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Mucosal Allergic Sensitization to Cockroach Allergens Is Dependent on Proteinase Activity and Proteinase-Activated Receptor-2 Activation

Narcy G. Arizmendi,* Melanie Abel,* Koichiro Mihara,[†] Courtney Davidson,*
 Danny Polley,[†] Ahmed Nadeem,* Tamer El Mays,[‡] Brendan F. Gilmore,[§]
 Brian Walker,[§] John R. Gordon,[¶] Morley D. Hollenberg,[†] and Harissios Vliagoftis*

We have shown that proteinase-activated receptor-2 (PAR₂) activation in the airways leads to allergic sensitization to concomitantly inhaled Ags, thus implicating PAR₂ in the pathogenesis of asthma. Many aeroallergens with proteinase activity activate PAR₂. To study the role of PAR₂ in allergic sensitization to aeroallergens, we developed a murine model of mucosal sensitization to cockroach proteins. We hypothesized that PAR₂ activation in the airways by natural allergens with serine proteinase activity plays an important role in allergic sensitization. Cockroach extract (CE) was administered to BALB/c mice intranasally on five consecutive days (sensitization phase) and a week later for four more days (challenge phase). Airway hyperresponsiveness (AHR) and allergic airway inflammation were assessed after the last challenge. To study the role of PAR₂, mice were exposed intranasally to a receptor-blocking anti-PAR₂ Ab before each administration of CE during the sensitization phase. Mucosal exposure to CE induced eosinophilic airway inflammation, AHR, and cockroach-specific IgG1. Heat-inactivated or soybean trypsin inhibitor-treated CE failed to induce these effects, indicating that proteinase activity plays an important role. The use of an anti-PAR₂ blocking Ab during the sensitization phase completely inhibited airway inflammation and also decreased AHR and the production of cockroach-specific IgG1. PAR₂ activation by CE acts as an adjuvant for allergic sensitization even in the absence of functional TLR4. We conclude that CE induces PAR₂-dependent allergic airway sensitization in a mouse model of allergic airway inflammation. PAR₂ activation may be a general mechanism used by aeroallergens to induce allergic sensitization. *The Journal of Immunology*, 2011, 186: 000–000.

A number of environmental allergens from diverse sources have proteinase activity, which has been suggested to skew the immune response toward the Th2 phenotype (1, 2) and allergic sensitization. Such proteinase activity may mediate allergic sensitization to the proteinases themselves (3) or other Ags encountered by the mucosal immune system at the same time (4).

A number of cockroach proteins are potent environmental aeroallergens. There is evidence that early life exposure (5) to cockroach allergens leads to allergic sensitization to cockroaches, which has been shown to have a strong correlation with the incidence of asthma (6) and asthma exacerbations (7, 8). Childhood sensitization to cockroach allergens also has been associated with an increased risk for persistent asthma and bronchial hyperresponsiveness and with a greater loss of lung function (9). In general, cockroach allergens are an important cause of asthma exacerbations in many parts of the world (10, 11).

Although most cockroach allergens are not serine proteinases, they are inhaled along with a variety of cockroach-derived proteins from the gastrointestinal track and feces that contain serine proteinase activity. Interestingly, cockroach extract (CE) activates airway epithelial cells in a proteinase-activated receptor-2 (PAR₂)-dependent fashion (12). German cockroach (*Blattella germanica*) extract induces PAR₂-mediated activation of airway epithelial cells, which leads to the release of TNF (13) and IL-8 (14). However, it is not known whether specific cockroach allergens activate PAR₂ or whether this activation is due to other cockroach-derived proteins inhaled at the same time as the allergens. At least one of the cockroach allergens, Per a 10, has trypsin-like serine proteinase activity (15) and therefore has the ability to activate PAR₂.

PAR₂ is a prime candidate to sense environmental exposure to serine proteinases and has been implicated in the development of inflammatory reactions (16–18). Previous reports have linked PAR₂ to human asthma (19, 20). The PAR₂ gene is located in 5q13, a genomic area that is linked to the development of allergy (21), and PAR₂ expression is increased in the airways of asthmatic

*Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada; [†]Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, Canada; [‡]Department of Pathology and Laboratory Medicine, University of Calgary, Calgary, Alberta, Canada; [§]Biomolecular Sciences Group, School of Pharmacy, Queen's University, Belfast, United Kingdom; and [¶]Immunology Research Group, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

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Address correspondence and reprint requests to Dr. Harissios Vliagoftis, Department of Medicine, University of Alberta, 550A Heritage Medical Research Center, Edmonton, Alberta T6G 2S2, Canada. E-mail address: hari@ualberta.ca

Abbreviations used in this article: ABP, activity-based probe; AHR, airway hyperresponsiveness; AMC, 7-amino-4-methylcoumarin; BAEE, N-benzoyl-L-arginine ethyl ester; BAL, bronchoalveolar lavage; Bio-PK-DPP4, biotin-linker-Pro-Lys-diphenyl phosphonate; CE, cockroach extract; 2-FLI, 2-furoyl-LIGRLO-NH₂; HCE, heat-inactivated cockroach extract; i.n., intranasally; KNRK, Kirsten murine sarcoma virus-transformed normal rat kidney cell line; KO, knockout; PAR₂, proteinase-activated receptor-2; PBST, isotonic PBS with Tween 20; Penh, enhanced pause; QAR-AMC, butoxycarbonyl-Gln-Ala-Arg-AMC-HCl; SBTI, soybean trypsin inhibitor; WT, wild-type.

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individuals (19). Moreover, PAR₂^{-/-} mice develop lower levels of airway hyperresponsiveness (AHR) and airway inflammation than normal mice (20). In addition, we have shown that PAR₂ activation in the airways leads to allergic sensitization to concomitantly inhaled Ags (22) and also to increased allergen-mediated airway inflammation and AHR development in sensitized mice (23).

In our previous studies (22, 23), we have activated PAR₂ with PAR₂-activating peptides administered together with OVA and analyzed allergic sensitization or airway inflammation as a result of Ags without proteinase activity. We propose that environmental allergens with proteinase activity and PAR₂ activation by such allergens function as an adjuvant and mediate allergic sensitization and inflammation. To test this hypothesis and study the role of PAR₂ in sensitization to common allergens, we developed a murine model of mucosal sensitization using CE. Using our model, we provide evidence that supports the hypothesis that PAR₂ activation by CE is involved in the development of allergic sensitization and inflammatory changes in murine airways and that this PAR₂-dependent adjuvant activity of CE is present, although to a lesser extent, even in the absence of functional TLR4.

