

ORIGINAL PAPER

Activation of protease-activated receptor-2 reduces airways inflammation in experimental allergic asthma

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Clinical and Experimental Allergy

Summary

Background Proteinase-activated receptors (PAR)-2 are members of the family of G-protein-coupled receptors activated by proteases. These receptors are widely expressed in several tissues and in virtually all cells involved in rhinitis and asthma. In particular, proteinases activating PAR-2 may affect airway functions and play a role in human diseases.

Objective Assessment of the role of PAR-2 in bronchoconstriction, airway responsiveness and immune response after allergic challenge, in rabbits sensitized to Par j 1, the major allergen of *Parietaria judaica* pollen.

Methods Evaluation of antigen challenge in rabbits treated with PAR-2-activating peptide (PAR-2AP) (SLIGRL) or the scrambled peptide LSIGRL or vehicle immediately before allergen exposure measuring airway responsiveness. Characterization of bronchoalveolar lavage (BAL) following histamine challenge and phenotype analysis of cells by flow cytometry and analysis of cytokine production by quantitative PCR.

Results PAR-2AP pre-treatment, but not the scrambled peptide, was able to significantly inhibit bronchoconstriction, airway hyper-responsiveness and to modulate the immune response induced by allergic challenge in sensitized rabbits. The phenotype analysis of the cells recovered from BAL showed an increase in RLA-DR-positive cells while RTLA-positive cells were unchanged. IFN- γ and IL-2 production were inhibited, with a concomitant increase in IL-10 of about 10-fold over the control values.

Conclusions In this experimental model, PAR-2 modulates bronchoconstriction interfering with antigen challenge-induced immune response in rabbits sensitized and challenged to Par j 1.

Keywords allergic asthma, bronchoconstriction, cytokines, PAR-2

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Introduction

Proteinase-activated receptor 2 (PAR)-2 belongs to a family of G-protein-coupled receptors activated by proteolysis [1]. A serine protease cleaves the N-terminal extracellular domain of this receptor at a specific site within the protein chain to expose an N-terminal tethered ligand domain, which binds to and activates the cleaved receptor. Four PARs have been identified so far: PAR-1, -3 and -4, activated by thrombin, and PAR-2, which is selectively activated by trypsin, mast cell tryptase and coagulation factors VIIa and Xa [2]. PARs couple to

multiple signalling events that mediate different tissue responses, such as injury, inflammation and repair [3, 4]. Therefore, proteases and their receptors may represent important therapeutic targets in several human diseases. These receptors are widely expressed on cells in blood vessels, connective tissue, leucocytes, epithelium and many airway cells [4]. PARs are also present virtually on all cells involved in rhinitis and asthma. Proteases are known to activate PAR-2 and they have been shown to exert major effects on airway functions [5]. It has to be noted that patients with asthma express an increased amount of PAR-2 on respiratory epithelial cells [6, 7].

Agonists of PAR-2, including proteases and peptides corresponding to the tethered ligand domain PAR-2-activating peptide (PAR-2AP), affect airway tone in experimental animals, although the effects depend on the species and the model [8].

However, there is conflicting evidence as to whether the activation of the PAR-2 promotes or opposes the progression of airway inflammatory responses. Schmidlin et al. [9] evaluated the role of PAR-2 in allergic inflammation of the airway using genetically modified mice either lacking or overexpressing PAR-2. These experiments showed that the lack of PAR-2 expression lowered the inflammatory infiltrate and reduced airway hyper-reactivity to allergen challenge in these animals. In contrast, Moffatt et al. [10] reported that a PAR-2AP SLIGRL inhibited bacterial lipopolysaccharide-induced neutrophil influx into mouse airways, suggesting that PAR-2 agonists may be useful anti-inflammatory therapeutic molecules in airway inflammatory diseases.

Although the role of protease agonists in allergic diseases is yet to be fully characterized, it is likely that protease-induced responses can be considered to be an important form of innate immunity [11].

Asthma induced by pollens is dramatically increasing in the western world [12] and *Parietaria judaica* (pellitory of the wall) accounts for most of the allergenic sensitization in the southern Mediterranean area [13], as well as in other countries [13, 14]. In this study, we investigated the role of PAR-2 in an animal model of allergenic sensitization using rabbits sensitized to *Parietaria*. In these animals, we measured the effects of PAR-2 activation on airway hyper-reactivity, cells and cytokine production in the bronchoalveolar lavage (BAL).

