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Pituitary adenylate cyclase-activating peptide receptor 1 mediates anti-inflammatory effects in allergic airway inflammation in mice

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Summary

Background—Bronchial asthma is characterized by airway inflammation and reversible obstruction. Since the gold standard of therapy, a combination of anti-inflammatory corticosteroids and bronchodilatory β_2 agonists, has recently been discussed to be related to an increased mortality, there is a need for novel therapeutic pathways.

Objective—A new experimental concept that encompasses the vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide (PACAP) family of receptors by demonstrating the anti-inflammatory effects of the PACAP receptor 1 (PAC1R) in a murine model of allergic asthma is described.

Methods—PAC1R expression was investigated in lung tissue and isolated dendritic cells (DCs) via real-time PCR. Ovalbumin (OVA)-induced asthma models were used in PAC1R-deficient mice and BALB/c mice treated with PAC1R agonist maxadilan (MAX). Bronchoalveolar lavages have been performed and investigated at the cellular and cytokine levels. Fluorescence staining of a frozen lung section has been performed to detect eosinophil granulocytes in lung tissue. Plasma IgE levels have been quantified via the ELISA technique. Lung function was determined using head-out body plethysmography or whole-body plethysmography.

Results—Increased PAC1R mRNA expression in lung tissue was present under inflammatory conditions. PAC1R expression was detected on DCs. In OVA-induced asthma models, which were applied to PAC1R-deficient mice (PAC1R^{−/−}) and to BALB/c mice treated with the specific PAC1R agonist MAX, PAC1R deficiency resulted in inflammatory effects, while agonistic stimulation resulted in anti-inflammatory effects. No effects on lung function were detected both

in the gene-depletion and in the pharmacologic studies. In summary, here, we demonstrate that anti-inflammatory effects can be achieved via PAC1R.

Conclusion—PAC1R agonists may represent a promising target for an anti-inflammatory therapy in airway diseases such as bronchial asthma.

Keywords

airway inflammation; allergy; asthma; mouse model; neuropeptides; PACAP; VIP

Introduction

Bronchial asthma is a chronic pulmonary disease that is characterized by airway inflammation and bronchoconstriction. Despite considerable advances in the understanding of the pathogenesis of this airway disease [1] effective treatment options remain essentially unchanged compared with the last decades, with few exceptions such as anti-leukotrienes. This is due to the great redundancy of pro-inflammatory pathways orchestrating the pathology of allergic asthma. With regard to the two main features of the disease, the gold standard of therapy is a combination of the two drug classes of inhaled bronchodilatory long-acting β agonists (LABA) and anti-inflammatory corticosteroids [2]. Using this combination, a global reduction of asthma mortality and morbidity was achieved over the past decades. However, the side effects of steroids are still debated by patients and there is a new concern that LABA may increase the risk of asthma mortality [3]. Therefore, alternative therapeutic options that encompass both bronchodilatory and anti-inflammatory effects need to be developed.

A potential pathway consisting solely of agonistic mechanisms is represented by the vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP) system [4, 5]. VIP and PACAP are neuropeptides that bind to three G protein-coupled receptors: PACAP receptor 1 (PAC1R), VIP and PACAP receptor 1 (VPAC1R), and VIP and PACAP receptor 2 (VPAC2R) [6]. Both VIP and PACAP share all three receptors of this system. Although it has been shown that PACAP binds to the PAC1R with a 1000-fold higher affinity than VIP [7, 8], functional analyses indicate a strong impact of VIP on PAC1R-induced effects regarding immune reactions [9]. This discrepancy might be explained by the at least 14 different isoforms of the PAC1R that differ in VIP- and PACAP-binding affinities [10].

VIP and PACAP are widely distributed throughout the respiratory tract [11–14]. Here, both neuropeptides exert a bronchodilatory effect on airway smooth muscles, modulate mucus secretion, and display anti-inflammatory characteristics [6, 15, 16]. We therefore consider the VIP/PACAP system as a promising target for future pharmacological interventions in the treatment of obstructive and inflammatory lung diseases like asthma [6].

