

The role of Foxp3⁺ T cells in long-term efficacy of prophylactic and therapeutic mucosal tolerance induction in mice

Background: Mucosal tolerance induction is suggested as treatment strategy for allergic diseases. Using a murine model of birch pollen (BP) allergy we investigated the long-term efficacy and the underlying mechanisms of mucosal tolerance induction with two structurally different molecules in a prophylactic and in a therapeutic set-up.

Methods: The three-dimensional major BP allergen Bet v 1 or a nonconformational hypoallergenic fragment thereof was intranasally applied before (prophylaxis) or after sensitization (therapy).

Results: In the prophylactic application both the Bet v 1 allergen and the fragment prevented allergic sensitization, and this effect lasted for 1 year. In the therapeutic approach established allergic immune responses were also suppressed after treatment with either of the molecules. However, a long-lasting curative effect (6 months) was only achieved with the Bet v 1 allergen but not with the Bet v 1 fragment. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of splenocytes revealed that tolerance induction with the Bet v 1 allergen was associated with enhanced expression of transforming growth factor (TGF)- β , interleukin (IL)-10, and Foxp3 mRNA in CD4⁺ T cells, whereas treatment with the fragment led to the induction of either Foxp3 (prophylaxis) or IL-10 (therapy) alone.

Conclusion: From these data we concluded (i) that the mechanisms underlying peripheral tolerance are linked to the conformation of the antigen, (ii) that mucosal tolerance is mediated by separate regulatory cell subsets, and (iii) that the long-term efficacy of immunosuppression is associated with the presence of Foxp3⁺ T cells.

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Specific immunotherapy (SIT) is an effective treatment in many cases of allergic diseases (1). Nevertheless SIT implies certain drawbacks because of the use of natural allergen extracts, such as the risk of anaphylactic side reactions and *de novo* sensitization against components within the allergen preparation (2). Therefore, application of only the disease-eliciting allergens in recombinant form, as well as a change to a less invasive route have been suggested for improvement of this treatment (3, 4).

Among the inhalant allergens birch pollen (BP) represents an important source for eliciting allergic symptoms.

The major BP allergen is Bet v 1, a 17 kDa molecule, to which 95% of the BP allergic patients display immunoglobulin (Ig)E-binding and to which about 60% react exclusively (5). Based on the fact that Bet v 1 occurs in different isoforms, some of them displaying reduced IgE-binding properties (6, 7), genetic engineering was used to synthesize similar recombinant hypoallergenic molecules: one approach was to disrupt the three-dimensional (3D) structure and to produce recombinant fragments comprising only a half of the complete molecule (fragment I: amino acids 1–74, fragment II: amino acids 75–160; 8). In allergic patients these fragments displayed a more than 100-fold reduced capacity to induce basophile histamine release and immediate type I skin reactions (9). Based on these findings hypoallergenic allergen variants have been used for improved/safer SIT of type I allergy (10).

Abbreviations: Al(OH)₃, aluminum hydroxide; BP, birch pollen; Fr-Bet, recombinant hypoallergenic fragment of Bet v 1; i.n., intranasal; i.p., intraperitoneal; NaCl, sodium chloride; RBL, rat basophile leukemia; Tr, regulatory T cells.

Regarding the change to a less invasive route of allergy treatment, mucosal tolerance induction was suggested as an alternative treatment strategy for type I allergies (4). In this respect we previously demonstrated in a mouse model of BP allergy that intranasal (i.n.) administration of Bet v 1 suppressed allergic sensitization in naive and in primed mice (11, 12). Using the nonconformational hypoallergenic fragments of Bet v 1 for tolerance induction we showed that only the fragment that contained the major T-cell epitope (fragment II: amino acids 75–160) exhibited similar tolerogenic capacities as the complete Bet v 1 allergen (13).

It has been described that the mechanisms of mucosal tolerance induction are differentially regulated in dependence of the conformation or the dose of the antigen (Ag; 14, 15). Besides anergy or deletion, regulatory T cells (Tr) play a central role, of which different subpopulations have been described (16). Among CD4⁺ cells Tr1 cells produce primarily interleukin (IL)-10 (17), whereas Th3 cells act via transforming growth factor (TGF)- β , IL-4, and/or IL-10 (18). TGF- β has been shown to be produced also by CD8⁺ and γ/δ cells (19, 20). Naturally occurring CD4⁺CD25⁺ Tr are characterized by the expression of Foxp3 (21, 22). Despite the detailed phenotypical characterization, the interaction of these different subsets is, however, still unclear.

