

ORIGINAL ARTICLE

Allergen immunotherapy decreases LPS-induced NF- κ B activation in neutrophils from allergic patients

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Abstract

Background: Allergen-specific immunotherapy (IT) is widely used to treat allergic diseases. The molecular mechanisms have not been clarified yet completely. The present work was undertaken to analyze the effect of IT in the activation of NF- κ B. **Methods:** Neutrophils from 15 pollen-allergic IT-treated patients, 10 untreated pollen-allergic patients, and 10 healthy donors were *in vitro* stimulated with LPS. NF- κ B activation (p65/p52) was measured in their nuclear extracts by enzyme-linked immunosorbent assay (ELISA). I κ B α phosphorylation, NF- κ B-repressing factor (NRF) activation, and thromboxane A₂ (TXA₂) and Interleukin-8 (IL-8) release were measured by ELISA.

Results: There was a positive correlation between the score of symptoms and NF- κ B activation in human neutrophils. IT significantly decreased NF- κ B activation levels in neutrophils compared with neutrophils from untreated patients. I κ B α phosphorylation and NRF activation levels were, respectively, significantly lower and higher in neutrophils from IT-treated patients than from untreated patients. IL-8 and TXA₂ release were significantly lower in neutrophils from IT-treated patients than from untreated patients.

Conclusions: IT positive effects are at least in part mediated by the negative regulation of NF- κ B activation in human neutrophils. These observations represent a novel view of neutrophils as possible cell target to treat IgE-dependent diseases through NF- κ B downmodulation.

There are three defined types of IgE receptors, all previously described in neutrophils (Fc ϵ RI, Fc ϵ RII/CD23 and galectin-3) (1–3) and reviewed in (4). We have previously shown that neutrophils isolated from patients with allergies produce a functional response to those allergens (Ags) that produce clinical symptoms. For example, in neutrophils, Ags induce the release of myeloperoxidase, IL-8, elastase, alpha defensins, the downmodulation of L-selectin, and the respiratory burst and intracellular increase in Ca²⁺ levels (5, 6) and reviewed in (4). In addition, they induce the activation of important inflammatory transcription factors, such as nuclear factor κ B (NF- κ B) and nuclear factor of activated T cells (NFAT) (6, 7), and the expression of allergic inflammatory mediators, such as cyclooxygenase-2, eosinophil cationic protein (ECP) (4, 8), and histamine (9). There is increasing evidence of the participation of neutrophils in allergic processes in general, and in asthma in particular (10–14), and reviewed in (4).

Allergen-specific immunotherapy (IT) is effective to treat allergic diseases (15, 16). This form of allergen desensitization results in reduced symptoms upon exposure to the allergen (17), and it is particularly effective in seasonal pollinosis (18, 19). In children, it prevents new sensitizations (20) and the progression from rhinitis to asthma (21). However, IT downmodulates the immune response by mechanisms that are not yet fully understood.

The transcription factor nuclear factor- κ B (NF- κ B) has pivotal roles in inflammatory disorders (22). There are several NF- κ B family members: NF- κ B₁ (also called p50), NF- κ B₂ (p52), RelA (p65), c-Rel, and RelB. NF- κ B resides in the cytoplasm in an inactive form and upon activation, translocates to the nucleus regulating the expression of genes such as TNF- α , IL-1 β , IL-6, IL-8, and cyclooxygenase 2 (COX-2) (22). RelA/p65 and NF- κ B₂/p52 are involved, respectively, in the classical and alternative NF- κ B activation pathways. Both

pathways share some functions but also have distinct regulatory roles (23). In the cytoplasm, NF- κ B is trapped in an inactive complex by I κ B, which upon cell activation is phosphorylated, polyubiquitinated, and degraded at the proteasome, leading to the NF- κ B translocation to the nucleus where it activates transcription. The best-characterized NF- κ B inhibitor is I κ B α , which binds avidly to RelA/p65 (22). However, it has been described as an I κ B-independent repression mechanism of NF- κ B (24). NF- κ B-repressing factor (NRF) is a nuclear transcription factor that binds to a specific DNA sequence in NF- κ B target promoters, interfering with NF- κ B transcriptional activity (24). The NRF-mediated silencing might be a general nuclear repression mechanism in NF- κ B activation (25).

The persistence of airway inflammation in asthma is likely to be due to the sustained transcription and production of inflammatory mediators. NF- κ B plays an important role in the airway inflammation, increasing the expression of key inflammatory mediators through activation of their transcription (26). IL-8 and COX-2 are two key inflammatory mediators in asthma (27, 28). We have previously found that LPS induce the activation of NF- κ B and the production of IL-8 and TXA₂ (a COX-2 product) dependent on NF- κ B in human neutrophils from allergic patients (6, 29).

The present work was undertaken to analyze the effect of IT on the activation of NF- κ B pathway and IL-8/ TXA₂ production by neutrophils from allergic patients with seasonal asthma.