Materials and Methods

Animals

Male BALB/c and C57BL/6NCrIBR mice were purchased from Charles River Laboratories and were used when they were between 6 and 8 wk of age. Male C.C3-TLR4Lps-d/J and the control BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at the same age. Male PAR₂^{-/-} mice on a C57BL/6 background were obtained from The Jackson Laboratory and bred at the animal facility at the University of Calgary. Wild-type and PAR₂^{-/-} C57BL/6 mice, for which breeding pairs were obtained as a gift through the courtesy of Dr. Patricia Andrade-Gorton (Johnson & Johnson Pharmaceutical Research & Development, Spring House, PA) (24), also were bred in-house and used interchangeably for comparison with the mice purchased from The Jackson Laboratory. All of the mice were housed in virus- and Ab-free conditions and maintained on a 12 h light/dark cycle. All of the experiments described were approved by the University of Alberta Health Sciences Laboratory Animal Ethics Committee (Edmonton, Alberta, Canada).

Cockroach extract

Lyophilized frozen whole-body German cockroach (*B. germanica*) extracts were obtained from Greer Laboratories (Lenoir, NC). Extracts were resuspended in 0.9% sterile saline solution at a concentration of 6 mg/ml of total protein. The trypsin-like activity of CE was determined with the fluorogenic peptide substrate butoxycarbonyl-Gln-Ala-Arg-7-amino-4-methylcoumarin (AMC)-HCl (QAR-AMC; Bachem, King of Prussia, PA). The activity of the cockroach powder was equivalent to 25–30 N-benzoyl-L-arginine ethyl ester (BAEE) units per milligram of trypsin-like activity, which corresponds to 0.75–0.90 IU/mg using QAR-AMC as the substrate. The QAR-AMC substrate assays were standardized with trypsin of known BAEE units per milligram (13,000–20,000 BAEE units per milligram) (Sigma-Aldrich Canada, Oakville, Ontario, Canada). The endotoxin content of CE was evaluated using a *Limulus* amebocyte lysate assay kit (Sigma-Aldrich, St. Louis, MO) with a sensitivity of 0.05 endotoxin units per milliliter. CE at a concentration of 100 mg/ml tested positive in this test, indicating that it contains >0.05 endotoxin units per milliliter.

Inhibition of CE proteinase activity with soybean trypsin inhibitor

The ability of soybean trypsin inhibitor (SBTI) to inhibit the proteolytic activity in CE was monitored by measuring the hydrolysis of the substrate described previously (QAR-AMC) in the absence and presence of increasing concentrations of SBTI (Sigma-Aldrich) (0.1 and 1.0 μ M). A total of 150 mU/ml CE was incubated with each concentration of SBTI in a total volume of 0.2 ml for 10 min at 24°C prior to the addition of the substrate. For in vivo use, to eliminate the proteinase activity, we either treated the cockroach extracts with 200 μ M SBTI for 30 min at 37°C or with heat at 65°C for 30 min. SBTI-treated or heat-treated CE was used for allergic sensitization and challenge of mice.

Intranasal administration of whole CE and anti-PAR₂ Ab

After light anesthesia with ketamine (75 mg/kg) and acepromazine (2.5 mg/kg), mice were given intranasally (i.n.) 50 μ g whole CE dissolved in 25 μ l of 0.9% sterile saline according to the protocol shown in Fig. 1A. Control mice were given saline solution only (saline/saline group).

To investigate the role of PAR₂ activation in the airways during allergic sensitization with CE, we administered i.n. a rabbit polyclonal receptor-blocking anti-PAR₂ Ab, B5 (25 μ l of a 1:500 dilution of antiserum) 30 min before i.n. administration of CE. B5 is known to have blocking activity in vivo (25). Control mice received the same amount of normal rabbit serum before CE administration. We also used the mouse anti-PAR₂ mAb SAM-11 (Santa Cruz Biotechnology, Santa Cruz, CA). In this case, we administered i.n. 10 ng SAM-11 or an isotype control (mouse IgG2a) dissolved in 25 μ l saline 30 min before CE administration.

Evaluation of AHR and allergic airway inflammation

Twenty-four hours after the final i.n. CE administration, we measured enhanced pause (Penh) to increasing doses of methacholine by noninvasive whole-body plethysmography (Buxco Electronics, Wilmington, NC) to determine AHR. Conscious mice were placed in whole-body plethysmographic chambers, and after 10 min of stabilization, dose–response curves to aerosolized methacholine (2–32 mg/ml) were generated.

Mice were euthanized 48 h after the last challenge. After blood collection by cardiac puncture, the trachea was exposed and intubated with a polyethylene catheter. Lungs were lavaged five times with 1 ml isotonic PBS (pH 7.4), and 5 ml of this bronchoalveolar lavage (BAL) fluid was collected. The BAL fluid was centrifuged at 300 $\times$ g for 5 min. Total cells were counted, and then cytopspins of 5000 cells were prepared and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI). Airway inflammation was assessed by counting the number of inflammatory cells in the BAL fluid as described previously (26).

Detection of cockroach-specific IgG1

Cockroach-specific IgG1 in mouse serum was measured by ELISA. Briefly, CE [200 μ g/ml in 0.1 M sodium carbonate buffer (pH 9.5)] was added onto 96-well Nunc MaxiSorp plates (Corning Life Sciences, Lowell, MA) and incubated overnight at 4°C. Plates were washed three times with 0.05% Tween 20 (Fisher Scientific, Fair Lawn, NJ) in isotonic PBS (pH 7.4) (PBST), blocked with PBS/10% FBS (Life Technologies Invitrogen, Grand Island, NY), and incubated for 1 h at 24°C. Plates were washed three times with PBST. Mouse serum diluted 1:200 with PBS was added and incubated for 3 h at 24°C. Plates were washed three times, and a 1:5000 dilution of biotin-labeled rat anti-mouse IgG1 (BD Pharmingen, Mississauga, Ontario, Canada) in PBS/10% FBS was added. Plates were incubated for 1 h at 24°C and then washed three times, followed by a 1:1000 dilution (in PBS/10% FBS) of HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Burlington, Ontario, Canada), and incubated for 30 min at 24°C. After incubation, plates were washed three times with PBST. Tetramethylbenzidine (BD Pharmingen) was added as a substrate and allowed 20 min at 24°C to develop. The reaction was stopped by the addition of 2 N H₂SO₄, and the absorbance was measured at 405 nm on a Power Wave XS (BioTek Instruments, Winooski, VT) ELISA reader. Results are shown as absorbance units.

Detection of total IgE

The total concentration of IgE in mouse sera was measured by sandwich ELISA using the Mouse IgE ELISA set (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. The total concentration of IgE in sera was calculated from a recombinant mouse IgE standard curve and expressed as nanograms per milliliter.