Materials and methods

Animals

New Zealand White germ-free rabbits (Harlan, Udine, Italy) of either sex were used throughout the study according to the Italian Legislative Decree 116/92.

Drugs and chemicals

Histamine (Sigma, Milano, Italy), diazepam (Roussel Pharma., Wiesbaden, Germany), hypnorm (Janssen Pharmaceutical Ltd., Grove, Oxfordshire, UK), aluminium hydroxide (Pierce, Boston, USA), biotinylated serum anti-rabbit IgG, streptavidin peroxidase, diamino-benzidine, SDS, and SLIGRL-NH₂ (PAR-2AP) were generous gifts from Prof. Caliendo of the Department of Medicinal Chemistry of Federico II University, Napoli, Italy. The monoclonal antibodies anti-T lymphocytes (Ken 5), MHC-Class II (2C4), CD4 (Ken-4), CD8 (12C6), CD14 (TUK-4) and CD25

(Kei-ALPHA1) were from Serotec (Oxford, UK). All solutions were prepared in saline.

Allergen extraction

Briefly, an allergenic extract was prepared from grains of *P. judaica* (Allergon, Angelhom, Sweden) as reported previously in detail [14].

Experimental protocol (Fig. 1)

On day 1, after measurement of baseline lung function, the anaesthetized rabbits were exposed to an aerosol of sterile saline for 2 min and lung function parameters were recorded. Airway responsiveness was then determined by exposing the animals to cumulative concentrations of aerosolized histamine (1.25–80 mg/mL; 2 min/concentration), administered directly to the lungs via an endotracheal tube and the following 10 breaths were recorded and the mean values of R_L and C_{dyn} were calculated. Immediately after histamine challenge, BAL was performed.

On day 2, antigen challenge was performed in rabbits pre-treated with aerosol of PAR-2AP (10 mg/kg), scrambled peptide LSIGRL (10 mg/kg) or vehicle immediately before allergen exposure. Each antigen challenge consisted of a 4-min aerosol of saline, followed by five consecutive aerosols of a 4-min duration (total of 20 min) of Par j 1 (500 µg/mL); then, the respiratory function was recorded as described above.

On day 3, airway responsiveness to histamine and BAL was determined as on day 1.

Aerosols of saline and antigen were generated by an ultrasonic nebulizer (De Vilbiss Health Care, UK Ltd., Heston, Middlesex, UK).

The provocation concentrations (PC) of histamine that produced a 100% increase in airway resistance (R_L) (PC₁₀₀) or a 50% decline in dynamic compliance (C_{dyn}) (PC₅₀) were determined for each animal by linear interpolation and were used as indices of airway responsiveness. Fluorescence activated cell sorting (FACS) analysis and cytokines production evaluation were performed on BAL recovered immediately after histamine challenge on days 1 and 3.

The immunization protocol, the assessment of positive sensitization to Par j 1, the pulmonary function measurement and BAL have been reported in detail previously [15]

Phenotypic analysis of cells recovered from bronchoalveolar lavage

Cells were recovered from BAL by centrifugation according to standard protocols; the phenotype analysis was performed using flow cytometry after staining the cells with conjugated monoclonal antibodies to rabbit T cell lymphocyte antigen (RTL) anti-CD4, CD8, IL-2 receptor and to rabbit Class II antigen (RLA) (Serotech, Raleigh,

