

A Novel Approach to Specific Allergy Treatment: The Recombinant Allergen-S-Layer Fusion Protein rSbsC-Bet v 1 Matures Dendritic Cells That Prime Th0/Th1 and IL-10-Producing Regulatory T Cells¹

Marianne Gerstmayr,* Nicola Ilk,[†] Irma Schabussova,* Beatrice Jahn-Schmid,* Eva M. Egelseer,[†] Uwe B. Sleytr,[†] Christof Ebner,[‡] and Barbara Bohle^{2*}

An ideal vaccine for allergen-specific immunotherapy of type I allergies should display reduced mediator-releasing capacity, induce maturation of APC, and modify the disease-eliciting Th2-dominated allergen-specific response to a more physiological response. We have previously shown that rSbsC-Bet v 1, the recombinant fusion protein of a bacterial surface (S-layer) protein of *Geobacillus stearothermophilus* ATCC 12980 and the major birch pollen allergen Bet v 1, exhibited reduced allergenicity and induced IFN- γ and IL-10 synthesis in Bet v 1-specific Th2 clones. In this study, we characterized the effects of rSbsC-Bet v 1 on immature monocyte-derived dendritic cells (mdDC) and the consequences for the polarization of naive CD4⁺ T lymphocytes isolated from the blood of birch pollen-allergic patients. mdDC responded to rSbsC-Bet v 1 with a significant up-regulation of costimulatory molecules, functional maturation, and the synthesis of IL-10 and IL-12. mdDC matured with rSbsC-Bet v 1 induced the differentiation of naive T cells into IFN- γ -producing cells. This effect was IL-12 dependent. In parallel, a substantial number of naive T cells developed into IL-10-producing CD25⁺Foxp3⁺CLTA-4⁺ cells capable of active suppression. Thus, rSbsC-Bet v 1 showed immune stimulatory capacity on DC, which then promoted the simultaneous differentiation of Th0/Th1 cells and regulatory T cells. These data further support that the concept of conjugating allergens to bacterial agents is a promising approach to improve vaccines for specific immunotherapy of atopic allergies. *The Journal of Immunology*, 2007, 179: 7270–7275.

Allergen-specific Th2 cells, i.e., CD4⁺ T lymphocytes that produce high levels of IL-4, IL-5, and IL-13, but low amounts of IFN- γ , are pivotal for the induction and maintenance of type I allergies (1). The only approved treatment that counterregulates the disease-eliciting allergen-specific Th2 response in allergic individuals is specific immunotherapy (SIT).³ Successful SIT significantly reduces allergen-induced T cell proliferation, indicating the induction of peripheral tolerance in allergen-specific T cells. In parallel, the shift from Th2 toward a more physiological Th0/Th1-like immune response, either by reduced IL-4 production or the induction of IFN- γ , has been observed

(2–5). More recently, the induction of IL-10-producing allergen-specific T cells has been associated with successful SIT (6–9). SIT-induced IL-10-producing CD4⁺CD25⁺ T cells suppress proliferation and cytokine production of allergen-specific T cells and are thus designated T regulatory (Treg) cells (10–13). Furthermore, the fine balance between IL-10-producing allergen-specific Treg and Th2 cells has been considered a relevant component of the physiological immune response to allergens (14).

One strategy to improve vaccines for SIT is the use of adjuvants that amplify the modulation of the allergen-specific Th2 response. In this respect, different agents from bacterial origin have been regarded as promising candidates. Synthetic lipopeptides derived from lipoproteins of Gram-negative bacteria have been reported to induce IFN- γ and IL-10 synthesis by human T cells (15). Monophosphoryl lipid A, a nontoxic derivative of LPS of *Salmonella minnesota*, mixed with grass pollen extract enhanced the production of IFN- γ and reduced the production of IL-5 in PBMC from grass pollen-allergic patients (16). Synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODN) derived from bacterial DNA promoted Th1-like cytokine production and decreased the spontaneous synthesis of IgE Abs in allergic individuals in vitro (17–20). This immunomodulatory capacity was even more pronounced when CpG-ODN were chemically linked to a single defined allergen (21–23). Both conjugated CpG-ODN and monophosphoryl lipid A have been successfully applied in clinical trials and revealed promising results regarding the treatment of type I allergies (24–26).