Serine proteinase activity-based probe

To detect trypsin-like enzymes in the cockroach extracts, we employed a strategy described previously for the identification of serine proteinases using biotinylated diphenyl phosphonate probes (27). On the basis of the use of probes with proline-lysine or asparagine-lysine sequences in the P2 and P1 enzyme target positions (28), we elected to synthesize the biotinylated probe with the PK sequence as the tryptic target (so-called compound 4, biotin-linker-Pro-Lys-diphenyl phosphonate, Bio-PK-DPP4).

The synthesis of the biotinylated probe was achieved by an expedited solid-phase approach that we have detailed recently for chymotrypsin-like and elastase-like serine proteinases (29). The utility of the probe was verified by its high reactivity with trypsin.

Monitoring trypsin-like serine proteinases present in CE using activity-based probe analysis

Active serine proteinases in CE were labeled covalently with a biotin-tagged serine proteinase-targeted activity-based probe (ABP) (28), as done previously for murine colon extracts (30). In brief, CE (1.5 U/ml trypsin-like activity) was pretreated or not with 0.1 or 1.0 μ M SBTI for 10 min at 24°C, followed by the addition of the ABP, Bio-PK-DPP4 (28). CE was reacted with 100 μ M ABP (dissolved in DMSO) and incubated for 60 min at 24°C in 50 mM Tris-HCl (pH 7.4) supplemented with 0.2% (v/v) Nonidet P-40 and 1.5 mM CaCl_2 in a reaction volume of 20 μ l. The reaction was terminated upon the addition of 20 μ l of 2 $\times$ electrophoresis sample buffer containing 2-ME, and the reaction products were heat-denatured for 3 min at 92°C. Proteins in the ABP-labeled CE samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride Hybond-P membranes (GE Healthcare, Piscataway, NJ). The membranes were blocked with 2% ECL Advanced blocking reagent (GE Healthcare) and 0.1% PBST (pH 7.4), washed in PBST, and reacted with VectaStain (Vector Laboratories, Burlington, Ontario, Canada) in PBST to detect ABP-biotinylated enzyme. The membranes were washed and treated with ECL Plus detection reagents (GE Healthcare), and ABP-biotinylated enzyme was detected by fluorescence emission using the Storm 860 imaging system (GE Healthcare). The blot signals visualized were quantified using ImageQuant software (GE Healthcare). A disappearance of the Western blot signal was taken as evidence for the neutralization of serine proteinase activity in CE by SBTI.

PAR₂-mediated calcium signaling triggered by CE

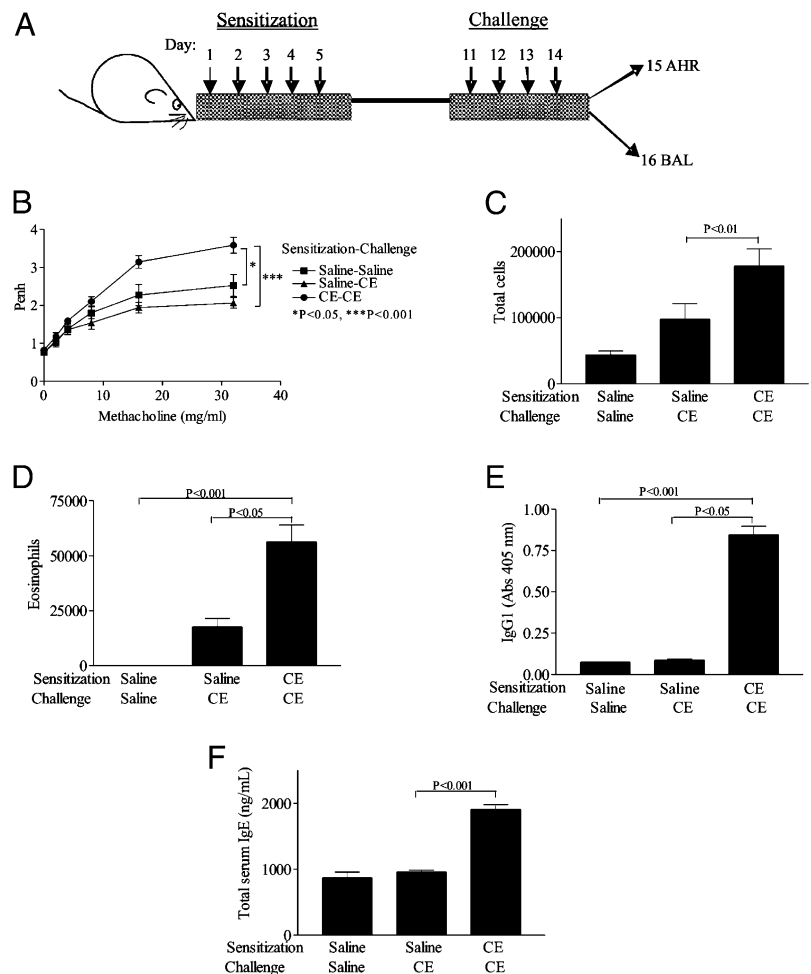
Calcium signaling via PAR₂ activation was monitored using a rat PAR₂-transfected Kirsten murine sarcoma virus-transformed normal rat kidney cell line (KNRK) cell line, as described previously (31, 32) using a fluo-3 dye calcium indicator. KNRK cells expressing rodent PAR₂ were grown to confluency in 75 cm² T flasks and split with trypsin-free dissociation medium. Disaggregated cells were pelleted by centrifugation and resuspended in 1 ml of DMEM/10% FCS for loading with the intracellular calcium indicator fluo-3-acetoxymethyl ester (Molecular Probes, Eugene,

OR) at a final concentration of 22 μ M (25 μ g/ml). Indicator uptake was established over 20–25 min at 24°C in the presence of 0.25 mM sulfinpyrazone, after which time cells were washed twice by centrifugation and resuspended with the buffer described below to remove excess dye. Fluo-3-loaded cells then were resuspended to yield a stock suspension ($\sim 6 \times 10^6$ cells/ml) in a buffer of the following composition: 150 mM NaCl, 3 mM KCl, 1.5 mM CaCl_2 , 20 mM HEPES, 10 mM glucose, and 0.25 mM sulfinpyrazone. Fluorescence measurements, reflecting elevations of intracellular calcium levels in response to CE (pretreated or not with SBTI), trypsin, or PAR₂-activating peptide, were conducted at 24°C using a fluorescence spectrometer (PerkinElmer Life Sciences, Boston, MA), with an excitation wavelength of 480 nm and an emission recorded at 530 nm. Cells (~ 2 ml of 3×10^5 cells per milliliter) were maintained in suspension in a stirred (magnetic bar) thermostatted cuvette (total volume, 4 ml), and CE, trypsin, or PAR₂-activating peptide stock solutions were added directly to the suspension to monitor agonist-induced changes in fluorescence. Because CE caused a slow PAR₂-independent elevation of intracellular calcium levels after a 3–5 min time period, the spike increase in fluorescence monitored during the first 2 min after agonist addition was taken as the magnitude of stimulation due to PAR₂ activation. CE (1.5 U/ml of trypsin-like activity) was treated or not with 1 or 10 μ M SBTI for 10 min prior to its addition to the cells for measurements of PAR₂-triggered calcium signaling. Signals generated by CE in PAR₂-expressing KNRK cells were compared with the effects of CE on KNRK cells transfected with vector alone. Calcium signals generated by PAR₂ activation were expressed as a percentage of the calcium signal generated in the same cells by 2 μ M of the calcium ionophore A23187. To verify that the calcium signals triggered by CE were due to PAR₂ activation, a cross-desensitization protocol was used (33). Cells first were desensitized with the PAR₂ agonist 2-furoyl-LIGRLO-NH₂ (2-fLL, two exposures of 10 μ M), and the disappearance of the calcium signal generated by CE was monitored.