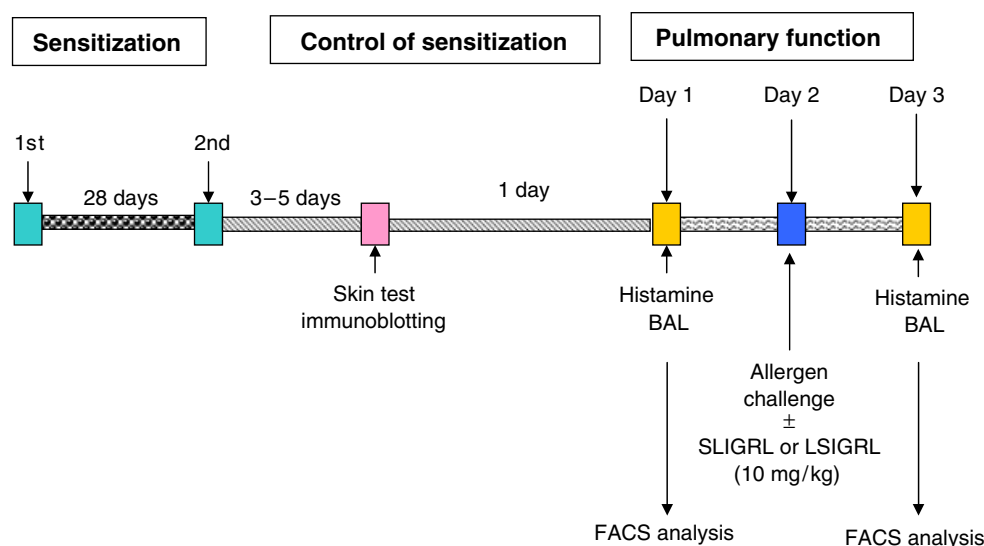


Fig. 1. Schematic representation of rabbit sensitization and the steps involved in pulmonary function determination following antigen stimulation and proteinase-activated receptors-2 activating peptide (SLIGRL) or scrambled peptide (LSIGRL) challenge.

USA). All the protocols were performed according to standard procedures using FACScalibur and Cell Quest software (Becton-Dickinson, Milan, Italy). We also studied the number of eosinophils present in the BAL according to standard protocols, as we used before [15, 16].

Analysis of cytokine production

RNA was extracted by means of a Trizol reagent according to the manufacturer's instructions. Reverse transcription was performed using MMLV, and 100 ng of each RNA sample was used for quantitative PCR. Samples were run in triplicate in 50- μ L reactions using an ABI PRISM 5700 sequence detector system (Applied Biosystems, Foster City, CA, USA); the cycling was 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. SYBR-green oligonucleotides were design to detect cytokines. In particular, we analyzed the expression of IFN- γ , IL-2 and IL-10 5700 System and software (Applied Biosystems). The differences in cDNA templates were corrected by normalizing signals obtained with primers specific for glyceraldehyde-3-phosphate dehydrogenase. The primers and probes for the experiments described here were designed by processing the sequences for the rabbit cytokines found in GeneBank and described previously [17]. The derived sequences were optimized for cycling and efficiency using Primer Express software (Applied Biosystems). All the experiments were performed in triplicate.

Statistical analysis

Bartlett's test for homogeneity of variances was used on all data to determine whether parametric or non-

parametric statistics were to be applied. For the lung function studies, statistical analysis was performed on log₁₀-transformed data (PC₁₀₀ and PC₅₀) in order to normalize the distribution of the data and to allow the application of parametric statistics. The paired *t*-test was used for the lung function data before and after allergen challenge. The results were considered to be significant if *P* < 0.05.

Results

Baseline lung function

Baseline absolute values of R_L or C_{dyn} were not significantly different between the third and first days. Furthermore, no significant difference was observed on the third experimental day in sensitized animal groups treated with PAR-2AP, LSIGRL or vehicle aerosol (data not shown).

Acute antigen-induced bronchoconstriction

In sensitized rabbits, aerosol of antigen caused an acute bronchoconstriction with an approximately two-fold greater increase in R_L (Fig. 2a) and decrease in C_{dyn} with respect to an aerosol of a saline solution (Fig. 2b). This antigen-induced acute bronchoconstriction was significantly reduced by PAR-2AP pre-treatment, but was unaffected by LSIGRL (Fig. 2).

Airway responsiveness to histamine

Effect of proteinase-activated receptors-2. As expected, antigen challenge exposure resulted 24 h later in an increase of airway responsiveness to inhaled histamine,

