



Pulmonary, gastrointestinal and urogenital pharmacology

Adalimumab ameliorates OVA-induced airway inflammation in mice: Role of CD4⁺ CD25⁺ FOXP3⁺ regulatory T-cellsMohamed G. Elsakkar^a, Olla A. Sharaki^b, Dina M. Abdallah^c, Dalia K. Mostafa^{a,*}, Fadia T. Shekondali^d^a Department of Clinical Pharmacology, Faculty of Medicine, Alexandria University, Alexandria, Egypt^b Department of Clinical and Chemical Pathology, Faculty of Medicine, Alexandria University, Alexandria, Egypt^c Department of Pathology, Faculty of Medicine, Alexandria University, Alexandria, Egypt^d Department of Pharmacology, Faculty of Medicine, EL-Zawia University, Libya

ARTICLE INFO

Article history:

Received 31 January 2016

Received in revised form

23 May 2016

Accepted 1 June 2016

Available online 2 June 2016

Keywords:

Adalimumab

Airway inflammation

Neutrophils

FOXP3⁺ Treg cells

Asthma

TNF- α

Chemical compounds studied in this article:

Adalimumab (PubChem SID: 50070208)

Ovalbumin (PubChem CID: 71311993)

Aluminum Hydroxide (PubChem CID:

6328211)

Phosphate buffered saline (PubChem CID:

24978514)

IL-10 (PubChem CID: 146070)

ABSTRACT

Asthma is a chronic inflammatory heterogeneous disorder initiated by a dysregulated immune response which drives disease development in susceptible individuals. Though T helper 2 (TH2) biased responses are usually linked to eosinophilic asthma, other Th cell subsets induce neutrophilic airway inflammation which provokes the most severe asthmatic phenotypes. A growing evidence highlights the role of T regulatory (Treg) cells in damping abnormal Th responses and thus inhibiting allergy and asthma. Therefore, strategies to induce or augment Treg cells hold promise for treatment and prevention of allergic airway inflammation. Recently, the link between Tumor necrosis factor- α (TNF- α) and Treg has been uncovered, and TNF- α antagonists are increasingly used in many autoimmune diseases. Yet, their benefits in allergic airway inflammation is not clarified. We investigated the effect of Adalimumab, a TNF- α antagonist, on Ovalbumin (OVA)-induced allergic airway inflammation in CD1 mice and explored its impact on Treg cells. Our results showed that Adalimumab treatment attenuated the OVA-induced increase in serum IgE, TH2 and TH1 derived inflammatory cytokines (IL-4 and IFN- γ , respectively) in bronchoalveolar lavage (BAL) fluid, suppressed recruitment of inflammatory cells in BAL fluid and lung, and inhibited BAL fluid neutrophilia. It also ameliorated goblet cell metaplasia and bronchial fibrosis. Splenocytes flow cytometry revealed increased percentage of CD4⁺ CD25⁺ FOXP3⁺ Treg cells by Adalimumab that was associated with increase in their suppressive activity as shown by elevated BAL fluid IL-10. We conclude that the beneficial effects of Adalimumab in this CD1 neutrophilic model of allergic airway inflammation are attributed to augmentation of Treg cell number and activity.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The inflamed airways in asthma undergo distinct structural and functional changes, leading to non-specific bronchial hyper-responsiveness and airflow obstruction that fluctuates over time (Holgate et al., 2008). Despite advanced understanding of the pathophysiology of asthma, available treatment is non curative, and affected patients continue to incur significant morbidity (Bosnjak et al., 2011; Peters et al., 2006). Studies addressing the pathogenesis of asthma uncovered the heterogeneous nature of the disease, where several distinct endotypes, including eosinophilic, neutrophilic and mixed eosinophilic/neutrophilic inflammations, have been identified; in

all, the CD4⁺ T helper cells apparently play the central role. Whilst Th2 lymphocytes, with its cytokines [Interleukin-4 (IL-4), IL-5, and IL-13], are mainly involved in the development of eosinophilic allergic asthma, Th1 and Th17 lymphocytes appears to play a key role in neutrophilic airway inflammation (Newcomb and Peebles, 2013). Activation of these immune cells is tightly regulated by counter regulatory circuits, mastered by the T-regulatory cells (Tregs) that dampen the overactive immune responses (Kushwah and Hu, 2010). The Tregs are characterized by high levels of CD25 expression and their immunomodulatory function appears to require the expression of the transcription factor, FOXP3. Tregs are able to inhibit the development of allergy via several mechanisms, including suppression of other effector Th1, Th2 and Th17 cells, via the production of transforming growth factor (TGF)- β and IL 10 (Ray et al., 2010; Palomares et al., 2010). This functional characterization of Tregs has led to a resurgence of interest in the therapeutic implication of Treg modulation in a number of immune mediated diseases (Buckner et al., 2010). The tumor necrosis

* Corresponding author.

E-mail address: dalia.kashishy@alexmed.edu.eg (D.K. Mostafa).

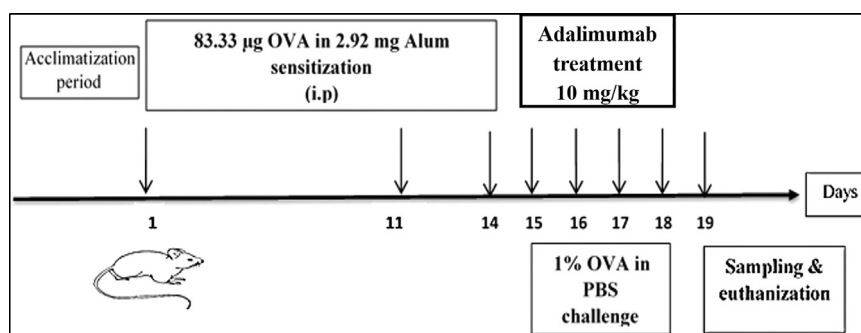


Fig. 1. Schematic illustration of the experimental schedule for induction of airway inflammation and drug administration.

factor (TNF)- α , which is a Th1 associated pro-inflammatory cytokine, was found to be essentially involved in the pathogenesis of various inflammatory and autoimmune diseases such as asthma (Berry et al., 2007; Blüml et al., 2012). Both TNF- α , and Interferon (IFN)- γ are elevated and may induce the influx of neutrophils that is characteristic of a severe asthma endotype (Pawankar et al., 2015).

The TNF- α signals mainly through its receptors, TNF receptor1 and TNF receptor2. The role of these receptors in modulation of Treg function is not well understood (Blüml et al., 2012). However, TNF- α mediated suppression of Treg function was demonstrated to be related to down regulation of FOXP3 expression (Valencia et al., 2006). Various studies suggested that TNF- α inhibitors have the potential to augment Treg cell numbers and function in many autoimmune diseases like rheumatoid arthritis (RA) (McGovern et al., 2012; Ricciardelli et al., 2008). However, in-vivo or in-vitro studies addressing their therapeutic benefits in asthma are limited.

The present study was therefore conducted to investigate the possible effect of Adalimumab, a TNF- α inhibitor, on OVA-induced allergic airway inflammation in mice. It also aimed to explore the involved mechanisms and whether they are related to modulation of Treg cells number and/or activity.

2. Materials and methods

2.1. Experimental animals

The present study was conducted on 36 adult male CD1 mice of body weights ranging from 20 to 25 g. The animals were purchased from the animal house of the Faculty of Medicine, Alexandria University. The mice were housed in standard conditions of light and temperature with free access to food and water in accordance with the EU Directive 2010/63/EU for animal experiments. All of the experimental procedures were approved and performed in compliance with the guidelines of the Local Ethics Committee of Alexandria Faculty of Medicine, University of Alexandria.

