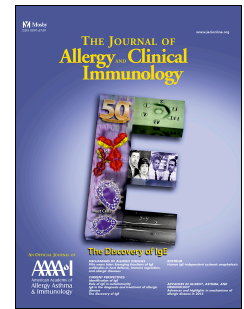


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Fusion proteins of flagellin and the major birch pollen allergen Bet v 1 show enhanced immunogenicity, reduced allergenicity and intrinsic adjuvanticity

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Clinical Implications

Bet v 1-flagellin fusion proteins represent promising vaccines for birch pollen AIT.

Capsule summary

Bet v 1-flagellin fusion proteins display several advantages over wild-type Bet v 1, namely reduced allergenicity, immunomodulatory capacity, enhanced immunogenicity and intrinsic adjuvanticity.

Keywords

Allergen-specific immunotherapy; adjuvant; Toll-like receptor 5; flagellin; Bet v 1

Abbreviations

aa, amino acid; Ag, Antigen; AIT, allergen-specific immunotherapy; alum, aluminium hydroxide; APC, antigen-presenting cell; BAT, basophil activation test; cpm, counts per minute; fliC, flagellin from *Salmonella typhimurium*; GFP, green fluorescent protein; i.p., intraperitoneal; mdDC, monocyte-derived dendritic cells; MPL-A, monophosphoryl-lipid A; O.D., optical density; PBMC, peripheral blood mononuclear cells; SD, standard deviation; SI, stimulation index; SLIT, sublingual immunotherapy; SN, supernatants; TCC, T cell clone; TCL, T cell line; TLR, Toll-like receptor;

ABSTRACT

Background: Recombinant fusion proteins of flagellin and antigens have been demonstrated to induce strong innate and adaptive immune responses. Such fusion proteins may enhance the efficacy of allergen-specific immunotherapy (AIT).

Objective: To characterize different fusion proteins of flagellin and the major birch pollen allergen Bet v 1 for suitability as allergy vaccines.

Methods: A truncated version of flagellin (NtCFIg) was genetically fused to the N- or C-terminus of Bet v 1. TLR5-binding was assessed with HEK293 cells expressing TLR5. Up-regulation of CD40, CD80, CD83, and CD86 on monocyte-derived dendritic cells (mdDC) from allergic patients was analyzed by flow cytometry. The T cell-stimulatory capacity of the fusion proteins was assessed with naïve and Bet v 1-specific T cells. IgE-binding was tested in inhibition ELISA and basophil activation tests (BAT). Mice were immunized with the fusion proteins in the absence and presence of aluminium hydroxide (alum). Cellular and antibody responses were monitored. Murine antibodies were tested for blocking capacity in BAT.

Results: Both fusion proteins matured mdDC via TLR5. Compared to Bet v 1 the fusion proteins showed stronger T cell-stimulatory and reduced IgE-binding capacity and induced murine Bet v 1-specific antibodies in the absence of alum. However, only antibodies induced by immunization with NtCFIg fused to the C-terminus of Bet v 1 inhibited the binding of patients' IgE antibodies to Bet v 1.

Conclusion: Bet v 1-flagellin fusion proteins show enhanced immunogenicity, reduced allergenicity and intrinsic adjuvanticity and thus, represent promising vaccines for birch pollen AIT. However, the sequential order of allergen and adjuvant within a fusion protein determines its immunological characteristics.

INTRODUCTION

Allergen-specific immunotherapy (AIT) is currently the only treatment for IgE-mediated allergic diseases that modifies the cellular and humoral response of allergic patients and leads to a long-term amelioration of the allergic symptoms. Conventional AIT is based on a high number of regular subcutaneous injections of allergen extracts for up to 3 years. The time consuming and long-lasting treatment schedule impairs patients' compliance¹. Using Toll-like receptor (TLR) ligands as adjuvants in AIT vaccines may dramatically reduce the number of injections by activation of the innate immune system and subsequent stronger counter-regulation of the disease-eliciting Th2 response²⁻⁴. For example, supplementation of allergen vaccines with the TLR4-ligand monophosphoryl-lipid (MLP)-A strongly promoted Th1-like responses⁵ and achieved clinical improvement of patients after only four pre-seasonal injections⁶⁻⁹.

Direct conjugation of TLR-ligands to allergens has been shown to amplify their immunomodulatory capacity¹⁰⁻¹². The TLR5-ligand flagellin, the main component of bacterial flagella, is used as adjuvant in various vaccines. As a protein, flagellin can be genetically fused to antigens and such hybrid proteins have been demonstrated to induce strong innate and adaptive immune responses¹²⁻¹⁴. Although TLR5 activation does not enhance the uptake of antigens⁴, a flagellin fusion protein with green fluorescent protein (GFP) has been shown to be efficiently taken up, processed and presented by dendritic cells (DC) inducing specific immune responses against the *per se* non-immunogenic GFP¹⁵. This enhanced immunogenicity has been referred to the TLR5-upregulated expression of MHC and costimulatory molecules on the surface of the APC that takes up and presents the antigen. Flagellin fusion proteins with allergens may represent immunogenic vaccines for AIT^{16, 17}. Indeed, it has been

demonstrated that the fusion protein of flagellin from *Listeria monocytogenes* and ovalbumin but not the mixture of both components suppresses the allergic Th2 response and allergy-related symptoms in a mouse model of intestinal allergy¹⁸⁻²⁰.

AIT with recombinant Bet v 1.0101 (Bet v 1) has achieved a similar clinical improvement of birch pollen allergy as AIT with birch pollen extract in a multicenter, randomized, double-blind, placebo-controlled trial²¹. Thus, Bet v 1 substituted natural birch pollen extract but *per se* did not exhibit a superior effectivity. To enhance its immunogenicity we fused Bet v 1 to flagellin from *Salmonella typhimurium* (fliC) as the immunostimulatory capacity of this flagellin has been proven in various studies²².

