

Cor a 1-reactive T cells and IgE are predominantly cross-reactive to Bet v 1 in patients with birch pollen-associated food allergy to hazelnut

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Background: IgE- and T-cell cross-reactivity contribute to the birch pollen-food syndrome.

Objectives: We performed a comprehensive analysis of T-cell cross-reactivity in primary cell cultures, facilitating the identification of allergen-specific T-cell subpopulations from individual patients.

Methods: Patients with birch pollen allergy and associated food allergy to hazelnuts, carrots, or both were analyzed for IgE cross-reactivity, T-cell responses, and T-cell cross-reactivity to recombinant Bet v 1.0101 (Bet v 1; birch), Cor a 1.0401 (Cor a 1; hazelnut), and Dau c 1.0104 (Dau c 1; carrot). A novel flow cytometry-based method using a 2-step staining process with fluorescent dyes was established to identify subpopulations of cross-reactive T cells.

Results: IgE-binding inhibition tests of individual sera revealed that the vast majority of Cor a 1-reactive IgE was cross-reactive to Bet v 1, whereas Bet v 1-reactive IgE was only partially inhibited by preincubation with Cor a 1. Primary stimulation of T cells with Bet v 1 or Cor a 1 resulted in a significant increase

in specific responses to Cor a 1 or Bet v 1 after secondary stimulation, respectively, indicating T-cell cross-reactivity between birch and hazelnut allergens in all patients of the study cohort. Preactivation with Dau c 1 induced less pronounced effects. A novel flow cytometry-based proliferation assay identified a predominant Cor a 1/Bet v 1-cross-reactive T-cell subpopulation within highly Bet v 1/Cor a 1-responsive T cells. **Conclusion:** Analysis of primary allergen-specific T cells combined with flow cytometry-based proliferation assays facilitates investigation of allergen-specific T-cell subpopulations in subjects and might be helpful to evaluate the effect of birch-specific immunotherapy on pollen-associated food allergies. (J Allergy Clin Immunol 2012;■■■■:■■■-■■■.)

Key words: T-cell cross-reactivity, birch pollen allergy, hazelnut, carrot, food allergy, Bet v 1, Cor a 1, Dau c 1, oral allergy syndrome, type I allergy, IgE, pathogenesis-related proteins

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Pollen-related food allergy represents the most frequent food allergy in adults in Europe.¹⁻⁵ On ingestion of fruits, nuts, and vegetables, up to 70% of patients with birch pollen allergy have oral allergy syndrome, which is characterized by immediate onset of oral itching, irritation of the oral mucosa, or angioedema.⁶⁻⁸ Occasionally, systemic reactions, ranging from urticaria to anaphylaxis, occur after consumption of the respective foods.^{6,7}

Bet v 1.0101 (Bet v 1) belongs to the pathogenesis-related protein family PR-10,^{2,9-14} which comprises, among others, Cor a 1.0401 (Cor a 1; hazelnut) and Dau c 1.0104 (Dau c 1; carrot).¹⁵⁻¹⁸ Cor a 1 and Dau c 1 share 67% and 38% amino acid sequence identity with Bet v 1, respectively, and display similar tertiary structures. The onset of birch pollen-related food allergy is believed to be induced by primary sensitization to pollen allergens and subsequent development of secondary food allergy caused by IgE cross-reactivity between homologous pollen and food allergens.^{2,11,13} Bet v 1-specific IgE antibodies were shown to recognize dietary proteins.⁵ In addition, cross-reactivity at the T-cell level has been demonstrated for certain homologues of Bet v 1 from, for example, apple, hazelnut, and carrot in studies using T-cell lines and clones.¹⁹⁻²⁵

Specific immunotherapy (SIT) for the treatment of allergy, such as birch pollen, has been proved to be mostly successful and is well established.²⁶ Nevertheless, severe side effects can occur, and data on the effects of SIT with pollen extracts on concomitant food allergy are inconsistent. Some authors demonstrated an improvement in the cross-reactive food allergen-induced symptoms, whereas others did not observe beneficial effects.²⁷⁻³⁰ Therefore, detailed analyses of humoral and cellular mechanisms of cross-reactivity are still elusive. Apart from analyzing IgE, analysis of T-cell cross-reactivity between pollen and food allergens in

Abbreviations used

Bet v 1: Bet v 1.0101
 CFSE: Carboxyfluorescein succinimidyl ester
 Cor a 1: Cor a 1.0401
 Dau c 1: Dau c 1.0104
 DC: Dendritic cell
 OVA: Ovalbumin
 SI: Stimulation index
 SIT: Specific immunotherapy

selected allergen-specific T-cell subpopulations of subjects might allow for elucidating the pathomechanism of clinical cross-reactivity in patients with birch pollen–food syndrome. These data might also contribute to the development of strategies for tolerance induction to food allergens on birch SIT at the T-cell level.

The present study was designed to perform a comprehensive analysis of IgE and T-cell cross-reactivity in individual patients with birch pollen–associated food allergy. We combined the use of allergen-specific primary T cells with a novel flow cytometry–based method to facilitate the identification of allergen-specific and cross-reactive T-cell subpopulations in individual patients.

METHODS**Allergic patients and healthy control subjects**

Patients with birch pollen allergy (clinical history, positive skin prick test response [ALK-Abelló, Hørsholm, Denmark], specific IgE, and ImmunoCAP [Thermo Fisher Scientific, Uppsala, Sweden] class ≥ 3), most of whom had concomitant food allergy to hazelnut, carrot, or both were included ($n = 20$; 13 female and 7 male subjects; median age, 44.5 years; age range, 28–59 years; see [Tables E1 and E2](#) in this article's Online Repository at www.jacionline.org). Healthy volunteers (negative clinical history, skin prick test response, and ImmunoCAP result; $n = 12$; 8 female and 4 male subjects; median age, 29 years; age range, 23–53 years) served as control subjects. PBMCs were isolated from whole blood with approval of the local ethics committee of Rhineland-Palatinate. Written informed consent was obtained from all participants.

Flow cytometry–based proliferation assay

CD4⁺ T cells of allergic patients were stained with a final concentration of 0.5 $\mu\text{mol/L}$ CellTrace Violet (Invitrogen, Darmstadt, Germany) before T-cell stimulation. On day 8 after primary stimulation, T cells were recovered, additionally stained with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) at a final concentration of 1 $\mu\text{mol/L}$, and subsequently restimulated as described. After primary and secondary stimulation, T-cell proliferation was detected (LSR II; BD Bioscience, Heidelberg, Germany) on days 0/1 and 4 after staining. Toxic effects of the dyes were excluded by using Annexin V/7-amino-actinomycin D staining, and maintenance of their proliferative capacity was proved in response to polyclonal activation by anti-CD3/CD28.

For description of allergen expression, flow cytometry, dendritic cell (DC) culture, CD4⁺ T-cell isolation, primary and restimulation experiments, cytokine ELISA, detection of specific IgE, quantitative IgE ELISA inhibition experiments, and statistical methods, see the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS**Study group: Characterization of the sensitization status by determination of IgE**

The study group comprised 20 allergic subjects who were selected according to birch pollen and hazelnut allergy with

positive skin prick test responses and a respective CAP class of 2 or greater for birch and hazelnut extract and the recombinant allergens Bet v 1 and Cor a 1 (see [Tables E1 and E2](#) and [Fig E1, A](#), in this article's Online Repository at www.jacionline.org).

