

# Development and characterisation of a novel and rapid lung eosinophil influx model in the rat

Melanie Werner-Klein<sup>1,2</sup>, Rolf Göggel<sup>2</sup>, Andreas Westhof, Klaus J. Erb<sup>\*</sup>

Department of Pulmonary Research, Boehringer Ingelheim Pharma GmbH & Co. KG, H91-02-01, Birkendorferstr. 65, D-88397 Biberach a.d. Riss, Germany

## ARTICLE INFO

### Article history:

Received 18 January 2008

Received in revised form

29 February 2008

Accepted 26 March 2008

### Keywords:

Asthma

Eosinophils

Rat

Steroids

Inflammation

Animal model

Therapeutics

## ABSTRACT

Eosinophils play a major role in the development and severity of asthma. Robust and rapid preclinical animal models are desirable to profile novel therapeutics inhibiting the influx of eosinophils into the airways. To develop a rapid, airway eosinophil recruitment model in the rat, Brown-Norway (BN) rats were immunised with ovalbumin (OVA)/alum on day 0, 1 and 2 and challenged with OVA aerosol on day 5 and 6. On day 7 bronchoalveolar lavage fluid (BALF) was analysed for eosinophil numbers, eosinophil peroxidase (EPO) activity and cytokines. Lung sections were also examined. The immunised animals showed a strong selective influx of eosinophils into the airways correlating with enhanced EPO activity, Interleukin (IL-4), IL-5 and monocytes chemo attractant protein levels in the BALF in comparison to sham-sensitised rats. In addition the immunised rats developed goblet cell metaplasia in the lung and showed OVA specific IgG1 and IgE levels in the serum but no airway hyperreactivity after metacholine challenge. Airway inflammation was suppressed by applying the steroids Budesonide (intra tracheally) and Prednisolone (per orally), Roflumilast a phosphodiesterase-4 inhibitor, and the H1 receptor antagonists Epinastine and Ketotifen. Montelukast, a Leukotriene receptor antagonist and Chromoglycate, a mast cell stabiliser, had no effect in this model. In summary, in this novel preclinical rat model therapeutics expected to inhibit the development of airway eosinophilia can rapidly be tested.

© 2008 Elsevier Ltd. All rights reserved.

## 1. Introduction

Asthma is characterised by chronic airway inflammation, airway hyper-responsiveness (AHR), and in the most severe state airway remodelling. The airway inflammation is dominated by activated eosinophils, which are present in large numbers in the lung parenchyma and the broncho-alveolar lavage fluid (BALF). Airway eosinophilia was found to correlate with asthma severity and AHR in both atopic and non-atopic asthma patients [1,2]. In addition, there is evidence suggesting that eosinophils play a major role in asthma exacerbations [3]. Eosinophils produce and release numerous toxic and pro-inflammatory mediators such as major basic protein, eosinophil cationic protein, eosinophil peroxidase (EPO), lipid mediators (leukotrienes, platelet activating factor), cytokines and chemokines (IL-1, IL-3, IL-4, IL-5, MCP-1, TNF- $\alpha$ ) that contribute to airway inflammation and cause damage of the airway tissue and nerves [1,4]. Furthermore, the eosinophil and its mediators seem to be involved in the development of

bronchial hyperreactivity as it significantly correlates with blood and airway eosinophilia [1].

Numerous animal studies support the hypothesis that eosinophils play an important role in the pathophysiology of asthma in humans. For example, mice immunised with OVA which show a strong reduction in eosinophil numbers [5,6], do not develop AHR [5,6] and show less severe airway remodelling in comparison to control animals [7,8]. These findings are supported by other studies indicating that eosinophils may activate fibroblasts in the inflamed tissue either directly or by the induction of transforming growth factor- $\beta$  (TGF- $\beta$ ), thereby inducing airway fibrosis [9–13]. These findings are supported by a recent human trial where patients treated with mepolizumab (anti-IL-5 antibody), correlating with a strong reduction of eosinophils in the blood and BAL of the patients, showed reduced amounts of tissue matrix proteins in the airways [14]. Interestingly, the FEV<sub>1</sub> in these patients did not improve, generally questioning the pathogenic role of eosinophils in human asthma. However, the finding that airway tissue eosinophil numbers were not reduced in these patients may explain the lack of improved FEV<sub>1</sub>.

Asthma models in rodents are commonly used to test and profile new therapeutics for the treatment of asthma in humans. The most commonly used models are based on different sensitisation/airway challenge schemes of mice and rats with

<sup>\*</sup> Corresponding author. Tel.: +49 07351 54 94005; fax: +49 7351 8394005.

E-mail address: [klaus.erb@bc.boehringer-ingelheim.com](mailto:klaus.erb@bc.boehringer-ingelheim.com) (K.J. Erb).

<sup>1</sup> Present address: University of Regensburg, Institute for Immunology, Franz-Josef Strauss Allee 11, 93053 Regensburg, Germany.

<sup>2</sup> These two authors have contributed equally to this work.

ovalbumin (OVA) or other allergens [15–18]. Most currently used rat asthma models use BN rats immunised with OVA/alum with or without heat-killed *Bordetella pertussis* as adjuvant followed by one to several OVA aerosol challenges [16–20]. These animals develop AHR and airway eosinophilia. When heat-killed *B. pertussis* is used as an adjuvant airway inflammation is more pronounced [21]. While these models are excellent tools to validate therapeutics and investigate features of asthma in rats they take at least 3–4 weeks and usually also have a significant neutrophilic inflammation in the airways. The aim of our current study was to develop a more rapid airway eosinophil-specific influx model in BN rats which takes only a week. We were successful and found that in this model a very strong selective influx of eosinophils into the airways occurs correlating with enhanced EPO activity in BAL and that these effects could be suppressed when the animals were treated with steroids, histamine 1 receptor (H1) and PDE-4 antagonists, indicating that this model may also be predictive for human disease.

## 2. Materials and methods

### 2.1. Animals

Specific pathogen-free male Brown Norway (BN) rats and Balb/c mice were purchased from Charles River, Germany at 7–9 weeks of age. Food and water were supplied *ad libitum*. Animal care and experiments were in accordance with institutional guidelines.

### 2.2. Immunisation and exposure to OVA

Mice and rats were immunised by intraperitoneal (i.p.) injection with a mixture of OVA and alum (1 mg/kg OVA, SERVA, Heidelberg, Germany; 200 mg/kg (mice) 13.3 mg/kg (rats) alum, Sigma-Aldrich, Taufkirchen, Germany) and challenged with OVA aerosol for 20 min as indicated. Negative control mice and rats were sham immunised with PBS/alum and then challenged with an aerosol of PBS or 0.9% NaCl, respectively. At indicated time points animals were sacrificed and analysed. In some experiments rats were immunised with OVA/alum subcutaneous (s.c.) and  $1 \times 10^8$  heat-killed *Bordetella pertussis* (generously provided by Dr. Hoymann, ITEM, Hannover, Germany) i.p. on days 0, 14 and 21. On day 28 and 29 the rats were challenged with OVA aerosol and analysed on day 30.