We substituted its hypervariable region, which is thought to promote the production of neutralizing antibodies (Ab)²³ that down regulate TLR5 signalling, with a short turn motif to reduce its size and to keep the close proximity of the N- and C-termini necessary for TLR5-binding. The deletion of the hypervariable region did not abrogate the adjuvant effect as the resulting truncated flagellin, NtCFlg, still activated TLR5 and was fused to either the N- or C-terminus of Bet v 1, termed NtCFlg-Bet v 1 and Bet v 1-NtCFlg, respectively, in order to assess possible position effects. Both fusion proteins were characterized and compared with regard to their ability i) to target TLR5, ii) to activate human monocyte-derived (md) DC, iii) to bind Bet v 1-specific IgE Ab and to induce IgE-mediated effector cell activation, and iv) to activate Bet v 1-specific T cells. In addition, we immunized mice with the fusion proteins in the absence or presence of aluminium hydroxide (alum) to investigate their intrinsic adjuvanticity and analyzed whether murine Ab were able to inhibit the binding of patient-derived IgE to Bet v 1.

METHODS

The cloning of constructs is described in the Online Repository.

TLR activation assays

HEK 293 cell lines stably transfected with human TLR5 (HEK-hTLR5) or TLR4, MD2 and CD14 (HEK-hTLR4/MD2-CD14) (both InvivoGen, San Diego, CA, USA) were cultured in DMEM High Glucose supplemented with 10% FCS (both from PAA Laboratories, Pasching, Austria) and selective antibiotics as described in the manufacturer's instructions (all InvivoGen). Cells (4×10^4 /96 well, Corning, New York, NY, USA) were stimulated with titrated amounts of Bet v 1 (Biomay, Vienna, Austria), recombinant fliC (InvivoGen), NtCFlg, Bet v 1-NtCFlg or NtCFlg-Bet v 1 for 20 h in triplicate. Secreted IL-8 was quantified by ELISA. For this, 96-well plates (NUNC Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA) were coated with anti-human IL-8 Ab M801 (Thermo Fisher Scientific) overnight and blocked for 1h at RT in 4% BSA in PBS-Tween 0.05%. Samples and standards (Thermo Fisher Scientific) were added in blocking buffer for 1h at RT. Bound IL-8 was detected with biotinylated IL-8 Ab (Thermo Fisher Scientific), poly-HRP streptavidin (Thermo Fisher Scientific) and TMB solution (Millipore, Billerica, MA, USA).

Patients

To be included, patients had to provide a doctor's diagnosis of birch-pollen allergy based on rhinoconjunctivitis in spring, positive skin prick tests to birch pollen extract (ALK-ABELLO, Hørsholm, Denmark) and Bet v 1-specific IgE levels of >5 kU_A/l (ImmunoCAP, Thermo Fisher Scientific; see Table E2 in the Online Repository). The

study was approved by the local ethics committee. Blood donors gave written informed consent.

Monocyte-derived dendritic cells

mdDC were generated as described ²⁴. In preliminary experiments, immature mdDC from 5 individuals were treated with titrated concentrations of Bet v 1-NtCFlg, NtCFlg-Bet v 1 and NtCFlg (100 nM – 0.01 nM) for 48 h and analyzed for upregulation of CD83, CD80, CD86 and CD40 using flow cytometry. The highest values of upregulation were found with 0.01 nM NtCFlg, 0.1 nM Bet v 1-NtCFlg and 1 nM NtCFlg-Bet v 1, respectively (data not shown). These concentrations were then used to stimulate mdDC from 14 birch pollen-allergic individuals. Bet v 1 (100 nM) and fliC (3 nM) served as controls.

Stimulation of T cells

Bet v 1-specific T cell lines (TCL) were generated from PBMC (1.5×10^6 /well) using 10 µg/ml of Bet v 1 as described ²⁵. Cryopreserved Bet v 1-specific T cell clones (TCC) with determined epitope specificity (Table II) were thawed and expanded. TCL and TCC were stimulated with titrated amounts of Bet v 1, NtCFlg, Bet v 1-NtCFlg or NtCFlg-Bet v 1 (300 nM – 7.5 nM) in duplicate in the presence of 1×10^5 autologous irradiated (60 Gy) PBMC. Incorporation of tritiated thymidine was measured after 48h. Stimulation indices (SI) were calculated as the ratio of counts per minute (cpm) of stimulated cultures to cpm of unstimulated cultures. Naïve CD4⁺ T cells were purified from PBMC using the Naïve CD4⁺ T cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in >90% CD4⁺CD45RA⁺ cells. These cells were cocultured for six days with irradiated, autologous mdDC that had been

stimulated with Bet v 1 (100 nM) or Bet v 1-NtCFlg (0.1 nM) for 24h⁴. Cytokine levels in SN were analyzed using the Luminex System 100 (Luminex, Texas, USA).

Inhibition ELISA

Sera were pre-incubated with equimolar concentrations of Bet v 1, NtCFlg, Bet v 1-NtCFlg or NtCFlg-Bet v 1 (300 nM to 480 pM) for 4h at RT and added to ELISA plates coated with Bet v 1 (1 µg/ml) overnight and saturated with 1% human serum albumin for 6h. Bound IgE was detected with alkaline phosphatase (AP)-conjugated anti-human IgE (BD Biosciences, Heidelberg, Germany) and PNPP (Sigma-Aldrich). Percentage of inhibition was calculated ($\% \text{ inhibition} = 100 - [\text{OD}(\text{serum with inhibitor}) / \text{OD}(\text{serum without inhibitor}) \times 100]$).

