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*J Immunol* 2002; 169:5315-5321; doi: 10.4049/jimmunol.169.9.5315
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Protease-Activated Receptor 2 Mediates Eosinophil Infiltration and Hyperreactivity in Allergic Inflammation of the Airway

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Trypsin and mast cell tryptase can signal to epithelial cells, myocytes, and nerve fibers of the respiratory tract by cleaving protease-activated receptor 2 (PAR2). Since tryptase inhibitors are under development to treat asthma, a precise understanding of the contribution of PAR2 to airway inflammation is required. We examined the role of PAR2 in allergic inflammation of the airway by comparing OVA-sensitized and -challenged mice lacking or overexpressing PAR2. In wild-type mice, immunoreactive PAR2 was detected in airway epithelial cells and myocytes, and intranasal administration of a PAR2 agonist stimulated macrophage infiltration into bronchoalveolar lavage fluid. OVA challenge of immunized wild-type mice stimulated infiltration of leukocytes into bronchoalveolar lavage and induced airway hyperreactivity to inhaled methacholine. Compared with wild-type animals, eosinophil infiltration was inhibited by 73% in mice lacking PAR2 and increased by 88% in mice overexpressing PAR2. Similarly, compared with wild-type animals, airway hyperreactivity to inhaled methacholine (40 μg/ml) was diminished 38% in mice lacking PAR2 and increased by 52% in mice overexpressing PAR2. PAR2 deletion also reduced IgE levels to OVA sensitization by 4-fold compared with those of wild-type animals. Thus, PAR2 contributes to the development of immunity and to allergic inflammation of the airway. Our results support the proposal that tryptase inhibitors and PAR2 antagonists may be useful therapies for inflammatory airway disease. The Journal of Immunology, 2002, 169: 5315–5321.

Certain proteases that are generated or released during trauma and inflammation can signal to cells by cleaving protease-activated receptors (PARs),² members of the family of G protein-coupled receptors (1, 2). Thrombin cleaves PAR1, PAR3, and PAR4, and trypsin, mast cell tryptase, and coagulation factors VIIa and Xa activate PAR2. These proteases cleave their receptors within the extracellular N terminus to expose a tethered ligand domain that binds to and activates the cleaved receptor. Synthetic peptides, which mimic the tethered ligand, can directly activate PAR1, PAR2, and PAR4 and are useful pharmacological tools.

Proteolytic activation of PARs is irreversible and cleaved receptors internalize and are degraded in lysosomes (3, 4). Therefore, PARs are single-use receptors that probably mediate signaling during pathophysiological or “emergency” conditions when PAR agonists are generated or released. For example, thrombin is generated in the blood during trauma and coagulation, and tryptase is released when mast cells degranulate during inflammation. These proteases cleave PARs on multiple cell types to activate signaling events that are mostly associated with inflammation, pain, and repair (1, 2).

In the present study, we examined the role of PAR2 in inflammation of the airways. It is important to understand the role of this receptor in the airways because inhibitors of tryptase, a possible agonist of PAR2, inhibit allergic inflammation in the lungs of experimental animals and have been proposed as novel therapeutics for asthma (5–7). PAR2 is expressed by airway epithelial cells, fibroblasts, myocytes, and sensory neurons and by endothelial cells and vascular smooth muscle (8–11). Although the agonists of PAR2 in the airways are yet to be unequivocally identified, tryptase from lung mast cells and trypsin-like proteases from the airway epithelium are possible candidates. However, there is controversy about whether PAR2 agonists protect or promote airway inflammation. In support of a protective role are observations that PAR2 agonists induce an eicosanoid-dependent bronchodilation in rats, mice, and guinea pigs (8, 12, 13), and that intranasal administration of a PAR2 agonist in mice reduces the infiltration of neutrophils into bronchoalveolar lavage (BAL) after treatment with LPS (11). PAR2 agonists also relax the guinea pig trachea and human bronchi by releasing eicosanoids and NO from the epithelium (9, 10). In support of a proinflammatory role, PAR2 agonists induce contraction of guinea pig and human bronchi and bronchioles, the main site of resistance to air flow (9, 10). Furthermore, PAR2 agonists administered i.v. or intraluminally cause increased airway resistance in the guinea pig by releasing tachykinins from sensory nerves (9). Tryptase also contracts human bronchi (10), induces plasma extravasation (14), infiltration of neutrophils and...
incorporated. Finally, the long arm of the vector (Sse Xho) was ligated in /H9262. A schematic to show the strategy for generation of targeting short arm of the vector (Xba-).