Identification of cross-reactive Bet v 1- and Cor a 1-reactive IgE in subjects

Individual sera from 20 donors with birch pollen and hazelnut allergy showed a significant correlation of specific IgE values to birch versus hazelnut extract and recombinant Bet v 1 versus Cor a 1, respectively (see [Fig E1, B](#)). Therefore, we analyzed the extent of IgE cross-reactivity between Bet v 1 and Cor a 1 using dose-related IgE-binding competition assays with sera of 11 individual patients selected based on high specific IgE levels. IgE binding to Bet v 1 was completely eliminated by 10 to 50 $\mu\text{g/mL}$ soluble Bet v 1, whereas IgE binding to Bet v 1 was only partially inhibited on preincubation with soluble Cor a 1 at the highest concentration (median, 43%; [Fig 1, upper panel](#)). In contrast, when Bet v 1 was applied as an inhibitor, it completely eliminated the IgE binding to Cor a 1 at low concentrations of 0.4 to 2 $\mu\text{g/mL}$ ([Fig 1, lower panel](#)). Analysis of the relative potency of Cor a 1 as an inhibitor in comparison with Bet v 1 resulted in a considerably lower relative potency for Cor a 1, suggesting a higher avidity of Cor a 1-reactive IgE to Bet v 1 than to Cor a 1 ([Fig 1, lower panel](#)). The data suggest that Cor a 1-reactive IgE constitutes a subgroup of Bet v 1-reactive IgE and support the theory that the majority of IgE epitopes of Cor a 1 (food allergen)^{25,31} are present on Bet v 1 (pollen allergen) but not *vice versa*.

T cells of allergic donors exhibit significant allergen-specific proliferation

Human monocyte-derived DCs have been used previously for the analysis of allergen-specific T-cell responses.^{32–36} We applied autologous human DCs as stimulators for the analysis of allergen-specific CD4⁺ T-cell responses and cross-reactivity between recombinant Bet v 1, Cor a 1, and Dau c 1. To reduce background proliferation and avoid exogenous cytokine-related bias, we performed coculture experiments of DCs and primary T cells without addition of cytokines or repeated stimulations.

In [Fig 2](#) T-cell proliferation is expressed as the stimulation index (SI) normalized to background proliferation in the presence of unloaded DCs. After primary activation, a significant specific T-cell proliferation in the group of patients with allergy to Bet v 1 (median SI, 2.1) and Cor a 1 (median SI, 1.6), but not Dau c 1 (median SI, 1.1), was determined compared with that in healthy volunteers ([Fig 2](#)).

T-cell responses of individual donors exhibited a considerable degree of variation in terms of the allergen used for stimulation. Nevertheless, analyses demonstrated a significant allergen-specific T-cell proliferation in all allergic patients compared with that seen in healthy control subjects. No correlation was found between specific IgE levels and specific T-cell proliferation of individual patients (see [Table E1](#) and [Figs E1 and E2](#) in this article's Online Repository at www.jacionline.org).

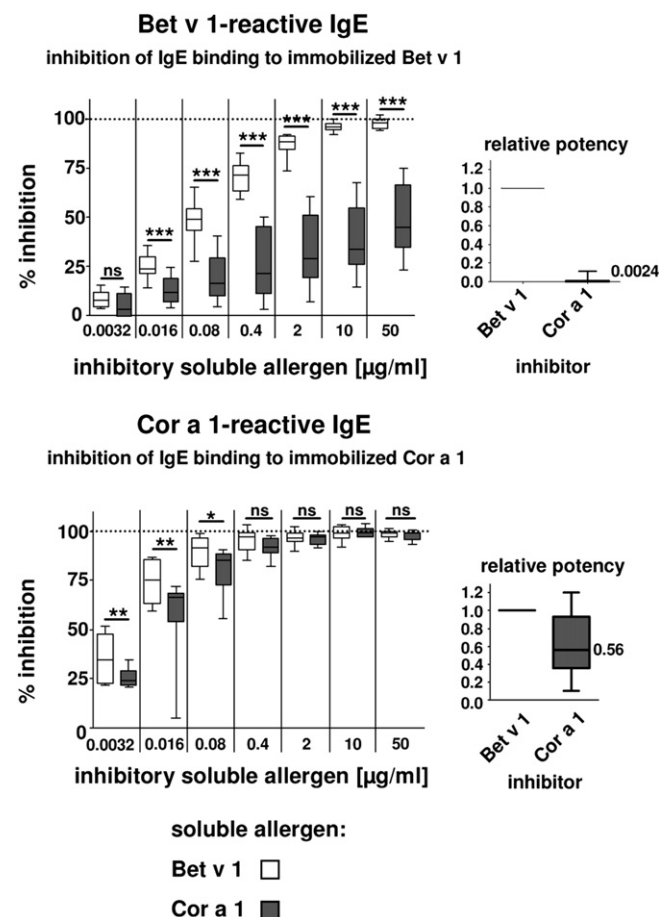


FIG 1. Cor a 1 only partly competes with specific Bet v 1-IgE interactions in contrast to Bet v 1, which completely abolishes IgE binding to Cor a 1. The sera of 11 donors were used in inhibition ELISA assays to analyze IgE reactivity to immobilized Bet v 1 (upper panel) and immobilized Cor a 1 (lower panel). The percentage of inhibition is presented as pooled results of 11 assays (medians and interquartile ranges). Relative potency of inhibition is calculated as described in the Methods section. Medians and interquartile ranges are depicted. Open boxes, Soluble Bet v 1; gray boxes, Cor a 1 used as a competitor. * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

Allergen-specific activation of T cells from allergic donors results in a strong T_H2 -biased cytokine profile

The allergen-specific cytokine response of allergic subjects at primary stimulation was clearly dominated by the T_H2 cytokine IL-5 accompanied by enhanced levels of IL-9 and IL-13 (Fig 3, A, and for details [ranges and medians, see Table E3 in this article's Online Repository at www.jacionline.org]). We observed a significant Bet v 1- and Cor a 1-specific increase and a tendency for a Dau c 1-specific increase in IL-5 and IL-13 levels in supernatants of allergen-stimulated $CD4^+$ T-cell cultures of allergic subjects. A significant allergen-specific enhancement in IL-9 production was found in response to all allergens: Bet v 1, Cor a 1, and Dau c 1 (Fig 3, A, and see Table E3). Allergen-induced secretion of IL-5, IL-9, and IL-13 was not detected in healthy control subjects (Fig 3, A, and see Table E3).

Production of the T_H1 cytokine IFN- γ was slightly but significantly increased in response to Bet v 1 in the allergic

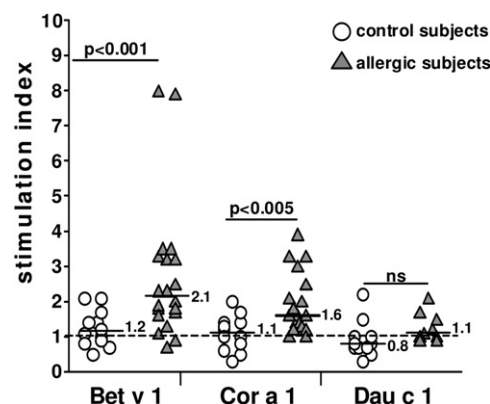


FIG 2. Allergen-specific T-cell proliferation of allergic donors to recombinant Bet v 1, Cor a 1, and Dau c 1. Primary T-cell proliferation of allergic patients and nonallergic control subjects induced by Bet v 1-, Cor a 1-, and Dau c 1-loaded DCs is shown. Compiled results of allergic patients ($n = 20$) and control subjects ($n = 12$) are shown. Median values for each group are indicated. The SI of allergen-specific proliferation was calculated by using normalization to proliferation induced by control DCs. $P < .05$. ns, Not significant.

group but not to Cor a 1 or Dau c 1 (Fig 3, B, and see Table E3). Amounts of IFN- γ in nonallergic control subjects were comparable with levels in allergic patients. Thus for allergic subjects, the mean IL-5/IFN- γ ratios for Bet v 1-, Cor a 1-, and Dau c 1-specific cytokine production, respectively, were significantly higher compared with the corresponding ratios for nonallergic control subjects, indicating a strong IL-5-dominated cytokine profile in allergic subjects only (Fig 3, C).

The experiments did not reveal any significant allergen-induced IL-10 production by T cells from allergic or nonallergic donors (Fig 3, B). Production of IL-2 and IL-4 was less than the detection limit for both the group of allergic and nonallergic subjects (data not shown).