2.2. Drugs, chemicals and kits

The following drugs and chemicals were used in the study: Adalimumab; (HUMIRA[®], Abbott laboratories, Abbott Park, IL, USA), Ovalbumin (OVA); (Sigma-Aldrich, Poole, U.K.), Aluminum hydroxide (Alum); (Serva, Heidelberg, Germany), Phosphate Buffered Saline (PBS) pH=7.4; (Medicago AB, Uppsala, Sweden), Roswell Park Memorial Institute media (RPMI); (Euro Clone S.p. A., Italy). Mouse Enzyme linked immunosorbent assay (ELISA) kits for OVA-specific IgE, IL-4, IL-10, IFN- γ and mouse Regulatory T Cell-Staining Kit were purchased from eBioscience, Vienna, Austria.

2.3. Experimental design and drug administration

After 1 week of acclimatization, animals were randomly divided into 3 groups, twelve mice each. Normal control group, OVA sensitized and challenged (OVA-S&C) group which served as positive control and Adalimumab-treated OVA-sensitized and challenged (Ada-OVA-S&C) group.

Induction of airway inflammation was done in all of mice, except for the normal control group, by an initial sensitization with i.p. injection of 83.33 μ g OVA in 2.92 mg Alum on day 1, 11 and 14 followed by challenge with OVA aerosol delivered by a nebulizer (Mirage air jet nebulizer-Casnat CO, Italy) in a concentration of 1% w/v OVA in PBS for 30 min, for four successive days beginning on day 15 to day 18 of the experiment (You et al., 2014). Schematic illustration of the experimental schedule for induction of airway inflammation and drug administration is shown in Fig. 1.

Ovalbumin used for sensitization was prepared by dissolving 10 mg OVA in 10 ml PBS. The formed solution was well mixed by vortex for 5 min at 1500g and stored at -20°C as separate OVA aliquots of 1 ml each until further use. On the days of i.p. injection (day 1, 11 and 14), the stored OVA aliquots were brought to room temperature and each aliquot was freshly added to 35 mg Alum and PBS up to a total volume of 6 ml in falcon tube. The formed suspension was gently homogenized by a shaker for 4 h and was directly used for i.p. injection of all of the groups of mice as 0.5 ml/mice, except for the control mice that received equal volume of PBS instead.

On the other hand, OVA used for challenge was prepared as 1% w/v OVA in PBS. The formed suspension was well mixed by vortex for 5 min at 1500g and stored at -20°C as aliquots of 4 ml each until further use. On days of challenge (days 15–18), the stored OVA aliquots were brought to room temperature and used for nebulization as 4 ml of OVA solution/session. Mice were nebulized in groups (12 mice each) with OVA suspension driven by compressed air at a rate of 6 L/min for 30 min. Mice in the control group were nebulized with an equal volume of PBS at the same rate instead.

Adalimumab 0.8 ml solution (40 mg) was diluted with PBS to a final concentration of 1 mg/ml. The prepared drug solution was administered as 1 ml/100 g b.wt, by intraperitoneal (i.p.) injection, 1 h before each challenge (days 15–18) (Catal et al., 2015; Nam et al., 2009). Mice in the OVA-S&C group received i.p. injection of an equal volume of PBS as a vehicle instead.

2.4. Experimental procedures

On the last day of the experiment, the animals were fasted overnight for 8 h. On the next day, mice were anesthetized by i.p. injection of 80 mg/kg of ketamine (Xu et al., 2007), and blood samples were collected from the abdominal aortae and centrifuged at 750g for 10 min for separation of serum that was stored

at -20°C until assayed for OVA specific IgE (Breslow et al., 2010). The bronchoalveolar lavage (BAL) fluid was then collected. Briefly, a small incision was made in the neck exposing the trachea, and a 22-gauge cannula was inserted and tied in place. PBS (1.5–2 ml) was carefully injected into the lungs with gentle chest massage and then the lavage fluid was aspirated. The procedure was repeated at least 3 times per mouse. The collected BAL fluids were centrifuged at 750g for 10 min and the supernatants were collected (Hsu et al., 2013) and stored at -20°C until used to assay the following cytokines: IL-4, IFN- γ and IL-10, while the cell pellets were collected and re-suspended for cytological examination (You et al., 2014; Taher et al., 2008).

The chest of each animal was opened and the lung was excised and fixed in 10% formalin for further histopathological examination (You et al., 2014). Finally, the spleen of each mouse was excised, minced into small pieces in RPMI culture media and crushed gently by compressing tissue fragment against mesh of 70- μm cell strainer for separation of splenocytes. The cell suspensions were then collected and centrifuged for 4–5 min at 1200-g. The splenocytes were then collected from the interface and were freshly processed and analyzed by flow cytometry for identification of Treg cells (Arora et al., 2014; Jang et al., 2012; Liu et al., 2011; Sun et al., 2010).

2.5. Biochemical estimates

Serum OVA specific IgE and BAL fluid cytokines (IL-4, IFN- γ and

IL-10) were determined using mouse ELISA kits (eBioscience) according to the manufacturer's protocol. The colour change was measured spectrophotometrically at a wavelength of 450 nm. The concentrations were calculated based on standards and expressed in ng/ml and pg/ml for the IgE and the cytokines, respectively.

2.6. Cytological examination of BAL fluid

After centrifugation of the BAL fluid, the BAL cells were collected and the total cell count of the fresh fluid specimen was determined by a hemocytometer. Finally, the cells were stained by Diff-Quick stain for differential counting (Han and Ziegler, 2013; You et al., 2014).

2.7. Histopathological examination of the lung

For histological examination, specimens of the lung tissues were fixed in buffered 10% formalin. They were processed for routine paraffin block preparation. Sections of 3 μm were cut and stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) and Masson's trichrome (MT), and examined under the light microscope for evaluation of lung inflammation, mucus gland hyperplasia and amount of collagen deposition, respectively (You et al., 2014). Semi-quantitative morphometric analysis was performed where the degrees of inflammation, mucus and collagen deposition were scored from 0 (absent) to 3 (severe), as modified from a previously described protocol (Stellari et al., 2015). For

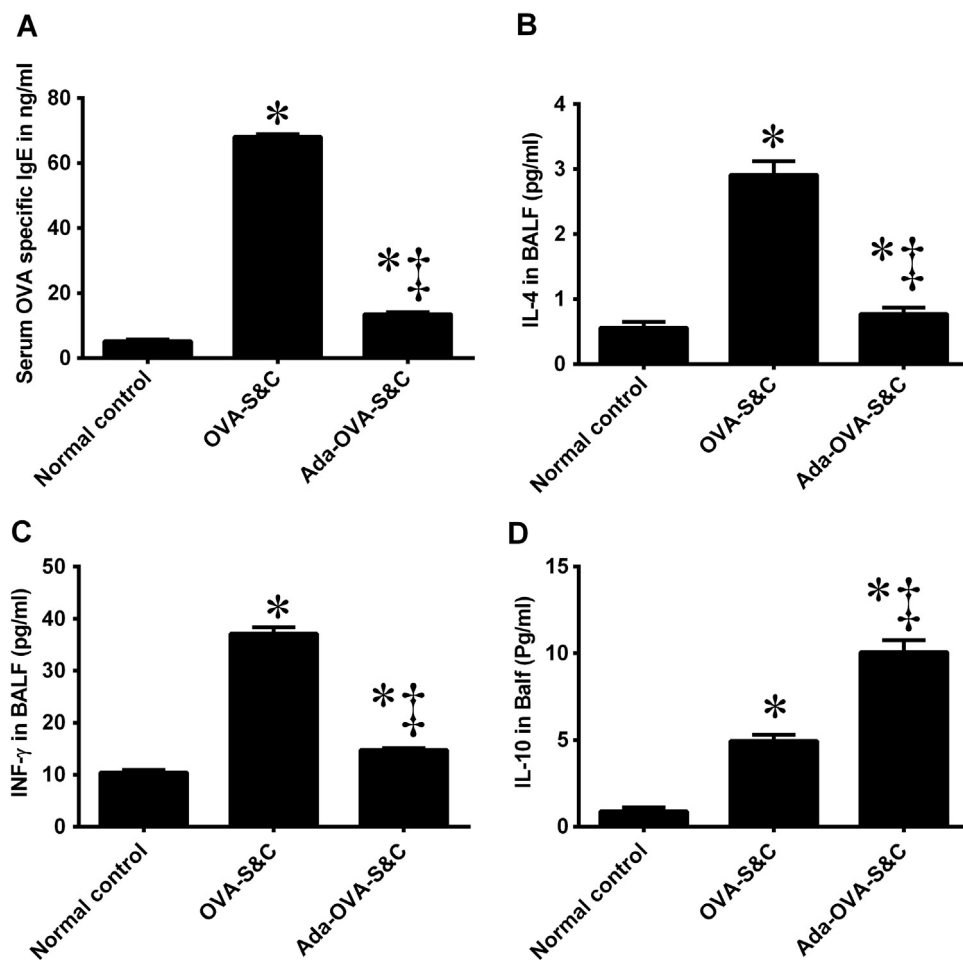


Fig. 2. Effect of Adalimumab on OVA-induced changes of serum OVA specific IgE in A and measured cytokines in bronchoalveolar lavage (BAL) fluid: IL-4 in B, IFN- γ in C and IL-10 in D. Data are expressed as means $\pm$ S.E.M. * $P < 0.05$ versus normal control and ‡ $P < 0.05$ versus untreated OVA-S&C mice. OVA-S&C: Ovalbumin sensitized and challenged, Ada: Adalimumab.