Four mouse genomic clones were isolated using mouse PAR2 primers. The endogenous human promoter and flornization no. 23C20) containing theenous human PAR2 gene is under the control of the endog-

Northern blot hybridization
Total RNA (10 µg/lane) was fractionated by electrophoresis on a 1% agarose/0.6 M formaldehyde gel, transferred to Nylon membrane (Hybond N+; Amersham, Piscataway, NJ), and hybridized with a 32P-riboprobe specific for the human PAR2 sequence. Blots were probed for the housekeeping gene GAPDH to control for loading.

RT-PCR
Total RNA (1 µg) was reverse transcribed at 42°C for 1 h using hexanucleotides and avian myeloblastoma virus reverse transcriptase (Promega, Madison, WI). To analyze expression of human PAR2 in transgenic mice, primers were selected to amplify a 525-bp product specific to human PAR2 gene (forward primer, 5'-CCCT TTT GTA TGT CTG GAA GCA GAC-3'; reverse primer, 5'-TTC CTT CGG TAT TGT TTG GGT GTC-3'). To analyze expression of mouse PAR2 in wild-type and KO animals, primers were selected to amplify a 531-bp product specific to the mouse PAR2 (forward primer, 5'-TAC GAG GAG GTT GCT AGG AGC-3'; reverse primer, 5'-TTC GGA TCT TTT TCC GAA CG). Cycling conditions were 92°C for 1.5 min, 53°C for 2 min, and 72°C for 1.5 min. Cycling under these conditions was repeated 30 times followed by a final extension step at 72°C for 8 min.

Localisation of immunoreactive PAR2
Lungs were placed in 4% paraformaldehyde in 100 mM PBS for 24 h at 4°C. Tissues were washed in PBS, placed in 25% sucrose for 24 h at 4°C, embedded in OCT, and frozen sections were prepared. Sections were per-

Immunochemistry and challenge protocols
Male and female mice (20–25 g) were sensitized by i.p. injection of OVA and saline (10 µg in 0.2 ml Al(OH)3) on days 0 and 14 (23). To examine infiltration of immune cells into BAL, mice were exposed to a single challenge by OVA aerosol (5%, 20 min) or PBS aerosol (100 mM, 20 min, control) on day 21 and were studied 24, 72, or 96 h later. To examine airway hyperreactivity, mice were challenged by OVA aerosol (5%, 20 min) or PBS for 4 consecutive days (21–24) and were studied 24 h after the final challenge. BAL was also examined in these animals 24 h after the final challenge.

Intranasal administration of PAR2 agonists
A peptide corresponding to the tethered ligand of mouse PAR2 (SLIGRKL-NH2, SL-NH2), or the reverse sequence that does not activate PAR2 (LIGGLS-NH2, LR-NH2) (both 10-4 M in 50 µl of saline), or saline (vehicle) was administered intranasally for 3 consecutive days to lightly anesthesitized mice (ketamine, 80 mg/kg; xylazine, 16 mg/kg, i.p.).

Bronchoalveolar lavage
Mice were anesthetized (urethane, 1/g/kg, i.p.). A cannula was inserted into the trachea and the lungs were lavaged with PBS (4 x 0.3 ml) (23). The total number of leukocytes in 20 µl of lavage was determined using a Coulter Counter (Coulter Electronics, Hialeah, FL). Differential leukocyte counts were made by counting 300 cells on stained (Diff-Quik; Dade Diagnostics, Aguada, Puerto Rico) cytospin preparations by light microscopy using standard morphological criteria.