S-layer proteins forming crystalline arrays on the cell surface of many bacteria and most archaea have already a long tradition in vaccine development (27–29). We have proposed to engineer allergy vaccines by genetic fusion of allergens with S-layer proteins:

*Department of Pathophysiology, Center for Physiology, Pathophysiology, and Immunology, Medical University of Vienna, Vienna, Austria; and [†]Center for Nano-Biotechnology, University of Natural Resources and Applied Life Sciences and [‡]Allergy Clinic Reumannplatz, Vienna, Austria

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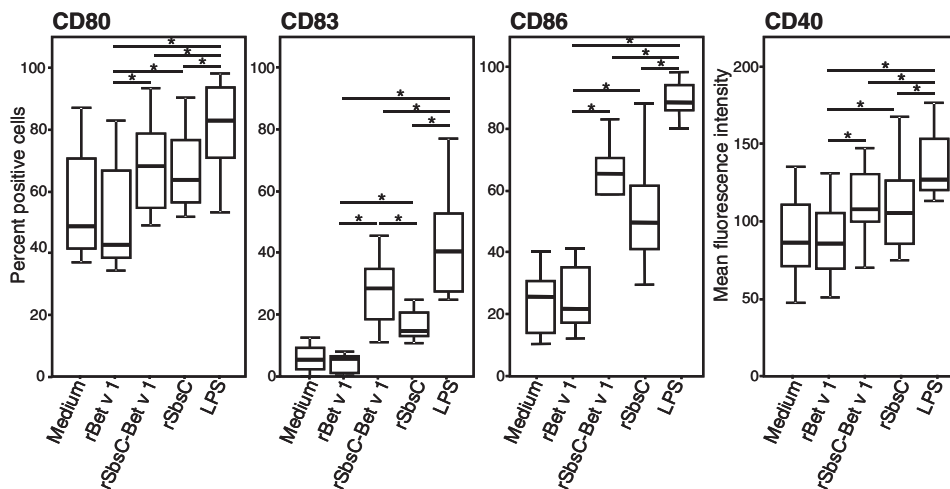
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² Address correspondence and reprint requests to Dr. Barbara Bohle, Department of Pathophysiology, Center for Physiology, Pathophysiology, and Immunology, Medical University of Vienna, Austria; AKH-3Q, Waehringer Guertel 18-20, Vienna, Austria. E-mail address: barbara.bohle@meduniwien.ac.at

³ Abbreviations used in this paper: SIT, specific immunotherapy; DC, dendritic cell; mdDC, monocyte-derived DC; B-DC, mdDC incubated with rBet v 1; CpG-ODN, oligodeoxynucleotides containing CpG motifs; Ct, threshold cycle; LB-CD, mdDC incubated with LPS and rBet v 1; SB-DC, mdDC incubated with rSbsC-Bet v 1; Treg, T regulatory.

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FIGURE 1. rSbsC-Bet v 1 induces DC maturation. Immature mdDC were left untreated or stimulated with rBet v 1, rSbsC-Bet v 1, rSbsC, or LPS. The percentage of DC expressing CD80, CD83, and CD86 surface markers and the mean fluorescence intensity of CD40 are shown. Data from seven different birch pollen-allergic donors were summarized in box plots. Each box represents the interquartile range containing 50% of the data. The line across the box indicates the median. The significances of differences to rBet v 1 were calculated using the Wilcoxon signed ranks test (*, $p < 0.05$).



the major birch pollen allergen Bet v 1 was fused with the S-layer protein from the nonpathogenic bacteria *Geobacillus stearothermophilus* ATCC 12980 (30, 31). The resulting allergen-S-layer fusion protein rSbsC-Bet v 1 induced IFN- γ and IL-10 production in PBMC and Bet v 1-specific Th2 clones from birch pollen-allergic donors (31). Moreover, rSbsC-Bet v 1 displayed a reduced IgE-binding capacity, and consequently a lower risk to induce adverse vaccine-related anaphylactic reactions (31). Thus, we consider rSbsC-Bet v 1 as promising hypoallergenic molecule to skew allergen-specific Th2 effector cells to both IFN- γ and IL-10 production. In this study, we investigated whether rSbsC-Bet v 1 affected the differentiation of naive CD4⁺ T cells from birch pollen-allergic individuals. We first analyzed the effects of rSbsC-Bet v 1 on monocyte-derived dendritic cells (mdDC) regarding maturation, functionality, and cytokine production. Next, mdDC stimulated with rSbsC-Bet v 1 were used to prime naive CD4⁺ T cells. T cell polarization was evaluated by measuring IL-4, IFN- γ , and IL-10. The suppressive capacity of IL-10-secreting T cells was tested in proliferation assays.

