IL-10-modulated dendritic cells from birch pollen- and hazelnut-allergic patients facilitate Treg-mediated allergen-specific and cross-reactive tolerance

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Abstract

**Background** Approximately 70 % of individuals allergic to birch pollen (Bet v 1.01 [Bet]) develop a secondary food allergy (e.g. hazelnut: Cor a 1.04 [Cor]), due to allergen cross-reactivity. However, standard immunotherapy for type I allergies often does not improve the food allergy sufficiently. We analyzed the allergen-specific and cross-reactive suppressive capacity of primary human regulatory T cells (Treg) induced by autologous IL-10-modulated dendritic cells (IL-10 DC) in vitro and in vivo. **Methods** CD4⁺ T cells of patients with birch pollen and associated hazelnut allergies were differentiated into Bet-specific or non-specific induced Treg (iTreg). After Bet- or Cor-specific restimulation the phenotype, proliferation and suppressive capacity of iTreg subsets were analyzed. iTreg function was further investigated in humanized mouse models of airway and intestinal allergy, generated by engraftment of peripheral blood mononuclear cells from allergic donors into immunodeficient animals. **Results** After IL-10 DC priming and allergen-specific restimulation (Bet or Cor) non-specific control iTreg remained anergic, whereas Bet-specific iTreg proliferated extensively and exhibited a regulatory phenotype (enhanced expression of CTLA-4, PD-1, TNFR2, IL-10). Accordingly, activated Bet-specific iTreg displayed a high capacity to suppress Bet- and Cor-induced responder T helper 2 cell responses in vitro, indicating induction of both allergen-specific (birch) and cross-reactive tolerance (hazelnut). In vivo, the beneficial effect of Bet-specific iTreg was verified in humanized mouse models of allergic airway and intestinal inflammation, resulting in reduced allergen-induced clinical symptoms and immune responses. **Conclusion** Human IL-10 DC-induced iTreg facilitate allergen-specific and cross-reactive tolerance. Therefore, they are potential candidates for regulatory cell therapy in allergic and autoimmune diseases.

Title Page

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Short title: Treg induced allergen specific- and cross-tolerance

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Methods

CD4+ T cells of patients with birch pollen and associated hazelnut allergies were differentiated into Bet-specific or non-specific induced Treg (iTreg). After Bet- or Cor-specific restimulation the phenotype, proliferation and suppressive capacity of iTreg subsets were analyzed. iTreg function was further investigated in humanized mouse models of airway and intestinal allergy, generated by engraftment of peripheral blood mononuclear cells from allergic donors into immunodeficient animals.

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After IL-10 DC priming and allergen-specific restimulation (Bet or Cor) non-specific control iTreg remained anergic, whereas Bet-specific iTreg proliferated extensively and exhibited a regulatory phenotype (enhanced expression of CTLA-4, PD-1, TNFR2, IL-10). Accordingly, activated Bet-specific iTreg displayed a high capacity to suppress Bet- and Cor-induced responder Th2 cell responses in vitro, indicating induction of both allergen-specific (birch) and cross-reactive tolerance (hazelnut). In vivo, the beneficial effect of Bet-specific iTreg was verified in humanized mouse models of allergic airway and intestinal inflammation, resulting in reduced allergen-induced clinical symptoms and immune responses.

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Human IL-10 DC-induced iTreg facilitate allergen-specific and cross-reactive tolerance. Therefore, they are potential candidates for regulatory cell therapy in allergic and autoimmune diseases.

Key words

- type I allergy
- Bet v 1.01
- tolerogenic IL-10 DC
- regulatory T cells
- cross-reactive tolerance

Abbreviations
Introduction

Allergic diseases are becoming the focus of attention, as their incidences as well as severity are increasing worldwide. Among the responsible allergens, birch pollen were identified as the third most-diagnosed allergen for respiratory allergy, and around 70% of patients allergic to birch also develop a secondary food allergy. The symptoms of this so-called pollen-associated food allergy (PFA) range from nasal, ocular and oral pruritus and angioedema to severe allergic asthma and anaphylaxis, which has a comprehensive impact on personal quality of life, work or school performance and the socio-economic burden.