Material and methods

Reagents

LPS was purchased from Sigma-Aldrich Co (L2630). Ficoll–Hypaque, phosphate-buffered saline, RPMI 1640, fetal bovine serum, and penicillin/streptomycin were purchased from Bio-Whittaker. All culture reagents used in this work (including allergens) had endotoxin levels of ≤ 0.01 ng/ml, as verified by the Coatest *Limulus* lysate assay (Chromogenix, Mölndal, Sweden).

Patients and controls

Three groups of Caucasian spanish children (aged 16–18 yr) were examined and compared (summarized in Table 1): IT-treated atopic patients with G₃ (*Dactylis glomerata*) seasonal allergic asthma (n = 15), non-IT-treated atopic patients with G₃ (*D. glomerata*) seasonal allergic asthma (n = 10), and healthy non-atopic volunteer controls (n = 10) (30). The atopic patients with intermittent bronchial asthma were included in their respective groups (with or without IT) randomly. Intermittent bronchial asthma was diagnosed on the basis of criteria previously described in detail (31). All subjects were lifelong non-smokers. The patients with G₃ seasonal allergic asthma had positive skin-prick test (SPT) (Diater Laboratorios, Madrid, Spain) and serum-specific IgE (HYTEC 288; Hycor Biomedical-IZASA, Barcelona, Spain) to G₃. The sensitivity of the assay was of 0.24 ng/ml to G₃. The non-IT-asthmatic group (n = 10) received no specific hyposensitization. The

IT-asthmatic group (n = 15) had been immunotherapy-treated with *D. glomerata* extract (Diater Laboratorios) for the previous 3 yrs and continued to receive a maintenance dose within the highest dose of the extract. The patients were not allowed to take any bronchodilators within 8 h before challenge of neutrophils *in vitro*. Oral bronchodilators and inhaled bronchial steroids were withheld for 24 h, and none of the subjects had taken oral corticosteroids, disodium cromoglycate, or nedocromil sodium in the previous week. The healthy group had no history of allergy or bronchial symptoms and were negative for the skin-prick and specific IgE tests to a battery of inhalant antigens (house-dust mites, pollens, molds, and animal danders). None of the studied subjects had experienced respiratory-tract infections in the 4 wks before blood sampling. The Hospital Universitario Virgen Macarena ethics committee approved the study.

Symptom diary cards

All patients recorded daily chest symptoms (breathlessness, wheeze, chest tightness, and cough) scores during the pollen season on a scale ranging from 0 to 3 (0 = none; 1 = slight, the symptom is clearly present but is not troublesome; 2 = moderate, the symptom is present, it is troublesome, but is not disabling or insufferable; and 3 = severe, the symptom is severe, disabling, and/or insufferable).

Neutrophils isolation and culture

Whole blood (30 ml) was collected by venipuncture into heparized tubes. Highly purified human neutrophils were isolated exactly as previously described (7), which is a modification of an earlier method (32). Briefly, after isolation, neutrophil preparations were further purified, depleting CD9⁺ cells (eosinophils), CD203c⁺ cells (basophils), and CD14⁺ cells (monocytes) using a magnetic cell-sorting system (MACS) by four rounds of incubation with mouse anti-human CD9, anti-human CD203c, and anti-human CD14 Abs, and then with anti-mouse IgG micromagnetic beads. The purity of the neutrophil preparations was determined as previously described (32). This purification method reduced contaminating eosinophils to 0.001–0.004% of the final cell population. The purity of neutrophils was on average >99%. Monocytes,

Table 1 Demographic characteristics of the study groups

Parameter	Controls (n = 10)	Non-IT treated patients (n = 10)	IT-treated patients (n = 15)
Age*	17.3 $\pm$ 0.82	17.3 $\pm$ 0.82	17.1 $\pm$ 0.83
Gender (male/female)	5/5	4/6	8/7
IgE (KU/l)*	9.5 $\pm$ 5.1	141.2 $\pm$ 58.7	124 $\pm$ 42
Caucasian (%)	100	100	100
Score of symptoms*	0	73 $\pm$ 16.7	30.3 $\pm$ 6.8

*Mean $\pm$ SD.

basophils and lymphocytes were not detected in the neutrophil preparations as previously showed (7). Neutrophils were cultured at a density of 5×10^6 cells/ml with LPS $1 \mu\text{g/ml}$ for 4 h in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin and maintained at 37°C in an atmosphere of 95% O_2 and 5% CO_2 . The dose of LPS was chosen based on a previous study from our laboratory (6, 7) and on pilot experiments. None of the reagents affected the viability of the cells at the concentrations used in this work, as confirmed by the trypan blue dye exclusion test.