### 2.3. Application of test compounds

The amount used for the different experiments is indicated in the figures or mentioned in the text as data not shown. 6- $\alpha$  Methylprednisolone 21-hemisuccinate (Sigma-Aldrich, Taufkirchen, Germany) and montelukast-natrium (Singulair-tablets, MSD Sharp+Dohme GmbH, Haar, Germany) were applied per orally (p.o.) in 0.5% Natrosol (Merck, Darmstadt, Germany). Roflumilast (synthesised by the chemical department of Boehringer-Ingelheim) was either micronised in PBS/0.1% Tween 80 for the intra tracheal (i.t.) application or given p.o. in 0.5% Natrosol. Budesonide (Sigma-Aldrich, Taufkirchen, Germany) was micronised in PBS/0.1% Tween 80 and applied i.t. into the lung of rats. All of these substances were applied on days 5 and 6, 1 h before the aerosol challenge with OVA (with the exception of Roflumilast which was applied p.o. or i.t. 2 h before OVA aerosol challenge). Epinastine (Boehringer Ingelheim Pharma, Ingelheim, Germany), Ketotifen (Sigma-Aldrich, Taufkirchen, Germany), and Cromoglycate (Sigma-Aldrich, Taufkirchen, Germany) were all applied p.o. together with 0.5% Natrosol on days 5 and 6 1 h before and 6 h after OVA

aerosol challenge. Sham immunised and aerosol challenged negative control rats were also p.o. or i.t. sham treated with the respective solvent used for the test compound.

### 2.4. Bronchoalveolar lavage

A bronchoalveolar lavage (BAL) was prepared as previously described [22]. An amount of 200  $\mu$ l aliquot of lavage fluids were spun down on glass slides, stained with May–Grünwald–Giemsa and 300 cells/slide were microscopically differentiated.

### 2.5. Lung histology

The lung of rats was removed, fixed in 4% formaldehyde/PBS, dehydrated, mounted in paraffin, sectioned and stained with Alican blue or hematoxylin and eosin (H&E).

### 2.6. Measurement of airway reactivity to methacholine

AHR to methacholine in mice and rats was assessed using whole-body-plethysmography (Buxco, Wilmington, NC, USA) as described previously [22].

### 2.7. Detection of ova-specific IgG<sub>1</sub> and IgE in the serum of rats

Allergen-specific IgE and IgG<sub>1</sub> antibody concentrations in serum were measured by ELISA as described previously [22]. OVA-specific immunoglobulin-titres are shown as the inverse of the dilution that yielded a  $\Delta OD_{405-495 \text{ nm}}$  two-fold higher than the  $\Delta OD_{405-495 \text{ nm}}$  at a  $\frac{1}{5}$  (IgE) or  $\frac{1}{100}$  (IgG<sub>1</sub>) dilution of a negative control serum (sham-sensitised and -challenged controls).

### 2.8. Detection of EPO activity in the BAL fluid

Human EPO (1  $\mu$ g/ml) was obtained from Planta Natural Products Life Science, USA. Serial dilutions in PBS were prepared containing 1–7.81 ng/ml. Substrate solution was prepared by adding 500  $\mu$ l 50 mM O-Phenylenediamine OPD (Sigma-Aldrich, Taufkirchen, Germany) to 24.25 ml Tris buffer (pH 8.0), 3  $\mu$ l 30% hydrogen peroxide (Merck, Darmstadt, Germany) and 25  $\mu$ l TritonX100 (Sigma-Aldrich, Taufkirchen, Germany). 100  $\mu$ l substrate solution was incubated for 30 min at room temperature together with 100  $\mu$ l cell free BAL-fluid or the EPO-standard dilutions. The reaction was stopped by adding 50  $\mu$ l 2 M H<sub>2</sub>SO<sub>4</sub>. Each sample was prepared in duplicate and the OD<sub>490 nm</sub> was determined by using a Power-Wave-HT from Biotek, USA. Specificity for EPO activity was controlled by adding 4  $\mu$ l 1,2,4-Triazol-3-Ylamin AMT (Sigma-Aldrich, Taufkirchen, Germany) to the samples and by subtracting the OD of the sample +AMT from the OD of the sample without AMT.

### 2.9. Detection of cytokines in the BAL fluid

BALF from two individual rats were pooled ( $n = 8$  samples per group) and analysed with a multiplex assay (LINCOplex, LINCO Research Inc., St. Charles, MO, USA) designed to detect simultaneously the following cytokines: IL-1 $\alpha$ , -1 $\beta$ , -2, -4, -5, -6, -10, -12p70, -18, GRO/KC, TNF- $\alpha$ , IFN- $\gamma$  and MCP-1. The assay was performed according to the manufacturer's instructions.

### 2.10. Flow cytometry

Cells from the BALF were stained with monoclonal antibodies specific for CD4 (Ox-35, FITC), CD8 (Ox-8, PE), CD45RA

(Ox-33, FITC) and HIS48 FITC (staining granulocytes) and RP-1, PE (staining neutrophils) as described previously [22]. All monoclonal antibodies were purchased from Becton Dickinson/Pharmingen (Mountain View, CA, USA).

### 2.11. Statistical analysis

Data were either analysed by unpaired students t-test, when only two samples were compared, or in all other cases by Dunnett's multiple comparison test following one way ANOVA (GraphPadPrism 4.03, GraphPad Software Inc. San Diego, USA). In case of heteroscedasticity data were log-transformed prior to analysis.  $P < 0.05$  was considered to be significant.

## 3. Results

### 3.1. Development of a rapid eosinophil influx model in rats

Balb/c mice immunised and challenged with OVA develop airway eosinophilia and AHR to metacholine challenge (data not shown and [15]). Next we used a similar sensitisation scheme in BN rats. We chose BN rats, because similar to Balb/c mice, this strain shows a stronger intrinsic tendency to develop Th<sub>2</sub> responses in comparison to other rat strains [23–25]. The animals were immunised with OVA on days 0, 14 and 21 and exposed to OVA on two (d26, 27) or three (d25, 26, 27) consecutive days. Alternatively, rats were immunised only twice on days 0 and 7 or days 0 and 14 and challenged six and seven days after the last challenge (d13, 14 or d26, 27). Although the number of infiltrating eosinophils into the airways and EPO activity in BALF was significantly increased in most of the protocols used in comparison to sham-treated controls, they remained relatively low (Fig. 1A and B). All the immunisations lead to increased numbers of neutrophils present in BALF. In OVA-immunised and challenged animals no increased AHR after metacholine challenge was detected (Fig. 1C).

