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A single mouse monoclonal antibody, E58 modulates multiple IgE epitopes on group 1 cedar pollen allergens

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Abstract

We recently described a dominant role for conformational epitopes on the group 1 allergen of the mountain cedar (*Juniperus ashei*, Cupressaceae), Jun a 1, in pollen hypersensitivity in South Central U.S.A. Since these epitopes are surface exposed and are likely to be flexible, they may be susceptible to molecular or physical perturbations. This may make Jun a 1 a potential target for new forms of therapy for cedar pollinosis. Here, we describe a mouse monoclonal antibody, termed E58, which binds to the group 1 allergens of cedar pollens from three highly populated regions of the world (central U.S.A., France and Japan). Upon binding to these allergens, E58 strongly reduces the binding of patient's IgE antibodies to these dominant allergens. This characteristic of E58, and potentially other similar antibodies, suggests an opportunity to identify preventative or therapeutic agents that may inhibit cedar pollen sensitization or prevent the allergic reactions.

Keywords

Allergen structure; cedar pollen allergy; conformational epitope; IgE epitope; Jun a 1; monoclonal antibody

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Conflict of interest

The authors declare no conflict of interest.

1. Introduction

The prevalence and morbidity of all allergic diseases have increased dramatically during the last 2–3 decades (Malmstrom *et al.*, 2007; McCoy *et al.*, 2005; Jorgensen *et al.*, 2000; Harrison *et al.*, 2005) to become the most common human disorders in developed countries; affecting up to 10–50% of some populations (Moorman *et al.*, 2007; Snijders *et al.*, 2007; Midoro-Horiuti *et al.*, 1992). Despite improvements in clinical management, allergic rhinitis remains a leading cause of missed work and school days (Bhattacharyya, 2012). Current disease management is based on avoiding allergen exposures, blunting the allergic inflammation and modulating immune responses to the allergens. Oral antihistamines, the most common treatments for allergic rhinitis, can often control some of the symptoms (itchy nose and eyes, runny nose and sneezing), but are not effective in managing the primary complaint (nasal congestion), which can be debilitating in many sufferers (Meltzer *et al.*, 2009). Intranasal steroids reduce the congestion, as well as the other symptoms; but are often used improperly, take several days to become fully effective, and can have bothersome side effects. However, none of these approaches can prevent or interrupt the binding of specific IgE antibodies to their cognate allergens. As in infectious diseases, vaccines that prevent allergic rhinitis are more likely to be effective than current therapeutic approaches. However, current vaccines for allergic diseases are limited by the need for numerous injections or ingestions. Thus, novel approaches and products that prevent or treat allergic rhinitis are needed.

Our laboratory has been exploring the molecular basis by which proteins from a relatively small number of protein families (pfams) induce allergic sensitization and subsequent reactions, using the dominant allergens from cedar pollens as models (Czerwinski *et al.*, 2005; Goldblum *et al.*, 2014; Ivanciuc *et al.*, 2009). We identified and characterized two major allergens from the mountain cedar pollen (Midoro-Horiuti *et al.*, 1999; Midoro-Horiuti *et al.*, 2000), which dominate the landscape of the South Central US and are responsible for fall and winter pollinosis, commonly called “cedar fever.” Similar allergens were recognized for Japanese cedar and subsequently cypress trees in southern Europe. Recent clinical studies from our lab, indicate that IgE antibodies against the single allergen Jun a 1, dominates (average of 93% of IgE reactivity) the allergic IgE antibody response to mountain cedar pollen (Goldblum *et al.*, 2014). This makes Jun a 1 and its homologues excellent therapeutic targets. Our recently published data (Goldblum *et al.*, 2014) suggests that human immune responses to Jun a 1 are predominantly (93–99%) directed against conformational epitopes.