Measurement of DNA-binding activity of NF- κ B, NRF nuclear localization, and I κ B α phosphorylation

Nuclear and cytosolic protein extracts of LPS-stimulated neutrophils were prepared as previously described (7). Neutrophils were stimulated with LPS ($1 \mu\text{g/ml}$) for 4 h to quantify p65/p52 activation (29, 33), I κ B α phosphorylation (29, 33), and NRF nuclear translocation (24). NF- κ B (p65 and p52) nuclear activation and the cytosolic active phosphorylated form of I κ B- α were quantified, respectively, using a Trans-AM Transcription Factor Assay Kit and a Functional I κ B- α ELISA kit (Active Motif North America, Carlsbad, CA, USA) according to the manufacturer's instructions. All the experiments were performed in triplicate and expressed as arbitrary units. Nuclear NRF quantification was performed using the NRF ELISA kit (Usen Life Science Inc., Atlanta, GA, USA) according to the manufacturer's protocol. All the experiments were performed in triplicate and expressed as ng/ml.

Measurement of TXA₂ and IL-8 release

The concentrations of the released IL-8 and thromboxane A₂ (TXA₂) were measured in the culture supernatants using, respectively, the IL-8 ELISA kit (Bender MedSystems, Vienna, Austria) and the TXB₂ enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's protocol, as previously described (6, 29). As TXA₂ has a short half-life (37s), it is rapidly hydrolyzed non-enzymatically to its stable derivative TXB₂. TXA₂ was measured indirectly by measuring free TXB₂ (29).

Statistics

All statistical analyses were performed using GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Normality distribution was first examined using Shapiro–Wilk normality test before analysis of statistical significance. The data are expressed as the mean $\pm$ s.e.m. A one-way ANOVA test was used, and statistical significance was defined at $p < 0.05$. Regression analysis was performed using Pearson rank correlation coefficients. A p-value < 0.05 was considered significant.

Results

Previous work has shown that expression of p65 in peripheral blood mononuclear cells (MNCs) correlates with the severity of

asthma (34). This prompted us to analyze whether p65 and p52 activation levels in neutrophils are correlated with the level of symptoms in asthmatic patients in season. As shown in Fig. 1, in LPS-stimulated neutrophils from allergic asthmatic patients ($n = 10$), the nuclear localization and DNA-binding activity of p65 and p52 were correlated with the severity of asthma symptoms ($p = 0.001$, $p = 0.003$).

Previous work has also shown that *Dermatophagoides pteronyssinus*-IT prevents NF- κ B nuclear translocation in $\text{CD4}^+ \text{CD25}^{\text{Hi}}$ T_{Reg} cells and T_{Reg}-depleted PBMCs (35). In agreement with this report, LPS-stimulated neutrophils from non-IT-treated patient showed significantly ($p < 0.001$) higher amount of p65 and p52 localized into the nucleus in an active manner than in neutrophils from IT-treated patients (Fig. 2a,b). As also shown in Fig. 2, the activation of NF- κ B is significantly higher in neutrophils from allergic patients during

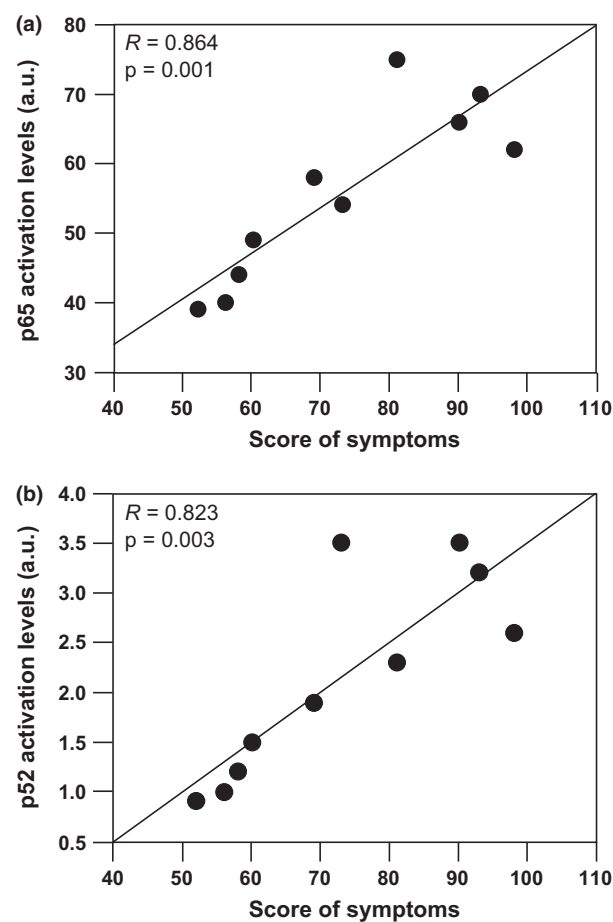


Figure 1 p65 and p52 activation levels are correlated with the severity of asthma. Neutrophils from allergic asthmatic patients sensitized to *G*₃ (*Dactylis glomerata*) ($n = 10$) (10^7 cells) were stimulated with LPS $1 \mu\text{g/ml}$ for 4 h. Nuclear protein extracts were extracted, and the levels of p65 (a) and p52 (b) were analyzed as described in materials and methods. All the experiments were expressed as arbitrary units (a.u.). Regression analysis was performed using Pearson rank correlation coefficients. A p-value < 0.05 was considered significant.

