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Modulation of Allergenicity of Major House Dust Mite Allergens Der f 1 and Der p 1 by Interaction with an Endogenous Ligand¹

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Although many allergens bind endogenous molecules other than Abs in the human body, whether the interaction can modulate allergenicity has been unknown. Here, we investigated the effect of the interaction of recombinant major mite group 1 allergens (Der f 1 and Der p 1), which belong to the papain-like cysteine protease family, with an endogenous protease inhibitor, cystatin A, on their allergenicity. Cystatin A bound reduced forms of the allergens, in which the cysteine residue at the catalytic center of the protease activity was reduced by treatment with L-cysteine, but did not bind oxidized forms. Cystatin A partially inhibited the binding of IgE in mite-allergic volunteers' sera to the reduced forms, but unexpectedly enhanced the basophil histamine-releasing activity. A catalytic site-mutant of Der f 1 behaved in terms of histamine release, similarly to the reduced form. Molecular modeling showed that cystatin A interacts with the allergens within a narrow area. The results indicate that interaction with cystatin A reduces the limited number of IgE epitopes of the allergens but enhances their biological activity to release histamine, suggesting a new concept, that interaction between allergens and their endogenous ligands modulates the allergenicity even toward enhancement in the effector phase. On the other hand, i.p. immunization without alum of mice with cystatin A-treated reduced Der f 1 induced less serum Der f 1-specific IgE than immunization with reduced Der f 1 alone, suggesting that endogenous protease inhibitors suppress the induction of allergen-specific IgE, which is dependent on the enzymatic activity of cysteine protease-allergens, in the sensitization process. *The Journal of Immunology*, 2009, 183: 7958–7965.

House dust mites of two species, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are major sources of allergens associated with allergic diseases such as asthma, rhinitis, and atopic dermatitis (1–4). Group 1 and group 2 allergens are major allergens derived from house dust mites based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract (4–11). The group 1 allergens, Der f 1 and Der p 1, belong to the papain-like cysteine protease family (12, 13) and actually exhibit cysteine protease activity (14, 15). Der f 1 and Der p 1 have 82% amino acid sequence identity with each other. IgE epitopes of Der f 1 and Der p 1 are dependent on the integrity of the conformational structure (8, 16–19), and high cross-reactivity between Der f 1 and Der p 1 has been reported (9, 11, 19, 20). The crystal structures of the Der p 1 precursor (21) and mature Der p 1 (22) were determined.

It has been proposed that the proteolytic activity of Der f 1 and Der p 1 is involved in the pathogenesis of allergies and sensitization to the production of IgE by reducing physical and biochemical tissue barriers, cleaving various molecules, and modulating the functions of various cells (23–37). We found that human sweat inhibited the proteolytic activities of recombinant Der f 1 and Der p 1 and used sweat as a skin-derived material for isolation of the inhibitor, which was subsequently determined to be cystatin A (31), also called stefin A (38, 39). Moreover, stimulation of keratinocytes with the cysteine protease activity of these allergens promoted the secretion of IL-8 and GM-CSF, and the addition of cystatin A blocked this (31, 40). Recently, a significant association of a cystatin A genotype with atopic dermatitis has been reported (41).

Some allergens including Der f 1 and Der p 1 are proteases (42). If protease inhibitors, which are present in the body (31, 43, 44), can interact with protease allergens, do they modulate their allergenicity? No study on the basis of this question has been reported. Here, we investigate the effect of the interaction of Der f 1 and Der p 1 with cystatin A on their allergenicity, in terms of IgE-binding capacity and basophil histamine-releasing activity using sera and peripheral blood obtained from mite-allergic volunteers. In addition, we examine the effect on IgE and IgG production in the in vivo sensitization process in mice.

Materials and Methods

Cystatin A and recombinant allergens

Recombinant cystatin A (R&D Systems) and natural cystatin A purified from human sweat were used (31). Concentration of cystatin A in sweat was determined by ELISA (31). Allergens used were recombinant mature forms of mite major group 1 allergens (15, 19, 45–47), Der f 1-N53Q and

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Der p 1-N52Q (rDer f 1³ and rDer p 1), which have molecular weights identical with those of natural allergens (19), those with yeast-derived hyperglycosylation (rDer f 1-WT and rDer p 1-WT), and a mutant of rDer f 1 (rDer f 1-C35S; Refs. 48 and 49), and a recombinant form of a mite major group 2 allergen, Der f 2 (50). A precursor form of rDer f 1-C35S was prepared by site-directed mutagenesis, secreted into the yeast culture supernatant, and converted to its mature form by yeast-derived protease activity within the supernatant by dialysis with an acidic buffer (48). The mature rDer f 1-C35S was purified with column chromatography, and the yeast-derived protease activity was removed.

Detection of cystatin A binding

Microtiter plates (MaxiSorp; Nunc) were coated with 50 μ l of Der f 1 and Der p 1 (2.5 μ g/ml) in PBS by incubation overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBST), remaining binding sites were blocked with BlockAce (Snow Brand; 0.25 dilution; 200 μ l/well) by incubation for 1.5 h at 37°C. The plates were washed and incubated with PBST with or without 5 mM L-cysteine (Sigma-Aldrich) and/or 10 μ M E-64 (Peptide Institute; 50 μ l/well) for 30 min at 37°C at neutral pH, and diluted recombinant cystatin A, cystatin A purified from sweat, or sweat (50 μ l/well) was added to the plate (total of 100 μ l/well). After incubation for 1 h at 37°C and washing, mouse anti-human cystatin A mAb (Oxford Biotechnology) was added (1/5000 dilution; 50 μ l/well). After further incubation for 1 h at 37°C and washing, HRP-labeled anti-mouse IgG Ab was added (1/1000 dilution; 50 μ l/well). After incubation for 1 h at 37°C and washing, binding of the conjugates was visualized with tetramethylbenzidine (Sigma-Aldrich) in citrate buffer (pH 5.0) containing 0.2% H₂O₂ (100 μ l/well) at room temperature. The color reaction was stopped by adding 2 N sulfuric acid (50 μ l/well), and the OD at 450 nm was measured. Cystatin A and the Abs were used after dilution with PBST with 10% BlockAce.

Detection of human IgE binding

After microtiter plates coated with Der f 1 and Der p 1 were blocked, L-cysteine and recombinant cystatin A, cystatin A purified from sweat, or sweat were added (50 μ l/well). After incubation for 0.5 h at 37°C, diluted serum (1/10 to 1/70 dilution; 50 μ l/well) was added, and the plates were incubated for 1.5 h at 37°C (total of 100 μ l/well). After washing, β -galactosidase-labeled anti-human IgE (RAST-FEIA kit; Pharmacia; 1/5 dilution) was added (50 μ l/well). After incubation for 1.5 h at 37°C and washing, bound conjugates were reacted with the fluorogenic substrate (RAST-FEIA kit; 1/7-diluted in PBS containing 1 mM MgCl₂; 75 μ l/well). The reaction was stopped by adding stop solution (RAST-FEIA kit; 75 μ l/well). The solution was moved to white microtiter plates (Optiplate; Corning; 120 μ l/well), and fluorescence at 355 nm excitation and 460 nm emission was measured on a fluorometer, Fluoroscan Ascent (LabSystems). In some experiments, each group datum of the OD was compared with a control without the inhibitors by unpaired *t* test (two-tailed) to evaluate the significance of differences. Percent inhibition was expressed as the relative reduction of the fluorescence intensity in each sample to that when no inhibitors were added.

