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EFFECT OF ANTI-IgE IN OCCUPATIONAL ASTHMA CAUSED BY EXPOSURE TO LOW MOLECULAR WEIGHT AGENTS

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ABSTRACT

Background: The role of immunoglobulin (Ig)-E in occupational asthma (OA) due to low molecular weight (LMW) agents is not well established compared to classical atopic asthma. In the present study we evaluate whether anti-IgE monoclonal antibody (mAb) has an effect in a mouse model of OA, using persulfate salts.

Methods: On days 1 and 8, BALB/C mice were dermally sensitized with 5% ammonium persulfate (AP) or dimethyl sulfoxide (DMSO). On days 15, 18 and 21 animals were injected intraperitoneally with anti-IgE mAb or PBS 6 hours before challenge with AP or saline. Airway hyperresponsiveness (AHR) using a methacholine test, airway inflammation in bronchoalveolar lavage (BAL) and lung tissue and total free IgE in serum samples were analyzed 24, 48 and 96 hours after the last challenge.

Results: Anti-IgE mAb treatment almost completely neutralized free serum IgE. In AP sensitized and challenged mice, anti-IgE mAb treatment abolished AHR 24h and 48h after the last challenge and significantly reduced the total number of eosinophils and neutrophils 48h and 96h after the last AP challenge compared with non-treated mice. Levels of interleukin (IL)-13 in BAL were also significantly decreased after anti-IgE administration 24h and 48h after the last AP challenge. Histological analysis of the lung sections from anti-IgE-treated mice revealed normal inflammatory patterns similar to control groups 48h after the last challenge.

Conclusions: Anti-IgE-treated mice showed a significant improvement in asthma features related to the AHR and airway inflammation. Anti-IgE mAb has positive effects in OA induced by persulfate salts.

INTRODUCTION

Immunoglobulin-E (IgE) plays a central role in the pathophysiology of allergic diseases such as asthma, rhinitis and food allergies ⁽¹⁾. It binds to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils, triggering a wide variety of inflammatory responses including a rapid release of preformed mediators (histamine, proteoglycans and proteases), proinflammatory cytokines and other active mediators (prostaglandins and leukotrienes), which result in clinical manifestations of type I hypersensitivity ⁽²⁻⁴⁾.

Because of this key role of IgE in allergic asthma, researchers have investigated the therapeutic potential of targeting this antibody. Omalizumab (OmAb) is a recombinant humanized monoclonal antibody (mAb) which binds to the C-epsilon-3 locus of the Fc (constant fragment) portion of free IgE

near the binding site for the high-affinity receptor FcεRI⁽⁵⁻⁸⁾ and can neutralize free IgE, reducing mediator release and allergic inflammation. Its clinical efficacy has been established in several clinical trials in adults and children with diagnoses ranging from moderate to severe allergic asthma⁽⁹⁾.

Although airway eosinophilic inflammation is an important feature of allergic asthma, there is increasing evidence that certain phenotypes of asthma are characterized by an influx of neutrophils in the airways⁽¹⁰⁾. This is especially the case for some types of occupational asthma (OA) in humans with chemical-induced OA⁽¹¹⁾, as well as in experimental animal models^(12;13). Frequently, these patients present difficult asthma with poor control despite high doses of corticosteroids and this makes them candidates for alternative treatments⁽¹⁴⁾.

The immunological mechanisms of OA due to low-molecular-weight (LMW) agents seem to differ from the typical IgE-mediated allergic response, yet high levels of total serum IgE have been reported in some patients with this type of OA^(15;16). To our knowledge only one study reported a positive effect of anti-IgE mAb therapy in patients with OA induced by LMW agents⁽¹⁷⁾. In this paper, Lavaudet *al.* demonstrated a clinical benefit with anti-IgE mAb therapy in OA even in patients with OA induced by chemical agents, suggesting an immunological mechanism⁽¹⁷⁾. Previously, a mouse model of OA using persulfate salts⁽¹²⁾, a known chemical asthmogenic agent⁽¹⁸⁾, showed high levels of total serum IgE, which might suggest a key role for IgE. Therefore, we hypothesize that mouse precursor of OmAb (anti-IgE mAb) can improve asthma characteristics in a mouse model of chemical-induced asthma. Hereby, we shed light on the role of IgE in this type of OA induced by persulfate salts.