T-cell cross-reactivity to the major allergens of birch, hazelnut, and carrot

T cells were recovered after primary stimulation and subsequently restimulated either with identical allergen, as in primary coculture experiments, or the respective homologous allergen to assess T-cell cross-reactivity. Fig 4, A, shows the compiled results of restimulation assays for allergic ($n = 15$; Fig 4, A, upper panel) and nonallergic ($n = 12$; Fig 4, A, lower panels) subjects. All data have been normalized to the respective control of 2 subsequent stimulations with autologous unloaded DCs and are expressed as the SI. Secondary stimulation with Bet v 1 of T cells that were preactivated with the identical allergen (Bet v 1 $\rightarrow$ Bet v 1; SI, 2.9), with the homologous Cor a 1 (Cor a 1 $\rightarrow$ Bet v 1; SI, 2.9), or with Dau c 1 (Dau c 1 $\rightarrow$ Bet v 1; SI, 2.3) resulted in a significant increase in T-cell proliferation in all approaches compared with control T cells (none $\rightarrow$ Bet v 1; SI, 1.5; Fig 4, A, upper left). In addition, the effect of preactivation with Bet v 1 and Dau c 1 on the resulting T-cell response of allergic donors induced by restimulation with Cor a 1 was analyzed (Fig 4, A, upper right). Here we found similar results as observed for secondary stimulation with Bet v 1 (Fig 4, A, upper right). Restimulation by Cor a 1 of T

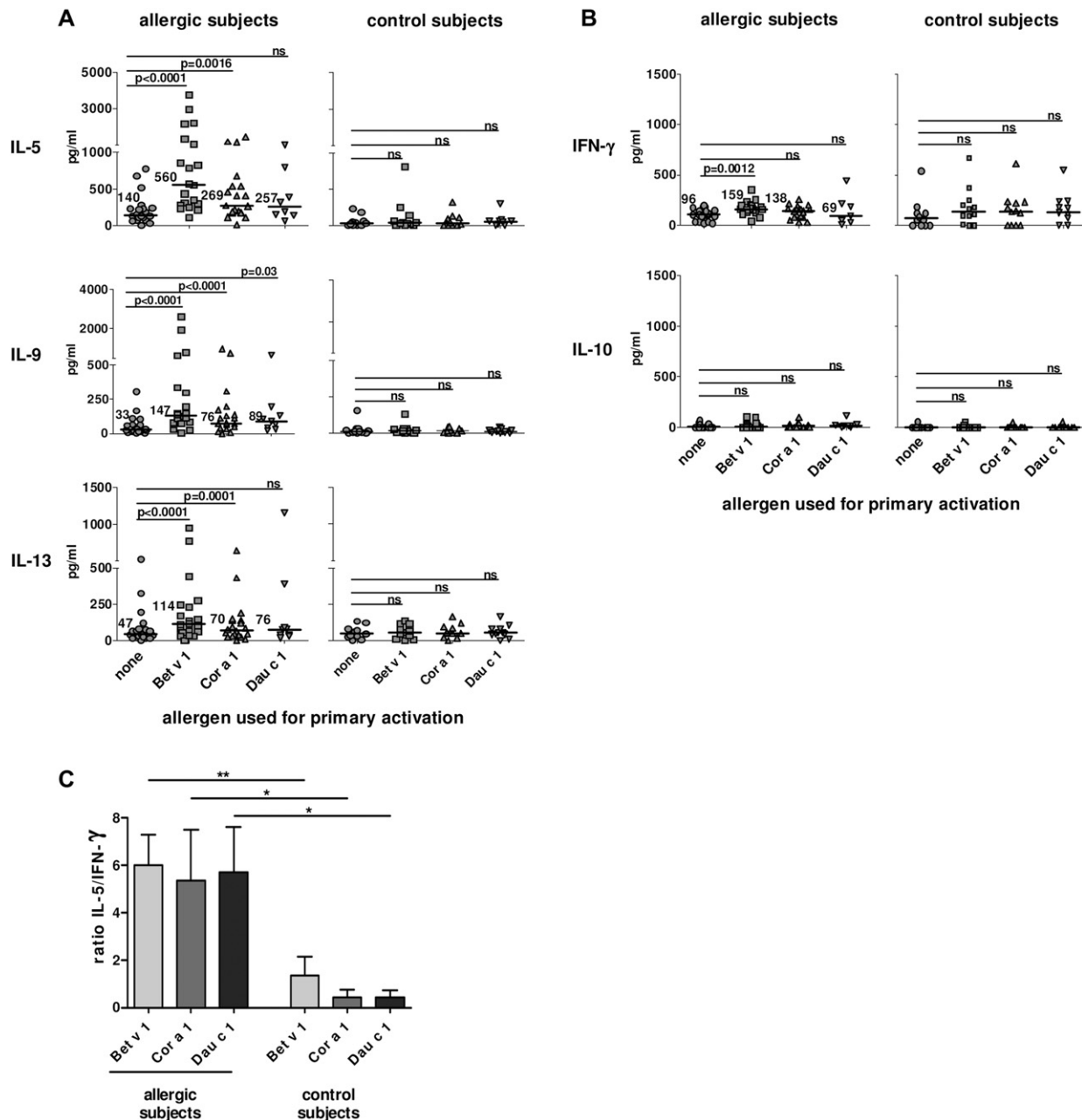


FIG 3. T_H2 cytokine profile of allergic patients in primary response to Bet v 1, Cor a 1, and Dau c 1. Supernatants of primary culture were obtained on day 3 and analyzed for cytokine production. **A**, IL-5, IL-9, and IL-13. **B**, IFN- γ and IL-10. **C**, Pooled results are shown for allergic patients ($n = 20$) and control subjects ($n = 12$). IL-5/IFN- γ ratios were calculated for individual subjects. Means $\pm$ SEMs are demonstrated. * $P \leq .05$ and ** $P \leq .01$. ns, Not significant.

cells preactivated by the identical allergen Cor a 1 (Cor a 1 $\rightarrow$ Cor a 1; SI, 2.7) or Bet v 1 (Bet v 1 $\rightarrow$ Cor a 1; SI, 2.5) exhibited a significantly enhanced proliferation compared with that seen in control T cells (none $\rightarrow$ Cor a 1; SI, 1.1). In contrast, primary activation with Dau c 1 (Dau c 1 $\rightarrow$ Cor a 1; SI, 1.1) had no effect (Fig 4, A, upper right). Analysis of healthy donors did not reveal any significant effect of preactivation (identical or homologous) on subsequent secondary allergen-specific T-cell responses (Fig 4, A, lower panels).

To gain more detailed insight into the respective effect of identical versus homologous preactivation in individual

responses, Fig 4, B, depicts the data of individual allergic subjects normalized to primary stimulation with unloaded DCs and restimulation with Bet v 1 (none $\rightarrow$ Bet v 1 = 100%, left panel) or Cor a 1 (none $\rightarrow$ Cor a 1 = 100%, right panel), respectively. In restimulation experiments with Bet v 1, preactivation by Cor a 1 or Dau c 1 resulted in a significant increase in T-cell proliferation in 11 (73%) of 15 or 5 (71%) of 7 patients, respectively (Fig 4, B, left panel). When Cor a 1 was applied for restimulation, preactivation with Bet v 1-loaded DCs induced a significantly enhanced T-cell response in 13 (87%) of 15 allergic subjects, whereas primary stimulation with Dau c 1 (Dau c 1 $\rightarrow$ Cor a 1) induced a relevant