Histamine release from human basophils

The allergen solution used was prepared as follows. Der f 1 and Der p 1 were incubated with L-cysteine for 10 min at 37°C, then serially diluted to various concentrations in incubation buffer with 0.03% human serum albumin included with HRT-Shionogi (Shionogi) (51), and incubated with recombinant cystatin A (2.5 μ g/ml) for 30 min at 37°C (24 μ l/well). After the incubation, they were diluted with the incubation buffer (1/5 dilution, total of 120 μ l/well) and moved to microplates coated with streptavidin (100 μ l/well). After the dilution, the concentration of cystatin A was 500 ng/ml. The method used to measure the amount of histamine released from basophils was as described previously (19, 50). Effective concentrations yielding half-maximal allergen response were determined from curves in the histamine-releasing assay by using Prism version 4.0 (GraphPad). The blood samples were collected from volunteers with informed consent.

Molecular modeling

The tertiary structure of Der f 1-cystatin A complex was modeled by using the automated comparative protein modeling server SWISS-MODEL (52). The template structure was produced by superimposing the Brookhaven Protein Data Bank entries 2AS8 (mature Der p 1; Ref.

22) and 1NB3 (cathepsin H-cystatin A complex; Ref. 53) and removing the cathepsin H portion.

Immunization of mice

Female CBA/J mice (7–10 wk old) purchased from Charles River Japan were maintained in a specific pathogen-free animal facility at Juntendo University, and used in accordance with the guidelines of the institutional committee on animal experiments. Before use, 4.5 μ g/1 μ l of rDer f 1 was incubated with 5 μ l of 50 mM L-cysteine (neutral pH; 5 min at 37°C) and then added with 45 μ l of PBS and immediately moved onto ice (reduced Der f 1); and 4.5 μ g/1 μ l of rDer f 1 was incubated with 1 μ l of 50 mM L-cysteine (5 min at 37°C) and then added with 4 μ l of 50 mM L-cysteine and 4.5 μ g/45 μ l of recombinant human cystatin A and incubated (30 min at 37°C; cystatin A-treated reduced Der f 1). The Abs were further diluted to 900 μ l with saline, 200 μ l of which contained 1 μ g of rDer f 1, and were kept at room temperature until used. Mice were given four weekly i.p. injections of 200 μ l of each of the Ag solutions (1 μ g of rDer f 1 with or without 1 μ g of cystatin A per head) without using any adjuvants. Peripheral blood was collected at 1 wk after the last injection. All sera were stored at –20°C before analysis for Ab content.

ELISA for murine serum Abs

Serum total IgE was measured by a sandwich ELISA as described previously (54). Der f 1-specific Abs were detected on plates coated with 5 μ g/ml rDer f 1 and blocked with ImmunoBlock (DS Pharma Biochemicals), and developed with HRP-conjugated Abs specific to the murine IgE and IgG subclasses as described previously (37, 54, 55) with some modifications as follows. Sera and detection Abs were diluted with solutions 1 and 2 of CanGetSignal (TOYOBO), respectively. Serum dilutions were 1/50, 1/1000, 1/200, and 1/2000 for detection of Der f 1-specific IgE, IgG1, IgG2a, and IgG2b, respectively. For detection of Der f 1-specific IgE, incubation with serum or a detection Ab was performed for 15 h at 4°C or for 2 h at 37°C, respectively. For detecting Der f 1-specific IgGs, incubation with serum or a detection Ab was performed for 1.5 h at 37°C.

Results

Cystatin A bound to Der f 1 and Der p 1 treated with L-cysteine

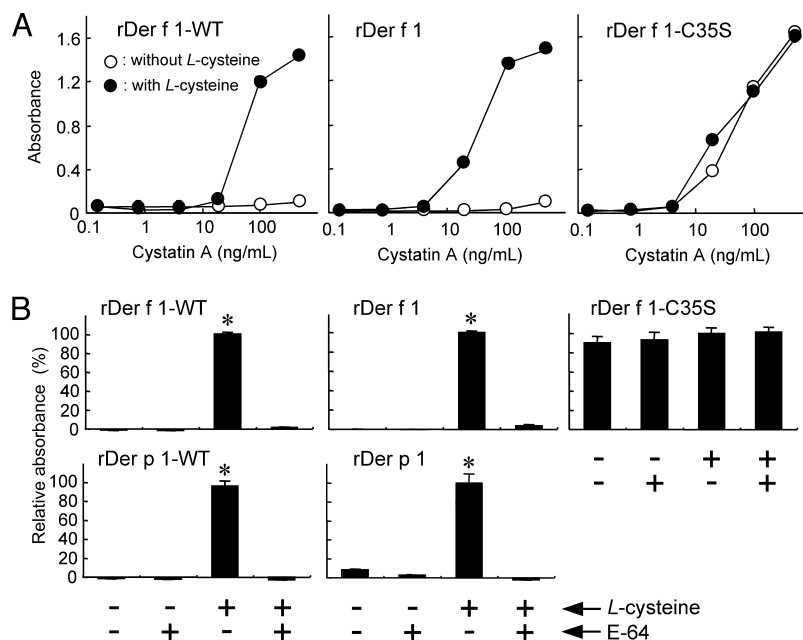
To reveal the mode of interaction of Der f 1 and Der p 1 with cystatin A, we analyzed the binding of cystatin A to plates coated with allergens with or without preincubation with a reducing reagent, L-cysteine, to regenerate the catalytic thiol group of these mite cysteine proteases. Results obtained with the use of recombinant cystatin A are shown (Fig. 1). Similar results were obtained with the use of cystatin A purified from sweat or crude sweat (our unpublished observations). After the reduction, cystatin A bound both rDer f 1-WT, which was hyperglycosylated at a molecular surface distant from the catalytic site, and rDer f 1 without the hyperglycosylation (19, 46) but did not bind them without the reduction (Fig. 1A, left two panels). Similar binding patterns were observed in the case of Der p 1, and the binding of cystatin A to the reduced forms of Der f 1 and Der p 1 was completely blocked by the covalent and irreversible binding of the cysteine protease inhibitor E-64 to the cysteine residue at the catalytic center of the cysteine proteases (Fig. 1B, left four panels). These results indicated that the state of these mite cysteine proteases, reduced or oxidized, corresponded respectively to the recognition or not of cystatin A.

Cystatin A bound to rDer f 1-C35S, a catalytic site mutant of Der f 1

We also analyzed the binding of cystatin A to a catalytic site-mutant of Der f 1 designated rDer f 1-C35S (Fig. 1, A, right, and B, right), in which the cysteine residue at the catalytic center was changed to a serine residue and no proteolytic activity was detectable after the removal of the yeast-derived protease activity (our unpublished observations). Cystatin A bound rDer f 1-C35S with or without treatment with L-cysteine. Thus, cystatin A bound reduced Der f 1, reduced Der p 1, and rDer f 1-C35S but did not bind oxidized forms and E-64 complexes of Der f 1 and Der p 1.

³ Abbreviations used in this paper: rDer f 1 and rDer p 1, unglycosylated recombinant Der f 1 and Der p 1; rDer f 1-WT and rDer p 1-WT, hyperglycosylated rDer f 1 and rDer p 1; rDer f 1-C35S, a catalytic site mutant of rDer f 1; PBST, PBS containing 0.05% Tween 20.

FIGURE 1. Cystatin A bound to Der f 1- and Der p 1-treated with L-cysteine. *A* and *B*, Results obtained with the use of recombinant cystatin A. In *B*, the concentration of cystatin A was 200 ng/mL, means + SEM of triplicate data on OD relative to that for L-cysteine-treated allergens are shown, and each group data was compared with the other groups by the Tukey post hoc test followed by one-way ANOVA to evaluate the significance of differences. *, $p < 0.05$.