2. Methods

2.1 Patient selection and serum collection

Eleven adult subjects, who lived in the Austin, Texas area, where mountain cedar is a major part of the flora and produces a severe fall to wintertime allergic rhinitis, were recruited based on their diagnosis of mountain cedar allergy (cedar pollinosis) from Dr. van Bavel’s clinical research facility (Isis Clinical Research) (Goldblum *et al.*, 2014). Ten and 14 subjects from France and Japan, where Italian cypress and Japanese cedar pollen cause similar symptoms, were recruited from Drs. Panzani’s and Kamijo’s clinics (Togawa *et al.*,

2006). Each subject provided serum samples under a University of Texas Medical Branch (UTMB) IRB Protocol (06–050). Subjects who had not been tested for their respective sensitivity to cedar allergens in the previous year had repeat skin prick testing for mountain cedar, Italian cypress or Japanese cedar pollen with commercial extracts of the appropriate cedar pollens. None of these subjects had been treated with allergen immunotherapy. Their diagnosis was established based on clinical symptoms of allergic rhinitis during the pollination season and their positive skin prick test results. The total and cedar specific IgE concentrations of the subjects are shown in Table 1.

2.2 Purification of Type 1 cedar allergens

Jun a 1, Cup s 1 and Cry j 1 were purified as we described previously (Midoro-Horiuti *et al.*, 1999).

2.3 Production, screening, selection of mAb E58

All of the mouse experiments were approved by the Institutional Animal Care and Use Committee at UTMB. Mouse IgE monoclonal antibody (mAb) E58 was induced in BALB/c mice by subcutaneous injections of Jun a 1 protein, as described (Oshiba *et al.*, 1996). Briefly, female BALB/c mice were immunized intraperitoneally with 10 µg of purified native Jun a 1 protein absorbed on 2.25 mg alum (Imject Alum; Pierce, Rockford, IL) two times, two weeks apart. One week after the last immunization, mice were euthanized, spleens were removed and mononuclear cells were fused with FO (SP2/0 myeloma cells, ATCC) according to a standard protocol (Fuller *et al.*, 2001). Supernatant from individual wells, containing a single focus of growing hybridoma cells were tested for antibodies to Jun a 1 by ELISA, using 2 µg/mL of Biotinylated-anti mouse IgE as the detector (BD Pharmingen, San Jose, CA) and Streptavidin-horseradish peroxidase (HRP, Zymed) and 3,3', 5,5'-Tetramethylbenzidine (TMB, KPL, Gaithersburg, MD) as the enzyme and its substrate. Hybridoma cells in wells that tested positive were subcloned at least 3 times, by limiting dilution. The reactivity of these antibodies to native and denatured Jun a 1 was analyzed by preincubating some of the Jun a 1 coated microtiter wells with 6 M Guanidine HCl for 30 min., before incubating both sets of wells with various concentration of E58 antibody. All wells were developed using HRP-anti mouse Ig (H&L) (Goldblum *et al.*, 2014).

2.4 Inhibition ELISAs for comparing the binding specificity of the IgE mAb E58 with those of four groups of IgG mAbs (G1–4), each of which recognize unique conformational epitopes on Jun a 1

We first tested the effects of preincubating Jun a 1-coated, microtiter plates with mAb E58 on the subsequent binding of four groups of mAbs (G1–4), each of which recognize unique conformational epitopes on Jun a 1 (Goldblum *et al.*, 2014). ELISA plates were coated with 3 µg/mL of purified Jun a 1 (Midoro-Horiuti *et al.*, 1999) in borate buffer, pH 8.2 overnight at 37 °C. Next, some of the Jun a 1 coated wells of these plates were preincubated with 20 µg/mL of E58 mAb, while other Jun a 1 coated wells were filled with buffer only, at room temperature, with shaking for one hour. Then, one µL of biotinylated G1–4 mAbs (1:100 dilutions) were added to separate sets of wells. After incubating with shaking for one hour, the binding of G1–4 mAbs was assessed by adding Streptavidin-HRP and incubating for 50 min, followed by TMB for 30 min. The effects of E58 preincubation on the binding of G1–4

mAbs to Jun a 1 were assessed by comparing the color development in wells on the same plate, with and without preincubation with E58. The percent inhibition produced by E58 on the binding of each IgG antibody concentration was computed based on the reduction in TMB product in the E58 treated wells (Fig. 1A).

