Molecular Immunology 138 (2021) 58–67

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

# Recombinant Bet v 1-BanLec chimera modulates functional characteristics of peritoneal murine macrophages by promoting IL-10 secretion

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ARTICLE INFO

*Keywords:*
Allergy
Bet v 1
Banana lectin
Immunotherapy
Macrophages

ABSTRACT

Allergen-specific immunotherapy (AIT) is a desensitizing treatment for allergic diseases that corrects the underlined pathological immune response to innocuous protein antigens, called allergens. Recombinant allergens employed in the AIT allowed the production of well-defined formulations that possessed consistent quality but were often less efficient than natural allergen extracts. Combining recombinant allergens with an adjuvant or immunomodulatory agent could improve AIT efficacy. This study aimed to perform structural and functional characterization of newly designed recombinant chimera composed of the Bet v 1, the major birch pollen allergen, and Banana Lectin (BanLec), TLR2, and CD14 binding protein, for the application in AIT. rBet v 1-BanLec chimera was designed *in silico* and expressed as a soluble fraction in *Escherichia coli*. Purified rBet v 1-BanLec (33.4 kDa) retained BanLec-associated biological activity of carbohydrate-binding and preserved IgE reactive epitopes of Bet v 1. The chimera revealed secondary structures with predominant β sheets. The immunomodulatory capacity of rBet v 1-BanLec tested on macrophages showed changes in myeloperoxidase activity, reduced NO production, and significant alterations in the production of cytokines when compared to both rBanLec and rBet v 1. Comparing to rBet v 1, rBet v 1-BanLec was demonstrated to be more efficient promoter of IL-10 production as well as weaker inducer of NO production and secretion of pro-inflammatory cytokines TNFα, and IL-6. The ability of rBet v 1-BanLec to promote IL-10 in together with the preserved 3D structure of Bet v 1 part implies that the construct might exert a beneficial effect in the allergen-specific immunotherapy.

## 1. Introduction

Type one allergy is one of the most common chronic immune disorders, affecting almost 30 % of the worldwide population (Han et al., 2021; Valenta et al., 2018). Pollen grains are one of the most common sources of aeroallergens. Bet v 1 is the prominent white birch pollen (*Betula verrucosa*) allergen, and it represents the leading cause of type one allergies (Ipsen and Løwenstein, 1983). The production of specific IgE (sIgE) targeting Bet v 1 upon the first contact with birch pollen (sensitization phase) is the hallmark of the birch pollen allergy. Bet v 1 sIgE antibodies bind to the high-affinity receptors on the effector cells (basophils and mast cells). Upon each new entrance of the allergen a cross-linking of anchored sIgE occurs, which triggers intracellular signaling and a release of biologically active mediators (histamine, leukotrienes, prostaglandins, and cytokines) in the effector phase. Over the years, several studies with different approaches have tried to induce allergy desensitization with recombinant Bet v 1 (rBet v 1) or its derivatives (Grönlund and Gafvelin, 2010; Klimek et al., 2015; Spertini et al., 2016; Tourdot et al., 2013; Vrtala et al., 2011). It has been demonstrated that treatment with rBet v 1 induced decrease in skin sensitivity to birch pollen extract (Pauli et al., 2008). However, patients treated with rBet v 1 experienced more side effects, and it was not as efficient as the treatment with birch pollen extract (Pauli et al., 2008). Available data regarding the length of the treatment and the risk of the side effects, imply a need for improvement in the efficacy of allergen-specific immunotherapy (AIT) of birch pollen allergy (Pointner et al., 2020).

AIT was introduced by Noon in 1911 (Noon, 1911). Until nowadays,

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https://doi.org/10.1016/j.molimm.2021.06.015
Received 16 January 2021; Received in revised form 27 May 2021; Accepted 22 June 2021
Available online 4 August 2021
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it is the only therapeutic option for the induction of long-term allergen tolerance. AIT induces disease-modifying effects, which lead to a decrease in disease severity, reduced drug usage, and prevention of further allergen sensitization (Akdis and Akdis, 2011). However, despite the benefits of AIT, it is necessary to improve therapeutic strategy in terms of clinical outcome, therapy duration, and side effects (such as anaphylaxis and sensitization to minor allergens from natural extracts) (Satitsuksanoa et al., 2018). The mechanism of AIT is not completely clear. AIT is associated with the induction of IL-10- and TGF-β-producing T regulatory cells (Tregs) and has the potential to inhibit Th2- type responses (Satitsuksanoa et al., 2018). During AIT, secretion of allergen-specific IgG4 antibodies also occurs. Specific IgG4 is essential since it competes with IgE for allergen binding, suppressing allergic response (Groh et al., 2017). It is also demonstrated that dampening of the expression of the CD66b and myeloperoxidase activity in human neutrophils contributes to AIT's positive effects (Aroca et al., 2014).

The first generation of formulations for AIT was mainly based on allergens' natural extracts (Bousquet et al., 1998). Replacement of allergens extracted from natural sources by recombinant ones allowed the production of well-defined formulations that possessed consistent quality but were often less efficient than natural extract. It is assumed that the improvement in AIT efficacy could be made by combining allergens with adjuvants or immunomodulatory agents (Johansen et al., 2005). Banana lectin (BanLec) is a mannose-specific protein that exerts an immunomodulatory effect (Gavrovic-Jankulovic et al., 2008; Sansone et al., 2016; Stojanović et al., 2010), including modulation of the functional characteristics of macrophages (Marinkovic et al., 2017). This modulation most likely occurs through the binding of oligosaccharide structures on TLR2 and CD14 (Marinkovic et al., 2017). Also, it has been demonstrated that BanLec induces secretion of IgG4 antibodies (Koshte et al., 1992), which are otherwise produced instead of IgE during the allergen-specific immunotherapy (Groh et al., 2017). Furthermore, the capacity of BanLec to promote balanced Th1 skewing of the immune response (Stojanović et al., 2010) might also contribute to the alleviation of the allergic reaction (Peng et al., 2019).

Recombinant chimera rBet v 1-BanLec was created in silico, produced in *E. coli*, and its structural and functional characteristics were evaluated. The immunomodulatory potential of rBet v 1-BanLec was examined *in vitro* on murine peritoneal macrophages, focusing on its potential application for AIT.

## 2. Materials and methods

### 2.1. Design of rBet v 1-BanLec chimera

The 3D-model structure of the rBet v 1-BanLec construct was designed from the crystal structure of a variant of the prominent birch pollen allergen Bet v 1, protein data bank (PDB) entry 4BK7 (Kofler et al., 2014) (chain A containing 159 amino acid residues) and the crystal structure of a recombinant sugar-binding protein - banana lectin, PDB entry 5EXG (Hopper et al., 2017) (chain A containing 142 amino acid residues) by combining the molecular modeling and molecular mechanics. A glycine-proline-glycine-proline (GPGP) linker is constructed between the Bet v 1 and BanLec to prevent steric interference. Simultaneously, LE residues derived from the restriction site and a 6His tag is added at the C-terminus of the BanLec protein for the purification purpose. rBet v 1-BanLec chimera with GPGP linker and the 6His tag were modeled in Discovery Studio 19.1.0. The computer-generated rBet v 1-BanLec chimeric structure is obtained by connecting the four fragments and adding the hydrogen atoms, followed by the full structural minimization in NAMD 2.9 (Phillips et al., 2005) with CHARMM22 force field (MacKerell et al., 1998) in the implicit water model for 100 ps.

