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Proteinase-Activated Receptor-2 Promotes Allergic Sensitization to an Inhaled Antigen through a TNF-Mediated Pathway[1]

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Proteinase-Activated Receptor-2 Promotes Allergic Sensitization to an Inhaled Antigen through a TNF-Mediated Pathway[1]

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The reason why particular inhaled Ags induce allergic sensitization while others lead to immune tolerance is unclear. Along with a genetic predisposition to atopy, intrinsic characteristics of these Ags must be important. A common characteristic of many allergens is that they either possess proteinase activity or are inhaled in particles rich in proteinases. Many allergens, such as house dust mite and cockroach allergens, have the potential to activate the proteinase-activated receptor (PAR)-2. In this study, we report that PAR-2 activation in the airways at the same time as exposure to inhaled Ags induces allergic sensitization, whereas exposure to Ag alone induces tolerance. BALB/c mice were administered OVA with a PAR-2 activating peptide intranasally. Upon allergen re-exposure mice developed airway inflammation and airway hyperresponsiveness, as well as OVA-specific T cells with a Th2 cytokine profile when restimulated with OVA in vitro. Conversely, mice given OVA alone or OVA with a PAR-2 control peptide developed tolerance. These tolerant mice did not develop airway inflammation or airway hyperresponsiveness, and developed OVA-specific T cells that secreted high levels of IL-10 when restimulated with OVA in vitro. Furthermore, pulmonary dendritic cell trafficking was altered in mice following intranasal PAR-2 activation. Finally, we showed that PAR-2-mediated allergic sensitization was TNF-dependent. Thus, PAR-2 activation in the airways could be a critical factor in the development of allergic sensitization following mucosal exposure to allergens with serine proteinase activity. Interfering with this pathway may prove to be useful for the prevention or treatment of allergic diseases. ***The Journal of Immunology,*** **2007, 179: 2910–2917.**

Atopy may be defined as a genetically and environmentally determined predisposition to the development of disorders such as allergic rhinitis, atopic dermatitis or eczema, and allergic asthma (1, 2). Atopy has increased dramatically in western societies over the last couple of decades. Although theories, such as the hygiene hypothesis, try to explain this increased prevalence, the main question still remains unanswered: why and how do people develop atopy?

The main route of sensitization to aeroallergens in humans is the respiratory tract. However, only a limited number of inhaled Ags actually become "allergens". This indicates that, in addition to the genetic predisposition to atopy, intrinsic characteristics of the allergens must also be important for the development of allergic sensitization. In animal models of mucosal exposure to Ags, it has been shown that the proteinase activity of particular allergens is important for their allergenic potential (3, 4). There are various theories on how the proteinase activity of allergens is involved in allergic sensitization. One proposes that allergens with proteinase activity may degrade the extracellular matrix of the airway mucosa, allowing them to be more accessible to dendritic cells (DCs)[3] (5). Another theory has these allergens cleaving cell surface molecules that are important in the regulation of the allergic response (6). Furthermore, a proteinase-mediated pathway for allergic sensitization, and subsequent development of allergic airway disease, has also been proposed (3). However, the exact mechanism by which these proteinases are involved in allergic sensitization is still unknown.

Many of the potent allergens associated with atopic diseases, and more specifically asthma, are serine proteinases. Allergens with proteinase activity include house dust mite (HDM) allergen (7), cockroach allergen (8), and fungal allergen whose proteinase activity is vital for the development of allergic sensitization (9). These are the same allergens that induce allergic sensitization in mice following mucosal exposure. Even potent allergens that have no proteolytic activity, such as pollen allergens, are packaged in particles that contain large quantities of proteinases (10). Finally, a number of occupational allergens also possess proteinase activity (11).

Among proteinases, serine proteinases have been found to have a specific receptor system (12) through which they can have effects on a variety of cell types. These receptors are called proteinase-activated receptors (PAR) and encompass a family of four receptors (PAR-1 to PAR-4). Proteinases cleave within the N terminus of the receptors and unmask a tethered ligand domain that folds back on the receptor for activation. One of the members of this family, PAR-2, has been implicated in the development of

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[3] Abbreviations used in this paper: DC, dendritic cell; PAR, proteinase-activated receptor; PAR-2AP, PAR-2 activating peptide; PAR-2CP, PAR-2 control peptide; AHR, airway hyperresponsiveness; HDM, house dust mite; BAL, bronchoalveolar lavage; i.n., intranasally.

inflammatory reactions (13–15), is expressed in a number of cell types in the airways (16–21), and can be activated by allergens possessing serine proteinase activity (22, 23). Therefore, PAR-2 is a prime candidate to sense environmental exposures to serine proteinases.

We have previously shown PAR-2 to be expressed in murine airways and that PAR-2 activation enhances allergen-mediated airway inflammation and airway hyperresponsiveness (AHR) (24). The goal of the current study was to determine whether the PAR-2 activating potential of proteolytic allergens promotes allergic sensitization. We hypothesize that PAR-2 activation by an allergen or a serine proteinase inhaled at the time of the encounter with the allergen is the general mechanism leading to allergic sensitization in genetically susceptible individuals. Because most of the aeroallergens are serine proteinases, or are presented in the airways together with serine proteinases, this hypothesis proposes a general mechanism of allergic sensitization. Even the susceptibility to allergic sensitization could be associated with polymorphisms in this pathway. Indeed one PAR-2 polymorphism that has been described decreases the ability of the receptor to become activated following interactions with serine proteinases (25).