Airway hyperreactivity
Bronchoconstriction in response to inhaled methacholine (MCh) was determined from changes in enhanced pause (Penh) that were measured by barometric plethysmography in conscious mice as described previously (24). Mice were placed in whole-body plethysmographs (Buxco, Troy, NY), exposed to PBS aerosol for 30 s, and the average Penh value was calculated during the next 5 min. After a 10-min recovery period, mice were challenged with increasing concentrations of MCh (2.5–40 µg/ml) by aerosol for 30 s at intervals of 20 min. The average Penh value for the 5 min after challenge was calculated.
**IgE assay**

Total IgE was measured in serum collected on day 21, 7 days after the second immunization with OVA. Blood was collected from euthanized animals by cardiac puncture and serum was stored at −80°C before assay. IgE was measured using a specific ELISA with IgE capture and detection Abs and purified mouse IgE standards (BD PharMingen, San Diego, CA). Samples were assayed in duplicate at several dilutions and OD was measured at 450 nm using a microplate reader (Molecular Devices, Mountain View, CA). Sample IgE concentrations were calculated with reference to a standard curve (1–200 ng/ml).

**Statistical analysis**

Results are expressed as mean and SE and were compared by ANOVA and Student’s *t* test. Differences were considered to be significant if the *p* < 0.05.

**Results**

**Generation and characterization of PAR2tg and PAR2ko mice**

PAR2tg and PAR2ko mice showed no spontaneously abnormal phenotype at the ages studied. In PAR2tg mice, Northern blot analysis indicated high levels of expression of human PAR2 in the liver, skin, and spleen and lower expression in the lung, heart, and testis (Fig. 2A), which closely matches the pattern of PAR2 expression in humans (25). A single PCR product of 525 bp, corresponding to human PAR2, was amplified from the lung of PAR2tg mice, confirming expression of the human transgene in this tissue (Fig. 3). PAR2 mRNA was undetectable by Northern blot analysis of liver tissue from PAR2ko mice and detectable at a reduced level in heterozygote animals compared with PAR2wt(ko) (Fig. 2). A single PCR product of 634 bp was amplified from the lungs of PAR2wt mice, but was not detected in PAR2ko animals, confirming the absence of PAR2 in this tissue (Fig. 3).

**Localization of PAR2 in the airway**

We used immunofluorescence to localize PAR2 in the mouse airway and to assess overexpression in PAR2tg mice and absence in PAR2ko animals. In PAR2wt(ko) and PAR2wt(tg) mice, immunoreactive PAR2 was detected in the airway epithelium and smooth muscle and in endothelial cells and vascular myocytes (Fig. 4, A and C). Similar staining patterns were observed using Abs to human (C-17, Fig. 4A) and rat (B5, Fig. 4C) PAR2. This result suggests that both Abs cross-react with mouse PAR2. Staining by B5 was abolished by preabsorption of the Ab with the receptor fragment that was used for immunization, confirming specificity (data not shown). Using the C-17 Ab, immunoreactive PAR2 was also detected in airway epithelium and smooth muscle as well as the vasculature of PAR2tg mice (Fig. 4B). This staining for human PAR2 in PAR2tg mice was markedly more intense compared with that of PAR2wt(tg) animals, confirming the overexpression of the human PAR2 transgene. There was no detectable B5 staining in tissues from PAR2ko mice (Fig. 4D), which verifies effective PAR2 deletion and confirms Ab specificity.

**Cellular infiltration in OVA-induced airway inflammation**

To assess directly the role of PAR2 in airway inflammation, we compared OVA-induced inflammation in mice lacking or overexpressing PAR2, and compared responses in wild-type animals of the appropriate strains.

Mice were sensitized with OVA and challenged with PBS or OVA 24 h later. In wild-type mice of both strains immunized with OVA, OVA challenge by aerosol stimulated a marked cellular infiltration into BAL compared with PBS-challenged or nonimmunized mice. The magnitude of the infiltrate was markedly increased 24 h after OVA challenge (Fig. 5), and the response persisted for 72 h (data not shown). Compared with PBS-challenged mice (Fig. 5, A and C), OVA challenge markedly increased numbers of eosinophils, neutrophils, and lymphocytes (Fig. 5, B and D). Cell numbers in the BAL after OVA challenge were greater in the PAR2wt(ko) mice than in the PAR2wt(tg) animals, which may reflect strain differences in this response (26, 27). The extent of the cellular infiltrate into BAL after OVA challenge was markedly diminished in PAR2ko mice compared with PAR2wt(ko) mice at 24 h (Fig. 5C), but not at 72 h after challenge (data not shown). Compared with PAR2wt(ko) mice, in PAR2ko mice the number of inflammatory cells in BAL was diminished by 64% for all cells, 73% for eosinophils, 71% for neutrophils, 49% for macrophages, and 59% for lymphocytes. In contrast, the extent of the cellular infiltrate into BAL was greater in PAR2tg mice compared with PAR2wt(tg) mice at 24 h (Fig. 5D), but not at 72 h after challenge (data not shown). The increase in BAL cell numbers in the PAR2tg mice was almost entirely a consequence of an enhancement of eosinophil influx (88% increase in PAR2tg compared with PAR2wt(tg)). Thus, deletion of PAR2 diminishes OVA-induced cellular infiltrate into BAL, whereas overexpression of PAR2 exacerbates the response.