The anti-inflammatory potential of VIP and PACAP has been tested in various animal models of inflammatory diseases like septic shock [17], Crohn's disease [18], colitis [19, 20], and arthritis [21]. The transfer of anti-inflammatory effects after VIP or PACAP stimulation is mediated through reduction of pro-inflammatory cytokines as well as co-stimulatory surface molecules on different cell types. In this context, T cells [22], macrophages [23], monocytes [24], and dendritic cell (DC) [25] have been identified. Assignment of these anti-inflammatory effects to single receptors of the VIP/PACAP system has partly been performed using specific receptor agonists. Both receptors, VPAC1R and VPAC2R, induce immunomodulatory effects [26, 27]. The specific activation of VPAC1R and VPAC2R led to an increased chance of survival in a murine model of septic shock. VPAC2R additionally exhibits strong bronchodilatory effects [28]. The role of PAC1R in

the lung, however, remains unclear. The breeding of PAC1R-deficient mice [29] and isolation maxadilan (MAX), a peptidergic-specific PAC1R agonist from the saliva of the sandfly *Lutzomyia longipalpis* [30, 31], represent effective tools for investigating PAC1R-induced effects in the lung.

Because of the need for new drugs in the treatment of severe asthma, here, we analysed the anti-inflammatory potency of the VIP/PACAP system in order to assess whether combined synthetic VIP/PACAP receptor agonists may provide a future drug class that drives both bronchodilatory and anti-inflammatory mechanisms.

In the study presented, PAC1R mRNA was investigated in homogenized lung tissue and isolated cell populations of the immune system via real-time PCR in a first approach. Because of the anti-inflammatory effects of the VIP/PACAP system, *in vivo* experiments in the study presented were performed under inflammatory conditions: allergic airway inflammation was induced in PAC1R-deficient and wild-type mice (WT). Airway hyper-responsiveness (AHR) was detected using non-invasive whole-body plethysmography in response to methacholine (MCh), and airway inflammation was monitored. In a second *in vivo* approach application of the specific PAC1R agonist MAX was performed in a similar murine model of allergic airway inflammation as before. In this approach, AHR was monitored via non-invasive head-out body plethysmography and the immune response was characterized at the cellular level of the bronchoalveolar lavage fluid (BALF) as well as at the cytokine level.

Methods

Isolation of T cells from the spleen, dendritic cell, and macrophages from lung tissue

T cells were isolated from spleens of naïve mice by negative magnetic cell separation using the pan T-cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) with LD columns. Cells were isolated according to the manufacturer's instructions.

Pulmonary DC and macrophages were isolated in two steps. Firstly, CD11c-positive cells were isolated from homogenized lung tissue of naïve mice by positive magnetic cell separation using mouse CD11c-specific microbeads (Miltenyi). Secondly, CD11c-positive cells were fluorescently labelled for MHCII and CD11c. Via FACS sort using FACS aria (BD Biosciences, San Jose, CA, USA), CD11c^{low} and MHCII^{low} cells were considered as macrophages and CD11c⁺ and MHCII^{high} cells were considered as DC. Cell populations with a purity of at least 95% after reanalysis were used for further experiments.

Animals

Transgenic mice (PAC1^{-/-}) and their counterparts (C57BL/6×129 Sv) were kindly provided by Dr Philippe Brabet (Institut des Neurosciences de Montpellier, France) [29]. Female BALB/c mice were obtained from Charles River, Sulzfeld, Germany, and used at the age of 8–10 weeks. All animals received ovalbumin (OVA)-free laboratory food and tap water *ad libitum* at regular 12-h dark/light cycles at 22 °C. The animals were acclimatized for at least 1 week and randomized 6 days before the experiments started. The studies were approved by the competent governmental authority and were performed in compliance with the German animal protection law.

Induction of airway inflammation in pituitary adenylate cyclase activating peptide receptor 1-deficient mice

WT and PAC1R-deficient mice were divided into two groups. The OVA-induced allergic asthma model was applied as described before [32]. Briefly, WT mice (WT OVA/OVA) and

PAC1R-deficient mice (PAC1^{-/-} OVA/OVA) were systemically sensitized by administration of 20 mg OVA (Sigma Aldrich, Munich, Germany, OVA grade VI) emulsified in 2 mg aluminium hydroxide (Alum, Pierce, Rockford, IL, USA) in 50 μ L phosphate-buffered saline (PBS) by intraperitoneally (i.p.) injections on days 0 and 14. For control, WT mice (WT PBS/PBS) and PAC1R-deficient mice (PAC1^{-/-} PBS/PBS) were sham-sensitized with 50 μ L PBS and 2 mg Alum (Pierce). Mice were exposed to 1% OVA aerosol for 20 min on days 28 and 29. Animals of the negative controls were exposed to a PBS aerosol.