MATERIAL AND METHODS

Animals

Male BALB/c mice (20 g, 5 weeks old) were obtained from Harlan (Horst; The Netherlands). The mice were housed in filter top cages in a conventional animal house with 12 h dark/light cycles and received slightly acidified water and pelleted food (Teklad 2014, Harlan Laboratories, Indianapolis, IN) ad libitum. All experimental procedures were approved by the Ethical Committee for Animal Experimentation of Hospital Universitari Vall d'Hebron, Barcelona, Spain (Assurance number CEEA 66/13).

Non-anaphylactogenic anti-mouse IgE 1-5 mAb

The rat IgG1 anti-mouse IgE 1-5 antibody (anti-IgE mAb) was provided by Novartis (Novartis Pharma AG, Basel, Switzerland) and was used for the treatment of mice, while PBS was administered as vehicle to non-treated mice. The rat IgG1 anti-mouse IgE 1-5 antibody is specific for IgE isotype and interacts with free IgE but does not recognize IgE bound to mast cells. This antibody recognizes an epitope within the FcεRI-binding region. As a result of this binding specificity, the antibody 1-5 does not induce histamine release from IgE-sensitized rat basophilic leukemia cells and in view of this property it is termed non-anaphylactogenic.

Mouse model of chemical-induced asthma

On days 1 and 8, the mice were dermally sensitized with 5% ammonium persulfate (AP) [(NH₄)₂S₂O₈, Sigma-Aldrich, Germany] or dimethylsulfoxide (DMSO) as a vehicle [Sigma-Aldrich, Germany] on the dorsum of each ear (20μl). On days 15, 18 and 21 animals received an intraperitoneal injection of 200μg of anti-IgE 1-5 mAb or PBS (100μl, final volume) followed 6h later by an intranasal challenge [40μl of 1%AP or saline (0.9% NaCl)], under light isoflurane [Forane®, Abbott Laboratories, Spain] anesthesia. The experimental groups are represented by three abbreviations: (DMSO/SAL)NT, (DMSO/SAL)T, (AP/AP)NT, (AP/AP)T. The first abbreviation identifies the agent used for dermal applications on days 1 and 8 (sensitization), the second identifies the agent administered via intranasal instillation (challenge), and the third identifies the antibody that was injected i.p. (T as treated, and NT as non-treated, according to the treatment with anti-IgE 1-5 mAb). Each group of mice (vehicle or AP) consisted of 5 to 12 mice at each time point. The experiments were performed on two separate time points for each group.

Airway hyperreactivity

Airway reactivity in response to methacholine was measured using a forced oscillation technique [FlexiVent 6, SCIREQ, Canada] 24h, 48h and 96h after last intranasal instillation. As previously described, mice were anesthetized with pentobarbital (70mg/kg body weight) (Nembutal®, Abbot Laboratories) and airway resistance (R) was measured using a “snapshot” protocol. For each mouse, R was plotted against methacholine concentration (0–20 mg/ml) and the area under the curve (AUC) was calculated ⁽¹⁹⁾.

Lung inflammation

Broncho-alveolar lavage

After functional airway measurements, mice were deeply anesthetized by an intraperitoneal injection of pentobarbital (90 mg/kg body weight). Broncho-alveolar lavage (BAL) was performed by three repeated lavages with 0.7ml of saline injected into the lungs via the trachea. Total cell counts were performed using a hemocytometer and BAL fluid was centrifuged (1000g, 10min). The supernatant was frozen (-80°C) until further analyses. For differential cell counts, cytopsins were prepared (10⁵ cells/ml; 1400 g, 6minutes) [Cytospin 3, Shandon, Thermo Scientific, United Kingdom). A total of 500 cells were counted for the number of macrophages, eosinophils, neutrophils and lymphocytes.

