

Natural Self-Assembly of Allergen-S-Layer Fusion Proteins Is No Prerequisite for Reduced Allergenicity and T Cell Stimulatory Capacity

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Key Words

Allergy • Antigen presentation • Major birch pollen allergen • Recombinant fusion protein • Specific immunotherapy • Vaccines

Abstract

Background: Recombinant allergen-S-layer fusion proteins display a strongly reduced IgE-binding activity and promote the induction of allergen-specific Th0/1 cells and regulatory T cells. Such fusion proteins show a natural capacity to self-assemble into mono- or double-layer sheets reaching particle-like dimensions of 0.5–2 μm . We were interested in finding out whether self-assembly was crucial for the immunological characteristics of allergen-S-layer fusion proteins.

Methods: The IgE-binding and mediator-releasing capacities of nonassembled and self-assembled rSbpA-Bet v 1, consisting of the major birch pollen allergen Bet v 1 and the S-layer protein SbpA, were compared in inhibition ELISA and basophil activation assays using sera from patients allergic to birch pollen. T cell stimulation was evaluated using Bet v 1-specific T cell clones reactive to distinct epitopes of Bet v 1. Autologous B lymphocytes, monocytes and monocyte-derived dendritic cells were employed to evaluate potential differences in uptake and processing by different antigen-

presenting cells. **Results:** Both rSbpA-Bet v 1 variants showed significantly less IgE-binding and mediator-releasing activity than Bet v 1. However, self-assembly further minimized the reduced allergenicity of nonassembled rSbpA-Bet v 1. Both rSbpA-Bet v 1 variants induced comparable proliferation in Bet v 1-specific T cell clones. B cells inappropriately presented either variant of rSbpA-Bet v 1. Self-assembly amplified the T cell stimulatory capacity of monocytes and dendritic cells. **Conclusions:** The promising characteristics of allergen-S-layer fusion proteins regarding their potential use for allergy treatment do not depend on the formation of particle-like structures.

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Introduction

Allergen-specific immunotherapy (SIT) is the only treatment of type I allergies that induces a modulation of the disease-eliciting immune response [1, 2]. Conventional SIT consists of multiple subcutaneous injections of crude allergen extracts into allergic individuals in order to induce a state of clinical tolerance to natural allergen exposure [3]. However, the administration of allergen extracts may induce undesired local and systemic

IgE-mediated side effects [4]. Therefore, different approaches were developed to improve the safety of allergy vaccines. During the past decades, a high number of relevant allergens has been cloned, sequenced and produced in recombinant form [5]. Recombinant allergens were modified into variants with less IgE-binding activity and consequently, a reduced risk of inducing undesired side effects [6–8]. In addition, hybrids consisting of allergens and other proteins were engineered. Such hybrids consisted either of (1) different allergens or allergenic peptides to cover the B and T cell epitope spectrum desired for immunotherapy [9–14]; (2) allergens and cytokines to foster desired immune responses [15] or (3) allergens and bacterial proteins to modulate the allergic response [16]. Each of these hybrids represents a new protein and needs to be analyzed regarding its immunological characteristics and the functionality of the contained allergens.

We have previously characterized rSbsC-Bet v 1, a hybrid consisting of the major birch pollen allergen Bet v 1 and a surface layer (S-layer) protein derived from the nonpathogenic Gram-positive *Geobacillus stearothermophilus* ATCC 12980 [17]. This fusion protein displayed less IgE-binding and reduced histamine-releasing capacity as compared to rBet v 1 and modulated the Th2-dominated cellular response to Bet v 1 towards a Th0/Th1-like response [18]. In addition, rSbsC-Bet v 1 differentiated immature dendritic cells (DCs) from patients allergic to birch pollen into a phenotype that primed naïve CD4⁺ T cells to develop into IL-10-producing T cells with suppressive capacity [19]. Thus, this allergen-S-layer fusion protein was regarded as a promising vaccine candidate for SIT. Isolated S-layer proteins have the intrinsic tendency to self-assemble into flat sheets, open-ended cylinders or closed vesicles and rSbsC-Bet v 1 retained this ability [17, 20]. To evaluate whether the formation of such macromolecular structures determined the immunological characteristics of allergen-S-layer fusion proteins we compared the nonassembled and self-assembled variant of an allergen-S-layer fusion protein regarding its IgE binding, mediator-releasing capacity and T-cell-activating capacity employing Bet v 1-specific T cell clones (TCCs) and different types of autologous antigen-presenting cells (APCs), namely B cells, monocytes and monocyte-derived DCs (mdDCs). In the present study, rSbpA-Bet v 1, containing Bet v 1 fused to a C-terminally truncated form of the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177, was employed because this hybrid shows the advantage of dirigible, calcium-ion-dependent self-assembly [21, 22].