Cystatin A partially inhibited IgE binding to Der f 1 and Der p 1 treated with L-cysteine

We examined the inhibition of IgE binding to the reduced Der f 1 and Der p 1 through the blocking of IgE epitopes by the binding of cystatin A (Fig. 2). Purified cystatin A (Fig. 2A) and four individual sweat samples (Fig. 2B; Ref. 31) exhibited equivalent inhibition curves in IgE binding to the reduced Der f 1 using a mite-allergic serum. Furthermore, we examined seven individual sera, where cystatin A purified from sweat and recombinant cystatin A were used as inhibitors (Fig. 2C). Cystatin A partially blocked the binding of IgE to Der f 1 and Der p 1. Level of inhibition differed among sera in the range of 10–40% in Der f 1 and 20–50% in Der p 1, suggesting that relative importance of the blocked IgE-epitopes to total IgE binding differed with the patient.

Cystatin A enhanced basophil histamine-releasing activity of Der f 1 and Der p 1 treated with L-cysteine

To investigate whether cystatin A modulates the allergenicity of the reduced Der f 1 and Der p 1 at the cellular level, the biological activities of these allergens in inducing the release of histamine from allergic volunteers' basophils were analyzed with and without preincubation with cystatin A (Fig. 3). We expected that cystatin A would decrease the histamine-releasing activity on the basis of the inhibition of IgE binding observed in Fig. 2. However, effective concentration yielding half maximal response of the allergens in the histamine-releasing assay was decreased by incubation with cystatin A in Der f 1 in four mite-allergic volunteers, volunteers 1–4, except for volunteer 5, to 1/6.2, 1/5.6, 1/8.1, and 1/2.7, respectively (Fig. 3A), and in Der p 1 in two volunteers,

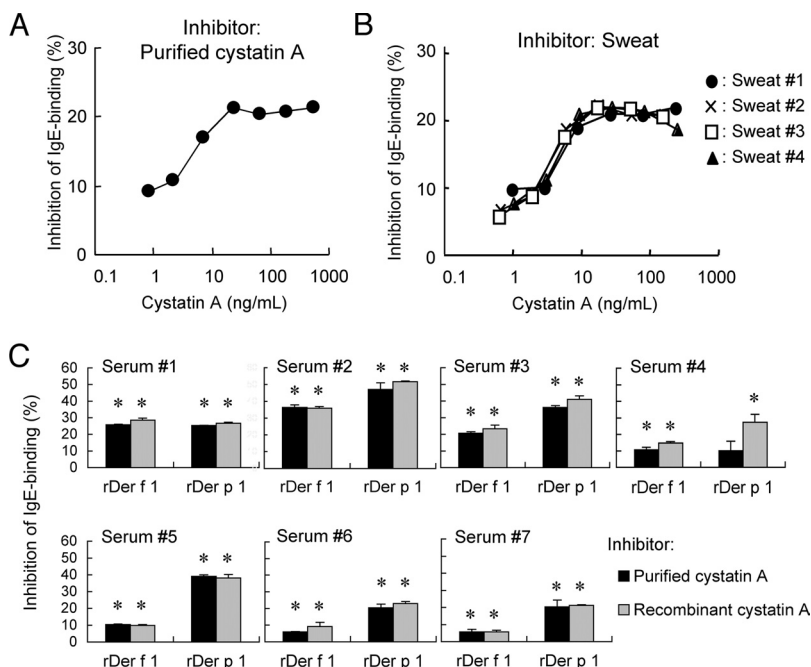
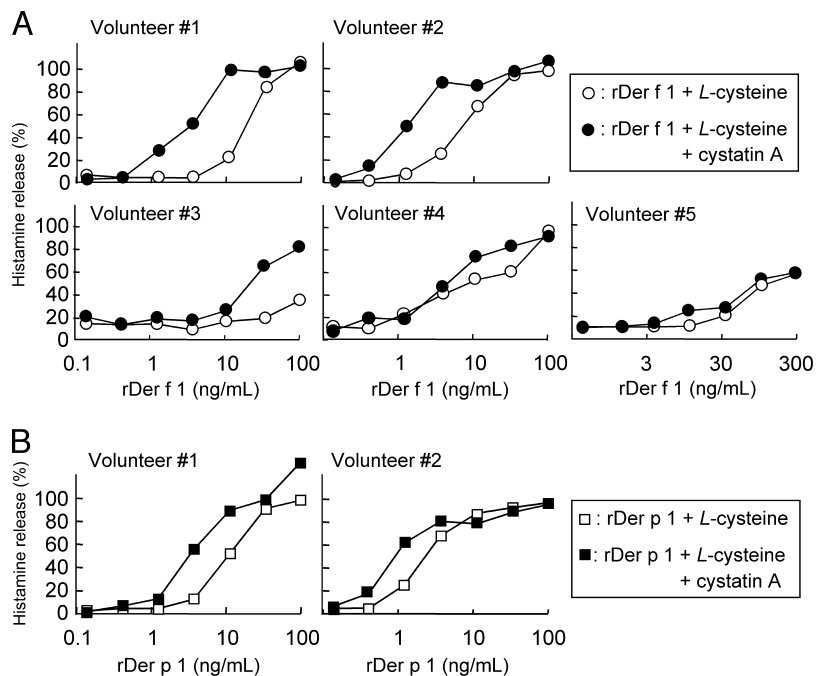


FIGURE 2. Cystatin A partially inhibited the binding of IgE to Der f 1 and Der p 1 treated with L-cysteine. Percent inhibition was expressed as the relative reduction of the fluorescence intensity in each sample to that when no inhibitors were added. *A* and *B*, serum 1 was used. Four individual sweat samples were used in *B*. *C*, Seven individual mite-allergic sera, sera 1–7, were used, the final concentration of cystatin A was 100 ng/mL, and means + SEM of triplicated data are shown. Each group datum of the OD was compared with a control without the inhibitors by unpaired *t* test (two-tailed) to evaluate the significance of differences. *, $p < 0.05$.

FIGURE 3. Cystatin A enhanced basophil histamine-releasing activity of Der f 1 and Der p 1 treated with L-cysteine. The rDer f 1 (A) and rDer p 1 (B) pretreated with L-cysteine were incubated with or without recombinant cystatin A and subjected to basophil histamine release assay. Basophils from five and two mite-allergic volunteers were tested. The final concentration of cystatin A was 500 ng/ml.



volunteer 1 and volunteer 2 to 1/2.5 (Fig. 3B). Cystatin A itself did not show histamine-releasing activity, and histamine-releasing activities of a recombinant form of another mite major allergen Der f 2 and polyclonal anti-IgE Ab were not enhanced by addition of cystatin A (our unpublished observations). Thus, the enhancement of allergenicity in terms of basophil histamine release by addition of cystatin A was a specific effect in the reduced Der f 1 and Der p 1.

Interaction with cystatin A was the cause of the enhancement of the histamine-releasing activity

To determine whether the interaction of cystatin A with these allergens was the cause of the unexpected enhancement of histamine-releasing activity, we checked the dose dependency of the effect and analyzed this phenomenon using reduced Der f 1, oxidized Der f 1, and the catalytic site mutant of Der f 1 (Fig. 4). The curve of histamine release shifted to the left dependent on the dose of cystatin A (Fig. 4A). Addition of cystatin A enhanced the histamine-releasing activity of reduced Der f 1 but did not affect that of oxidized Der f 1 in two volunteers, volunteers 1 and 3 (Fig. 4B). On the other hand, the catalytic site mutant, rDer f 1-C35S, showed enhanced activity on addition of cystatin A with or without the reducing reagent in three volunteers, volunteers 1, 3, and 4, except for volunteer 5 (Fig. 4C). These results indicated that the enhancement of the histamine-releasing activity in these volunteers was caused by the interaction of cystatin A with these allergens demonstrated in Fig. 1 but not by inhibition by cystatin A of self-degradation of the allergens.

Oxidized Der f 1 showed moderate histamine-releasing activity more than reduced Der f 1 in volunteer 1 (Fig. 4B), suggesting that the oxidized thiol group contributed to the number and/or affinity of IgE epitopes and/or that a structural difference between the oxidized and reduced forms existed.