peribronchial inflammatory cell infiltrate, absent or rare infiltrate was scored as 0, 1 denotes 'Mild' (only in a focal place), 2: 'Moderate' (< 5 cell lines deep) and 3: 'Severe' (> 5 lines of cells deep). The same semi-quantitative scoring system were adopted for the abundance of PAS-positive goblet cells, where 0 corresponds to absent findings, while 1, 2, and 3 denote mild (1–30% of the bronchial lining in the examines sections), moderate (31–60%) and severe (> 60) involvement, respectively. The Masson's trichrome stain was used to evaluate the amount of collagen deposition around the airways. Sections were semi-quantitatively scored where: 0, absent; 1: mild (1–30% of structures involved); 2: moderate (31–60% of structures involved); 3: severe (> 60% of structures involved).

The score values for these individual parameters were added to form a composite inflammatory score ranging from 0 to 9. The histological slides were coded and the investigator was unaware of the origin of the material during scoring. For each mouse, 5 airway sections that were randomly distributed throughout the left lung were analyzed, and their average scores were calculated.

2.8. Flow cytometric analysis of Treg cells

The CD4⁺ CD25⁺ FOXP3⁺ Treg cells were identified by a flow cytometry, where the collected splenocytes were surface stained with anti-Mouse CD4 FITC and anti-Mouse CD25 PE, then fixed and permeabilized using the FOXP3/Transcription Factor Staining Buffer Set. Subsequently, cells were stained with 0.5 µg of Anti-Mouse/Rat Foxp3 PE-Cy5. The cells in the lymphocyte gate were used for analysis using a Flow cytometer equipped with two lasers, 488 nm blue and a 635 nm red laser (Jang et al., 2012). The intensity was measured by a FACS Aria II flow cytometer and analyzed using cell Quest software.

2.9. Data analysis

Statistical analyses were performed using the Statistical Package of Social Science, SPSS version 20-software package. The variables for each group were analyzed by calculating means and standard errors of the means (S.E.M.). The ANOVA one way test was used for comparing means between groups after testing normal distribution using one sample Lilliefors test for normality. The Post Hoc test (Duncan's new multiple range test) was used for comparing means of two independent groups after a significant ANOVA. Semi-quantitative morphometric data were analyzed using Kruskal-Wallis one way ANOVA followed by the Dunn - Sidák method for multiple comparison and were expressed as median values. A level of $P < 0.05$ was defined as being statistically significant.

3. Results

3.1. Effect of Adalimumab on OVA-induced elevation of OVA-specific serum IgE

OVA sensitization and challenge was associated with a significant increase in OVA-specific serum IgE compared with the normal control group. Treatment with Adalimumab significantly decreased this OVA-induced elevation of serum IgE, compared with the untreated OVA-S&C group (Fig. 2(A)).

3.2. Effect of Adalimumab on OVA-induced changes in BAL fluid cytokines

All the measured cytokines (IL-4, IL-10 and INF- γ) were significantly elevated in the BAL fluid of untreated OVA-S&C mice

compared with the normal control group. As shown in Fig. 2(B)–(D), treatment with Adalimumab was associated with significant decrease in BAL fluid IL-4 and INF- γ compared with the positive control group. Conversely, treatment with Adalimumab was associated with a significant elevation of IL-10 in comparison to untreated OVA-S&C mice.

3.3. Effects of Adalimumab on OVA-induced recruitment of inflammatory cells in BAL fluid and on OVA-induced BAL fluid neutrophilia

Sensitization and challenge with OVA was associated with marked leukocyte recruitment in BAL fluid, which was significantly higher relative to the number of cells that could be harvested from BAL fluid of the normal control mice (Fig. 3(A)).

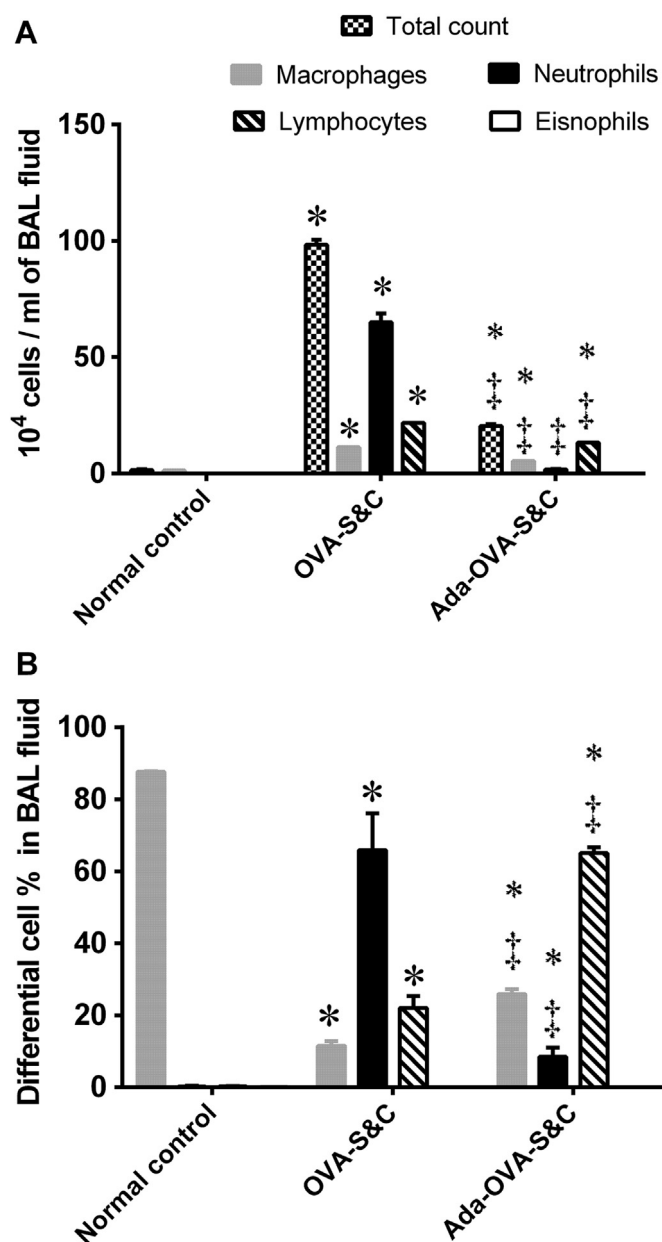


Fig. 3. Effect of Adalimumab on OVA-induced change in percentage of CD4⁺ CD25⁺ FOXP3⁺ Treg cells as identified by flow cytometric analysis of isolated splenocytes. Data are expressed as means $\pm$ S.E.M. * $P < 0.05$ versus normal control and $\dagger P < 0.05$ versus untreated OVA-S&C mice. OVA-S&C: Ovalbumin sensitized and challenged, Ada: Adalimumab.