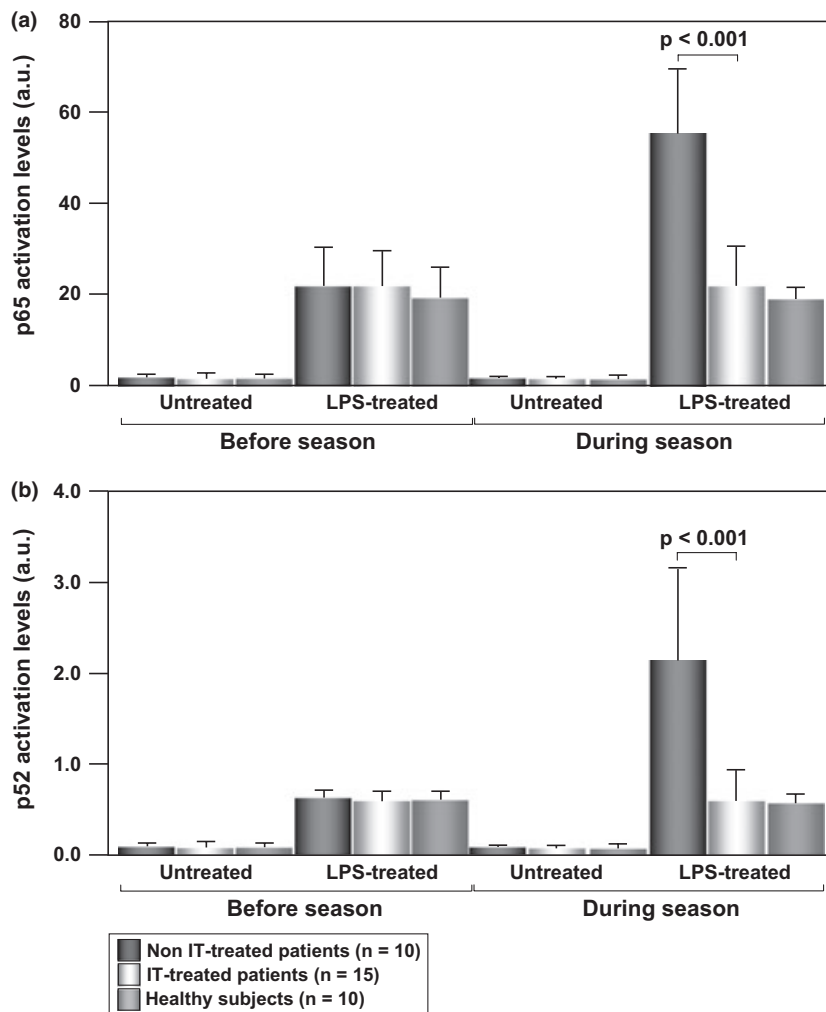


Figure 2 Allergen-specific immunotherapy downmodulates p65 and p52 activation levels. Neutrophils from IT-treated allergic asthmatic patients ($n = 15$) and non-IT-treated allergic asthmatic patients sensitized to G_3 (*D. glomerata*) ($n = 10$) (10^7 cells) were stimulated with LPS $1 \mu\text{g/ml}$ for 4 h. Nuclear protein extracts were extracted, and the levels of p65 (a) and p52 (b) were analyzed as described in materials and methods. All the experiments were expressed as arbitrary units (a.u.). Normality distribution was first examined using Shapiro–Wilk normality test before analysis of statistical significance. The data are expressed as the mean $\pm$ s.e.m. A one-way ANOVA test was used and statistical significance was defined at $p < 0.05$.

season than those from allergic patients before season and healthy donors, before and during season. There are no significant differences between the levels of NF- κ B in neutrophils from healthy donors before and during season, allergic patients before season, and IT-treated patients before and during season. In concordance with these results, the amount of cytosolic phosphorylated I κ B α were significantly decreased ($p < 0.001$) in LPS-stimulated neutrophils from IT-treated patients compared with neutrophils from non-IT-treated patients (Fig. 3a). In addition, LPS-stimulated neutrophils from IT-treated patient had higher nuclear levels of NRF than neutrophils from non-IT-treated patients ($p < 0.001$) (Fig. 3b).

IL-8 and TXA₂ are two important inflammatory mediators that we have previously found to be produced by neutrophils from allergic patients in response to LPS by a NF- κ B-dependent mechanism (6, 29). In concordance with the previous results, IT decreases the release of these two mediators (IL-8, $p < 0.001$; TXA₂, $p < 0.001$) by LPS-stimulated neutrophils from allergic asthmatic patients (Fig. 4a,b).