To confirm that PAR2 activation is an important determinant of cellular infiltrate into BAL, we administered the PAR2 agonist SL-NH₂, the inactive peptide LS-NH₂, or saline intranasally to PAR2wt(tg) mice. At 24–72 h after treatment, we assessed cellular infiltrate into the BAL. Compared with animals receiving LS-NH₂...
or saline. SL-NH₂ stimulated a 2-fold increase in BAL macrophages after 48 h, although there was no difference at other time points or in other cell types (Fig. 6). Thus, PAR2 agonists can cause infiltration of inflammatory cells into the airway lumen.

Airway hyperreactivity in OVA-induced airway inflammation

We examined MCh-induced bronchoconstriction in OVA-immunized mice that had been challenged for 4 consecutive days with nebulized PBS or OVA. In both strains of wild-type mice, MCh caused a concentration-dependent increase in Penh at 24 h after the final PBS or OVA challenge (Fig. 7). The responses to increasing concentrations of inhaled MCh were greater in OVA-challenged (Fig. 7, B and D) than in PBS-challenged (Fig. 7, A and C) animals, confirming that the sensitization and OVA challenge procedure caused hyperreactivity to MCh. Hyperreactivity to high doses of MCh (>20 μg/ml) showed a trend to be diminished in PAR2ko mice compared with that of PAR2wt(ko) mice at 24 h (Fig. 7B), but differences did not attain statistical significance. Compared with PAR2wt(ko) mice, in PAR2ko mice the response was diminished by 30% for 20 μg/ml and 38% for 40 μg/ml MCh. In contrast, hyperreactivity was significantly greater in PAR2tg mice.
compared with that of PAR2wt(tg) mice at 24 h (Fig. 7D). Compared with PAR2wt(tg) mice, in PAR2tg mice the response was increased by 57% for 20 µg/ml and 52% for 40 µg/ml MCh. Thus, deletion of PAR2 tends to diminish OVA-induced airway hyper-reactivity, whereas overexpression of PAR2 exacerbates the response.

We also examined BAL in these mice 1 h after challenge with MCh. In both wild-type strains, challenge with OVA for 4 consecutive days induced a marked cellular infiltrate into BAL compared with animals challenged with PBS (Fig. 8). However, deletion or overexpression of PAR2 did not affect the composition of the cellular infiltrate in OVA-challenged animals compared with wild-type animals expressing PAR2 at normal levels (Fig. 8, B and D). Thus, PAR2 participates in the early but not the late phases of allergic inflammation of the airways.

**IgE responses in OVA-sensitized mice**

Differences in cellular infiltration and airway hyperreactivity in mice with altered expression of PAR2 may be due to differences in the inflammatory response to OVA challenge or to alterations in the responsiveness of animals to sensitization with OVA. To evaluate OVA sensitization, we measured IgE levels in the serum of animals on day 21, 7 days after the second immunization with OVA. Animals were not challenged with OVA by aerosol, since local OVA challenge does not affect circulating IgE levels (P. R. Gater, unpublished observation). IgE levels were 4-fold higher in PAR2wt(ko) animals (4.2 ± 0.4 µg/ml) than in PAR2ko mice (1.0 ± 0.2 µg/ml, p < 0.05, n = 8). IgE levels in PAR2wt(tg) mice (4.5 ± 0.9 µg/ml) were similar to those in PAR2tg animals (6.6 ± 1.2 µg/ml) and the same as in PAR2wt(ko). These results suggest that the absence of PAR2 also affects the development of immunity, whereas overexpression of PAR2 has little effect on this response.