The differential staining of these cells revealed the presence of neutrophilic cell predominance ($65.9 \pm 10.15\%$) in comparison to the normal control mice, where macrophages constituted the majority of cells encountered ($87.60 \pm 0.84\%$). Treatment with Adalimumab, not only was associated with significant attenuation of cellular recruitment (up to 80% decrease in cell count), but also it was accompanied with a marked change in the differential staining of these cells. This is shown as a significant increase in percentage of lymphocytes and to lesser extent in macrophages on the expense of neutrophil cell percentage that was $8.5 \pm 2.59\%$ of cells in the BAL fluid of Ada-OVA-S&C group compared with the untreated OVA-S&C group of mice (Fig. 3(B)).

3.4. Effect of Adalimumab on the OVA-induced change in $CD4^+CD25^+FOXP3^+$ Treg cells percentage (%) in the spleen

Flow cytometric analysis of the harvested splenocytes revealed a slight but significant increase in $CD4^+CD25^+FOXP3^+$ Treg cell percentage in OVA-S&C mice compared with the normal control group. Further significant increase in the percentage of these cells was observed with Adalimumab treatment compared with the untreated OVA-S&C group (Fig. 4).

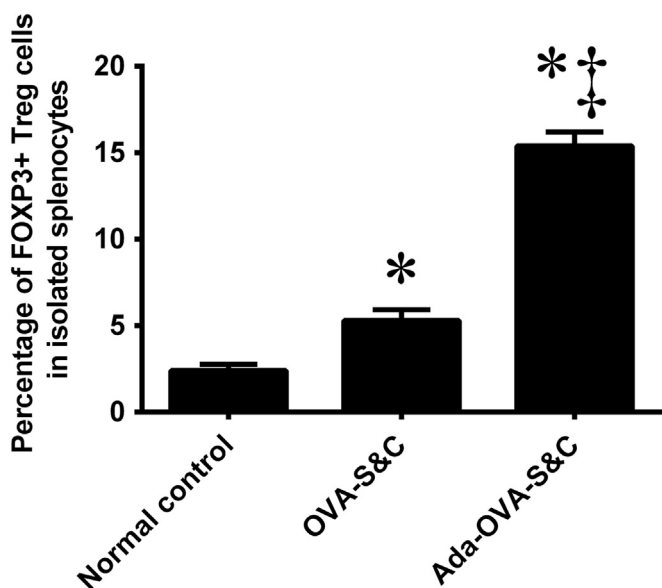


Fig. 4. Effect of Adalimumab on OVA-induced recruitment of inflammatory cells in bronchoalveolar lavage (BAL) fluid. A: Total and differential cell count, B: differential percentage of different cells encountered in BAL fluid. Data are expressed as means $\pm$ S.E.M. * $P < 0.05$ versus normal control and † $P < 0.05$ versus untreated OVA-S&C mice. OVA-S&C: Ovalbumin sensitized and challenged, Ada: Adalimumab.

3.5. Effects of Adalimumab on OVA-induced lung inflammation, mucus hyperplasia and fibrosis

Examination of H&E stained lung sections obtained from normal control mice revealed normal lung parenchyma with very few inflammatory cells in the inter-alveolar septa. Normal appearance of goblets cells with normal airway wall thickness and absence of fibrotic changes were also confirmed with special stains; PAS and MT (Fig. 5(A)–(C)).

Examination of the lungs from untreated OVA-S&C mice showed several key findings characteristic of allergic airway inflammation. In particular, the bronchial tissue of this group revealed marked inflammatory cellular infiltration and penetration into the surrounding alveolar walls and alveolar spaces, including mainly neutrophils and lymphocytes. This was associated with an

increased thickness of small airways wall that encroaches on airway lumen. PAS staining showed severe goblet cell hyperplasia of the bronchial lining as depicted from the significant increase in mucin secretion score, while a significant increase in small airway wall thickness and marked fibrosis in the bronchial wall were detected in MT stained sections (Fig. 5(D)–(F), Fig. 6). Treatment with Adalimumab effectively alleviated these asthma-like features with a significant decrease in the individual as well as the composite inflammatory scores compared with untreated OVA-S&C group. Tissues from Adalimumab-treated mice showed very few neutrophilic and lymphocytic cell infiltrations in the interalveolar septa, marked reduction of goblet cell metaplasia with almost no fibrosis (Fig. 5(G)–(I), Fig. 6).

4. Discussion

The TNF- α is a multi-potent pro-inflammatory mediator that plays a critical role in immune regulation of asthma. When elevated, TNF- α may induce the influx of neutrophils (rather than eosinophils) or a mixed granulocytic airway infiltrate that characterize severe asthma endotypes (Barnes et al., 2008; Pawankar et al., 2015; Wenzel et al., 2013). Moreover, TNF- α over expression has been linked to glucocorticoid insensitivity by reducing glucocorticoid receptor nuclear translocation (Dejager et al., 2015). Thus, antagonizing TNF- α may offer a new strategy for therapeutic intervention in severe and glucocorticoid-resistant asthma. Nevertheless, the anti-inflammatory effects of Adalimumab, one of the TNF- α inhibitors, on allergic airway inflammation are not widely investigated before, and the data about its potential benefits are limited.

In the present study, we investigated the effect of Adalimumab on OVA-induced allergic airway inflammation in CD1 mice. In contrast to the BALB/c and C57BL/6 mice, the most commonly used strains for modelling allergic airway inflammation, which develop a good Th2 biased immunological response with predominant eosinophilic response (Holmes et al., 2011; McAlees et al., 2015), the CD1 mice are not widely used in this field. Recently, Abdel-Aziz et al. (2014) established a murine model of allergen-induced inflammation in male CD1 mice; however, the type of cells infiltrating the lung tissues was not clarified. In the current study, we document for the first time, to our knowledge, the development of neutrophilic airway inflammation in CD1 mice. This was elucidated by the differential staining of BAL fluid cells from OVA-S&C mice; where a shift towards neutrophilic predominance was observed at the expense of macrophages that constituted the majority of cells in lavage fluids from normal control mice. At the same time, eosinophilic cells were nearly absent in the BAL fluids from OVA-S&C mice. This is further documented by the marked infiltration of neutrophils and lymphocytes into the interalveolar septa on histopathological examination of lung tissues from OVA-S&C mice.

The two distinct inflammatory patterns, the eosinophilic and the neutrophilic, were identified in several previous studies (Liou et al., 2014; McAlees et al., 2015; Nabe et al., 2011). In the present study, mice were sacrificed 24 h after the fourth challenge with OVA. This timing may explain the neutrophilic infiltration found in the BAL fluid and histological sections. Nabe et al. (2011) reported that the first through the third challenges are responsible for establishing the airway inflammation that is characterized by eosinophils and $CD4^+$ T cells infiltrates. Subsequently, after the fourth challenge neutrophil infiltration into the airway is induced, which contributes importantly to the induction of the late allergic response (LAR). Furthermore lipopolysaccharides (LPS) that contaminate most preparations of OVA may contribute to the neutrophilia (Jung et al., 2006; Reed and Milton, 2001; Schwartz,