Materials and Methods

Patients, allergens, and reagents

Birch pollen-allergic patients with a typical case history, positive skin prick tests, and CAP/RAST scores >3 (Pharmacia) to birch were included. Sensitization to Bet v 1 was assessed by means of immunoblotting. The study was approved by the local medical ethics committee (Vienna, Austria). rSbsC-Bet v 1 and rSbsC were produced, as described (30). Endotoxin levels in rSbsC-Bet v 1 and rSbsC were below 0.4 EU/mg, as determined by *Limulus* amoebocyte lysate assay (BioWhittaker). rBet v 1 was purchased from Biomay, and LPS from Sigma-Aldrich.

Generation of mdDC

Monocytes were isolated from PBMC by magnetic cell sorting using anti-CD14 microbeads (Miltenyi Biotec). The isolated fraction contained $>95\%$ CD14⁺ cells, as determined by flow cytometry using FITC-conjugated anti-CD14 mAb (BD Pharmingen). CD14⁺ cells (1×10^6 /ml) were cultured in 24-well plates (Corning Glass) in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 2 mM L-glutamine, 170 mg/L gentamycin sulfate (both Sigma-Aldrich), human rIL-4 (1000 U/ml; Strathmann Biotec), and GM-CSF (50 ng/ml; PeproTech) in the presence of either 3% autologous plasma or 10% heat-inactivated FCS (PAA Laboratories) for 7 days. The resulting dendritic cell (DC) population was homogeneously CD14⁺, and $>70\%$ of the cells reacted with PE-Cy5-conjugated anti-CD1a mAbs (BD Pharmingen). Immature DC were either left untreated or stimulated with rBet v 1 (10 μ g/ml), rSbsC-Bet v 1 (59 μ g/ml containing 10 μ g/ml rBet v 1), and corresponding amounts of rSbsC and LPS (1 μ g/ml), respectively. After 48 h, surface expression of DC maturation markers on CD14⁺CD1a⁺ cells was analyzed using PE-conjugated anti-CD80, anti-CD83 mAb (both from BD Pharmingen), anti-CD86 mAb

(Caltag Laboratories), and FITC-conjugated anti-CD40 mAb (provided by O. Majdic, Institute of Immunology, Vienna, Austria).

Mixed leukocyte reaction

Differently stimulated DC were harvested after 48 h, irradiated (60 Gy), and added to 1×10^5 allogeneic PBMC from nonallergic donors in different ratios. Cells were cultured in triplicates in 96-well plates (Nunc; Nunc) in serum-free UC medium (BioWhittaker) supplemented with 2 mM L-glutamine and 2×10^{-5} M 2-ME for 6 days. Proliferation was assessed by adding [^{3} H]thymidine (0.5 μ Ci/well) during the last 16 h of culture and measuring the incorporated radioactivity by scintillation counting.

Real-time PCR

DC were incubated in 24-well plates (Corning Glass) with different stimuli for 14 h. RNA was isolated using the RNeasy Mini Kit (Qiagen) and reversely transcribed with TaqMan reverse-transcription reagents using random hexamers (Applied Biosystems). Analysis of 18S rRNA (control housekeeping gene), IL-10, and IL-12p40 was performed with cDNA-specific Assays-on-Demand (Applied Biosystems) using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). A negative control without template and a positive control with a template of known amplification were included in each run. All amplifications were performed in triplicates. Relative quantification and calculation of the range of confidence were performed by using the comparative threshold cycle ($\Delta\Delta C_t$) method (Applied Biosystems). The fold induction of cytokines was calculated by the formula $2^{-\Delta\Delta C_t} = 2^{-(\Delta C_t \text{ for unstimulated culture} - \Delta C_t \text{ for stimulated culture})}$.