Levels of interleukin (IL) IL-2, IL-4, IL-5, IL-13, IL-10, IL-12p40, IL-17A and IFN γ , were measured in the first fraction of undiluted BAL fluid by a mouse cytokine magnetic bead panel according to the manufacturer's instructions (Bio-PlexPro Mouse Cytokine plex Assay, Bio-Rad Laboratories S.A.; Madrid, Spain). Lower limits of detection were 3.41, 6.11, 1.85, 4.00, 1.26, 1.58, 3.02 and 1.56 pg/mL for IL-2, IL-4, IL-5, IL-13, IL-10, IL-12p40, IL-17A and IFN γ , respectively.

Lung pathology

After BAL, lungs were instilled with formaldehyde 3.7–4.0% until all lobes were deemed to be fully inflated on visual inspection. Evaluation of lung injury on slides stained by hematoxylin and eosin (H&E) and Masson's trichrome was performed in a blinded manner by an experienced pathologist. A semi-quantitative scoring system was used to grade the severity and extent of inflammation, the presence of alveolar macrophages and bronchiolar epithelium hyperplasia on H&E stained sections. We graded the thickness of the infiltrate in the interalveolar septa and hyperplasia as follows: 0 (normal) = absence of inflammatory cells; 1 (mild) = 1–2 layers of inflammatory cells; 2 (moderate) = 3–5 layers; 3 (severe) = more than 5 layers (20). The same scoring was used to grade the other histopathological parameters.

Serum IgE

Blood was taken by cardiac puncture and levels of total serum IgE were measured by DELFIA immunoassay (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay). Indicated microplates for fluorescence were coated with rat anti-mouse IgE 4B3-39 (provided by Novartis) and incubated

with diluted serum samples (1/5 dilution for control and treated-mice, and 1/10 dilution for treated-mice). Bound mouse IgE was detected with rat anti-mouse IgE 3-11 (provided by Novartis) previously biotinylated according to standard procedure [EZ-Link Sulfo-NHS-Biotinylation Kit, Thermo Fisher Scientific, Massachusetts, United States] and a fluorescent DELFIA chelate (Europium) was used as the readout. Antibodies 4B3-39 and 3-11 recognize different epitopes on the IgE molecule than the non-anaphylactogenic anti-IgE antibody 1-5, without inducing steric hindrance. Thus, the combination of anti-mouse IgE antibodies chosen for this assay resulted in a highly sensitive and isotype-specific detection of mouse IgE, which is not affected even by a large excess of anti-IgE 1-5 mAb in the serum sample.

Data analysis

A normal distribution of the data was assessed by D'Agostino & Pearson omnibus normality test. All data are presented as means and 95% Confidence Interval (CI), except for the AUC data of airway hyperresponsiveness (AHR), which are shown as individual data and group means. For the cytokine levels, values were normalized to assay controls and expressed as ratio or fold-changes relative to control samples (from (DMSO/SAL) groups). All data were analysed using the non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparison test, or one-way ANOVA followed by a Bonferroni test in the case of data normally distributed (AHR and IL-13) (Graphpad Prism 6.01, Graphpad Software Inc, San Diego, USA). A level of $p < 0.05$ (two-tailed) was considered significant.

RESULTS

Serum IgE

Treatment of mice with anti-IgE mAb 6h before each AP challenge completely neutralized serum IgE 24h, 48h and 96h after the last challenge (figure 1).

Airway Hyperreactivity

The AHR over time after the last challenge with AP or vehicle is shown in figure 2A, as well as the individual AUC for each group (figure 2B). There was a significant increase in airway reactivity to methacholine in non-treated mice [(AP/AP)NT] 24h and 48h after the last AP challenge. Treatment

with anti-IgE mAb abolished the AHR induced by AP sensitization and challenge when analyzed 24h and 48h after the last challenge. At the last time point (96h after the last challenge) AHR decreased in AP-nontreated group [(AP/AP)NT] and no significant differences were found when anti-IgE mAb was administered.