Mucosal exposure of mice to HDM allergens, which are able to activate PAR-2, leads to allergic sensitization (26), whereas mucosal exposure to OVA has been shown to lead to immune tolerance (27). To test our hypothesis, we used a murine system with mucosal exposure to OVA as an Ag and to a PAR-2 activating peptide (PAR-2AP) to mimic the potential of the allergen or other inhaled proteinases to activate PAR-2. Using this system, we reproduce the PAR-2 activating potential of an allergen with serine proteinase activity, but avoid the PAR-2-independent effects of proteolytic enzymes. A scrambled peptide with the same amino acid composition as the PAR-2AP was used as a PAR-2 control peptide (PAR-2CP). We further hypothesized that the mechanism by which PAR-2 activation leads to sensitization is through the release of inflammatory mediators into the airways, which are able to influence the resulting immune response. In the present study we show that PAR-2 activation in the airways at the time of mucosal administration of OVA leads to allergic sensitization, whereas animals that encounter OVA alone develop tolerance. Furthermore, this allergic sensitization depends on TNF released in the airways as a result of PAR-2 activation.

Materials and Methods

Animals

Male BALB/c mice (18–20 g) were purchased from Charles River Laboratories. All mice were housed in virus- and Ab-free conditions and maintained on a 12-h light-dark schedule. The University of Alberta Health Sciences Laboratory Animal Ethics Board (Edmonton, Alberta, Canada) approved all experiments described.

Intranasal administration of OVA and PAR-2 peptides

Following light anesthesia with ketamine (75 mg/kg) and acepromazine (0.6×10^{-2} mg/kg), groups of mice were intranasally (i.n.) administered 50 or 100 μg of OVA (grade V; Sigma-Aldrich) dissolved in 25 μl of 0.9% sterile saline solution five times once every fifth day or on three consecutive days, respectively. For PAR-2 activation, we i.n. administered a PAR-2AP (SLIGRL-NH_2, 25 μl of 100-μM solution in saline) at the time of OVA administration. Other mice received PAR-2CP (LSIGRL-NH_2, 25 μl of 100-μM solution in saline) or saline with OVA. All experiments were performed using four to five mice per group. Experiments were reproduced at least three times. The total number of mice in every experiment is indicated.

OVA i.p. immunization

Where indicated, animals were immunized with an i.p. injection of 0.9% sterile saline solution (0.5 ml) containing 10 μg of OVA and 2 mg of $Al(OH)_3$.

Determining AHR

Twenty-four hours after the final administrations of OVA with or without PAR-2 peptides, or 24 h following aerosolized OVA challenge (5% OVA in 5 ml of saline for 5 min), we measured enhanced pause (Penh) using whole-body plethysmography (Buxco Electronics) to determine AHR to methacholine (2–32 mg/ml) as described (28).

In vitro T cell proliferation

Two spleens from each experimental group were excised from animals after sacrifice, homogenized in HBSS, pooled, and passed through a 70-μm nylon mesh (BD Pharmingen) to obtain a single-cell suspension. RBC were lysed with 20 mM Tris and 140 mM NH_4Cl, and the single-cell suspension was incubated on nylon wool columns at 37°C for 1 h to isolate T cells. Purity of T cells was ≥90% as determined by flow cytometry for CD3 (anti-CD3 Ab from BD Pharmingen). T cells (2.5×10^5) were subsequently cultured in a 96-well flat-bottom plate in vitro with 2.5×10^5 irradiated spleen cells as a source of APCs in the presence of OVA (100 and 1000 μg/ml) in 0.2 ml of DMEM containing 10% FBS, 4 mmol/L L-glutamine, and 100 μg/ml penicillin/streptomycin for 4 days. T cell proliferation was assessed using the CellTiter 96 MTS colorimetric cell proliferation assay (Promega).

Cytokine assays

IL-4, IL-5, IL-10, and IL-13 were measured in the supernatants of T cell and irradiated APC cocultures by ELISA as described (29).

OVA-specific IgE, IgG1, and IgG2a

OVA-specific IgE, IgG1, and IgG2a were measured in the serum of OVA challenged mice by ELISA as previously described (30).

Airway and lung inflammation

Airway inflammation was assessed by counting the number of inflammatory cells in the bronchoalveolar lavage (BAL) fluid as described (28). In other experiments, lung inflammation was assessed by determining the number of eosinophils following lung digestion in HBSS containing 1.7 mg/ml collagenase type III (Worthington Biochemical) at 37°C for 1 h.

Flow cytometry following Alexa Fluor 488 OVA administration

Following light anesthesia with ketamine (75 mg/kg) and acepromazine (0.6×10^{-2} mg/kg), groups of mice were i.n. administered 100 μg of Alexa Fluor 488 OVA (Molecular Probes) suspended in 25 μl of saline alone or OVA with PAR-2AP (100 μM). Control mice received PAR-2CP (100 μM) with Alexa Fluor 488 OVA, whereas unlabeled OVA was administered to other mice as a control. After 24 h, spleen, cervical lymph nodes and bronchial lymph nodes were digested in 1.7 mg/ml collagenase type IV (Worthington Biochemical) and passed through a 70-μm nylon mesh. Cells from lymph nodes were pooled, and 1×10^6 cells stained with PE-conjugated anti-CD11c Ab. Analytical flow cytometry was conducted with a FACSCalibur (BD Biosciences) to determine the number of double positive cells. Data were processed with CellQuest software (BD Biosciences).