We also tested the use of heat-killed *Bordetella pertussis* as an adjuvant in combination with OVA/alum (see methods). We found, that although this protocol increased the amounts of eosinophils detected in BALF and enhanced the AHR to methacholine, large amounts of neutrophils were also detected (data not shown). For this reason and since the model takes more than 4 weeks, we decided to try and develop a more rapid and selective eosinophil influx model. For this purpose we immunised BN rats with OVA/alum on days 0, 1 and 2 and challenged the animals on day 5 with OVA aerosol. Fig. 2A shows that this protocol resulted in high eosinophil numbers detected in the BALF. However, the EPO-level was not significantly increased as compared to sham-treated animals (Fig. 2B). A second OVA-exposure on day 6 resulted in a doubling of eosinophil numbers with an even higher increase in EPO activity detected in the BAL fluid (Fig. 2A and B). Importantly, no increase in neutrophil numbers was observed. To determine whether less than three immunisations with OVA/alum would be sufficient to induce this phenotype, animals were immunised on day 0 only, days 0 and 1 or days 0 and 2. In addition, to exclude that the amount of antigen on day 0 might be the limiting factor, the antigen and alum amount of all three applications was given on day 0 at once. Fig. 2A and B shows that the number of eosinophils and the amount of EPO activity in the BALF was not significantly increased as compared to the control if the immunisation was performed only once on day 0. Immunisations on two days (days 0 and 1 or days 0 and 2) lead to a significant increase in eosinophils in BALF compared to sham-treated controls (Fig. 2A). The highest numbers of eosinophils

were obtained when the rats were immunised on days 0, 1 and 2. The results for the EPO activity measurement were similar (Fig. 2B). Taken together our results clearly show that the protocol shown in Fig. 2C yielded the best results.

### 3.2. Immunological characterisation of the eosinophil influx model in the BN rat

Next we characterised this model in more detail. Fig. 3A shows that the protocol shown in Fig. 2C induces significant levels of OVA-specific IgE and IgG<sub>1</sub> in the serum of the rats. Furthermore, increased levels of IL-4, IL-5 and MCP-1 in BALF as compared to sham-treated rats were observed (Fig. 3E). In contrast, IL-1 $\alpha$ , -1 $\beta$ , -2, -6, -10, -12p70, -18, GRO/KC, TNF- $\alpha$  and IFN- $\gamma$  could not be detected (data not shown). Although eosinophils and macrophages were the most prominent cell types present in the BALF, flow cytometric analysis revealed that the number of T cells (in particular CD4<sup>+</sup>) and B cells was significantly elevated as compared to control-animals (Fig. 3B and D). By using a combination of an anti-RP-1 (binds to neutrophils but not eosinophils) and anti HIS48 mAb (binds to neutrophils and eosinophils) neutrophils could readily be distinguished from eosinophils (Fig. 3D). No increased AHR in response to metacholine was detected one day after the last challenge with OVA (Fig. 3C). Lung histology revealed that OVA immunised and challenged rats showed peribronchial airway inflammation (Fig. 4B vs. A) characterised by large numbers of infiltrating eosinophils (data not shown). Furthermore, goblet cell hyperplasia was observed in OVA immunised and challenged rats in comparison to control animals (Fig. 4D and F vs. C and E). Animals immunised with OVA and challenged with PBS or sham immunised with PBS and challenged with OVA showed no allergen-specific Th2 responses in the airways (data not shown).

### 3.3. Pharmacological substances reducing the influx of eosinophils into the airways

To further characterise and validate the model we tested several reference compounds which have been shown or may be expected to reduce the development of airway eosinophilia both in patients or other rodent models of asthma. For this purpose rats were treated with the different substances at day 5 and 6 as described in material and methods. Fig. 5 shows that airway eosinophilia and EPO activity in the BALF was suppressed when the animals were pre-treated before allergen challenge with the steroids Prednisolone (p.o.) and Budesonide (i.t.). The PDE-4 inhibitor Roflumilast (p.o. or i.t.) and the H1 receptor antagonist Epinastine (p.o.) also significantly reduced the influx of eosinophils into the airways and EPO activity in the BALF. The second H1 receptor antagonist Ketotifen (p.o.) also reduced airway eosinophilia, but to a weaker degree. In contrast, treating the rats with the Leukotriene receptor antagonist Montelukast or the mast cell stabiliser Cromoglycate had no suppressive effects at a dose of 10, 30 or 100 mg/kg. Table 1 shows the ED<sub>50</sub> values of the different pharmacological compounds tested.

## 4. Discussion

The aim of our current study was to attempt to develop a selective and rapid eosinophil recruitment model in the BN rat. Firstly we immunised and challenged the rats with OVA using a protocol which is well established in Balb/c mice. We found, that in contrast to the mice, the rats did not develop an AHR after metacholine challenge using full body plethysmography and also