### 2.2. Expression and purification of the recombinant Bet v 1- BanLec chimera

For the design of the recombinant construct rBet v 1-BanLec sequences encoding Bet v 1a isoform of the birch pollen allergen (GenBank: CAB02159.1) and recombinant Banana Lectin (GenBank: ADW77219.1) were used. The resulting construct gene was purchased in pUC57 vector from Synbio Technologies (New Jersey, USA), and was cloned into the pET23b vector using NdeI and XhoI restriction endonucleases (ThermoFisher Scientific, Waltham, Massachusetts, USA). Gene insertion was confirmed by restriction analysis of the recombinant plasmid (pET23b-Bet v 1-BanLec) using the same set of restriction enzymes.

Expression of rBet v 1-BanLec was done in BL 21-CodonPlus (DE3)-Ripl cells (Agilent Technologies Inc., La Jolla, USA) in Luria-Bertani liquid medium (LB medium) (10 g/L tryptone (Institute of Virology, Vaccines and Sera Torlak, Belgrade, Serbia), 5 g/L yeast extract (Institute of Virology, Vaccines and Sera Torlak, Belgrade, Serbia), 5 g/L NaCl (Beta Hem, Belgrade, Serbia), pH 7.5) supplemented with 100 mg/L ampicillin (Carl Roth Germany), 25 mg/L kanamycin (Carl Roth Germany), 25 mg/L chloramphenicol (Carl Roth Germany). A single colony containing pET23b-rBet v 1-BanLec vector was inoculated in 5 mL of LB medium containing antibiotics. The culture was left overnight at 37 °C in a shaking incubator (Bio San, Medical-Biological, Research and Technology, Riga, Latvia). Five mL of the overnight culture was inoculated in 1 L of sterile LB medium with respective antibiotics at 37 °C under constant shaking (250 rpm). When absorbance at 600 nm (OD600) reached 0.7, the medium temperature was lowered to 22 °C and expression was induced using 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (ThermoFisher Scientific, Waltham, Massachusetts, USA). Cells were grown at 22 °C overnight under constant shaking (250 rpm).

After harvesting the cells by centrifugation (3000 ×, 25 min at 4 °C, Eppendorf centrifuge 5430R, Hamburg, Germany), cells were resuspended in lysis buffer (20 mM Tris-HCl (Merck KGaA, Darmstadt, Germany)), 150 mM NaCl, 5 mM glucose (Beta Hem, Belgrade, Serbia), pH 7.8). To extract soluble protein fraction it was sufficient to mechanically disrupt cells by sonication in an ice water bath (10 × 10 s at 30 W, Branson sonifier 150 (Branson Ultrasonic SA, Carouge, Switzerland)). BanLec can bind glycans, such as mannose and glucose. For purification of the rBet v 1-BanLec Sephadex G-75 (branched α-1,6 and α-1,3 glucan polymer) was used. This purification strategy provided better purity and protein yield than immobilized metal affinity chromatography (IMAC). Soluble protein fraction was applied onto affinity matrix Sephadex G-75 (GE Healthcare Life Sciences, Tokyo, Japan) in batch for 1 h at room temperature. The matrix was previously equilibrated with 20 mM Tris-HCl, 150 mM NaCl, 5 mM glucose, pH 7.8, in batch for 1 h at room temperature. The elution of the protein was done on a homemade column (12 mm × 20 mm). The bound fraction was eluted using 20 mM Tris-HCl, 150 mM NaCl, 0.3 M glucose, pH 7.8. After SDS-PAGE under reducing conditions, eluted fractions were pulled together and dialyzed against 20 mM Tris-HCl, 150 mM NaCl, 5 mM glucose, pH 7.8. After that, rechromatography was performed under the same conditions as chromatography. The level of protein purity was analyzed by SDS-PAGE under reducing conditions.

The concentration of the purified chimera was measured spectrophotometrically at 280 nm (A280). The extinction coefficient (26,360 M-1 cm-1) and molecular mass of the rBet v 1-BanLec (33458.67 Da) were calculated based on the amino acid sequence using online tools (https://web.expasy.org/protparam/). Fractions containing purified protein were dialyzed against 20 mM ammonium bicarbonate buffer, pH 8.0. Lyophilized protein was stored at −20 °C until further experiments.

Recombinant BanLec (rBanLec) was produced according to the previously published procedure (Gavrovic-Jankulovic et al., 2008). Recombinant Bet v 1 (rBet v 1) was produced in *E. coli* as a His-tagged protein and immobilized metal affinity chromatography (IMAC) was

used for purification (Spriestersbach et al., 2015).

### 2.3. Circular dichroism

Circular dichroism (CD) was performed to evaluate the overall secondary structures in the purified recombinant chimera. CD spectra were recorded at 25 °C using Jasco J-815 A spectrophotometer (Jasco, Japan). The measurement was performed in a far-ultraviolet region (180−260 nm) in a 0.5 mm wide cuvette. Data were collected at a rate of 50 nm/min. The spectra represent the average of three accumulations, and the baseline was corrected by subtraction of blank buffer. The proteins samples were prepared by rehydration of lyophilized proteins in 20 mM Tris-HCl buffer, pH 8.0. The concentrations of the rBet v 1-BanLec, rBanLec and rBet v 1 were 1 mg/mL.

### 2.4. Size exclusion chromatography

Size-exclusion chromatography was performed using a Superdex 75 PC 3.2/30 column (V = 2.4 mL, GE Healthcare, Little Chalfont, UK) connected to Äkta Purifier (GE Healthcare, Little Chalfont, UK). Equilibration and elution were performed using the same eluent (20 mM Tris-HCl, 150 mM NaCl, 0.3 M glucose, pH 8.0) with the flow rate 0.02 mL/min column pressure limit 2.4 MPa, at room temperature. The sample was prepared by dialysis against the eluent. The concentration of the chimera after dialysis was 1 mg/mL. The elution profile was monitored at 280 nm.

The standard samples: BSA (66,5 kDa) (Serva Electrophoresis GmbH, Heidelberg, Germany), ovalbumin (OVA 43 kDa) (Merck KGaA, Darmstadt, Germany) and lysozyme (14.4 kDa) (Merck KGaA, Darmstadt, Germany) were separated under the same conditions. Using elution volumes, and molecular weight of the standards (BSA, ovalbumin and lysozyme) the calibration curve was constructed.