Using our mouse model of BP allergy, the present study was performed to investigate the influence of the Ag conformation on the long-term efficacy of tolerance induction as well as the mechanisms focusing on CD4⁺ and CD8⁺ T cell populations. For this purpose the complete Bet v 1 allergen or the hypoallergenic Bet v 1 fragment harboring the major T cell epitope (Fr-Bet) was i.n. applied in a prophylactic and a therapeutic treatment regime. The results indicate that the long-term efficacy of immunosuppression is associated with the induction of Foxp3⁺ Tr.

Methods

Animals

Female, 6-week-old BALB/c mice were obtained from Charles River (Sulzfeld, Germany). All experiments were approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Education, Science and Culture.

Antigens

Recombinant (r)Bet v 1 was obtained from Biomay GmbH (Vienna, Austria). The Bet v 1 fragment (Fr-Bet), comprising amino acids 75–160 of Bet v 1, was expressed in the *Escherichia coli* strain BL21 (DE3) and purified as described (8). Measurement of bacterial endotoxin contamination was performed using the LAL ENDO-CHROME test (Charles River Endosafe, Wilmington, DE, USA), revealing lipopolysaccharide (LPS) levels ranging from 0.015 to 0.05 EU/ μ g protein per protein fragments. Birch pollen extract (Allergon, Ängelholm, Sweden) was produced and characterized as previously described (13).

Experimental design

Sensitization. For systemic sensitization 1 μ g rBet v 1 absorbed to aluminum hydroxide [(Al(OH)₃], 2 mg per animal; Serva, Heidelberg, Germany] was intraperitoneally (i.p.) injected three times at 14-day intervals (11).

Prophylaxis – immediate effects ($n = 5–9$ mice per group). Mice were i.n. pretreated with rBet v 1 or Fr-Bet (three times at 7-day intervals, i.e. $3 \times 10 \mu$ g), or sham-pretreated with NaCl, and 10 days later i.p. sensitized as described above.

Prophylaxis – long-term efficacy ($n = 4–7$ mice per group). Mice were i.n. pretreated and sham-pretreated as described for prophylaxis (immediate effects), and 10 days later once i.p. injected with rBet v 1/Al(OH)₃. Booster immunizations were given either 6 months or 1 year later. In all prophylactic protocols blood and spleens were taken 7 days after sensitization.

Therapy – immediate effects ($n = 5–13$ mice per group). Mice were i.p. sensitized with rBet v 1/Al(OH)₃ and 1 week later i.n. treated with rBet v 1 or Fr-Bet (three times at 4-day intervals, i.e. $3 \times 50 \mu$ g), or sham-treated with NaCl. One week after i.n. treatment the mice were challenged with an aerosol of BP extract as described (12).

Therapy – long-term efficacy ($n = 5–10$ mice per group). Mice were sensitized and i.n. treated as described for therapy (immediate effects). Six months after i.n. treatment mice were challenged with a BP aerosol. In all therapeutic protocols blood, lungs, and spleens were taken 3 days after the challenge.

Determination of allergen-specific antibodies

Allergen-specific antibody (Ab) levels in sera were measured by enzyme-linked immunosorbent assay (ELISA) and were calculated as mean arbitrary ELISA units (EU) after subtraction of baseline levels in preimmune sera as described (12).

Rat basophile leukemia cell mediator release assay

The rat basophile leukemia (RBL) assay was performed as previously described (23). RBL-2H3 cells were incubated with sera obtained from treated and sham-treated sensitized mice. Degranulation of RBL cells was induced by adding 0.03 μ g of rBet v 1 diluted in 100 μ l Tyrode's buffer and supernatant (SN) were analyzed for β -hexosaminidase activity. Results are reported as percentages of total β -hexosaminidase released after addition of 1% Triton X-100 and are shown after subtraction of baseline release levels (1.13 ± 0.81) obtained with preimmune sera.

Cytokine production in spleen and lung cell cultures

At the time of killing spleen and lung cell cultures were prepared as described (11), and lungs were pooled within groups because of low cell counts. Cell suspensions were cultured with BP (25 μ g per well) and SN were collected 48 h after Ag stimulation for measurement of cytokine production. Levels of IL-4, IL-5, and IL-13 were measured with mouse ELISA kits (sensitivity < 5 pg/ml; Endogen, Cambridge, MA, USA), and interferon (IFN)- γ levels were determined as previously described (sensitivity < 15 pg/ml) (12). Results reflect cytokine levels after subtraction of baseline levels measured in nonstimulated cultures.