Basophil activation test (BAT)

Heparinized blood of 7 birch pollen-allergic donors was incubated with titrated amounts of Bet v 1, NtCFlg, Bet v 1-NtCFlg or NtCFlg-Bet v 1 (600 nM – 6 pM) in HEPES Calcium Buffer pH 7.4 containing 2 ng/ml IL-3 for 15 min at 37°C. For inhibition experiments, Bet v 1 (1.2 and 0.12 ng in 2 µl buffer) was incubated with pooled mouse sera (5 µl) for 1 h at 37°C. The reaction was stopped with HEPES/EDTA (20 mM) buffer and cells were stained with CD123–FITC, CCR3-APC and CD63-PE (all BioLegend, San Diego, CA, USA), followed by erythrocyte lysis. Basophils were defined as CD123⁺CCR3⁺ cells. Basophil activation was expressed as percentage of CD63⁺ cells in reference to untreated cells²⁶.

Immunization of mice

50 female BALB/c mice (6-8 weeks, Charles River, Sulzfeld, Germany) were immunized five times by biweekly intraperitoneal (i.p.) injections with either Bet v 1 (5 µg) or equimolar amounts of fliC, NtCFlg, Bet v 1-NtCFlg or NtCFlg-Bet v 1 in PBS alone or adsorbed to 100 µl alum (Al(OH)₃, Serva GmbH, Heidelberg, Germany) in a total volume of 150 µl. Prior to each immunization blood was collected by tail bleeding. Seven days after the final immunization, mice were sacrificed. All mouse experiments were approved by the Animal Experimentation Ethics Committee of the Medical University of Vienna and the Ministry of Science and Research (BMWF 66009/0215-II/3b/2010).

Murine immune responses

ELISA plates were coated with Bet v 1 (1 µg/ml) in carbonate buffer (pH=9.6). Sera were diluted 1:1000 for detection of IgG1 and IgG2a and 1:20 for detection of IgE Ab. Rat anti-mouse IgE, IgG1, IgG2a (all BD Pharmingen, San Jose, CA, USA), HRP-conjugated goat anti-rat IgG (GE Healthcare, Vienna, Austria) and ABTS (Sigma Aldrich) were used for detection. Absorbance was measured at 405 nm. Single cell suspensions from spleens (1x10⁶/ml) were stimulated in triplicates with Bet v 1 (10 µg/ml) and proliferation was assessed by incorporation of tritiated thymidine after 72h. Moreover, splenocytes (8x10⁶/ml) were stimulated with Bet v 1 (10 µg/ml). SN were collected after 48h and IL-4 and IFN-γ levels were measured using the Luminex system 100.

Statistics

Data were analyzed using IBM SPSS 20.0 (SPSS, Chicago, IL, USA). Statistical differences were determined by the Wilcoxon signed ranks test or paired sample t-

218 test and considered statistically significant for $P < 0.05$. Data from duplicate and
219 triplicate experiments are shown as means with standard deviation (SD).

220

RESULTS

NtCFlg, Bet v 1-NtCFlg and NtCFlg-Bet v 1 bind TLR5 and induce maturation of mdDC

We replaced the hypervariable domain (aa 177-400) of fliC (50 kDa) with the hairpin turn sequence RGITVNGKTYGR (see Figure E1 in the Online Repository). The resulting 31 kDa protein NtCFlg induced higher levels of IL-8 in HEK-hTLR5 cells than full length fliC (Fig. 1A). NtCFlg was fused to either the N- or C-terminus of Bet v 1 termed NtCFlg-Bet v 1 and Bet v 1-NtCFlg, respectively. Both 48 kDa proteins induced IL-8 secretion in HEK-hTLR5 cells with slightly stronger responses to Bet v 1-NtCFlg (Fig. 1 A). None of the proteins induced IL-8 secretion in HEK-hTLR4/MD2-CD14 cells demonstrating that the recombinant proteins were endotoxin-free (data not shown).

Next, immature mdDC were stimulated with optimal concentrations of each protein as indicated in Methods. Bet v 1 did not induce maturation of mdDC whereas Bet v 1-NtCFlg, NtCFlg-Bet v 1, NtCFlg, and fliC significantly enhanced the expression of CD83, CD80, CD86 and CD40 ($p < 0.001$, Fig. 1 B). Notably, 10 fold less Bet v 1-NtCFlg than NtCFlg-Bet v 1 was sufficient to achieve the same expression levels of the surface markers on mdDC.

Bet v 1-NtCFlg and NtCFlg-Bet v 1 show T cell-activating capacity

Bet v 1-specific TCL from 6 birch pollen-allergic patients were stimulated with equimolar amounts of Bet v 1, Bet v 1-NtCFlg, NtCFlg-Bet v 1 or NtCFlg. At all concentrations used, proliferative responses to Bet v 1-NtCFlg were more pronounced than to Bet v 1 and NtCFlg-Bet v 1, respectively (Table I). To assess whether antigen processing of Bet v 1-NtCFlg produced the most relevant T cell-

activating regions²⁷ we employed 11 Bet v 1-specific TCC from 5 different birch pollen-allergic donors specific for epitopes distributed over the entire sequence of Bet v 1 (Table II). All TCC except for TCC9 responded with a significantly higher proliferation to Bet v 1-NtCFlg than to Bet v 1 ($p < 0.005$). As expected, NtCFlg did not induce proliferative responses in Bet v 1-specific T cell cultures. We also analyzed the levels of IL-4, IL-10 and IFN- γ in Bet v 1-specific TCC. However, none of these presumably end-differentiated clones changed their cytokine profile in response to a one-time stimulation with Bet v 1-NtCFlg (data not shown).