To elucidate the underlying mechanism of our unusual observation, that a single mAb (E58) reduced the binding of antibodies to at least four unique epitopes on Jun a 1 (G1–4), we reversed the order of addition of E58 and the mAbs to each of the four conformational mAbs (Goldblum *et al.*, 2014). Individual wells on Jun a 1 coated microtiter plates were preincubated with 20 µg/mL of G1–4 mAbs, at room temperature, with shaking for one hour. One µL of biotinylated E58 antibody (diluted 1:100) was added to each well and incubated with shaking for another hour. Binding of E58 was assessed after incubation with Streptavidin-HRP for 50 min and TMB for 30 min. The relative binding of E58 to Jun a 1 coated wells with and without prior incubation with G1–4 mAbs was computed. Positive and negative controls included wells, without any G series mAbs, and wells with unlabeled E58 antibody.

2.5 ELISAs for assessing the effect of E58 on the subsequent binding of human IgE antibodies to Jun a 1

To determine whether E58 can reduce the subsequent binding of patient's IgE antibodies to other group 1 cedar pollen allergen, we developed another set of ELISA inhibition assays. Microtiter plates were coated with either Jun a 1, Cup s 1 or Cry j 1 (3 µg/mL), as described (Goldblum *et al.*, 2014). Some of the wells on each of these plates were preincubated with 20 µg/mL of E58 at room temperature for 30 min. Next, serial dilutions of sera from patients from each of the three geographical area were added to plates coated with their regional allergen and incubated at room temperature for 1 hour. IgE binding was detected with biotinylated-goat anti-human IgE (Vector, Burlingame, CA), followed by HRP-streptavidin conjugates and TMB. The degree to which preincubation with E58 inhibited the binding of patient serum IgE to their group 1 allergens were assessed by comparing their IgE binding to wells that had or had not been preincubated with E58. The results are shown in Fig. 1C.

2.6 Mapping the potential sites of E58 epitope

In order to begin to exploring the mechanisms by which the binding of E58 reduces the subsequent IgE binding to all three group 1 allergen, we first searched for similar surface exposed regions on the three allergens that might represent their E58 epitopes. In this search, we also considered that the E58 epitopes of the three allergens are likely to be structurally different, given the differences in inhibitory activity of E58 on the three allergens. We thus aligned the sequences the three allergens and predicted their surface exposures. We next looked for differences that might explain differences in reactivity of E58 to Jun a 1, Cup s 1 and Cry j 1. We used the IEDB analysis resource (<http://tools.iedb.org/bcell/>) for this modeling, which is shown in Fig. 3.

2.7 Generation of recombinant single chain, mAb from E58 (scFv E58) for use in mast cell assays and subsequently conversion into a pFab E58 for testing in surface plasmon resonance studies

ScFv E58, a single chain three domain protein was generated and expressed as described (Krebber *et al.*, 1997; Jeong *et al.*, 2007; Mabry *et al.*, 2005). Briefly, we used the primer set designed by Krebber *et al.* to clone the Vh and VL domains of E58 Ab, using the total mRNA from multiply subcloned E58 hybridoma as templates. The appropriate cDNA from these segments were excised by restriction enzymes and cloned into the pMoPac16 vector (a kind gift from Dr. George Georgiou) which expresses a three domain monovalent protein consisting of mouse Vh, Vl and Ckappa domains. The same Vh and Vl domains were subsequently cloned into the pFab vector, which expresses a four domain structure, which includes a human Ck and Ch1 domains (Gibson *et al.*, 2009). Both vectors were expressed in *E. coli* BL21 and their proteins purified via their His-tags.

2.8 Mast cell degranulation assay

In order to more closely model the potential of E58 mAb to prevent allergic reactions to group 1 cedar allergen, we developed a cell-based assay using a rat basophil cell line (RBL) with characteristics of mast cells, that were transfected with a plasmid expressing human FcεRIα, β and γ chains (RBL-SX 38) (Wiegand *et al.*, 1996), a kind gift from Dr. J-P Kinet. Sera from three patients were selected, based on their high-titer of IgE anti-Jun a 1 antibodies and availability of adequate volume for these studies.