Discussion

We have previously shown that neutrophils isolated from patients with allergies produce a functional response to those allergens that produce clinical symptoms (4–9, 29) and reviewed in (4). There is increasing evidence of the participation of neutrophils in allergic processes in general, and in asthma in particular. Grass pollen immunotherapy has been shown to be highly effective treating hay fever, even in patients that did not respond to conventional pharmacotherapy (19, 36–38), and it may confer long-term benefit for at least 3 yrs after discontinuation (16).

The molecular mechanisms regulated by IT are not completely understood. It has been described a decrease in the number of effector cells, including mast cells, basophils, and eosinophils, at the inflammation focus in IT-treated patients together with a decrease in T-cell responsiveness to allergens. IT also modifies the balance T_H2/T_H1 toward T_H1 differentiation and results in a new population of circulating T cells – IL-10⁺CD4⁺CD25⁺ – after allergen restimulation (18, 37, 38).

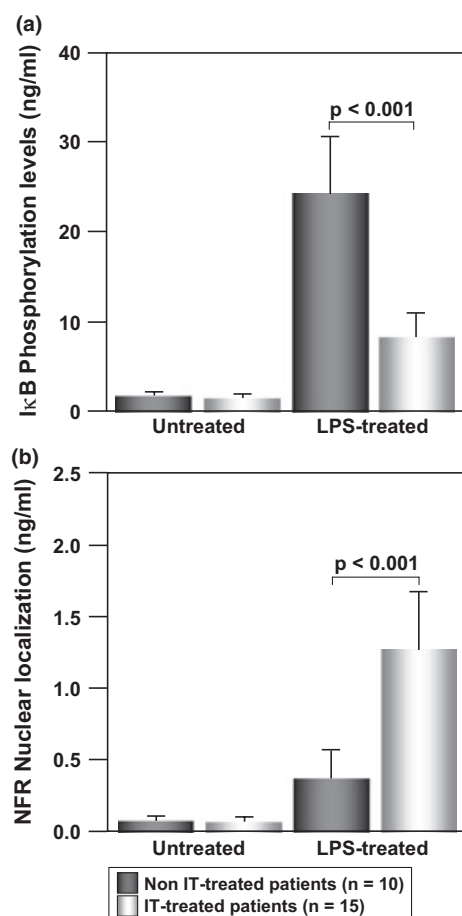


Figure 3 Allergen-specific immunotherapy downmodulates I κ B α phosphorylation and upregulates NFR activation levels. Neutrophils from IT-treated allergic asthmatic patients (n = 15) and non-IT-treated allergic asthmatic patients sensitized to *G*₃ (*D. glomerata*) (n = 10) (10^7 cells) were stimulated with LPS 1 μ g/ml for 4 h. Cytosolic protein extracts were extracted, and the levels of I κ B α (a) and NFR (b) were analyzed as described in materials and methods. Normality distribution was first examined using Shapiro–Wilk normality test before analysis of statistical significance. The data are expressed as the mean $\pm$ s.e.m. A one-way ANOVA test was used and statistical significance was defined at $p < 0.05$.

NF- κ B has a key role in the regulation of proinflammatory gene expression, leading to the synthesis of cytokines, adhesion molecules, chemokines, growth factors, and enzymes (39). These mediators play important roles in a variety of inflammatory diseases. NF- κ B expression is increased in the airways of patients with asthma, and this expression is reduced by IT (34, 40). Moreover, the inhibition of NF- κ B ameliorates allergy symptoms (41, 42). Therefore, a possible IT-target could be NF- κ B. To date, the activation of the classical (p65) and the alternative (p52) NF- κ B pathways has not been evaluated in IT-treated asthmatic patients. Here, we show for the first time that the level of activation of two subunits of NF- κ B in neutrophils, p65 and p52, is correlated with the severity of asthma symptoms. We also show that the levels of activation