Purification and stimulation of naive CD4⁺ T cells

Peripheral CD4⁺ T cells were purified using the CD4 Negative Isolation Kit from Dynal Biotech. From the resulting CD4⁺ population, CD45RA⁺ cells were isolated by magnetic cell sorting using anti-CD45RA microbeads (Miltenyi Biotec). A purity of $>92\%$ CD4⁺CD45RA⁺ T cells

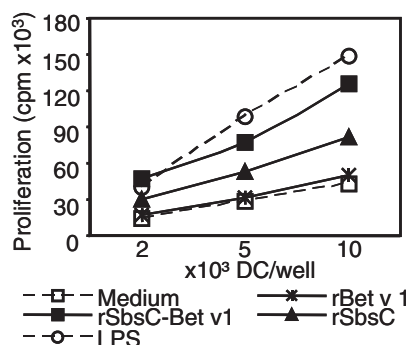
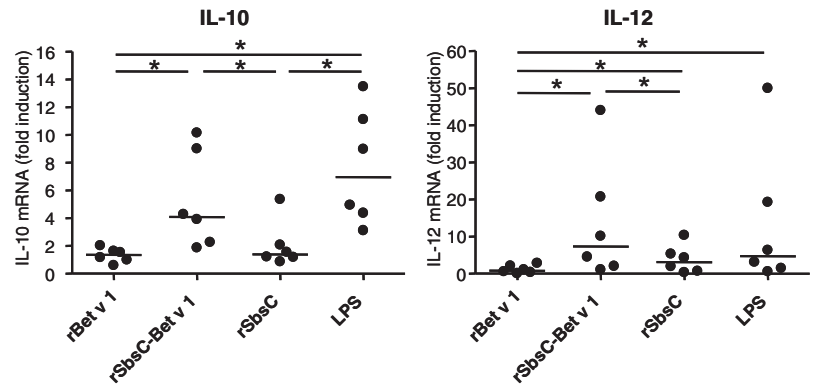


FIGURE 2. rSbsC-Bet v 1 induces functional DC. Increasing numbers of untreated mdDC or mdDC incubated with rBet v 1, rSbsC-Bet v 1, rSbsC, or LPS were cultured with 1×10^5 allogeneic PBMC. Mean cpm of triplicates of one experiment of three are shown.

FIGURE 3. rSbsC-Bet v 1 induces IL-10 and IL-12 in DC. Immature mdDC from six patients were left untreated or stimulated with rBet v 1, rSbsC-Bet v 1, rSbsC, or LPS. The relative expression of IL-10 and IL-12 mRNA was individually calculated as compared with the mRNA expression in unstimulated cells. Lines indicate the median. *, $p < 0.05$ (Wilcoxon signed ranks test).



was achieved. Autologous immature DC were stimulated with either rBet v 1 (10 μ g/ml), rSbsC-Bet v 1 (59 μ g/ml), or LPS (100 ng/ml) plus rBet v 1 (10 μ g/ml). After 24 h, 1×10^6 CD4⁺CD45RA⁺ T cells were added (day 0). At day 7, T cells were restimulated with the respective DC population and analyzed for cytokine production and proliferation at day 14. In some experiments, a neutralizing anti-IL-12 mAb (50 μ g/ml; R&D Systems) was added at days 0 and 7.

Phenotyping of primed CD4⁺ T cells

T cells were stained with PE-conjugated anti-CD25, PE-Cy5-conjugated anti-CD152 mAb (both from BD Pharmingen), and a FITC-conjugated anti-human Foxp3 (eBioscience). Intracellular cytokine detection in T cells was performed using FITC-, PE-, PerCP-labeled anti-IFN- γ , anti-IL-4, anti-CD3 mAbs, and respective isotype control Abs, as described (31). To quantify cytokine levels, 1×10^5 T cells were stimulated in triplicates with anti-CD3 (1 μ g/ml OKT3) and anti-CD28 (1 μ g/ml; Sanquin) in the presence of irradiated PBMC (60 Gy) in 96-well plates for 48 h. Levels of IL-10, IFN- γ , and TGF- β in supernatants were analyzed by ELISA using matched Ab pairs (Pierce) and a TGF- β 1 ELISA kit (IBT) (31).

Evaluation of suppressive capacity of IL-10-secreting T cells

Purified CD4⁺ T cells (1×10^6) were stimulated with immobilized anti-CD3 (0.5 μ g/ml) and soluble anti-CD28 (1 μ g/ml) in 48-well plates. After 18 h, IL-10-secreting cells were isolated using the IL-10 Secretion Assay-Cell Enrichment and Detection Kit (Milteny Biotec), according to the manufacturer's protocol. Autologous CD4⁺CD25⁺ cells were cocultured with IL-10-secreting cells in different ratios in the presence of anti-CD3 (OKT3 0.5 μ g/ml) or without any stimulus. After 72 h, proliferation was assessed.

Statistical analysis

Statistical significance of differences was determined using the Wilcoxon signed ranks test. Differences were considered statistically significant for $p < 0.05$.

