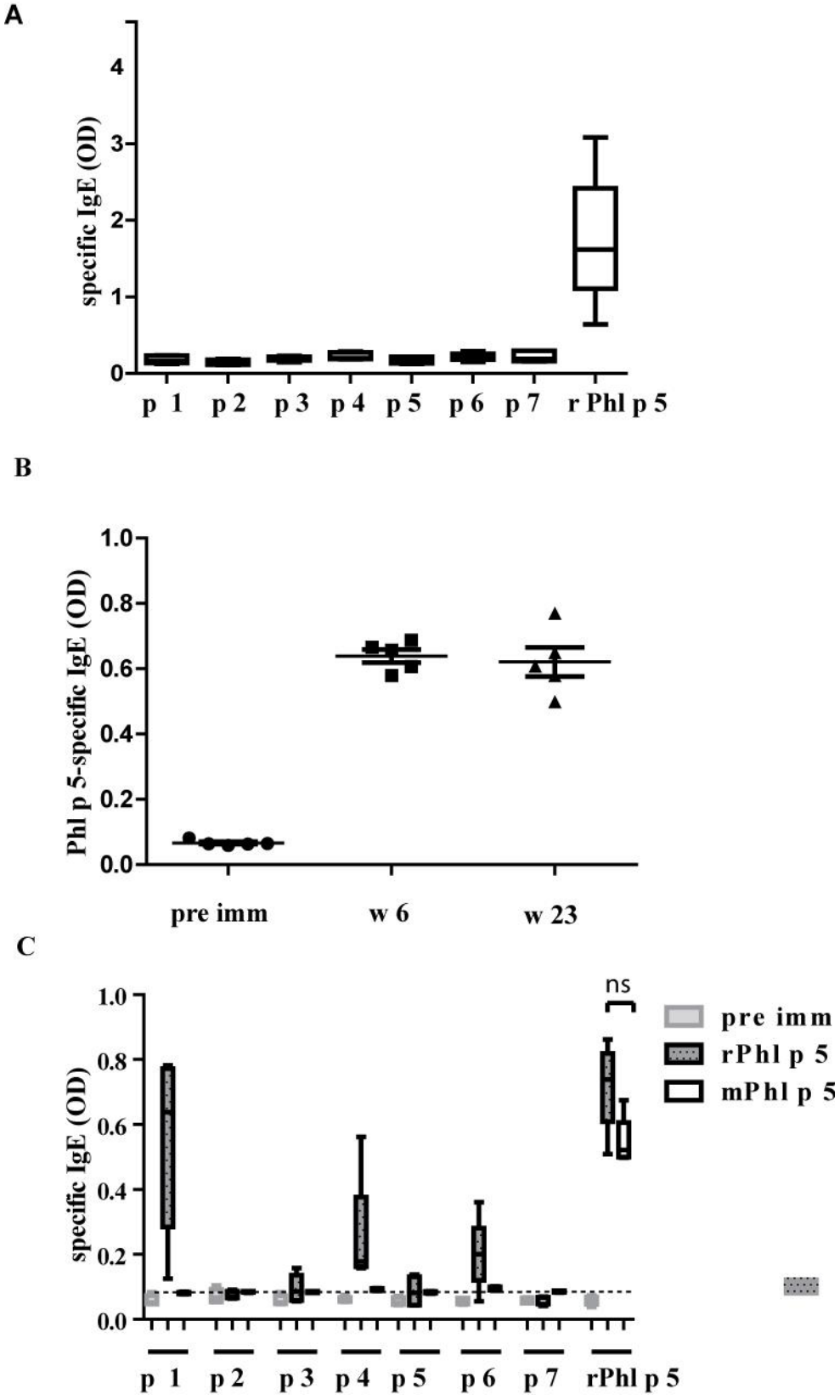
A
specific IgE (OD)
0
1
2
3
4
p 1
p 2
p 3
p 4
p 5
p 6
p 7
r Phl p 5
B
Phl p 5-specific IgE (OD)
0.0
0.2
0.4
0.6
0.8
1.0
pre imm
w 6
w 23
C
specific IgE (OD)
0.0
0.2
0.4
0.6
0.8
1.0
ns
pre imm
rPhl p 5
mPhl p 5
p 1
p 2
p 3
p 4
p 5
p 6
p 7
rPhl p 5