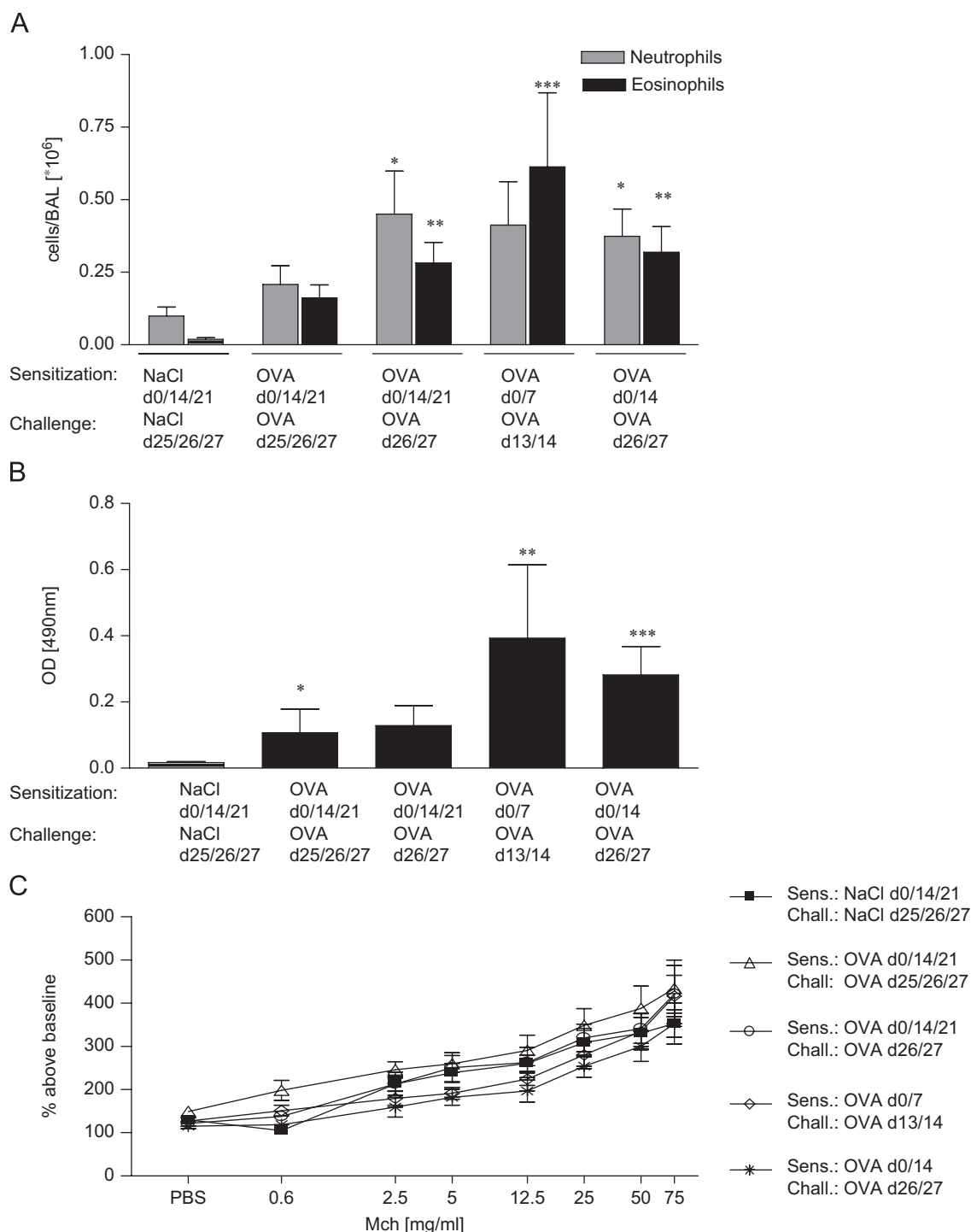


**Fig. 1.** Different OVA immunisation and challenge protocols in BN rats. BN rats were immunised and challenged with OVA or PBS as indicated. Absolute cell numbers of eosinophils (black) and neutrophils (grey) in BALF one day after the last aerosol challenge with OVA are shown (A). EPO activity in BALF one day after the last OVA challenge (B). Airway reactivity to metacholine (Mch) of OVA-immunised and -challenged or sham-treated rats (C). Shown is the mean increase  $\pm$  SEM above baseline in % measuring enhanced pause ( $P_{\text{enh}}$ ) of  $n = 8$ /group.  $P < 0.05^*$ ,  $P < 0.01^{**}$  and  $P < 0.001^{***}$  in comparison to the PBS control group.

did not show a strong influx of eosinophils into the airways. Furthermore, the rats also developed a neutrophilia to the same extent as an eosinophilia.

Next we tried several variations of the immunisation protocol to induce a stronger and selective airway eosinophilia. We found that rats immunised on day 0, 1, 2 with OVA/alum i.p. and challenged with OVA aerosol on day 5 and 6 developed a strong airway eosinophilia correlating with strongly enhanced levels of EPO detected in the BALF on day 7. The number of

eosinophils detected in BALF were enhanced or similar to the numbers published by other investigators using different and longer immunisation protocols [16–18,26–29]. The very short immunisation scheme we used seems to favour the generation of a selective eosinophilia since only very low numbers of neutrophils were detected. The neutrophilia observed in the other models may be due to OVA specific  $\text{Th}_1$  responses which may take longer to develop than the allergen-specific  $\text{Th}_2$  responses.