Next, we co-cultured naïve T cells from three birch pollen-allergic patients with autologous mdDC stimulated with Bet v 1 and Bet v 1-NtCFlg, respectively, and assessed their production of IL-4, IL-5, IL-10, IL-13 and IFN- γ . However, IL-4 and IL-10 were not detected in any of the cultures. The levels of IL-5, IL-13 and IFN- γ are summarized in Table III and revealed that mdDC stimulated with the fusion protein displayed a more pronounced polarizing capacity than Bet v 1-stimulated mdDC. Furthermore, Bet v 1-NtCFlg-stimulated mdDC promoted the production of IFN- γ by T cells which was marginal in cultures polarized with Bet v 1-stimulated mdDC.

Bet v 1-NtCFlg and NtCFlg-Bet v 1 are hypoallergenic

Sera from 11 birch pollen-allergic patients were pre-incubated with the fusion proteins or Bet v 1 to assess the inhibition of IgE-binding to plate-bound Bet v 1 (Fig. 2A). The fusion proteins displayed a significantly lower inhibitory capacity than Bet v 1. Bet v 1-NtCFlg bound significantly less IgE than NtCFlg-Bet v 1 at low concentrations.

The efficacy to induce IgE-mediated effector cell activation was tested in BAT of 7 birch-pollen allergic donors (Fig. 2B). Bet v 1-NtCFlg and NtCFlg-Bet v 1 induced

significantly less basophil activation than Bet v 1 at 6, 60 and 600 pM ($p < 0.05$). No significant difference between the fusion proteins was observed.

Bet v 1-NtCFlg and NtCFlg-Bet v 1 show intrinsic adjuvanticity

BALB/c mice were immunized 5 times i.p. with equimolar doses of Bet v 1, Bet v 1-NtCFlg, NtCFlg-Bet v 1, or the mixture of Bet v 1 and NtCFlg without or adsorbed to alum. Sera were regularly taken and assessed for Bet v 1-specific IgG1, IgG2a and IgE levels. After 2-3 immunizations with alum, all three Bet v 1-specific Ab subclasses were detected in response to all proteins (Fig. 3A). In the absence of alum, both fusion proteins also induced Bet v 1-specific IgG1 and IgE Ab. However, only Bet v 1-NtCFlg induced noticeable Bet v 1-specific IgG2a Ab (Fig. 3A). Compared to Bet v 1-NtCFlg the Ab response induced by NtCFlg-Bet v 1 was delayed. Five immunizations with the mixture of Bet v 1 and NtCFlg were necessary to induce Bet v 1-specific IgG1 whereas Bet v 1-specific IgG2a and IgE levels remained negligible. Immunization with Bet v 1 in the absence of alum induced no detectable Ab responses.

To compare allergen-specific cellular responses in the different groups, splenocytes were stimulated with Bet v 1 and proliferative and cytokine responses were assessed. Figure 3B shows that Bet v 1-induced proliferative responses were more pronounced in mice immunized with the fusion proteins compared to those immunized with Bet v 1 and the mixture of Bet v 1 and NtCFlg. Allergen-specific proliferation was higher in all groups immunized without alum except for mice immunized with wild-type Bet v 1. Similar IFN- γ /IL-4 ratios were found in all groups when immunized with and without alum except for mice immunized with Bet v 1-NtCFlg. These mice showed a significantly higher IFN- γ /IL-4 ratio in the absence of

alum indicating a more Th1-like response that matches with the induction of Bet v 1-specific IgG2a Ab.

Murine antibodies raised against Bet v 1-NtCFIg show blocking capacity

Bet v 1 was pre-incubated with pools of mouse sera collected before or after 5 immunizations with either Bet v 1, Bet v 1-NtCFIg or NtCFIg-Bet v 1 adsorbed to alum and used to activate basophils from 6 birch pollen-allergic patients. Figure 3B shows that, compared to pre-immune sera, sera from mice immunized with Bet v 1 and Bet v 1-NtCFIg significantly reduced basophil activation ($p < 0.05$). In contrast, sera from mice immunized with NtCFIg-Bet v 1 had no inhibitory effect.

DISCUSSION

To enhance the immunogenicity of Bet v 1 for improved efficacy of AIT, we genetically fused it N- and C-terminally with a truncated variant of the TLR5-ligand fliC which is used as adjuvant in various human vaccines^{28, 29}. Bet v 1 and NtCFlg do not mutually interact as NtCFlg has no influence on functional IgE-binding of Bet v 1 and Bet v 1 does not alter the TLR5-activating capacity of NtCFlg (see Figure E2 in the Online Repository). Both fusion proteins triggered TLR5 and caused the maturation of mdDC. TLR5 is expressed on the surface of many types of APC similarly to TLR4³⁰. Consequently, we expect that the use of such fusion proteins may reduce the number of injections similar to AIT vaccines containing MPL-A^{6, 8}. Beyond that, fliC has been shown to directly stimulate CD4⁺ T cells leading to enhanced proliferation and IFN- γ production in memory T cells³¹ and a higher suppressive potential in regulatory T cells³². Indeed, Bet v 1-NtCFlg displayed a stronger capacity in the activation of naïve and allergen-specific effector T cells than wild-type Bet v 1. Bet v 1-NtCFlg also polarized naïve T lymphocytes to the production of IFN- γ . Antigen processing of Bet v 1-NtCFlg retained the relevant T cell epitopes of Bet v 1 which is a necessary prerequisite to target all types of allergen-specific T cells during AIT. In addition to the enhanced immunogenicity, both fusion proteins displayed significantly lower functional IgE-binding capacity indicating that their administration to allergic patients will trigger less IgE-mediated side effects. Finally, the fusion proteins induced Bet v 1-specific humoral and cellular responses in mice even when not adsorbed to alum which were not observed with wild-type Bet v 1 and clearly less pronounced with the mixture of Bet v 1 and NtCFlg. In the absence of alum, Bet v 1-NtCFlg induced Bet v 1-specific IgG2a Ab and a higher IFN- γ /IL-4

ratio indicating a Th1-like response. These findings suggest that a vaccine based on this fusion protein can be devoid of alum which additionally promotes the counter-regulation of Th2-like responses during AIT.