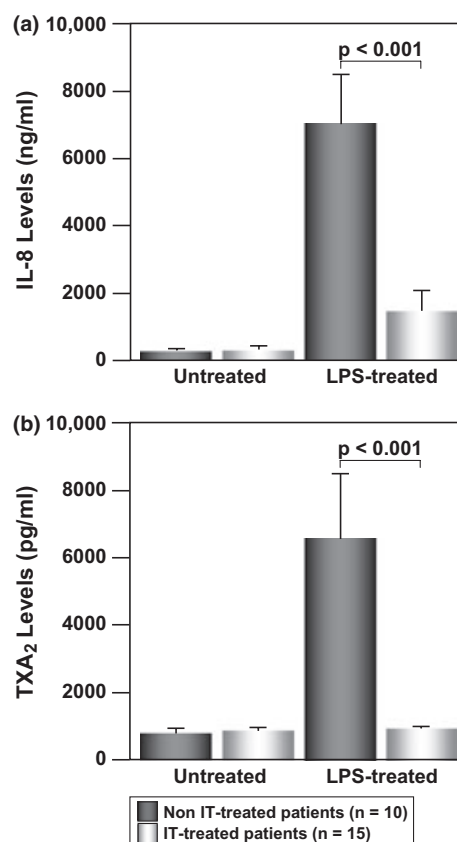


Figure 4 Allergen-specific immunotherapy downmodulates the levels of IgE-dependent IL-8 and TXA₂ production. Neutrophils from IT-treated allergic asthmatic patients (n = 15) and non-IT-treated allergic asthmatic patients sensitized to *G*₃ (*D. glomerata*) (n = 10) (10^7 cells) were stimulated with LPS 1 μ g/ml for 24 h. The levels of IL-8 (a) and TXA₂ (b) were measured in the culture supernatant as described in materials and methods. Normality distribution was first examined using Shapiro–Wilk normality test before analysis of statistical significance. The data are expressed as the mean $\pm$ s.e.m. A one-way ANOVA test was used and statistical significance was defined at $p < 0.05$.

of p65 and p52 are reduced in IT-treated asthmatic patients compared with non-IT-treated asthmatic patients. In concordance with these results, we found that IT decreases the levels of p-I κ B α whereas upregulates the nuclear amount of NRF in neutrophils of IT-treated patients compared with neutrophils from non-IT-treated patients. Furthermore, IT decreased IL-8 production by neutrophils as previously described for MNCs (43). However, opposite results have also been shown (44), (45). Thus, these differences may account to the cell type or the cell culture conditions. Another goal of this study is that we found for the first time that IT decreased the levels of TXA₂ production by neutrophils. The abundance of novel data is promising, but comes along with the awareness that this study has some limitations. Firstly, the sample size is relatively small. Secondly, there were not included experiments pre- and post-treatment. In conclusion, further cohort studies are needed to confirm our results in a large population-based, prospective

placebo-controlled study examining NF- κ B activation in neutrophils before, during, and after immunotherapy.

Neutrophils are usually viewed as terminal, short-lived, innate effector cells that remove microbes and cellular debris at sites of infection or inflammation by mediating phagocytosis and releasing proteolytic enzymes, antimicrobial proteins, and reactive oxygen species. Over the last decade, a number of studies have shown that neutrophils have a lifespan longer than originally thought and mediate diverse immune functions. Although the importance of the quantification of neutrophil half-lives and kinetics is evident, there is still many uncertainty about neutrophil turnover in bone marrow and circulation. Both old and new labeling experiments suffer from technical limitations, such as the toxicity of the tracers that were used, *ex vivo* manipulation affecting neutrophil kinetics and migration, and the inability to distinguish between labeled cells residing in the blood and those that are released by the bone marrow. Alternative approaches that are based on the quantification of neutrophil production in the bone marrow are hampered by the lack of consistent data (46). In the search of the typical neutrophil circulatory half-life, different methods have led to different conclusions. The current paradigm is that neutrophils have a circulatory half-life of only 6–18 h. This short half-life arises from the fact that bloodstream neutrophils constitutively undergo apoptosis. In contrast to the short half-life of circulating cells, neutrophils recruited into tissues have a much longer survival time (several days) because they become rescued from the constitutive apoptotic pathway (47, 48). Multiple studies support the view that neutrophil apoptosis and/or clearance may be defective in patients with asthma and show survival factors in the airway lining fluid, which are specifically enriched in the context of severe asthma (48, 49). Mast cells can enhance the survival of neutrophils due to produced factors, such as tumor necrosis factor α (TNF- α) and granulocyte macrophage colony-stimulating factor (GM-CSF),

which delay their apoptosis (49). Perturbation of the normal apoptosis pathway of tissue neutrophils can enhance their survival times and thereby extend their capacity to secrete their products. On the other hand, in contrast to the traditional view of neutrophils as unsophisticated cells of the phagocytic system, neutrophils have emerged recently as an important component of the effector and regulatory circuits that control the magnitude and quality of an immune response. By establishing bidirectional interactions with dendritic cells, monocytes, macrophages, natural killer cells, B and T cells, neutrophils modulate innate and adaptive immune responses in health and disease states. In allergy, neutrophils may act as primary or secondary effectors of these reactions (50–52).

In conclusion, here we report a novel molecular mechanism of suppression of NF- κ B activation by IT, acting through both the classical – p65 – and the alternative – p52 – pathways. This mechanism of suppression is translated into a reduced production of two important NF- κ B-dependent inflammatory mediators, IL-8 and TXA₂. This mechanism could explain, at least in part, the effectiveness of IT. The understanding of the cells and of the cellular signaling pathways that are involved in IT not only helps us to understand the pathogenesis of asthma, but is also important for the identification of new therapeutic targets. Additional studies are required to illuminate this important matter.