Lung Inflammation

Figure 3 shows the total cell count (A) and the total number of macrophages (B), neutrophils (C), eosinophils (D) and lymphocytes (E) in the BAL fluid 24h, 48h and 96h after the challenge. In AP/AP mice, a predominantly neutrophilic inflammation was found with a peak response at 24h time point. Although airway neutrophilia remained increased compared with control groups [(DMSO/SAL)NT] 48h and 96h after the last challenge, the total number of neutrophils decreased significantly over time in AP-nontreated mice [(AP/AP)NT] ($p=0.0208$ and $p<0.0001$ respectively), much the same as in AP-treated mice [(AP/AP)T] ($p=0.0014$ and $p<0.0001$ respectively). Moreover, in AP-nontreated mice [(AP/AP)NT] there were small but significant influxes of eosinophils (48h after the last challenge) and lymphocytes (24h, 48h and 96h after the last challenge). Treatment with anti-IgE mAb [(AP/AP)T] reduced the total number of eosinophils 48h (figure 3D) and neutrophils 96h (figure 3C) after the last challenge compared to AP-nontreated mice [(AP/AP)NT].

Levels of cytokines IL-5, IL-10 and IL-13 were detected in BAL fluid and are shown in figure 4. The levels of IFN γ , IL-2, IL-4, IL-12p40 and IL-17A were not detected in BAL fluid (data not shown). This study showed significant fold-increases in IL-5 in AP-nontreated mice [(AP/AP)NT] 24h after the last challenge compared with controls [(DMSO/SAL)NT] and although not significant, anti-IgE mAb administration tends towards a decrease in IL-5 levels compared with non-treated mice [(AP/AP)NT] (figure 4A). The ratio of IL-10 was significantly reduced in asthmatic mice groups (AP/AP) compared with control groups (DMSO/SAL) regardless of treatment 24h after the last challenge, and it returned to baseline values 48h after the last challenge. At this time point, AP-treated mice [(AP/AP)T] showed significant increases in IL-10 compared to AP-nontreated mice [(AP/AP)NT] (figure 4B). There was a significant fold-increase in the levels of IL-13 in non-treated mice [(AP/AP)NT] 24h and 48h after the

last AP challenge. Treatment with anti-IgE mAb [(AP/AP)T] significantly reduced the ratio of IL-13 compared with AP-nontreated [(AP/AP)NT] mice when analyzed 24h and 48h after the last challenge (figure 4C).

The inflammatory patterns were also observed in lung tissue sections by a blinded histopathological examination (figure 5). There was mild to moderate inflammatory cell infiltration, and presence of alveolar macrophages (grade 1-2, mild to moderate) as well as mild basal cell hyperplasia (grade 1, mild) in AP-nontreated mice [(AP/AP)NT] 48h after the last AP challenge (figure 5E and 5F) compared with AP-treated mice [(AP/AP)T] examined at the same time point, which showed normal/absent levels of inflammation parameters similar to control groups (grade 0, absent/normal) (figure 5G and 5H). There were no significant differences in airway inflammation 24h and 96h after the last challenge. In this multiple challenge model of OA, no collagen deposition was observed in the lung sections stained with Masson's trichrome (data not shown).

DISCUSSION

In this study, we evaluated the effects of anti-IgE mAb treatment in a mouse model of chemical-induced asthma due to persulfate salts ⁽¹²⁾. In the study, anti-IgE treatment significantly improved asthma features in mice, such as non-specific AHR and lung inflammation. This suggests that anti-IgE mAb may have beneficial effects on OA caused by LMW agents.

Agents causing OA can be classified into high molecular weight (HMW) compounds, which normally act by producing specific IgE antibodies and typical allergic responses, and LMW compounds, whose mechanisms for inducing OA are less well known. For certain LMW agents (platinum salts, trimellitic anhydride, and other acid anhydrides), the development of OA is accompanied by the production of specific IgE antibodies, probably acting as haptens and combining with a body protein to form functional antigens ⁽²⁰⁾. However, most LMW agents induce asthma through mechanisms other than IgE ⁽²¹⁾, despite a phenotype suggesting sensitization ⁽²²⁾. In the case of diisocyanates, for example, serum-specific IgE to toluene diisocyanate has only been detected in 40% of patients, suggesting the presence of more than one immunological mechanism ⁽²³⁾.