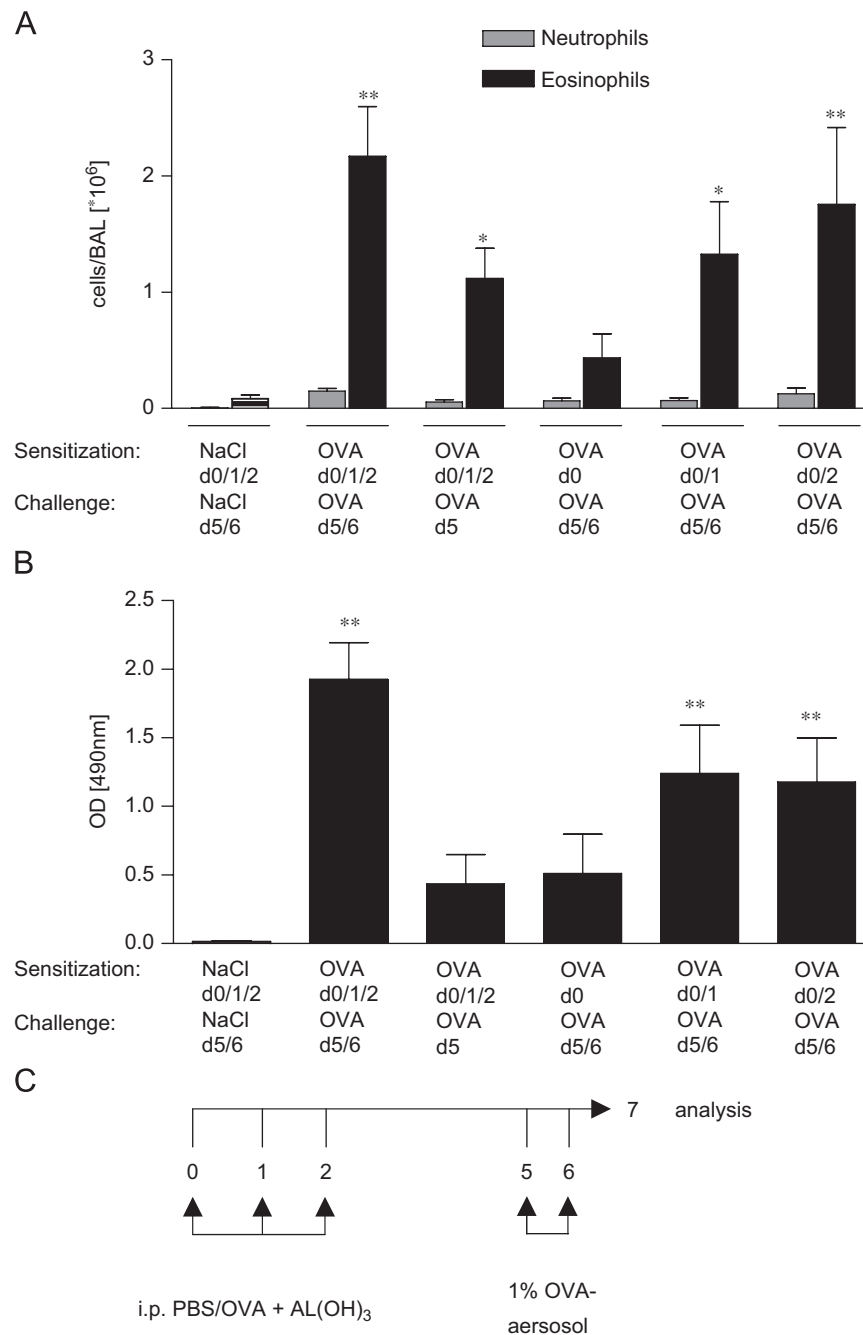


**Fig. 2.** Rapid allergen-induced airway eosinophil recruitment models in BN rats. BN rats were immunised and challenged with OVA or PBS on different days as indicated. Analysis was performed on day 7 after the first immunisation with OVA or PBS. Absolute cell numbers (A) of eosinophils (black) and neutrophils (grey) and EPO activity (B) in BALF are shown. Mean  $\pm$  SEM values ( $n = 8$  per group) are shown. The immunisation protocol yielding the highest eosinophil numbers in the BAL is shown in (C). Data from the first two groups are representative for at least five independent experiments.  $P < 0.05^*$  and  $P < 0.01^{**}$  in comparison to the PBS control group.

We further characterised our model in greater detail and found that surprisingly our short immunisation protocol was able to induce detectable levels of OVA-specific IgG<sub>1</sub> and IgE in the serum, increased numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells in the BALF and goblet cell metaplasia in the airways. Enhanced levels of IL-4, IL-5 and MCP-1 were also detected in the BALF. These findings suggest that although the immunisation protocol is very short, it is sufficient to induce allergen-specific Th<sub>2</sub>-type responses in the airways. The presence of OVA-specific IgE and IgG<sub>1</sub> in the serum supports this view. The most likely explanation for the development of the detected airway eosinophilia is that the IL-5 produced by Th<sub>2</sub> cells in the lung was responsible for the

recruitment of the eosinophils into the airways, since IL-5 is both a survival and recruitment factor for eosinophils [30]. However, other factors may also contribute.

Using our short immunisation protocol we could not detect an increased airway reactivity to methacholine, which is in contrast to protocols using the same sensitisation scheme [16] (on three consecutive days), but with exposure to OVA-aerosol two weeks later than in our setting (day 21 vs. day 7). We can only speculate why in our model no AHR develops. The most likely explanation is that in our short model eosinophils may not be present long enough in the lung to cause AHR. Supporting this view is that both in mice and rats AHR usually correlates with the presence of