Results

rSbsC-Bet v 1 induces phenotypic and functional maturation of DC

Immature mdDC from seven different birch pollen-allergic patients were left untreated or incubated with rBet v 1, rSbsC-Bet v 1, and rSbsC for 48 h (Fig. 1). As positive control, LPS, a well-characterized agent to mature mdDC, was used. Incubation of mdDC with rBet v 1 showed no effects as compared with medium alone. Incubation with rSbsC-Bet v 1 and rSbsC induced a significant up-regulation of CD80 ($p = 0.018$, $p = 0.018$), CD83 ($p = 0.018$, $p = 0.018$), CD86 ($p = 0.018$, $p = 0.018$), and CD40 ($p = 0.018$, $p = 0.028$) as compared with rBet v 1 (Fig. 1). rSbsC-Bet v 1 induced a significantly higher expression of CD83 than rSbsC ($p = 0.043$) (Fig. 1). LPS-stimulated mdDC displayed significantly higher levels of all surface markers as compared with rSbsC-Bet v 1 (CD80, $p = 0.018$; CD83, $p = 0.043$; CD86, $p = 0.018$; CD40, $p = 0.018$). To test whether the differently matured mdDC were capable of activating T cells, MLR were performed. The allostimulatory capacity of mdDC reflected the observed

changes of surface marker expression (Fig. 2). In comparison with unstimulated or rBet v 1-stimulated mdDC, mdDC incubated with rSbsC-Bet v 1 or rSbsC induced enhanced proliferative responses in allogeneic T cells. LPS-stimulated mdDC induced a more pronounced T cell proliferation as compared with rSbsC-Bet v 1 (Fig. 2).

rSbsC-Bet v 1 induces IL-10 and IL-12 production in DC

IL-10 and IL-12 mRNA expression levels in response to the different stimuli were assessed by real-time PCR in mdDC generated from six different birch pollen-allergic donors (Fig. 3). The medium control was used as baseline for calculating the fold induction of IL-10 and IL-12, respectively. The major birch pollen allergen marginally enhanced IL-10 and IL-12 mRNA expression in mdDC. Compared with rBet v 1, rSbsC-Bet v 1 induced a significantly higher mRNA expression for both cytokines ($p = 0.028$, $p = 0.028$) as did LPS ($p = 0.046$, $p = 0.028$; Fig. 3), and stimulation with rSbsC resulted in a significant increase of IL-12 mRNA expression ($p = 0.046$). These results obtained at the mRNA level were confirmed by ELISA of supernatants derived from mdDC stimulated for 48 h (data not shown).

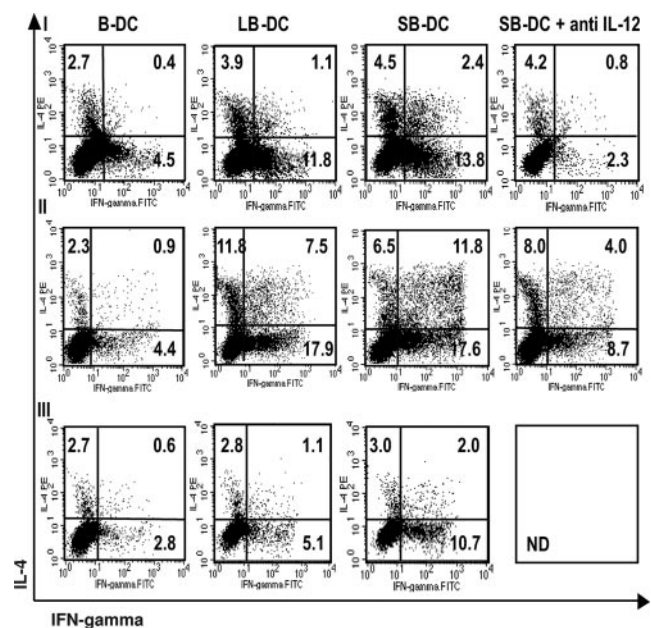


FIGURE 4. DC matured with rSbsC-Bet v 1 promote a Th0/Th1-like response. Naive CD4⁺ T cells were primed with autologous B-DC, LB-DC, and SB-DC in the absence or presence of anti-IL-12. Intracellular synthesis of IFN- γ and IL-4 was determined. Three experiments (patients I–III) of 10 are shown.