### 2.5. IgE reactivity of Bet v 1 -BanLec

Approval for this research was obtained by the Ethics Committee of University Children's Hospital, University of Belgrade, Belgrade (Approval number 1575/7). To test IgE reactivity of rBet v 1-BanLec chimera in immunoblot, the purified protein was resolved onto 14 % polyacrylamide gel and transferred onto nitrocellulose (NC) membrane (Whatman, Maidstone, United Kingdom) at 2 mA/cm$^2$, 45 min. The membrane was cut into stripes and blocked in 2% bovine serum albumin (BSA) (Serva Electrophoresis GmbH, Heidelberg, Germany), 50 mM glucose in tTBS (20 mM Tris-HCl, 150 mM NaCl, 0,2% Tween 20, pH 7.4), overnight at room temperature. The membrane strips were incubated with sera from seven patients allergic to birch pollen, diluted with TBS to final IgE concentration of approximately 6 kU/L), one serum from a non-atopic patient as a negative control, and one strip was incubated in TBS only as control of secondary antibody. Synopsis of history details and allergy diagnostic tests of patients are presented in Table 1. Specific IgE was detected using monoclonal anti-human IgE-alkaline phosphatase (AP) antibody (A3076 Merck KGaA, Darmstadt, Germany) for 2 h at room temperature. IgE reactivity toward recombinant chimera was detected using nitro-blue tetrazolium and 5-Bromo-4-chloro-3-indolyphosphate (BCIP/NBT, Serva, Heidelberg, Germany) in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.5).

As a positive control, immunoblot was performed under the same conditions with rBet v 1.

### 2.6. Enzyme-Linked lectin assay (ELLA)

The recombinant Bet v 1- BanLec construct, rBanLec, and rBet v 1 were added to the 96- wells MaxiSorp (MaxiSorp; Nunc, Roskilde, Denmark) microtiter plate, respectively. The plate was previously coated with 10 μg per well of horseradish peroxidase glycoprotein (HRP; Serva, Heidelberg, Germany) in 15 mM $Na_2CO_3$/35 mM $NaHCO_3$, pH 9.5, overnight at 4 °C. After blocking with 1 % BSA (Serva Electrophoresis GmbH, Heidelberg, Germany) in 0.1 %, Tween 20 in PBS (tPBS) for one hour at 37 °C, different concentrations of the chimera, rBanLec or rBet v 1 were added (10−10$^{-4}$ μg per well) to the plate. Incubation lasted for 1 h at room temperature. Using rabbit polyclonal anti-BanLec antisera (1:5000) and anti-rabbit antisera produced in goat labeled with AP (1:1000, Merck KGaA, Darmstadt, Germany), the interaction with HRP carbohydrate moieties was detected. Visualization was obtained by incubation with a substrate for alkaline-phosphatase p-nitrophenyl phosphate (1 mg/mL in diethanolamine buffer pH 9.6) for 1 h at 37 °C. The reaction was stopped with 1 M NaOH. The absorbance was measured at wavelength 405 nm ($A_{405}$) using an ELISA reader (Multiskan Ascent, Labsystems, Helsinki, Finland). After each incubation step, the plate was washed with tPBS four times five minutes each.

**Table 1**
Patient's characteristics.

| # | Age | Sex | History of birch pollen allergy | sIgE birch (kU/L) | Skin-prick test birch (nm) | Symptoms |
|---|---|---|---|---|---|---|
| 1. | 18 | Male | Last 5 years | class IV (32.7) | 8 × 20 | Allergic rhinitis |
| 2. | 18 | Female | Last 6 years | class IV (19.5) | 5 × 28 | Allergic rhinitis |
| 3. | 10 | Female | Last 2 years | class VI (102.7) | 15 × 30 | Allergic rhinitis |
| 4. | 15 | Female | Last 5 years | class IV (46.2) | 10 × 20 | Allergic rhinitis |
| 5. | 17 | Male | Last 3 years | class II (3.04) | 3 × 15 | Allergic rhinitis |
| 6. | 14 | Male | Last 5 years | class V (53.4) | 10 × 25 | Allergic rhinitis |
| 7. | 14 | Female | Last 5 years | class III (13.4) | 5 × 15 | Allergic rhinitis |
| 8. | 27 | Female | – | – | – | – |

### 2.7. Functional testing: stimulation of peritoneal macrophages

Functional testing of the produced rBet v 1-BanLec chimera was performed in the culture of peritoneal macrophages of BALB/c mice. Resident (RM) and thioglycolate-elicited (TGM) peritoneal macrophages were collected according to the previously described procedure (Marinkovic et al., 2017). All interventions conformed to the Serbian laws and European regulations on animal welfare and were approved by the Ethics Committee for the Welfare of Experimental Animals (Approval No. 323-07-03902/2017-05/1). RMs and TGMs were plated into 24-well flat-bottomed tissue culture plates (1 × 10$^6$ cells/mL, 1 mL per well) and allowed to adhere (37 °C, 5% $CO_2$, overnight). Non-adherent cells were discarded by washing the plates with warm RPMI 1640 medium. Fresh RPMI 1640 medium containing specific stimulator (rBanLec, rBet v 1 or rBet v 1-BanLec in defined concentration) was added into the wells (1 mL per well). All stimulators were assessed in three concentrations: 13.7 nM (equal to 0.20 μg/mL rBanLec, 0.26 μg/mL rBet v 1 and 0.46 μg/mL rBet v 1-BanLec), 68.6 nM (equal to 1.00 μg/mL rBanLec, 1.28 μg/mL rBet v 1 and 2.29 μg/mL rBet v 1-BanLec), and 342.9 nM (equal to 5 μg/mL rBanLec, 6.40 μg/mL rBet v 1 and 11.45 μg/mL rBet v 1-BanLec). Five samples of RMs and TGMs were stimulated by each stimulator in defined concentration. RMs (n = 5) and TGMs (n = 5) incubated in 1 mL per well RPMI 1640 medium without any stimuli were also included in the test. All stimulators, in working concentrations, were determined by limulus amoebocyte lysate assay (Charles River Laboratories, USA) to contain endotoxins in concentration below 0.5 ng/mL (0.43 ng/mL for rBanLec, 0.47 ng/mL for rBet v 1, and 0.4 ng/mL for rBet v 1-BanLec, respectively).

After 48 h long incubation, supernatants of stimulated and non-stimulated RMs and TGMs were collected and further used to analyze cytokines' production (IL-10, IL-6, and TNFα) and NO. Each supernatant

was analyzed in duplicate in a specific test. The concentration of IL-10, IL-6, and TNFα in supernatants were determined by sandwich ELISA, using cytokine-specific commercially available monoclonal antibodies (Marinković et al., 2016). NO production, an indicator of nitric oxide synthase activity, was quantified by Griess reagent (Green et al., 1982). Assessment of myeloperoxidase (MPO) upon specific stimulation of RMs and TGMs was performed in a flat-bottom 96-well tissue culture plates. Hence, all previously described solutions (the suspensions of cells, RPMI medium with or without stimulators) were added in volume 100 μL per well, following the above-described procedures. After 48 h long incubation, the supernatant was discarded, and plates were rinsed twice (200 μL per well warm PBS followed by centrifugation for 5 min at 250 × g). Enzymatic activity of MPO was measured in cell lysates (50 μL per well 0.1 % Triton X-100 / PBS containing protease inhibitors, shaking gently for 30 min at room temperature) of specifically stimulated RMs and TGMs. The MPO activity assessment was based on the oxidation of *o*-phenylenediamine (OPD; Merck KGaA, Darmstadt, Germany). OPD-containing solution (1 mg/mL OPD, 0.01 % hydrogen peroxide, 50 mM citric acid, pH 5) was added into the wells. The reaction was allowed to proceed at room temperature for 30 min and then was stopped by adding 1 M sulfuric acid. The absorbance was measured at wavelength 492/620 nm ($A_{492/620}$) using an ELISA reader (Multiskan Ascent, Labsystems, Helsinki, Finland).