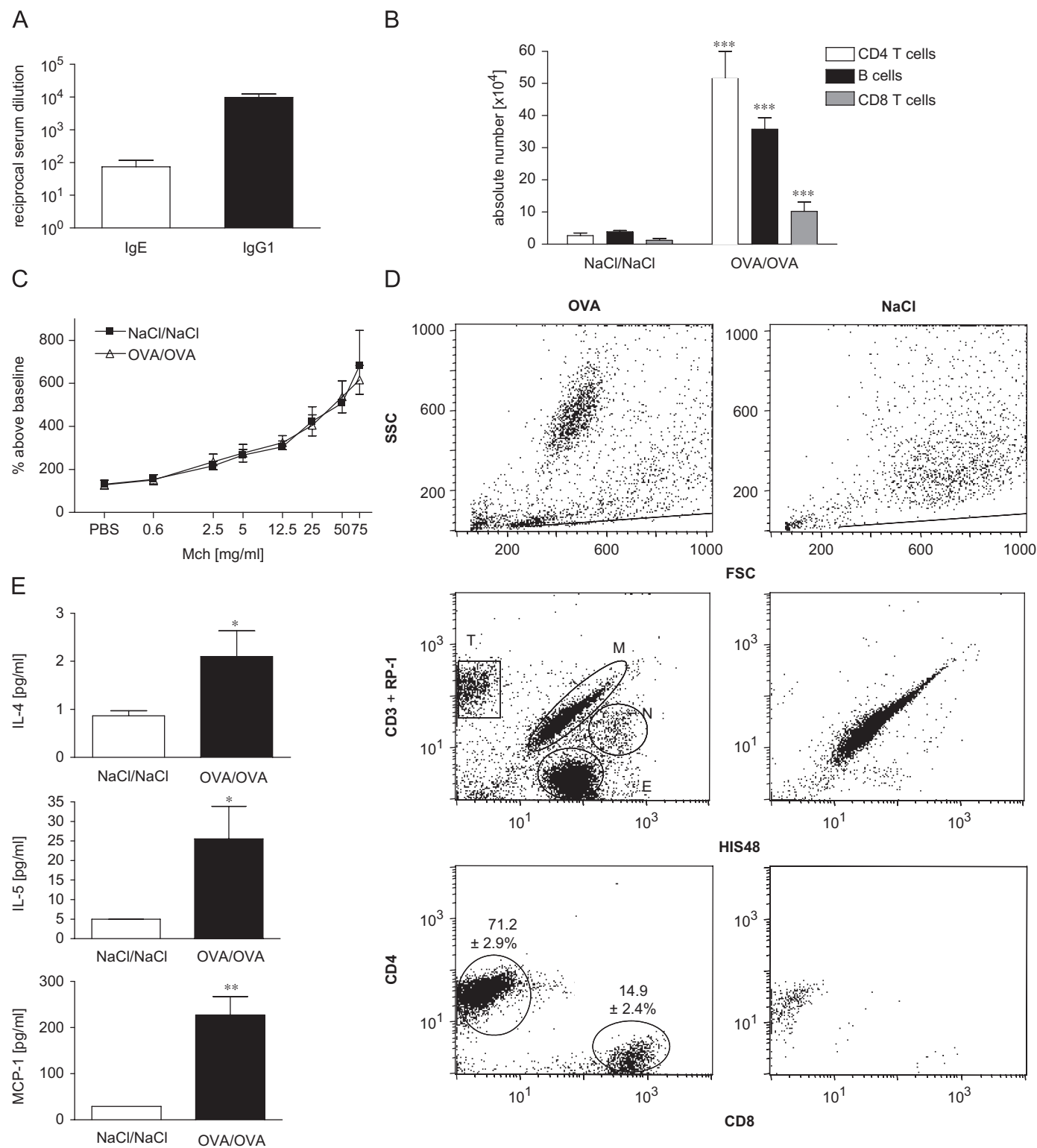


**Fig. 3.** Immunological characterisation of the established eosinophil influx model in BN rats. BN rats were immunised, challenged and analysed as shown in Fig. 2C. (A) Reciprocal OVA-specific IgE and IgG<sub>1</sub> titres in the serum (mean ± SEM ( $n = 8$ ), representative for three independent experiments). (D) Flowcytometric analysis of cells from the BALF. Data shown are representative of two separate measurements of two pooled BAL-samples (the cells from eight rats/group were pooled). Cells were stained with a combination of HIS48-FITC, CD3 $\epsilon$ -PE and RP-1 PE or with CD3 $\epsilon$ -APC, CD4-FITC and CD8 $\alpha$ -PE. E = Eosinophils; M = Macrophages; N = Neutrophils; T = CD3<sup>+</sup> T cells are indicated. The stainings showing CD4 and CD8 positive cells were gated on CD3<sup>+</sup> cells and total numbers of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and B cells detected in the BAL are shown in (B). (C) Airway reactivity to metacholine (Mch) of OVA-immunised and -challenged or sham-treated rats. Shown is the mean increase ± SEM above baseline in % measuring enhanced pause ( $P_{enh}$ ) of  $n = 8$ /group. (E) IL-4, IL-5 and MCP-1 levels detected in BALF. Shown are the mean values ± SEM of  $n = 8$  per group (the BALF of two rats/group was pooled resulting in four samples/group being analysed).  $P < 0.05^*$  and  $P < 0.01^{**}$  in comparison to the NaCl control group.

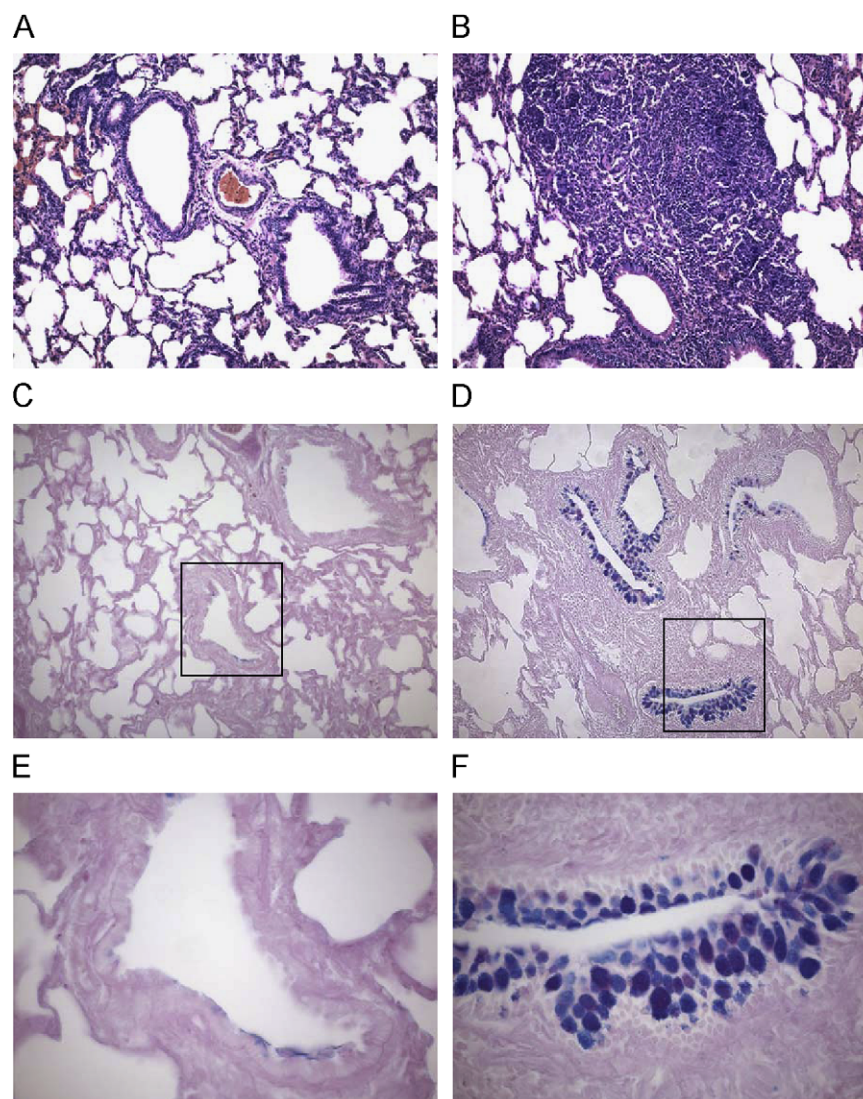


**Fig. 4.** Rats immunised and challenged with OVA develop goblet cell metaplasia and airway inflammation. BN rats were immunised and challenged as indicated in Fig. 2C. Shown are the lung sections subjected to H&E (A and B) or Alcian blue (C–F) staining from NaCl (A, C and E) or OVA (B, D and F) immunised mice. Representative examples of eight rats/group are shown (A–D  $\times 10$ ; E and F  $60\times$  magnification).

eosinophils and a therapeutic intervention reducing eosinophils also reduces AHR [5–8,15–21]. A further possibility is that the neutrophilia, which develops after 21 days but not in our model, may cause the AHR. Supporting this hypothesis is a report by Leung et al. [28] where AHR to methacholine was found when both neutrophils and eosinophils were present in the BALF. However, a recent study in mice indicates that airway neutrophilia induced after allergen-challenge did not affect AHR [31]. A further possibility is that our used immunisation protocol is too short to induce sufficient amounts of OVA-specific Ig and OVA specific Th<sub>2</sub> cells needed to induce an AHR after methacholine challenge. Furthermore, the short immunisation protocol, associated with relatively low levels of allergen-specific antibodies, might also explain why the rats did not develop a neutrophilia, in comparison to using other longer protocols, since a neutrophilia was shown to be mediated by Fc $\gamma$ RIII and dependent on the presence of allergen-specific antibodies in a murine model of experimental asthma [32].