Control sera were derived from normal subjects who had undetectable levels of IgE antibodies to the relevant cedar allergens from their region. Each of these sera was used to sensitize the RBL-SX 38 cells (Zaitzu *et al.*, 2007; Narita *et al.*, 2007). This cell line was maintained in DMEM media containing 1.2 mg/ml of G418 (Geneticin, Cellgro, Manassas, VA). The cells (1×10^5 /well) were distributed into the wells of 96-well microtiter plates and grown for 48 hours in DMEM, in the absence of G148. Patient sera were added to the experimental wells to achieve a final dilution of 1:10 and incubated overnight. This allowed the patient's serum IgE to bind to the human FcεRIα chain, thereby sensitizing the rat mast cells. Wells containing control serum were treated similarly. After washing with Tyrode's buffer three times, 100 μL of Tyrode's was added to each well. The mast cells were then stimulated by adding varying concentrations (0.1–100 ng/mL) of purified Jun a 1, alone or Jun a 1 mixed with either 2 or 20 μg/mL of E58 mAb. After 30 minutes of incubation at 37°C, the release of β-hexosaminidase was quantified, as we have described (Zaitzu *et al.*, 2007; Narita *et al.*, 2007) and the results were calculated as the percent inhibition of mediator release by each concentration of E58. Ca²⁺ ionophore (10^{-5} M) and serum-sensitized cells, cross-linked with anti-human IgE antibodies (Sigma, St. Louis, MO) were used as positive controls and spontaneous release was assessed in wells without cross-linking agents.

2.9 Biophysical analysis

We used surface plasmon resonance (Biacore T100, GE Healthcare) analyses to compare the affinity of binding of the native bivalent E58 mAbs, produced by our hybridoma cells, and our monovalent molecular constructs (E58 scAb and E58 Fab) to Jun a 1. Jun a 1 was

immobilized by amine coupling on a CM5 to a final response of over 200 RUs. Biophysical analysis was performed using HBS EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20, GE Health Life Science) as running buffer. Analytes included either native E58, E58 single chain Ab (scAb) or E58 fragment antigen-binding (Fab) were injected at a flow rate of 30 μ L/min for a contact time of 600 s followed by a dissociation time of 600 s. 10 mM Glycine pH 2.5, used as regeneration solution, was injected after each cycle for 60 s at 30 μ L/min, followed by a stabilization period of 120 s. Each analyte was injected at several different concentrations and equilibrium constants determined by steady affinity analysis, available within the Biacore T100 analysis software.

2.10. Statistical analyses

Student t test was used to compare the data between indicated groups. Differences at $p < 0.05$ were defined as statistically significant.

3. Results and Discussion

3.1 ELISA inhibition by mAbs

During the process of producing mouse IgE mAbs to Jun a 1, we discovered that one Ab, termed E58, displayed a unique activity. There were no significant differences in the reactivity of E58 to native and guanidine denatured Jun a 1, suggesting E58 binds to the linear epitope (Fig. 1A). However, preincubating Jun a 1-coated wells with E58 mAb, substantially reduced the subsequent binding of all four groups of IgG mAbs, each of which recognize independent, conformational epitopes of Jun a 1 (G1–4, Fig. 1B) (Goldblum *et al.*, 2014). However, when we reversed the order of the addition of these mAbs, by first incubating the Jun a 1-coated wells with each of the IgG mAbs (G1–4) and then adding E58, none of these four IgG mAbs inhibited the binding of E58 (Fig. 1C). This unilateral pattern of inhibition suggests that the binding of E58 to Jun a 1 alters its conformation, stabilizes a non-allergic structure, or forms an unusual complex with Jun a 1, which substantially reduces the subsequent binding of each mAbs (G1–4) (Goldblum *et al.*, 2014).