**Discussion**

We evaluated the role of PAR2 in allergic inflammation of the airway by using genetically modified mice either lacking or overexpressing PAR2. Our results show that OVA challenge of wild-type animals triggers the infiltration of inflammatory cells into the lumen of the airway and induces hyperreactivity in response to a MCh challenge. Overexpression of PAR2 exacerbates both the infiltration of eosinophils cells into the lumen and the hyperreactivity of the airway. In contrast, deletion of PAR2 diminishes inflammatory cell infiltration and reduces airway hyperreactivity. PAR2 deletion also diminishes the IgE response to OVA sensitization, suggesting a role for PAR2 in the genesis of the immune response. Together, these results support the hypothesis that PAR2 mediates allergic inflammation of the mouse airway. Overexpression of PAR2, which can occur during inflammation (21), may exacerbate cellular infiltration and hyperreactivity.

**PAR2 mediates allergic inflammation of the airways**

Tryptase, a potential agonist of PAR2 (28–33), plays an important role in airway inflammation, and tryptase inhibitors are under development to treat asthma (6, 7, 34). Therefore, it is important to understand the role of PAR2 in inflammation and hyperreactivity of the airway. The lack of selective agonists or antagonists of PAR2 precludes a traditional pharmacological approach. To circumvent this difficulty, we used genetic techniques to manipulate PAR2 expression.

By using RT-PCR, Northern blotting, and immunofluorescence, we verified altered expression of PAR2 mRNA and protein in the
airways of transgenic and KO mice. In wild-type animals, PAR2 mRNA was readily detected in the lungs, and immunoreactive PAR2 was localized to airway epithelium and smooth muscle, as well as vascular smooth muscle and endothelium. PAR2 has been localized to similar cells in the guinea pig and human airways, which supports our results (8–11). In PAR2ko mice, both PAR2 mRNA and immunoreactivity were absent from the lungs and airways. These results confirm the effectiveness of the deletion and verify specificity of the PAR2 Ab. The tissue-specific pattern of expression of human PAR2 in the PAR2tg animals (e.g., high expression in skin and liver) resembles that of PAR2 in humans (25). Moreover, analysis by RT-PCR indicated high levels of expression of the human PAR2 transgene in the mouse lung. Immunoreactive PAR2 was more strongly expressed in the expected cell types in the airways of PAR2tg mice compared with PAR2wt(tg) animals, as detected with an Ab to human PAR2, which confers overexpression of the protein. Although the PAR2tg animals expressed human rather than mouse PAR2, the cleavage site for trypsin and trypstatin are identical for the human and rodent receptors, both of which respond to trypsin and tryptase (25, 31, 32, 35).

Infiltration of inflammatory cells, especially eosinophils, is a characteristic of allergic inflammation of the airways of experimental animals and of asthma in humans (36). Deletion of PAR2 markedly diminished infiltration of eosinophils (75% reduction), whereas overexpression greatly exaggerated this response (88% increase). These effects were apparent at 24 h after OVA challenge but not at later time points or after multiple OVA challenges, indicating an involvement of PAR2 in the acute response to Ag challenge. IgE levels in sensitized animals were not altered by overexpression of PAR2, but were markedly diminished by deletion of PAR2, suggesting that PAR2 contributes both to the development of immunity and the inflammatory response. Additional experiments are required to directly assess the role of PAR2 in the genesis of an immune response. The suggestion that PAR2 plays a role in the early phases of inflammatory cell infiltration is supported by our observation that intranasal administration of a selective PAR2 agonist, SL-NH₂, stimulated macrophage infiltration only after 2 days of treatment. Notably, whereas a PAR2 agonist stimulated macrophage infiltration, altered PAR2 expression mainly influenced eosinophil infiltration in the inflamed airway. Thus, the role of PAR2 may depend on the experimental system. To our knowledge, our results provide the first evidence that PAR2 plays an important role in mediating the infiltration of leukocytes in the acute phases of allergic inflammation of the airways. In support of our results, perivascular application of PAR2 agonists promotes the rolling and firm adhesion of lymphocytes in rat mesenteric vessels (37), and surgically induced lymphocyte rolling and adhesion is diminished in PAR2ko mice different from those used in the current investigation (38). The intraplantar administration of PAR2 agonists in rats also causes granulocyte infiltration (32). The molecular mechanism of these effects remains to be determined, but probably involves altered expression of adhesion molecules on endothelial and epithelial cells that are known to express PAR2.