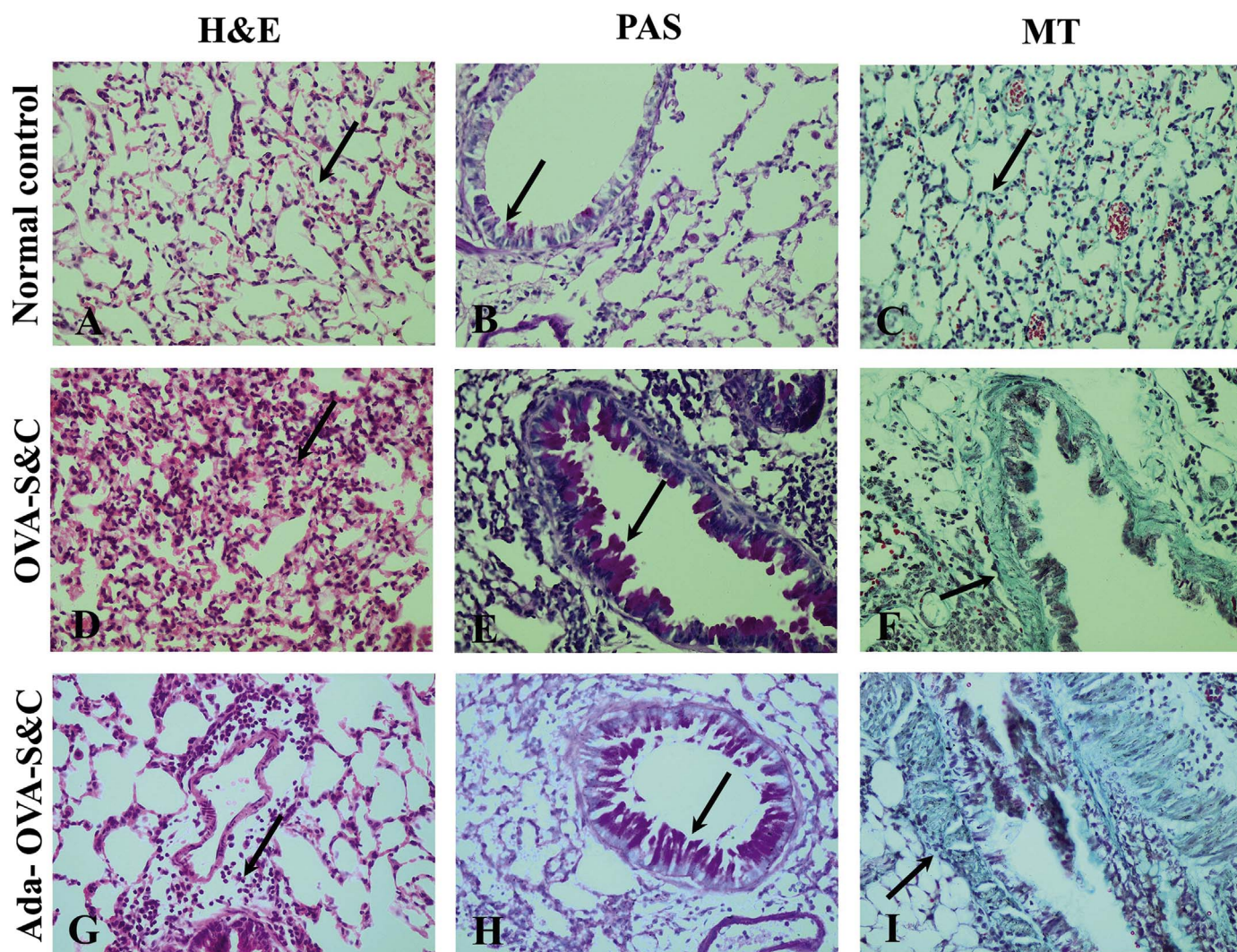


Fig. 5. Representative photomicrographs of H&E, PAS and MT stained lung tissue sections. (A–C): Photomicrograph of lung sections of normal control mice showing (→) very few neutrophilic and lymphocytic cell infiltration in the interalveolar septa (A), normal mucus glands of bronchial lining without any evidence of metaplasia (B), and no fibrosis in the bronchial wall (C). (D–F): Photomicrograph of lung sections of untreated OVA-sensitized and challenged mice showing (→) marked neutrophilic and lymphocytic cell infiltration in the interalveolar septa (D), moderate mucus gland metaplasia of bronchial lining (E) and marked fibrosis in the bronchial wall (F). (G–I): Photomicrograph of lung sections of Adalimumab-treated OVA-sensitized and challenged mice showing (→) a few neutrophilic and lymphocytic cell infiltration in the interalveolar septa (G), mild mucus gland metaplasia of bronchial lining (H) and minimal fibrosis in the bronchial wall (I). Mag. $\times 400$. H&E: Hematoxylin and eosin, PAS: periodic acid Schiff and MT: Masson's trichrome. OVA-S&C: Ovalbumin sensitized and challenged, Ada: Adalimumab.

2001). Airway exposure to LPS-containing allergens induces stimulation of Th17 that secrete IL-17, which in turn produce neutrophilic airway inflammation (Kim et al., 2009). It is not only the LPS contamination, but also the quantity of the LPS inhaled at the time of allergic sensitization is critical for the development of neutrophilic response. Using LPS in very low doses ($\leq 10^{-3}$ μg) with the OVA produced modest amounts of Th2 cytokines response, with associated airway eosinophilia. However, the use of higher dose of LPS (10^{-1} μg) was associated with initial Th2 responses as well as an associated neutrophilia. These findings might account in part for the heterogeneity of inflammatory infiltrates seen in lungs of asthmatics (Whitehead et al., 2014). Species-specific differences in anatomical, physical and immunological responses may be also sharing factors (Eisenbarth et al., 2002; Kumar et al., 2008; Kurucz and Szelenyi, 2006).

Apart from the inflammatory cellular infiltrations, the PAS and MT staining revealed severe apparent goblet cell hyperplasia of the bronchial lining and thickening and fibrosis of the walls of the small airways. These changes may be secondary to the observed increase in IL-4. Similar previous reports also suggested that the

increase in IL-4 expression is responsible for goblet cell hyperplasia, airway hyper-responsiveness (AHR) and fibrosis in allergic airway inflammations (Lee et al., 2012; Liou et al., 2014; Temann et al., 2007; You et al., 2014).

On the other hand, IL-4 was also documented to be responsible for immunoglobulin isotype switching towards IgE predominance. In the contrary, IFN- γ and IL-21 are important inhibitors of IgE isotype switching (Steinke, 2010). Herein, we observed an increase in both IL-4 and IFN- γ ; therefore the increase in the IgE isotype is probably the net result of IL-4 and IFN- γ influences.

The differentiation of naïve T cells (T0) to Th2 and Th1 cells after OVA-S&C can be predicted from the demonstrated increase in IL-4 and IFN- γ expressions in the BAL fluids, respectively. Although other immune cells can produce these cytokines, T-helper cells are the main sources (Berry et al., 2006; Truyen et al., 2006).

The observed upregulation of FOXP3⁺ Treg cells in OVA-S&C mice, in our study, seems to be a trial from the immune system to suppress the OVA-induced T helper cells over activity. One of the important tools by which the immune system inhibit allergic inflammation is the increased expression of IL-10 in the BAL fluid as