TNF neutralization experiments

Mice received 100 μg of goat anti-mouse TNF polyclonal Ab (R&D Systems) or 100 μg of normal goat IgG (R&D Systems) 24 h before the first and 4 h before the second administration of OVA with PAR-2CP or OVA with PAR-2AP. After 15 days, some mice were sacrificed, and T cell proliferation was determined using MTS reagent. Other mice were challenged 16 days following the initial i.n. administration with OVA four times on alternating days and lung inflammation and AHR were assessed.

Statistical analysis

Statistical differences in the mean values among treatment groups were determined by using a one-way ANOVA. The paired Student t test was used to compare the mean between two groups. Differences in AHR were determined by F test analysis that compared values over the entire curve between each treatment group. From this F score, a value for p was generated. In all cases, a value for $p < 0.05$ was considered statistically significant.

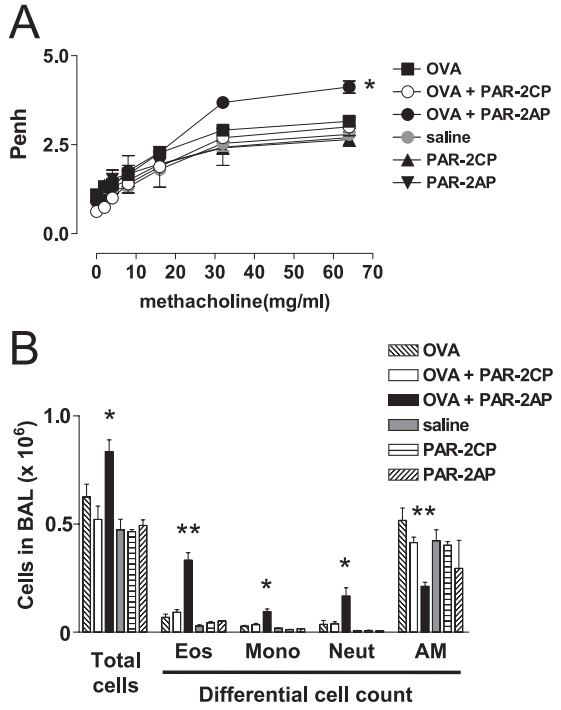

FIGURE 1. PAR-2 activation concurrently with mucosal exposure to OVA promotes AHR and airway inflammation. Mice were given 50 μg of OVA alone or OVA with PAR-2AP or PAR-2CP (25 μl of 100-μM solution) i.n. five times once every fifth day. Saline, PAR-2CP, or PAR-2AP were administered alone as controls. Twenty-four hours after the final administration, AHR and airway inflammation were assessed. *A*, Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge ($n = 9$ mice). $*$, $p < 0.05$ compared with saline, PAR-2CP administered alone, PAR-2AP administered alone, or OVA alone, or OVA with PAR-2CP. *B*, Airway inflammation was determined by differential inflammatory cell counts in BAL fluid ($n = 11$ mice). AM, alveolar macrophages; Eos, eosinophils; Mono, mononuclear cells; Neut, neutrophils. $*$, $p < 0.05$; $**$, $p < 0.01$ compared with saline, PAR-2CP administered alone, PAR-2AP administered alone, OVA alone, or OVA with PAR-2CP. Data are mean $\pm$ SEM.

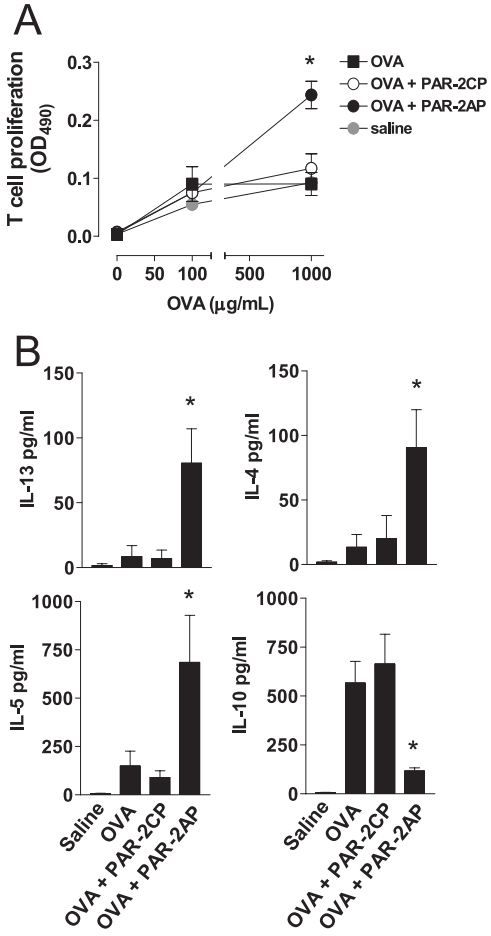

FIGURE 2. PAR-2 activation concurrently with mucosal exposure to OVA induces allergic sensitization. Mice were given 50 μg of OVA alone or OVA with PAR-2AP or PAR-2CP (25 μl of 100-μM solution) i.n. five times once every fifth day. Saline administered alone acted as a control for T cell proliferation. Five days later splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 μg/ml) and irradiated APCs in vitro for 4 days, after which proliferation and secreted cytokines were assessed. *A*, T cell proliferation was determined using MTS colorimetric cell proliferation assay read at 490 nm ($n = 3$). $*$, $p < 0.05$ compared with saline, OVA administered alone, or OVA with PAR-2CP. *B*, IL-4, IL-5, IL-10, and IL-13 were measured in the supernatants of in vitro T cell proliferation cultures stimulated with 1000 μg/ml OVA in the presence of APCs by ELISA ($n = 3$). $*$, $p < 0.05$ compared with OVA alone or with PAR-2CP. Data are mean $\pm$ SEM.