Material and Methods

Patients

Birch-pollen-allergic, Bet v 1-sensitized individuals with allergic rhinoconjunctivitis during spring, specific IgE immunocAP class >3 to birch (Phadia Diagnostics, Uppsala, Sweden) and positive skin prick test reactions to birch pollen (wheal diameter >5 mm, Soluprick; ALK Abello, Hørsholm, Denmark) were included. All participants gave written consent and the study was approved by the local Ethics Committee of the Medical University of Vienna, Austria (EK029/2006).

Proteins

rBet v 1 was purchased from Biomay (Vienna, Austria). rSbpA-Bet v 1 and rSbpA were produced as described [21, 22]. The absence of calcium ions prevents self-assembly of rSbpA and keeps the S-layer protein in the form of monomeric and oligomeric precursors. One day before each set of experiments, nonassembled rSbpA-Bet v 1 was centrifuged at 50,000 g for 20 min and the supernatant containing monomers was divided into two aliquots [21, 22]. One aliquot was substituted with the respective volume of buffer without calcium. CaCl₂ (10 mM final) was added to the second aliquot and was shaken at 1,400 rpm overnight at room temperature to induce self-assembly. Self-assembly was analyzed by electron microscopy [22]. Only preparations showing 100% self-assembly were used as self-assembled samples. Dissolving precursors in culture medium did not induce self-assembly. Equimolar amounts of Bet v 1 in rBet v 1 and rSbpA-Bet v 1 were used, considering that 45.3 µg of rSbpA-Bet v 1 contained 2.5 µg of Bet v 1.

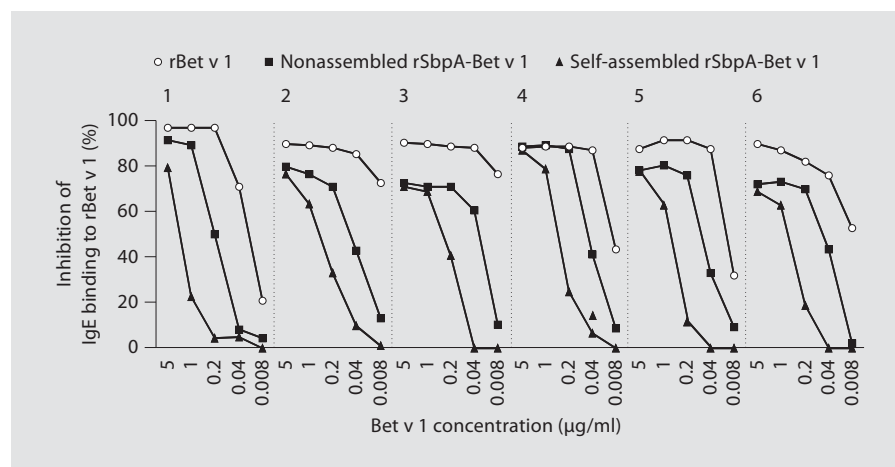
IgE Inhibition ELISA

IgE inhibition ELISA was performed as described previously [18]. Briefly, sera of 5 birch-pollen-allergic individuals and a serum pool of 10 Bet v 1-sensitized individuals with a CAP/RAST >6 to birch pollen were preincubated overnight with different concentrations of rBet v 1 (0.008–5 µg/ml) or nonassembled or self-assembled rSbpA-Bet v 1 containing equivalent amounts of Bet v 1. Adjusted concentrations of nonassembled or self-assembled rSbpA or BSA (20 µg/ml) served as controls. ELISA plates (Nunc, Roskilde, Denmark) were coated with rBet v 1 (1 µg/ml). After saturation with 1% human serum albumin preincubated sera and controls were added and incubated overnight. Bound IgE antibodies were detected by using an alkaline-phosphatase-conjugated mouse anti-human IgE antibody (Ab; BD PharMingen, San Diego, Calif., USA). All tests were performed in duplicates.