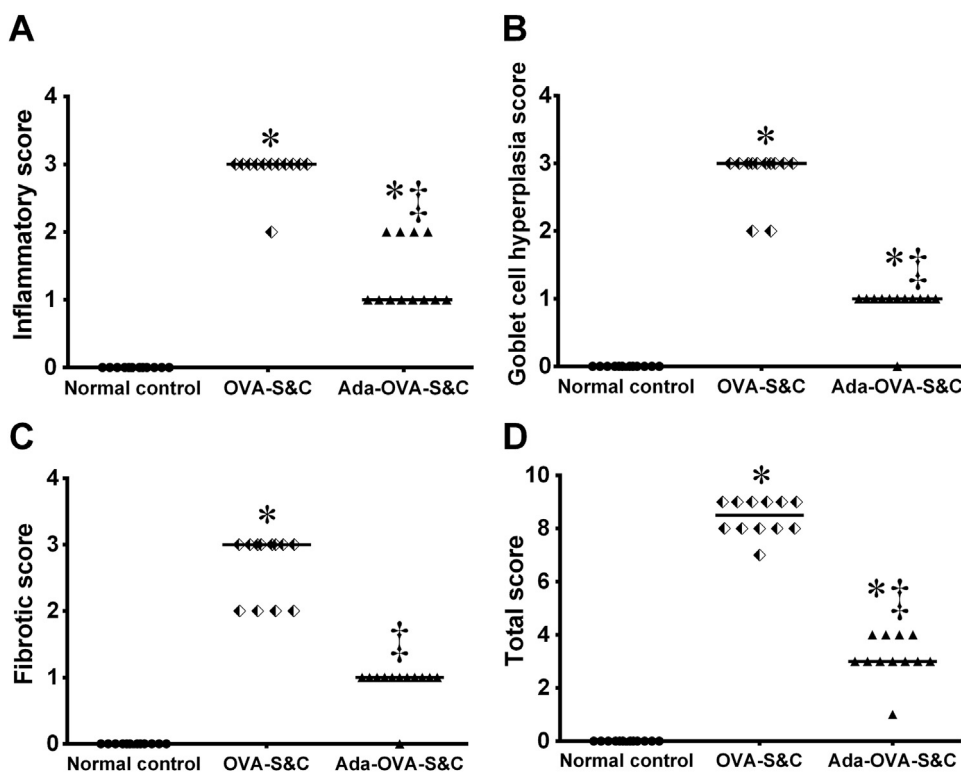


Fig. 6. Scatter plot of $n=12$ with the median values (line) for each experimental group, representing the semi-quantitative morphometric data analysis of histopathologically examined lung sections for (A) inflammation, (B) bronchiolar mucin secretion, (C) peribronchiolar fibrosis where 0, 1, 2, 3 represent absent/rare, mild, moderate and severe, respectively. The score values for these individual parameters were added to form a composite inflammatory score ranging from 0 to 9 in (D). * $P < 0.05$ versus normal control and † $P < 0.05$ versus untreated OVA-S&C mice. OVA-S&C: Ovalbumin sensitized and challenged, Ada: Adalimumab.

shown in our study. However, it seems that the suppressive effect of these Treg cells and its secreted IL-10 was not enough to inhibit the OVA-induced inflammation. In accordance with the present study, Robinson (2009) reported a defective or over ridden suppressive capability of Treg cells expressing the transcription factor FOXP3, on the overactive T-helper responses in patients with an allergic disease.

In the present study, antagonizing TNF- α with Adalimumab induced a significant decrease in the total number of inflammatory cells in the BAL fluids and especially the neutrophil counts. This was coincident with alleviation of asthma-like pathology in the bronchial tissue as shown by the reduction of inflammatory cell infiltrations mainly of the neutrophils, decrease in the mucus gland metaplasia as well as inhibition of the fibrosis and sub-epithelial collagen deposition in the airways.

There are no previous data about the effect of Adalimumab on inflammatory cells in BAL fluid, but Deveci et al. (2008) observed that the use of infliximab, another TNF- α inhibitor, resulted in significant decrease in the number of BAL fluid neutrophils and eosinophils, as well as lung eosinophils in both low and high doses. Moreover, similar data were obtained with etanercept, another TNF- α inhibitor, that was demonstrated to reduce OVA-induced airway remodeling and mucus gland metaplasia (Dejager et al., 2015). Only a single study has reported a reduction of the inflammatory cellular infiltrations in the bronchial tissue and a decrease in alveolar wall thickness in Adalimumab treated mice (Catal et al., 2015). However, the study about the effect of Adalimumab on the cytokine profile in the previous model was deficient. At the same time, there are no available data about the effect of Adalimumab on OVA-induced goblet cell metaplasia and sub-epithelial fibrosis in lung tissues, which is shown in the present study.

Our novel finding that Adalimumab treatment was associated

with increased number as well as the activity of FOXP3⁺ Treg cells, as depicted from the marked increase in IL-10 in BAL fluid, may explain its beneficial effects in this model of allergic airway inflammation.

TNF- α inhibitors were proven to increase Treg cell number and IL-10 levels in patients with immune diseases like RA and Crohn's disease (Ehrenstein et al., 2004; McGovern et al., 2012; Nadkarni et al., 2007; Ricciardelli et al., 2008). However, to the best of our knowledge, no experimental study has addressed the effect of any of the TNF- α inhibitors on Treg cells in a similar model of allergic lung inflammation.

In light of the present findings, Adalimumab treatment can be assumed to augment Treg-mediated inhibition of Th2 cells as shown by the decrease in the inflammatory cytokine, IL-4 and in serum OVA specific IgE. In fact, the studies addressing the effect of Adalimumab on inflammatory cytokines (IL-4 and IFN- γ) and serum OVA specific IgE in murine asthma models seem to be deficient; almost this is the first study investigating this point. However, Nam et al. (2009) demonstrated a significant decrease in Th2-derived cytokine levels including IL-4 in BAL fluid in mice treated with soluble TNF- α receptor (sTNFR) during OVA challenge. Later, infliximab was shown to decrease the local and systemic Th2 cytokine IL-4 production and serum total and OVA specific IgE levels in a mouse model of allergic rhinitis (Mo et al., 2011).

Since it is known that IL-13 and especially IL-4 facilitates IgE production (Robinson et al., 2009; Truyen et al., 2006), the inhibition of IL-4 production may explain the observed decrease in level of OVA specific IgE in our model. Moreover, the encountered reduction in INF- γ by Adalimumab treatment in this model may also point to the inhibitory effect of Treg on its main cellular source, the Th1 cells. In support of this finding, Dejager et al. (2015) has recently reported a suppressive effect of etanercept, a

TNF- α inhibitor, on many cytokines including INF- γ in a severe neutrophilic murine model of asthma.

Although the aforementioned studies demonstrated positive effects of other TNF- α inhibitors on IgE, IL-4 and INF- γ levels, it was worthy to investigate the effect of Adalimumab because of the differential clinical efficacy for different TNF- α inhibitors. This may be partially explained by different mechanisms of action of these compounds (Jin et al., 2010).

In the light of our findings, it can be concluded that the beneficial effects of Adalimumab in this model of neutrophilic inflammation can be mainly attributed to its positive effect on Treg cells that predominantly suppress the other T-effector cells.

Remarkably, Th1-driven airway neutrophilic inflammation was found to be more susceptible to natural Treg (nTreg) cell-dependent suppression than Th2-driven eosinophilic inflammation. This suggests that nTreg-based therapeutic strategies might be more effective in patients with predominant neutrophilic airway inflammation based on deregulating Th1 response (Dehzad et al., 2011). Treatment with corticosteroids in these cases may not be beneficial in the light of the findings that corticosteroids inhibit neutrophil apoptosis, thereby prolonging the survival of these inflammatory cells (Saffar et al., 2011). Thus, novel anti-neutrophil therapies like Adalimumab may be a promising candidate for treating severe neutrophilic asthma phenotypes that is usually steroid resistant.

Available data suggest that anti-TNF therapy is associated with a small but significant overall risk of serious infections (Galloway et al., 2011). However, the risk-benefit balance have to be considered during treatment of the severe, corticosteroid resistant, neutrophilic airway inflammations.

5. Conclusions

At the end, the firm conclusion that could be withdrawn from the present study is that Adalimumab treatment ameliorated all of the OVA-induced biochemical, cytological and structural alterations in CD1 mice, which present a good model for neutrophilic airway inflammation. Activation of Treg cells may stand behind the observed benefit of Adalimumab in this model of allergic inflammation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by EL-Zawia University, Libya which had no further role in the study.