Fig. E2. Phl p 5-specific IgE upon immunization with mPhl p 5 splenecytes resembles Phl p 5 recognition of sera of allergic individuals. (A) IgE in sera of Phl p 5-allergic patients (n=5) towards linear peptides (p1-7) and whole rPhl p 5 determined in ELISA. (B) Phl p 5 IgE levels in sera of mPhl p 5 immunized BALB/c mice (n=5). (C) Phl p 5-peptide specific IgE (p1-p7) was determined in sera of mice immunized with r Phl p 5+alum (rPhl p 5, n=5) or m Phl p 5 splenocytes (mPhl p 5, n=5). Pre-immune sera served as baseline control (n=5) (pre imm). Results are demonstrated as box or scatter plots with median and standard deviation

Figure E3

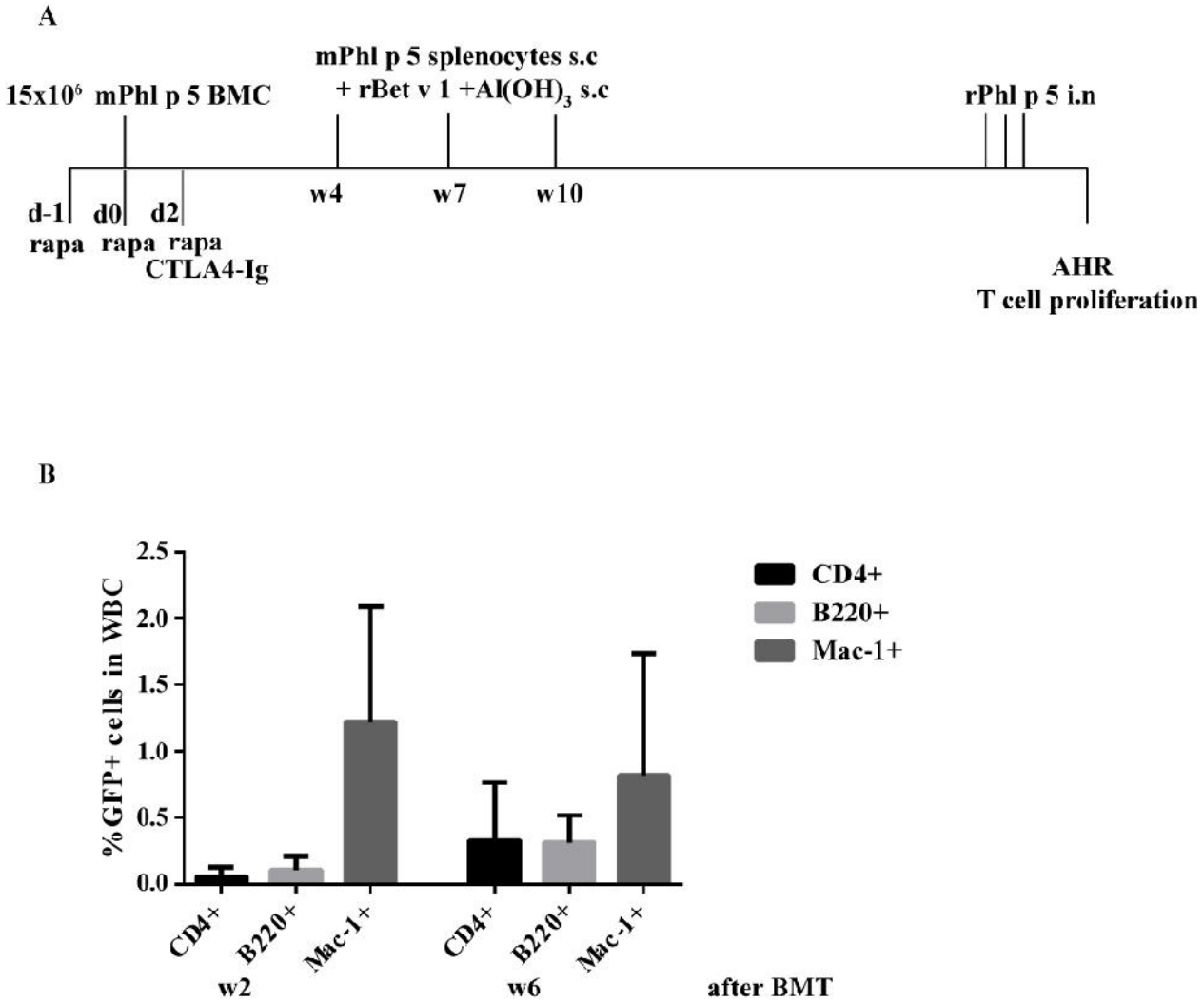

Fig. E3: (A) Timeline demonstrating the tolerogenic protocol. Scheme is not to scale. (B) Chimerism levels (i.e. percentage of CD4+, B220+ and Mac-1+ and GFP double positive cells within blood cells) are demonstrated 2 and 6 weeks (w) after BMT. GFP and lineage marker positive cells percentages are shown as mean and error bars are indicated (n=4).