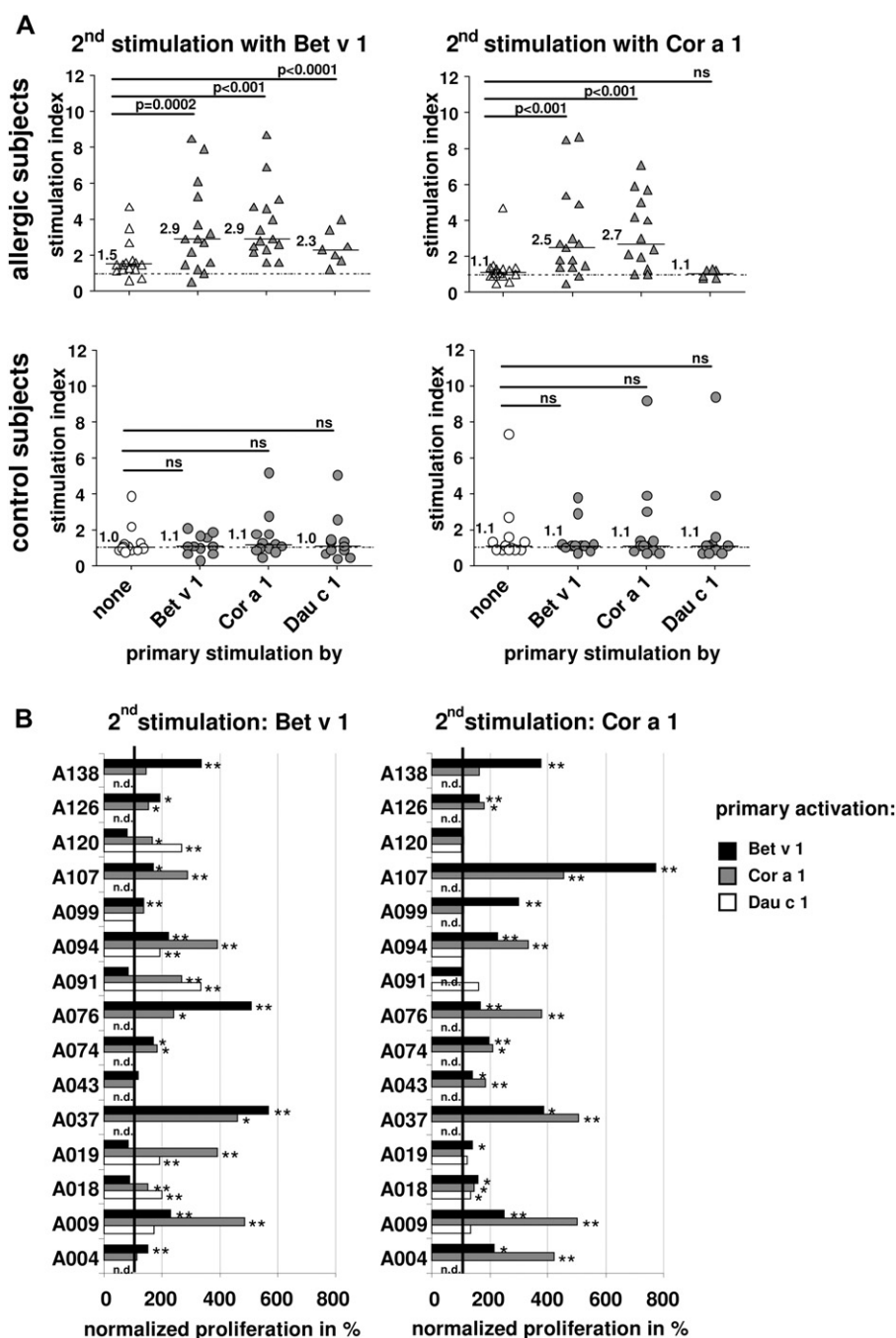


FIG 4. T-cell cross-reactivity of major allergens of birch, hazelnut, and carrot. **A**, Pooled results of T-cell proliferation of allergic patients ($n = 15$) and control subjects ($n = 12$) are demonstrated for restimulation with Bet v 1 (left panel) and Cor a 1 (right panel). **B**, Individual T-cell proliferation induced by restimulation with Bet v 1 (left panel) or Cor a 1 (right panel), respectively, is shown. Tritiated thymidine incorporation data are normalized to T-cell proliferation induced by the respective allergen in restimulation experiments after primary culture with unloaded DCs (= 100%, black lines). * $P < .05$ and ** $P < .01$. n.d., Not done.

T-cell response in 1 (17%) of 6 allergic patients (Fig 4, B, right panel).

In summary, 15 (100%) of 15 patients tested showed T-cell cross-reactivity between Bet v 1 and Cor a 1 at least in 1 experimental setting (Bet v 1 $\rightarrow$ Cor a 1) or *vice versa* (Cor a 1 $\rightarrow$ Bet v 1). In line with IgE and clinical history, these data suggest Bet v 1/Cor a 1–T-cell cross-reactivity in all patients of our study cohort.

Cor a 1/Bet v 1–cross-reactive T cells are a predominant, highly proliferative subpopulation of allergen-responsive T cells

To address the issue of T-cell monoreactivity or cross-reactivity in defined T-cell subpopulations, we combined the above-described culture system for primary T cells with a novel flow cytometry–based dye-dilution proliferation assay. Briefly, T cells were stained before primary stimulation with CellTrace Violet

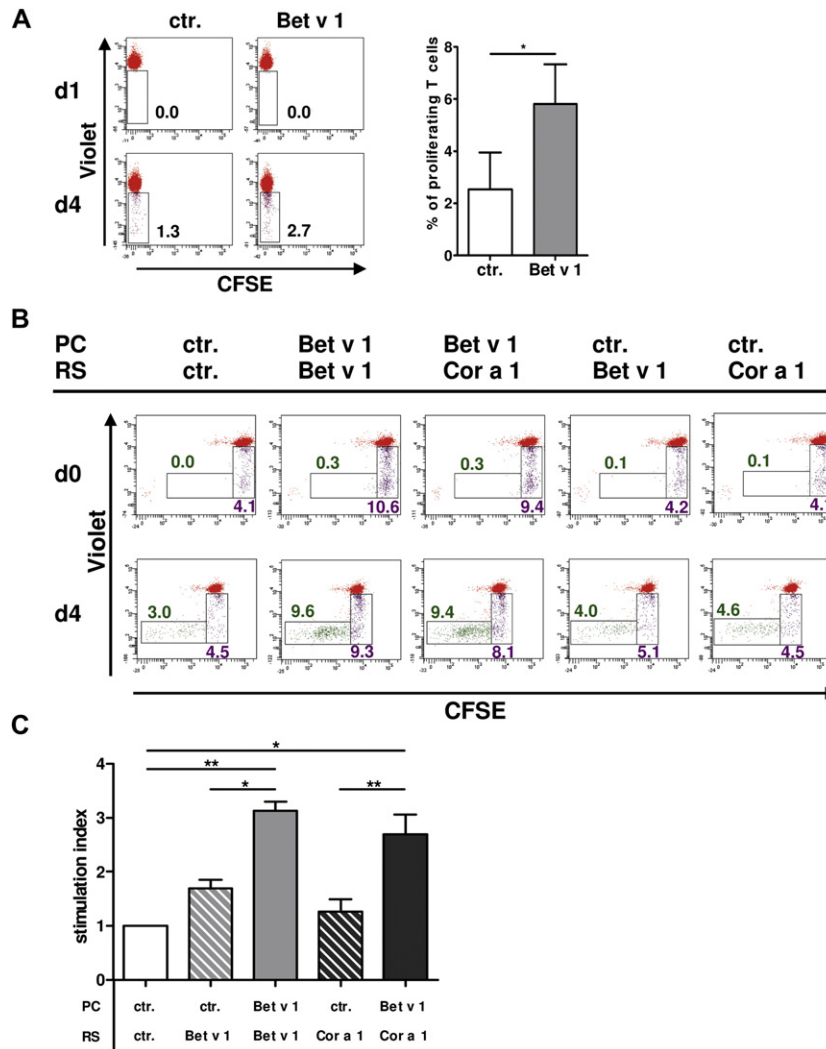


FIG 5. Identification of a Bet v 1/Cor a 1-cross-reactive T-cell subpopulation. T cells of allergic patients were primarily stimulated (primary culture [PC]) with Bet v 1-loaded, Cor a 1-loaded, or control DCs (unloaded and OVA loaded), respectively, and restimulated (RS) as indicated. **A-C**, Bet v 1 → Cor a 1; **D-F**, Cor a 1 → Bet v 1 (and corresponding controls). T-cell proliferation was detected by means of flow cytometry with fluorescent dye-dilution assays. Fig 5, A and D, Primary proliferation of T cells on day 1 and day 4 after CellTrace Violet staining of 1 representative donor (left) and pooled results (right) of 4 (Fig 5, A) or 5 (Fig 5, D) donors. Fig 5, B, C, E, and F, Secondary proliferation was assessed after subsequent staining with CFSE of T cells before restimulation on days 0/1 and 3/4. One representative result (Fig 5, B and E) and pooled results of 3 (Fig 5, C) and 4 (Fig 5, F) independent experiments are depicted. Means ± SEMs are demonstrated. * $P \leq .05$ and ** $P \leq .01$.

and before restimulation with CFSE to assess proliferation after primary stimulation (Violet) and after restimulation (Violet/CFSE). This method allows for discrimination of T cells dividing after either primary or secondary stimulation only and T cells dividing in response to both.