Results

PAR-2 activation promotes allergic sensitization

To model the effects of an allergen that has PAR-2 activating potential we administered OVA with a PAR-2AP. A scrambled version of PAR-2AP was used as control (PAR-2CP). Mice were administered OVA (100 μg) alone or in combination with either PAR-2AP or PAR-2CP (25 μl of 100-μM solution) i.n. once every fifth day for a total of five times (control mice received saline, PAR-2AP, or PAR-2CP without OVA). Twenty-four hours after the final administration, mice were assessed for AHR and airway inflammation by whole-body plethysmography and BAL, respectively. Mice receiving saline, PAR-2CP alone, PAR-2AP alone, OVA, or OVA with PAR-2CP did not develop AHR or airway inflammation. However, mice given OVA with PAR-2AP developed both AHR and airway inflammation (Fig. 1).

Other groups of mice were sacrificed 5 days after the last i.n. administration, and splenic T cells were cocultured in vitro with irradiated APCs from the spleens of naive mice in the presence of OVA. T cell proliferation and cytokine release from these T cells was assessed 4 days later. Splenic T cells from mice given OVA alone or OVA with PAR-2CP proliferated poorly (Fig. 2*A*), produced small amounts of IL-4, IL-5, and IL-13, and a large amount of IL-10 (Fig. 2*B*). In contrast, splenic T cells from mice given OVA with PAR-2AP proliferated to OVA (Fig. 2*A*) and produced large amounts of IL-4, IL-5, and IL-13 and low amounts of IL-10 (Fig. 2*B*), indicating the development of OVA-specific Th2 cells under these conditions.

To confirm that PAR-2AP administration promoted allergic sensitization, memory responses to OVA and the production of OVA-specific Igs were assessed in mice treated as described. Two weeks after the last administration of OVA with PAR-2AP, the number of eosinophils in the BAL returned to baseline (data not shown). At this time we administered aerosolized OVA (5% OVA in 5 ml of saline for 5 min) on three consecutive days to mice from all groups. AHR did not differ from that seen in mice initially administered OVA alone or OVA with PAR-2CP or PAR-2AP (Fig. 3*A*). However, mice given OVA with PAR-2AP initially showed enhanced eosinophilic airway inflammation after aerosolized OVA challenge (Fig. 3*B*). In contrast, mice given OVA with PAR-2CP initially did not exhibit signs of AHR or airway inflammation after

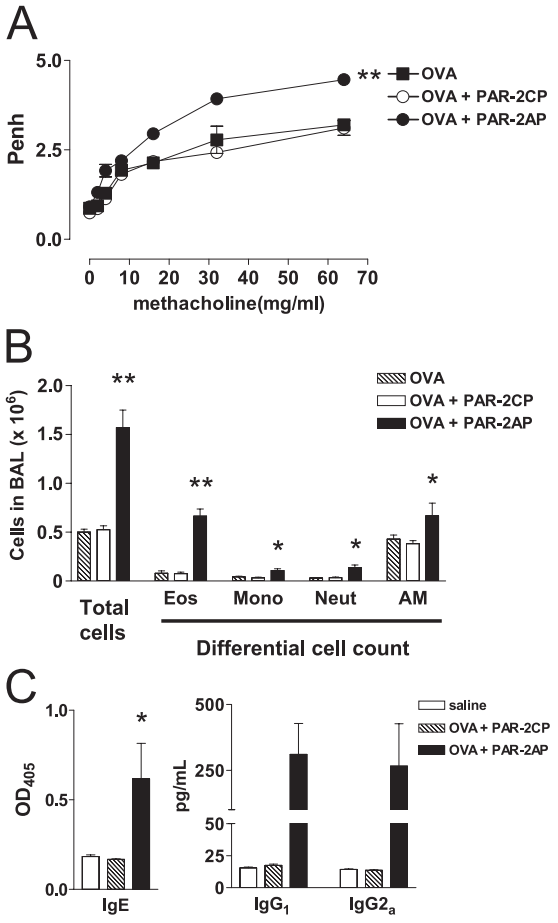

FIGURE 3. Memory response to OVA and measurement of OVA-specific Igs following PAR-2 activation concurrently with mucosal exposure to OVA. Mice were given 50 μg of OVA alone or OVA with PAR-2AP or PAR-2CP (25 μl of 100-μM solution) i.n. five times once every fifth day followed 2 wk later by aerosolized OVA challenge (5% OVA in 5 ml of saline for 5 min). Twenty-four hours after the final challenge, AHR and airway inflammation were assessed. *A*, Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge ($n = 5$ mice). *, $p < 0.05$ compared with OVA administered alone or OVA with PAR-2CP. *B*, Airway inflammation was determined by differential inflammatory cell counts in BAL fluid: AM, alveolar macrophages; Eos, eosinophils; Mono, mononuclear cells; Neut, neutrophil ($n = 5$ mice). *, $p < 0.05$; **, $p < 0.01$ compared with saline, OVA alone, or OVA with PAR-2CP. *C*, OVA-specific IgE, IgG1, and IgG2a in the serum of mice were determined by ELISA ($n = 8$ mice treated with OVA plus PAR-2CP or PAR-2AP, $n = 4$ mice treated with saline). *, $p < 0.05$ compared with saline or OVA with PAR-2CP. Data are mean ± SEM.