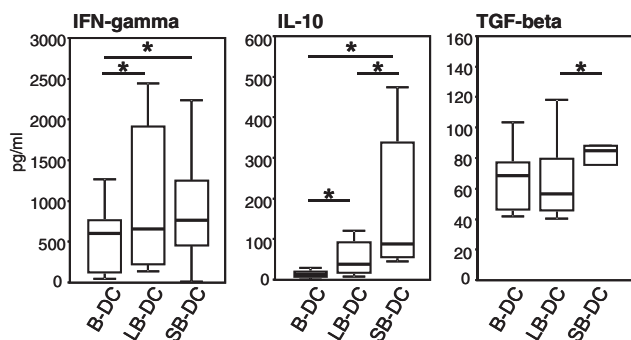


FIGURE 5. T cells primed with mdDC matured with rSbsC-Bet v 1 produce IFN- γ , IL-10, and TGF- β . Naive CD4 $^{+}$ T cells were primed with autologous B-DC, LB-DC, and SB-DC. After stimulation with anti-CD3 plus anti-CD28, supernatants were assessed for cytokines by ELISA. Data from 10 different donors were summarized in box plots. *, $p < 0.05$ (Wilcoxon signed ranks test).

rSbsC-Bet v 1-matured DC promote IL-12-dependent Th0/Th1 differentiation of naive CD4 $^{+}$ T cells

CD4 $^{+}$ CD45RA $^{+}$ cells derived from 10 birch pollen-allergic patients were stimulated twice with autologous mdDC incubated with either rBet v 1 (B-DC), rSbsC-Bet v 1 (SB-DC), or simultaneous addition of LPS and rBet v 1 (LB-DC). T cell polarization was determined by intracellular cytokine detection (Fig. 4). Compared with B-DC, both SB-DC and LB-DC induced significantly higher numbers of IFN- γ -producing T cells ($p = 0.005$; $p = 0.013$) and enhanced numbers of IL-4-producing T cells. Similar to LB-DC, SB-DC induced significantly more IFN- γ^{+} than IL-4 $^{+}$ T cells ($p = 0.005$), reflecting a Th1-like response. Interestingly, significantly higher numbers of IL-4 $^{+}$ IFN- γ^{+} double-positive T cells ($p = 0.017$) were detected in SB-DC-primed T cells as compared with LB-DC-stimulated cultures. The simultaneous addition of neutralizing anti-IL-12 mAb and SB-DC to CD4 $^{+}$ CD45RA $^{+}$ cells drastically reduced the number of IFN- γ -producing CD4 $^{+}$ T cells (Fig. 4).

rSbsC-Bet v 1-matured DC promote the differentiation of regulatory T cells

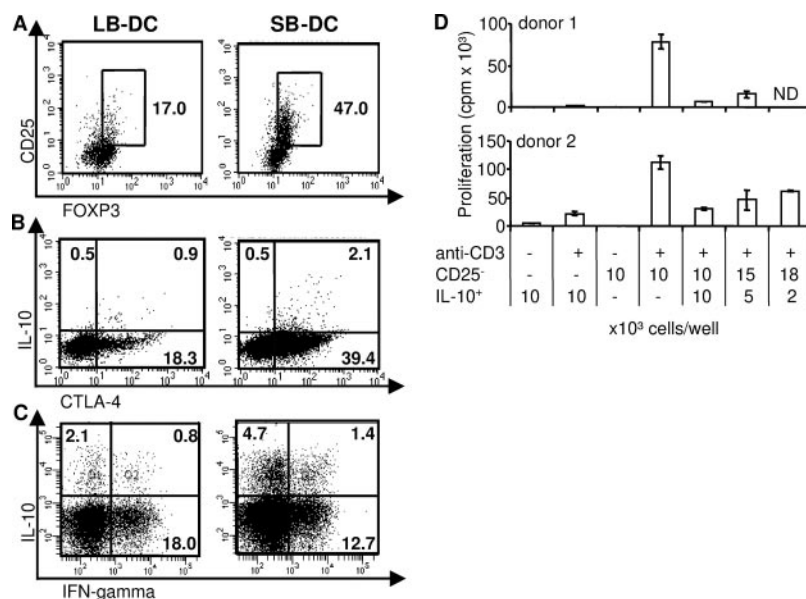
To investigate whether the different DC maturation induced by rSbsC-Bet v 1 and LPS plus rBet v 1 translated into different Th