After Bet v 1-specific primary culture, we observed a significant increase in allergen-specific proliferation (CellTrace Violet) at day 4 compared with that seen in control T cells (Fig 5, A). Subsequently, restimulation experiments (CFSE staining) revealed that only a minor percentage of control T cells (prestimulated with unloaded control DCs: [control → control], [control → Bet v 1], and [control → Cor a 1]) exhibited an allergen-specific proliferation to Bet v 1- or Cor a 1-specific restimulation, respectively (Fig 5, B and C). We identified T cells dividing

vigorously in response to the identical allergen (Bet v 1 → Bet v 1) and a Bet v 1/Cor a 1 cross-reactive T-cell population (Bet v 1 → Cor a 1). The vast majority of both T-cell populations resided within T cells already strongly proliferating in primary stimulation in response to Bet v 1 (Violet^{dim}CFSE⁺; Fig 5, B and C). We did not detect Cor a 1-monospecific T cells after restimulation with the hazelnut allergen. Pooled data confirmed these results and demonstrated significant differences between controls (control → control, control → Bet v 1, control → Cor a 1) and identical (Bet v 1 → Bet v 1) or homologous (Bet v 1 → Cor a 1) restimulation, respectively (Fig 5, C).

To confirm the existence of a dominant Cor a 1/Bet v 1-cross-reactive T-cell population, we used the food allergen Cor a 1 for primary stimulation and the pollen allergen Bet v 1 for

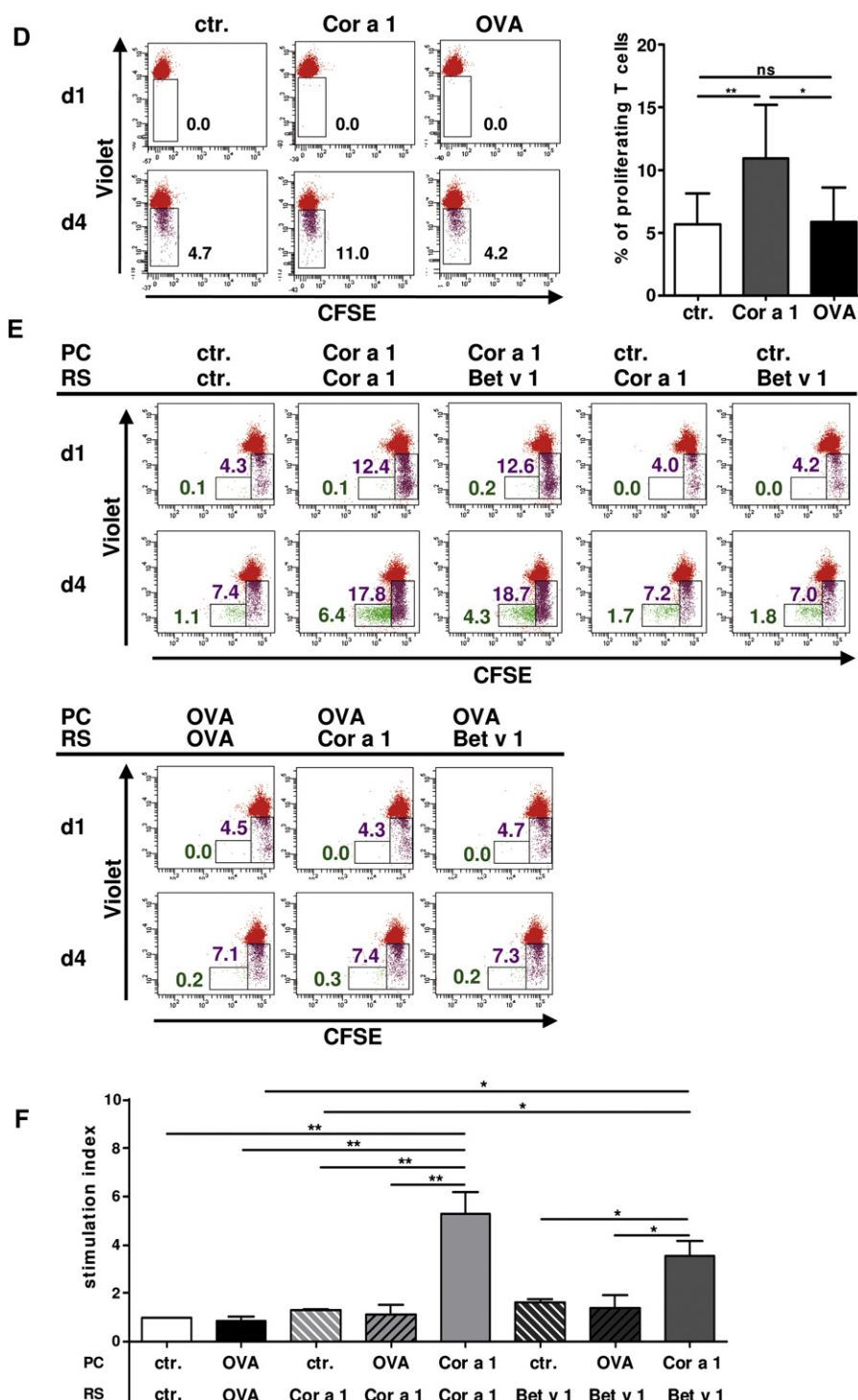


FIG 5. (Continued)

restimulation experiments (Cor a 1 → Bet v 1; Fig 5, D and E). Unloaded and ovalbumin (OVA)-loaded DCs, a food allergen that is not homologous to Cor a 1 or Bet v 1, served as controls. After primary activation with Cor a 1, significant specific T-cell proliferation was induced compared with that seen in control groups (Fig 5, D). In line with our previous results, after restimulation, we identified a dominant Cor a 1/Bet v 1-cross-reactive T-cell population (Cor a 1 → Bet v 1) within the highly proliferative

Cor a 1 subpopulation (Fig 5, E and F). As demonstrated for identical restimulation with Bet v 1, primary stimulation and restimulation with Cor a 1 induced a strong Cor a 1-specific T-cell proliferation (Fig 5, E and F). Pooled data of these experiments revealed significant differences between unloaded controls (control → control, control → Bet v 1, control → Cor a 1) and homologous (Cor a 1 → Bet v 1) or identical (Cor a 1 → Cor a 1) restimulation (Fig 5, E and F). Moreover, we did not observe

significant T-cell proliferation after primary stimulation with OVA as a control food allergen independently of the allergen used for restimulation (OVA → OVA, OVA → Bet v 1, OVA → Cor a 1), excluding an unspecific T-cell activation caused by primary activation by (food) allergen-loaded DCs (Fig 5, F). These experiments identified a predominant Cor a 1/Bet v 1–cross-reactive T-cell population within highly proliferative allergen-reactive T cells in patients with birch pollen allergy and associated hazelnut allergy.