3.2 ELISA inhibition by patient sera

Next we tested the effect of pre-incubating Jun a 1 coated wells with E58 on the subsequent binding of IgE antibodies from the sera of highly sensitized, mountain cedar pollinosis subjects. The results, as in the case of mAb to Jun a 1, indicate that the prior addition of E58 caused a substantial reduction in the binding of the patient's IgE antibodies to Jun a 1 (left panel, Fig. 1D). The magnitude and consistency of this effect among the subjects make it more likely that E58 extensively alters the molecular structure of Jun a 1, rather than just causing steric hindrance of the binding of serum IgE. These findings are also consistent with our recent report that G1–4 mAbs bind to individual epitopic regions that are also recognized by antibodies in sera of patients who are sensitized to Jun a 1 (Goldblum *et al.*, 2014). Thus, the inhibition by E58, of binding of all four mouse IgG mAb (Fig. 1B) suggests that E58 alters the display of a spectrum of conformational, human IgE epitopes on Jun a 1.

We then asked whether E58 has similar inhibitory effects on the binding of IgE antibodies from patients sensitized to homologous group 1 allergens from the Italian cypress (Cup s 1; *Cupressus sempervirens*, Cupressaceae) or to the Japanese cedar (Cry j 1; *Cryptomeria japonica*, Taxodiaceae) pollens (Midoro-Horiuti *et al.*, 2006). The results of these experiments, suggests that E58 binds to structurally conserved region(s) in all three group 1 allergens (Fig. 1C). While the extent to which E58 inhibited the binding of IgE antibodies from patients from France and Japan was less than that for our Texas patients. This is most likely due to lower affinity of E58 for Cup s 1 and Cry j 1, than for Jun a 1, as substantiated by the homologous inhibition experiments (closed squares at top of each scattergram) in which the extent of inhibition of the binding of labeled E58 by Jun a 1 was inhibited by non-labeled E58 antibodies that was greater than the heterologous inhibition. However, the partial conservation of the E58 epitope between species may provide a basis for additional innovative immunotherapy, which would down modulate the display of multiple IgE epitopes on the group 1 cedar allergens from several geographic regions.

3. 3 Inhibition of mast cell degranulation by E58

In order to extend our observations of E58 inhibitory activity to a more functional assay, we next tested whether E58 also inhibits mast cell degranulation. However, since complete, bivalent E58 mAb, by itself, could cross-link individual Jun a 1 molecules bound to IgE on the surfaces of the mast cells and enhance their degranulation, we produced a monovalent single chain recombinant E58 (rE58 scFv) (Krebber *et al.*, 1997; Jeong *et al.*, 2007; Mabry *et al.*, 2005). Fig. 2 shows the results of experiments using a mouse mast cell line (RBL SX-38) that expresses the human Fc ϵ R1 α chain (Goldblum *et al.*, 2014) and then sensitized with IgE from the sera of mountain cedar allergic patients. These mast cells were then exposed to varying concentration of either purified Jun a 1 alone, or Jun a 1 that had been preincubated with 10 μ g/mL of E58-derived scAb. The results indicate rE58 scAb significantly inhibited the ability of Jun a 1 to induce degranulation of IgE sensitized mast cells. While the extent of inhibition of mast cell degranulation was less than that of IgE binding in ELISA (Fig. 1C), this is most likely due to the stronger binding of bivalent full E58 mAb to Jun a 1, compared to that for E58 scAb (Mabry *et al.*, 2005).

3.4 Potential epitope of E58

Based on the combined data analysis, E58 most likely binds near the N-terminal of the group 1 cedar allergens (Fig. 3).

3.4 Biacore analyses

Binding to Jun a 1 of native E58, E58 scAb and E58 Fab was analyzed using surface plasmon resonance technology (Biacore T100). Dissociation constants (K_D) for native E58, E58 scAb and E58 Fab were determined by steady state affinity analysis to be 9.39×10^{-7} M, 1.14×10^{-6} M and 3.55×10^{-7} M, respectively (Fig. 4). Thus, rE58 scAb, and rE58 Fab were determined to have similar affinities to Jun a 1 as native E58, (in the range of 0.3 to 1.2 μ M). This finding suggests that, under the conditions of these analyses, intact E58 mAb only bind to a single epitope on Jun a 1, which may narrow the possible mechanisms by which E58, or monomeric fragments, inhibit subsequent binding of human IgE.