responses, the production of IFN- γ , IL-10, and TGF- β was quantified in supernatants of anti-CD3-stimulated T cells primed with the differently stimulated mdDC by ELISA (Fig. 5). Compared with B-DC-primed T cells, SB-DC- as well as LB-DC-primed T cells produced significantly higher levels of IFN- γ ($p = 0.037$; $p = 0.047$) and IL-10 ($p = 0.005$; $p = 0.009$). SB-DC-primed T cells synthesized comparable levels of IFN- γ and significantly higher amounts of IL-10 ($p = 0.022$) and TGF- β ($p = 0.028$) than LB-DC-primed T cells. Because these cytokines are typically synthesized by Treg cells, T cells from six individuals were stained for CD25 and intracellular expression of Foxp3 7 days after the second addition of DC. SB-DC-primed T cell cultures contained significantly higher numbers of CD25 $^{+}$ Foxp3 $^{+}$ cells than LB-DC-primed T cells ($p = 0.028$). One representative experiment is shown in Fig. 6A. Parallel staining for CTLA-4 revealed that CD25 $^{+}$ Foxp3 $^{+}$ cells were all CTLA-4 positive (data not shown). To analyze whether IL-10-producing cells expressed these Treg markers, T cells needed to be stimulated before intracellular cytokine detection. Most T cells became Foxp3 positive in response to PMA/ionomycin, whereas the expression of CTLA-4 was hardly affected (data not shown). Therefore, we costained T cells for IL-10 and CTLA-4 and observed that all IL-10 $^{+}$ cells were CTLA-4 $^{+}$. One representative example is shown in Fig. 6B. Next, T cells from four individuals were stained for their anti-CD3-induced secretion of IL-10 and IFN- γ . The majority of IL-10-secreting cells constituted a population different from IFN- γ -secreting cells, but also cells secreting both cytokines were detected. One example is depicted in Fig. 6C. Subsequently, we isolated IL-10-secreting cells from SB-DC-primed T cell cultures from two patients. Proliferative responses of these cells were markedly lower as compared with autologous CD4 $^{+}$ CD25 $^{-}$ cells (Fig. 6D). Coculturing IL-10-secreting T cells with autologous CD4 $^{+}$ CD25 $^{-}$ cells in the presence of irradiated autologous PBMC and anti-CD3 Abs resulted in the suppression of the proliferation of CD4 $^{+}$ CD25 $^{-}$ cells in a dose-dependent manner (Fig. 6D). In two independent experiments, a mean suppression of 83% was observed at a suppressor:responder cell ratio of 1:1 and 74% at a ratio of 1:3, respectively.

Discussion

We have previously shown that genetic fusion of the major birch pollen allergen and a bacterial S layer resulted in a recombinant protein exhibiting reduced mediator-releasing capacity, retained

FIGURE 6. rSbsC-Bet v 1-matured mdDC prime IL-10-producing Treg cells. Naive CD4 $^{+}$ T cells were primed with autologous LB-DC or SB-DC and stained for A, CD25 and intracellular Foxp3; B, intracellular IL-10 and CTLA-4; and C, secretion of IL-10 and IFN- γ assessed by cytokine secretion assays. One representative experiment of each staining is shown. D, SB-DC-induced IL-10-secreting cells were isolated from two donors and cocultured with autologous CD4 $^{+}$ CD25 $^{-}$ cells in the presence of irradiated autologous PBMC and anti-CD3. Mean cpm and SD of triplicates are depicted.



Bet v 1-T cell epitopes, and the potency to induce IFN- γ and IL-10 production in Bet v 1-specific Th2 clones (31). In this study, we analyzed the effects of rSbsC-Bet v 1 on immature DC and naive CD4⁺ T cells isolated from the blood of birch pollen-allergic patients. We demonstrate that DC matured with rSbsC-Bet v 1 promote the simultaneous differentiation of Th0/Th1 cells and IL-10-producing Treg cells. These data further support the concept that recombinant fusion of allergens and S-layer proteins is a promising approach to improve vaccines for SIT of atopic allergies.

In a first step, the effects of rSbsC-Bet v 1 and rSbsC on immature mdDC were analyzed. Stimulation with these proteins resulted in DC maturation, increased T cell stimulatory property, and the production of IL-10 and IL-12 (Figs. 1–3). Thus, the recombinant S-layer protein from *G. stearothermophilus* ATCC 12980 stimulates the innate immune system of allergic patients. These findings are in line with the effects of natural S layers isolated from *Clostridium difficile* on monocytes and mdDC of nonallergic individuals (32). Stimulation of DC with rBet v 1 alone resulted neither in activation nor maturation, indicating that an additional stimulus is required to induce strong immune responses to the recombinant allergen. This activity was introduced by recombinant fusion of rBet v 1 to the S-layer protein. The fact that rSbsC-Bet v 1 displayed stronger effects than rSbsC may be referred to the involvement of Ab receptor-mediated uptake of rSbsC-Bet v 1. We have previously shown that the fusion protein contains relevant IgE-binding epitopes of Bet v 1 (31). It may well be possible that rSbsC-Bet v 1 was recognized by Bet v 1-specific IgE Abs bound to the high affinity receptor Fc ϵ RI on APC, resulting in IgE-mediated uptake in addition to phagocytosis (33).