Ag sequences have been added to fliC at the N- or C-terminus or within the hypervariable region without any loss of signaling *via* TLR5^{29, 33, 34}. Also our fusion proteins retained TLR5-activity and matching previous observations with flagellin from *Listeria monocytogenes*¹⁸⁻²⁰, the physical linkage of Bet v 1 and NtCFlg resulted in stronger immune responses than the mixture of both reagents (Fig. 3). However, the N-terminal variant displayed a clearly lower capacity to trigger TLR5 than the C-terminal fusion protein. The impaired capacity of NtCFlg-Bet v 1 to activate TLR5 was overcome by the addition of NtCFlg (see Figure E3 in the Online Repository). In contrast, NtCFlg did not affect TLR5-activation by Bet v 1-NtCFlg demonstrating that this fusion protein fully activated TLR5. Further comparison of both hybrids revealed that Bet v 1-NtCFlg was also superior to NtCFlg-Bet v 1 in the activation of mdDC and allergen-specific T cells. Both fusion proteins bound significantly less Bet v 1-specific IgE from birch pollen-allergic patients but still induced the production of Bet v 1-specific Ab in mice. Again, slightly higher responses to Bet v 1-NtCFlg were observed. In the absence of alum Bet v 1-NtCFlg induced an earlier and stronger Bet v 1-specific IgG1 and IgG2a response than NtCFlg-Bet v 1. Testing the capacity of murine Bet v 1-specific Ab to inhibit the binding of patients' IgE revealed a major difference between both fusion proteins. Sera from mice immunized with Bet v 1-NtCFlg inhibited the Bet v 1-mediated activation of basophils from birch pollen-allergic patients to the same degree as sera from mice immunized with Bet v 1. In contrast, sera from mice immunized with NtCFlg-Bet v 1 lacked this blocking capacity. These data suggest that solely a Bet v 1-NtCFlg-based vaccine will induce

allergen-specific IgG Ab which inhibit IgE-mediated reactions. Bet v 1-NtCFlg was superior to NtCFlg-Bet v 1 in the inhibition of IgE-binding to Bet v 1 and in basophil activation when used at low concentrations (Fig. 2). From these findings we deduce that Bet v 1-NtCFlg contains more IgE-epitopes of Bet v 1 and thereby induces diverse Bet v 1-specific IgG Ab with sufficient affinity to block IgE-binding. Thus, the sequential order of allergen and fliC within fusion proteins is of major importance for the induction of blocking Ab and needs to be carefully evaluated for each variant.

In summary, Bet v 1-NtCFlg displays several advantages over wild-type Bet v 1, namely reduced allergenicity, enhanced immunogenicity and intrinsic adjuvanticity. Therefore, we consider Bet v 1-NtCFlg a promising vaccine candidate for AIT which may be particularly interesting for sublingual immunotherapy (SLIT). Only recently, SLIT with recombinant Bet v 1 has been demonstrated to be safe and efficacious in patients with birch pollen-induced rhinoconjunctivitis ³⁵. As previously observed for subcutaneous administration ²¹, sublingual administration of Bet v 1 showed an efficacy consistent with the efficacy of SLIT with birch pollen extract. However, several patients dropped out of the trial due to treatment-associated IgE-mediated adverse side effects. To improve this situation novel vaccines for AIT should be hypoallergenic and incorporate adjuvants which effectively stimulate the innate immune response and thereby enhance adaptive immune responses. Bet v 1-NtCFlg combines all these requirements with the additional advantages of recombinant protein production, e.g. easy standardization, batch-to-batch consistency, a constant allergen-adjuvant ratio and consequently, invariant immunological characteristics. Furthermore, fusion proteins containing fliC and the otherwise poorly immunogenic influenza antigen M2e proved to be safe and immunogenic in clinical trials ^{36, 37}.

380 Beyond that, fusion proteins of fliC and the influenza hemagglutinin antigen induced
381 seroprotection in elderly humans³⁸. Consequently, we propose Bet v 1-NtCFIg to be
382 a promising vaccine candidate for conventional and sublingual AIT of birch pollen
383 allergy.

384

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522

Table I: Proliferation of Bet v 1-specific TCL in response to Bet v 1, NtCFlg and the fusion proteins

TCL	Bet v 1 [nM]			Bet v 1-NtCFlg [nM]			NtCFlg-Bet v 1 [nM]			NtCFlg [nM]		
	300	150	75	300	150	75	300	150	75	300	150	75
TCL 1	18 ^a	8.1	5.2	29	23	25	5.1	5.3	4.3	1.2	1.3	1.2
TCL 2	16	15	10	26	21	23	1.9	7.1	8.9	2.1	1.1	1.2
TCL 3	12	10	10	11	12	10	2.7	10	7.8	1.4	1.4	1.9
TCL 4	11	11	10	17	18	18	6.9	8.2	11	0.4	0.4	0.8
TCL 5	22	21	20	35	35	36	13	24	29	0.9	3.2	2.9
TCL 6	9.1	8.9	8.8	10	11	10	4.2	6.2	7.9	0.3	0.4	0.3

^aStimulation indices are shown.