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In the case of persulfate salts, the mechanisms by which these substances induce sensitization and OA are complex. Some authors have suggested that mast cells and T cells may play an important role in the pathogenesis of the asthmatic reactions following inhalation of persulfate salts^(16;24). Positive skin-prick tests for persulfate salts have been reported, and elevated total serum IgE levels have been observed in 50% of patients, suggesting the involvement of an IgE-dependent mechanism^(15;16). In addition to studies with patients, experimental murine research on chemical-induced asthma due to persulfate salts showed increased levels of total serum IgE⁽¹²⁾. In these experimental studies, AHR, neutrophilic airway inflammation, increases in Th, Tc, and B-lymphocytes, a mixed Th1/Th2 immune response derived from cytokine profiles in local draining lymph nodes and BAL fluid, and serum IgG1/IgG2a levels were reported after AP sensitization and challenge^(12;25). Moreover, we were able to show that lymphocytes from sensitized mice could be directly triggered to proliferate by *in vitro* re-stimulation with the sensitizing agent. These results suggest that OA due to persulfate salts may be caused by a complex interaction of an innate and a mixed Th1/Th2 adaptive immune response.

Although OmAb is indicated for the treatment of allergic disease, and its efficacy has been proven in patients with allergic asthma and rhinitis⁽²⁶⁾, it is not clear that it is effective for chemical-induced OA. The present study demonstrates that treatment of mice with anti-IgE mAb completely neutralized serum IgE after challenge, and that this neutralization of serum IgE abolished AHR and reduced cellular airway inflammation. These findings are consistent with the proven effects of blocking free IgE with OmAb in the treatment of IgE-mediated allergic processes, that is, reductions in cell-bound IgE, mediator release and allergic inflammation^(5;27). With a specific multiple challenge protocol, eosinophilic airway inflammation was found. Anti-IgE mAb administration induced a decrease in the number of eosinophils, similar to previous reported in clinical studies targeting the severe eosinophilic asthma phenotype⁽²⁸⁻³⁰⁾. In the case of persulfate salts, the eosinophilic airway inflammation seems to be regulated by a Th2 immune response, as demonstrated by the presence of increased levels of total serum IgE as well as significantly increased levels of IL-5 in AP-nontreated mice⁽¹⁾.

The presence of neutrophils is an important feature of certain types of OA^(10;22) and previously studies have also demonstrated an increase in the total number of airway neutrophils in the mouse model of chemical-induced asthma^(12;25). Neutrophils are the first cells to migrate toward the site of inflammation during an innate immune response to tissue damage, and they have been implicated in

the initiation phase (T- and B- lymphocyte activation in lymph nodes) and in the effector phase (AHR, lung inflammation and epithelial damage)⁽³¹⁾. To our knowledge, the present study is the first report of an OmAb effect on neutrophils. Identifying the molecular mechanisms by which innately recruited neutrophils contribute to the airway inflammatory response and the pathophysiology of OA is an area of active research.

Moreover, it has recently been reported that OmAb may reduce or even reverse airway remodeling, conferring a persistence of the mAb effects on asthmatic patients⁽³²⁾. In the present study, we did not observe smooth muscle hyperplasia or collagen deposition in the histopathological sections, due to the limited number of airway exposures and the shortness of the model. Yet, we found that anti-IgE mAb was able to act in airway structures and to reverse the bronchial epithelial hyperplasia and the inflammatory cell infiltration.