In a second step, we used rSbsC-Bet v 1-matured DC (SB-DC) to prime naive CD4⁺ T cells from birch pollen-allergic donors and investigated the polarization of the T cell response. To induce an allergen-specific Th1 response, mdDC were primed with rBet v 1 plus LPS (LB-DC). Similar to this positive control, priming of T cells with SB-DC resulted in significantly more IFN- γ than IL-4⁺ cells (Fig. 4). In line with our previous data showing that S-layer-induced IFN- γ synthesis in effector T cells was mediated by IL-12 (34), SB-DC-induced T cell polarization toward IFN- γ production also depended on the presence of this cytokine (Fig. 4). Interestingly, significantly higher numbers of IL-4⁺IFN- γ ⁺ double-positive T cells were detected in SB-DC-primed T cells as compared with LB-DC-primed T cells. These cells may reflect a Th0 phenotype, simultaneously synthesizing Th1 and Th2 cytokines. Thus, rSbsC-Bet v 1 may have the advantage of not promoting exclusive Th1-like immune responses. Nevertheless, potential risks of in vivo administration of rSbsC-Bet v 1 need to be carefully evaluated.

SB-DC primed naive CD4⁺ T cells to synthesize significantly more IL-10 and TGF- β as compared with LB-DC (Fig. 5). Furthermore, higher numbers of IL-10⁺CTLA-4⁺Foxp3⁺CD25⁺ cells were detected in SB-DC-primed cultures (Fig. 6, A–C). Coculturing IL-10-secreting T cells isolated from SB-DC-primed T cells suppressed proliferative responses of autologous CD4⁺CD25⁺ T cells (Fig. 6D). Together, these data provide strong evidence that SB-DC primed naive T cells toward a population of IL-10-producing Treg cells in addition to Th0/Th1 cells. Preliminary studies in our laboratory indicated that rSbsC-Bet v 1 targets TLR2, because HEK293 cells stably transfected with TLR2 produced IL-8 in response to rSbsC-Bet v 1, whereas HEK293 cells transfected with TLR1 or TLR3–9 did not (data not shown). The involvement of TLR2 may promote Treg cells through the induction of IL-10 production in DC (35, 36). However, to date, we observed comparable IL-10 levels produced by DC stimulated with rSbsC-Bet v 1 or LPS at the mRNA (Fig. 3) and protein level.

In contrast, the significantly lower expression of CD40, CD80, and CD86 on the surface of SB-DC as compared with LB-DC may indicate that rSbsC-Bet v 1 does not induce a fully matured DC phenotype (Fig. 1). Immature and semimature DC have been demonstrated to induce IL-10-producing Treg cells (37–39). Whether other potential influences, e.g., enhanced expression of inhibitory molecules of the B7 family or DC-derived TGF- β , are involved in rSbsC-Bet v 1-induced development of Treg cells is currently under investigation.

Our previous (31) and present data strongly suggest that rSbsC-Bet v 1 meets several desired requirements for an ideal allergy vaccine: less IgE-binding and reduced mediator-releasing capacity; immune stimulatory effects on DC; immune modulatory effects on naive and allergen-specific effector T cells; and a constant ratio between allergen and adjuvant that should improve consistency of the product used for SIT. In addition to the induction of a Th0/Th1-like T cell response, rSbsC-Bet v 1 also promoted IL-10-producing Treg cells. Both immune deviation and increased numbers of circulating IL-10-secreting CD4⁺CD25⁺ cells have been associated with successful allergen SIT. Our in vitro data indicate that rSbsC-Bet v 1 will simultaneously support these two immune mechanisms. Due to its hypoallergenic features (31), this fusion molecule may also reduce the risk of IgE-mediated side effects. Therefore, allergens genetically fused to S-layer proteins are promising future vaccines for SIT of atopic allergies.

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Disclosures

The authors have no financial conflict of interest.

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