Statistical analysis

Values are expressed as mean $\pm$ SEM. Statistical differences in the mean values among treatment groups were determined using a one-way ANOVA

FIGURE 1. Mucosal sensitization to cockroach allergens. *A*, Model of mucosal sensitization to CE. *B*, Mice were administered i.n. saline or CE (sensitization phase) followed by i.n. challenge with saline or CE (saline-saline, $n = 14$; saline-CE, $n = 12$; CE-CE, $n = 15$). Twenty-four hours after the final administration, Penh was measured by whole-body plethysmography using increasing doses of methacholine. *C* and *D*, Allergic airway inflammation was assessed 24 h after AHR by total cell numbers (*C*) and total eosinophils (*D*) present in BAL fluid. *E* and *F*, Serum from these mice was analyzed by ELISA for the presence of cockroach-specific IgG1 (*E*) and total IgE (*F*) Abs. Values shown in *B–F* are means $\pm$ SEM.



and Tukey's multiple comparison test. The paired Student *t* test was used to compare the mean between two groups. Differences in AHR were determined by *F* test analysis, which compares values over the entire curve between each treatment group. From this *F* score, a value for *p* was generated. In all of the cases, a *p* value < 0.05 was considered statistically significant.

Results

Intranasal administration of CE to BALB/c mice induces allergic sensitization

To study the role of proteinases in mucosal allergic sensitization, we developed a model for mucosal allergic sensitization using i.n. exposure to CE, which is known to have serine proteinase activity. Mice received i.n. 50 µg CE on five consecutive days (sensitization phase), rested for 5 d, and then received a daily i.n. challenge for four consecutive days with 50 µg CE (challenge phase) (Fig. 1A). Mice were assessed for AHR using whole-body plethysmography (Penh) 24 h after the last challenge and for allergic airway inflammation according to the analysis of the inflammatory cell content of the BAL fluid 48 h after the last challenge. Mice sensitized and challenged with CE developed AHR in response to methacholine (Fig. 1B) and showed increased total cell numbers (Fig. 1C) and total eosinophil numbers (Fig. 1D) in the airways compared with those of mice that received saline during the sensitization phase and were challenged with CE or those of mice that received saline during both the sensitization and the challenge phases. CE-treated mice also exhibited increased levels of cockroach-specific IgG1 and total IgE Abs (Fig. 1E, 1F, respectively). We then tested the dose-dependency of CE administered to the mice during the sensitization phase. In three separate groups of BALB/c mice, we administered 2, 10, or 50 µg CE daily during the sensitization phase and then challenged all of the animals with 50 µg CE daily for four more days as in the previous protocol. All three groups of mice showed similar levels of airway inflammation (total cells and eosinophils) in the BAL fluid (data not shown). This result strengthens our assumption that the first 5 d of CE administration function as a sensitization phase and not as an inducer of allergic inflammation, because the dose of Ag used during this phase did not affect the degree of inflammation.

CE induces allergic sensitization in the absence of TLR4

CEs that we used have measurable LPS activity (data not shown), and it is known that low amounts of LPS can mediate allergic sensitization to inhaled Ags through TLR4 activation (34). Furthermore, recent work illustrates cooperativity between TLR4 and PAR₂ in terms of signaling via NF-κB (35). To assess a possible role for TLR4 in terms of the action of LPS present in the extract or via cooperativity with PAR₂, we used TLR4 knockout (KO) mice and BALB/cJ control mice with the same genetic background.

TLR4 KO mice showed increased numbers of total cells and increased accumulation of eosinophils in the BAL fluid after mucosal sensitization and challenge with CE (Fig. 2A, 2B, respectively) compared with those of mice that received only saline. Total cell and eosinophil accumulation in TLR4 KO mice was significantly lower than accumulation seen in wild-type (WT) mice. TLR4 KO mice also developed cockroach-specific IgG1 Abs after mucosal sensitization and challenge with CE (Fig. 2C). The difference in levels between KO and WT mice was also statistically significant. These results indicate that CE has adjuvant activity distinct from that of LPS, which participates in the development of allergic sensitization and allergic airway inflammation, although the presence of TLR4 is required for the full effect of mucosal sensitization to CE.

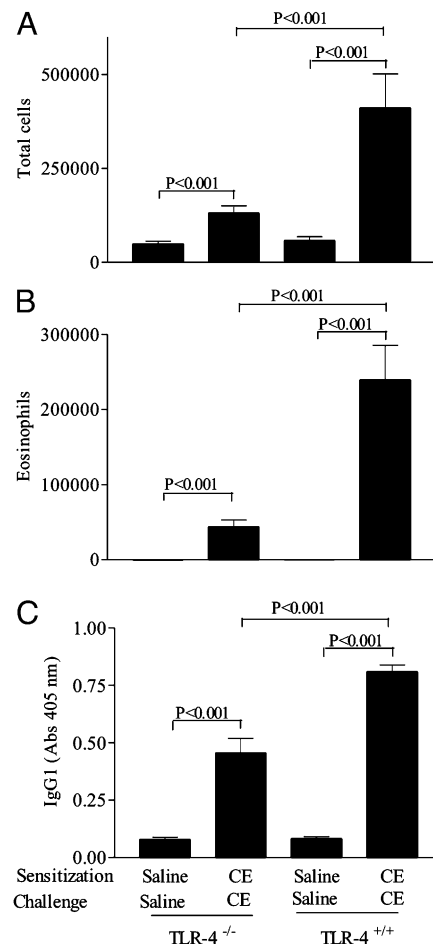


FIGURE 2. Sensitization of TLR4 KO mice. TLR4 KO and WT mice received i.n. saline or 50 µg CE according to the protocol shown in Fig. 1A (for both TLR4 KO and WT mice, saline-saline, *n* = 10; CE-CE, *n* = 9). A and B, Allergic airway inflammation was assessed 48 h after the last challenge by BAL fluid analysis for total cells (A) and total eosinophils (B). C, Allergic sensitization was evaluated by measuring serum cockroach-specific IgG1 Abs. Values are means ± SEM.