Cell sorting

Spleen cell suspensions were prepared 3 days after the first i.p. sensitization (prophylaxis) or 3 days after aerosol challenge (therapy) and pooled within the groups. Cells (3×10^7 per group) were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 and phycoerythrin (PE)-labeled anti-CD8 monoclonal Ab ($10 \mu\text{g}/3 \times 10^7$ cells; Pharmingen, Mississauga, Canada). CD4+ and CD8+ T cells were sorted by the EPICS Altra (Beckman Coulter, Fullerton, CA, USA) equipped with a water-cooled Coherent Enterprise laser using EXPO32 ADC Software (standard Mode 3 option). Sorted cells were of >99% purity as determined by fluorescence-activated cell sorter (FACS) analysis and were used for real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Real-time RT-PCR

Total RNA was isolated from sorted CD4+ and CD8+ splenocytes using RNeasy Minikit (Quiagen, Valencia, CA, USA), treated with DNase (Quiagen) and thereafter reverse-transcribed into cDNA using random hexamers (GeneAmp RT-PCR kit, Perkin Elmer, Boston, MA, USA). Gene expression of TGF- β , IL-10, and Foxp3 was determined in triplicates by quantitative real-time PCR using predesigned TaqMan[®] Gene Expression Assays according to the manufacturer's protocol on an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Amplification of the endogenous control 18S rRNA (Applied Biosystems) was performed in order to standardize the amount of sample cDNA added, and relative quantification was performed using the standard curve method (Applied Biosystems). The thermal cycle conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min.

Statistical methods

Data are expressed as mean $\pm$ SEM from three independent experiments. Normality was assessed by Kolmogorov–Smirnov tests, homogeneity of variance by Levene's tests. In cases of a significant ($P < 0.01$) deviation in one or both of these tests, Kruskal–Wallis test and *post hoc* comparisons of treatment/prophylaxis groups with sham treatment/prophylaxis group were performed. In all other cases ANOVA with linear contrasts was applied. Two-sided *P*-values of below 0.05 were considered significant.

Results

Prophylaxis: short-term and long-term efficacy of intranasal pretreatment with rBet v 1 and Fr-Bet

Intranasal treatment with rBet v 1 and Fr-Bet prior to sensitization prevented the development of Bet v 1-specific Ab levels (IgE, IgG1, IgG2a, $P < 0.0001$; Table 1), inhibited IgE-dependent basophile degranulation and significantly suppressed the production of IL-5 and IFN- γ in spleen cell cultures ($P < 0.01$ to $P < 0.001$; Fig. 1A) (13).

Investigating the long-term efficacy of tolerance induction, 6 months after rBet v 1- and Fr-Bet-pretreatment all mice still displayed significantly suppressed Ab levels ($P < 0.05$ to $P < 0.01$; Table 1), IgE-dependent basophile degranulation and IL-5 production in spleen cell

Table 1. Prophylaxis: Bet v 1-specific serum Ab levels

	Ab levels (EU) $\ddagger$		
	IgE	IgG1	IgG2a
Immediate effects $\dagger$			
NaCl i.n./sens	211.3 $\pm$ 61.4	1749.9 $\pm$ 175.8	74.5 $\pm$ 59.3
rBet i.n./sens	4.7 $\pm$ 7.4****	42.8 $\pm$ 94.8****	ND
Fr-Bet i.n./sens	5.9 $\pm$ 10.1****	5.0 $\pm$ 9.5****	ND
Long-term efficacy (6 months) $\dagger$			
NaCl i.n./sens	321.0 $\pm$ 375.0	1377.8 $\pm$ 225.9	179.2 $\pm$ 135.9
rBet i.n./sens	77.5 $\pm$ 132.3*	190.4 $\pm$ 272.8**	18.0 $\pm$ 17.5*
Fr-Bet i.n./sens	17.8 $\pm$ 29.0*	229.7 $\pm$ 239.2**	5.1 $\pm$ 5.8**
Long-term efficacy (1 year) $\dagger$			
NaCl i.n./sens	225.9 $\pm$ 101.8	1055.0 $\pm$ 362.9	127.4 $\pm$ 82.3
rBet i.n./sens	118.7 $\pm$ 50.1	5.0 $\pm$ 8.7**	0.5 $\pm$ 0.9**
Fr-Bet i.n./sens	20.5 $\pm$ 5.3**	262.8 $\pm$ 295.5*	13.3 $\pm$ 11.9*

* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ pretreated vs sham-pretreated sensitized control mice.