IgE-Mediated Mediator Release Assays

Rat basophil leukemia (RBL) cells transfected with the human ϵ receptor I (Fc ϵ RI, RBL-30/25) were used for mediator release assays as described [23]. Briefly, RBL-30/25 cells were incubated overnight with sera from birch-pollen-allergic individuals. After washing, titrated concentrations (1–10^{–5} µg/ml) of rBet v 1, nonassembled or self-assembled rSbpA-Bet v 1 or rSbpA were added to trigger mediator release. The release of β -hexosaminidase was determined according to published protocols [24]. All tests were performed in triplicates.

Fig. 1. IgE inhibition ELISA. Sera of 5 birch-pollen-allergic individuals (1–5) and a serum pool (6) were preincubated with titrated concentrations of rBet v 1 and adjusted concentrations of nonassembled or self-assembled rSbpA-Bet v 1.



Bet v 1-Specific T Cell Lines and Clones

Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood of patients allergic to birch pollen by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation and Bet v 1-specific T cell lines (TCLs) were generated according to published protocols [25]. All cell experiments were performed in serum-free Ultra Culture Medium (BioWhittaker) supplemented with 2 mM glutamine and 2×10^{-5} M β -mercaptoethanol. Briefly, 1.5×10^6 PBMCs were stimulated with 10 μ g/ml rBet v 1 in 24-well culture plates (Costar, Cambridge, Mass., USA). After 5 days, 10 U/ml human rIL-2 (Boehringer Mannheim, Germany) were added and at day 7, T cell blasts were enriched by density gradient centrifugation and seeded into 96-well plates (Nunc, Nunc, Denmark). Irradiated PBMCs (60 Gy) and rIL-2 were added at day 7 and day 14. At day 24, TCLs (0.5×10^5 cells/well) were incubated in triplicates with rBet v 1 (2.5 μ g/ml) in the presence of either autologous B cells (0.5×10^5 per well), monocytes (0.2×10^5 per well) or mDCs (0.05×10^5 per well) [26]. APCs alone with and without stimuli and APCs plus T cells without stimuli served as controls. After 48 h, proliferation was assessed by ^{3} H-thymidine incorporation (0.5 μ Ci per well; Amersham, Little Chalfont, UK). Delta count per minute (dpm) denote counts per minute in stimulated minus counts per minute in unstimulated cultures. All TCCs specific for Bet v 1 were available from previous studies [27, 28]. Cryopreserved TCCs were thawed and expanded at weekly intervals with irradiated PBMCs and rIL-2. TCCs were stimulated with rBet v 1 (2.5 μ g/ml), corresponding amounts of nonassembled or self-assembled rSbpA-Bet v 1 in the presence of either 5×10^4 irradiated autologous PBMCs or the different types of isolated APCs as indicated for TCLs for 48 h in duplicates. The stimulation index (SI) was calculated as ratio between counts per minute obtained in cultures with TCCs and APCs plus stimulators and counts per minute obtained in cultures containing TCCs and APCs.

Isolation of APCs

Five days prior to testing of allergen-specific T cell cultures, 100×10^6 PBMCs were isolated from each individual and incubated with magnetic microbeads coated with anti-CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the man-

ufacturer's instructions. Two consecutive separation columns yielded populations containing >95% CD14+ cells as determined by flow cytometry. From these cells, mDCs were generated using IL-4 and GM-CSF cells as described [19]. After 5 days of culture, the resulting mDC population was homogeneously CD14+HLA-DR+ CD1a+. On the day of testing of allergen-specific T cell cultures, peripheral monocytes and B lymphocytes were prepared from 200×10^6 freshly isolated PBMCs. For this purpose, 100×10^6 PBMCs each were incubated with either anti-CD14-coated microbeads or anti-CD19-coated microbeads. The obtained cell fractions consistently contained >95% CD14+ and >97% CD19+ cells.