### 2.8. Statistics

Results are presented as a mean value ± standard error (n = 5). Statistical significances of changes in the production of cytokines and NO and MPO activity were determined by one-way ANOVA test followed by Bonferroni's multiple comparisons test. $P < 0.05$ was set as a limit of significance.

## 3. Results

### 3.1. Design of rBet v 1-BanLec chimera

The molecular modeling and design of the rBet v 1-BanLec construct is done in silico, and the depiction of the final minimized structure is made in Discovery Studio 19.1.0 (Fig. 1).

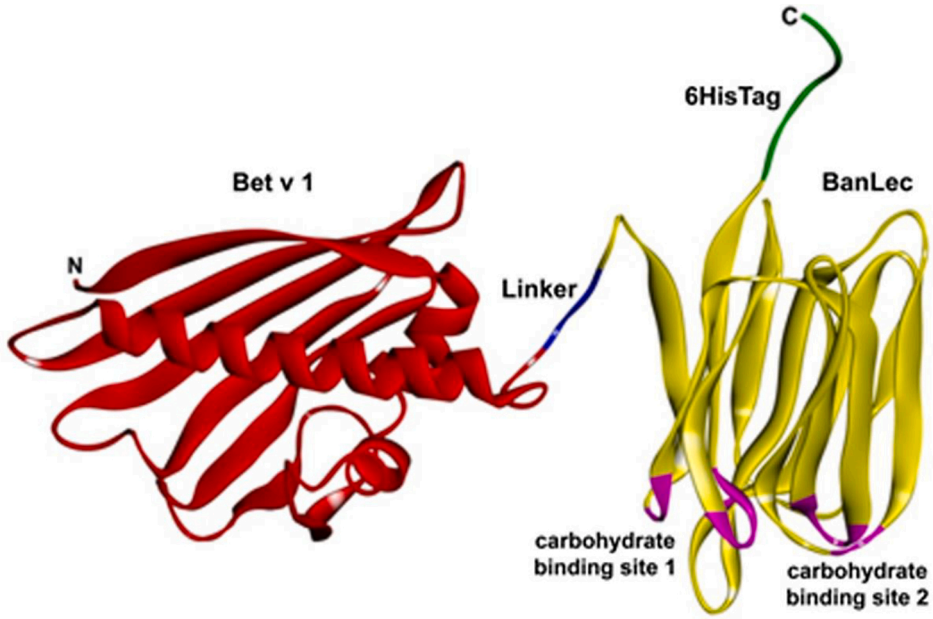
The optimized resulting structure is strain-free, fully minimized, and relaxed without any bond, angle, torsion angle restraints, and van der Waals (VdW) atomic clashes. The final Bet v 1-BanLec structure (Fig. 1) comprises 313 amino acids, encoded for the Bet v 1 pollen allergen, linker GPGP, and BanLec with the 6His tag C terminus.

The short linker part in the rBet v 1-BanLec chimera efficiently keeps the two protein domains distant, preventing a considerable distortion of the secondary and tertiary structure due to the strong electrostatic and VdW (hydrophilic and hydrophobic) interactions.

Additionally, we have highlighted all residues that belong to the rBanLec domain's carbohydrate-binding sites for further analysis and discussion, as indicated by Meagher et al. (Meagher et al., 2005). There are no visible steric hindrances and atomic clashes between the two main protein domains.

### 3.2. Expression and purification of recombinant Bet v 1-BanLec chimera

Binding of the recombinant protein to the Sephadex G-75 (branched α-1,6 and α-1,3 glucan polymer) matrix at low glucose concentration in




A)

MGVFNYETETTSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFPEG
FPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNKYHT
KGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYNGPGPMNGAIKVGAWGGNGG
SAFDMGPAYRIISVKIFSGDVVDAVDVTFTYYGKTETRHFGGSGGTPHEIVLQEGEYLVG
NKGEFGNYHGVVVVGKVGFSTNKKSYGPFGNTGGTTFSLPIAAGKISGFFGRGGDFIDAI
GVYLEPLEHHHHHH

B)

**Fig. 1. A)** Model structure of rBet v 1-BanLec Bet v 1 is shown in red, BanLec in yellow, GPGP linker in blue, carbohydrate-binding sites in purple, and six His colors in green. **B)** Amino acid sequence of the rBet v 1-BanLec.

the buffer, and its elution using a buffer with high glucose concentration (described in materials and methods) was also a confirmation that the protein retained structure and function of lectin domain. Purified rBet v 1-BanLec was resolved by SDS-PAGE, where only one band of about 33 kDa was detected (Fig. 2A). The yield of the recombinant rBet v 1-BanLec was 0.3 g per L of the expression. Size-exclusion chromatography addresses whether the recombinant chimera rBet v 1-BanLec forms a monomeric or multimeric structure in solution. The chromatogram showed a single sharp peak (Fig. 2B). Elution volumes of the standard proteins were used to create a calibration curve (Fig. 2C) to calculate the rBet v 1-BanLec molecular weight. The size-exclusion chromatography revealed that rBet v 1-BanLec has a molecular mass of approximately 67 kDa, while SDS-PAGE under reducing conditions showed a band of about 33 kDa. These results indicate that rBet v 1-BanLec forms a dimer in a solution.