$\dagger$ Immediate effects, long-term efficacy (6 months), and long-term efficacy (1 year) of mucosal pretreatment with rBet v 1 and Fr-Bet.

$\ddagger$ Bet v 1-specific serum Ab levels were measured 7 days after completed sensitization.

EU, mean arbitrary ELISA units $\pm$ SEM; ND, not detectable; Ab, antibody; Ig, immunoglobulin; Fr-Bet, recombinant hypoallergenic fragment of Bet v 1.

cultures ($P < 0.01$; Fig. 1B). Even 1 year after i.n. pretreatment with either of the molecules the animals showed significantly lower IgG1, IgG2a Ab levels ($P < 0.05$ to $P < 0.01$; Table 1) and IgE-dependent basophile degranulation than sensitized controls ($P < 0.05$; Fig. 1C). In spleen cell cultures IL-5 production remained significantly suppressed in both pretreated groups ($P < 0.01$), only IFN- γ reached similar levels as those of the sensitized controls (Fig. 1C).

Prophylaxis: TGF- β , IL-10, and Foxp3 mRNA expression on CD4+ and CD8+ T cells

In order to dissect the mechanisms underlying prophylactic mucosal tolerance induction we measured mRNA expression of TGF- β , IL-10, and Foxp3 in sorted CD4+ and CD8+ splenocytes. Mice i.n. pretreated with rBet v 1 displayed strongly upregulated mRNA levels of TGF- β in CD4+ and CD8+ T cells. Additionally, IL-10 and Foxp3 expression was markedly enhanced in CD4+ T cells compared with levels of sensitized controls (Fig. 2). In contrast, i.n. pretreatment with Fr-Bet led to a moderate increase of Foxp3 in CD4+ splenocytes alone (Fig. 2).

Therapy: short-term efficacy of intranasal treatment with rBet v 1 and Fr-Bet

To investigate the immunosuppressive effects of mucosal treatment in already primed mice, sensitized mice were i.n. treated with rBet v 1 or Fr-Bet and thereafter challenged with a BP aerosol (12).

After rBet v 1-treatment mice displayed significantly suppressed IgG1 titers [$P < 0.01$; IgG1 (EU): NaCl i.n. 1501.9 ± 324.7 , rBet v 1 i.n. 830.8 ± 451.7 , Fr-Bet i.n.

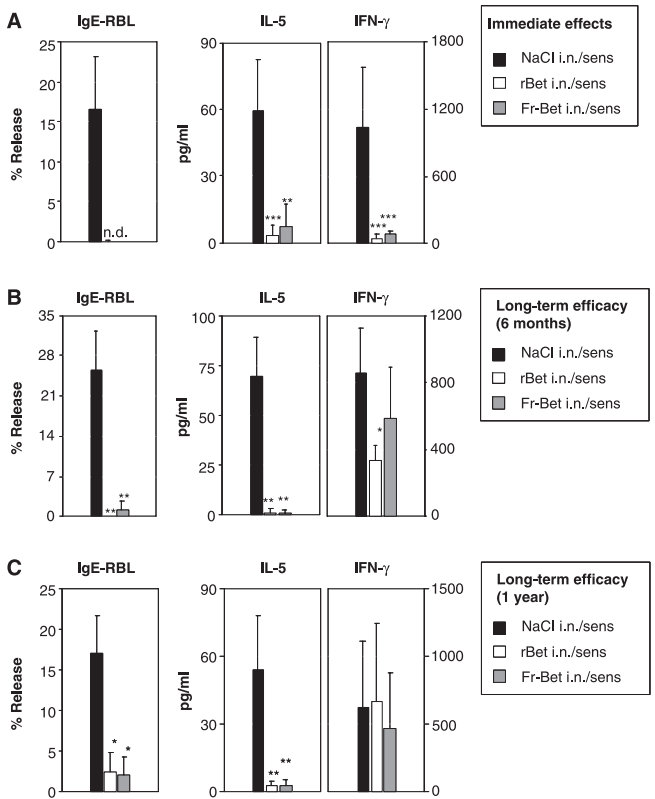


Figure 1. Prophylaxis: immunoglobulin (Ig)E-dependent Bet v 1-specific basophile degranulation [rat basophile leukemia (RBL) assay] and cytokine production in spleen cell cultures. (A) Immediate effects, (B) long-term efficacy (6 months), and (C) long-term efficacy (1 year) of mucosal pretreatment with rBet v 1 (white bars) and Fr-Bet (gray bars) were determined 7 days after completed sensitization. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ pretreated vs sham-pretreated sensitized control mice (black bars; n.d. = not detectable).