Statistical Analysis

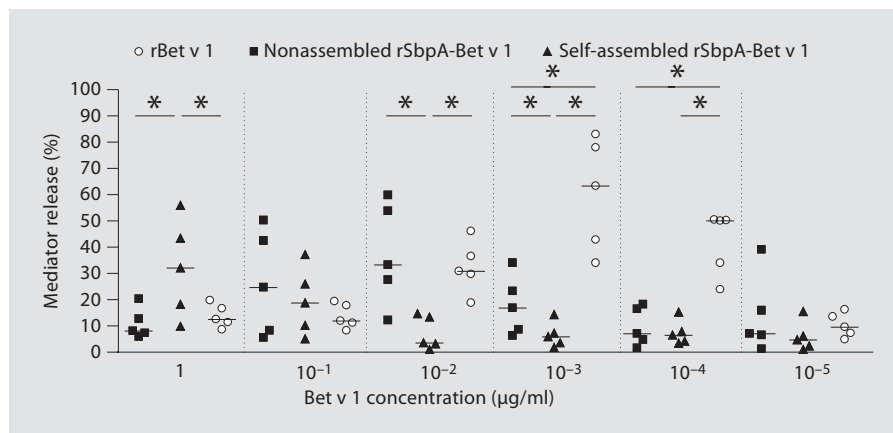
Statistical significance of differences between the individual stimuli was determined by the Wilcoxon signed rank test (SPSS for Windows, version 10.0.7). Differences were considered statistically significant for $p < 0.05$.

Results

IgE-Binding Capacity of Nonassembled and Self-Assembled rSbpA-Bet v 1

Sera were preincubated with different concentrations of rBet v 1 or adjusted amounts of nonassembled and self-assembled rSbpA-Bet v 1 and the inhibition of IgE-binding to rBet v 1 was assessed by ELISA. Overall, each rSbpA-Bet v 1 variant showed a significantly reduced inhibition capacity as compared to free rBet v 1 (fig. 1). Compared to rBet v 1, the p values for nonassembled rSbpA-Bet v 1 were $p = 0.046$ at a concentration containing 5 and 1 μ g/ml of Bet v 1 and $p = 0.027$ at all other concentrations. The p values for self-assembled rSbpA-Bet v 1 were $p = 0.027$ at all concentrations studied. Moreover, at all concentrations, the self-assembled rSbpA-Bet

Fig. 2. Mediator release assays. RBL assays were performed with sera from 5 patients allergic to birch pollen. The percentage of mediator release induced by rBet v 1 or equivalent amounts of nonassembled or self-assembled rSbpA-Bet v 1 are shown. Lines indicate median values (Wilcoxon signed rank test). * $p < 0.05$.



v 1 showed significantly less IgE-binding capacity than the nonassembled fusion protein (all $p = 0.027$). Preincubation of sera with nonassembled and self-assembled rSbpA or BSA as controls had no effect on Bet v 1-specific IgE binding (data not shown).

Mediator-Releasing Capacity of Nonassembled and Self-Assembled rSbpA-Bet v 1

RBL cells expressing the human FcεRI were sensitized with IgE present in sera from patients allergic to birch pollen. For mediator release, Bet v 1 was added at a previously determined optimum concentration range ($1\text{--}10^{-5}$ µg/ml) [23] and compared with nonassembled and self-assembled rSbpA-Bet v 1 containing equivalent amounts of Bet v 1. Consistent with our previous studies, Bet v 1 induced optimum RBL cell degranulation (median: 63.3%) at a concentration of 10^{-3} µg/ml (fig. 2). At this concentration, both variants of rSbpA-Bet v 1 induced significantly less mediator release as compared to rBet v 1 (nonassembled: $p = 0.042$; self-assembled: $p = 0.043$). Both variants also induced significantly less basophil activation at a concentration of 10^{-4} µg/ml of Bet v 1 (both $p = 0.043$), whereas at a concentration of 10^{-2} µg/ml of Bet v 1 only self-assembled rSbpA-Bet v 1 induced significantly less RBL cell degranulation ($p = 0.043$). Optimum mediator release of nonassembled rSbpA-Bet v 1 was observed at a concentration of 10^{-2} µg/ml of Bet v 1 (median: 33.2%). At this concentration self-assembled rSbpA-Bet v 1 induced significantly less mediator release as compared to nonassembled rSbpA-Bet v 1 and free rBet v 1 (both $p = 0.043$). Self-assembled rSbpA-Bet v 1 induced the highest mediator release (median: 32.0%) at the concentration of 1 µg/ml of Bet v 1. At a concentration of 10^{-5} µg/ml, none of the stimuli induced considerable ba-