Asthma protocol in a pharmacological study

Female BALB/c mice were sensitized to OVA as described before [33]. Briefly, sensitization was achieved by an i.p. administration of 10 μ g OVA and 1.5 mg Alum emulsified in 50 μ L saline on days 0, 14, and 21 (OVA/OVA). For control, mice were sham-sensitized with 50 μ L saline and 1.5 mg Alum (NaCl/OVA). The challenge of mice to 1% OVA aerosol was performed for 20 min on days 27 and 28 using a jet-driven aerosol exposure system as described recently [34].

Treatment in the pharmacological study

Animals were treated with 2.5 μ g MAX in 50 μ L saline intranasally (i.n.) (OVA/OVA +MAX) and sham-treated with saline 2 h before challenge, respectively. The i.n. administration was performed under halothane (Sigma-Aldrich) narcosis. Treatment control mice received three i.p. injections of dexamethasone (Ratiopharm, Ulm, Germany) (OVA/OVA+DEX) 16 and 1 h before the first and 1 h before the second challenge.

Measurement of airway hyperresponsiveness

Twenty-four hours after OVA challenge, airway AHR was monitored using barometric whole-body plethysmography in response to aerosols from 3 to 50 mg/mL MCh (Sigma-Aldrich) as described before [35] or using head-out body plethysmography in response to aerosols from 2.5 mg/mL up to 40 mg/mL MCh as described before [34, 36]. MCh aerosolic provocation was performed using between 2.5 and 40 mg/mL. Percent decrease in tidal midexpiratory flow (EF₅₀) or increase in enhanced pause (PenH) in relation to baseline was determined.

Animal dissection

Twenty-four hours after AHR measurement, mice were sacrificed by an overdose of Narcoren (0.1 mg Narcoren in 300 μ L NaCl i.p.). Blood and BALF were collected as described before [37]. Part of the lung organs was either filled with/embedded in OCT and snap-frozen or fixed in 4% paraformaldehyde (50% of the animals of each group). Other parts of the lungs were frozen in liquid nitrogen.

Cell count

The total BALF cells and the number of macrophages, eosinophils, neutrophils, and lymphocytes were determined as described before [37].

Cytokine quantification

Cytokines in the BALF (IL-4, IL-5, IL-10, eotaxin-2, and IFN- γ) were quantified via the ELISA technique (R&D Systems, Wiesbaden, Germany) according to the manufacturers' instructions. Detection limits were assigned at 16 pg/mL for IL-4 and eotaxin-2 and at 32 pg/mL for IL-5, IL-10, and IFN- γ .

Histological staining

Giemsa staining of BALF cells was performed as described before [37]; trichrome staining was performed according to Masson Goldner. Immune fluorescence staining was performed in Shandon chamber slides. Sections (6 μm) were placed in Shandon chambers, rehydrated in PBS, and blocked with a 4% goat serum (Dianova, Hamburg, Germany). The primary antibody (rat anti-mouse major basic protein) (MBP), kindly provided by Dr J. Lee (Mayo Clinic Scottsdale, AZ, USA), was added to the slides for 60 min. Slides were washed, blocked with 4% donkey serum (Dianova), and incubated with the secondary antibody (alexa 594 donkey anti-rat IgG) (Molecular Probes, Eugene, OR, USA) for 60 min. Slides were washed, covered with vectashield, and stored overnight at 4 °C. Pictures were taken the next day.