In the pathogenesis of PFA, patients are first sensitized towards a pollen allergen, such as Bet v 1 (Bet), a birch pollen allergen belonging to the pathogenesis-related (PR)-10 protein family. PR-10 proteins share a high degree of amino acid sequence identity as well as structural homology and facilitate cross-reactivity of pollen-specific IgE antibodies and T cells with food allergens, which initiates the development of secondary food allergies. PR-10 proteins are found in different plant pollen and in edible plant parts like hazelnut (Cor a 1, Cor), apple (Mal d 1) and carrot (Dau c 1). Interestingly, the hazelnut allergen isoform Cor a 1.04 is more similar to the birch pollen isoallergen Bet v 1.01 than to the hazel pollen isoallergen Cor a 1.01.

In contrast to the ongoing spread of allergic diseases due to cross-reactivities, the selection of efficient and long-lasting therapies that improve pollen and cross-reactive food allergies together are sparse. The only disease-modifying therapy available is allergen-specific immunotherapy (AIT), which might achieve a 20-50% reduction of combined symptom and medication scores. But several years of therapy are needed to maintain the therapeutic success and placebo-controlled studies showed that pollen AIT have little to no clinical effect on the associated food allergy. Therefore, the current state of the art regarding treatment of PFA remains unsatisfying and novel therapeutic strategies have to be explored.

Autologous tolerogenic dendritic cells (tolDC) are promising candidates for cell-based immunomodulatory therapies, as they can be differentiated from peripheral blood cells of patients ex vivo based on standardized protocols. After loading with the desired antigen, they are reintroduced into the patients, where they are generally well tolerated without any severe side effects. In our group, we found that Interleukin (IL)-10-modulated DC (IL-10 DC) are exceptionally suited for tolerance-inducing therapies, as they displayed a tolerogenic phenotype, with strong migratory and suppressive functions. Induced Treg (iTreg) primed by these IL-10 DC exhibited a suppressive phenotype resulting in highly efficient antigen-specific regulatory functions. In this study, we combined our expertise in IL-10 DC and iTreg biology demonstrating the capacity of IL-10 DC as inducers of iTreg which facilitated allergen-specific and cross-reactive tolerance in birch pollen allergic patients with associated hazelnut allergy in vitro and in a humanized mouse model of allergy in vivo.

Methods

All experiments with human samples were conducted according to approval of the local ethics committee of Rhineland Palatinate (authorization no. 837.303.13 and 837.054.17 (10888)) and written informed consent was obtained from all participants. Murine experiments were in accordance with current federal, state, and
Methods are described in brief, for further details see Supplementary Methods.

**Induction of iTreg and effector T cells (Teff)**

Immature dendritic cells (DC) from donors with birch pollen and hazelnut allergies were generated by culture of plastic-adherent peripheral blood mononuclear cells (PBMC) with IL-4 and GM-CSF for 6 days and were then differentiated into mature DC (mDC) by addition of a maturation cocktail or into IL-10 DC by addition of the maturation cocktail and IL-10 (Supplementary Figure 1). Bet-loaded mDC (mDCBet) or IL-10 DC (IL-10 DCBet) were generated by addition of rBet v 1.0101 during DC differentiation. Autologous CD4\(^+\) T cells were cocultured with mDC or IL-10 DC to prime effector T cells (Teff) or iTreg, respectively.

**Suppressor assay (SA)**

CD4\(^+\)CD25\(^{\text{low}}\) responder T cells (Tresp) from donors with birch pollen and associated hazelnut allergy were stimulated with autologous Bet- or Cor- loaded mDC (control, ctrl). iTreg primed by IL-10 DCBet (iTregBet) or IL-10 DC0 (iTreg0) were added in 1:1 or 1:2 Tresp:iTreg ratios (SA 1:1 / SA 1:2), respectively. Tresp, mDC and iTreg were stained with different cell proliferation dyes for identification and assessment of T cell proliferation.

**Humanized mouse model of type I allergy**

As described previously\(^{40,41}\), NOD.CB17-Prkdc\(^{scid}\)/J\(^{Ye^-}\) mice were engrafted with PBMC from donors with birch pollen and associated hazelnut allergy and were simultaneously injected with birch pollen extract (=PBMC + birch, allergic positive control) or without (=PBMC, negative control). iTregBet were co-injected at day 0 into animals treated like the allergic positive control. After three weeks, blood samples for human IgE analysis were taken, the mice were challenged rectally with birch pollen or hazelnut extract and the allergen-induced intestinal inflammation was scored endoscopically. On the next day the mice were challenged intranasally with birch pollen extract and allergen-induced airway inflammation was evaluated by measurement of methacholine (MCh)-provoked airway resistance.