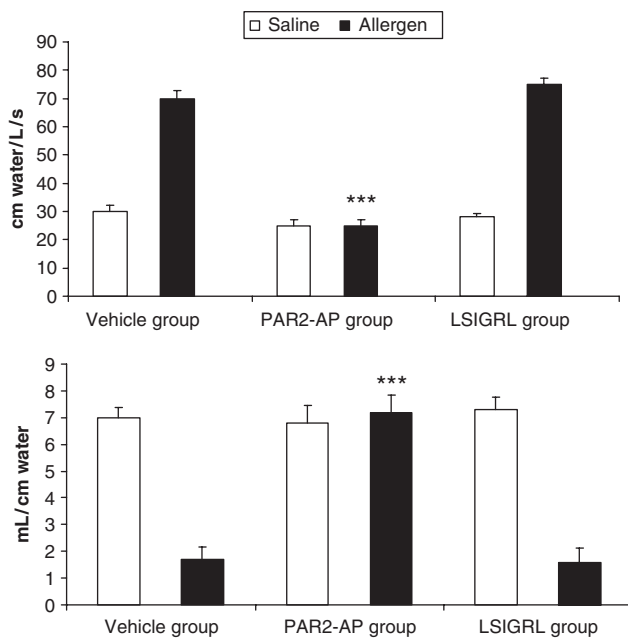


Fig. 2. Effect of Par j 1 or saline challenge on R_L and C_{dyn} in sensitized rabbits ($n = 5$) with vehicle (open column) or proteinase-activated receptors-2 activating peptide (PAR-2AP) (SLIGRL 10 mg/mL) or scrambled peptide (LSIGRL 10 mg/mL) treatment. All values are expressed as mean $\pm$ SEM, $n = 5$ per group. *** $P < 0.01$ vs. vehicle-treated group.

leading to an increase of the airway responsiveness to inhaled histamine about fourfold ($P < 0.01$) when compared with saline challenge in sensitized rabbits (Fig. 3). The pre-treatment with PAR-2AP (10 mg/mL) significantly reduced the antigen-induced airway hyper-responsiveness (AHR) to inhaled histamine in terms of both R_L PC₁₀₀ and C_{dyn} PC₅₀ (Fig. 3). The experiments conducted with the scrambled peptide LSI GRL (10 mg/mL) did not affect airway responsiveness (Fig. 3).

Effect of proteinase-activated receptors-2 activating peptide on cell populations recovered from bronchoalveolar lavage. FACS analysis of the cells recovered from BAL of a rabbit undergoing allergen challenge at day 1 and at day 3 in the absence and presence of PAR-2AP is shown in Fig. 4. For characterization, we used antibodies specific for CD4, T cell receptor, RLA, IL-2 receptor and CD8. The population recovered from BAL was identified to be CD4⁺; thus, the entire successive analysis has been performed using a gate on CD4⁺ cells and excluding dead cells and debris. From the FACS analysis shown in Fig. 4, it is evident that after 1 day there is a consistent increase of CD4⁺/DR⁺, TCR-negative (see lower panel, Fig. 4) cells in BAL.

BAL fluid recovered from antigen-sensitized and challenged rabbits contained elevated numbers of eosinophils. In our experiments, the numbers of BAL eosinophils were significantly reduced by PAR-2AP treatment but were not affected by LSI GRL treatment (Fig. 5).

Effect of proteinase-activated receptors-2 activating peptide on cytokine production in bronchoalveolar lavage. In order to better characterize the function, we also analysed IL-10, IL-2 and IFN- γ mRNA expression by real-time PCR in cells obtained from BALs (Fig. 6). From this analysis, it appeared that levels of IL-2 and IFN- γ , as expected, are boosted by antigen challenge while IL-10 levels are unchanged. Treatment with PAR-2AP (10 mg/mL) reduced IL-2 and IFN- γ levels below the basal values, while IL-10 levels were about 12-fold increased (Fig. 6). In contrast, LSI GRL did not modify the cytokine levels (data not shown). These latter data correlate well with the functional data where PAR-2 confers protection against the early bronchoconstriction and AHR induced by the allergen (Figs 2 and 3).

Discussion

The role of protease activation in allergic inflammation still remains controversial. Indeed, there are reports indicating that the activation of protease receptors may either increase or reduce airway inflammation [9, 10].

In an established animal model of pollen allergy [15], we studied the effects of pre-treatment with PAR-2AP on the allergic reactions induced by *Parietaria judaica* (Par j). Rabbits were first sensitized to Par j 1 and subsequently received an aerosol challenge of Par j with or without treatment PAR-2AP. The animal group that received SLIGRL showed reduced bronchoconstriction, lower number of eosinophils and a reduction in AHR.