Serum immunoglobulin E quantification

The total IgE and OVA-specific IgE levels of the blood serum were quantified using ELISA sets (R&D Systems; MD Biosciences, Zurich, Switzerland) appropriate to the targets. Detection limits were assigned at 1.6 ng/mL for total IgE and 7.8 pg/mL for OVA-specific IgE.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from homogenized lung tissue or isolated cells using the RNeasy RNA separation kit (Qiagen, Hilden, Germany) as described before [38]. The concentration of isolated RNA was quantified photometrically. Amounts of 800 ng total RNA were used for transcription into cDNA using the Omniscript RT kit (Qiagen). Two μL of transcribed cDNA were used as a template for quantitative RT-PCR with the Light Cycler system (Roche Applied Science, Mannheim, Germany) using the Light Cycler FastStart DNA Master SYBR green I (Roche Applied Science). All kits were used according to the manufacturers' instructions. Quantification of expression was performed via the RelQuant software normalized to the house keeping gene porphobilinogen deaminase (PBGD). All primers were designed to be intron-spanning to avoid the contamination of genomic DNA-derived PCR products. PCR products were verified via sequencing, gel electrophoresis, and melting curves. The primer sequences used for PAC1R and PBGD were: PBGD: sense: ATCCAGGT CCCTGTTCAGCAAGAA, antisense: CATTAAGCTGCCGTG CAACATCCA, (181 bp); PAC1: sense: CATAGGGGATTCTG GCTTTG, antisense: GCCTTCACCGACAGGTAGTAA, (183 bp).

Statistical methods

Data in figures are given as mean $\pm$ SEM. Gaussian distribution was checked via the Kolmogorov–Smirnov test. When $\alpha = 0.05$, Gaussian distribution was assumed. Statistics was performed using the unpaired two-tailed Student's *t*-test or one-way ANOVA with the Dunnett or the Bonferroni post-test. In the case of a non-parametric distribution, the Man–Whitney *U*-test was used to detect differences. *P*-values ≤ 0.05 were considered to be significant.

Results

Gene expression experiments

To investigate the expression of PAC1R in the lung, mRNA analyses were performed under physiological and inflammatory conditions in homogenized murine lung tissue. The PBGD-normalized expression of PAC1R was found to be increased under inflammatory conditions, when compared with physiological conditions (Fig. 1).

Pituitary adenylate cyclase activating peptide receptor 1 expression in isolated immune cells

To identify possible PAC1R-dependent effector cells, different leucocyte populations of naive mice were isolated from either lung tissue or spleen and investigated for PAC1R expression at the RNA level. According to the current literature, macrophages, T cells, and DC had been chosen as the most probable candidate immune cells to respond to MAX stimulation [22, 39, 40]. While no PAC1R expression was found in macrophages (Fig. 2a) and T cells (Fig. 2b), isolated DC appeared to express PAC1R (Fig. 2c).

Pituitary adenylate cyclase activating peptide receptor 1 depletion study

To investigate the impact of PAC1R on the pathogenesis of airway inflammation, a protocol for the induction of asthma-like symptoms in the respiratory tract was run with PAC1R-deficient mice and WT mice.

For determination of the allergic inflammation in the lung, BALF samples were collected and characterized at the cellular level. PAC1R-deficient mice that had been sensitized and challenged with OVA displayed significantly increased concentrations of total cells in the BALF, when compared with the WT control (Fig. 3a). Differentiation of BALF cells revealed significant increases in eosinophils (Fig. 3b). Both the total cell counts and the eosinophil cell counts were elevated over 200% in PAC1R-deficient animals, when compared with WT animals.

To assess the impact of PAC1R deficiency on lung function, PenH was measured under MCH provocation. The determination of PenH did not show any effects of PAC1R depletion on airway function (data not shown).

Pharmacological study

The PAC1R agonist MAX was used to modulate pathogenesis in a murine model of allergic asthma. To prove OVA sensitization, the IgE concentrations in the blood plasma were quantified. Total IgE (Fig. 4a) and OVA-specific IgE (Fig. 4b) were increased in the positive control group, the dexamethasone-treated group, and the MAX-treated group (Fig. 4). IgE levels were not influenced by MAX or dexamethasone treatment.

To determine the status of the allergic inflammation in the lung, BALF cells and cytokines were analysed. MAX treatment led to significant decreases in the total cell numbers (Fig. 5a) and eosinophil cell numbers (Fig. 5b) in the BALF in comparison with the positive control group and this decrease was of the same magnitude as with the treatment control dexamethasone. Total cells were reduced by about 50% and eosinophils by about 80%, when compared with the positive control. Neutrophil cell numbers were not reduced (Fig. 5c).