**Results**

**Induction of Bet-specific iTreg from birch-pollen allergic patients with associated hazelnut allergy by autologous IL-10 DC**

As previously demonstrated by us, human IL-10 DC induce iTreg with a high capacity to suppress T cell responses in an antigen-specific manner.\(^{35,38,42,43}\) In this study, we wanted to investigate whether these tolerogenic IL-10 DC are able to induce an allergen-specific as well as cross-reactive tolerance in patients suffering from a pollen (birch, Bet v 1) and associated food (hazelnut, Cor a 1) allergy (for patients’ details see Supplementary Table 1). For this purpose, we used our previously established *in vitro* model with human DC to analyze allergen-specific and cross-reactive T cell responses.\(^{12}\)

IL-10 DC obtained from birch pollen allergic patients with associated hazelnut allergy were loaded with Bet or left unloaded (IL-10 DC\(_{\text{Bet}}\)/IL-10 DC\(_{0}\)) and cocultured with autologous CD4\(^+\) T cells to induce Bet-specific or control iTreg, respectively (iTregBet/iTreg0). In addition, mDC (mDC\(_{\text{Bet}}\)/mDC\(_{0}\)) were used to generate Bet-specific and non-specific Teff (Teff\(_{\text{Bet}}\)/Teff\(_{0}\)) as controls (see Methods, Supplementary Methods and Supplementary Figure 1).

After primary culture, Teff\(_{\text{Bet}}\) exhibited a significantly increased proliferative capacity compared to Teff\(_{0}\), demonstrating an allergen (Bet)-specific T cell response (Figure 1A). In contrast, stimulation with IL-10 DC\(_{0}\) or IL-10DC\(_{\text{Bet}}\) resulted in a significantly reduced T cell proliferation of both iTreg0 and iTregBet.

In line with the data of allergen-specific T cell proliferation, analysis of cytokine production after primary culture revealed significantly increased levels of Th2 cytokines (IL-5, IL-9 and IL-13) and of IL-2 (T cell activation) in supernatants of mDC\(_{\text{Bet}}\) activated Teff\(_{\text{Bet}}\) compared to control Teff\(_{0}\), demonstrating highly stimulated Bet-specific Teff (Figure 1B-E). In contrast, supernatants of iTreg primed by IL-10 DC\(_{0}\) or IL-10
DC_{Bet}, respectively, exhibited reduced amounts of T\textsubscript{H}2 cytokines in comparison to Bet-specific control Teff, confirming the diminished activity of iTreg after primary culture. However, we observed very high IL-10 concentrations produced by iTreg\textsubscript{Bet} compared to control Teff\textsubscript{Bet}/Teff\textsubscript{0} as well as to iTreg\textsubscript{0}, suggesting the immunosuppressive cytokine IL-10 as mediator of allergen-specific iTreg suppressor function (Figure 1F).

**Bet-specific iTreg induced by IL-10 DC exhibited an activated phenotype after Bet-and Cor-specific restimulation**

Several studies revealed an anergic phenotype of CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+} natural Treg and iTreg, in particular of iTreg induced by IL-10 DC.\textsuperscript{35,38,42,44} In order to prove the allergen-specific and cross-reactive induction of T cell anergy in our system, we have performed restimulation experiments with autologous mDC\textsubscript{0}, mDC\textsubscript{Bet} or mDC\textsubscript{Cor}, respectively (see Supplementary Methods). Compared to control T cells (Teff\textsubscript{0}), restimulation of Teff\textsubscript{Bet} with mDC\textsubscript{Bet} led to a significantly increased and in the case of mDC\textsubscript{Cor} slightly enhanced Teff proliferation, demonstrating the induction of both a Bet-specific and cross-reactive, Cor-specific T cell response (Figure 2A). However, also restimulation of iTreg\textsubscript{Bet} with mDC\textsubscript{Bet} or mDC\textsubscript{Cor} resulted in a very pronounced and significantly enhanced T cell proliferation, compared to Teff\textsubscript{0}. iTreg\textsubscript{Bet} proliferated even stronger compared to Teff\textsubscript{Bet}, indicating that allergen-specific iTreg did not display an anergic but activated phenotype after specific (Bet) or cross (Cor)-specific restimulation (Figure 2A). In contrast to iTreg\textsubscript{Bet}, iTreg\textsubscript{0} exhibited a significantly lower proliferation and remained anergic regardless of the allergen-specific or cross-specific restimulation with mDC\textsubscript{Bet} or mDC\textsubscript{Cor} (Figure 2B).