As mentioned above, treatment with anti-IgE mAb abolished the AHR. The anti-IgE mAb blocks the interaction between IgE and its high-affinity receptor FcεRI, inhibiting the mast cell and basophil activation and the subsequent release of their inflammatory mediators, which result in bronchoconstriction⁽⁵⁾. The complete removal of AHR in AP-treated mice was accompanied by a reduction in the levels of IL-13 in BAL fluid. The cytokine IL-13 has an essential role in the induction of AHR in allergic asthma⁽³³⁾. Walter *et al.* showed that IL-13 was essential for the induction of AHR in a mouse model of asthma, and demonstrated that airway inflammation alone, as defined by the presence of large quantities of peribronchiolar eosinophils and lymphocytes, was not enough for the development of significant AHR in the absence of IL-13⁽³⁴⁾. Moreover, the effects on IL-13 after OmAb treatment have been described, in the form of significant reductions in the number of IL-13+ T-lymphocytes and in circulating levels of IL-13 after OmAb administration in moderate-to-severe asthma⁽³⁵⁾.

The reduction of Th2 cytokines other than IL-13 such as IL-4 and IL-5 after OmAb treatment has been described in only a few studies^(29,36). This reduction may be mainly the result of the decreasing numbers of Th2-cytokine-producing cells, possibly via suppression of proliferation and/or accumulation of Th2 cells in the bronchial wall⁽²⁹⁾. Some studies suggest that the reduction of allergen-driven Th2 cell proliferation may be due to the attenuation of the activity of antigen-

presenting cells such as dendritic cells (DCs). By neutralizing IgE, the expression of FcεRI on DCs seems to be decreased, thus weakening their antigen-presenting ability and leading to decreased levels of Th2 cytokines⁽³⁷⁾. On the other hand, the levels of IL-10 in asthmatic mice were lower than those in control groups 24h after the last challenge, and increased 48h after the last challenge in AP-treated mice. IL-10 has anti-inflammatory properties and can inhibit proinflammatory synthesis from macrophages and T-cells⁽³⁸⁾; therefore, the increase in the levels of IL-10 observed in AP-treated mice may be a consequence of reductions in AHR and airway inflammation as a result of anti-IgE mAb treatment.

Patients with severe OA are difficult to treat as they do not usually respond to the conventional treatment approaches proposed in the guidelines specifically for OA⁽³⁹⁾. In view of the socio-economic outcomes for these patients and the consequences for their health, it is important to establish new therapeutic approaches to minimize the negative impact for both companies and workers. Complete elimination of the exposure is the most straight-forward approach to reducing the burden of the disease and it is the most commonly applied in the management of OA. Nevertheless, complete avoidance of workplace exposure improves asthma symptoms in approximately 33% of patients⁽⁴⁰⁾, and the long-term health effects of reducing rather than eliminating exposure to the causal agent remain uncertain⁽¹⁴⁾.

Currently, only one study reported a positive effect in three of four patients with OA induced by LMW agents. These patients were able to keep on working after receiving OmAb⁽¹⁷⁾. The causal agents of two of these patients were isocyanates and only one presented specific-IgE antibodies. OmAb allowed control of exacerbations in both patients, even when specific IgE antibodies were not detected. Similarly, the patient exposed to tetrachloroethylene, a chemical agent widely used in dry-cleaning and reported to cause OA^(41;42), was able to continue working in the same workplace after OmAb treatment with low levels of total serum IgE. However, OmAb administration was unable to control asthma in the fourth patient who was exposed to acrylate.

A limitation of this study is the lack of an additional control group in which mice are treated with non-specific IgG anti-body. Yet, previously it has been demonstrated that IgG has no effect in animal models where therapeutic and modulating effects of monoclonal antibodies were tested⁽⁴³⁾.

In conclusion, the present study has demonstrated the efficacy of OmAb in a mouse model of OA induced by persulfate salts and suggests that OmAb could result in positive effects in the treatment of this type of OA. Future studies with patients should be performed to corroborate these findings.

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FIGURE LEGENDS

Figure 1. Total serum immunoglobulin (Ig)-E. Total serum free IgE was measured using a DELFIA immunoassay. For group coding, the first digit represents the agent used for sensitization (days 1 and 8), the second digit identifies the agent administered via intranasal instillation; each group is identified as T (treatment) or NT (no treatment) according to the treatment with anti-IgE mAb. Mean with 95% CI of total serum IgE is represented for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. AP, ammonium persulfate; CI, confidence intervals; NT, no treatment; T, treatment.