1215.8 ± 93.8], whereas IgE and IgG2a levels from rBet v 1- and Fr-Bet-treated mice did not differ from those of the sham-treated controls [IgE (EU): NaCl i.n. 148.7 ± 87.9 , rBet v 1 i.n. 111.5 ± 40.0 , Fr-Bet i.n. 54.1 ± 31.5 ; IgG2a (EU): NaCl i.n. 71.6 ± 76.0 , rBet v 1 i.n. 101.7 ± 69.9 , Fr-Bet i.n. 52.9 ± 21.4]. The IgE-dependent basophile degranulation was significantly decreased in mice treated with Fr-Bet ($P < 0.01$), but did not differ between rBet v 1-treated and control mice (Table 2).

In terms of cellular immune responses, rBet v 1- and Fr-Bet-treated mice displayed significantly reduced IL-4, IL-5, and IL-13 levels in spleen cell cultures compared with the sham-treated controls ($P < 0.05$ to $P < 0.0001$; Fig. 3A). In addition to these curative effects at the systemic sites of inflammation, i.n. treatment with rBet v 1 and Fr-Bet significantly suppressed lung inflammation, as determined by IL-5 levels and eosinophilic recruitment in the bronchoalveolar lavages [IL-5 (pg/ml): NaCl i.n. 11.4 ± 5.8 , rBet v 1 i.n. 2.2 ± 0.3 ($P < 0.01$), Fr-Bet i.n. 0 ± 0 ($P < 0.01$); eosinophils (% total cell count): NaCl i.n. 11.2 ± 10.0 , rBet v 1 i.n. 4.3 ± 2.2 ($P < 0.05$), Fr-Bet i.n. 0.4 ± 0.5 ($P < 0.01$)]. Additionally, in lung cell cultures from both treated groups IL-4, IL-5, and IL-13 levels were lower than in those from sensitized controls (Table 2).

Therapy: long-term efficacy of intranasal treatment with rBet v 1 and Fr-Bet

The long-term efficacy of therapy was tested up to 6 months after i.n. treatment with rBet v 1 and Fr-Bet. The IgE-dependent basophile degranulation did not significantly differ between the treated and control mice, although there was a tendency towards lower levels after rBet v 1 treatment (Table 2). Bet v 1-specific serum Ab levels from rBet v 1- and Fr-Bet-treated mice remained unchanged compared with sham-treated control animals [IgE (EU): NaCl i.n. 92.5 ± 83.8 , rBet v 1 i.n. 39.0 ± 27.2 , Fr-Bet i.n. 87.7 ± 64.4 ; IgG1 (EU): NaCl

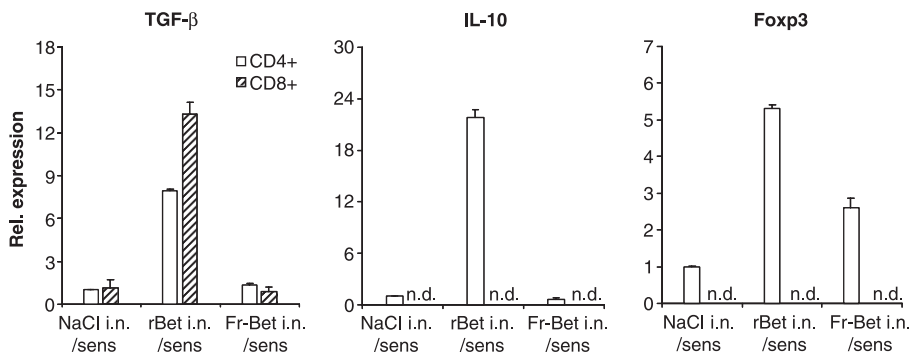


Figure 2. Prophylaxis: transforming growth factor (TGF)-β, interleukin (IL)-10, and Foxp3 mRNA expression in CD4+ (white bars) and CD8+ (hatched bars) splenocytes from rBet v 1- and Fr-Bet-pretreated mice shown as relative values in comparison with sham-pretreated sensitized controls (n.d. = not detectable).