These effects were not obtained on administration of a control peptide, LSI GRL. We used LSI GRL as a control peptide because, although it has charge and compositional properties similar to SLIGRL, it lacks PAR-2-activating activity [18, 19].

The peptide SLIGRL is the tethered ligand sequence for murine PAR-2 and its selectivity for PAR-2 has been demonstrated in different experimental systems. For example, *in vivo* studies have shown that SLIGRL induces responses in PAR-2 (+/+) mice but not in PAR-2 (-/-) mice [19]. Furthermore, structure – function studies using immortalized murine cell lines transfected with PARs indicate that SLIGRL selectively activates PAR-2, but not other PARs, such as PAR-1 [20]. Among PARs, PAR-2 is the receptor that has been shown to be more closely involved in inflammatory events as opposed to coagulation. Indeed, PAR-2 can be up-regulated by several stimuli including LPS and cytokines such as TNF- α [1]. Taken together, the findings reported here suggest that SLIGRL-induced stimulation of airway PAR-2 activates pathways able to inhibit airway inflammation and AHR.

These results are in agreement with other papers documenting a protective role of PAR-2 stimulation against inflammation and AHR in the lung. In fact, it has been reported in the mouse, rat and guinea-pig that

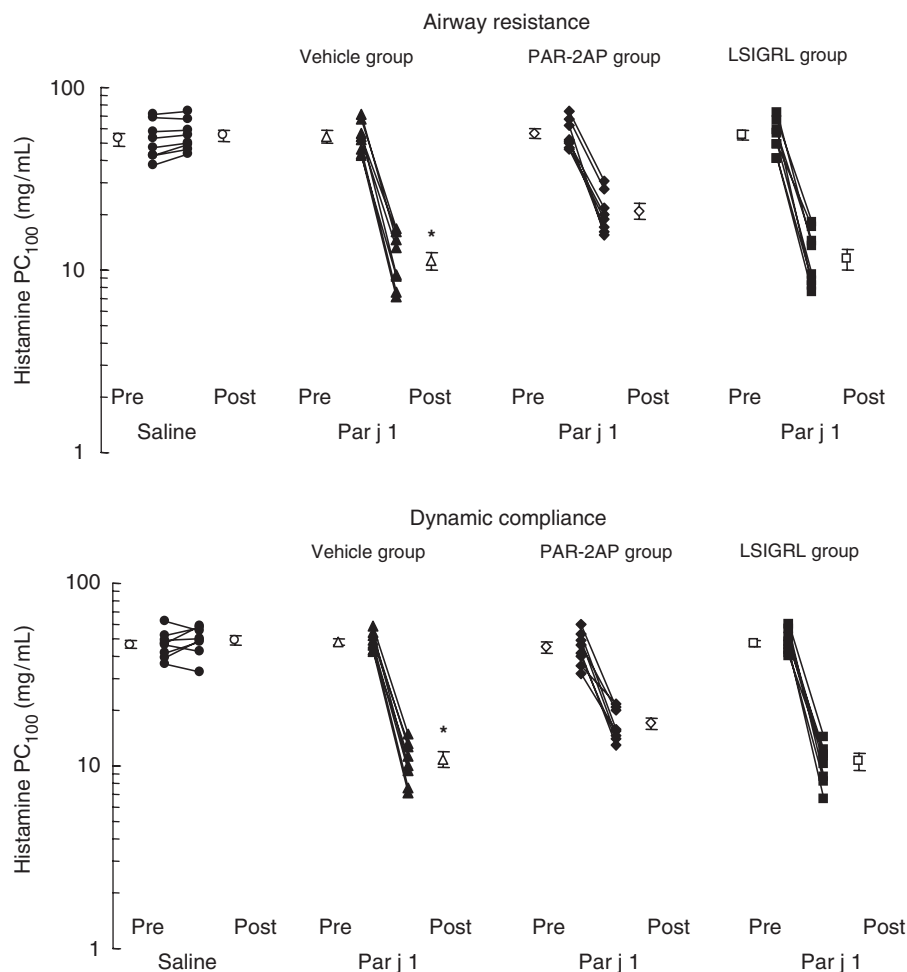


Fig. 3. Airway responsiveness to histamine 24 h before (pre) and 24 h after (post) saline or Par j 1 challenge in sensitized rabbits ($n = 5$) with vehicle or proteinase-activated receptors-2 activating peptide (PAR-2AP) (SLIGRL 10 mg/mL) or scrambled peptide (LSIGRL 10 mg/mL) treatment. * $P < 0.05$ Par j 1 vehicle group vs. Par j 1 PAR-2AP group.

administration of PAR-2 agonists partially reverses serotonin- and histamine-induced bronchoconstriction [11, 21], and in mice a PAR-2 agonist reduces the infiltration of neutrophils into BAL after treatment with LPS [11].