Another major feature of allergic inflammation of the airway in animals and of asthma in humans is bronchial hyperreactivity (34). Using the same model of allergic inflammation of the airways, we found that overexpression of PAR2 markedly increased hyperreactivity to MCh compared with wild-type animals by 57% (to 20 µg/ml). In contrast, deletion of PAR2 diminished MCh-induced hyperreactivity, although the effect was not statistically significant. These findings suggest that exaggeration of PAR2-mediated responses is a major determinant of the proinflammatory allergic phenotype in the mouse airway. These changes in airway hyperreactivity were not associated with altered infiltration of inflammatory cells into BAL fluid although measurements of leukocytes in the lung tissue were not made. Multiple daily challenges of sensitized animals with aerosolized OVA are needed to produce airway hyperreactivity to MCh. Our data suggest that PAR2 is mostly involved in the acute phase inflammation but the elevated hyperreactivity of transgenic animals supports a role for PAR2 in this more chronic response. Our results are supported by the observations that administration of PAR2 agonists to intact guinea pigs markedly increases airway resistance (9) and by the reports that PAR2 agonists stimulate contraction of guinea pig and human bronchioles in vitro (9, 10). The findings that tryptase inhibitors diminish airway hyperreactivity in experimental animals and humans also support a role for PAR2 in this response (6, 7, 34).

Our results are in contradiction to reports that PAR2 agonists can protect against inflammation. In the mouse, rat, and guinea pig, PAR2 agonists partially reverse serotonin- and histamine-induced bronchoconstriction by stimulating the release of eicosanoids (8, 12, 13), and in mice a PAR2 agonist reduces the infiltration of neutrophils into BAL after treatment with LPS (11). PAR2 agonists also stimulate release-protective eicosanoids in the intestinal mucosa (22) and protect against gastric ulcers by stimulating secretion of mucus (39). In contrast, other studies indicate that PAR2 agonists can induce inflammation. Thus, PAR2 agonists induce inflammation of the skin (32, 37, 40) and the gastrointestinal tract (N. W. Bunnett, unpublished observations). In these tissues, as in the airways, the inflammatory effects are mediated partly by neurogenic mechanisms through the release of substance P and calcitonin gene-related peptide from sensory nerves. Thus, PAR2 may play a role both in protecting against inflammation or in mediating inflammation, depending on the species and experimental model. However, PAR2 agonists are not always selective, and there are no PAR2 antagonists that would permit evaluation of agonist selectivity or investigation of the role of PAR2 in experimental models of human disease. Our observations using genetically modified animals provide direct evidence of a role for PAR2 in enhancing early events in allergic inflammation of the airways.

Tryptase and trypsin may activate PAR2 in the inflamed airway

Although our results indicate that PAR2 contributes to allergic inflammation of the airway, the agonists remain to be identified. One candidate is mast cell tryptase, which is elevated in the lungs of asthmatics (41–43). Tryptase inhibitors block allergen-induced airway hyperreactivity (5–7). Moreover, tryptase has many proinflammatory effects in the airways (10, 14–20), which could be mediated by PAR2. Tryptase from human lung and skin cleaves and activates PAR2 in transfected cell lines overexpressing PAR2 (31) as well as in cells that naturally express the receptor (29, 30, 32). These responses to tryptase are desensitized by selective PAR2 agonists, suggesting that tryptase activates PAR2. Moreover, the hyperalgesic effects of tryptase in the skin are diminished in PAR2ko mice (33). A recent report has questioned the capacity of tryptase to activate PAR2 (44). The explanation of this discrepancy is unknown. However, alterations in the extent of receptor glycosylation dramatically affect the ability of tryptase to cleave and activate PAR2 (28). Thus, deglycosylation in vivo could regulate tryptase activation of PAR2.

Proteases other than tryptase may also activate PAR2 in the airway. Tryptsin is the most potent PAR2 agonist, and trypsin-like enzymes have been detected in airway epithelial cells (8, 11) and in airway secretions (11, 45). The purification and characterization of these enzymes is required to ascertain their effectiveness as PAR2 agonists.

In summary, our observations in mice either lacking or overexpressing PAR2 show that this receptor plays an important role in
allergic inflammation of the airway. They suggest that antagonists of PAR2 or inhibitors of proteases that activate this receptor may be valuable therapies for asthma.

References