Figure 2. Airway reactivity to methacholine expressed as area under the curve (AUC) of the resistance (R). Experimental groups are the same as in figure 1. **A.** Mean with 95% CI of AUC of R against methacholine concentrations (0 to 20 mg/ml) over time. ** $p < 0.01$ (DMSO/SAL)NT *versus*

(AP/AP)NT; +p<0.05 (DMSO/SAL)T *versus* (AP/AP)NT; øp<0.05 (AP/AP)NT *versus* ((AP/AP)T. **B.** Mean individual values of AUC 24h, 48h and 96h after the last challenge. *p<0.05 and **p<0.01. No significant differences were found in the other groups. AHR, airway hyperresponsiveness; AP, ammonium persulfate; CI, confidence intervals; NT, no treatment; R, resistance; T, treatment.

Figure 3. Total cell count and total number of macrophages, neutrophils, eosinophils and lymphocytes in BAL. Experimental groups are the same as in figure 1. Mean with 95% CI of total cell count (**A**), macrophages (**B**), neutrophils (**C**), eosinophils (**D**) and lymphocytes (**E**) 24h, 48h and 96h after the last challenge. *p<0.05, **p<0.01 and ***p<0.001. No significant differences were found in the other groups. AP, ammonium persulfate, BAL, bronchoalveolar lavage; CI, confidence intervals; NT, no treatment; T, treatment.

Figure 4. Levels of interleukin (IL)-5, IL-10 and IL-13 in BAL fluid. Experimental groups are the same as in figure 1. Mean with 95% CI of ratios (normalized to control groups) of IL-5 (**A**), IL-10 (**B**) and IL-13 (**C**) concentrations (pg/ml). *p<0.05, **p<0.01 and ***p<0.001. No significant differences were found in the other groups. AP, ammonium persulfate; BAL, bronchoalveolar lavage; CI, confidence intervals; IL, interleukin; NT, no treatment; T, treatment.

Figure 5. Lung histopathology. Representative images of hematoxylin and eosin stained histological lung sections are shown at low (**A**, **C**, **E** and **G**) and high magnification (**B**, **D**, **F** and **H**) 48h after the last challenge. Experimental groups are the same as in figure 1. AP, ammonium persulfate; NT, no treatment; T, treatment.