Table 2. Therapy: IgE-dependent basophile degranulation and cytokine production in lung cell cultures

	IgE-RBL (% release)‡	Cytokine levels/lungs (pg/ml)§			
		IL-4	IL-5	IL-13	IFN-γ
Immediate effects†					
sens/NaCl i.n./aerosol	24.9 ± 8.1	77.8	2654.0	519.7	266.2
sens/rBet i.n./aerosol	25.6 ± 8.4	36.1	1345.0	326.5	256.6
sens/Fr-Bet i.n./aerosol	9.5 ± 7.6**	10.1	960.9	80.5	0.0
Long-term efficacy (6 months)†					
sens/NaCl i.n./aerosol	16.9 ± 8.7	73.4	2073.9	551.5	220.7
sens/rBet i.n./aerosol	11.2 ± 6.5	10.3	569.1	50.4	0.0
sens/Fr-Bet i.n./aerosol	18.9 ± 10.7	351.3	2246.9	1414.6	0.0

** $P < 0.01$ treated vs sham-treated sensitized control mice.

†Immediate effects and long-term efficacy (6 months) of mucosal treatment with rBet v 1 and Fr-Bet.

‡IgE-dependent Bet v 1-specific basophile degranulation (RBL assay).

§Cytokine levels in lung cell cultures were determined 3 days after the aerosol challenge.

Ig, immunoglobulin; RBL, rat basophile leukemia; IL, interleukin; IFN, interferon; Fr-Bet, recombinant hypoallergenic fragment of Bet v 1.

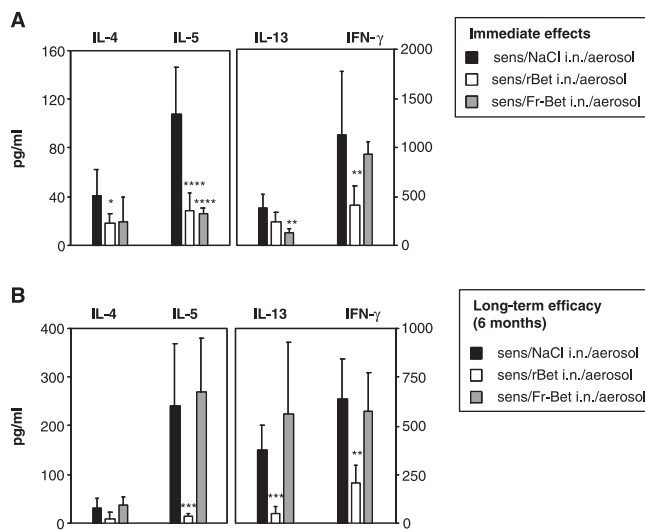


Figure 3. Therapy: cytokine production in spleen cell cultures. (A) Immediate effects and (B) long-term efficacy (6 months) of mucosal treatment with rBet v 1 (white bars) and Fr-Bet (gray bars) were determined 3 days after the aerosol challenge. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ treated vs sham-treated sensitized control mice (black bars).

i.n. 1177.4 ± 887.1 , rBet v 1 i.n. 1188.5 ± 501.8 , Fr-Bet i.n. 641.0 ± 200.6 ; IgG2a (EU): NaCl i.n. 61.8 ± 55.0 , rBet v 1 i.n. 159.7 ± 216.3 , Fr-Bet i.n. 228.9 ± 172.6].

In spleen cell cultures from rBet v 1-treated mice IL-4, IL-5, IL-13, and IFN-γ levels were still significantly suppressed after 6 months ($P < 0.01$ to $P < 0.001$), whereas in splenocytes from Fr-Bet-treated animals cytokine production was no longer reduced compared with the sensitized controls (Fig. 3B). Similar effects were observed with respect to Th2 cytokine levels in lung cell

cultures. Treatment with rBet v 1 persistently suppressed IL-4, IL-5, and IL-13 production whereas treatment with Fr-Bet did not induce long-lasting effects on these parameters (Table 2).