Modeling of Der f 1-cystatin A complex

Molecular modeling showed that cystatin A interacts with Der f 1 within a narrow area (Fig. 5). In the lower part of the cystatin A structure, an N-terminal hook and two hairpins form a sharp edge of the wedge-shaped structure. The N-terminal peptide with the hook tightly binds to part of the substrate binding cleft of Der f 1

in a substrate-like manner and then turns away from the active site, preventing proteolytic processing, as reported for the cathepsin H-cystatin A complex (53) and carboxymethylated papain-cystatin B complex (56).

Cystatin A suppressed induction of Der f 1-specific IgE in the sensitization process in mice immunized with reduced Der f 1

In addition to the investigation of modulation of allergenicity of the mite cysteine protease allergens by their interaction with cystatin A using sera and basophils from mite-allergic volunteers, which focused on the effector phase in allergies (Figs. 2–4), we examined the effect of cystatin A on induction of IgE and IgG in mice immunized with Der f 1, which focused on the sensitization process (Fig. 6).

Without the use of any adjuvants, mice were immunized i.p. with unreduced Der f 1 without treatment with L-cysteine, reduced Der f 1 in which the catalytic thiol group was regenerated with L-cysteine or with cystatin A-treated reduced Der f 1 (Fig. 6). Mice immunized with reduced Der f 1 produced significantly higher levels of serum Der f 1-specific IgE than unreduced Der f 1 and cystatin A-treated reduced Der f 1 (Fig. 6B, left). There was no statistical significance of differences among the three forms of Der f 1 in production of total IgE (Fig. 6A) and Der f 1-specific IgGs (Fig. 6B, right three panels), although reduced Der f 1 showed the highest OD mean of the tested mice in total IgE and Der f 1-specific IgG1 and IgG2a.

Discussion

Cystatin A is an endogenous protease inhibitor, which can block the cysteine protease activity of house dust mite major allergens, Der f 1 and Der p 1 (31). In this study, we analyzed the modulation of the allergenicity of Der f 1 and Der p 1 caused by the binding of cystatin A using sera and basophils from mite-allergic volunteers, focusing on the effector phase in allergies (Figs. 2–4). The concentration of cystatin A in vivo in the skin is considered to be much higher than those used in Figs. 1–4 (0.5 μ g/ml or less) because even those in human sweat determined in our previous study were 1–5 μ g/ml (31). We showed that the binding of cystatin A partially inhibited the binding of IgE to Der f 1 and Der p 1 (Fig. 2)

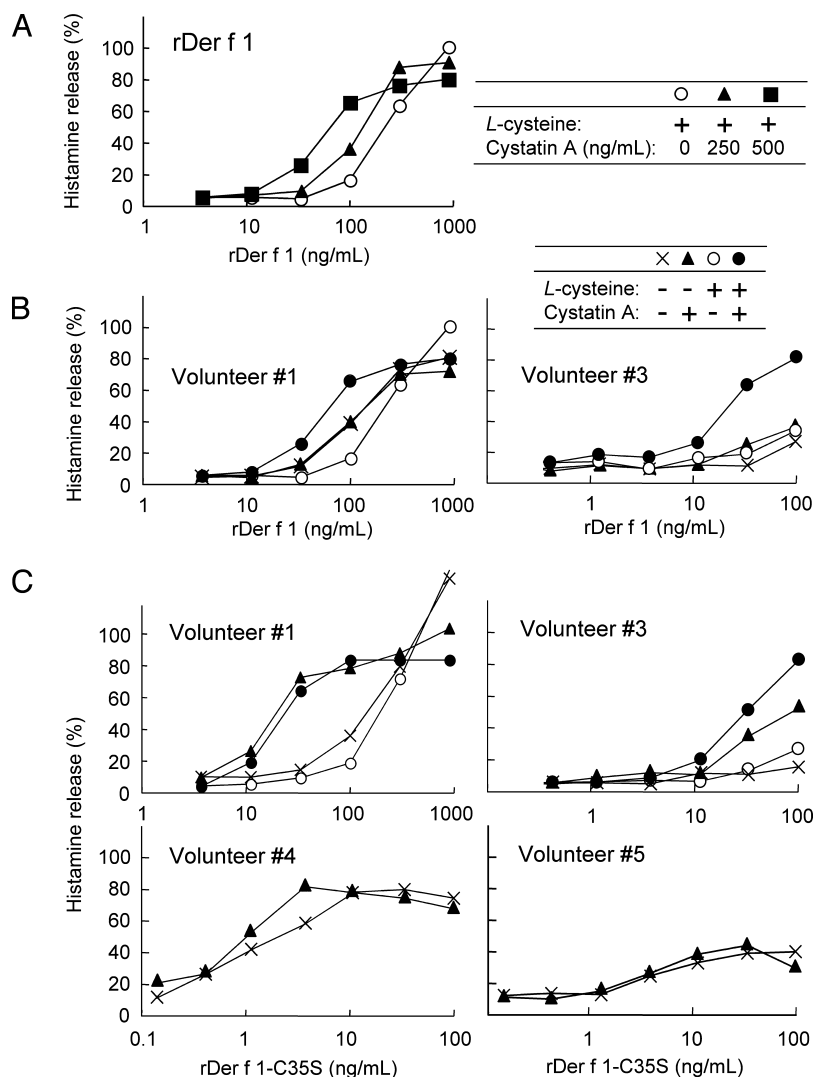


FIGURE 4. Enhancement of basophil histamine-releasing activity of Der f 1 by cystatin A was dependent on interaction with cystatin A. The rDer f 1 and rDer f 1-C35S pretreated with or without L-cysteine were incubated with or without recombinant cystatin A and subjected to basophil histamine release assay. *A*, Dose dependency for reduced rDer f 1. *B*, Results for reduced or oxidized rDer f 1. *C*, Results for rDer f 1-C35S with or without treatment with L-cysteine. The final concentration of cystatin A was 250 ng/ml or 500 ng/ml in *A* and 500 ng/ml in *B* and *C*.

but unexpectedly enhanced basophil histamine-releasing activity (Figs. 3 and 4). In contrast, cystatin A showed a suppressive effect on induction of Der f 1-specific IgE in the sensitization process in mice (Fig. 6).

Cystatin A inhibits the proteolytic activity of cysteine proteases and therefore is considered to bind active cysteine proteases. We demonstrated that cystatin A bound reduced forms of Der f 1 and Der p 1, which had been treated with L-cysteine, and a catalytic site mutant of Der f 1, rDer f 1-C35S, with or without L-cysteine treatment, but did not bind to oxidized forms and E-64 complexes of Der f 1 and Der p 1 (Fig. 1). These results suggest the importance of a wider space surrounding the ...S-H of the catalytic cysteine residue, Cys³⁵ of Der f 1 and Cys³⁴ of Der p 1, and the ...O-H of Ser³⁵ of rDer f 1-C35S for cystatin A binding. That there was no inhibition of the binding of cystatin A to rDer f 1-C35S by E-64 can be explained by the fact that E-64 cannot covalently and irreversibly bind Ser³⁵ of rDer f 1-C35S. Molecular modeling showed that cystatin A interacts with the protease allergens within a narrow area, supporting the partial inhibition of IgE binding to Der f 1 and Der p 1 (Fig. 5).