Figure E4

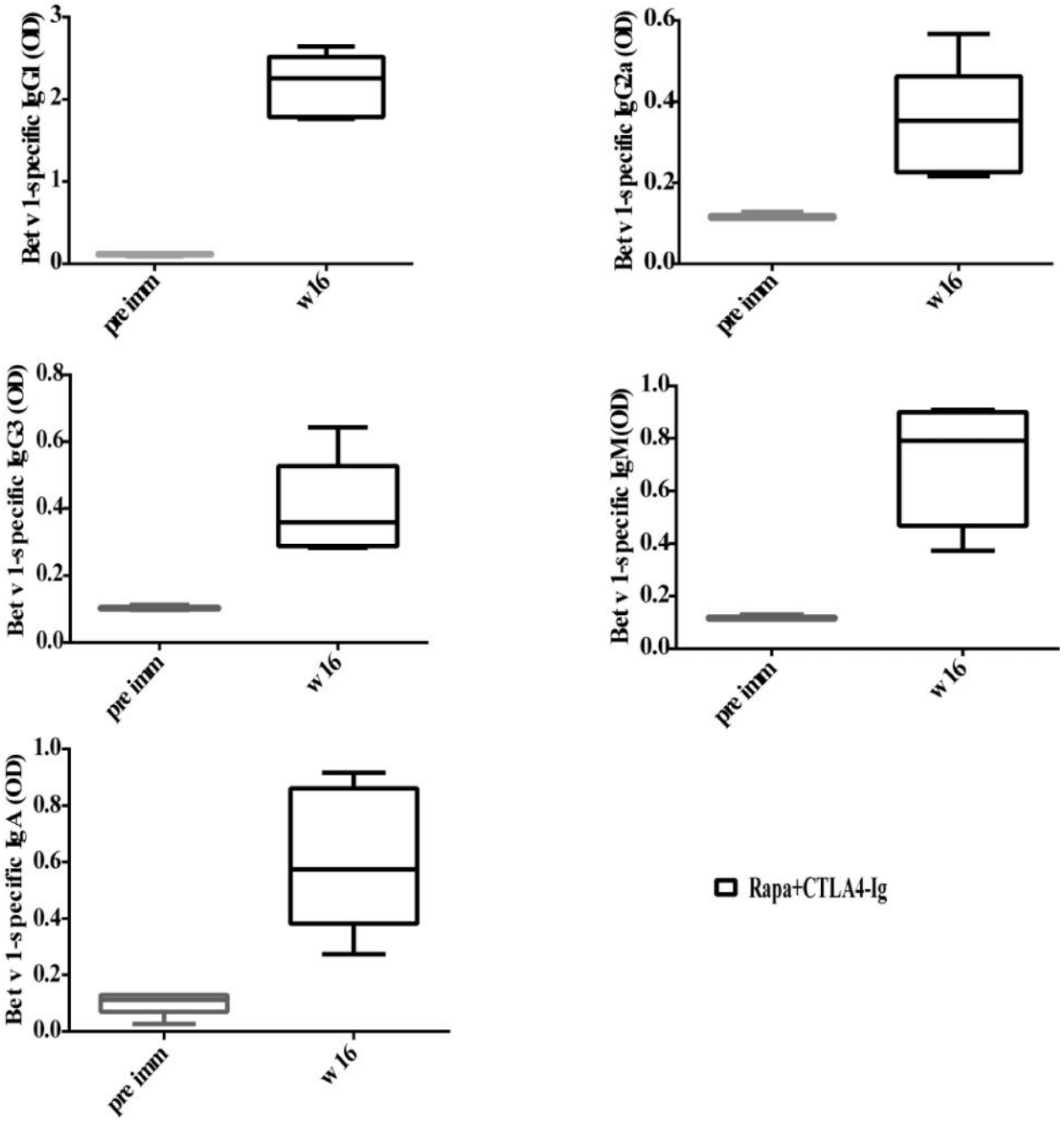

Fig. E4: Bet v 1-specific isotypes in Phl p 5-tolerized recipients. Bet v 1-specific antibody levels are demonstrated at week (w) 16 after BMT and compared to preimmune sera. (n=5). Antibody levels are demonstrated as box blots and median are shown.

Figure E5

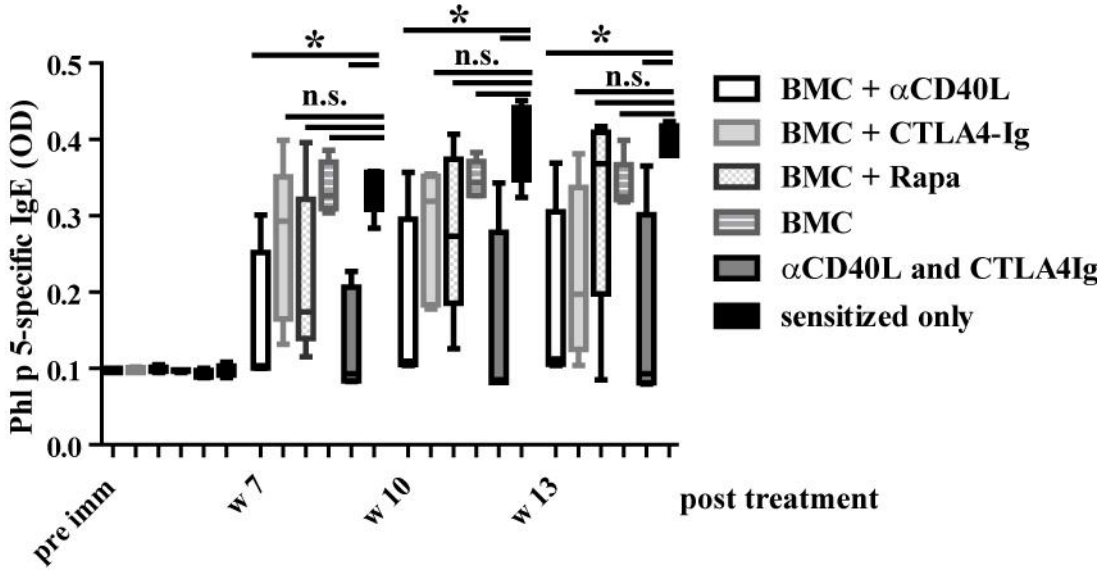

Fig. E5: Combination of BMCs with CTLA4Ig or rapamycin alone does not effectively prevent Phl p 5-specific IgE development. Phl p 5-specific IgE levels determined in ELISA in mice receiving $15x10^6$ m Phl p 5 BMC and anti-CD40L (BMC + αCD40L), $15x10^6$ mPhl p 5 BMC and CTLA4-Ig (BMC + CTLA4-Ig), $15x10^6$ mPhl p 5 BMC and rapamycin (BMC + Rapa), $15x10^6$ mPhl p 5 BMC (BMC), anti-CD40L and CTLA4-Ig, or non-treated immunized mice (sensitized only) (n=5 each group). Results are demonstrated as box blots and medians are shown. * $p< 0.05$. n.s-not significant.