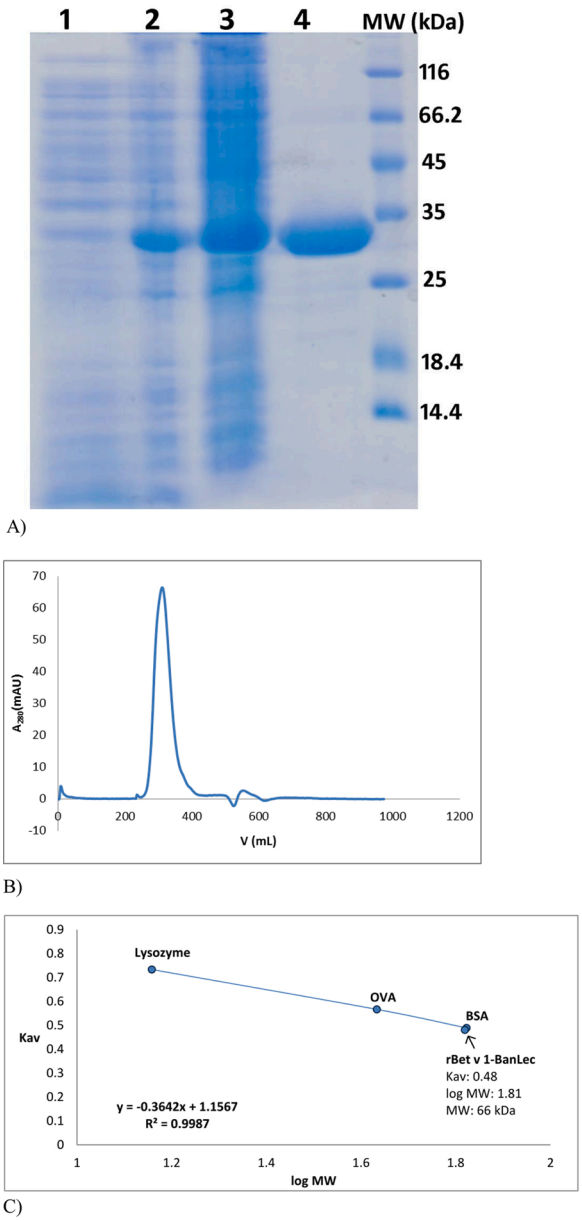



**Fig. 2. A)** SDS-PAGE of the recombinant Bet v 1-BanLec chimera. Samples were separated on 14 %, reducing SDS-PAGE, after which the gel was stained with CBB R 250. Lines: 1) cell lysate before the addition of IPTG, 2) cell lysate after overnight expression, 3) supernatant after sonication of cells, 4) purified protein after chromatography on Sephadex G-75 matrix, MW) unstained protein molecular weight marker (ThermoFisher Scientific, Waltham, Massachusetts, USA). **B)** Size-exclusion chromatogram after separation of rBet v 1-BanLec chimera carried out onto Superdex 75 PC 3.2/30.3.3. **C)** Standard curve obtained by BSA, ovalbumin, and lysozyme as standard proteins.

### 3.3. CD spectra analyses

CD was employed to analyze secondary structures of the purified protein. An obtained spectrum, measured in the far UV region, is characteristic of the protein-containing β-sheets. The CD spectra (Fig. 3) revealed a minimum of about 215 nm and a maximum of 197 nm. These values indicate the presence of β- strands in the structure of the recombinant chimera. Structural data for rBet v 1 obtained using X-ray diffraction and NMR spectroscopy revealed the presence of seven antiparallel β-sheets that wraps around α-helix at the C-terminus (Gajhede et al., 1996). BanLec comprises twelve β-sheets organized into three groups with four antiparallel β-sheets, and every group represents one side of a prism with pseudo-three-fold symmetry (Meagher et al., 2005).

### 3.4. IgE reactivity of rBet v 1-BanLec chimera in immunoblot

IgE reactivity of the recombinant rBet v 1-BanLec was tested in immunoblot using the serum of patients between 10–18 years old, suffering from birch pollen allergy. The recruited patients are in the age group in which the introduction of AIT is essential to avoid asthma development. The level of specific IgE antibodies to birch pollen is presented in Table 1, while the profile of IgE reactivity is shown in Fig. 4. The intensity of bands indicating serum IgE binding to rBet v 1-BanLec (Fig. 4A) correlates to the level of specific IgE detected in the patient's sera and the profile obtained with Bet v 1 as a positive control (Fig. 4B). The strong IgE reactivity was found for sera no. 1-4 and no. 6. Sera no. 5 and no. 7 showed weak IgE reactivity as they contained a relatively low level of specific IgE (3.04 kU/L, class 2 and 13,4 kU/L, class 3, respectively). The obtained results indicate that the antigenic structure of Bet v 1 is preserved in rBet v 1-BanLec chimera.

### 3.5. rBet v 1-BanLec binding to the oligosaccharide structures on the HRP

Enzyme-linked lectin assay (ELLA) was performed to test the rBet v 1-BanLec carbohydrate-binding activity. HRP glycoprotein was coupled to the microtiter plate. After adding increasing concentrations of the proteins, their bindings were detected using rabbit anti-BanLec antibodies, and alkaline phosphatase labeled goat anti-rabbit antibodies. We observed for both rBet v 1-BanLec and rBanLec an increased absorbance in a dose-dependent manner. On the other hand, there was no change in absorbance for rBet v 1 (Fig. 5).

### 3.6. Myeloperoxidase activity and production of NO by peritoneal macrophages upon rBet v 1-BanLec stimulation

Analyses of MPO activity in peritoneal macrophages stimulated by a

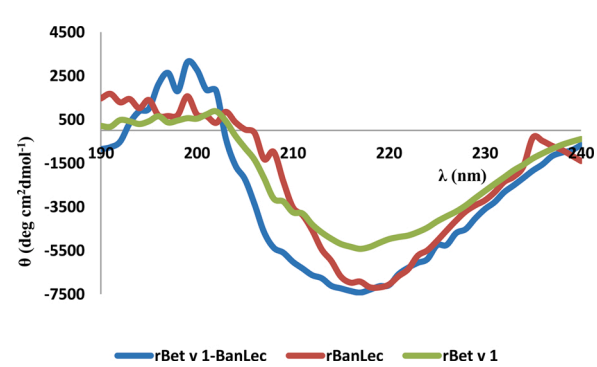



**Fig. 3.** CD spectra of rBet v 1-BanLec (blue line), rBanLec (red line) and rBet v 1 (green line) in the far ultraviolet region (180-260 nm).