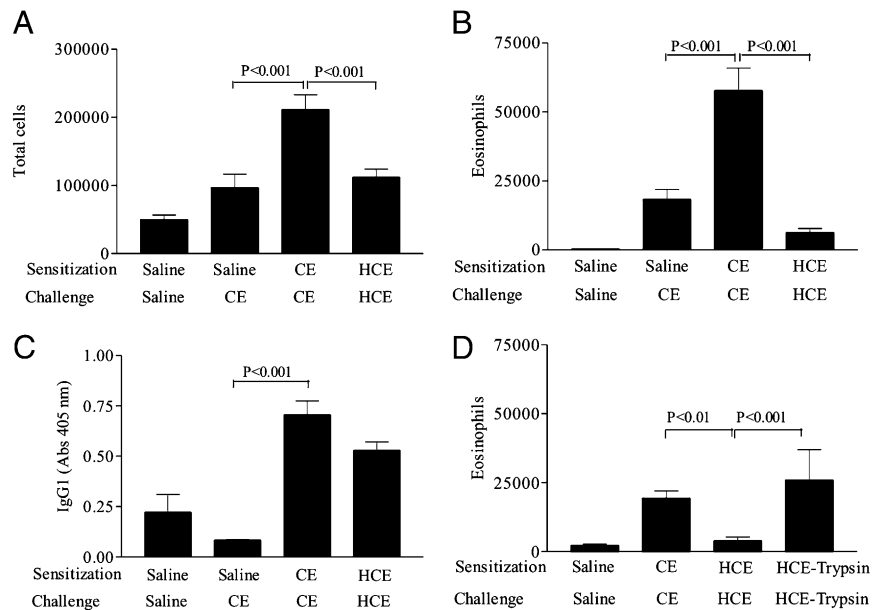
Allergic sensitization to CE is proteinase-dependent

It is known that the proteinase activity of Ags participates in the development of mucosal allergic sensitization (4). Thus, we examined whether mucosal allergic sensitization to CE is dependent on proteinase activity. To this end, we neutralized CE proteinase activity using two different approaches: 1) heat inactivation and 2) treatment with SBTI.

We first heat-inactivated CE for 30 min at 65°C. This procedure decreased trypsin-like proteinase sp. act. by >90% (from 29 to 1.8 BAEE units per milligram trypsin-like activity). Mice that were sensitized and challenged with heat-inactivated CE (HCE; 50 µg) showed significantly lower numbers of total infiltrating cells in the BAL fluid (Fig. 3A) with dramatically lower eosinophilic infiltration (Fig. 3B) compared with those of mice sensitized and challenged with CE. Cockroach-specific IgG1 in animals sensitized and challenged with HCE showed a trend toward lower levels compared with those of mice sensitized and challenged with CE (Fig. 3C).

To verify that these differences were the result of inhibiting the trypsin-like proteinase activity of CE, we repeated the experiment while supplementing the trypsin-like activity in the HCE with trypsin. We added 0.2 µg trypsin to 50 µg HCE to reconstitute the serine protease activity to the same level as that contained in 50

FIGURE 3. Allergic sensitization using HCE. Mice were sensitized and challenged with saline, CE, or HCE according to the protocol shown in Fig. 1A (saline-saline, $n = 12$; saline-CE, $n = 14$; CE-CE, $n = 14$; HCE-HCE, $n = 14$). A and B, Allergic airway inflammation was assessed 48 h after the last challenge by BAL fluid analysis for total cells (A) and eosinophils (B). C, Allergic sensitization was evaluated by measuring serum cockroach-specific IgG1 Abs. D, In some mice, trypsin-like activity in HCE was reconstituted with trypsin, and the level of eosinophils in the BAL fluid was evaluated 48 h after the last challenge. Values are means $\pm$ SEM.



μ g CE that was not heat-inactivated. This supplemented HCE induced the same level of eosinophilia in the airways as the native noninactivated CE (Fig. 3D).

For our second approach, the trypsin-like activity in CE was inhibited efficiently by SBTI, because low concentrations of SBTI (0.1–1 μ M) reduced enzyme activity by >95%, indicating a K_i well below 0.1 μ M for inhibition of the cleavage of the substrate, QAR-AMC (Fig. 4A). In parallel, using the activity-based serine proteinase probe reagent Bio-PK-DPP4, we were able to identify three biotin-labeled enzymes with serine proteinase activity in the molecular mass range between 32 and 36 kDa in CE (Fig. 4B); the labeling of all three of these enzymes was inhibited by SBTI (0.1–1 μ M; Fig. 4B). To neutralize CE to be administered in our in vivo studies, we used 200 μ M SBTI, two orders of magnitude greater

than the K_i for enzyme inhibition. CE was treated or not with 200 μ M SBTI for 30 min at 37°C prior to i.n. administration to mice.

In keeping with the results obtained with HCE, mice sensitized and challenged with SBTI-neutralized CE showed a marked decrease (>50%) in the total infiltrating cell counts (Fig. 5A) and an even greater (>70%) decrease in eosinophils (Fig. 5B) compared with those of mice sensitized and challenged with CE. There was, however, no difference in the IgG1 levels between animals treated with either CE or SBTI-neutralized CE (Fig. 5C). SBTI admin-

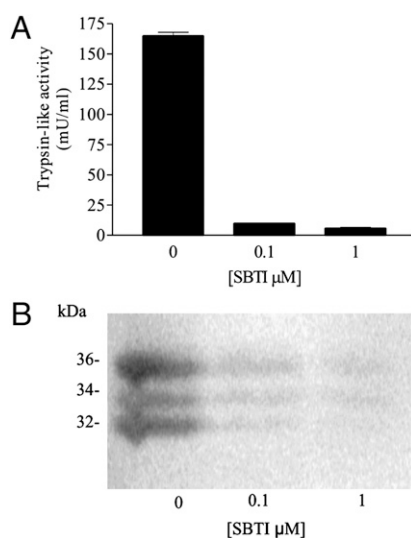


FIGURE 4. Inhibition of trypsin-like activity of CE by SBTI. CE was treated with SBTI (0.1 or 1 μ M) for 10 min at 24°C followed by the addition of an ABP. A, Trypsin-like activity of CE was inhibited by pre-treatment with SBTI. B, Western blot analysis showed that the ABP Bio-PK-DPP4b was able to label three enzymes in CE in the molecular mass range of 32–36 kDa; SBTI treatment showed dose-dependent inhibition of binding of these three serine proteinases to the probe.