**Profound capacity of Bet-specific iTreg to suppress allergen-specific and cross-reactive T cell responses in vitro**

In order to analyze the function of iTreg with regard to their suppressive capacity on allergen-specific and cross-reactive responses, we investigated the suppressive capacity of iTreg\textsubscript{Bet} and iTreg\textsubscript{0} on Bet- or Cor-stimulated responder T cells (Tresp) obtained from birch-pollen allergic patients with associated hazelnut allergy in a flow cytometry-based suppressor assay.\textsuperscript{35} (see Methods and Supplementary Methods; gating strategy in Supplementary Figure 2).

Coculture with iTreg\textsubscript{Bet} resulted in a significantly reduced proliferation of allergen (Bet)-specific, and cross-reactive, Cor-specific Tresp, respectively, indicating the induction of allergen-specific (birch) and cross-reactive (hazelnut) tolerance (Figure 3A). Further analysis revealed that higher numbers of iTreg (ratio 1:2) led to a significantly more impaired Tresp proliferation regardless of the allergen-specificity, demonstrating a dose-dependency of the iTreg suppressive capacity (Figure 3A). We also listed the results from each individual allergic donor (Supplementary Figure 3A).

We further compared the suppressive activity of iTreg\textsubscript{Bet} with non-specific iTreg\textsubscript{0}, on antigen-specific and cross-reactive T cell immune responses (Figure 3B). The experiments revealed a moderately impaired Tresp proliferation after coculture with iTreg\textsubscript{0}. However, iTreg\textsubscript{Bet} exhibited a significantly higher suppressive potential for both Bet- and Cor-specific T cell responses, indicating the strong potential of antigen-specific stimulated iTreg\textsubscript{Bet} as inducers of allergen-specific and cross-reactive tolerance in birch pollen- and hazelnut-allergic patients. Further evidence is depicted in Supplementary Figure 3B, which presents the corresponding data of each individual donor. In 8 out of 10 (80%) experiments with Bet-stimulation, and 6 out of 8 (75%) with Cor-stimulation, iTreg\textsubscript{Bet} showed a stronger capacity to reduce Tresp proliferation compared to iTreg\textsubscript{0}, suggesting their specific and cross-reactive suppressive capacity as superior to non-specific iTreg. Thus, the data demonstrated the high, specific and dose-dependent suppressive capacity of iTreg\textsubscript{Bet} to suppress allergen-specific (birch) as well as cross-reactive (hazelnut) T cell responses, highlighting the importance of allergen-specific iTreg induction.

To support our findings, we harvested the supernatants of suppressor assay samples after Bet- or Cor-specific mDC-stimulation and analyzed the T cell cytokine profile (Figure 3C,D). The presence of iTreg\textsubscript{Bet} – but not non-specific iTreg\textsubscript{0} – resulted in significantly reduced levels of the T\textsubscript{H}2 cytokine IL-13 (Figure 3C) compared to pronounced cytokine levels produced by control allergen-specific (both, Bet or Cor-stimulated) responder T cells. These results strongly confirmed our T cell proliferation data (Figure 3A,B) and indicated
an allergen-specific and cross-reactive downregulation of TH2 immunity mediated by Bet-specific iTreg (iTregBet).

We also found a profound and significant upregulation of the immunosuppressive cytokine IL-10 in suppressor assays with cocultured Bet-specific, but not with non-specific iTreg, when compared to control (Figure 3D). Production of additional TH2 (IL-5, IL-9) and TH1 (IFN-γ, TNF-α) cytokines were mostly unchanged (see Supplementary Figure 4).

In addition to T cell proliferation and cytokine production, we analyzed the phenotype of Tresp after presence or absence of iTregBet in suppressor assays (Supplementary Figure 5A). Coculture of Bet-stimulated Tresp with iTregBet in suppressor assays resulted in a significantly impaired activation (reduction of CD25, HLA-DR) and differentiation (reduced CD45RO / increased CD45RA expression) of Bet-stimulated Tresp. Similar results were observed for Cor-stimulated Tresp (Supplementary Figure 5B), confirming the data of the allergen-specific as well as cross-reactive suppressive capacity of iTregBet on Tresp proliferation and IL-13 production.

iTregBet displayed an activated and suppressive phenotype after allergen-specific and cross-reactive stimulation