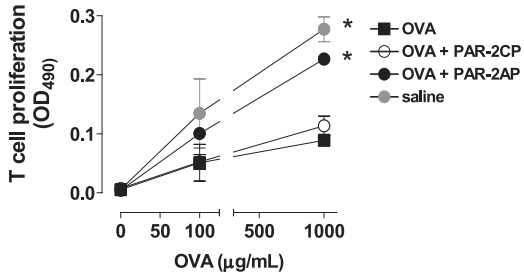

FIGURE 4. PAR-2AP administrations with OVA prevents i.n. OVA-induced mucosal airway tolerance. Mice were given 50 μg of OVA alone or OVA with PAR-2CP or PAR-2AP (25 μl of 100-μM solution) i.n. five times once every fifth day followed by an OVA and aluminum hydroxide immunization as a test for OVA-specific T cell tolerance. Five days after the immunization, splenic T cells were isolated from mice and cultured with increasing doses of OVA (100 and 1000 μg/ml) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. T cell proliferation was determined using MTS colorimetric cell proliferation assay read at 490 nm ($n = 3$). *, $p < 0.05$ compared with OVA alone or OVA with PAR-2CP. Data are mean ± SEM.

challenge with aerosolized OVA (Fig. 3, *A* and *B*). Furthermore, mice given OVA with PAR-2AP initially exhibited the production of OVA-specific IgE in their serum as well as slight production of OVA-specific IgG1 and IgG2a (Fig. 3*C*). The enhanced airway inflammation indicates an OVA-specific memory response in PAR-2AP-treated mice. This response, along with the production of OVA-specific IgE, strongly indicates the possibility that PAR-2 mediated allergic sensitization.

The high levels of IL-10 secreted by T cells from mice given OVA alone or with PAR-2CP (Fig. 2*B*) suggested the development of immune tolerance as shown previously (31). To confirm the presence of immune tolerance, we immunized mice with an i.p. injection of OVA and $Al(OH)_3$ 5 days after the last i.n. administration of OVA with or without PAR-2AP. Splenic T cells from the mice that received OVA alone or OVA with PAR-2CP before i.p. sensitization proliferated poorly when stimulated with OVA and APCs in vitro, whereas splenic T cells from mice given OVA with PAR-2AP proliferated vigorously (Fig. 4). These results confirmed the existence of immune tolerance in mice treated with OVA alone or OVA with the PAR-2CP, as these mice did not develop proliferating OVA-specific T cells even following i.p. immunization with OVA and $Al(OH)_3$.

To determine whether PAR-2-mediated allergic sensitization is independent of the specific model used, we replicated these experiments in an accepted model of tolerance (32). Mice were i.n. administered 100 μg of OVA alone, OVA with PAR-2AP, or OVA with PAR-2CP (25 μl of 100-μM solution) for three consecutive days. Control mice received saline i.n. Ten days later, all mice received an i.p. immunization with OVA and $Al(OH)_3$. Some mice were sacrificed 5 days later and splenic T cells were cultured in vitro with different concentrations of OVA and irradiated APCs isolated from naive mice. T cell proliferation was assessed 4 days later. T cells isolated from mice, given i.n. OVA alone or OVA with PAR-2CP initially, proliferated poorly following in vitro stimulation with OVA, whereas T cells from mice given saline or OVA with PAR-2AP proliferated vigorously (Fig. 5*A*). Other groups of mice were challenged with OVA twice on alternate days starting 10 days after i.p. immunization, and AHR and lung inflammation assessed 24 h after the second challenge. AHR was measured by plethysmography and lung inflammation was evaluated by the number of eosinophils in lung digests. Mice administered OVA alone or OVA with PAR-2CP initially did not develop AHR or lung inflammation following OVA challenge, whereas mice given saline or OVA with PAR-2AP did (Fig. 5, *B* and *C*). These experiments showed that mice receiving OVA with PAR-2AP did not develop tolerance, in contrast to mice receiving OVA alone or OVA with PAR-2CP.

To study whether OVA with PAR-2AP also induced allergic sensitization in this model, we repeated the protocol of i.n. sensitization on three consecutive days, without subsequent i.p. immunization, and assessed allergic sensitization by T cell proliferation to OVA. T cells isolated from mice that were i.n. administered OVA with PAR-2AP proliferated vigorously, indicating that these animals were sensitized to the OVA. T cells from mice given OVA alone or OVA with PAR-2CP proliferated poorly (Fig. 6).