We also tested several compounds recommended for the treatment of asthma in humans (see “Global Initiative for Asthma” guidelines [33] to investigate which pharmacological mechanisms contributed to the induced airway eosinophilia. The

steroid Prednisolone (applied p.o.) strongly reduced eosinophilia. The steroid Budesonide (applied i.t.) had the same efficacy but had an approximately 100 times higher potency. The two tested H1 antagonists Epinastine and Ketotifen showed a different profile of activity in our experiments. Epinastine showed a strong anti-inflammatory effect on eosinophil recruitment into the lung whereas Ketotifen showed the same efficacy but a lot weaker potency. This finding confirms previously published results comparing Epinastine with Ketotifen [34]. Epinastine also binds to serotonin receptors (5HT<sub>2</sub>) and has other modes of action apart from its antihistaminic activity which also may explain its superiority over Ketotifen [35]. The mast cell stabiliser Cromoglycate did not show any effect in our experiments and was reported to have only weak anti-inflammatory effects in humans [36]. We also tested Montelukast, a leukotriene antagonist, and found that the drug was also not effective in reducing airway eosinophilia. This finding is in line with clinical studies showing only weak effects of Leukotriene receptor antagonists as mono-therapy on airway inflammation [33]. Finally we tested Roflumilast, a new drug inhibiting PDE-4 and previously shown to be effective in the treatment of asthma in animal models [37,38]. Roflumilast given either orally or i.t. strongly reduced OVA induced airway

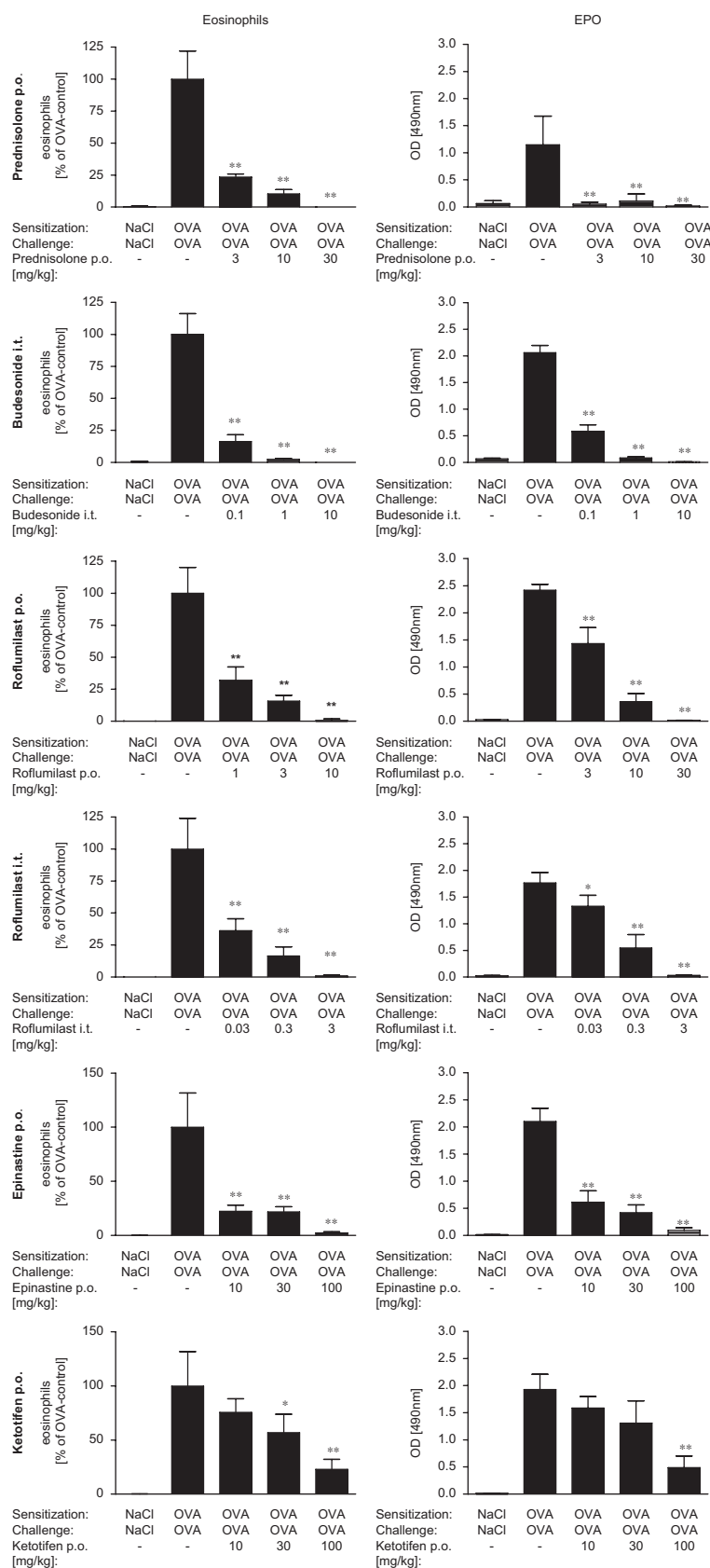


**Fig. 5.** Reference substances inhibiting the recruitment of eosinophils into the airways of rats. Rats were immunised and challenged as indicated in Fig. 2C and treated with the different test substances as indicated and described in the methods part. The mean percent inhibition  $\pm$  S.E.M in eosinophil influx or EPO activity in the BALF of eight rats per group is shown.  $P < 0.05^*$ ,  $P < 0.01^{**}$ ,  $P < 0.001^{***}$  in comparison to the OVA control group (0% inhibition).

**Table 1**

Effective dose of the different pharmacological substances tested reducing eosinophil numbers and EPO activity in the BALF by 50% in comparison to the values obtained in the positive control group (ED50)

| Reference compound | Eosinophils (mg/kg) | EPO (mg/kg) |
|--------------------|---------------------|-------------|
| Prednisolone p.o.  | <3                  | <3          |
| Budesonide i.t.    | 0.02                | 0.05        |
| Roflumilast p.o.   | <1                  | <1.2        |
| Roflumilast i.t.   | <0.03               | <0.1        |
| Epinastine p.o.    | <10                 | <10         |
| Ketotifen p.o.     | 34.4                | 46.1        |
| Cromoglycate p.o.  | >100                | >100        |
| Montelukast p.o.   | >100                | >100        |

eosinophilia, with the direct application of the drug into the lung being much more effective. Taken together these data indicate that the airway eosinophilia induced in this model was steroid sensitive and dependent upon PDE-4 and points at a minor mast cell component. Leukotrienes appear to play no role in our model, however, we cannot completely rule out the possibility that we would need to apply Montelukast more often to have an effect.

In conclusion, we developed a rapid airway eosinophil influx model suitable to test anti-inflammatory compounds, targeting eosinophils, for the treatment of asthma. The findings that steroids, H1 antagonists and the PDE-4 inhibitor Roflumilast were very efficacious in our model suggest that this model may also be predictive for the human disease.