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References

- Gounni AS, Lamkhieou B, Koussih L, Ra C, Renzi PM, Hamid Q. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. *FASEB J* 2001; **15**: 940–9.
- Truong MJ, Gruart V, Kusnier JP, et al. Human neutrophils express immunoglobulin E (IgE)-binding proteins (Mac-2/epsilon BP) of the S-type lectin family: role in IgE-dependent activation. *J Exp Med* 1993; **177**: 243–8.
- Yamaoka KA, Arock M, Issaly F, Dugas N, Le Goff L, Kolb JP. Granulocyte macrophage colony stimulating factor induces Fc epsilon RII/CD23 expression on normal human polymorphonuclear neutrophils. *Int Immunol* 1996; **8**: 479–90.
- Monteseirín J. Neutrophils and asthma. *J Investig Allergol Clin Immunol* 2009; **19**: 340–54.
- Monteseirín J, Chacon P, Vega A, et al. L-selectin expression on neutrophils from allergic patients. *Clin Exp Allergy* 2005; **35**: 1204–13.
- Monteseirín J, Chacon P, Vega A, et al. Human neutrophils synthesize IL-8 in an IgE-mediated activation. *J Leukoc Biol* 2004; **76**: 692–700.
- Vega A, Chacon P, Monteseirín J, et al. Expression of the transcription factor NFAT2 in human neutrophils: IgE-dependent, Ca²⁺ and calcineurin-mediated NFAT2 activation. *J Cell Sci* 2007; **120**: 2328–37.
- Monteseirín J, Vega A, Chacon P, et al. Neutrophils as a novel source of eosinophil cationic protein in IgE-mediated processes. *J Immunol* 2007; **179**: 2634–41.
- Alcañiz L, Vega A, Chacon P, et al. Histamine production by human neutrophils. *FASEB J* 2013; **27**: 2902–10.
- Bucova M, Suchankova M, Dzurilla M, et al. Inflammatory marker sTREM-1 reflects the clinical stage and respiratory tract obstruction in allergic asthma bronchiale patients and correlates with number of neutrophils. *Mediators Inflamm* 2012; **2012**: 628754.
- Ciepiela O, Zawadzka-Krajewska A, Kotula I, Pyrzak B, Demkow U. Influence of sublingual immunotherapy on the expression of Mac-1 integrin in neutrophils from asthmatic children. *Adv Exp Med Biol* 2013; **756**: 73–80.
- Hoskins A, Reiss S, Wu P, et al. Asthmatic airway neutrophilia after allergen challenge is associated with the glutathione S-transferase M1 genotype. *Am J Respir Crit Care Med* 2013; **187**: 34–41.
- Mansson A, Bachar O, Adner M, Björnsson S, Cardell LO. Leukocyte phenotype changes induced by specific immunotherapy in patients with birch allergy. *J Investig Allergol Clin Immunol* 2010; **20**: 476–83.
- Monteseirín J, Bonilla I, Camacho J, Conde J, Sobrino F. Elevated secretion of