The binding of cystatin A enhanced the basophil histamine-releasing activity (Figs. 3 and 4). No dimerization or aggregation of the cystatin A used in the assays was detected by size exclusion column chromatography (our unpublished observation). Although no global structural difference has been reported between cysteine

proteases and their complexes with inhibitors of the cystatin family (53, 56, 57), a small structural change of these allergens induced by the binding of cystatin A is a possible cause of the enhancement of allergenicity at the cellular level. Allergic individuals, who showed enhanced histamine release by addition of cystatin A, might be sensitized to such IgE epitopes stably structured by binding of cystatin A. Recently, a research group reported that mature Der p 1 forms a homodimer in crystals and speculated that the homodimer might also be formed in solution on the basis of their observation that Der p 1 showed abnormal behavior in an analysis with size exclusion gel chromatography (22). The interface of the homodimer overlaps with the binding site for cystatin A and therefore another possible explanation for the enhancement of histamine-releasing activity could be that the binding of cystatin A prevented the homodimer from forming. The level of the enhancement was different among volunteers and between Der f 1 and Der p 1, suggesting that the number and/or affinity of IgE epitopes affected by binding of cystatin A are different among individuals and between these homologous and cross-reactive allergens.

It has been proposed that the proteolytic activity of Der f 1 and Der p 1 is involved in the pathogenesis of allergies and sensitization to the production of IgE by reducing physical and biochemical tissue barriers, cleaving various molecules, and modulating the functions of various cells (23–37). Cystatin A is the dominant biochemical skin barrier that eliminates the enzymatic activity of

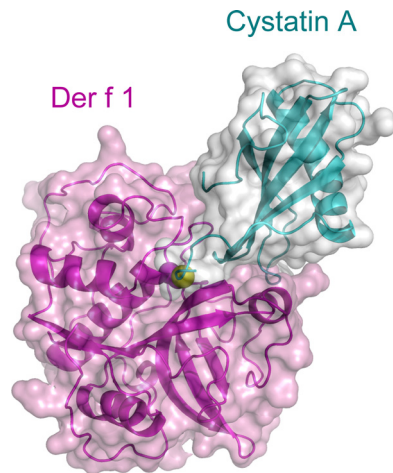


FIGURE 5. Molecular model of Der f 1-cystatin A complex on the basis of the crystal structures of mature rDer p 1 and a cathepsin H-cystatin A complex. Magenta ribbon and pale magenta molecular surface, Der f 1. Cyan ribbon and transparent molecular surface, cystatin A. Yellow sphere, the sulfur atom of the cysteine residue at the catalytic center of Der f 1, Cys35. The figure was made by PyMOL (DeLano Scientific).

these mite cysteine proteases and prevents them from stimulating keratinocytes (31, 40). In the present study, we demonstrated the suppressive effect of cystatin A on induction of Der f 1-specific IgE in the sensitization process in mice (Fig. 6*B, left*). As far as we know, this is the first demonstration that an endogenous protease inhibitor suppresses *in vivo* IgE-inducing immunogenicity of a cysteine protease allergen. On the other hand, the results shown in Figs. 2–4, which focused on the effector phase, suggest that once ones were sensitized to produce IgE specific to Der f 1 and Der p 1, cystatin A binding, in turn, would enhance the allergenicity of these allergens in terms of the degranulation of basophils and possibly also mast cells and might contribute to the enhancement of clinical allergic symptoms. On the contrary, such enhancement of allergenicity in the effector phase by the endogenous inhibitor

might be beneficial for mammals in IgE-dependent parasite expulsion.

Although cystatin A binds Der f 1 and Der p 1 treated with L-cysteine but not those oxidized during purification, these allergens could be converted to reduced forms *in vivo* by endogenous reducing factors such as glutathione as speculated by Herbert et al. (25) on the basis of their *in vitro* results and might be present in part as reduced or differentially oxidized forms, which could bind to the inhibitor (58), in fresh mite bodies or fecal pellets. In our observations, with or without pretreatment with L-cysteine, recombinant mite group 1 allergens can activate primary cultured human keratinocytes *in vitro*, and the activation can be blocked with cystatin A (40) and can induce Ag-specific IgE production when concentrated on alum in a manner dependent on the potential of the catalytic thiol group to be regenerated (our unpublished observations), suggesting that their cysteine protease activity could be regenerated with interaction with the cells, factors in the culture supernatant, or *in vivo* environment. Although almost no Der f 1-specific IgE was detected in mice immunized *i.p.* with unreduced Der f 1 without using any adjuvants (Fig. 6*B, left*), different doses, time schedule, and/or routes for administration of the unreduced Der f 1 might enable more efficient induction of Der f 1-specific IgE.

Machado et al. (32) reported that preparations containing mast cells and basophil-enriched leukocyte preparations, which were obtained from healthy donors, released histamine by stimulation with Der p 1 at a high concentration (10 $\mu\text{g/ml}$), suggesting IgE-independent and possibly protease activity-dependent histamine release from human mast cells and basophils. Our results that binding of cystatin A to rDer f 1 and rDer p 1 enhanced the histamine release indicate that the possible protease activity-dependent histamine release is smaller than the enhancing effect by cystatin A binding and/or is negligible at the lower concentrations tested in this study (<100 ng/ml in Fig. 3 and <1 $\mu\text{g/ml}$ in Fig. 4). Interestingly, while this article was under revision, Sokol et al. (59) reported that cysteine protease activity of papain, an occupational allergen homologous to Der f 1 and Der p 1, initiates Th2 sensitization *in vivo* in mice via activation of basophils. At a high

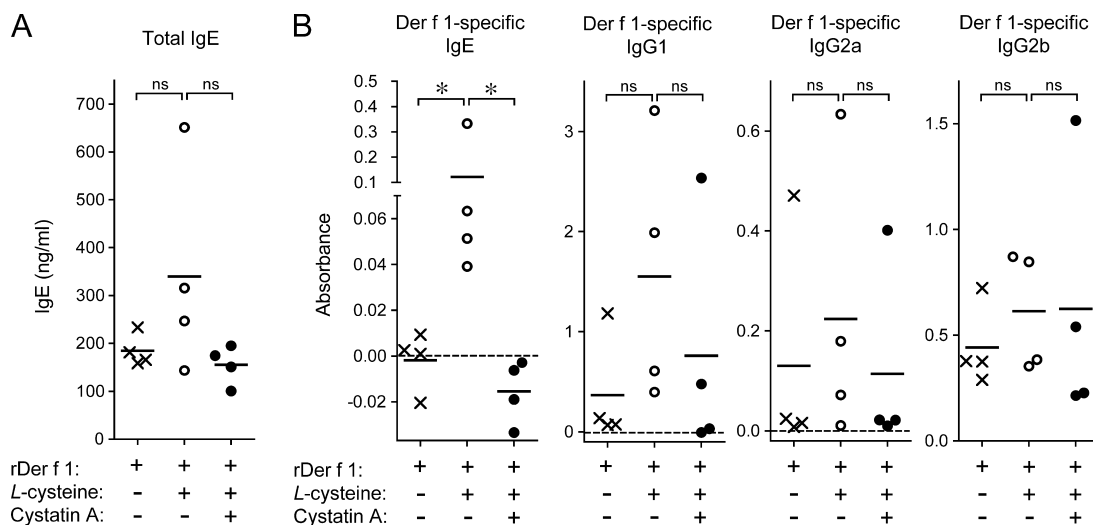


FIGURE 6. Cystatin A suppressed induction of Der f 1-specific IgE in the sensitization process in mice immunized with reduced Der f 1. Without the use of any adjuvants, mice were given four weekly *i.p.* injections with three differently treated Der f 1 (unreduced Der f 1, reduced Der f 1 in which the catalytic thiol group was regenerated with L-cysteine, and cystatin A-treated reduced Der f 1; 1 $\mu\text{g/head}$) with or without treatment with L-cysteine or cystatin A (1 $\mu\text{g/head}$). Sera were collected at 4 wk. A, Serum total IgE. B, Serum Der f 1-specific IgE and IgGs. Serum dilution factors were 50, 1000, 200, and 2000 for detecting Der f 1-specific IgE, IgG1, IgG2a, and IgG2b, respectively. Data are for four mice per group. Bars, means. *, $p < 0.05$ by the Mann-Whitney *U* test (two-tailed). ns, No significant difference. Data are representative of two independent experiments with similar results.

concentration of papain (100 $\mu\text{g/ml}$), its cysteine protease activity stimulated murine bone marrow-derived basophils in vitro to produce Th2-inducing cytokines including IL-4 but not release histamine. Cysteine protease activity of our preparations of rDer p 1 and rDer f 1 also crucially commits to the sensitization process in mice (37). Although Phillips et al. (60) reported that Der p 1 stimulated purified human basophils to release IL-4, whether its cysteine protease activity is responsible for the basophil activation is unknown because their Der p 1 preparation also contained serine proteases other than Der p 1 (28, 61). Whether the rDer f 1 and rDer p 1, which show cysteine protease activity but no serine protease activity (15), could stimulate basophils to produce Th2-inducing cytokines should be addressed in a future study.