Figure E6

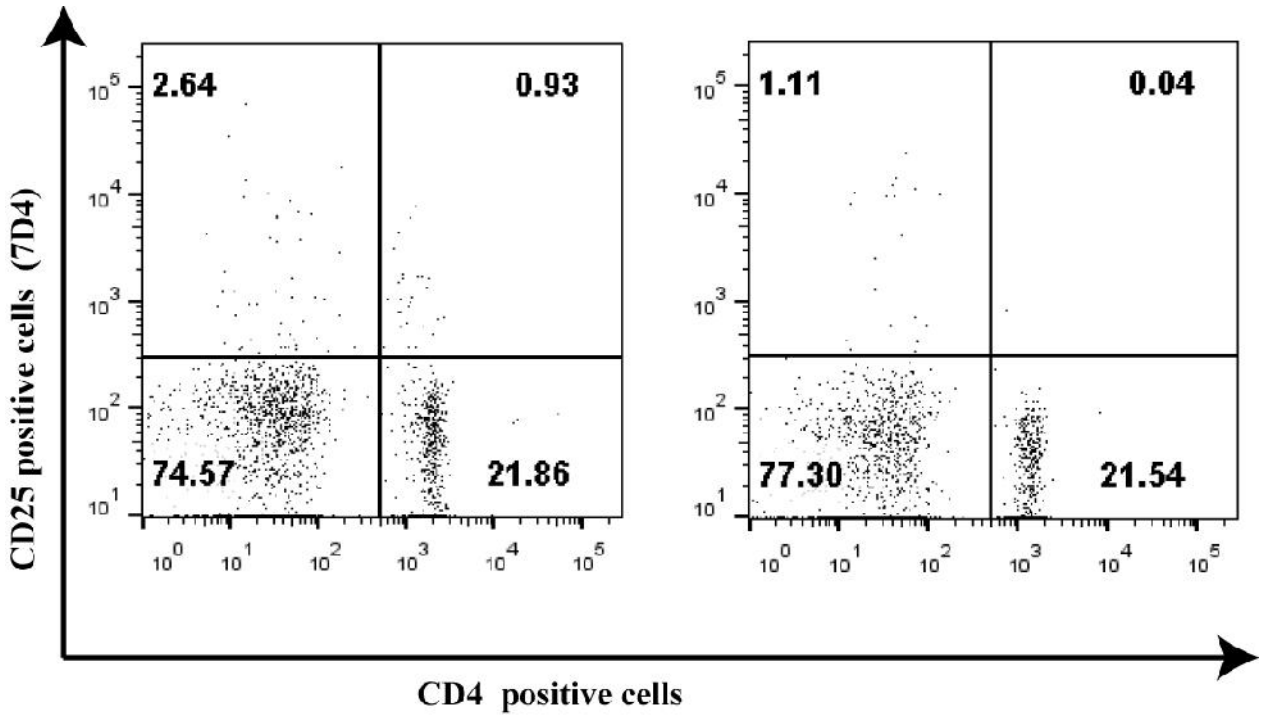

Fig. E6: Confirmation of Treg depletion in FCM. Dot blots of peripheral blood of non PC61-depleted recipient (left panel) and PC61 treated recipients (right panel). Blots show one representative of each group (n=4).