## References

- Gleich GJ. Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol* 2000;105:651–63.
- Woodruff PG, Khashayar R, Lazarus SC, Janson S, Avila P, Boushey HA, et al. Relationship between airway inflammation, hyperresponsiveness, and obstruction in asthma. *J Allergy Clin Immunol* 2001;108:753–8.
- Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;360:1715–21.
- Kingham PJ, Costello RW, McLean WG. Eosinophil and airway nerve interactions. *Pulm Pharmacol Ther* 2003;16:9–13.
- Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996;183:195–201.
- Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, et al. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 2004;305:1773–6.
- Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, et al. Inhibition of airway remodeling in IL-5-deficient mice. *J Clin Invest* 2004;113:551–60.
- Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, et al. A critical role for eosinophils in allergic airways remodeling. *Science* 2004;305:1776–9.
- Gharaee-Kermani M, Phan SH. The role of eosinophils in pulmonary fibrosis (review). *Int J Mol Med* 1998;1:43–53.
- Levi-Schaffer F, Garbuzenko E, Rubin A, Reich R, Pickholz D, Gillery P, et al. Human eosinophils regulate human lung- and skin-derived fibroblast properties in vitro: a role for transforming growth factor beta (TGF-beta). *Proc Natl Acad Sci USA* 1999;96:9660–5.
- Montesano R, Orci L. Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. *Proc Natl Acad Sci USA* 1988;85:4894–7.
- Phipps S, Ying S, Wangoo A, Ong YE, Levi-Schaffer F, Kay AB. The relationship between allergen-induced tissue eosinophilia and markers of repair and remodeling in human atopic skin. *J Immunol* 2002;169:4604–12.
- Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 1999;160:1001–8.
- Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, et al. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest* 2003;112:1029–36.
- Epstein MM. Do mouse models of allergic asthma mimic clinical disease? *Int Arch Allergy Immunol* 2004;133:84–100.
- Haczku A, Moqbel R, Elwood W, Sun J, Kay AB, Barnes PJ, et al. Effects of prolonged repeated exposure to ovalbumin in sensitized brown Norway rats. *Am J Respir Crit Care Med* 1994;150:23–7.
- Schneider T, van VD, Moqbel R, Issekutz AC. Kinetics and quantitation of eosinophil and neutrophil recruitment to allergic lung inflammation in a brown Norway rat model. *Am J Respir Cell Mol Biol* 1997;17:702–12.
- Underwood SL, Haddad E, Birrell MA, McCluskie K, Pecoraro M, Dabrowski D, et al. Functional characterization and biomarker identification in the brown Norway model of allergic airway inflammation. *Br J Pharmacol* 2002;137:263–75.
- Eidelman DH, Bellofiore S, Martin JG. Late airway responses to antigen challenge in sensitized inbred rats. *Am Rev Respir Dis* 1988;137:1033–7.
- Eynott PR, Salmon M, Huang TJ, Oates T, Nicklin PL, Chung KF. Effects of cyclosporin A and a rapamycin derivative (SAR943) on chronic allergic inflammation in sensitized rats. *Immunology* 2003;109:461–7.
- Dong W, Selgrade MK, Gilmour MI. Systemic administration of *Bordetella pertussis* enhances pulmonary sensitization to house dust mite in juvenile rats. *Toxicol Sci* 2003;72:113–21.
- Trujillo-Vargas CM, Werner-Klein M, Wohlleben G, Polte T, Hansen G, Ehlers S, et al. Helminth-derived products inhibit the development of allergic responses in mice. *Am J Respir Crit Care Med* 2007;175:336–44.
- Hylkema MN, Hoekstra MO, Luinge M, Timens W. The strength of the OVA-induced airway inflammation in rats is strain dependent. *Clin Exp Immunol* 2002;129:390–6.
- Singh P, Daniels M, Winsett DW, Richards J, Doerfler D, Hatch G, et al. Phenotypic comparison of allergic airway responses to house dust mite in three rat strains. *Am J Physiol Lung Cell Mol Physiol* 2003;284:L588–98.
- Renzi PM, Olivenstein R, Martin JG. Effect of dexamethasone on airway inflammation and responsiveness after antigen challenge of the rat. *Am Rev Respir Dis* 1993;148:932–9.
- Elwood W, Lotvall JO, Barnes PJ, Chung KF. Characterization of allergen-induced bronchial hyperresponsiveness and airway inflammation in actively sensitized brown-Norway rats. *J Allergy Clin Immunol* 1991;88:951–60.
- Haczku A, Chung KF, Sun J, Barnes PJ, Kay AB, Moqbel R. Airway hyperresponsiveness, elevation of serum-specific IgE and activation of T cells following allergen exposure in sensitized Brown-Norway rats. *Immunology* 1995;85:598–603.
- Leung SY, Eynott P, Nath P, Chung KF. Effects of ciclesonide and fluticasone propionate on allergen-induced airway inflammation and remodeling features. *J Allergy Clin Immunol* 2005;115:989–96.
- Schuster M, Tschernig T, Krug N, Pabst R. Lymphocytes migrate from the blood into the bronchoalveolar lavage and lung parenchyma in the asthma model of the brown Norway rat. *Am J Respir Crit Care Med* 2000;161:558–66.
- Rothenberg ME, Hogan SP. The eosinophil. *Annu Rev Immunol* 2006;24:147–74.
- Taube C, Nick JA, Siegmund B, Duez C, Takeda K, Rha YH, et al. Inhibition of early airway neutrophilia does not affect development of airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 2004;30:837–43.
- Taube C, Dakhama A, Rha YH, Takeda K, Joetham A, Park JW, et al. Transient neutrophil infiltration after allergen challenge is dependent on specific antibodies and Fc gamma III receptors. *J Immunol* 2003;170:4301–9.
- GINA Report (Global Strategy for Asthma Management and Prevention). <www.ginasthma.com>, 2007.
- Tasaka K. Epinastine: an update of its pharmacology, metabolism, clinical efficacy and tolerability in the treatment of allergic diseases. *Drugs Today* 2000;36:735–57.
- Meade CJ. The mechanism by which epinastine stops an adenosine analog from contracting BDE rat airways. *Am J Respir Crit Care Med* 1998;157:522–30.
- Szeffler SJ, Nelson HS. Alternative agents for anti-inflammatory treatment of asthma. *J Allergy Clin Immunol* 1998;102:S23–35.
- Christie P. Roflumilast: a selective phosphodiesterase 4 inhibitor. *Drugs Today* 2005;41:667–75.
- Fan CK. Phosphodiesterase inhibitors in airways disease. *Eur J Pharmacol* 2006;533:110–7.