3.5 Comparison of E58 with other IgE modulating antibodies

MABs that altered the subsequent binding of human IgE to the major allergen of birch pollen, Bet v 1 were described previously (Laffer *et al.*, 1996; Lebecque *et al.*, 1997). However, the effects of these mAbs to Bet v 1 on the subsequent binding of patients' serum IgE varied between their human subjects. Thus, the same mAbs that decreased the binding of IgE from some patients, increased the binding of IgE from other patients. In contrast, our current findings indicate that E58 consistently decreases the binding of patients' IgE Abs. Further, we were able to show that E58 reduced the IgE binding to multiple epitopes on Jun a 1 and had similar inhibitory activity on the binding of IgE from sera of patients who were sensitized to other group 1 cedar allergens, Cup s 1 and Cry j 1, (Fig. 1D). Together, our finding suggests that the activity of E58 may be unique in its mechanism and the extent to which it's binding modifies surface structures on Jun a 1, and its homologous allergens.

Another approach for using mAbs to reduce the allergenicity of the experimental allergen ovalbumin was recently described, in a sensitized mouse model of allergic rhinitis by Matsuoka et al. (Matsuoka *et al.*, 2014). In their studies, monovalent (Fab) fragments of an IgG1 anti-OVA mAb were instilled into the noses of systemically OVA-primed mice. They found that this topical pretreatment with the IgG1 "fragments" prevented the immediate (sneezing) response to nasal challenges with OVA, presumably by inhibiting access of intact anti-OVA antibodies to the same OVA epitope. Importantly, they showed that repeated instillation of IgG1 Fab fragments prior to intact OVA on sneezing persisted and increased through four daily challenges. Their protocol significantly reduced the activity of the IgE anti-OVA antibodies, relative to those in the mice that got intact OVA alone. This suggests that monomeric anti- OVA fragments induced a long-term suppression of the allergic response to OVA. Thus instillations of monovalent ligands for Jun a 1, such as our E58 scAb or E58 Fab, may prevent sensitization, as well as the acute manifestations of exposing the nasal tissue to group 1 allergens of cedar pollens.

3.5 Potential clinical application

Putting these observations together suggests that mAbs with E58-like activity might be harnessed for clinical applications. These applications may include E58 nasal sprays that would be used during the pollination season to reduce the intensity of nasal pollinosis symptoms or even prevent primary sensitization (Matsuoka *et al.*, 2014) to cedar or other pollens. This approach might be effective in at least three heavily populated areas of the world. Finally, it seems likely that selective screening of hybridomas against IgE epitopes of other allergens may identify additional mAbs with similar protective or therapeutic capacity.

Further a better understanding of the mechanism by which antibodies like E58 may alter the course of development and manifestations of allergic reactions could lead to new preventative or therapeutic approaches to these very common diseases. For instance, while the experiments we described here strongly suggest that when mAb E58, and recombinant, monovalent constructs thereof bind to Jun a 1, they can reduce Jun a 1's reactivity with soluble and mast cell associated IgE. However, the molecular basis for the activity of such antibody remains to be elucidated. Thus, our discovery opens the possibility for preventing or at least decreasing the intensity of allergic reactions to other groups of seasonal or

perineal allergens. The specific products and modes of administration however remain to be developed, but may include antibody-containing nasal sprays that could be applied topically either as a therapeutic agent used prior to pollen exposure, or as a seasonal preventative to reduce sensitization.

3.6. Conclusion

The mAb E58 has a unique function in modulating the binding of several other antibodies to Jun a 1 and its congeners. This suggests that monovalent forms of E58 and other antibodies with similar suppressive activity might be used to prevent the acute response to cedar exposure. Further, instillation of a nasal formulations of E58 mAb might also preventive the development of pollen allergies. Further, understanding the mechanism of E58's function may lead to the development of additional therapeutic or preventative agents that can neutralize or disarm other allergens.