References

- Abdel-Aziz, M., Abass, A., Zalata, K., Abd Al-Galel, T., Allam, U., Karrouf, G., 2014. Effect of dexamethasone and Nigella sativa on inducible nitric oxide synthase in the lungs of a murine model of allergic asthma. *Iran J. Allergy Asthma Immunol.* 13 (5), 324–334.
- Arora, P., Kharkwal, S.S., Porcelli, S.A., 2014. Isolation and in-vivo transfer of antigen presenting cells. *Bio-Protocol* 4 (19), e1260 (<http://dx.doi.org/bio-protocol.org/e1260>).
- Barnes, P.J., 2008. The cytokine network in asthma and chronic obstructive pulmonary disease. *J. Clin. Investig.* 118 (11), 3546–3556. <http://dx.doi.org/10.1172/JCI36130>.
- Berry, M., Brightling, C., Pavord, I., Wardlaw, A., 2007. TNF-alpha in asthma. *Curr. Opin. Pharmacol.* 7 (3), 279–282.
- Berry, M.A., Hargadon, B., Shelley, M., Parker, D., Shaw, D.E., Green, R.H., Bradding, P., Brightling, C.E., Wardlaw, A.J., Pavord, I.D., 2006. Evidence of a role of tumor necrosis factor alpha in refractory asthma. *N. Engl. J. Med.* 354 (7), 697–708.
- Blüml, S., Scheinecker, C., Smolen, J.S., Redlich, K., 2012. Targeting TNF receptors in rheumatoid arthritis. *Int. Immunol.* 24 (5), 275–281. <http://dx.doi.org/10.1093/intimm/dxs047>.
- Bosnjak, B., Stelzmueller, B., ErbK, J., Epstein, M.M., 2011. Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respir. Res.* 12, 114. <http://dx.doi.org/10.1186/1465-9921-12-114>.
- Breslow, R.G., Rao, J.J., Xing, W., Hong, D.I., Barrett, N.A., Katz, H.R., 2010. Inhibition of Th2 adaptive immune responses and pulmonary inflammation by leukocyte immune-globulin like receptor B4 on dendritic cells. *J. Immunol.* 184 (2), 1003–1013. <http://dx.doi.org/10.4049/jimmunol.0900877>.
- Buckner, J.H., 2010. Mechanisms of impaired regulation by CD4⁺CD25⁺FOXP3⁺ regulatory T cells in human autoimmune diseases. *Nat. Rev. Immunol.* 10 (12), 849–859. <http://dx.doi.org/10.1038/nri2889>.
- Catal, F., Mete, E., Tayman, C., Topal, E., Albayrak, A., Sert, H., 2015. A human monoclonal anti TNF- α antibody (Adalimumab) reduces airway inflammation and ameliorates lung histology in a murine model of acute asthma. *Allergol. Immunopathol. (Madr)* 43 (1), 14–18. <http://dx.doi.org/10.1016/j.aller.2013.11.002>.
- Dehzad, N., Bopp, T., Reuter, S., Klein, M., Martin, H., Ulges, A., Stassen, M., Schild, H., Buhl, R., Schmitt, E., Taube, C., 2011. Regulatory T cells more effectively suppress Th1-induced airway inflammation compared with Th2. *J. Immunol.* 186 (4), 2238–2244. <http://dx.doi.org/10.4049/jimmunol.1002027>.
- Dejager, L., Dendoncker, K., Eggermont, M., Souffriau, J., Van Hauwermeiren, F., Willart, M., Van Wouterghem, E., Naessens, T., Ballegeer, M., Vandevyver, S., Hammad, H., Lambrecht, B., De Bosscher, K., Grooten, J., Libert, C., 2015. Neutralizing TNF- α restores glucocorticoid sensitivity in a mouse model of neutrophilic airway inflammation. *Mucosal Immunol.* 8 (6), 1212–1225. <http://dx.doi.org/10.1038/mi.2015.12>.
- Deveci, F., Muz, M.H., Ilhan, N., Kirkil, G., Turgut, T., Akpolat, N., 2008. Evaluation of the anti-inflammatory effect of Infliximab in a mouse model of acute asthma. *Respirology* 13 (4), 488–497. <http://dx.doi.org/10.1111/j.1440-1843.2008.01278.x>.
- Ehrenstein, M.R., Evans, J.G., Singh, A., Moore, S., Warnes, G., Isenberg, D.A., Mauri, C., 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF- α therapy. *J. Exp. Med.* 200 (3), 277–285.
- Eisenbarth, S.C., Piggott, D.A., Huleatt, J.W., Visintin, I., Herrick, C.A., Bottomly, K., 2002. Lipopolysaccharide enhanced, toll-like receptor 4 dependent Th2 cell type responses to inhaled antigen. *J. Exp. Med.* 196 (12), 1645–1651.
- Galloway, J.B., Hyrich, K.L., Mercer, L.K., Dixon, W.G., Fu, B., Ustianowski, A.P., Watson, K.D., Lunt, M., Symmons, D.P., BSRBR Control Centre Consortium, British Society for Rheumatology Biologics Register, 2011. Anti-TNF therapy is associated with an increased risk of serious infections in patients with rheumatoid arthritis especially in the first 6 months of treatment: updated results from the British Society for Rheumatology Biologics Register with special emphasis on risks in the elderly. *Rheumatology* 50 (1), 124e31. <http://dx.doi.org/10.1093/rheumatology/keq242>.
- Holgate, S., Bisgaard, H., Bjermer, L., Haahtela, T., Haughney, J., Horne, R., McIvor, A., Palkonen, S., Price, D.B., Thomas, M., Valovirta, E., Wahn, U., 2008. The Brussels declaration: the need for change in asthma management. *Eur. Respir. J.* 32 (6), 1433–1442. <http://dx.doi.org/10.1183/09031936.00053108>.
- Holmes, A.M., Solari, R., Holgate, S.T., 2011. Animal models of asthma: value, limitations and opportunities for alternative approaches. *Drug Discov. Today* 16 (15–16), 659–670. <http://dx.doi.org/10.1016/j.drudis.2011.05.014>.
- Hsu, D., Liu, C., Chu, P., Li, Y., Liu, M., 2013. Sesame oil attenuates ovalbumin induced pulmonary edema and bronchial neutrophilic inflammation in mice. *Biomed. Res. Int.* 2013, 905670. <http://dx.doi.org/10.1155/2013/905670>.
- Jang, S.O., Kim, H.J., Kim, Y.J., Kang, M.J., Kwon, J.W., Seo, J.H., Kim, H.Y., Kim, B.J., Yu, J., Hong, S.J., 2012. Asthma prevention by *Lactobacillus rhamnosus* in a mouse model is associated with CD4⁺CD25⁺FOXP3⁺ T cells. *Allergy Asthma Immunol. Res.* 4 (3), 150–156. <http://dx.doi.org/10.4168/aiir.2012.4.3.150>.
- Jung, Y.W., Schoeb, T.R., Weaver, C.T., Chaplin, D.D., 2006. Antigen and lipopolysaccharide play synergistic roles in the effector phase of airway inflammation in mice. *Am. J. Pathol.* 168 (5), 1425–1434.
- Jin, J., Chang, Y., Wei, W., 2010. Clinical application and evaluation of anti-TNF-alpha agents for the treatment of rheumatoid arthritis. *Acta Pharmacol. Sin.* 31 (9), 1133–1140. <http://dx.doi.org/10.1038/aps.134>.
- Kim, Y.S., Hong, S.W., Choi, J.P., Shin, T.S., Moon, H.G., Choi, E.J., Jeon, S.G., Oh, S.Y., Gho, Y.S., Zhu, Z., Kim, Y.K., 2009. Vascular endothelial growth factor is a key mediator in the development of T cell priming and its polarization to type 1 and type 17 T helper cells in the airways. *J. Immunol.* 183 (8), 5113–5120. <http://dx.doi.org/10.4049/jimmunol.0901566>.
- Kumar, R.K., Herbert, C., Foster, P.S., 2008. The classical ovalbumin challenge model of asthma in mice. *Curr. Drug Targets* 9 (6), 485–494.
- Kurucz, I., Szelenyi, I., 2006. Current animal models of bronchial asthma. *Curr. Pharm. Des.* 12, 3175–3194.
- Kushwah, R., Hu, J., 2010. Dendritic cell apoptosis: regulation of tolerance versus immunity. *J. Immunol.* 185, 795–802. <http://dx.doi.org/10.4049/jimmunol.1000325>.
- Lee, S.H., Eren, M., Vaughan, D.E., Schleimer, R.P., Cho, S.H., 2012. A plasminogen activator inhibitor-1 inhibitor reduces airway remodeling in a murine model of chronic asthma. *Am. J. Respir. Cell. Mol. Biol.* 46 (6), 842–846. <http://dx.doi.org/10.1165/rcmb.2011-0369OC>.
- Liou, C.J., Cheng, P.Y., Huang, W.C., Chan, C.C., Chen, M.C., Kuo, M.L., Shen, J.J., 2014. Oral lovastatin attenuates airway inflammation and mucus secretion in