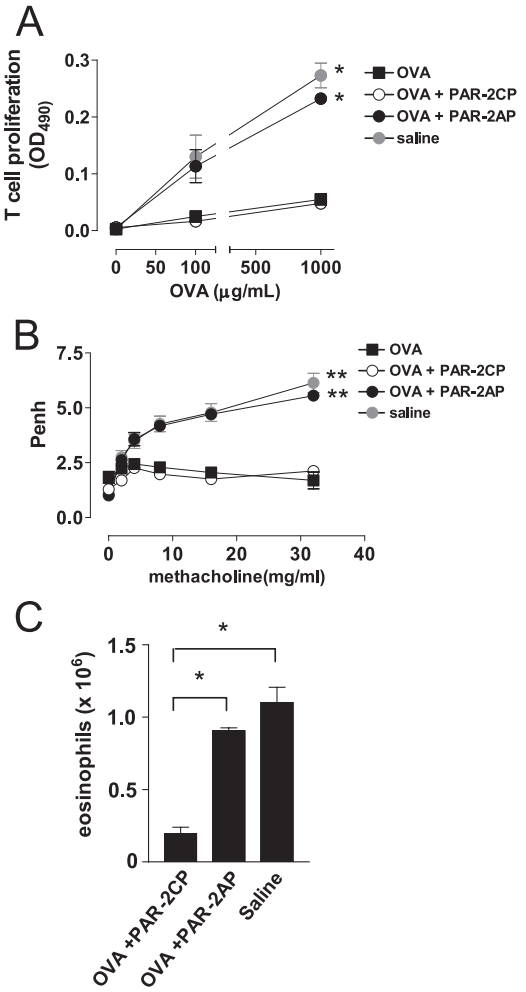

FIGURE 5. PAR-2 activation prevents mucosal airway tolerance to OVA. Mice were given saline, 100 μg of OVA alone, or OVA with PAR-2AP or PAR-2CP (25 μl of 100-μM solution) i.n. on three consecutive days followed by i.p. OVA and aluminum hydroxide immunization. Five days after the immunization splenic T cells were isolated from mice and cultured with increasing doses of OVA (100 and 1000 μg/ml) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. *A*, T cell proliferation was determined using MTS colorimetric cell proliferation assay read at 490 nm ($n = 3$). *, $p < 0.05$ compared with OVA alone or OVA with PAR-2CP. Ten days after the immunization, mice were challenged twice i.n. with 50 μg of OVA and assessed for AHR 24 h after the second challenge. *B*, Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge ($n = 9$ mice). **, $p < 0.01$ compared with OVA administered alone or OVA with PAR-2CP. *C*, Airway inflammation was determined by the number of eosinophils isolated following lung digestion ($n = 4$ mice). *, $p < 0.05$ compared with OVA administered with PAR-2CP.

PAR-2 activation in the airways alters pulmonary DC trafficking

Inhaled Ag is taken up and processed by CD11c$^+$ DCs and presented to naive T cells in the draining lymph nodes and the spleen. To investigate the effects of PAR-2 activation in the airways on DC activation status, we studied Ag uptake by DC and their accumulation in the cervical and bronchial lymph nodes and the spleen using Alexa Fluor 488-labeled OVA. Unlabeled OVA acted as a control. Alexa Fluor 488-labeled OVA was administered alone or in combination with PAR-2AP or PAR-2CP (25 μl of 100-μM solution) and 24 h later spleens and pooled cervical and bronchial lymph nodes were digested. Cell suspension was stained for CD11c as a DC marker and analyzed by flow cytometry. PAR-2

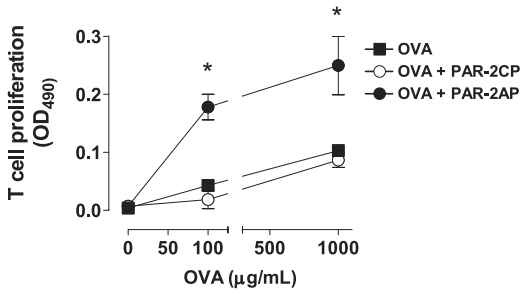

FIGURE 6. PAR-2 activation concurrently with mucosal exposure to OVA induces sensitization. Mice were given 100 μg of OVA alone or OVA with PAR-2AP or PAR-2CP (25 μl of 100-μM solution) i.n. on three consecutive days and 15 days later splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 μg/ml) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. T cell proliferation was determined using MTS colorimetric cell proliferation assay ($n = 3$). *, $p < 0.05$ compared with OVA administered alone or OVA with PAR-2CP. Data are mean ± SEM.

activation in the airways enhanced the uptake of Alexa Fluor 488-labeled OVA by CD11c$^+$ DCs in both the spleen and lymph nodes when compared with Alexa Fluor 488-labeled OVA given alone or with PAR-2CP (Fig. 7*A*). Twenty-four hours after PAR-2 activation in the airways, the number of CD11c$^+$ DCs containing Alexa Fluor 488-labeled OVA increased in the spleen, but not in the lymph nodes (Fig. 7*B*). Therefore, PAR-2 activation in the lungs augments Ag uptake by DCs and increases DC numbers residing in the spleen 24 h after Ag uptake.