In order to identify the phenotype of allergen-specific iTreg in more detail, we performed a flow cytometric analysis of iTregBet, thereby gating on proliferating (activated) and non-proliferating iTregBet in the setting of suppressor assays after Bet- and Cor-specific stimulation (by allergen-loaded mDC) (Figure 4, gating strategy see Supplementary Figure 6). Allergen (Bet or Cor)-specifically restimulated proliferating iTregBet populations exhibited significantly higher percentages of CD45RO+ (enhanced differentiation into a memory phenotype) and activated CD25+ and HLA-DR+ cells compared to Bet- or Cor-specifically stimulated non-proliferating iTregBet or Tresp, respectively (Figure 4A). Compared to non-proliferating iTreg and to Tresp, the proliferating iTregBet population was characterized by a significantly enhanced expression of CTLA-4, TNFR2, PD-1, IL-10 and ICOS, molecules known to be involved in the immunosuppressive capacity of regulatory T cells, confirming the regulatory phenotype of allergen-stimulated iTreg (Figure 4B). These results emphasized the activation state induced by allergen-specific (mDCBet) or cross-reactive (mDCCor) stimulation as prerequisite for iTreg-mediated suppressive activity. For further characterization, the expression of CD49b and LAG3 as parameters for Tr1 differentiation and Treg function were investigated (Figure 4C).

Compared to Tresp, iTregBet showed an increase in CD49b+LAG3+ cells after mDCBet stimulation, although this was not significant under mDCCor stimulation.

Bet-specific iTreg ameliorated allergic symptoms in humanized mouse models of allergen-induced airway and intestinal inflammation

Humanized mice are a remarkable investigative tool and preclinical study system, which close the gap between exclusively murine and human studies.49 Here, we used well-established and standardized humanized mouse models of type I allergy to analyze the function of iTregBet on allergic symptoms in allergen-induced intestinal and airway inflammation.41,50,51 An overview of the reconstitution and challenge protocol is visualized in Figure 5A (see also Methods and Supplementary Methods). Briefly, immunodeficient mice (NOD.CB17-Prkdcscid/Jc−/−) were engrafted with human PBMC from birch pollen allergic donors with associated hazelnut allergy and injected with birch pollen extract +/- iTregBet as indicated.41,52 After allergen-specific (birch) rectal challenge the intestinal inflammation was evaluated by a clinical score, that revealed a significantly increased inflammatory reaction of animals serving as allergic positive controls (PBMC + birch) compared to negative controls (PBMC), thereby demonstrating the validity of the humanized model and the development of an allergic immune reaction (Figure 5B, C). Intriguingly, injection of iTregBet resulted in a significant abrogation of the allergen-specific immune reaction as shown by a reduced intestinal inflammation (Figure 5B, C).

For induction of allergic asthma, the animals were engrafted and boosted as described above and were challenged intranasally with birch pollen extract as published previously.40,53 Subsequently, the airway resistance was assessed as outcome of the allergic immune reaction.40,51 In these experiments, allergic positive controls...
(PBMC + birch) showed a significantly enhanced airway hyperreactivity in contrast to negative control groups (PBMC) (Figure 5D). Importantly, co-injection of iTregBet curtailed the development of allergic asthma symptoms, as the airway resistance was significantly decreased compared to allergic asthma positive control animals (Figure 5D).

We also analyzed the serum concentrations of human birch-specific IgE (Figure 5E) as typical immunological parameter of type I allergic reactions prior to allergen challenge. Compared to enhanced amounts of birch-specific IgE in allergic positive controls (PMBC + birch), treatment with iTregBet significantly reduced birch-specific IgE concentrations in vivo, confirming our data of significantly reduced clinical symptoms of allergen-induced intestinal and airway inflammation after iTregBet application.

In order to investigate the induction of cross-reactive tolerance in vivo, the immunodeficient mice were engrafted and boosted as described above, but were challenged rectally with hazelnut extract prior to assessment of the intestinal inflammation (Figure 6). In these experiments, we found a less severe allergic immune response in the hazelnut-challenged compared to the birch-challenged control group (Figure 5C), likely due to lack of in vivo booster with hazelnut extract and/or to the donors’ less severe sensitization towards the food allergen. However, even after hazelnut challenge co-injection of iTregBet resulted in a pronounced inhibition of intestinal allergic symptoms compared to control animals, which was shown with data of individual experiments (Figure 6A). In addition, we observed reduced human hazelnut-specific IgE levels after iTregBet application and hazelnut challenge compared to the allergic positive control (Figure 6B). These data indicate the induction of allergen-specific (birch) as well as cross-reactive tolerance (hazelnut) in vivo through IL-10 DC-induced iTregBet.