Additionally, immunohistochemical staining of eosinophils by fluorescence-labelled MBP-specific antibodies in lung sections supported these data (Figs 6a–d). In sections from MAX-treated animals, only a few detached eosinophils could be found, while staining of the positive control group revealed a diffuse fluorescent signal in the area of blood vessels and airways, in which single eosinophils could not be distinguished. This difference compared with the other experimental groups might be due to a degranulation of eosinophils induced by allergen contact. In addition, the concentrations of eotaxin-2 and IL-4 in BALF were significantly reduced in MAX-treated animals (Figs 7a and b). Further cytokine quantifications of IL-5, IL-10, and IFN- γ did not reveal any significant changes induced by MAX treatment, or cytokine levels were below the detection limit (data not shown).

The impact of agonistic stimulation of PAC1R on lung function was investigated by head-out body plethysmography. MAX did not induce any effect on AHR in response to MCh (Fig. 8).

In contrast, dexamethasone induced anti-inflammatory effects and additionally led to a complete inhibition of AHR.

Discussion

The major findings of this study are, firstly, the expression of PAC1R in pulmonary DC and up-regulation of PAC1R in murine lung during inflammation, secondly, the increase in allergic inflammation in the lung by PAC1R depletion, and thirdly, the reduction of allergic inflammation in the lung by treatment with the PAC1R agonist MAX.

Assignment of the VIP/PACAP-induced effects to the different receptors appears to be difficult because of the binding of both neuropeptides to all three receptors. Some effects have already been attributed to single receptors using specific receptor agonists or transgenic models with receptor depletions. Regarding the impact of the VIP/PACAP system on inflammatory diseases, anti-inflammatory and bronchodilatory effects have been linked with the VPAC1R and VPAC2R receptors [26], while the role of PAC1R remains unknown.

The PAC1R is expressed in various tissues of the mammalian body. Apart from a high expression in the central nervous system, it can be found in the eye [41], endocrine glands [42, 43], gonads [44], urinary tract [44], digestive system [44], lymphoid tissues [44], and the respiratory tract [45]. The expression of PAC1R in an immunomodulatory context has been described in peritoneal macrophages of rats, while lymphocytes were tested negatively for PAC1R [46, 47]. In the study presented, PAC1R expression was observed neither in lymphocytes nor in macrophages. However, pulmonary DC expressed PAC1R RNA – a finding supported by a study in which *in vitro* stimulation of DC with MAX resulted in modifications of surface molecule expression and cytokine release pattern [39]. The discrepancy with regard to former studies might be explained by alternative receptor expression due to differences between pulmonary macrophages and peritoneal macrophages or a different level of activation due to the different isolation techniques. Furthermore, Pozo and colleagues identified peritoneal macrophages by cell size rather than by surface molecules. In the study presented, macrophages were differentiated from DC by a different expression pattern of MHC class II molecules. The cells, which were isolated from rat peritoneum and characterized via cell size, might have been contaminated by PAC1R-positive DC.

The increase in PAC1R mRNA in lung tissue under inflammatory conditions indicates an involvement of PAC1R in inflammatory processes. Because of the very limited data on quantitative PAC1R expression under inflammatory and physiological conditions in the lung and cells of the immune system, it is not clear whether this effect is due to an increase in PAC1R expression in resident cells of the lung or due to the influx of PAC1R-positive cells into the lung.

In the OVA-induced model of allergic asthma, PAC1R deficiency resulted in a significant increase in allergic inflammation in the lung, which is demonstrated by increased total cell counts and eosinophil cell counts. Eosinophils are a marker for allergic airway inflammation in asthma and play a central role in the pathogenesis of asthma [48–50]. Therefore, the increased eosinophilia indicates a more severe allergic reaction in the respiratory tract of PAC1R-deficient mice, when compared with WT mice. This finding is supported by the study by Wheat et al. [39], in which a reduced release of pro-inflammatory cytokines like IFN- γ and TNF- α from DC and a modification of surface molecules were found after