- myeloperoxidase by neutrophils from asthmatic patients: the effect of immunotherapy. *J Allergy Clin Immunol* 2001; **107**: 623–6.
15. Bousquet J, Maasch H, Martinot B, Hejjaoui A, Wahl R, Michel FB. Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. II. Comparison between parameters assessing the efficacy of immunotherapy. *J Allergy Clin Immunol* 1988; **82**: 439–46.
 16. Durham SR, Walker SM, Varga EM, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999; **341**: 468–75.
 17. Practice parameters for allergen immunotherapy. Joint Task Force on Practice Parameters, representing the American Academy of Allergy, Asthma and Immunology, the American College of Allergy, Asthma and Immunology, and the Joint Council of Allergy, Asthma and Immunology. *J Allergy Clin Immunol* 1996; **98**: 1001–11.
 18. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998; **102**: 558–62.
 19. Varney VA, Gaga M, Frew AJ, Aber VR, Kay AB, Durham SR. Usefulness of immunotherapy in patients with severe summer hay fever uncontrolled by antiallergic drugs. *BMJ* 1991; **302**: 265–9.
 20. Pajno GB, Barberio G, De Luca F, Morabito L, Parmiani S. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. *Clin Exp Allergy* 2001; **31**: 1392–7.
 21. Moller C, Dreborg S, Ferdousi HA, et al. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol* 2002; **109**: 251–6.
 22. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 2001; **107**: 7–11.
 23. Nishikori M. Classical and alternative NF-kB activation pathways and their roles in lymphoid malignancies. *J Clin Exp Hematopathol* 2005; **45**: 15–24.
 24. Ho SC, Lee KY, Chan YF, et al. Neutrophil elastase represses IL-8/CXCL8 synthesis in human airway smooth muscle cells through induction of NF-kappa B repressing factor. *J Immunol* 2009; **183**: 411–20.
 25. Nourbakhsh M, Hauser H. Constitutive silencing of IFN-beta promoter is mediated by NRF (NF-kappaB-repressing factor), a nuclear inhibitor of NF-kappaB. *EMBO J* 1999; **18**: 6415–25.
 26. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; **336**: 1066–71.
 27. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am J Respir Crit Care Med* 2000; **161**: 769–74.
 28. Profita M, Sala A, Bonanno A, et al. Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *J Allergy Clin Immunol* 2003; **112**: 709–16.
 29. Vega A, Chacon P, Alba G, El Bekay R, Martin-Nieto J, Sobrino F. Modulation of IgE-dependent COX-2 gene expression by reactive oxygen species in human neutrophils. *J Leukoc Biol* 2006; **80**: 152–63.
 30. National Heart, Lung, and Blood Institute. Global strategy for asthma management and prevention. NIH Publication No. 96-3659B. November 1998.
 31. American Thoracic Society. Definitions and classifications of chronic bronchitis, asthma and emphysema. *Am Rev Respir Dis* 1962; **85**: 762–8.
 32. Sur S, Glitz DG, Kita H, et al. Localization of eosinophil-derived neurotoxin and eosinophil cationic protein in neutrophilic leukocytes. *J Leukoc Biol* 1998; **63**: 715–22.
 33. Stacey MA, Sun G, Vassalli G, Marini M, Bellini A, Mattoli S. The allergen Der p1 induces NF-kappaB activation through interference with IkappaB alpha function in asthmatic bronchial epithelial cells. *Biochem Biophys Res Commun* 1997; **236**: 522–6.
 34. La Grutta S, Gagliardo R, Mirabella F, et al. Clinical and biological heterogeneity in children with moderate asthma. *Am J Respir Crit Care Med* 2003; **167**: 1490–5.
 35. Tsai YG, Chiou YL, Chien JW, Wu HP, Lin CY. Induction of IL-10 + CD4 + CD25 + regulatory T cells with decreased NF-kappaB expression during immunotherapy. *Pediatr Allergy Immunol* 2010; **21**: e166–73.
 36. Bousquet J, Lockey RF, Malling HJ, eds. Allergen Immunotherapy: Therapeutic vaccines for allergic diseases. WHO position paper. *Allergy* 1998; **53**(Suppl 44): 4–42.
 37. Wilson DR, Nouri-Aria KT, Walker SM, et al. Grass pollen immunotherapy: symptomatic improvement correlates with reductions in eosinophils and IL-5 mRNA expression in the nasal mucosa during the pollen season. *J Allergy Clin Immunol* 2001; **107**: 971–6.
 38. Frew AJ, Powell RJ, Corrigan CJ, Durham SR. Efficacy and safety of specific immunotherapy with SQ allergen extract in treatment-resistant seasonal allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 2006; **117**: 319–25.
 39. Baldwin AS Jr. Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 2001; **107**: 3–6.
 40. Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *Am J Respir Crit Care Med* 1998; **158**: 1585–92.
 41. Donovan CE, Mark DA, He HZ, et al. NF-kappa B/Rel transcription factors: c-Rel promotes airway hyperresponsiveness and allergic pulmonary inflammation. *J Immunol* 1999; **163**: 6827–33.
 42. Yang L, Cohn L, Zhang DH, Homer R, Ray A, Ray P. Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *J Exp Med* 1998; **188**: 1739–50.
 43. Akoum H, Duez C, Vorng H, et al. Early modifications of chemokine production and mRNA expression during rush venom immunotherapy. *Cytokine* 1998; **10**: 706–12.
 44. Hsieh KH, Chou CC, Chiang BL. Immunotherapy suppresses the production of monocyte chemotactic and activating factor and augments the production of IL-8 in children with asthma. *J Allergy Clin Immunol* 1996; **98**: 580–7.
 45. Wang JY, Hsieh KH. The effect of immunotherapy on the in vitro productions of histamine, prostaglandin E2 and leukotriene C4 in asthmatic children. *Asian Pac J Allergy Immunol* 1989; **7**: 119–24.
 46. Tak T, Tesselaar K, Pillay J, Borghans JA, Koenderman L. What's your age again? Determination of human neutrophil half-lives revisited. *J Leukoc Biol* 2013 Apr 26. [Epub ahead of print], doi: 10.1189/jlb.1112571.
 47. Edwards SW, Moulding DA, Derouet M, Moots RJ. Regulation of neutrophil apoptosis. *Chem Immunol Allergy* 2003; **83**: 204–24.
 48. Yang EJ, Choi E, Ko J, Kim DH, Lee JS, Kim IS. Differential effect of CCL2 on constitutive neutrophil apoptosis between normal and asthmatic subjects. *J Cell Physiol* 2012; **227**: 2567–77.
 49. Uddin M, Nong G, Ward J, et al. Prosurvival activity for airway neutrophils in severe asthma. *Thorax* 2010; **65**: 684–9.
 50. Cerutti A, Puga I, Magri G. The B cell helper side of neutrophils. *J Leukoc Biol* 2013; doi: 10.1189/jlb.1112596.
 51. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011; **11**: 519–31.
 52. Schuster S, Hurrell B, Tacchini-Cottier F. Crosstalk between neutrophils and dendritic cells: a context-dependent process. *J Leukoc Biol* 2012 Dec 18. [Epub ahead of print], doi: 10.1189/jlb.1012540.