Moreover, PAR-2 agonists stimulate eicosanoids' release into the intestinal mucosa [22] and protect against gastric ulcers by stimulating the secretion of mucus [23]. In many instances, the effects produced by PAR-2APs within the airways seem to be mediated by COX products. For example, bronchorelaxant effects induced by SLIGRL in mouse isolated trachea and bronchi [11, 24], in anaesthetized, ventilated mice [21] and ovalbumin (OVA)-sensitized mice [25] were abolished in the presence of a dual COX-1/-2 inhibitor indomethacin. It has been documented that PGE₂ is produced in cultured airway smooth muscle cells [11, 24] and in several cells within human airways (i.e. epithelial cells, follicular dendritic cells, fibroblasts, monocytes and alveolar macrophages) after expression of the inducible form of COX-2 [26]. In allergen-induced asthma, PGE₂ inhibits pulmonary

inflammation [27] and exerts bronchoprotective effects in human airways, such as the inhibition of bronchoconstriction [28].

Conversely, other studies indicate that PAR-2 agonists can induce inflammation [29]. In fact, it has been reported that administration of exogenous PAR-2 agonists causes inflammation of the skin, [30] oedema in the rat paw [23] and increased rolling of leucocytes [31]. Moreover, Schmidlin et al. [9] reported that elevated levels of PAR-2 expression are associated with worsened airway pathology in allergic mice; therefore, they speculate that PAR-2 mediates proinflammatory effects. However, the effects of exogenous activation of PAR-2, such as those induced by SLIGRL, on allergic-inflammatory responses in these experiments have not been reported, and therefore it is unclear what modulatory influences PAR-2 stimulants could have in these mice.

These controversial reports may suggest that PAR-2 can overall play a role both in protecting against inflammation or in mediating inflammation. These conflicting