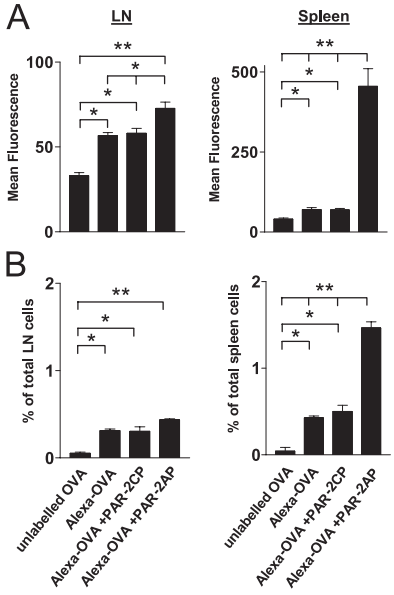

FIGURE 7. DC Ag uptake and trafficking following PAR-2 activation. *A*, Mean fluorescent intensity of Alexa Fluor 488 OVA (FL-OVA) in double positive CD11c$^+$ Alexa Fluor 488 OVA-positive DCs from the cervical and bronchial lymph nodes and spleen 24 h following administration of 100 μg of Alexa Fluor 488 OVA alone or OVA with PAR-2AP or PAR-2CP (25 μl of 100-μM solution). Unlabeled OVA acts as a control ($n = 3$ mice). *, $p < 0.05$; **, $p < 0.01$ compared with Alexa Fluor 488 OVA administered alone, OVA with PAR-2CP, or unlabeled OVA. Data are mean ± SEM. *B*, Percentage of CD11c$^+$, Alexa Fluor 488 OVA-positive double positive cells in the lymph nodes and spleen 24 h following administration of 100 μg of Alexa Fluor 488 OVA alone or OVA with PAR-2AP or PAR-2CP ($n = 3$ mice). *, $p < 0.05$; **, $p < 0.01$ compared with Alexa Fluor 488 OVA administered alone, or OVA with PAR-2CP, or unlabeled OVA. Data are mean ± SEM.

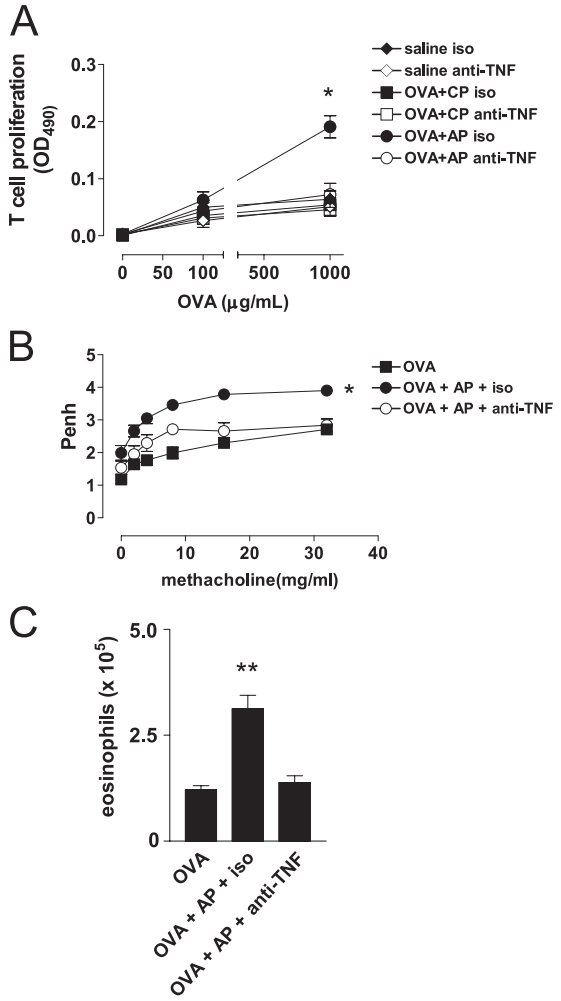

FIGURE 8. PAR-2-induced allergic sensitization is TNF-dependent. Mice were i.p. administered neutralizing TNF Ab or isotype control Ab 24 h before the first and 4 h before the second of three i.n. administrations of OVA with PAR-2AP or PAR-2CP. Fifteen days later, splenic T cells were isolated and cultured with increasing doses of OVA (100 and 1000 μg/ml) and irradiated APCs in vitro for 4 days, after which proliferation was assessed. *A*, T cell proliferation was determined using MTS colorimetric cell proliferation assay ($n = 3$). *, $p < 0.05$ compared with other treatments groups. Other groups of mice were challenged four times, starting 16 days after the initial administrations, on alternating days i.n. with 50 μg of OVA, and assessed for AHR 24 h after the fourth challenge. *B*, Enhanced pause (Penh) was measured by whole-body plethysmography to determine AHR in response to methacholine challenge ($n = 5$ mice). *, $p < 0.05$ compared with OVA administered alone or OVA with PAR-2AP and neutralizing Ab. *C*, Airway inflammation was determined by the number of eosinophils isolated following lung digestion ($n = 5$ mice). **, $p < 0.01$ compared with OVA administered alone or OVA with PAR-2AP and neutralizing Ab. Data are mean ± SEM.

PAR-2 activation promotes allergic sensitization through the release of TNF

These results indicate that PAR-2 activation in the airways alters DC activation status. We have previously shown that PAR-2 activation induces TNF release in the airways (24). Because TNF can activate DCs (33–35), we hypothesized that TNF released, as a result of PAR-2 activation, may alter pulmonary DC activation resulting in allergic sensitization instead of tolerance. To study the role of TNF in PAR-2-mediated allergic sensitization, we administered a TNF-neutralizing Ab to mice that were also given OVA with the PAR-2AP. Allergic sensitization in these mice was assessed by splenic T cell proliferation. Additionally, sensitization of other mice was assessed by the number of eosinophils recruited into the lungs and the presence of AHR following challenge with OVA 16 days postsensitization. The TNF-neutralizing Ab had no effect on T cell proliferation in mice receiving OVA alone or with PAR-2CP. However, TNF neutralization prevented T cell proliferation in mice given PAR-2AP with OVA (Fig. 8*A*), indicating that the TNF-neutralizing Ab prevented allergic sensitization. As expected, from the proliferation results we have shown in Fig. 6, mice initially given OVA and PAR-2AP with the control Ab developed airway inflammation and AHR after challenge. Conversely, mice given OVA and PAR-2AP with anti-TNF-neutralizing Ab showed neither eosinophil recruitment into the lungs nor AHR, indicating the absence of sensitization (Fig. 8, *B* and *C*). These mice had eosinophils numbers and AHR that were similar to mice that developed tolerance. Thus, we concluded that TNF released as a result of PAR-2 activation in the airways mediates allergic sensitization to concurrently inhaled Ag.