Table II: Proliferation of Bet v 1-specific TCC in response to Bet v 1, Bet v 1-NtCFlg and NtCFlg

TCC	Epitope specificity	Stimulus [300 nM]		
	Amino acid residue	Bet v 1	Bet v 1-NtCFlg***	NtCFlg
TCC1	4-18	303 ^a	415	0.8
TCC2	4-18	139	228	0.9
TCC3	4-18	29	47	0.3
TCC4	4-18	2.3	28	1.3
TCC5	4-18	2.4	10	1.2
TCC6	61-75	11	21	1.4
TCC7	82-96	84	140	n.t.
TCC8	82-96	27	85	n.t.
TCC9	112-123	52	49	1.8
TCC10	142-153	157	205	0.9
TCC11	142-153	102	200	0.3

^a Stimulation indices are shown, n.t., not tested; ***p<0.005, (paired samples t-test)

533 Table III. T cell-polarizing activity of Bet v 1-NtCFlg

	IL-5 [ng/ml]			IL-13 [pg/ml]			IFN- γ [pg/ml]		
Donors	1	2	3	1	2	3	1	2	3
Bet v 1	74.9	117.7	12.7	173.3	144.3	582.4	<0.1	<0.1	17.0
Bet v 1-NtCFlg	86.1	69.0	25.1	2246	1518	2153	1610	1156	119.9

534

535 Cytokine levels in unstimulated cultures were subtracted from levels in stimulated cultures.

Figure legends**Figure 1: NtCFlg and the Bet v 1-fusion proteins activate TLR5**

(A) HEK293-hTLR5 were left untreated (▲) or stimulated with Bet v 1-NtCFlg (◇), NtCFlg-Bet v 1 (●), NtCFlg (■) or fliC (□) for 20h. IL-8 secretion in one representative experiment of seven is shown. (B) mdDC from 14 birch pollen-allergic patients were left untreated or incubated with Bet v 1 (100 nM), Bet v 1-NtCFlg (0.1 nM), NtCFlg-Bet v 1 (1 nM), NtCFlg (0.01 nM), or fliC (3 nM). After 48h surface markers were analyzed (***, $P < 0.001$, Wilcoxon signed ranks test)

Figure 2: Bet v 1-NtCFlg and NtCFlg-Bet v 1 are hypoallergens

(A) Sera of 11 birch pollen-allergic patients were pre-incubated with equimolar amounts of Bet v 1 (charcoal), Bet v 1-NtCFlg (dark grey), or NtCFlg-Bet v 1 (light grey) for 4h. The percentage of inhibition of IgE-binding to Bet v 1 in ELISA is shown. (B) Basophils from 7 birch pollen-allergic donors were stimulated with Bet v 1 (charcoal), Bet v 1-NtCFlg (dark grey) or NtCFlg-Bet v 1 (light grey). The percentage of CD63⁺ basophils is shown (* $P < 0.05$, Wilcoxon signed ranks test).

Figure 3: Bet v 1-NtCFlg induces Bet v 1-specific blocking antibodies

(A) BALB/c mice ($n=5$ for each group) were immunized (day 0=first, day 56=5th immunization) with equimolar amounts of Bet v 1, Bet v 1-NtCFlg, NtCFlg-Bet v 1 or a mixture of Bet v 1 and NtCFlg without or adsorbed to alum. Sera collected at the indicated days were tested for Bet v 1-specific IgG1, IgG2a (diluted 1/1000) and IgE (diluted 1/20) Ab. Mean and SD of each group are shown. OD, optical density

559 (B) Bet v 1-induced proliferation and cytokine responses of splenocytes. Results
560 show mean values of n=5 animals per group. Dpm = counts per minute (cpm) of
561 stimulated cultures minus cpm of unstimulated cultures. n.d. = not detected.

562 (C) Bet v 1 was pre-incubated with pooled sera from mice immunized with either Bet
563 v 1 (charcoal), Bet v 1-NtCFIg (dark grey) or NtCFIg-Bet v 1 (light grey) prior to
564 stimulation of basophils from 6 birch pollen-allergic donors. Pre-immune sera (white)
565 served as control (*P<0.05, Wilcoxon signed ranks test).