Although the binding of murine Fab fragments against the birch major allergen Bet v 1 was reported to enhance the allergenicity of Bet v 1 in IgE binding and skin tests (62), no endogenous ligand has been reported to enhance the allergenicity of an allergen. Some allergens are proteases, and some endogenous inhibitors or substrates could interact with them (31, 40, 42–44, 63, 64). Moreover, other ligands against allergens are also present in the human body. For example, some allergens are enzymes and interact with corresponding substrates and inhibitors, and others actually or presumably bind lipids or molecules other than proteins (65–70). A mite major group 2 allergen, Der f 2, is homologous to a mammalian cholesterol-binding protein, NPC2/HE1 (71, 72), and has a structural relationship with MD-2 (68, 69, 73), which associates TLR4 on the cell surface and is part of the mammalian innate immune system involved in the recognition of LPS from Gram-negative bacteria. IgE-binding and basophil histamine-releasing activities of Der f 2 were decreased by mutations to a potential ligand-binding region (74). Very recently, Trompette et al. (75) reported surprising results that another mite group 2 allergen, Der p 2, can bind TLR4 and reconstitute LPS-driven TLR4 signaling in the absence of MD-2, and we have demonstrated LPS binding to Der f 2 and analyzed the binding sites (69). Thus, ligand binding to allergens, such as cystatin A binding to the mite group 1 allergens and binding of LPS (69) and TLR4 (75) to the mite group 2 allergens, modulates IgE-inducing immunogenicity in the sensitization process (Fig. 6 and Ref. 75) and might also modulate the allergenicity of the allergen in the effector phase or exacerbation in allergic diseases (Figs. 2–4 and Ref. 74).

In this study, we found the first example of the allergenicity of allergens being enhanced by the binding of an endogenous ligand. In addition, we demonstrated the first example that an endogenous protease inhibitor attenuated in vivo IgE-inducing immunogenicity of a cysteine protease allergen. The interaction between environmental allergens and their ligands present in the human body might be clinically important in both the sensitization process and effector phase/exacerbation in allergic diseases. Investigation with combinations of allergens and their ligands into their biological significance in vivo and clinical importance should be examined in future studies.

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Disclosures

The authors have no financial conflict of interest.

References

- Voorhorst, R., F. T. M. Spieksma, M. J. Vanekamp, M. J. Leupen, and A. W. Lyklema. 1967. The house dust mite (*Dermatophagoides pteronyssinus*) and the allergens it produces: identity with the house dust allergen. *J. Allergy* 39: 325–328.
- Miyamoto, T., S. Oshima, T. Ishizaki, and S. H. Sato. 1968. Allergenic identity between the common floor mite (*Dermatophagoides farinae* Hughes, 1961) and house dust as a causative antigen in bronchial asthma. *J. Allergy* 42: 14–28.
- Platts-Mills, T. A., and M. D. Chapman. 1987. Dust mites: immunology, allergic disease, and environmental control. *J. Allergy Clin. Immunol.* 80: 755–775.
- Thomas, W. R., W. A. Smith, B. J. Hales, K. L. Mills, and R. M. O'Brien. 2002. Characterization and immunobiology of house dust mite allergens. *Int. Arch. Allergy Immunol.* 129: 1–18.
- Chapman, M. D., and T. A. Platts-Mills. 1980. Purification and characterization of the major allergen from *Dermatophagoides pteronyssinus*-antigen P1. *J. Immunol.* 125: 587–592.
- Tovey, E. R., M. D. Chapman, and T. A. Platts-Mills. 1981. Mite faeces are a major source of house dust allergens. *Nature* 289: 592–593.
- Lind, P. 1985. Purification and partial characterization of two major allergens from the house dust mite *Dermatophagoides pteronyssinus*. *J. Allergy Clin. Immunol.* 76: 753–761.
- Yasueda, H., H. Mita, Y. Yui, and T. Shida. 1986. Isolation and characterization of two allergens from *Dermatophagoides farinae*. *Int. Arch. Allergy Appl. Immunol.* 81: 214–223.
- Heymann, P. W., M. D. Chapman, and T. A. Platts-Mills. 1986. Antigen Der f I from the dust mite *Dermatophagoides farinae*: structural comparison with Der p I from *Dermatophagoides pteronyssinus* and epitope specificity of murine IgG and human IgE antibodies. *J. Immunol.* 137: 2841–2847.
- van der Zee, J. S., P. van Swieten, H. M. Jansen, and R. C. Aalberse. 1988. Skin tests and histamine release with P1-depleted *Dermatophagoides pteronyssinus* body extracts and purified P1. *J. Allergy Clin. Immunol.* 81: 884–896.
- Yasueda, H., H. Mita, Y. Yui, and T. Shida. 1989. Comparative analysis of physicochemical and immunochemical properties of the two major allergens from *Dermatophagoides pteronyssinus* and the corresponding allergens from *Dermatophagoides farinae*. *Int. Arch. Allergy Appl. Immunol.* 88: 402–407.
- Chua, K. Y., G. A. Stewart, W. R. Thomas, R. J. Simpson, R. J. Dilworth, T. M. Plozza, and K. J. Turner. 1988. Sequence analysis of cDNA coding for a major house dust mite allergen Der p 1: homology with cysteine proteases. *J. Exp. Med.* 167: 175–182.
- Dilworth, R. J., K. Y. Chua, and W. R. Thomas. 1991. Sequence analysis of cDNA coding for a major house dust mite allergen. *Der f I. Clin. Exp. Allergy* 21: 25–32.
- Ando, T., Y. Ino, M. Haida, R. Honma, H. Maeda, H. Yamakawa, M. Iwaki, and H. Okudaira. 1991. Isolation of cysteine protease in the crude mite extract, *Dermatophagoides farinae*. *Int. Arch. Allergy Appl. Immunol.* 96: 199–205.
- Takai, T., T. Kato, Y. Sakata, H. Yasueda, K. Izuhara, K. Okumura, and H. Ogawa. 2005. Recombinant Der p 1 and Der f 1 exhibit cysteine protease activity but no serine protease activity. *Biochem. Biophys. Res. Commun.* 328: 944–952.
- Lombardero, M., P. W. Heymann, T. A. Platts-Mills, J. W. Fox, and M. D. Chapman. 1990. Conformational stability of B cell epitopes on group I and group II *Dermatophagoides* spp. Allergens: effect of thermal and chemical denaturation on the binding of murine IgG and human IgE antibodies. *J. Immunol.* 144: 1353–1360.
- Collins, S. P., G. Ball, E. Vonarx, C. Hosking, M. Shelton, D. Hill, and M. E. Howden. 1996. Absence of continuous epitopes in the house dust mite major allergens Der p 1 from *Dermatophagoides pteronyssinus* and Der f 1 from *Dermatophagoides farinae*. *Clin. Exp. Allergy* 26: 36–42.
- Yasuhara, T., T. Takai, T. Yuuki, H. Okudaira, and Y. Okumura. 2001. Cloning and expression of cDNA encoding the complete prepro-form of an isoform of Der f 1, the major group 1 allergen from house dust mite *Dermatophagoides farinae*. *Biosci. Biotechnol. Biochem.* 65: 563–569.
- Takai, T., T. Kato, H. Yasueda, K. Okumura, and H. Ogawa. 2005. Analysis of the structure and allergenicity of recombinant pro- and mature Der p 1 and Der f 1: major conformational IgE epitopes blocked by prodomains. *J. Allergy Clin. Immunol.* 115: 555–563.
- Lind, P., O. C. Hansen, and N. Horn. 1988. The binding of mouse hybridoma and human IgE antibodies to the major fecal allergen, Der p I, of *Dermatophagoides pteronyssinus*: relative binding site location and species specificity studied by solid-phase inhibition assays with radiolabeled antigen. *J. Immunol.* 140: 4256–4262.
- Meno, K., P. B. Thorsted, H. Ipsen, O. Kristensen, J. N. Larsen, M. D. Spangfort, M. Gajhede, and K. Lund. 2005. The crystal structure of recombinant proDer p 1, a major house dust mite proteolytic allergen. *J. Immunol.* 175: 3835–3845.
- de Halleux, S., E. Stura, L. VanderElst, V. Carlier, M. Jacquemin, and J. M. Saint-Remy. 2006. Three-dimensional structure and IgE-binding properties of mature fully active Der p 1, a clinically relevant major allergen. *J. Allergy Clin. Immunol.* 117: 571–576.
- Shakib, F., A. M. Ghaemmaghami, and H. F. Sewell. 2008. The molecular basis of allergenicity. *Trends Immunol.* 29: 633–642.
- Chapman, M. D., S. Wunschmann, and A. Pomes. 2007. Proteases as Th2 adjuvants. *Curr. Allergy Asthma Rep.* 7: 363–367.
- Herbert, C. A., C. M. King, P. C. Ring, S. T. Holgate, G. A. Stewart, P. J. Thompson, and C. Robinson. 1995. Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *Am. J. Respir. Cell Mol. Biol.* 12: 369–378.