Table 1. Proliferative responses of Bet v 1-specific TCCs

TCCs	Amino acid residues	Stimulation			
		rBet v 1	nonassembled rSbpA-Bet v 1	self-assembled rSbpA-Bet v 1	rSbpA
WI	4–18	2.3 ¹	2.1	2.7	0.5
SI	4–18	9.2	5.1	5.2	n.d.
MI	19–23	26.2	116.2	194.1	1.2
WIII	34–48	2.2	2.1	2.0	1.5
SII	76–90	4.9	4.6	4.2	0.9
MII	97–111	8.8	7.6	8.8	n.d.
WII	142–153	3.8	2.4	3.0	n.d.
SIV	139–156	5.0	6.7	13.0	n.d.

¹ Stimulation indices are shown. Background values of medium controls ranged from 120 to 543 cpm.

sophil activation. No mediator release was found in response to the nonassembled and self-assembled controls rSbpA at any concentration used (data not shown).

Nonassembled and Self-Assembled rSbpA-Bet v 1 Contain All Relevant T Cell Epitopes of Bet v 1

To test whether self-assembly altered processing and presentation of Bet v 1, 8 cryopreserved Bet v 1-specific TCCs from 3 different patients allergic to birch pollen reactive to relevant epitopes located in different regions of the allergen were thawed, expanded and stimulated with rBet v 1 or nonassembled and self-assembled rSbpA-Bet v 1, respectively (table 1). Self-assembled rSbpA served as negative control. All Bet v 1-specific TCCs proliferated in response to rBet v 1 and to both rSbpA-Bet v 1-variants

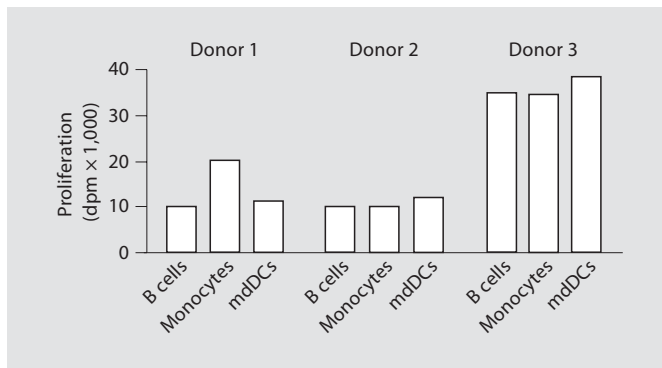


Fig. 3. Proliferative responses of Bet v 1-specific T cells to rBet v 1 in the presence of autologous B cells, monocytes or mdDCs. Background values of medium controls ranged from 2,460 to 7,454 cpm.

but not to rSbpA. Using PBMCs as APCs nonassembled and self-assembled rSbpA-Bet v 1 induced comparable proliferative responses in each TCC (table 1).

Uptake and T Cell Stimulation of Nonassembled and Self-Assembled rSbpA-Bet v 1 by Different APCs

To evaluate whether the formation of molecular lattices affected the uptake of allergen-S-layer fusion proteins by different APCs, we sought to stimulate Bet v 1-specific TCCs with nonassembled or self-assembled rSbpA-Bet v 1 in the presence of purified autologous B cells, monocytes or mdDCs. As the number of expandable Bet v 1-specific TCCs was limited, we adjusted the varying capacity of antigen presentation of these different types of APC by employing Bet v 1-specific TCCs. These cultures were stimulated with rBet v 1 in the presence of autologous B cells (0.5×10^5 per well), monocytes (0.2×10^5 per well) or mdDCs (0.05×10^5 per well), cell numbers that had previously been reported to be optimal for T cell activation [26]. Bet v 1-reactive TCCs and sufficient numbers of APCs of all cell types were obtained from 3 out of 10 birch-pollen-allergic individuals (fig. 3). In general, similar proliferative responses to rBet v 1 were observed, indicating that the chosen cell numbers induced similar T cell activation also in our test system. These results were further supported by data from additional Bet v 1-specific TCCs that could be stimulated with either mdDCs and monocytes or mdDCs and B cells (data not shown).