DISCUSSION

In this study we characterized allergen-specific, T_H2-dominated T-cell responses and T-cell cross-reactivity in a group of patients with birch pollen allergy with associated food allergy to hazelnut and carrot. By using primary T cells, a strong allergen-specific T-cell activation and T_H2 response (dominated by IL-5), mainly to Bet v 1, Cor a 1, and, to a lesser extent, Dau c 1, was detected, reflecting the clinical and diagnostic data of the individual patients.³⁷

To address T-cell cross-reactivity, we applied 2 complementary approaches for assessment of T-cell proliferation: one standard method of tritiated thymidine incorporation and a novel approach with a flow cytometry–based 2-step dye-dilution assay. This second method allows us to gain more detailed insights into T-cell proliferation in response to a second allergen (Cor a 1) after previous activation by a homologous allergen (Bet v 1). Our data demonstrated significant T-cell cross-reactivity among Bet v 1, Cor a 1, and, to a lesser extent, Dau c 1 at the level of primary T cells. In addition, we identified a predominant Cor a 1/Bet v 1–cross-reactive T-cell subpopulation within the highly proliferating allergen-specific T-cell pool in these patients. Performing IgE cross-inhibition assays, we found evidence that Bet v 1–reactive and the vast majority of Cor a 1–reactive IgE exhibits a cross-reactivity to Cor a 1 or Bet v 1, respectively, supporting the view that pollen-derived allergens act as the sensitizing agent for food allergens.^{25,31}

The allergens Cor a 1 (hazelnut) and Dau c 1 (carrot) have a molecular mass of 17.4 kDa (160 amino acids) and 16.1 kDa (153 amino acids) and share 80% and 58% sequence similarity with Bet v 1 and 56% similarity (36.4% amino acid identity) between each other, respectively.¹⁹ Two groundbreaking studies demonstrated T-cell cross-reactivity among birch pollen and food allergens to hazelnut and carrot through analysis of T-cell lines and T-cell clones from patients with birch pollen allergy and oral allergy syndrome.^{19,21} In addition, Bet v 1₁₄₂₋₁₅₆ was identified as the dominant T-cell epitope of the major birch pollen allergen and as of particular importance for cross-reactivity in patients with Bet v 1–related food allergy.¹⁹ The majority or at least a considerable percentage of the 11 generated Bet v 1₁₄₂₋₁₅₆–reactive T-cell clones displayed cross-reactivity to Cor a 1 (54.5%), Dau c 1 (45.5%), or both (36%).¹⁹ In the second study Bohle et al²¹ demonstrated 8 of 18 Cor a 1.04–specific T-cell clones to be cross-reactive to Bet v 1, whereas 10 did not proliferate in response to Bet v 1, but 8 of 9 tested were reactive to Dau c 1.

Our experiments with the novel approach of primary allergen-specific T cells confirm the results of these former studies. In addition, our study identifies the predominance of both cross-reactive T-cell populations and cross-reactive IgE to hazelnut in individual allergic patients with birch pollen–associated food allergy to hazelnut. This is the first study showing cross-reactivity

between both allergens by using dose-dependent IgE cross-inhibition assays. In line with former studies, we found that a high percentage of allergic patients with clinically relevant birch pollen–food syndrome exhibited T-cell cross-reactivity between Bet v 1 and the food allergen of hazelnut (Cor a 1) through the use of tritiated thymidine assays. In a second complementary approach of a novel flow cytometry–based assay, a predominant Cor a 1/Bet v 1–cross-reactive T-cell subpopulation within highly allergen-reactive T cells was identified. Nevertheless, one still has to keep in mind that these results do not exclude the existence of minor populations of Cor a 1–specific T cells lacking reactivity to Bet v 1 or *vice versa*, which might be beyond the detection limits of the method used.

The results obtained are in accordance with the theory that both exposition of the immune system to homologous allergens from seasonal airborne pollen and nonseasonal food can drive cross-specific reactions after initial sensitization by the pollen allergen. Recurrent exposition to the Bet v 1 homologues in food might selectively favor the expansion of cross-reactive Bet v 1–specific T cells and might drive the affinity maturation of cross-reactive IgE.

Some studies monitoring SIT described an improvement of concomitant food allergy in successfully SIT-treated patients with birch pollen allergy, whereas other studies reported no effects or even the induction of hypersensitivity reactions.²⁷⁻³⁰ These inconsistencies, apart from IgE-related mechanisms, might be due to a variation in the quality and quantity of cross-reactive T-cell repertoires in individual patients. Thus, identification of monoreactive or cross-reactive T-cell subpopulations, respectively, in the context of SIT can result in improved immunotherapeutic protocols. Therefore, analysis of T-cell cross-reactivity in primary unaffected T cells and the visualization of cross-reactivity in allergen-specific T-cell subpopulations might allow us to monitor the response to immune-modulating approaches at the level of individual allergic patients.

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Clinical implications: Our study supports the pivotal role of T-cell cross-reactivity in the pathogenesis of pollen-associated secondary food allergy and might facilitate the evaluation of SIT efficacy on the level of allergen-specific T-cell subpopulations.

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METHODS

Heterologous expression and purification of allergens

Bet v 1.0101 (GenBank accession no. X15877), Cor a 1.0401 (GenBank accession no. AF136945), and Dau c 1.0104 (GenBank accession no. Z81362) were overexpressed in *Escherichia coli* by using the plasmids pET15b-Betv1, pET15b-Cora1, and pET16b-Dauc1. Histidine-tagged proteins were purified from cell lysates (Dau c 1) or inclusion bodies (Cor a 1 and Bet v 1) by using affinity chromatography (Ni-NTA metal chelate chromatography), as described previously.^{E1} Endotoxin was depleted by means of second affinity chromatography with EndoTrap columns (Hyglos, Regensburg, Germany) or size exclusion chromatography and monitored by using the LAL test (Pyroquant Diagnostik GmbH, Walldorf, Germany; endotoxin <0.01 EU/μg). For some experiments, Cor a 1 was obtained from Blirt (Gdansk, Poland; endotoxin <0.0001 EU/μg). OVA (endotoxin <0.001 EU/μg) was obtained from Hyglos (Bernried, Germany).

Flow cytometric analysis

The following mAbs were used for DC and T-cell staining: CD2, CD14, CD19, CD80, CD83, CD4, CD8 mouse IgG₁ (all Beckman Coulter, Krefeld, Germany); CD86 and HLA-DR (AbD Serotec, Oxford, United Kingdom); mouse IgG₁, IgG_{2a}, and IgG_{2b} and rat IgG_{2a} subclass-specific isotypes (all from BD PharMingen, San Diego, Calif); fluorescein isothiocyanate-conjugated goat anti-rat IgG (BioLegend, San Diego, Calif); and phycoerythrin-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, Bar Harbor, Me).

Generation of allergen-loaded DCs and isolation of CD4⁺ T cells

DCs and CD4⁺ T cells (purity >95%) were generated from whole blood, as previously described.^{E2} On day 6 of DC culture, nonadherent cells were obtained, and a maturation cocktail was added for 2 days to induce final maturation.^{E2} Simultaneously, DCs (1×10^6) were loaded with 20 μg/mL of the respective recombinant allergen or remained unloaded as a control. Dose-response experiments revealed the optimal amounts of the respective allergen (20 μg/mL for Bet v 1, Cor a 1, and Dau c 1 and 5 μg/mL for OVA) for DC loading and excluded a detrimental or modulating effect on maturation of DCs (data not shown).