- ovalbumin induced murine model of asthma. *Allergy Asthma Immunol. Res.* 6 (6), 548–557. <http://dx.doi.org/10.4168/aa.2014.6.6.548>.
- Liu, Z., 2011. In vivo BrdU incorporation and detection in mouse. *Bio-Protocol* 101, E81 (<http://www.bio-protocol.org/e81>).
- McAlees, J.W., Whitehead, G.S., Harley, I.T., Cappelletti, M., Rewerts, C.L., Holdcroft, A.M., Divanovic, S., Wills-Karp, M., Finkelman, F.D., Karp, C.L., Cook, D.N., 2015. Distinct Tlr4-expressing cell compartments control neutrophilic and eosinophilic airway inflammation. *Mucosal Immunol.* 8 (4), 863–873. <http://dx.doi.org/10.1038/mi.2014.117>.
- McGovern, J.L., Nguyen, D.X., Notlry, C.A., Mauri, C., Isenberg, D.A., Ehrenstein, M.R., 2012. Th17 cells are restrained by Treg cells via the inhibition of IL-6 in patients with rheumatoid arthritis responding to anti-TNF antibodies therapy. *Arthritis Rheum.* 64 (10), 3129–3138. <http://dx.doi.org/10.1038/mi.2014.117>.
- Mo, J.H., Kang, E.K., Quan, S.H., Rhee, C.S., Lee, C.H., Kim, D.Y., 2011. Anti-tumor necrosis factor alpha treatment reduces allergic responses in an allergic rhinitis mouse model. *Allergy* 66 (2), 279–286. <http://dx.doi.org/10.1111/j.1398-9995.2010.02476.x>.
- Nabe, T., Hosokawa, F., Matsuya, K., Morishita, T., Ikeda, A., Fujii, M., Mizutani, N., Yoshino, S., Chaplin, D.D., 2011. Important role of neutrophils in the late asthmatic response in mice. *Life Sci.* 88 (25–26), 1127–1135. <http://dx.doi.org/10.1016/j.lfs.2011.04.003>.
- Nadkarni, S., Mauri, C., Ehrenstein, M.R., 2007. Anti-TNF- α therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF- β . *J. Exp. Med.* 204 (1), 33–39.
- Nam, H.S., Lee, S.Y., Kim, S.J., Kim, J.S., Kwon, S.S., Kim, Y.K., Moon, H.S., Song, J.S., Park, S.H., Kim, S.C., 2009. The soluble tumor necrosis factor-alpha receptor suppresses airway inflammation in a murine model of acute asthma. *Yonsei Med. J.* 50 (4), 569–575. <http://dx.doi.org/10.3349/ymj.2009.50.4.569>.
- Newcomb, D.C., Peebles Jr., R.S., 2013. Th17-mediated inflammation in asthma. *Curr. Opin. Immunol.* 25 (6), 755–760. <http://dx.doi.org/10.1016/j.coi.2013.08.002>.
- Palomares, O., Yaman, G., Azkur, A.K., Akkoc, T., Akdis, M., Akdis, C.A., 2010. Role of Treg in immune regulation of allergic diseases. *Eur. J. Immunol.* 40 (5), 1232–1240. <http://dx.doi.org/10.1002/eji.200940045>.
- Pawankar, R., Hayashi, M., Yamanishi, S., Igarashi, T., 2015. The paradigm of cytokine networks in allergic airway inflammation. *Curr. Opin. Allergy Clin. Immunol.* 15 (1), 41–48. <http://dx.doi.org/10.1097/ACI.0000000000000129>.
- Peters, S.P., Ferguson, G., Deniz, Y., Reisner, C., 2006. Uncontrolled asthma: a review of the prevalence, disease burden and options for treatment. *Respir. Med.* 100 (7), 1139–1151.
- Ray, A., Khare, A., Krishnamoorthy, N., Qi, Z., Ray, P., 2010. Regulatory T cells in many flavors control asthma. *Mucosal Immunol.* 3, 216–229. <http://dx.doi.org/10.1038/mi.2010.4>.
- Reed, C.E., Milton, D.K., 2001. Endotoxin-stimulated innate immunity: a contributing factor for asthma. *J. Allergy Clin. Immunol.* 108 (2), 157–166.
- Ricciardelli, L., Lindley, K.J., Londei, M., Quarantino, S., 2008. Anti tumour necrosis alpha therapy increases the number of FOXP3 regulatory T cells in children affected by Crohn's disease. *Immunology* 125 (2), 178–183. <http://dx.doi.org/10.1111/j.1365-2567.2008.02839.x>.
- Robinson, D.S., 2009. Regulatory T cells and asthma. *Clin. Exp. Allergy* 39 (9), 1314–1323. <http://dx.doi.org/10.1016/j.immuni.2009.08.007>.
- Saffar, A.S., Ashdown, H., Gounni, A.S., 2011. The molecular mechanisms of glucocorticoids-mediated neutrophil survival. *Curr. Drug Targets* 12 (4), 556–562.
- Schwartz, D.A., 2001. Does inhalation of endotoxin cause asthma? *Am. J. Respir. Crit. Care Med.* 163 (2), 305–306.
- Steinke, J.W., 2010. Current prospective of Anti-IL-4, IL-9 and IL-13 therapies in allergic disease. *Recent Pat. Inflamm. Allergy Drug Discov.* 4, 222–230.
- Stellari, F., Sala, A., Ruscitti, F., Carnini, C., Mirandola, P., Vitale, M., Civelli, M., Villetti, G., 2015. Monitoring inflammation and airway remodeling by fluorescence molecular tomography in a chronic asthma model. *J. Transl. Med.* 13, 336. <http://dx.doi.org/10.1186/s12967-015-0696-5>.
- Sun, L.Z., Elsayed, S., Aasen, T.B., Van, D.T., Aardal, N.P., Florvaag, E., Vaali, K., 2010. Comparison between ovalbumin and ovalbumin peptide 323–339 responses in allergic mice: humoral and cellular aspects. *Scand. J. Immunol.* 71 (5), 329–335. <http://dx.doi.org/10.1111/j.1365-3083.2010.02382.x>.
- Taher, Y.A., van Esch, B.C., Hofman, G.A., Henrickx, P.A., van Oosterhout, A.J., 2008. 1-alpha, 25-dihydroxyvitamin D3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF- β . *J. Immunol.* 180 (8), 5211–5221.
- Temann, U.A., Laouar, Y., Eynon, E.E., Homer, R., Flavell, R.A., 2007. IL-9 leads to airway inflammation by inducing IL-13 expression in airway epithelial cells. *Int. Immunol.* 19 (1), 1–10.
- Truyen, E., Coteur, L., Dilissen, E., Overbergh, L., Dupont, L.J., Ceuppens, J.L., Bullens, D.M., 2006. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 61, 202–208.
- Valencia, X., Stephens, G., Goldbach-Mansky, R., Wilson, M., Shevach, E.M., Lipsky, P. E., 2006. TNF down modulates the function of human CD4⁺CD25^{hi} T-regulatory cells. *Blood* 108 (1), 253–261.
- Wenzel, S.E., 2013. Complex phenotypes in asthma: current definitions. *Pulm. Pharmacol. Ther.* 26 (6), 710–715. <http://dx.doi.org/10.1016/j.pupt.2013.07.003>.
- Whitehead, G.S., Thomas, S.Y., Cook, D.N., 2014. Modulation of distinct asthmatic phenotypes in mice by dose-dependent inhalation of microbial products. *Environ. Health Perspect.* 122 (1), 34–42. <http://dx.doi.org/10.1289/ehp>.
- Xu, Q., Ming, Z., Dart, A.M., Du, X.J., 2007. Optimizing dosage of ketamine and xylazine in murine echo-cardiography. *Clin. Exp. Pharmacol. Physiol.* 34, 499–507.
- You, H., Chen, S., Mao, L., Li, B., Yuan, Y., Li, R., Yang, X., 2014. The adjuvant effect induced by di-(2-ethylhexyl) phthalate (DEHP) is mediated through oxidative stress in a mouse model of asthma. *Food Chem. Toxicol.* 71, 272–281. <http://dx.doi.org/10.1016/j.fct.2014.06.012>.