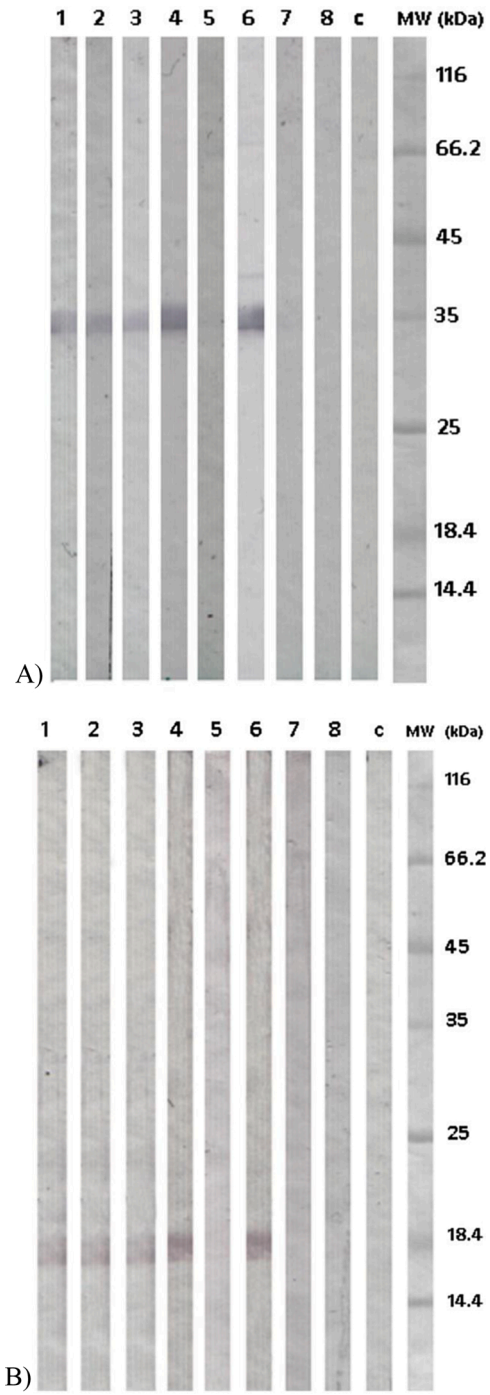



**Fig. 4.** IgE reactivity of the sera from birch pollen allergic patients to **A)** rBet v 1-BanLec, **B)** rBet v 1, 1-7) patients sera presented in Table 1, 8) non-atopic sera, C) control of the secondary antibody (monoclonal anti-human IgE-AP), MW) unstained protein molecular weight marker (ThermoFisher Scientific, Waltham, Massachusetts, USA).

defined amount of rBet v 1-BanLec chimera revealed some, but not statistically significant, differences in responses due to functional characteristics of macrophages (Fig. 6A; P > 0.05 for comparisons between RMs and TGMs cultures stimulated by rBet v 1-BanLec in same concentration). Contrary to the stimulation with rBanLec that exerted a potential to enhance MPO activity in RMs in a dose-dependent manner (non-stimulated vs. 342.9 nM rBanLec P > 0.05), stimulation of RMs by rBet v 1-BanLec chimera did not significantly alter MPO activity comparing to non-stimulated RMs. Although an increase in MPO activity

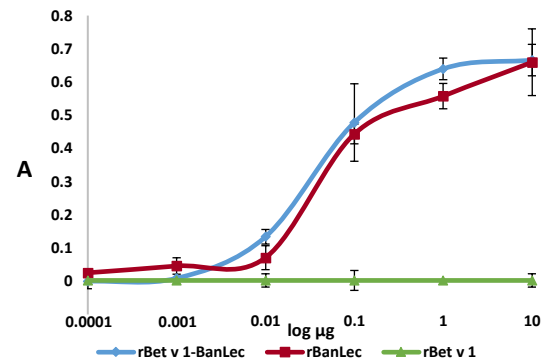



**Fig. 5.** Binding of rBet v 1-BanLec (blue line), rBanLec (red line) and rBet v 1 (green line) to HRP glycoprotein in ELLA. Results are presented as means +/- standard deviation.

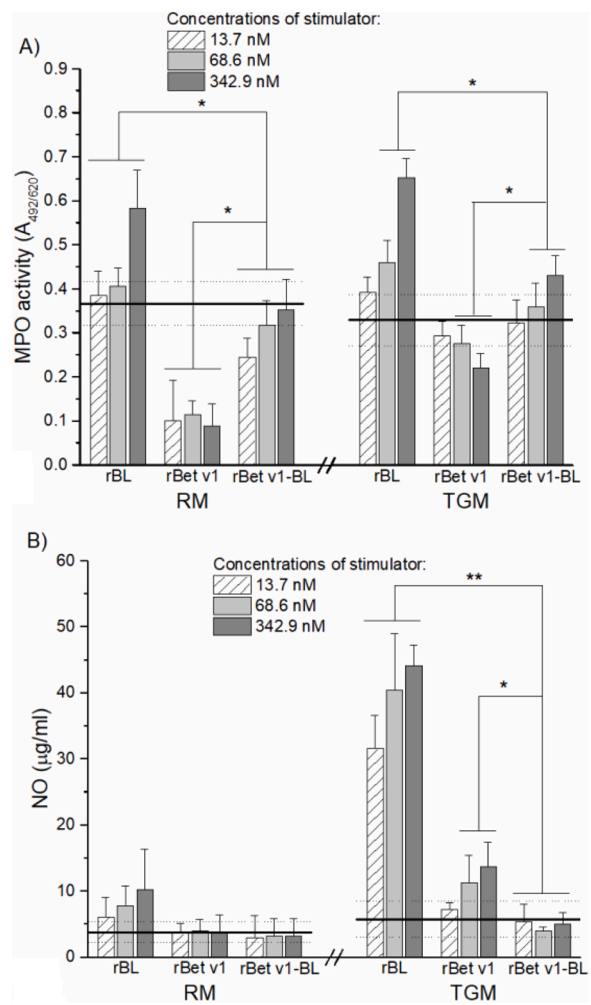



**Fig. 6.** The activity of MPO (**A**) and production of NO (**B**) by the resident (RM) and thioglycolate-elicited (TGM) peritoneal macrophages after stimulation with rBanLec (rBL), rBet v 1, or rBet v 1-BanLec (rBet v 1-BL) for 48 h. Results are presented as means ± standard error (n = 5). A solid black line indicates levels of MPO activity and NO production in non-stimulated RMs and TGMs (mean value) and dotted lines (mean ± standard error values; n = 5). Statistical significance of changes promoted by specific stimulation was determined by one-way ANOVA followed by Bonferroni's multiple comparisons test (* $P<0.05$, ** $P<0.005$). The comparisons between corresponding (same type of macrophages) rBet v 1-BanLec-stimulated (referent) and rBet v 1- or rBanLec-stimulated cultures are presented. The lowest levels of significance per stimulator are indicated.

with rBet v 1-BanLec-stimulated TGMs was recorded, it was significantly lower than in corresponding (same concentration of stimulators) rBanLec-stimulated TGMs cultures (rBet v 1-BanLec vs. rBanLec: $P < 0.05$ for TGMs stimulated by 68.6 nM and 342.9 nM). Besides, rBet v 1-BanLec was a more potent promoter of MPO activity than rBet v 1 in both RMs and TGMs (Fig. 6A). Still, MPO activity in all rBet v 1-BanLec-stimulated cultures did not significantly exceed the activity recorded in corresponding (same type of macrophages) non-stimulated cultures.

NO production was used as an indicator of NOS activity. 48 h long stimulation by rBet v 1-BanLec in defined concentrations did not significantly alter NO production in both RMs and TGMs comparing to corresponding non-stimulated cultures (Fig. 6B). There were no significant differences in NO production between RMs stimulated by rBet v 1-BanLec and corresponding (same concentration of stimulator) rBanLec- and rBet v 1-stimulated RMs. However, in TGMs stimulated by rBanLec or rBet v 1, NO production significantly exceeded the NO production recorded in corresponding rBet v 1-BanLec-stimulated TGMs (Fig. 6B).