Primary stimulation and restimulation of allergen-specific CD4⁺ T cells

Allergen-loaded and control DCs (unloaded or OVA-loaded, 2.5×10^5 cells/well) were cocultured with 2.5×10^6 cells/well CD4⁺ T cells for 5 days for primary *in vitro* stimulation. After a resting period of 3 days, allergen-specific and control T cells (0.5×10^5 cells/well) were recovered and restimulated with syngeneic mature DCs (0.25×10^4 cells/well), either loaded with allergen or unloaded. After 4 (primary culture) or 3 (restimulation) days, T-cell proliferation was assessed based on tritiated thymidine incorporation (in triplicates). SI primary T-cell proliferation was calculated based on normalization to background proliferation in the presence of unloaded DCs. In restimulation experiments the SI of pooled data was normalized to the control T-cell proliferation induced by unloaded DCs in both primary and secondary experiments. Data of 20 patients on primary response and 15 patients on restimulation were included in the analyses. Reduction was due to some patients dropping out of the study, the limitation of cell numbers, or both.

Cytokine ELISA

Supernatants of primary culture were obtained at day 3. ELISA kits were used according to the instructions of the manufacturer: IL-2 and IL-5 from BD PharMingen; IL-4, IL-10, IL-13, and IFN-γ from ImmunoTools (Friesoythe, Germany); and IL-9 from eBioscience (San Diego, Calif).

Detection of allergen-specific IgE

Specific IgE concentrations to allergen extracts from birch (t3), hazelnut (f17), and carrot (f31) and the major allergens Bet v 1 and Cor a 1 were determined by using the commercial ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). Dau c 1-specific IgE values were determined by using experimental ImmunoCAP (Thermo Fisher Scientific) tests.^{E3} Specific IgE values are expressed as kilounits of allergen per liter as follows: CAP class 0 (negative), less than 0.35 kU_A/L; CAP class 1, 0.35 to 0.7 kU_A/L or less; CAP class 2, greater than 0.7 to 3.5 kU_A/L or less; CAP class 3, greater than 3.5 to 17.5 kU_A/L or less; CAP class 4, greater than 17.5 to 50 kU_A/L or less; CAP class 5, greater than 50 to 100 kU_A/L or less; and CAP class 6, greater than 100 kU_A/L.

Quantitative IgE ELISA inhibition experiments

The sera of 11 donors (A004, A009, A018, A037, A074, A076, A091, A099, A107, A120, and A138) were used for ELISA cross-inhibition assays. IgE cross-reactivity between Bet v 1 and Cor a 1 was determined by using ELISA cross-inhibition assays. Briefly, wells were coated for 3 hours with the purified allergens (2 μg/mL) and then blocked with 2% BSA in PBST (PBS plus 0.05% Tween 20) for 1 hour. Afterward, 50 μL of diluted patient serum plus 50 μL of the respective inhibitor, either Bet v 1 or Cor a 1 (0.0032–50 μg/mL), respectively, was incubated overnight at 4°C. Subsequently, wells were washed and incubated with 100 μL of peroxidase-labeled anti-human IgE antibody (SBA, Birmingham, Ala). The colorimetric reaction was started by adding tetramethylbenzidine substrate buffer and stopped with 3 mol/L sulfuric acid, and the absorbance was measured at 450 nm. Statistical data are represented as combined box-and-whisker plots (GraphPad Prism version 5.00 for Windows; GraphPad Software, La Jolla, Calif), indicating the comparison of maximum inhibition values in percentages or relative potencies from inhibition curves of Cor a 1 related to the inhibition curves of Bet v 1. The latter were calculated with SoftMax Pro 5.2 software (Molecular Devices, Sunnyvale, Calif) according to a method first described by Gottschalk and Dunn.^{E4} For relative potency determinations, the curves are interpolated at the 50% effective dose. Combined box-and-whisker plots (GraphPad Prism version 5) demonstrate the relative potencies from inhibition curves of Cor a 1 related to the inhibition curves of Bet v 1 (set to factor = 1) coated with Bet v 1 or Cor a 1, respectively. The 25th and 75th percentiles and medians are depicted in the figures. Error bars above and below the box indicate minimum and maximum percentiles.

Statistical methods

We computed means, medians, SDs, SEMs, minimums, maximums, and quartiles for quantitative variables. Results are displayed in box plots to assess pairwise association of variables. Respective proliferation data of each donor were analyzed by using the paired Student *t* test, as indicated. ImmunoCAP values obtained with extract and the corresponding recombinant allergen were compared by using the Wilcoxon signed-rank test. Statistical analyses were performed with SAS 9.2 software (SAS Institute, Cary, NC), and mixed linear models were fitted with PROC MIXED. *P* values less than or equal to .05 are considered significant.

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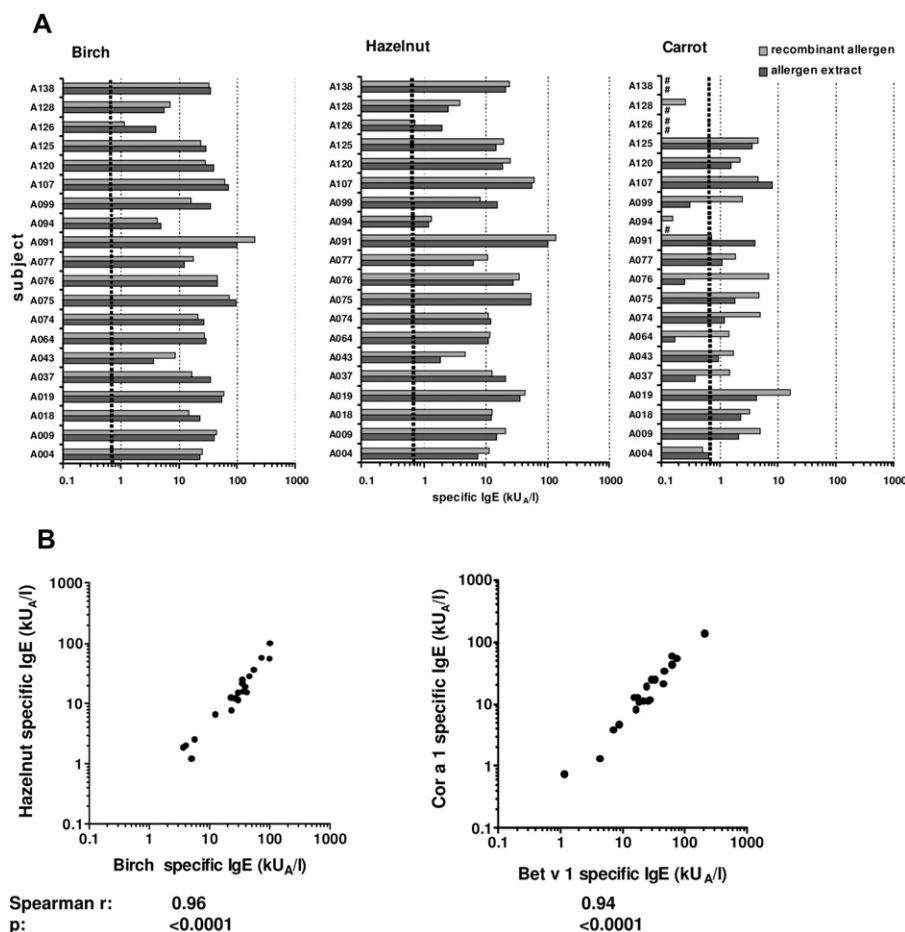


FIG E1. Specific IgE levels to birch, hazelnut, and carrot allergen extracts and recombinant proteins of allergic patients. **A**, Sera of allergic patients ($n = 20$) with proved birch pollen allergy (based on clinical history, skin prick test response, specific IgE level, and CAP class ≥ 2) and secondary food allergy to hazelnut, carrot, or both were tested for IgE reactivity to extracts and recombinant allergens by using ImmunoCAP. The respective results of ImmunoCAP assays to birch, hazelnut, and carrot are shown as the concentration of specific IgE (in kilounits of allergen-specific IgE per liter). The threshold for CAP class 2 is indicated as a line. #<0.1 kU_A/L. **B**, The results of the IgE tests of the 20 patients shown in Fig E1 were plotted as follows: IgE reactive to birch extract versus IgE reactive to hazelnut extract (*left*) and Bet v 1-reactive IgE versus Cor a 1-reactive IgE (*right*). Correlation (Spearman r) and significance are indicated.