26. Nakamura, T., Y. Hirasawa, T. Takai, K. Mitsuishi, M. Okuda, T. Kato, K. Okumura, S. Ikeda, and H. Ogawa. 2006. Reduction of skin barrier function by proteolytic activity of a recombinant house dust mite major allergen Der f 1. *J. Invest. Dermatol.* 126: 2719–2723.
27. Wan, H., H. L. Winton, C. Soeller, E. R. Tovey, D. C. Gruenert, P. J. Thompson, G. A. Stewart, G. W. Taylor, D. R. Garrod, M. B. Cannell, and C. Robinson. 1999. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J. Clin. Invest.* 104: 123–133.
28. Brown, A., K. Farmer, L. MacDonald, N. Kalsheker, D. Pritchard, C. Haslett, J. Lamb, and J. M. Sallenave. 2003. House dust mite Der p 1 downregulates defenses of the lung by inactivating elastase inhibitors. *Am. J. Respir. Cell Mol. Biol.* 29: 381–389.
29. Takai, T., T. Kato, M. Ota, H. Yasueda, T. Kuhara, K. Okumura, and H. Ogawa. 2005. Recombinant Der p 1 and Der f 1 with in vitro enzymatic activity to cleave human CD23, CD25 and α_1 -antitrypsin, and in vivo IgE-eliciting activity in mice. *Int. Arch. Allergy Immunol.* 137: 194–200.
30. King, C., S. Brennan, P. J. Thompson, and G. A. Stewart. 1998. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J. Immunol.* 161: 3645–3651.
31. Kato, T., T. Takai, K. Mitsuishi, K. Okumura, and H. Ogawa. 2005. Cystatin A inhibits IL-8 production by keratinocytes stimulated with Der p 1 and Der f 1: biochemical skin barrier against mite cysteine proteases. *J. Allergy Clin. Immunol.* 116: 169–176.
32. Machado, D. C., D. Horton, R. Harrop, P. T. Peachell, and B. A. Helm. 1996. Potential allergens stimulate the release of mediators of the allergic response from cells of mast cell lineage in the absence of sensitization with antigen-specific IgE. *Eur. J. Immunol.* 26: 2972–2980.
33. Miike, S., and H. Kita. 2003. Human eosinophils are activated by cysteine proteases and release inflammatory mediators. *J. Allergy Clin. Immunol.* 111: 704–713.
34. Grunstein, M. M., H. Veler, X. Shan, J. Larson, J. S. Grunstein, and S. Chuang. 2005. Proasthmatic effects and mechanisms of action of the dust mite allergen, Der p 1, in airway smooth muscle. *J. Allergy Clin. Immunol.* 116: 94–101.
35. Comoy, E. E., J. Pestel, C. Duez, G. A. Stewart, C. Vendeville, C. Fournier, F. Finkelman, A. Capron, and G. Thyphronitis. 1998. The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN- γ . *J. Immunol.* 160: 2456–2462.
36. Gough, L., O. Schulz, H. F. Sewell, and F. Shakib. 1999. The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J. Exp. Med.* 190: 1897–1902.
37. Kikuchi, Y., T. Takai, T. Kuhara, M. Ota, T. Kato, H. Hatanaka, S. Ichikawa, T. Tokura, H. Akiba, K. Mitsuishi, et al. 2006. Crucial commitment of proteolytic activity of a purified recombinant major house dust mite allergen Der p 1 to sensitization toward IgE and IgG responses. *J. Immunol.* 177: 1609–1617.
38. Turk, V., and W. Bode. 1991. The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett.* 285: 213–219.
39. Henskens, Y. M., E. C. Veerman, and A. V. Nieuw Amerongen. 1996. Cystatins in health and disease. *Biol. Chem. Hoppe Seyler* 377: 71–86.
40. Ogawa, T., T. Takai, T. Kato, T. Kikuchi, F. Niyonsaba, S. Ikeda, K. Okumura, and H. Ogawa. 2008. Upregulation of release of granulocyte-macrophage colony-stimulating factor from keratinocytes stimulated with cysteine protease activity of recombinant major mite allergens, Der f 1 and Der p 1. *Int. Arch. Allergy Immunol.* 146: 27–35.
41. Vasilopoulos, Y., M. J. Cork, D. Teare, I. Marinou, S. J. Ward, G. W. Duff, and R. Tazi-Ahmini. 2007. A nonsynonymous substitution of cystatin A, a cysteine protease inhibitor of house dust mite protease, leads to decreased mRNA stability and shows a significant association with atopic dermatitis. *Allergy* 62: 514–519.
42. Reed, C. E., and H. Kita. 2004. The role of protease activation of inflammation in allergic respiratory diseases. *J. Allergy Clin. Immunol.* 114: 997–1008.
43. Smith, P. K., and J. I. Harper. 2006. Serine proteases, their inhibitors and allergy. *Allergy* 61: 1441–1447.
44. Bode, W., and R. Huber. 2000. Structural basis of the endoprotease-protein inhibitor interaction. *Biochim. Biophys. Acta* 1477: 241–252.
45. Takai, T., R. Mineki, T. Nakazawa, M. Takaoka, H. Yasueda, K. Murayama, K. Okumura, and H. Ogawa. 2002. Maturation of the activities of recombinant mite allergens Der p 1 and Der f 1, and its implication in the blockade of proteolytic activity. *FEBS Lett.* 531: 265–272.
46. Takai, T., E. Mizuuchi, Y. Kikuchi, T. Nagamune, K. Okumura, and H. Ogawa. 2006. Glycosylation of recombinant proforms of major house dust mite allergens Der p 1 and Der f 1 decelerates the speed of maturation. *Int. Arch. Allergy Immunol.* 139: 181–187.
47. Yasuhara, T., T. Takai, T. Yuuki, H. Okudaira, and Y. Okumura. 2001. Biologically active recombinant forms of a major house dust mite group 1 allergen Der f 1 with full activities of both cysteine protease and IgE binding. *Clin. Exp. Allergy* 31: 116–124.
48. Takahashi, K., T. Takai, T. Yasuhara, T. Yokota, and Y. Okumura. 2001. Effects of site-directed mutagenesis in the cysteine residues and the N-glycosylation motif in recombinant Der f 1 on secretion and protease activity. *Int. Arch. Allergy Immunol.* 124: 454–460.
49. Kato, T., T. Takai, T. Fujimura, H. Matsuoka, T. Ogawa, K. Murayama, A. Ishii, S. Ikeda, K. Okumura, and H. Ogawa. 2009. Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes. *Allergy* 64: 1366–1374.
50. Takai, T., M. Takaoka, H. Yasueda, K. Okumura, and H. Ogawa. 2005. Dilution method to refold bacterially expressed recombinant Der f 2 and Der p 2 to exhibit the secondary structure and histamine-releasing activity of natural allergens. *Int. Arch. Allergy Immunol.* 137: 1–8.