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Abbreviations

Cup a 1	the major allergen of Italian cypress (<i>Cupressus sempervirens</i>)
Cry j 1	the major allergen of Japanese cedar (<i>Cryptomeria japonica</i>)
HRP	horse radish peroxidase
Jun a 1	the major allergen of mountain cedar (<i>Juniperus ashei</i>)
pfam	protein family

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Highlights

- We discovered a unique mouse monoclonal antibody, termed E58, which binds to the group 1 allergens of cedar pollens from three highly populated areas in the world (central U.S.A., France and Japan).
- Upon binding to these allergens, E58 reduces the binding of patient's IgE antibodies to group 1 allergens of cedar pollen.
- The characteristic of E58, and potentially other similar antibodies, suggests a new opportunity to develop preventative or therapeutic molecules for cedar pollen hypersensitivity.

Fig 1. E58 inhibits the binding of other mAbs in ELISAs

(A) Denaturation of Jun a 1 with 6M guanidine did not significantly reduced the reactivity of E58 to Jun a 1. N = 3, mean and SE were shown. N.S.: not significant. (B) However, preincubating Jun a 1 with mAb E58 inhibits the subsequent binding of 4 other mAbs (G1–4) to their conformational epitopes of Jun a 1. N = 3, mean and SE were shown. *: $p < 0.05$ compared to negative control, +: $p < 0.05$ compared to inhibition by E58. (C) The same mAbs G1–4 do not inhibit the binding of E58 to Jun a 1. *: $p < 0.05$ compared to negative control, +: $p < 0.05$ compared to inhibition by E58. (D) ■: E58 inhibition of E58 binding to each

allergen, —: mean $\pm$ SD. E58 also inhibits the binding of IgE antibodies from sera of patients' sensitive to other group 1 cedar allergens, Cup s 1 and Cry j 1. N.S.: not significant, *: $p < 0.05$ between indicated groups.

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Fig 2. rE58 scFv inhibits degranulation of human serum IgE-sensitized mast cells
 rE58 scFv (a univalent Ab) inhibits Jun a 1-induced degranulation of mast cells sensitized with three different mountain cedar patients' sera. N = 3, mean $\pm$ SD were shown. *: $p < 0.05$ compared to no antibody control.

Author Manuscript	Jun a 1	DNPIDSCWRG	DSNWDQNRMK	LADCAVGFGS	STMGGKGGD ⁴⁰ F	YTVTS ⁴⁶ TDDNP	VNPTPGTLRY	60
	Cup s 1	DNPIDSCWRG	DSNWDQNRMK	LADCAVGFGS	STMGGKGGDI	YTATS ⁴⁶ AEDNP	VNPTPGTLRY	
	Cry j 1	DNPIDSCWRG	DSNWAQNRMK	LADCAVGFGS	STMGGKGGDL	YTVTNS ⁴⁶ DDDP	VNPAPGTLRY	
		*****	*****	*****	*****;	*,*,:*:*	***:*****	
Author Manuscript	Jun a 1	GATREKALWI	IFSQNMNIKL	KMPLYVAGHK	TIDGRGADVH	LGNGGPCLFM	RKVSHVILHS	120
	Cup s 1	GATREKALWI	IFSQNMNIKL	KMPLYVAGHK	TIDGRGADVH	LGNGGPCLFM	RKVSHVILHG	
	Cry j 1	GATRDRPLWI	IFSGNMNIKL	KMPMYIAGYK	TFDGRGAQVY	IGNGGPCVFI	KRVSNVIIHG	
		****,*:***	*** *****	***:*:*:*	*:*****:	:*****:*	::**,*:*:	
Author Manuscript	Jun a 1	LHIHGCNTSV	LGDVLVSESI	GVEPVHAQDG	DAITMRNVTN	AWIDHNSLSD	CSDGLIDVTL	180
	Cup s 1	LHIHGCNTSV	LGDVLVSESI	GVEPVHAQDG	DAITMRNVTN	AWIDHNSLSD	CSDGLIDVTL	
	Cry j 1	LHLYGCSTSV	LGNVLINESF	GVEPVHPQDG	DALTLRTATN	IWIDHNSFSN	SSDGLVDVTL	
		:*:*,*	*:*:*:*:	***	***	***:*:*,*	*****:	*****:***
Author Manuscript	Jun a 1	GSTGITISNN	HFFNHHKVML	LGHDDTYDDD	KSMKVTVAFN	QFGPNAGQRM	PRARYGLVHV	240
	Cup s 1	SSTGITISNN	HFFNHHKVML	LGHDDTYDDD	KSMKVTVAFN	QFGPNAGQRM	PRARYGLVHV	
	Cry j 1	SSTGVTISNN	LFFNHHKVML	LGHDDAYSDD	KSMKVTVAFN	QFGPNCGQRM	PRARYGLVHV	
		.,***:*****	*****	*****:*,**	*****	*****	*****	
Author Manuscript	Jun a 1	ANNNYDPWNI	YAIGGSSNPT	ILSEGNSFTA	PSESYKKEVT	KRIGCESPSA	CANWVWRSTR	300
	Cup s 1	ANNNYDQWNI	YAIGGSSNPT	ILSEGNSFAA	PNENYKKEVT	KRIGCESTSA	CANWVWRSTR	
	Cry j 1	ANNNYDPWTI	YAIGGSSNPT	ILSEGNSFTA	PNESYKKQVT	IRIGCKTSSS	CSNWVWQSTQ	
		***** *	*****	*****:*	*,*,*:*:*	*****:	*:*:*:*:*	
Author Manuscript	Jun a 1	DAFINGAYFV	SSGKTEETNI	YNSNEAFKVE	NGNAAPQLTK	NAGVVT	346	
	Cup s 1	DAFSNGAYFV	SSGKTEETNI	YNSNEAFKVE	NGNLAPQLTK	NAGVVA		
	Cry j 1	DVFYNGAYFV	SSGKYEGGNI	YTKKEAFNVE	NGNATPQLTK	NAGVLT		
		, *****	***** *	*,*:*:*:*	*** ;*****	*****:		