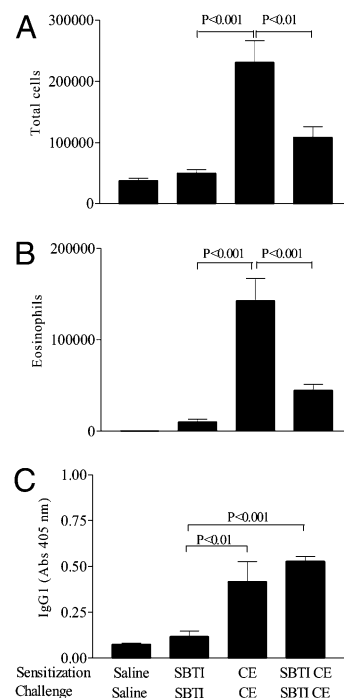


FIGURE 5. Allergic sensitization using SBTI-treated CE. A–C, Groups of mice were sensitized and challenged with SBTI-neutralized or non-neutralized CE (saline-saline, $n = 9$; CE-CE, $n = 8$; SBTI CE-SBTI CE, $n = 9$; SBTI-SBTI, $n = 4$) and then analyzed for total cells in the BAL fluid (A), total eosinophils in the BAL fluid (B), and serum cockroach-specific IgG1 (C). Values are means $\pm$ SEM.

istered i.n. without Ag had no effect on the development of inflammation or the presence of cockroach-specific IgG1 (Fig. 5A–C).

These results indicated that the presence of heat-sensitive and SBTI-sensitive serine proteinase activity in CE plays an important role in the development of allergic airway inflammation, particularly in terms of eosinophil infiltration.

CE activates calcium signaling via PAR₂, an effect blocked by SBTI

Given the trypsin-like activity in CE and the ability of other CEs to activate PAR₂ (12), we hypothesized that the CE that we were using would activate PAR₂. We therefore tested the ability of enzymes contained in CE to signal (calcium transients) via PAR₂ and to be neutralized by SBTI. In the absence of SBTI, CE caused a robust increase in calcium signaling in the PAR₂ KNRK cells (Fig. 6A and left tracing in Fig. 6B). Increasing amounts of SBTI blocked the calcium signal (Fig. 6A, 6B). This calcium signal was absent in receptor-expressing cells that had been PAR₂-desensitized initially by exposure to an excess of the PAR₂ agonist 2-fLI (Fig. 6C, compare middle and right tracings with left tracing).

Decreased effect of CE in PAR₂^{-/-} mice

To study whether PAR₂ activation plays a role in allergic sensitization after i.n. exposure to CE, we repeated the previous experiments using PAR₂^{-/-} mice and compared mucosal sensitization of PAR₂^{-/-} mice to sensitization of WT mice. The genetic background of PAR₂^{-/-} mice was C57BL/6, so the WT mice used for these experiments were also C57BL/6. PAR₂^{-/-} mice showed lower Penh after sensitization and challenge with CE compared with that of WT animals (Fig. 7A). PAR₂^{-/-} mice sensitized and challenged with CE showed increased total cell numbers (Fig. 7B) and total eosinophils (Fig. 7C) in the BAL fluid compared with those of mice sensitized and challenged with saline. However, the increase in total cells and in eosinophilic inflammation seen in PAR₂^{-/-} mice was lower than those seen in

similarly challenged WT mice (Fig. 7B, 7C). CE sensitized and challenged PAR₂^{-/-} mice also generated cockroach-specific IgG1 (Fig. 7D), but the levels of the Abs were lower than those in WT mice. In conclusion, PAR₂ is required for efficient mucosal sensitization to CE, although a lower degree of sensitization can develop in the absence of PAR₂.

Allergic sensitization to CE is PAR₂-dependent

We have shown that CE activates PAR₂, as also has been shown previously (12, 13). We also have shown that PAR₂ activation at the time of an encounter with an Ag leads to mucosal sensitization instead of immune tolerance (22). We sought to determine whether PAR₂ activation is the mechanism used by CE to induce allergic sensitization and allergic airway inflammation. To block the ability of serine proteinases in CE to activate PAR₂, we used a polyclonal receptor-blocking anti-PAR₂ Ab (B5) and a mAb (SAM11). They both bind PAR₂ at the serine proteinase cleavage site and have been shown to inhibit the ability of serine proteinases to activate PAR₂ (25).

Thus, we administered i.n. the B5 anti-PAR₂ Ab (25 µl at a 1:500 dilution of antiserum) or normal rabbit serum (25 µl at a 1:500 dilution) 30 min before administering CE to mice daily for the 5 d of sensitization. Mouse groups were challenged as described for previous experiments. Mice sensitized i.n. in the presence of rabbit serum developed AHR in response to methacholine challenge comparable to that of mice given only CE (data not shown). Mice sensitized to CE after pretreatment with B5 showed lower AHR than that of mice sensitized to CE in the presence of rabbit serum (Fig. 8A). The anti-PAR₂ Ab reduced the total cell accumulation in the airways (Fig. 8B) and completely inhibited the accumulation of eosinophils (Fig. 8C). Our results also showed a significant decrease of IgG1 levels in the sera of animals receiving the anti-PAR₂ Ab compared with those in the mice receiving normal rabbit serum (Fig. 8D, $p < 0.001$),