51. Nishi, H., S. Nishimura, M. Higashiura, N. Ikeya, H. Ohta, T. Tsuji, M. Nishimura, S. Ohnishi, and H. Higashi. 2000. A new method for histamine release from purified peripheral blood basophils using monoclonal antibody-coated magnetic beads. *J. Immunol. Methods* 240: 39–46.
52. Schwede, T., J. Kopp, N. Guex, and M. C. Peitsch. 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* 31: 3381–3385.
53. Jenko, S., I. Dolenc, G. Guncar, A. Dobersek, M. Podobnik, and D. Turk. 2003. Crystal structure of Stefin A in complex with cathepsin H: N-terminal residues of inhibitors can adapt to the active sites of endo- and exopeptidases. *J. Mol. Biol.* 326: 875–885.
54. Takai, T., Y. Ochiai, S. Ichikawa, E. Sato, T. Ogawa, T. Tokura, T. Kuhara, H. Kawai, H. Hatanaka, S. Takahashi, H. Ogawa, and K. Okumura. 2009. Enzyme-linked immunosorbent assays with high sensitivity for antigen-specific and total murine IgE: a useful tool for the study of allergies in mouse models. *Allergol. Int.* 58: 225–235.
55. Kikuchi, Y., T. Takai, M. Ota, T. Kato, K. Takeda, K. Mitsuishi, S. Ikeda, K. Okumura, and H. Ogawa. 2006. Application of immunoreaction enhancer solutions to an enzyme-linked immunosorbent assay for antigen-specific IgE in mice immunized with recombinant major mite allergens or ovalbumin. *Int. Arch. Allergy Immunol.* 141: 322–330.
56. Stubbs, M. T., B. Laber, W. Bode, R. Huber, R. Jerala, B. Lenarcic, and V. Turk. 1990. The refined 2.4 Å x-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. *EMBO J.* 9: 1939–1947.
57. Bode, W., R. Engh, D. Musil, U. Thiele, R. Huber, A. Karshikov, J. Brzin, J. Kos, and V. Turk. 1988. The 2.0 Å x-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. *EMBO J.* 7: 2593–2599.
58. Bjork, I., and K. Ylinenjarvi. 1989. Interaction of chicken cystatin with inactivated papains. *Biochem. J.* 260: 61–68.
59. Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* 9: 310–318.
60. Phillips, C., W. R. Cowad, D. I. Pritchard, and C. R. A. Hewitt. 2003. Basophils express a type 2 cytokine profile on exposure to proteases from helminths and house dust mites. *J. Leukoc. Biol.* 73: 165–171.
61. Hewitt, C. R., A. P. Brown, B. J. Hart, and D. I. Pritchard. 1995. A major house dust mite protease allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by anti-proteases. *J. Exp. Med.* 182: 1537–1544.
62. Denepoux, S., P. B. Eibensteiner, P. Steinberger, S. Vrtala, V. Visco, A. Weyer, D. Kraft, J. Banchereau, R. Valenta, and S. Lebecque. 2000. Molecular characterization of human IgG monoclonal antibodies specific for the major birch pollen allergen Bet v 1. Anti-allergen IgG can enhance the anaphylactic reaction. *FEBS Lett.* 465: 39–46.
63. Sakata, Y., K. Arima, T. Takai, W. Sakurai, K. Masumoto, N. Yuyama, Y. Suminami, F. Kishi, T. Yamashita, T. Kato, et al. 2004. The squamous cell carcinoma antigen 2 inhibits the cysteine proteinase activity of a major mite allergen, Der p 1. *J. Biol. Chem.* 279: 5081–5087.
64. Seto, T., T. Takai, N. Ebihara, H. Matsuoka, X. L. Wang, A. Ishii, H. Ogawa, A. Murakami, and K. Okumura. 2009. SLPI prevents cytokine release in mite protease-exposed conjunctival epithelial cells. *Biochem. Biophys. Res. Commun.* 379: 681–685.
65. Thomas, W. R., B. J. Hales, and W. A. Smith. 2005. Structural biology of allergens. *Curr. Allergy Asthma Rep.* 5: 388–393.
66. Holt, P. G., and W. R. Thomas. 2005. Sensitization to airborne environmental allergens: unresolved issues. *Nat. Immunol.* 6: 957–960.
67. Ichikawa, S., H. Hatanaka, T. Yuuki, N. Iwamoto, S. Kojima, C. Nishiyama, K. Ogura, Y. Okumura, and F. Inagaki. 1998. Solution structure of Der f 2, the major mite allergen for atopic diseases. *J. Biol. Chem.* 273: 356–360.
68. Ichikawa, S., T. Takai, T. Inoue, T. Yuuki, Y. Okumura, K. Ogura, F. Inagaki, and H. Hatanaka. 2005. NMR study on the major mite allergen Der f 2: its refined tertiary structure, epitopes for monoclonal antibodies and characteristics shared by ML protein group members. *J. Biochem.* 137: 255–263.
69. Ichikawa, S., T. Takai, T. Yashiki, S. Takahashi, K. Okumura, H. Ogawa, D. Kohda, and H. Hatanaka. 2009. Lipopolysaccharide binding of the mite allergen Der f 2. *Genes Cells* 14: 1055–1065.
70. Derewenda, U., J. Li, Z. Derewenda, Z. Dauter, G. A. Mueller, G. S. Rule, and D. C. Benjamin. 2002. The crystal structure of a major dust mite allergen Der p 2, and its biological implications. *J. Mol. Biol.* 318: 189–197.
71. Thomas, W. R., and K. Y. Chua. 1995. The major mite allergen Der p 2: a secretion of the male mite reproductive tract? *Clin. Exp. Allergy* 25: 667–669.
72. Friedland, N., H. L. Liou, P. Lobel, and A. M. Stock. 2003. Structure of a cholesterol-binding protein deficient in Niemann-Pick type C2 disease. *Proc. Natl. Acad. Sci. USA* 100: 2512–2517.
73. Inohara, N., and G. Nunez. 2002. ML: a conserved domain involved in innate immunity and lipid metabolism. *Trends Biochem. Sci.* 27: 219–221.
74. Nakazawa, T., T. Takai, H. Hatanaka, E. Mizuuchi, T. Nagamune, K. Okumura, and H. Ogawa. 2005. Multiple-mutation at a potential ligand-binding region decreased allergenicity of a mite allergen Der f 2 without disrupting global structure. *FEBS Lett.* 579: 1988–1994.
75. Trompette, A., S. Divanovic, A. Visintin, C. Blanchard, R. S. Hegde, R. Madan, P. S. Thorne, M. Wills-Karp, T. L. Gioannini, J. P. Weiss, and C. L. Karp. 2009. Allergic reaction resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457: 585–588.