Four Bet v 1-specific TCCs from 3 patients allergic to birch pollen (M., S., W.) could be expanded for stimulation with nonassembled or self-assembled rSbpA-Bet v 1

and the different types of autologous APCs (fig. 4). B cells, monocytes and mdDCs were available from patient M. to test TCC MII and from patient S. to test TCC SIV. TCC MIII could be tested with mdDCs. As no mdDCs were available from patient W., only freshly isolated B cells and monocytes could be used to stimulate TCC WII. In all experiments, self-assembled rSbpA-Bet v 1 induced stronger proliferative responses than its nonassembled variant (fig. 4). While this difference was marginal when B cells or mdDCs were used as APCs, monocytes promoted a considerably stronger proliferation of all Bet v 1-specific TCCs after incubation with self-assembled rSbpA-Bet v 1. The availability of each type of APC for TCC MI and TCC SIV allowed a ranking of their capacity to present rSbpA-Bet v 1 and revealed an increase in T cell stimulation in the following order: B cells < monocytes < mdDCs. In line, B cells induced less proliferation of TCC SIV as compared to monocytes. This ranking applied to nonassembled as well as self-assembled rSbpA-Bet v 1 (fig. 4).

Discussion

Allergen-S-layer conjugates that self-assemble into monomolecular structures of 0.5–2 μm were shown to display reduced allergenicity and immunomodulatory capacity and are therefore considered as promising candidate vaccines for SIT [18, 19, 29, 30]. However, for therapeutic application, repeated subcutaneous injections would be necessary and the administration of particulate self-assembled allergen-S-layer fusion proteins might induce undesired local side effects such as granuloma formation. Since structural modifications of allergens markedly influence their Ab-binding capacity and the induction of T cell responses due to the preferential uptake by different types of APC [31], we evaluated whether self-assembly was crucial for the immunological characteristics of allergen-S-layer fusion proteins.

Nonassembled and self-assembled rSbpA-Bet v 1 bound significantly less IgE antibodies than equivalent amounts of rBet v 1, indicating that IgE epitopes of the allergen were less accessible within the hybrid molecule independent of self-assembly. Accordingly, both rSbpA-Bet v 1-variants induced significantly less basophil activation as compared to free rBet v 1. Nonassembled rSbpA-Bet v 1 showed an approximately 10-fold and self-assembled rSbpA-Bet v 1 an approximately 1,000-fold reduced mediator-releasing capacity as compared to rBet v 1 (fig. 2). Thus, self-assembly further minimized the al-

ready reduced allergenicity of allergen-S-layer fusion proteins potentially due to the more fixed position of the allergen within the macromolecular structure, resulting in additional steric hindrance of allergen-specific Ab binding.

To compare processing and presentation of nonassembled and self-assembled rSbpA-Bet v 1, Bet v 1-specific TCCs reactive to epitopes located in different regions of the allergen were stimulated with both variants and rBet v 1 (table 1). Comparable proliferative and cytokine responses were observed in all Bet v 1-specific TCCs, indicating that irrespectively of the structure of rSbpA-Bet v 1, all relevant T cell epitopes of Bet v 1 were presented and resulted in T cell activation [25].

To address potential differences in T cell stimulation of nonassembled and self-assembled rSbpA-Bet v 1 by different APCs, autologous B lymphocytes, monocytes or mdDCs were incubated with both variants and Bet v 1-specific TCCs. Beforehand, the cell numbers of the individual APCs were adjusted to obtain comparable proliferative responses to rBet v 1 (fig. 3). Although B cells presented rBet v 1 as efficiently as monocytes and mdDCs, this cell type was rather inappropriate to present either variant of rSbpA-Bet v 1 (fig. 3, 4). The marginal ability of B cells to present allergen-S-layer fusion proteins may indicate that even nonassembled precursors with a molecular weight of 127.5 kDa were not efficiently internalized by pinocytosis. Furthermore, both rSbpA-Bet v 1-variants showed a reduced ability to bind Bet v 1-specific IgE antibodies (fig. 1), impairing IgE-facilitated uptake of allergen-S-layer fusion proteins via allergen-specific IgE bound to the FcεRII (CD23) expressed on the surface of B cells [32, 33]. In accordance, B cells have been shown to fail to present allergoids, which are chemically modified allergens of high molecular weight and reduced IgE-binding capacity [26]. In contrast to B cells, monocytes and immature mdDCs efficiently presented nonassembled and self-assembled rSbpA-Bet v 1, resulting in substantial proliferation of Bet v 1-specific TCCs (fig. 4). These APCs use phagocytosis to internalize antigens [34]. Incubation of monocytes with self-assembled rSbpA-Bet v 1 resulted in markedly enhanced T cell activation as compared to nonassembled rSbpA-Bet v 1 (fig. 4). Monocytes are known to be highly effective in internalizing particulate antigens, e.g. yeast derivatives, killed or live bacteria and apoptotic cells [35]. Apparently, self-assembly into lattices of 0.5–2 µm resulted in a ‘particle effect’ on this type of APC. However, this effect was less evident on immature DCs, which are the most effective APCs for antigen capture and T cell stimulation [36].