### *3.7. Cytokine secretion pattern of peritoneal macrophages stimulated by rBet v 1-BanLec*

Peritoneal macrophages were used as a model system to evaluate the impact of rBet v 1-BanLec stimulation on the production of pro-inflammatory cytokines TNFα and IL-6 and anti-inflammatory cytokine IL-10. Among tested stimulators, rBanLec was the most potent promoter of TNFα and IL-6. A positive dose-dependent enhancement of TNFα (Fig. 7A) and IL-6 (Fig. 7B) production was marked upon rBanLec stimulation in both RMs (TNFα: non-stimulated vs. 342.9 nM rBanLec $P < 0.005$; IL-6: production in non-stimulated cultures was below limit of detection) and TGMs (TNFα: non-stimulated vs. 342.9 nM rBanLec $P < 0.05$; IL-6: production in non-stimulated cultures was below limit of detection). rBet v 1 in concentrations 68.6 nM and 342.9 nM also exerted potential to promote production of proinflammatory cytokines in RMs (TNFα: non-stimulated vs. 342.9 nM rBet v 1 $P < 0.005$) and TGMs (TNFα: non-stimulated vs. 342.9 nM rBet v 1 $P < 0.05$). Comparing to rBanLec and rBet v 1, rBet v 1-BanLec was a significantly weaker promoter of TNFα and IL-6 production (Fig. 7A and B, respectively) in both RMs and TGMs. Furthermore, the production of TNFα by rBet v 1-BanLec-stimulated RMs and TGMs did not exceed TNFα production by non-stimulated macrophages under the same conditions.

All tested stimulators in defined concentrations stimulated IL-10 production in both RMs and TGMs (Fig. 7C). However, IL-10 production upon rBet v 1-BanLec stimulation significantly exceeded ones marked in corresponding cultures (same type of macrophages) stimulated by rBet v 1 in the same concentration (in all cases $P < 0.05$). Concentrations of IL-10 in rBet v 1-BanLec stimulated cultures were comparable to those recorded upon rBanLec stimulation of RMs and TGMs.

## 4. Discussion

Results presented herein show that major structural characteristics of rBet v 1 and rBanLec are well preserved within rBet v 1-BanLec chimera and imply that rBet v 1-BanLec might exert an IL-10-mediated anti-inflammatory effect.

The chimera rBet v 1-BanLec was designed *in silico*. The rBet v 1-BanLec chimera was produced as a soluble protein in *E. coli*. The protein yield in this prokaryotic expression system was around 0.3 g per L of the cell culture. CD spectral analysis of the chimera revealed well-defined secondary structures in rBet v 1-BanLec, mainly consisting of β-sheets, which is in line with literature data reported on Bet v 1 and BanLec (Gajhede et al., 1996; Meagher et al., 2005). The result of the size-exclusion chromatography revealed that recombinant rBet v 1-BanLec forms a dimer in the solution. Besides, successful purification by affinity chromatography on Sephadex G-75 and binding to HRP glycoprotein indicate that the lectin domain of rBet v 1-BanLec retained

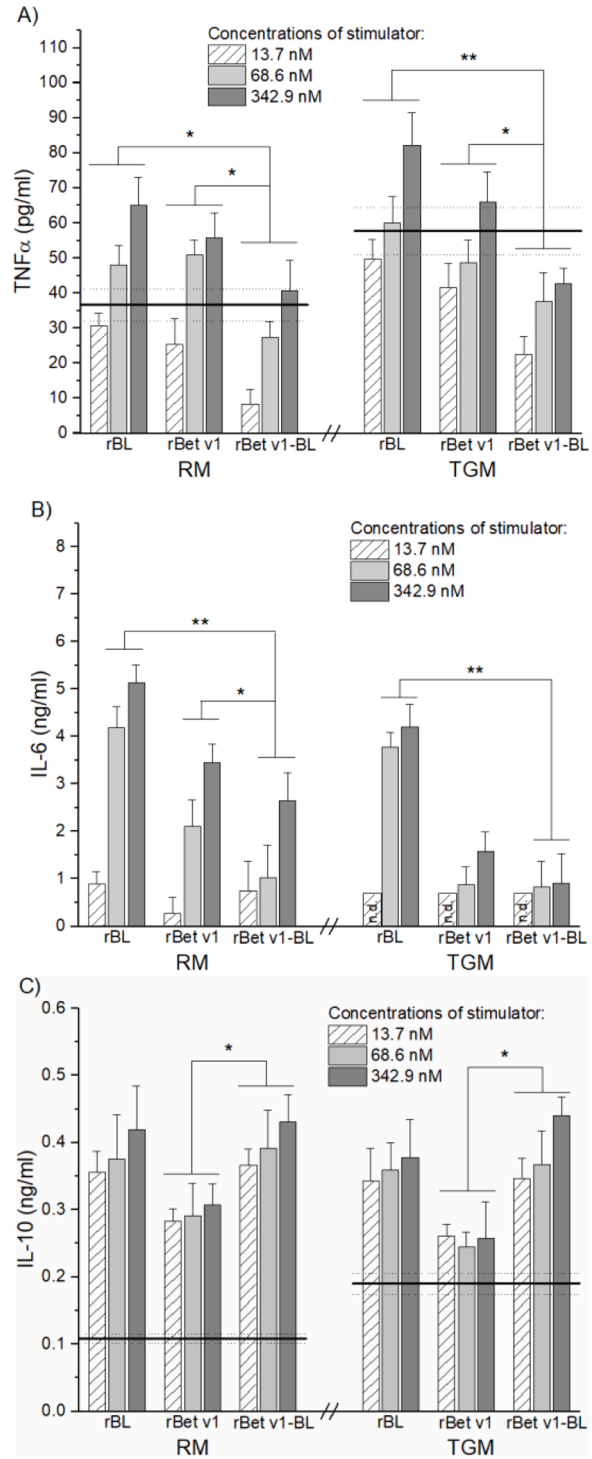



**Fig. 7.** The production of TNFα **(A)**, IL-6 (**B**), and IL-10 (**C**) by the resident (RM) and thioglycolate-elicited (TGM) peritoneal macrophages after stimulation with rBanLec (rBL), rBet v 1, or rBet v 1-BanLec (rBet v 1-BL) for 48 h. Results are presented as means ± standard error (n = 5). A solid black line indicates levels of tested cytokines in non-stimulated RMs and TGMs (mean value) and dotted lines (mean ± standard error values; n = 5). Statistical significance of changes promoted by specific stimulation was determined by one-way ANOVA followed by Bonferroni's multiple comparisons test (* $P<0.05$, ** $P<0.005$). The comparisons between corresponding (same type of macrophages) rBet v 1-BanLec-stimulated (referent) and rBet v 1- or rBanLec-stimulated cultures are presented. The lowest levels of significance per stimulator are indicated. n.d. - not detected.

specific mannose-binding capacity essential for its biological activity. Furthermore, immunoblot analysis results suggest that IgE reactive epitopes of the Bet v 1 were preserved in rBet v 1-BanLec.