Therapy: TGF-β, IL-10, and Foxp3 mRNA expression on CD4+ and CD8+ T cells

Similar to the prophylactic set-up, i.n. treatment with rBet v 1 of already sensitized mice led to upregulation of IL-10 and Foxp3 mRNA in CD4+ T cells and an increase of TGF-β mRNA in CD8+ T cells compared with those of sensitized controls (Fig. 4). In contrast, treatment with Fr-Bet was only associated with moderately enhanced IL-10 mRNA expression in CD4+ T cells, whereas TGF-β and Foxp3 remained unchanged compared with sensitized controls (Fig. 4).

Discussion

In the present study, we demonstrate that immunosuppression induced with either the complete Bet v 1 molecule – a conformational Ag – or the nonconformational Bet v 1 fragment lasted up to 1 year, as depicted by significantly suppressed IgE-dependent basophile degranulation, specific IgG1 and IgG2a titers as well as IL-5 production *in vitro* (Fig. 1A–C, Table 1). These results demonstrate that long-lasting effects of prophylactic tolerance induction were achievable with both molecules, independent of their structural condition. Even though the clinical efficacy was equally sustained by either of the molecules, several studies indicated that distinct mechanisms might operate in tolerance induction with conformational and nonconformational molecules/peptides (15, 16). Indeed, our own previous data revealed that tolerance were transferable only with splenocytes from Bet v 1- but not from fragment-pretreated mice (13).

In the present study, we addressed the underlying regulatory mechanisms in more detail and determined mRNA expression of TGF-β, IL-10, and Foxp3 in sorted T-cell populations of splenocytes. While Bet v 1-pretreated animals displayed strongly increased mRNA levels of TGF-β in both CD4+ and CD8+ T cells, and enhanced levels of IL-10 and Foxp3 in CD4+ T cells, pretreatment with the fragment led to increase of Foxp3 in CD4+ T cells alone (Fig. 2). It has been described that low-dose tolerance induction is mediated by TGF-β produced by both CD4+ and CD8+ Tr (18–20). In line with our previous studies showing that tolerance was transferable with CD4+ and CD8+ splenocytes from Bet v 1- but not from fragment-pretreated mice (13), we assume that the capacity to transfer tolerance is closely related to the production/function of TGF-β. This is further supported by others showing that the transferability of tolerance was abrogated after *in vivo* application of neutralizing anti-TGF-β Ab (19).

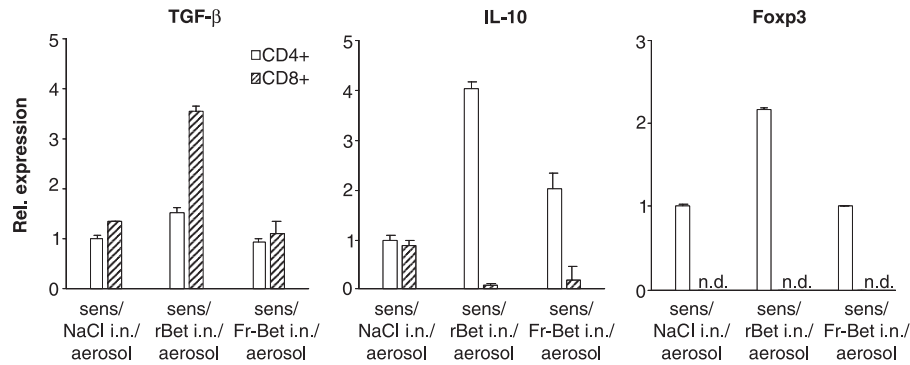


Figure 4. Therapy: transforming growth factor (TGF)- β , interleukin (IL)-10, and Foxp3 mRNA expression in CD4+ (white bars) and CD8+ (hatched bars) splenocytes from rBet v 1- and Fr-Bet-treated mice shown as relative values in comparison with sham-treated sensitized controls (n.d. = not detectable).