MAX stimulation *in vitro*. They showed reduced expression of CCR7 receptor, a receptor that is involved in DC homing to the draining lymph nodes. Additionally, effects on co-stimulatory molecules like CD80 and CD83 were described, resulting in reduced T cell activation *in vitro*. These results represent a possible mechanism explaining the results observed in the present study. Lacking the effect of endogenous ligands, regulatory effects on allergic inflammation are inhibited and co-stimulatory molecules and adhesion molecules might be overexpressed in PAC1R-deficient mice, resulting in an exacerbation of the allergic inflammation as compared with WT controls. PACAP-positive nerve fibres have been identified in the respiratory tract of diverse mammals, including guinea-pigs, sheep, and ferrets, mainly colocalized with VIP [12]. Although PACAP-positive nerve fibres have not been identified in the respiratory tract of mice and yet it may be assumed that cholinergic and sensible neurons innervating from intrinsic airway ganglia, which have been identified as being VIP and PACAP positive in different mammals, may represent an endogenous source of PACAP also in mice [14, 13, 51].

The same publication provides a possible explanation for the impact of MAX in the OVA-induced asthma model. Treatment with MAX led to a significant reduction of airway inflammation in the model of allergic asthma used in our *in vivo* approach. The amount of eosinophils in BALF was reduced by >80% by MAX treatment. This was about as effective as the use of dexamethasone. The T-helper type 2 (Th2)-cytokine IL-4 was significantly reduced by MAX treatment, as well as the chemokine eotaxin-2, which is involved in the recruitment of eosinophils into lung tissue. IL-4 is produced mainly by Th2 cells [52]. It is therefore likely that MAX affects either Th-2 cell recruitment or function.

As described before, PAC1R mediated a reduction in T cell activation and a reduction of adhesion molecules after agonistic stimulation [39]. By modification of surface molecules of DC, T cell recruitment might be inhibited by both a delayed homing of DC to the draining lymph nodes and a reduced ability of DC in T cell activation. Because of the lack of activated and matured Th2 cells, the secretion of Th2 cytokines is reduced, resulting in a diminished allergic inflammation.

Although a reduction of airway inflammation was suggested to be associated with reduced AHR [53], it was not significantly influenced either by PAC1R depletion or by MAX treatment. Dexamethasone treatment leads to a reduction of both the inflammation and AHR. A closer look at the cell counts in BALF provides a possible explanation. While the recruitment of eosinophils was strongly inhibited by MAX treatment, neutrophils were not reduced. In contrast, dexamethasone led to a reduction of both eosinophils and neutrophils. A possible explanation would be that the anti-inflammatory effects of MAX were directed only against T cell-mediated inflammatory mechanisms, while the anti-inflammatory effects of dexamethasone are directed against T cell-mediated and pro-inflammatory effects. The increase of neutrophils in the lung of MAX-treated animals might have an impact on the AHR as described by Park et al. [54].

The data presented show strong anti-inflammatory effects induced by the PAC1R, which demonstrates its potential use in the treatment of inflammatory diseases like asthma. The fact that these effects are induced by agonistic stimulation represents a great benefit compared with approaches in which disadvantageous effects are inhibited. A large variety of new compounds that aimed to antagonize single immune mechanisms, including antibodies against IL-5 [55] or recombinant IL-12 [56], failed to reach widespread clinical use in the treatment of bronchial asthma over the past few years. When considering the enormous research efforts directed towards single immune mechanisms and the relatively disappointing history of drug development, current approaches to develop new strategies should be reappraised carefully. In this respect, it has become apparent that the redundancy

of pro-inflammatory cascades hampers the efficacy of single antagonists and that there are far more than only immune cell-secreted mediators involved in the pathogenesis of asthma [57].

Therefore, here, we suggest that the anti-inflammatory effects mediated by agonistic stimulation of PAC1R in combination with bronchodilatory effects for instance mediated by VPAC2R might represent an alternative to the standard treatment of bronchial asthma with LABA and corticosteroids.

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Fig. 1.

Quantitative analysis of PAC1R expression on the RNA level via real-time RT-PCR in homogenized lung tissue under physiological (NaCl/OVA) and inflammatory conditions (OVA/OVA), normalized to the housekeeping gene PBGD; statistical analyses were performed using the Mann–Whitney *U*-test; **P* 0.05; *n* = 7. OVA, ovalbumin; PAC1R, PACAP receptor 1; PACAP, pituitary adenylate cyclase activating peptide; PBGD, porphobilinogen deaminase.