Discussion

Although much is known about the immune mechanisms leading to allergic sensitization (36), little is known about the specific characteristics of inhaled Ags that mediate or facilitate allergic sensitization. Our results point toward a pathway in which Ags with serine proteinase activity activate PAR-2 in the airways and induce TNF release, which in turn, biases the immunological outcome toward allergic sensitization. This study, therefore, implicates PAR-2 activation as a vital step in allergic sensitization to the major allergens associated with asthma.

Previous reports have shown evidence of a proteinase-mediated pathway for allergic sensitization. Fungal extracts with proteinase activity prevented inhalation-induced tolerance to OVA and led to allergic sensitization to OVA and subsequent development of allergic airway disease (3). In a similar fashion, HDM extracts can facilitate allergic sensitization to simultaneously administered OVA (26). However, these studies did not define the mechanism of proteinase-induced allergic sensitization. For the first time in this study we have shown a specific receptor that may mediate these effects, namely PAR-2, and also that PAR-2-mediated allergic sensitization is dependent on TNF release.

Ags with serine proteinase activity, such as HDM, have been shown to induce allergic sensitization of mice following mucosal exposure (37). Our study indicates that mucosal sensitization to HDM may be the result of PAR-2 activation in the airways. Studies to test this hypothesis will improve our understanding of allergic sensitization, but they are not easy to perform. The use of PAR-2 knockout mice could show lack of mucosal sensitization to HDM in the absence of PAR-2, but there are potential problems with this approach. PAR-2 knockout mice develop significantly lower AHR and airway inflammation compared with normal controls (38), and these mice may have DC developmental defects and, therefore, Ag presentation may be greatly hindered in vivo (39). Thus, one would be unable to discern whether an apparent lack of allergic sensitization is due to the absence of PAR-2 in the airways or defects in Ag presentation.

We used a synthetic peptide to activate PAR-2 in the airways. This PAR-2AP is specific for PAR-2, as has been determined in vivo through the use of PAR-2 knockout mice (40–42). We activated PAR-2 with 2.5 nM PAR-2AP. This amount is similar to that used in other in vivo studies (43), including a recent one by our laboratory showing that PAR-2 activation during allergen challenge enhances AHR and airway inflammation (24). Along with this activating peptide, we administered OVA, an Ag that is unable to activate PAR-2 and promotes the development of tolerance

when administered in the airways. We used such a system to reconstitute the effects of proteolytic allergens that are able to activate PAR-2 and avoid other proteinase effects of the allergens on the airways.

We have recently shown that PAR-2 is expressed on epithelial cells, smooth muscle, and alveolar macrophages in the airways of BALB/c mice (24). Alveolar macrophages are an attractive target for further study because they are situated throughout the airways, are one of the first cell types to encounter inhaled serine proteases, and can produce large amounts of TNF (44). TNF may have direct effects on DCs in the airways (45), or it may induce the release of other inflammatory mediators from the epithelium (46), which could then mediate DC activation. One such mediator, GM-CSF, has been shown to activate DCs (47) and induce allergic sensitization to OVA when administered directly or as transgene expression in the airways (48).

However, other cell types, such as mast cells, have been shown to express PAR-2 in other tissues (49). Therefore, another source of TNF following PAR-2 activation could be mast cells situated throughout the airways (50). More interestingly, it has been shown that TNF derived from mast cells can enhance DC migration (45) and therefore affect the subsequent immune response. Therefore, TNF may be secreted following PAR-2 activation of one or more of the cell types discussed in this study. Alternatively, TNF could be released by other cells activated by proinflammatory products induced by PAR-2 activation.

Our results indicate that PAR-2 activation in the airways by proteolytic allergens modifies local immune responses. PAR-2 may serve a biological purpose similar to that of TLRs (51). By recognizing conserved protein "patterns" of varying degrees of heterogeneity, PAR-2 senses "danger signals" in the environment, albeit serine proteinases instead of bacterial or viral components, and its activation influences the development of both innate immune responses, namely inflammation, and adaptive immune responses, namely the decision of the immune system to respond to a foreign Ag with immunologic tolerance or allergic sensitization. Furthermore, it has been shown that LPS-activating TLR4 in the airway is also capable of promoting allergic sensitization to inhaled proteins (52). Therefore, the activation of both PAR-2 and TLRs are capable of linking innate immunity with allergic immune responses. For this reason we were careful to ensure that our PAR-2AP and OVA preparations had undetectable LPS levels, to exclude possible TLR involvement in our results.

The majority of protein Ags encountered in the airways induce Ag-specific immune tolerance. We have shown that PAR-2 activation in the airways at the time of Ag encounter is capable of shifting the resulting immune outcome toward allergic sensitization and development of asthma. PAR-2 antagonists, or neutralization of inflammatory mediators released in the airways following PAR-2 activation, may therefore be a very attractive therapeutic strategy to block sensitization toward major asthma-causing allergens. By blocking allergic sensitization, a tolerogenic pathway should be induced to these proteolytic allergens, which would be protective during subsequent environmental exposure to the same allergen. This mechanism would prevent the development of allergic disease.

Disclosures

The authors have no financial conflict of interest.

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