Besides TGF- β , IL-10 has been described as an important regulatory cytokine produced by a further subset of Tr, i.e. Tr1 (17). In a model of wasp venom allergy we recently demonstrated the significant role of this cytokine, as neutralization of the IL-10R *in vitro* abrogated allergen-induced immunosuppression (24). Likewise in the present study, i.n. treatment with Bet v 1 was associated with enhanced levels of IL-10 mRNA in CD4+ T cells (Fig. 2). In contrast, after mucosal treatment with the fragment none of these two cytokines was upregulated in the prophylactic set-up (Fig. 2). However, for immunosuppression and in particular for its maintenance the presence of Foxp3+ cells seemed to be crucial, as this marker was elevated in both Bet v 1- and Fr-Bet-tolerized mice (Fig. 2). This presumption might be reasonable since Foxp3, originally described as a transcription factor in naturally arising thymic CD4+CD25+ T cells, is linked to maintenance of immunologic self-tolerance *in vivo* (21, 22). Recently, it has been shown that injection of *in vitro*-converted Foxp3+ cells prevented house dust mite-induced allergic pathogenesis in the lungs in a murine asthma model (25). Our study provides evidence for the first time that Foxp3+ T cells can be generated *in vivo* by mucosal treatment with allergens and allergen derivatives. As several studies indicate that Foxp3-linked immunosuppression is due to cell-cell contact-dependent mechanisms (16, 26), it seems plausible that fragment-induced immunosuppression occurred in a cytokine-independent manner. Thus, most likely, Foxp3+, IL-10-, and/or TGF- β -producing cells represent separate subsets of Tr in our model of tolerance induction.

It is well known that tolerance induction in naive animals is easier to achieve than in primed animals (27, 28); however, regarding the clinical situation the therapeutic approach is of particular importance. In the present study, we demonstrated that i.n. treatment with either of the molecules led to significant suppression of cytokine production in spleen cell cultures and of allergic

lung inflammation also in already sensitized mice (Fig. 3A). In terms of humoral immune responses only the treatment with the fragment led to significant suppression of IgE-reactivity (Table 2). This discrepancy of Bet v 1 and Fr-Bet treatment might be explained by the consideration that B cells in immunized mice have been already primed and undergo further expansion when encountering their particular allergen (28), while in case of the fragment, lacking all B cell epitopes of Bet v 1, B-cell stimulation is missing.

Similar to the prophylactic approach, mice treated with Bet v 1 after sensitization displayed enhanced levels of IL-10 and Foxp3 in CD4+ T cells and of TGF- β in CD8+ T cells (Fig. 4). In terms of treatment with the fragment only IL-10 expression in CD4+ T cells was enhanced (Fig. 4). These data indicate that IL-10 were primarily responsible for the curative effects in already primed animals, which similar as previously shown in a mouse model of inflammatory bowel disease (29). Moreover, IL-10-producing Tr1 has been described to migrate to inflamed tissues and suppress lung inflammation (30–33). In our model both molecules also successfully suppressed airway inflammation in the short-term observation (Table 2).

Concerning the long-term efficacy of therapeutic treatment only immunosuppression induced with the complete Bet v 1 allergen lasted for 6 months and was even more pronounced compared with the immediate effects. The IL-4, IL-5, IL-13, and IFN- γ levels were significantly suppressed in spleen cell cultures and lung inflammation was still diminished (Fig. 4B, Table 2). Moreover, mice treated with Bet v 1 in the long-term set-up displayed slightly decreased IgE-dependent basophile degranulation and Ab production (Table 2). Again, long-term efficacy of treatment was associated with elevated Foxp3 levels in CD4+ T cells (Fig. 4), a similar relation as described in the prophylactic set-up (Fig. 2). In case of treatment with the fragment the beneficial curative effects were limited to a short period, which might be explained by recent studies

showing that IL-10-producing Tr were only able to continue their protective effects after cell-cell contact with other subsets of Tr (29, 34). Moreover, one might speculate that in case of the full-length conformational allergen, Ag presentation and Ag persistence take place more effectively via Ag-Ab complexes on the surface of Ag-presenting cells in regional lymph nodes and/or spleen (35, 36), thereby continuously providing the signals for long-lasting activation of Tr. Thus, hypoallergenic molecules with conserved 3D structures (6, 7) instead of allergen fragments, which lack B-cell epitopes, might be favored for prophylactic but particularly for therapeutic tolerance induction.

Taken together our data demonstrate that i.n. treatment with allergens and hypoallergenic derivatives induced profound immunosuppression in naive and in sensitized mice, thus encouraging mucosal tolerance

induction as feasible treatment strategy against hypersensitivities. In dependence of the structure of the applied allergens, mucosal treatment might be mediated by different Tr, of which the interaction or mutual relationship remains to be elucidated. Nevertheless, the generation of distinct subsets may influence the clinical efficacy, as the induction of Foxp3⁺ Tr seems to be crucial for the maintenance of immunosuppression *in vivo*.

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