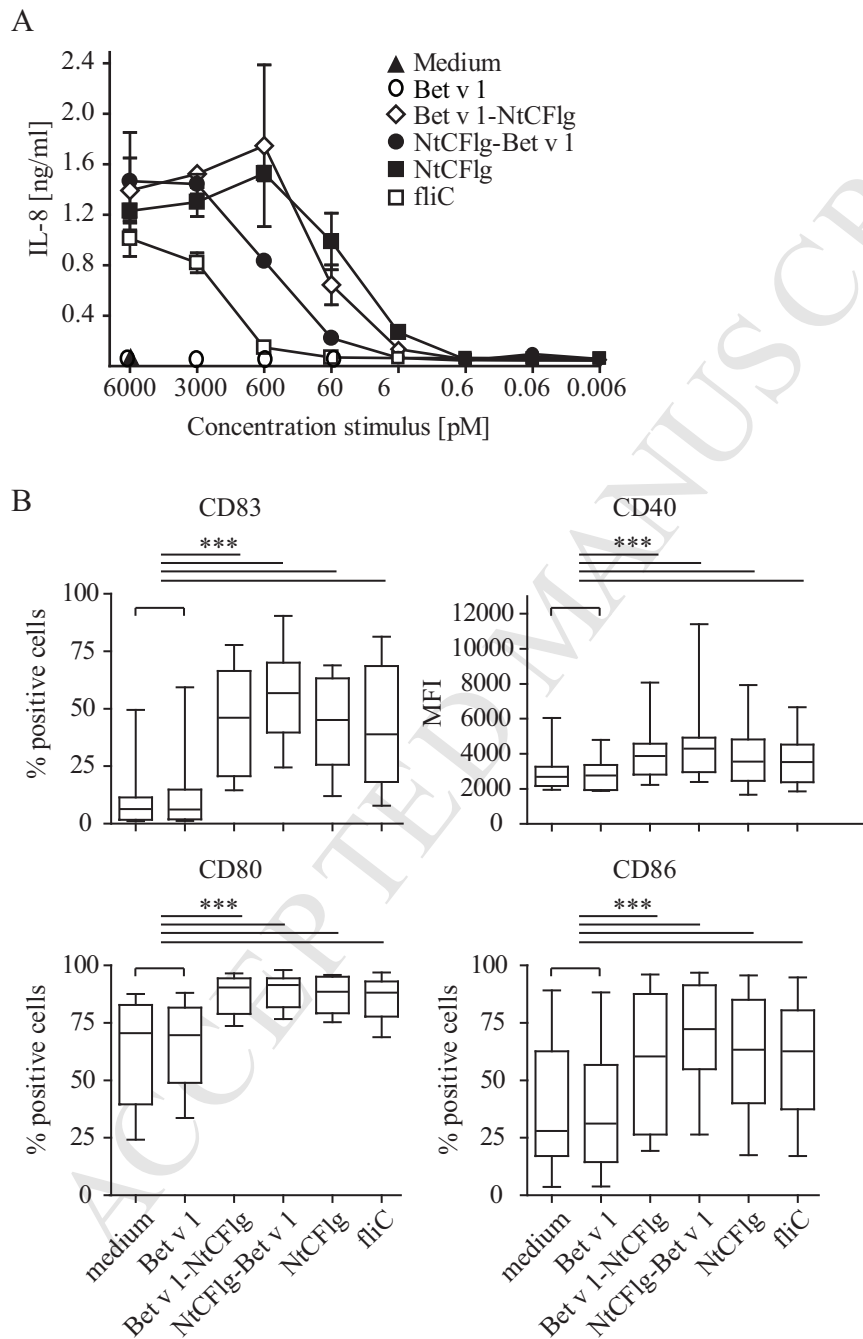


Figure 1

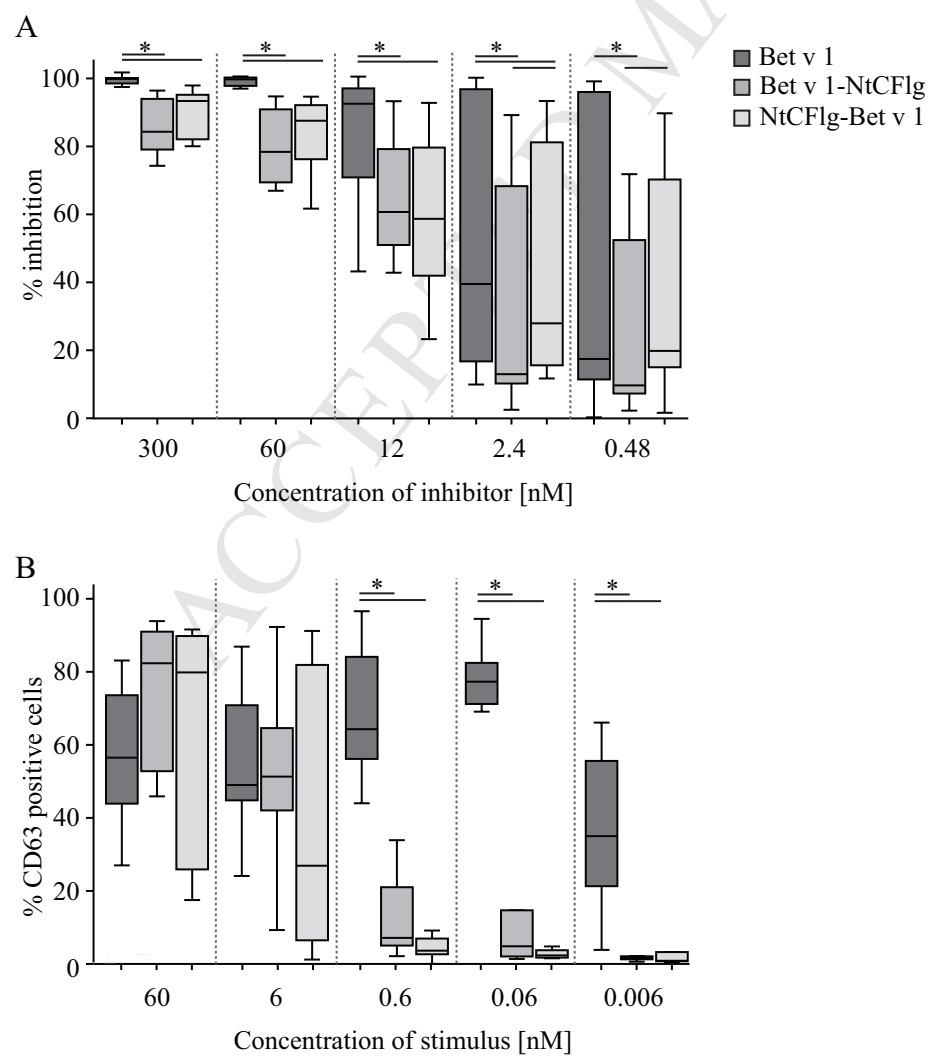


Figure 2

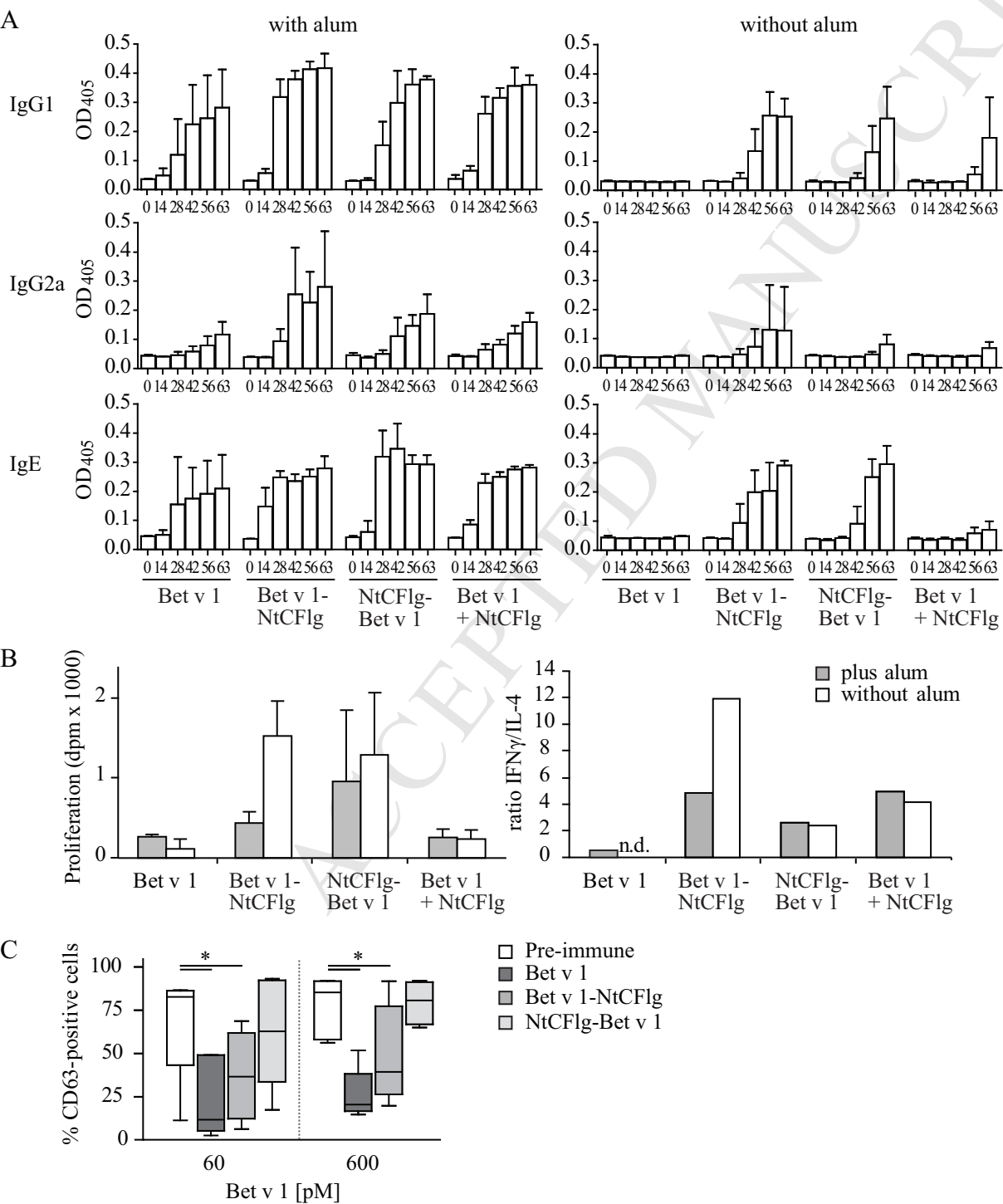


Figure 3

NtCFlg MSYYHHHHHDYDIPTTENLYFQGAMALGTAIERLSSGLRINSKDDAAGQAIANRFTAN 60
 S.t.Fl g --MAQVINTNSLSLLTQNNLN-KSQSALGTAIERLSSGLRINSKDDAAGQAIANRFTAN 57
 : : . . : * : * : . *****

NtCFlg IKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDLSIQAEIT 120
 S.t.Fl g IKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDLSIQAEIT 117

NtCFlg QRLNEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQ- 180
 S.t.Fl g QRLNEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQ 177

NtCFlg -----