Figure E7

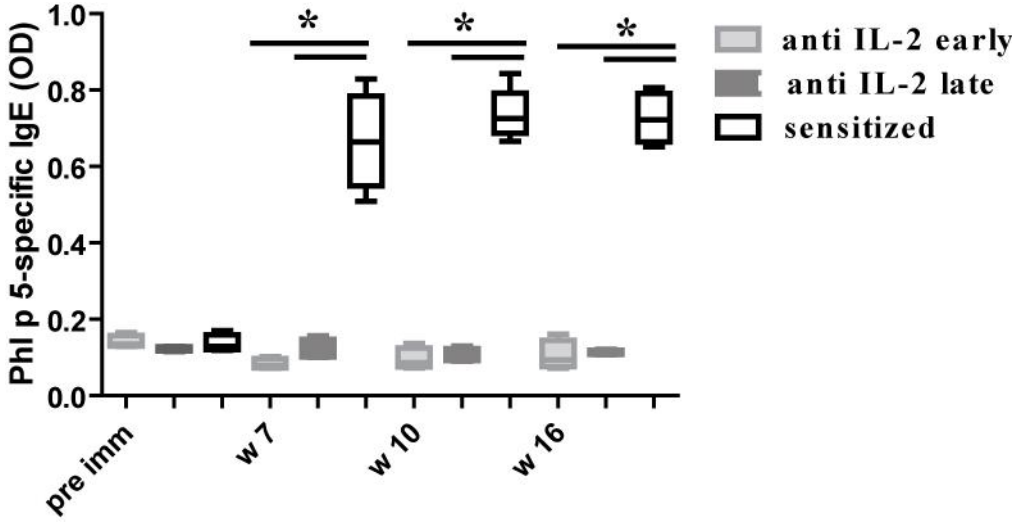

Fig. E7: Treatment of recipients with anti-IL2 antibody does not break B-cell tolerance. Recipients were treated with anti-IL-2 mAb before (n=5) or after BMT (n=5). Results are demonstrated as box blots and median are shown. * $p < 0.05$. n.s-not significant.

Figure E8

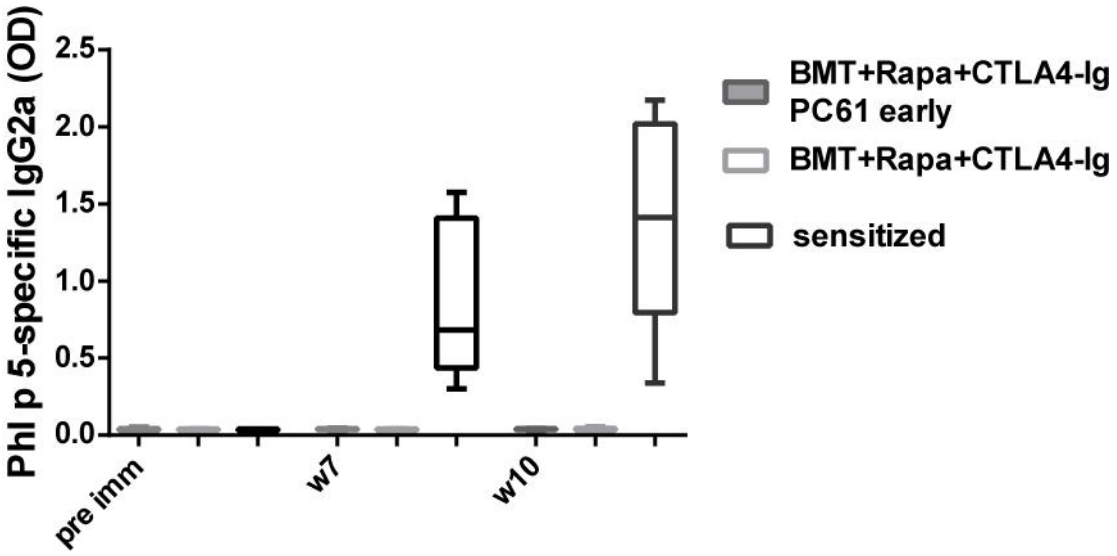

Fig. E8: Phl p 5-specific Th1 isotypes are not induced after depletion of Tregs. Phl p 5-specific IgG2a levels. Recipients (BMT + Rapa+ CTLA4Ig) were treated with anti-CD25 mAb (PC61) before BMT (BMT+Rapa+CTLA4-Ig PC61 early) (n=8) or untreated (sensitized) (n=5)