Discussion

Although PFA is a widespread allergic disorder, the only available disease-modifying therapy for pollen allergy has a rather limited effect on the associated food allergy. Therefore in this study, human monocyte-derived IL-10 DC were investigated with regard to their potential to induce allergen-specific (birch) and cross-reactive (hazelnut) tolerance in birch pollen allergic patients with associated hazelnut allergy in vitro and in vivo. We found that IL-10 DC induce Bet-specific iTreg which show a regulatory phenotype and strong suppressive capacities to inhibit allergen-specific and cross-reactive immune responses in vitro. In addition, Bet-specific iTreg were able to ameliorate allergic symptoms in vivo in a humanized mouse model of allergic intestinal and airway inflammation.

PFA results from highly conserved protein structures of pollen (e.g. Bet) and food allergens (e.g. Cor), which was shown to facilitate cross-reactions on IgE level and in T cell clones. In a previous study, we confirmed the data of cross-reactivity between pollen and food allergens in primary T cells directly obtained from patients with allergies to birch pollen and associated food allergens. Here, we focused on the induction of allergen-specific and cross-reactive iTreg to modulate the primary and secondary allergic immune response in patients suffering from PFA.

Bet-specifically stimulated iTreg but not non-specific iTreg underwent vigorous proliferation towards Bet- and Cor-induced restimulation, suggesting activation as prerequisite for suppressive activity. In this context, Pellerin et al. investigated peanut-specific Tr1 cells induced in vitro by IL-10 DC from allergic subjects. They found a highly proliferative phenotype with Th2-cytokine profile upon peanut-specific restimulation and, in contrast to our data, suggested a functional impairment of the peanut-specific Tr1 subset. Our experiments revealed that IL-10 DC-induced Bet-specific iTreg did have the ability to suppress allergen-specific responder T cell proliferation, displayed an activated and suppressive phenotype, even though they were highly proliferative. This discrepancy might be due to (1) different protocols for IL-10 DC culture and Treg generation, (2) different allergen-specific immune responses and/or (3) lack of functional assays in the study by Pellerin et al. Anergy has been initially described as a fundamental characteristic of functional Treg, but this idea has hence been revised: although breaking Treg anergy can be accompanied by loss of suppressive function, this is not always the case. In fact, it was shown that proliferating Treg can suppress T cell responses in vivo and Treg that have been stimulated to proliferate can even display an enhanced
suppressive capacity.57–59

One very crucial aspect of therapeutic tolerance induction is the allergen-specificity. We are therefore thrilled to report that Bet-specific iTreg showed significantly greater abilities to suppress allergen-specific responder T cell proliferation than non-specific iTreg, as was seen in in vitro suppressor assays from up to 80% of allergic donors. These results were strongly supported by the T cell cytokine profile in suppressor assays. Here, we found that Bet-specific iTreg significantly decreased levels of the Th2 cytokine IL-13, which was not achieved with non-specific iTreg. IL-13 is an IgE-promoting Th2 cytokine, which contributes to airway inflammation and food-induced anaphylaxis in asthma and type 1 allergies.60,61 In line with these data, amounts of the immunosuppressive cytokine IL-10 were significantly increased in the presence of Bet-specifically primed iTreg, which was not the case for non-specific iTreg. Accordingly, IL-10 is well known for its suppressive function in regulatory T cell activity, and particularly in control of Th2-driven allergic diseases.62 As the described cytokine shift was observed for both, Bet- and Cor-specific responder T cells after coculture with Bet-stimulated iTreg - but not with non-specific iTreg - these data underlined the induction of an allergen-specific (birch) and cross-reactive tolerance (hazelnut) through IL-10 DC-induced Bet-specific iTreg priming.

In addition, Bet-specific iTreg were able to ameliorate asthmatic and intestinal allergic symptoms provoked by challenge with birch extract and reduced birch-specific IgE in allergic mice in vivo. These combined pieces of evidence strongly suggest an allergen-specific tolerance induction in vitro and in vivo by iTreg which have been stimulated by allergen-loaded tolerogenic IL-10 DC. Intriguingly, Bet-specific iTreg also reduced the allergic gut inflammation and hazelnut-specific IgE levels in vivo after challenge with the hazelnut extract in mice engrafted with PBMC from birch pollen allergic patients with associated hazelnut allergy, facilitating cross-reactive tolerance.