Fig. 2. Representative agarose gel pictures of PCR products from isolated DC (a), macrophages (b), and T cells (c) of two experiments; *L* = 100 bp DNA-ladder, lanes 1 and 2: PBGD DNA, lane 3: PBGD RNA control, lanes 4 and 5: PAC1R DNA, lane 6: PAC1R RNA control. DC, dendritic cell; PAC1R, PACAP receptor 1; PACAP, pituitary adenylate cyclase activating peptide; PBGD, porphobilinogen deaminase.

Fig. 3.

BALF cell counts of the total cell numbers (a) and eosinophil cell numbers (b) for WT mice (WT) and PAC1R-deficient mice (PAC1^{-/-}) under physiological conditions (PBS/PBS) and inflammatory conditions (OVA/OVA); statistical analyses were performed using the one-way ANOVA and the Bonferroni post-test; **P* 0.05, ****P* 0.001; *n* = 5 to 8. PAC1R, PACAP receptor 1; PACAP, pituitary adenylate cyclase activating peptide; WT, wild type; OVA, ovalbumin; PBS, phosphate-buffered saline.

Fig. 4.

Blood plasma total IgE levels (a) and OVA-specific IgE levels (b) for non-sensitized negative controls (NaCl/OVA), OVA-sensitized and -challenged positive controls (OVA/OVA), OVA-sensitized and -challenged dexamethasone-treated group (OVA/OVA+DEX), OVA-sensitized and -challenged MAX-treated animals (OVA/OVA+MAX); statistical analyses were performed using the Mann–Whitney *U*-test; ***P* 0.01 compared with the positive control group; *n* = 6. OVA, ovalbumin; MAX, maxadilan.

Fig. 5.

BALF cell counts of the total cell numbers (a), eosinophil cell numbers (b), and neutrophil cell numbers (c) for non-sensitized negative controls (NaCl/OVA), OVA-sensitized and -challenged positive controls (OVA/OVA), OVA-sensitized and -challenged dexamethasone-treated treatment controls (OVA/OVA+DEX), OVA-sensitized and -challenged MAX-treated animals (OVA/OVA+MAX); statistical analyses were performed using the Mann-Whitney *U*-test; **P* 0.05, ****P* 0.001; compared with the positive control group; *n* = 16. OVA, ovalbumin; MAX, maxadilan.

Fig. 6. Representative pictures of immune fluorescent staining of eosinophils in paraffin sections. Non-sensitized negative controls (NaCl/OVA) (a), OVA-sensitized and -challenged positive controls (OVA/OVA) (b), OVA-sensitized and -challenged dexamethasone-treated treatment controls (OVA/OVA+DEX) (c), and OVA-sensitized and -challenged MAX-treated animals (OVA/OVA+MAX) (d). OVA, ovalbumin; MAX, maxadilan.

Fig. 7.

BALF eotaxin-2 quantification at the protein level. Non-sensitized negative controls (NaCl/OVA), OVA-sensitized and -challenged positive controls (OVA/OVA), OVA-sensitized and -challenged dexamethasone-treated treatment controls (OVA/OVA+DEX), OVA-sensitized and -challenged MAX-treated animals (OVA/OVA+MAX); statistical analyses were performed using the one-way ANOVA and the Dunnett post-test compared with the positive control group; * P 0.05, ** P <0.01, *** P <0.001; n = 6. OVA, ovalbumin; MAX, maxadilan.

Fig. 8.

Lung function measurement via head-out body plethysmography under MCh provocation to assess AHR. Non-sensitized negative controls (NaCl/OVA), OVA-sensitized and -challenged positive controls (OVA/OVA), OVA-sensitized and -challenged dexamethasone-treated treatment controls (OVA/OVA+DEX), OVA-sensitized and -challenged MAX-treated animals (OVA/OVA+MAX); effective inhalational dose (ED₅₀) of MCh in µg required to decrease the midexpiratory flow by 50%; statistical analyses were performed using the Mann–Whitney *U*-test; **P* 0.05 compared with the positive control group; *n* = 16. AHR, airway hyperresponsiveness; OVA, ovalbumin; MAX, maxadilan; MCh, methacholine.