Fig. 3. Potential epitope of E58

Amino acid sequences of Jun a 1, Cup s 1 and Cry j 1 were aligned. Surface exposed amino acids are highlighted with gray and prediction of linear B cell epitopes were underlined. There were only two amino acids 40 and 46, which were surface exposed and predicted as linear B cell epitopes that differ between these three allergens. This region may also be part of the E58 epitopes.

Fig 4. Biacore analyses

Surface plasmon resonance analysis of the binding of: (A) native E58, (B) E58 scAb, (C) E58 Fab to Jun a 1. Graphs display Response Units (RU) as a function of the molar concentration of each analyte. The line through the points is the resulting fit from the steady state analysis procedure (part of the Biacore T100 analysis software).

Table 1

US patient IgE (IU/mL)				
No.	total	Jun a	Cup s	Cry j
1	159	20	<0.35	<0.35
2	1,209	122	88	87
3	1,020	45	<0.35	<0.35
4	226	45	<0.35	<0.35
5	142	35	<0.35	<0.35
6	127	14	<0.35	<0.35
7	691	>100	<0.35	<0.35
8	316	49	<0.35	<0.35
9	490	20	<0.35	<0.35
10	144	14	<0.35	46
11	224	8	<0.35	38
French patient IgE (IU/mL)				
No.	total	Jun a	Cup s	Cry j
1	2,400	92	9	>100
2	140	<0.35	<0.35	<0.35
3	398	<0.35	<0.35	<0.35
4	86	26	<0.35	<0.35
5	500	<0.35	<0.35	<0.35
6	60	<0.35	<0.35	<0.35
7	310	34	<0.35	<0.35
8	675	<0.35	<0.35	<0.35
9	256	48	52	16
10	500	68	38	60
Japanese patient IgE (IU/mL)				
No.	total	Jun a	Cup s	Cry j
1	4,007	<0.35	<0.35	38
2	2,669	<0.35	<0.35	81
3	39	<0.35	<0.35	21
4	454	<0.35	<0.35	84
5	232	<0.35	<0.35	9
6	937	<0.35	<0.35	5
7	32	<0.35	<0.35	36
8	438	<0.35	<0.35	154
9	1,362	<0.35	<0.35	8
10	86	<0.35	<0.35	13

11	2,519	66	<0.35	61
12	995	77	<0.35	79
13	86	36	<0.35	8
14	79	<0.35	<0.35	16

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