Aiming to replace general immunosuppressive therapies, tolerogenic DC (tolDC) have been applied as antigen-specific immune-suppressors in numerous phase 1 clinical trials for multiple sclerosis, type 1 diabetes, rheumatoid arthritis and organ transplantation.32,34,63–65 In all studies, tolDC had negligible adverse effects and did not worsen disease symptoms. Clinical outcomes were only investigated in a few trials so far but preliminary evidence for antigen-specific tolerance induction was found.

In a comparative study by Boks et al. IL-10 modulation for human tolDC generation was identified as the protocol most suited for tolDC vaccination.66 We developed a protocol for IL-10 DC that resulted in a subpopulation of tolerogenic CD83highCCR7+ IL-10 DC that exhibit a high migratory activity, stability to pro-inflammatory stimuli and profound capacity to induce iTreg with a strong suppressive function.35 In our current study, we did show that human IL-10 DC through priming of allergen-stimulated iTreg are able to induce specific- and cross-reactive tolerance in vitro and in vivo and, therefore are promising candidates to modulate pollen as well as associated food allergies.

Combined with previous findings by us and other groups,35,51,66–68 our study results might further support the development of DC-based tolerance-inducing therapies for allergic and autoimmune diseases.

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Figure Legends
Figure 1: iTreg induced by unloaded or Bet-loaded IL-10 DC displayed an anergic phenotype after primary culture. PBMC for DC generation and T cells were obtained from birch-pollen allergic patients with associated hazelnut allergy. CD4+ T cells were primed with autologous unloaded IL-10 DC (IL-10 DC0) and Bet-loaded IL-10 DC (IL-10 DCBet) or mDC (mDC0/mDCBet), respectively, to induce non-specific and Bet-specific iTreg (iTregBet/iTreg0) and Teff (TeffBet/Teff0, as controls). (A) After 3 days of iTreg induction, the coculture was pulsed with [3H]TdR for 16-18 h to assess T cell proliferation which is shown as stimulation index (SI, mean ± SD) normalized to T cells stimulated with mDC0 (SI = 1). The data are pooled from 34 independent experiments. (B-F) Cytokine concentrations (B IL-5, C IL-9, D IL-13, E IL-2 and F IL-10) in the supernatants of primary cultures are depicted as mean ± SD normalized to ctrl (=1). P values calculated with paired student’s t-test are depicted as asterisks: **** p < 0.0001, ** p < 0.01, * p < 0.05, ns = not significant (p > 0.05). PC: primary culture (induction)

Figure 2: Bet-specific iTreg lost their anergic phenotype after Bet- and Cor-induced restimulation. In primary culture (PC), Teff0, TeffBet, iTreg0 and iTregBet were primed for five days by coculture of CD4+ T cells with autologous unloaded and Bet-loaded mDC or IL-10 DC, respectively. After a subsequent resting phase of 3 days, T cells were restimulated (RS) with unloaded, Bet-loaded or Cor-loaded mDC and were pulsed with [3H]TdR for 16-18 h on day 3. T cell proliferation is presented as SI (mean ± SD) normalized to Teff0 stimulated with mDC0(SI = 1). (A) Bet- and Cor-stimulated T cell proliferation of Teff0, TeffBet and iTregBet was pooled from 8 independent experiments. (B) Data of 9 independent experiments were pooled demonstrating T cell proliferation of iTregBet and iTreg0 restimulated with mDCBet or mDCCor, respectively. P values calculated with paired student’s t-test are depicted as asterisks: ** p < 0.01, * p < 0.05, ns = not significant (p > 0.05). PC: primary culture (induction), RS: restimulation