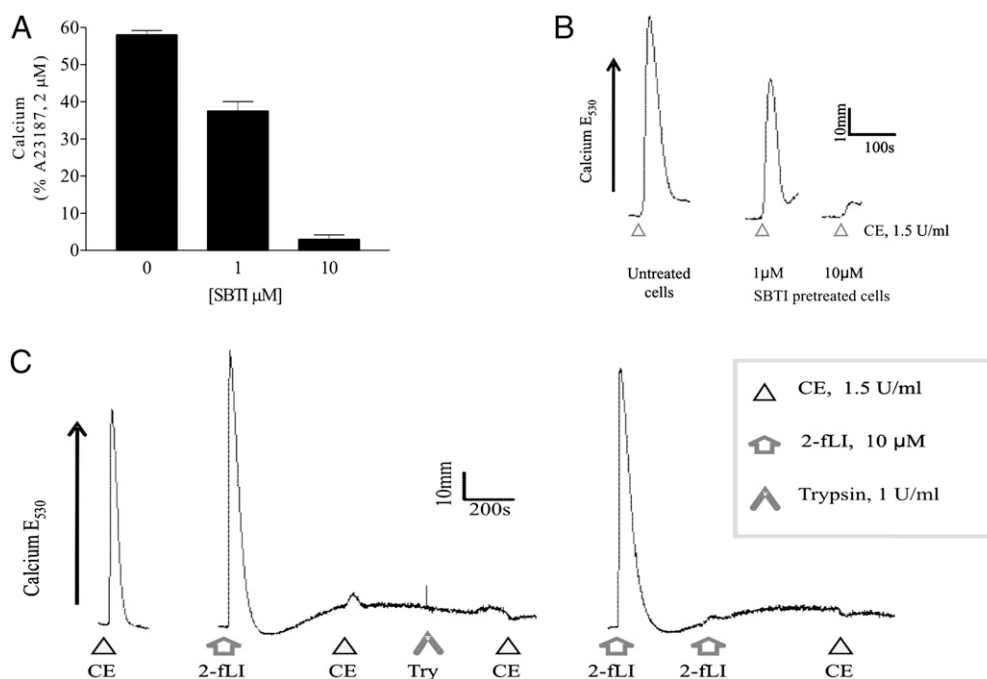
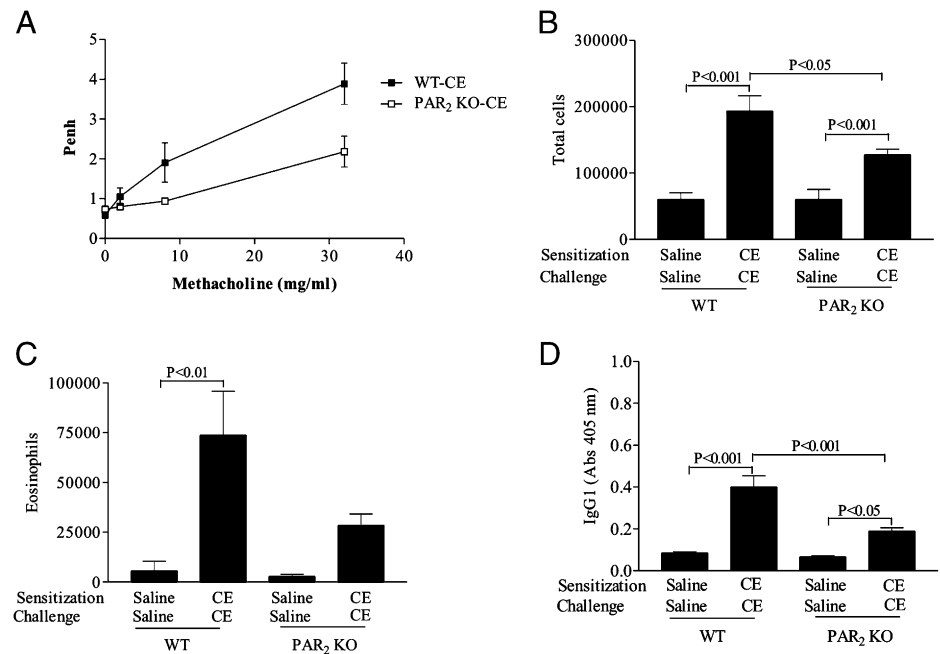


FIGURE 6. CE activates calcium signaling via PAR₂ in KNRK PAR₂ cells. KNRK cells expressing rodent PAR₂ were loaded with the intracellular calcium indicator fluo-3 (22 µM), and fluorescence measurements reflecting elevations of intracellular calcium levels in response to CE pretreated or not with SBTI, trypsin, or PAR₂-activating peptide were conducted using a fluorescence spectrometer. A and B, Increasing amounts of SBTI blocked the initial calcium signal (A and middle and right tracings in B). C, CE-induced calcium signaling in previously untreated PAR₂ KNRK cells (C, left tracing) was absent in cells that had been PAR₂-desensitized initially by exposure to an excess of the PAR₂ agonist 2-fLI (C, middle and right tracings).

FIGURE 7. Mucosal allergic sensitization in $\text{PAR}_2^{-/-}$ mice. $\text{PAR}_2^{-/-}$ and WT mice received i.n. saline or 50 μg CE according to the protocol shown in Fig. 1A (for both $\text{PAR}_2^{-/-}$ and WT mice, saline-saline, $n = 5$; CE-CE, $n = 5$). A, Penh was measured by whole-body plethysmography using increasing doses of methacholine 24 h after the last challenge. B and C, Allergic airway inflammation was assessed 48 h after the last challenge by BAL fluid analysis for total cells (B) and total eosinophils (C). D, Allergic sensitization was evaluated by measuring serum cockroach-specific IgG1 Abs. Values are means $\pm$ SEM.



suggesting that allergic sensitization to CE is dependent or modulated by PAR_2 activation. A similar polyclonal Ab from the serum of a different rabbit (A5) and a mAb (SAM-11) showed similar results (data not shown). Altogether, these data indicate that cockroach allergens use the PAR_2 receptor system to mediate allergic sensitization.

Discussion

We have shown that i.n. administration of whole CE leads to the development of allergic sensitization in mice. CE is able to induce mucosal allergic sensitization even in the absence of functional TLR4, indicating that LPS, known to function as an adjuvant for mucosal allergic sensitization (36), is not required for sensitization to cockroach allergens. Furthermore, we have shown that allergic sensitization to CE and the resultant allergic airway inflammation depend on the ability of the extract to activate PAR_2 .

Cockroach frass already has been used for mucosal sensitization (37, 38), although in those studies frass was administered intra-

tracheally whereas we administered i.n. cockroach allergens. The results from such studies are similar to ours except that, unlike our study, the authors of those studies were unable to sensitize C57BL/6 mice using cockroach frass. Although the two models have many other differences, it is possible that different proteinases or different amounts of allergens contained in the two preparations may explain this difference. A comparative study between cockroach frass and whole-body CE may allow us to identify proteinases that are important for allergic sensitization.

The role of proteinase activity in the development of allergic sensitization has been shown for cockroach frass (37), house dust mite (39), pollen, and fungal proteinases (4, 40), but the exact mechanisms of this effect were not studied. Our data indicate that PAR_2 activation by cockroach allergens may be the mechanism. A recent paper also has proposed that PAR_2 activation is important for sensitization to cockroach allergens after intratracheal administration (38). We propose that natural aeroallergens with serine proteinase activity induce PAR_2 activation, which functions