The immunomodulatory capacity of rBet v 1-BanLec was tested *in vitro* on peritoneal macrophages. Among the immune cells, macrophages have an essential role in shaping the allergic immune response. Activation of macrophages via TLRs can initiate the production of bactericidal molecules (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) and enhance their antigen-presenting capacity (Herb and Schramm, 2021). In atopic patients, alternatively activated macrophages secrete IL-13, contributing to the differentiation of Th2 lymphocytes and eosinophil infiltration into the lungs (Nazimek et al., 2013). Besides, activated macrophages are also a source of pro-inflammatory cytokines such as TNFα and IL-6 and anti-inflammatory cytokine IL-10, which can suppress inflammation and promote differentiation of Treg.

As macrophages' functional characteristics could influence the outcome of particular stimulation, the stimulatory capacity of rBet v 1-BanLec was assessed on RMs and TGMs, populations of macrophages found in the peritoneal cavity in resting conditions and upon exposure to specific inflammatory stimuli, respectively. RMs and TGMs have distinct phenotypic and functional characteristics shaped by the particular milieu they were exposed (Cassado et al., 2015). Besides, macrophages' responsiveness and the activation profile acquired due to specific stimuli might be highly influenced by the genetic background (Marinkovic et al., 2017). The mice strain we used, BALB/c, is a Th2-prone strain with some characteristics similar to those recorded in allergic patients (Cooley et al., 2015). They are shown as susceptible to sensitization with various allergens, even without adjuvant, and are widely used as a model system for allergy-related disorders (Chen et al., 2017; Zhou et al., 2016). Besides, in our previous research, we demonstrated the good responsiveness of BALB/c mice to rBanLec (Marinkovic et al., 2017; Stojanović et al., 2010), a portion of rBet v 1-BanLec we supposed to be mainly responsible for the immunomodulatory activity of the chimera.

Obtained results show that the rBet v 1-BanLec retained the capacity of rBanLec to promote IL-10 production. On the contrary, rBet v 1-BanLec power to promote an inflammatory milieu was reduced compared to rBanLec. That is mirrored in negligible production of NO, the limited activity of MPO, and reduced production of inflammatory cytokines (IL-6 and TNFα) in rBet v 1-BanLec-stimulated cultures. We have demonstrated that rBet v 1-BanLec is a homodimer in solution, while rBanLec is a homotetramer (Hopper et al., 2017). Each polypeptide chain contains two carbohydrate-binding sites derived from BanLec. This indicates that recombinant chimera has four carbohydrate-binding sites, while BanLec includes eight. TLR2 receptor builds a heterodimer with TLR1 or TLR6 glycoproteins (Mariano et al., 2014). Since macrophage activation may be induced through different signaling pathways by targeting other glycosylated receptors (Mariano et al., 2014), more carbohydrate-binding sites from rBanLec might differently stimulate macrophage activation than rBet v 1-BanLec. Nonetheless, it is necessary to examine the signaling pathways stimulated after macrophage activation by rBet v 1-BanLec or rBanLec.

Compared to rBet v 1, rBet v 1-BanLec was demonstrated to be a more efficient promoter of IL-10 production and weaker inducer of NO production and secretion of pro-inflammatory cytokines. The reduced production of inflammatory cytokines, together with the IL-10 production, might be of immense importance for the potential use rBet v 1-BanLec in the therapy of Bet v 1-induced allergies. Although allergies are considered a Th2-driven inflammatory state, there is also growing evidence on non-Th2 cytokines' role in shaping the allergic immune response (Deo et al., 2010). The contribution of IL-6 to the development of allergy-related pathology was demonstrated. The genetic inactivation of IL-6 resulted in the attenuation of Th2 response, eosinophils-mediated inflammation, and IgE production in the murine model of house dust mite-induced asthma. In the same study, macrophages and dendritic cells (DC) were identified as the primary sources of IL-6 (Gubernatorova et al., 2018). Besides, IL-6 has been suggested as a potential target for allergic asthma treatment as it is essential for the uptake of allergens by DC, their maturation, and initiation of Th2/Th17-mediated airway inflammation (Lin et al., 2016). The murine model of allergic rhinitis shows a lack of TNFα inhibited pathology (Iwasaki et al., 2003; Mo et al., 2011). TNFα promotes adhesion molecules' expression, allowing eosinophil efflux and being an inducer of production of specific IgE and Th2 cytokines and chemokines (Iwasaki et al., 2003). Some of these TNFα-promoted effects were recorded in human nasal mucosa (Widegren et al., 2007). The local rise in the concentration of soluble TNFα is a pivotal event that precedes eosinophils and neutrophiles' recruitment to the allergic inflammatory site (Ahmad et al., 2018). A local increase in MPO activity and NO production occur primarily due to an influx of neutrophils (Nabe, 2013; Widegren et al., 2008) and the priming of epithelial and resident immune cells by an inflammatory milieu. Furthermore, NO-mediated mechanisms (Widegren et al., 2008) and TNFα are essential for producing eosinophils-attracting chemokines and expression of adhesion molecules to recruit eosinophils to the allergic inflammatory site (Ahmad et al., 2018). The capacity to promote IL-10 production in macrophages irrespective of their functional state (either physiological or inflammatory conditions) implies that rBet v 1-BanLec construct might benefit allergy treatment. Namely, IL-10 is critical for the differentiation of Tregs, which further, through IL-10-dependant and IL-10-independent mechanisms, prevent the establishment of an allergic immune response (Akdis and Akdis, 2014; Hawrylowicz, 2005). Treg cells' expansion is one of the fundamental mechanisms leading to allergy symptoms' amelioration due to allergen-specific immunotherapy (Boonpiyathad et al., 2019).

In conclusion, preserved antigenic characteristics of Bet v 1 part of chimera, together with the higher capacity of rBet v 1-BanLec to promote a dominant anti-inflammatory response in macrophages in comparison to Bet v 1, strongly imply that rBet v 1-BanLec would be worth testing as a potential therapeutic of Bet v 1-induced allergy. Getting a complete insight into the therapeutic potential of rBet v 1-BanLec and its eventual limitation will be the aim of our further research. The achievement of the goal mentioned above will require evaluation in specific animal models where a palette of tissue-specific factors will be evaluated in the context of distinct genetic backgrounds.

## CRediT authorship contribution statement

**Isidora Protić-Rosić:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft. **Andrijana Nešić:** Methodology. **Ivana Lukić:** Methodology, Validation, Formal analysis. **Radmila Miljković:** Methodology, Validation. **Dragan Popović:** *In silico* modeling, Writing –Review & Editing. **Marijana Stojanović:** Conceptualization, Methodology, Validation, Supervision, Writing - Review & Editing. **Marina Atanasković-Marković:** Methodology, Validation, Formal analysis, Supervision. **Marija Gavrović-Jankulović:** Conceptualization, Resources, Supervision.

## Declaration of Competing Interest

The authors declare that they have no competing interests.

## Acknowledgments

Ministry of Education, Science and Technological Development of Republic of Serbia Contract number: 451-03-68/2021-14/200168, Contract number: 451-03-68/2021-14/200177 and, Contract number: 451-03-68/2021-14/200026.

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