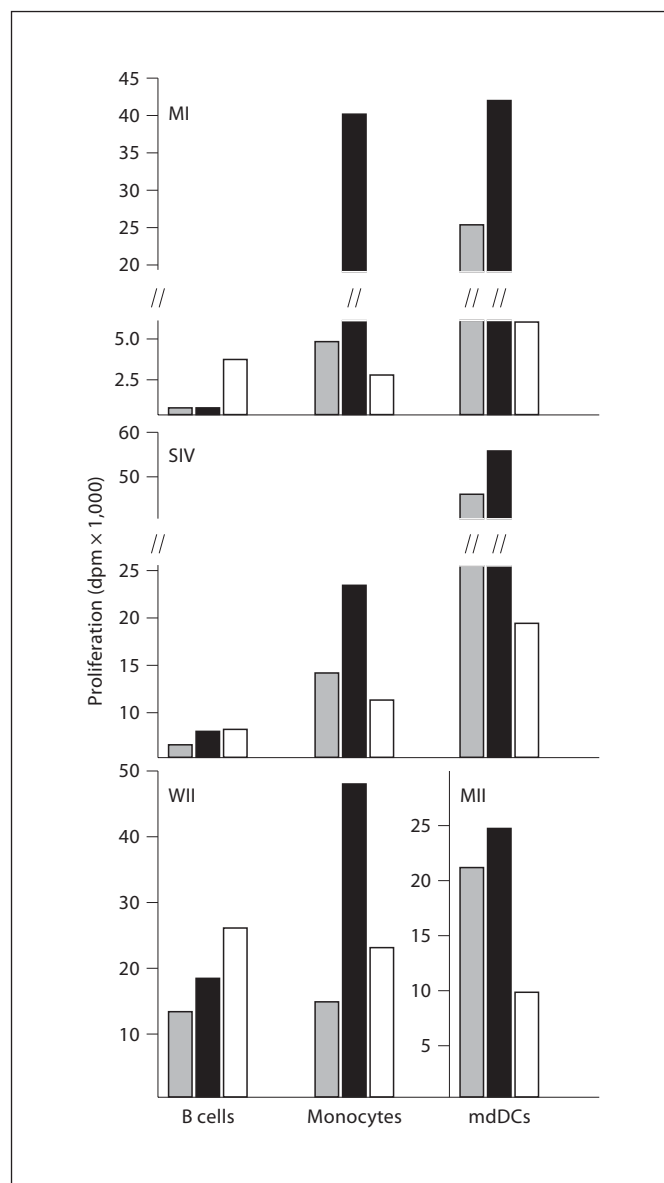


Fig. 4. Proliferative responses of Bet v 1-specific TCCs to nonassembled (white bars) and self-assembled rSbpA-Bet v 1 (gray bars) in the presence of autologous B cells, monocytes and mdDCs. Background values of medium controls ranged from 242 to 483 cpm.

This study provides first evidence that nonassembled and self-assembled allergen-S-layer fusion proteins show similar immunological characteristics. Thus, during SIT, allergen-S-layer fusion proteins may not necessarily have to be administered in self-assembled form in order to show reduced allergenicity and T-cell-stimulatory capacity. Furthermore, our data indicate that different S-layer pro-

teins can be used to engineer allergen-S-layer fusion proteins. rSbsC-Bet v 1 used in previous studies is based on the S-layer protein from *Geobacillus stearothermophilus* ATCC 12980 and rSbpA-Bet v 1 used in the present work contains an S-layer protein derived from *Bacillus sphaericus* CCM 2177. Although both S-layer proteins share less than 36% of amino acid sequence homology, they so far demonstrated comparable immunological characteristics. At present the different allergen-S-layer variants are tested in animal models in order to verify our in vitro results in vivo and to identify the best candidate for SIT.

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