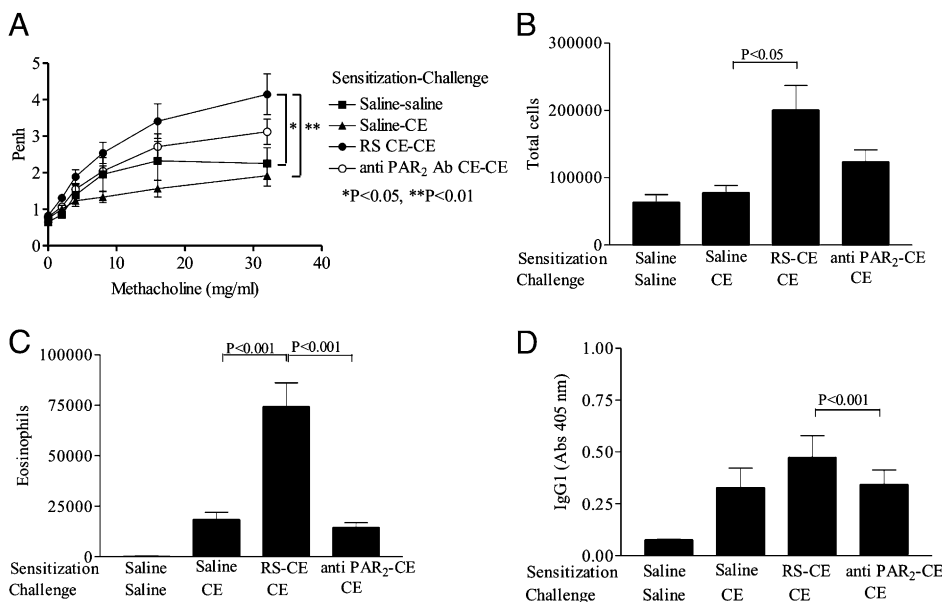


FIGURE 8. Allergic sensitization to CE is PAR_2 -dependent. During the sensitization phase, an anti- PAR_2 polyclonal Ab (B5, 1:500 dilution of anti-serum, $n = 17$) or normal rabbit serum (1:500 dilution, $n = 19$) were administered i.n. to mice prior to administration of CE. Mice then were challenged with CE as in previous experiments. A–D, Mice were analyzed for AHR (A) 24 h after the last challenge and for total cells in BAL fluid (B), total eosinophils in BAL fluid (C), and serum cockroach-specific IgG1 Abs 48 h after the last challenge. Values are means $\pm$ SEM.

as an adjuvant and alters the immune response from immune tolerance to allergic sensitization. The adjuvant activity that leads to allergic sensitization is not known, although we have shown previously that PAR₂ activation in the airways may act as a mucosal adjuvant through the release of TNF (22). We (41) and others (42) also have shown that PAR₂ activation of the epithelium leads to the release of GM-CSF, which has the potential to activate dendritic cells. PAR₂ activation also induces CCL20 release (43), which through activation of CCR6 can attract immature dendritic cells to mucosal surfaces (44). Each one of these pathways may be important for the development of allergic sensitization to cockroach allergens through PAR₂ activation.

One issue that has impeded the elucidation of the role for PAR₂ in vivo is the lack of potent and specific small molecule inhibitors of PAR₂ activation. Unfortunately, we found that the PAR₂ inhibitor ENMD-1068 (45) exhibits trypsin-inhibitory activity (data not shown), and this compound was therefore not of use for our study of trypsin-like PAR₂-activating activity in CE. Thus, to assess a role for PAR₂ activation, we used three different PAR₂-targeted Abs, two separately raised polyclonal Abs (A5 and B5) and a mAb (SAM11), to inhibit PAR₂ activation as has been done both in vitro (46) and also in vivo (45) in a model of arthritis. In our experiments, we administered i.n. the PAR₂ Abs during the sensitization phase. The Ab administered this way was restricted to the nasal cavity, and inhibition of PAR₂ activation should take place in that area. A small percentage of the Ab might have reached the lower airways and inhibited PAR₂ activation in that area. However, because we did not use systemic administration of the Ab, we suggest that the effects were due to local action within the nasal cavity or the lower airways. We postulate that PAR₂ expressed on nasal and airway epithelial cells could have been the main targets of the Ab and that it is likely that factors released from these epithelial cells are the source of the "adjuvant activity" induced by PAR₂ activation. However, direct activation of nasal or airway mucosal dendritic cells (47), activation of alveolar macrophages (48), or even activation of mast cells (49) also may be part of the mechanism.

Both heat-inactivating CE and inhibiting serine proteinases with SBTI significantly decreased allergic sensitization but did not completely abolish it. Furthermore, supplementing the HCE with trypsin fully restored the allergenic activity of the extract. However, because neither of the two approaches were able to completely inhibit the serine proteinase activity in the extract, it is possible that the remaining activity was enough to induce the small effect seen. Hence, incomplete inhibition of the extract may explain our results, because significantly lower amounts of CE (2 µg) than those used in this study (50 µg) are sufficient to induce sensitization.

It is possible that other mechanisms in addition to PAR₂ activation are responsible for the allergenic effect. For example, low LPS activity of the extract may be enough to induce allergic sensitization, although not to the levels seen when sensitization occurs in the presence of PAR₂ activation. It is interesting that CE can induce eosinophilic airway inflammation and cockroach-specific IgG1 Abs in TLR4 KO mice, although at significantly diminished levels as compared with those of normal mice. It is known that i.n. administration of innocuous Ags normally leads to immune tolerance (50) and that LPS can act as an adjuvant and mediate TLR4-dependent allergic sensitization under the same conditions (34). Our previous work suggests that PAR₂ activation in the airways may have adjuvant effects (22) similar to those seen with LPS. A recent article has shown that PAR₂ activation leads to TLR4 transactivation and TLR4- and MyD88-dependent NF-κB activation (35). Also, PAR₂ expression remained elevated with simultaneous exposure to LPS and PAR₂ agonists in airway epi-

thelial cells, demonstrating that there is coordinated action of two important mediators of inflammation, PAR₂ and TLR4, which contributes to the enhancement of inflammatory signaling in airways (51). Our data indicate that PAR₂ activation can act as an adjuvant for immunization without the need for TLR4 signaling.

If PAR₂ is important for allergic sensitization to natural aero-allergens, it would be expected that changes in its expression or polymorphisms of the receptor could contribute to the development of allergy and/or asthma. There is evidence that 5q13, the genomic area where *F2RL1*, the gene that encodes PAR₂, is located, may be linked to the development of allergy (21). Further work in this area will shed light on the role of PAR₂ in human allergic diseases.

Although the presence of PAR₂-activating proteinases in CE has been described (13), we have shown for the first time, to our knowledge, that several enzymes that may be able to activate PAR₂ are present in CE. Whether the ABP-labeled constituents represent different molecular forms of the same enzyme (e.g., glycosylated forms or catalytically active proteolytic fragments) or different enzymes remains to be determined. Any of the three enzymes may be able to induce the allergenic response, because all three are SBTI-neutralized, as is the allergenic response, and may be capable of activating PAR₂ (data not shown). The identity of the three enzymes labeled by the ABP probe remains a topic for future work. Many different cockroach allergens have been isolated, and at least one of them, Per a 10, from the American cockroach is known to have trypsin-like activity (15). It is possible that one of the three proteins that we have identified is Per a 10, but further investigation is needed to test this hypothesis.

In conclusion, we provide evidence indicating that the adjuvant activity of CE, which allows it to induce mucosal allergic sensitization is, at least in part, a direct effect of PAR₂ activation. Other allergens may share common features regarding PAR₂ activation, which can account for their allergenic potential.

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Disclosures

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