Figure 1

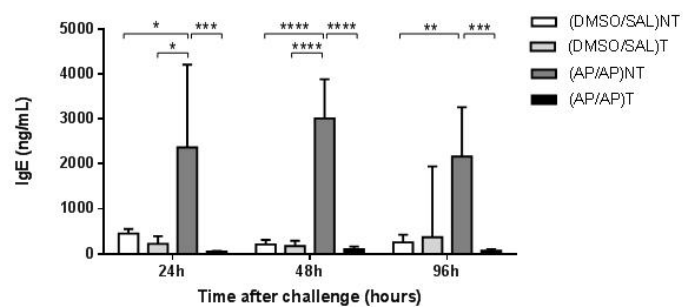


Figure 2

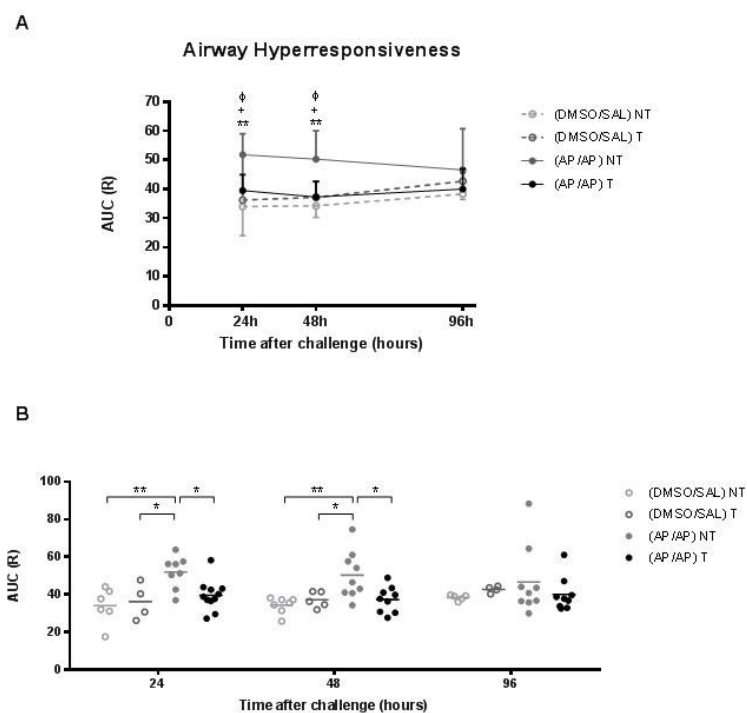


Figure 3

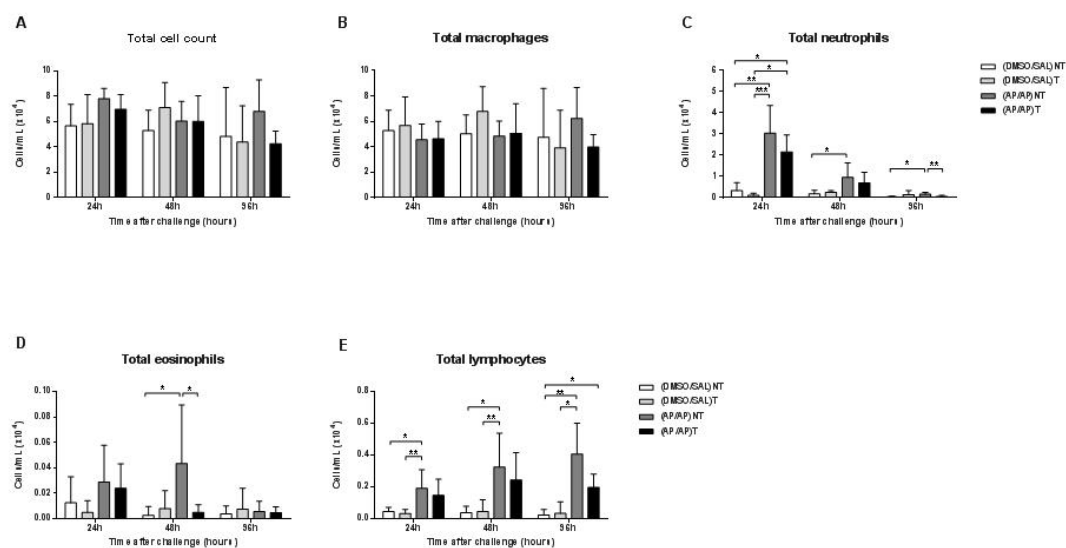


Figure 4

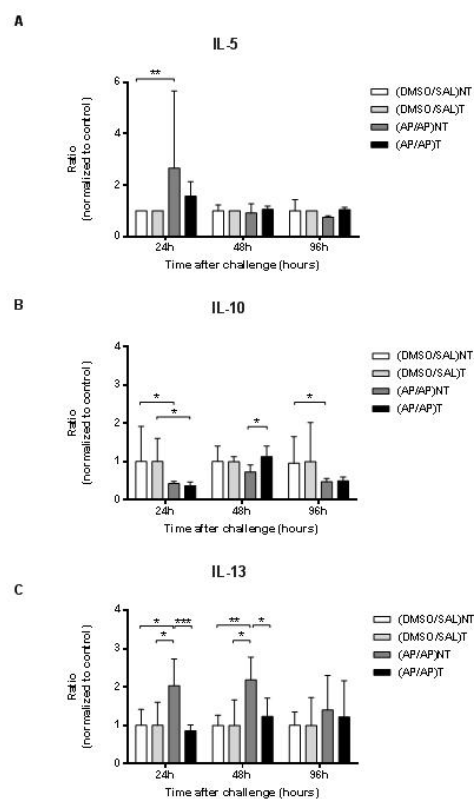


Figure 5