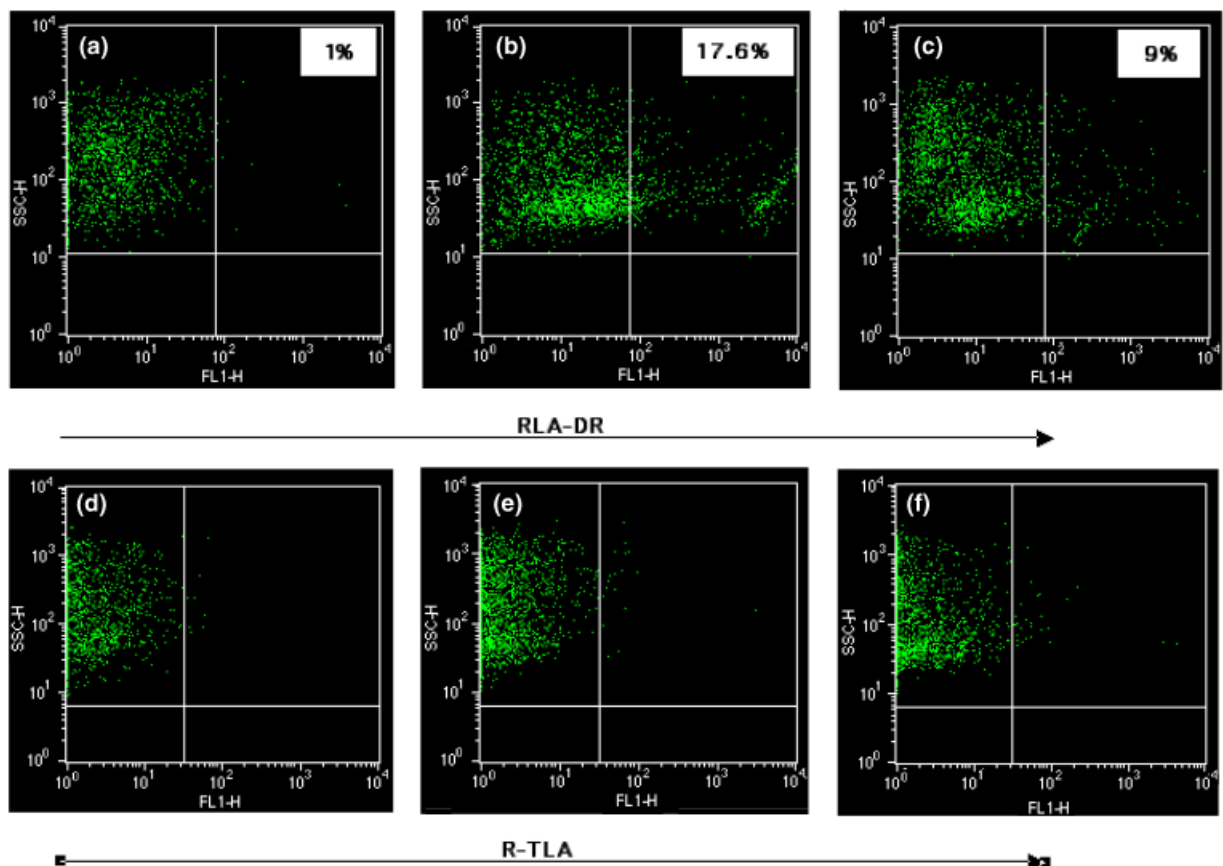


Fig. 4. Expression of RLA-DR (rabbit Class II antigen) (upper panel) and TLA (T lymphocyte antigen) (lower panel) on the surface of cells recovered from BAL of rabbits in the basal condition (a and d), after antigen challenge, before (b and e) and after proteinase-activated receptors-2 activating peptide pre-treatment (c and f). Data generated on $n = 5$ animals for each group.

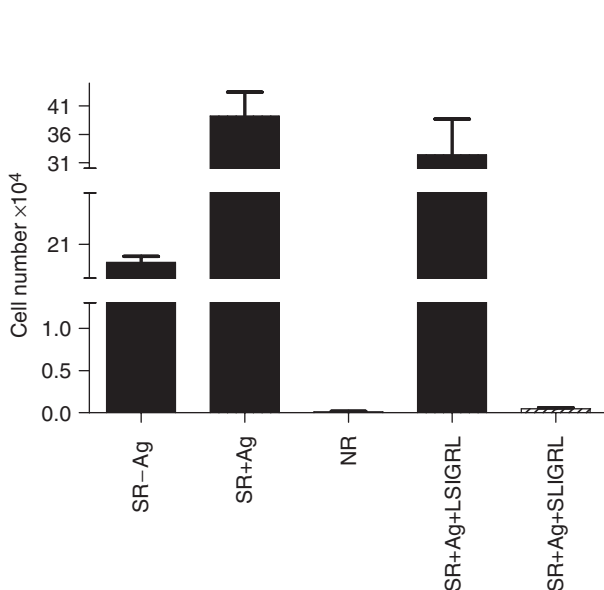


Fig. 5. Determination of eosinophil count in bronchoalveolar lavage of sensitized (SR) and unsensitized animals (NR) before (-) and after (+) Ag challenge, in the presence of a scrambled peptide (+LSIGRL) or proteinase-activated receptors-2 activating peptide (PAR2-AP) (+SLIGRL). Data generated on $n = 5$ animals.

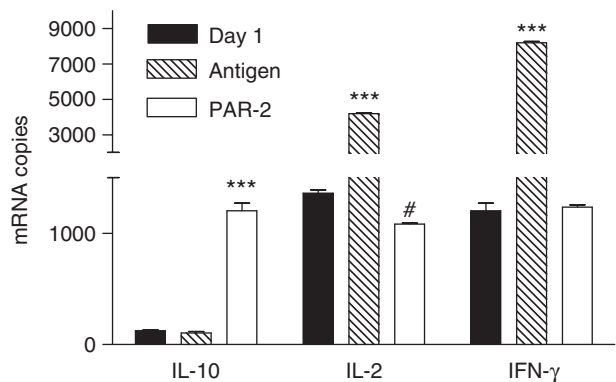


Fig. 6. mRNA expression of IL-10, IL-2 and IFN- γ in the basal and stimulated conditions as well as in proteinase-activated receptors-2 activating peptide-treated rabbits. # $P < 0.01$; *** $P < 0.001$.