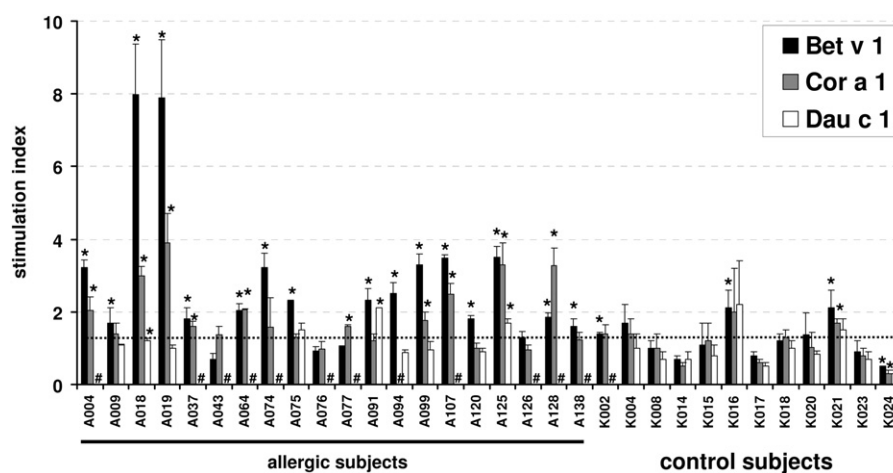


FIG E2. Individual allergen-specific T-cell proliferation of allergic patients and healthy control subjects. Primary T-cell proliferation of allergic patients and nonallergic control subjects induced by Bet v 1-, Cor a 1-, and Dau c 1-loaded DCs is shown. Individual results of allergic patients ($n = 20$) and control subjects ($n = 12$) are demonstrated. The SI of allergen-specific proliferation was calculated by normalization to T-cell proliferation induced by control DCs, as described in the Methods section. Significance ($*P \leq .05$) is related to T-cell responses induced by unloaded control DCs. #Not done.

TABLE E1. Characterization of allergic patients

Allergic subject	Sex	Age (y)	Birch				Hazelnut				Carrot			
			History	Skin prick test response	CAP (kU _A /L)	Class	History	Skin prick test response	CAP (kU _A /L)	Class	History	Skin prick test response	CAP (kU _A /L)	Class
A004	F	55	+	NA	23.1	4	+	NA	7.7	3	—	NA	0.7	1
A009	F	33	+	+++	41.7	4	+	+++	15.4	3	+	+	2.1	2
A018	F	35	+	++	22.7	4	—	+	12.6	3	+	++	2.4	2
A019	F	29	+	+	53.9	5	+	+	36.2	4	+	+	4.3	3
A037	M	53	+	+++	35.7	4	+	+++	21.5	4	—	—	0.4	1
A043	F	40	+	+++	3.6	3	+	NA	1.9	2	—	NA	1.0	2
A064	M	44	+	++	29.9	4	+	++	11.4	3	—	++	0.2	0
A074	M	46	+	++	27.0	4	+	+	12.2	3	+	+	1.2	2
A075	F	49	+	++	97.9	5	—	++	56.0	5	+	+	1.9	2
A076	M	51	+	++++	45.8	4	+	+++	28.5	4	+	++	0.3	0
A077	F	29	+	+++	12.4	3	—	++	6.5	3	+	+++	1.1	2
A091	F	41	+	+++	>100	6	+	+++	>100	6	+	+	4.1	3
A094	F	55	+	+	5.0	3	+	+	1.2	2	+	NA	0.0	0
A099	F	59	+	+++	35.7	4	+	++	15.9	3	+	++	0.3	0
A107	F	45	+	++	72.6	5	+	++	57.7	5	+	++	8.1	3
A120	F	26	+	++	38.7	4	+	++	18.9	4	+	+	1.6	2
A125	F	50	+	+	29.5	4	+	+++	15.0	3	+	++	3.6	3
A126	M	45	+	++	4.0	3	—	+	2.0	2	—	NA	0.0	0
A128	M	29	+	+++	5.6	3	+	+	2.5	2	—	NA	0.0	0
A138	M	28	+	++	35.0	4	+	+++	21.5	4	—	NA	0.0	0
Median		44.5												
Range		28-59												
Mean		42												

F, Female; M, male; NA, not available.

TABLE E2. Characterization of allergic patients

Allergic subject	Clinical symptoms*							Other allergies*										
	Birch			Hazelnut		Carrot		Pollen allergy	Birch pollen-related food allergies									
	Rhinitis	Conjunctivitis	Severe systemic reaction	OAS	Severe systemic reaction	OAS	Severe systemic reaction		Hazel	Apple	Cherry	Peach	Apricot	Plum	Pear	Kiwi	Celeriac	Soy
A004	2	3	0	2	0	0	0	+	+	+	−	+	−	−	−	−	+	
A009	1-3	1	0	1-2	0	1	0	+	+	+	−	−	−	−	−	+	+	
A018	2	2	0	0	0	1	0	+	+	+	−	−	−	−	−	−	−	
A019	2	0	2-3	3	3	3	3	+	+	+	+	+	+	+	+	+	−	
A037	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
A043	3	3	0	3	2	0	0	−	−	−	+	+	+	−	−	−	−	
A064	3	2	0	1-2	2	0	0	NA	+	+	+	+	−	+	−	−	−	
A074	3	3	0	3	0	3	0	+	+	+	+	+	+	+	+	+	−	
A075	3	3	0	0	0	0	0	+	−	+	−	−	−	−	−	+	−	
A076	3	3	0	1	0	0	0	+	+	−	−	−	−	−	−	−	−	
A077	1	1	3	0	0	3	3	+	+	−	+	−	−	−	+	+	−	
A091	1	3	2	2	0	2	0	+	+	+	+	+	+	+	+	−	−	
A094	3	3	0	3	3	3	3	+	+	−	+	+	−	−	−	−	−	
A099	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
A107	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
A120	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
A125	1-2	1	0	2	0	2	0	+	+	+	+	+	−	−	+	−	−	
A126	2-3	2-3	1-2	0	0	0	0	−	−	−	−	−	−	−	−	−	−	
A128	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
A138	3	3	0	1	0	0	0	+	+	+	−	−	−	+	+	−	+	

Severity of symptoms: 0, no symptoms; 1, mild symptoms; 2, distinct symptoms; and 3, severe symptoms.

NA, Not available; OAS, oral allergy syndrome.

*Based on case history.

TABLE E3. Cytokine production of T cells after primary stimulation with allergens

Cytokine	Baseline		Bet v 1			Cor a 1			Dau c 1		
	Median (pg/mL)	Range (pg/mL)	Median (pg/mL)	Range (pg/mL)	Significance vs baseline	Median (pg/mL)	Range (pg/mL)	Significance vs baseline	Median (pg/mL)	Range (pg/mL)	Significance vs baseline
Allergic subjects											
IL-5	140	<15-770	560	111-3760	$P < .0001$	269	<15-1452	$P = .0016$	257	65-1004	NS
IL-9	33	<3-305	147	<3-2594	$P < .0001$	76	<3-958	$P < .0001$	89	<3-660	$P = .03$
IL-13	47	<4-516	114	<4-943	$P < .0001$	70	<4-634	$P < .0001$	76	18-1156	NS
IFN- γ	96	19-200	159	41-355	$P = .0012$	138	26-253	NS	69	<15-441	NS
Control subjects											
IL-5	31	<15-228	40	<15-811	NS	27	<15-322	NS	54	<15-301	NS
IL-9	12	<3-156	15	<3-128	NS	15	<3-44	NS	11	<3-40	NS
IL-13	52	<4-132	54	<4-136	NS	52	<4-165	NS	55	<4-166	NS
IFN- γ	72	<15-536	138	<15-670	NS	137	<15-610	NS	124	<15-549	NS

Baseline, Stimulated with unloaded DCs; NS, not significant.