Figure 3: Bet-specific iTreg inhibited allergen-specific and cross-reactive responder T cell responses in vitro. Bet- and Cor-specific proliferation of autologous CD4+CD25low responder T cells (Tresp) obtained from birch-pollen allergic donors with associated hazelnut allergy was induced by stimulation with mDCBet (green) or mDCCor (brown), respectively. For analysis of the suppressive activity iTreg (iTregBet or iTreg0) were added in a Tresp:iTreg ratio of 1:1 or 1:2, respectively. DC, Tresp and iTreg were stained with different proliferation dyes for cell identification and assessment of proliferation by flow cytometry (see Supplementary Figure 2 for gating strategy). (A, B) Percentage of proliferating Tresp are shown from one representative experiment (top) and as pooled data (mean ± SD) relative to control ( = 100%) from independent experiments (bottom, number of independent experiments indicated below). (A) Percentage (mean ± SD) of proliferating Bet- (upper panel, green) and Cor-stimulated (lower panel, brown) Tresp, cocultured with iTregBet in the ratios 1:1 and 1:2, respectively, are demonstrated (Pooled data: Bet-stimulation: n=15; Cor-stimulation: n=14). (B) Function of iTregBet was compared to iTreg0 in suppressor assays with Tresp:iTreg = 1:2 and percentages (mean ± SD) of proliferating Bet- and Cor-stimulated Tresp are depicted as pooled data (Bet-stimulation: n=10; Cor-stimulation: n=8). (C) IL-13 (n=5-9) and (D) IL-10 concentrations (n=5-10) in the supernatants of suppressor assay samples are depicted as mean ± SD normalized to ctrl (=1). P values calculated with paired student’s t-test are depicted as asterisks: **** p < 0.0001, *** p < 0.01, * p < 0.05, ns = not significant (p > 0.05).

Figure 4: Bet- and Cor-specifically stimulated iTreg showed an activated and suppressive phenotype. Prior to flow cytometry analysis of suppressor assays, T cell populations were stained for expression of extra- and intracellular markers and with different cell proliferation dyes (see supplementary Figure 5 for gating strategy) to distinguish between proliferating and non-proliferating T cells. (A) Activation and (B) immunosuppressive parameters are pooled from independent experiments as indicated (CD45RO n=5, CD25 n=9, HLA-DR n=10, CTLA-4 n=10, TNFR2 n=9, PD-1 n=10, IL-10 n=5, ICOS n=5) and expression is shown as percentage (mean ± SD), except for ICOS which is presented as the mean fluorescence intensity (mean ± SD). (C) Five independent experiments were pooled to show the percentage (mean ± SD) of CD49b+LAG3+ T cells. P values calculated with paired student’s t-test are depicted as asterisks: **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05, ns = not significant (p > 0.05).

Figure 5: Bet-specific iTreg abrogated birch-specific allergic symptoms in humanized mouse
models of allergen-induced intestinal and airway inflammation. (A) Immunodeficient mice were engrafted with human PBMC from allergic donors suffering from birch pollen and associated hazelnut allergy and were boosted twice (d0, d8) with birch pollen extract to induce allergic immune responses. iTreg were co-injected to analyze their impact on allergy development. After three weeks, blood samples for IgE analysis were collected and subsequently the mice were challenged with birch pollen extract either rectally for induction of allergic intestinal inflammation or intranasally for allergic asthma induction. (B) The allergic intestinal inflammation was monitored by mini-endoscopy and scoring of colitis activity. One representative set of pictures is shown. (C) Quantitative endoscopic assessment of colitis activity in all groups is shown as mean ± SD from 8 independent experiments. (D) Results of airway resistance as parameter of allergic asthma were pooled from four independent experiments and are depicted as relative changes to baseline in % (mean ± SD). (E) The concentration of birch-specific IgE was obtained from 7 independent experiments as indicated and are presented in kU/L. P values calculated with paired student’s t-test are depicted as asterisks: **** p < 0.0001, ** p < 0.01, * p < 0.05. Figure 5A was created using Servier Medical Art (http://smart.servier.com/).

Figure 6: Bet-specific iTreg facilitated a cross-tolerance to hazelnut in a humanized mouse model of allergic gut inflammation. Immunodeficient mice were treated as described in Figure 5A. After three weeks, the mice were challenged rectally with either birch pollen or hazelnut extract, respectively, 2 h prior to scoring of the intestinal inflammation by mini-endoscopy. (A) The endoscopic score of the allergic intestinal inflammation of four independent experiments is depicted as pooled data (mean ± SD). (B) Blood samples were collected and human hazelnut-specific IgE was analyzed and is shown as mean value ± SD. Lines connect values from individual experiments. P values calculated with paired student’s t-test are shown as asterisks: *** p < 0.001, ** p < 0.01, ns = not significant (p > 0.05)

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Fig. 3

A) Restimulation of iTregBet

B) Restimulation of iTreg0

C) IL-13

D) IL-10
Fig. 4

**Activation parameters**

- % of CD86+ cells
- % of CD28+ cells
- % of FAS+ cells

**Immune-suppressive parameters**

- % of TGF-β cells
- % of IL10 cells
- % of IL6 cells

**Treg population markers**

- % of CD4+ cells
- % of CD25+ cells
- % of FoxP3+ cells