 S.t.Fl g KYKVSdTAAVTVTGYADTTIALDNSTFKASATGLGGTDQKIDGDLKFDDTTGKYAKVTVT 237

NtCFlg ----- 184
 S.t.Fl g GGTGKGdgyyEVSVDKtNGEVTLAGGATSPLTGGLPATATEDVKNVQVANADLTEAKAALT 297

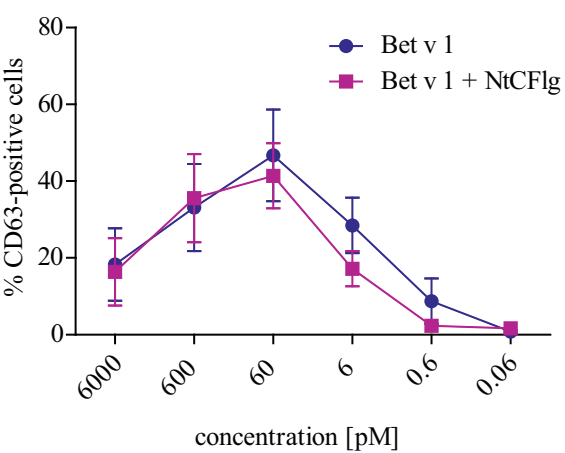
NtCFlg -----
 S.t.Fl g AAGVTGTASVVKMSYTDNNGKTIDGGLAVKVGDDYYSATQNKDGSISINTTKYTADDGTS 357

NtCFlg -----GIQRGITVNGKTYGRS-----TENPLQKIDAALA 209
 S.t.Fl g KTALNKLGGADGKTEVVSIGGKTYAASKAEGHNFKAQPDLAEEAAATTTENPLQKIDAALA 417

NtCFlg QVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIEDSDYATEVSNMSRAQILQQAGT 269
 S.t.Fl g QVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIEDSDYATEVSNMSRAQILQQAGT 477

NtCFlg SVLAQANQVPQNVL SLLR 287
 S.t.Fl g SVLAQANQVPQNVL SLLR 495

A



B