effects may be due to several reasons, such as the doses of SLIGRL used in the different studies and the lack of highly selective agonists and antagonists, which does not allow pharmacological dissection of PAR-2 effects in the modulation of the endogenous activation of the receptor. Furthermore, Schmidlin et al. [9] used OVA to study the

effect of PAR-2 on bronchoconstriction; here, we used a rabbit model of asthma that, according to many authors, may be considered a model closer to the human disease. Here, we used the major allergen of *P. judaica* to sensitize rabbits; this allergen, unlike OVA, is an allergen that has been shown to elicitate vigorous allergenic responses *in vitro* and *in vivo* [32–35].

In the last few years, considerable attention has been focused on the role of PARs in allergic respiratory diseases. PAR-2 expression is increased in the epithelium of patients with asthma [6], and proteases from mites and moulds appear to act through receptors similar to PARs. Viral infection of the respiratory tract was found to be associated with elevated epithelial PAR-2 expression; in these experiments, administration of SLIGRL caused enhanced PAR-2-mediated bronchodilator function in infected mice [18].

In the light of this evidence, our data suggest that under our experimental conditions, PAR-2 activation plays a protective role. In order to better characterize the meaning of our findings, we analysed BAL fluid obtained by rabbits at day 1 before the antigen challenge and at day 3, 24 h after the antigen challenge. Inflammatory cells, as expected, were significantly elevated in the BAL recovered after antigen challenge. The phenotype analysis performed by cytofluorimetry of the cells recovered from BAL of sensitized rabbits after allergen challenge showed an increase in RLA-DR⁺-cells, while RTLA⁺-cells were unchanged. First, these results indicate a major involvement of inflammatory cells other than T cells in the inflammatory event leading to cell exudation in the lung. It is indeed intriguing that, in these experiments, the triggering of PAR-2 changed the number of RLA-DR⁺ cells and eosinophils. It has been described that monocytes and dendritic cells express different patterns of PARs on the surface depending on the maturation stage and the activity [36]. Differentiation of monocytes into macrophages by either macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) increases the expression of PAR-1, -2 and -3. These two cytokines are abundantly produced in the presence of an allergenic stimulation, such as the stimulation with Par j, used in these experiments. In eosinophils, it has been shown that PAR-2 predominantly has an intracellular localization, but once the few receptors on the cell surface become activated, PAR-2 is redistributed from intracellular stores to the surface and have pleiotropic effects, directly and indirectly, on eosinophils' functions [37]. Therefore, we may speculate that the PAR-2 agonist here is able to promote the maturation of peculiar RLA-DR⁺ T lymphocyte antigen-negative cells and, at the same time, to inhibit eosinophils' recruitment.

Because no rabbit antibodies are available, to further address the function of these RLA-DR⁺ cells, we analysed their cytokine production by quantitative real-time PCR.

The results show that following antigen challenge, there is an increase in IL-2 and IFN- γ production with a three-fold increase in IL-2 and a six-fold increase in IFN- γ , coupled with low levels of IL-10. Strikingly, aerosol administration of PAR-2AP brings IFN- γ and IL-2 production to control levels with a concomitant increase in IL-10 of about 10-fold over the control values. Thus, treatment with PAR-2AP causes a strong shift in BAL cells towards IL-10 production, suggesting that PAR-2 stimulation leads to the production of anti-inflammatory cytokine.

In this context, the role of IL-2, IFN- γ and IL-10 in airway immune response is well documented [38]. Moreover, it has been shown that lamina propria lymphocytes isolated from a mice model of colitis, treated *in vitro* with PAR-2AP before specific stimulation through a T cell receptor, display an impaired IFN- γ production [39].

Here, we showed that PAR-2AP inhibited the development of bronchoconstriction, airway eosinophilia and hyper-responsiveness in an animal model of pollen asthma. Analysis of BAL lavage indicates that PAR-2 could lower the airway immune response induced by pollen exposure by switching the cytokine pattern towards an anti-inflammatory response. These findings for the first time suggest that exogenous substances that activate PAR-2 can impair allergic inflammation, thus providing new approaches for the treatment of these diseases.

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