



Non-antibiotic properties of tetracyclines as anti-allergy and asthma drugs

Rauno Joks^{a,*}, Helen G. Durkin^{a,b}

^a Department of Medicine, Center for Allergy and Asthma Research at SUNY Downstate, State University of New York Downstate Medical Center, Brooklyn, NY 11203, USA

^b Department of Pathology, Center for Allergy and Asthma Research at SUNY Downstate, State University of New York Downstate Medical Center, Brooklyn, NY 11203, USA

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ABSTRACT

All available therapies for human allergic disease target IgE mediated pathologic responses after IgE has been produced. We are developing tetracyclines as anti-allergy drugs to prevent IgE production, based on our findings that minocycline or doxycycline treatment of allergic asthmatic humans significantly improves their asthma symptoms, reduces their oral steroid requirements, and strongly suppresses their ongoing IgE responses (ELISA, mast cell mediated cutaneous late phase responses); the tetracyclines also strongly suppress peak IgE responses of BPO-KLH sensitized mice (ELISPOT assay, ELISA, skin tests). The antibiotic activity of the tetracyclines is not required for suppression of IgE responses; inclusion of minocycline or doxycycline in sterile culture prevents anti-CD40/IL-4 mediated induction of memory IgE responses by PBMC of allergic asthmatic patients (ELISA), and induction of specific memory IgE responses by spleen cells of BPO-KLH sensitized mice (ELISPOT assay, ELISA). The tetracyclines affect an epsilon specific pathway because IgM, IgG and IgA responses did not decrease. Further, in humans, DTH responses to recall antigens did not decrease. In related studies, we found that two distinct T cell subsets: CD4+CD60 negative and CD8+CD60+ (CD60 is a ganglioside) (humans) and CD4+ Asialo GM1 ganglioside negative and CD8+Asialo GM1 ganglioside+ (mice), both are required for induction of memory IgE responses. Phosphorylated (phos) p38 MAP kinase, but not phos ERK or phos JNK expression by CD4+ and CD8+, including CD8+CD60+, T cells is increased in allergic asthmatic humans, as is IL-4 and IL-10 production. The tetracyclines appear to target T cell pathways to induce suppression of IgE responses because they suppress phos p38 MAP kinase expression by both CD4+ and CD8+, including CD8+CD60+, T cell subsets, and IL-4 and IL-10, while upregulating IL-2 and IFN gamma, and suppressing IgE responses. Our finding that tetracyclines do not require antibiotic activity to suppress IgE responses opens the door to development of new tetracycline-based and other therapeutics for human allergic disease.

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1. Introduction

Allergic disorders including asthma, rhinoconjunctivitis, food allergy, and atopic dermatitis affect a substantial proportion of the general population [1]. The therapeutic armamentarium to treat these conditions has been limited for many years. Recent studies of the effects of tetracyclines on allergic responses have

opened a new door to the potential treatment of allergy and asthma.

1.1. Increasing prevalence of allergic disease

The prevalence of allergic disorders has doubled in ten to fifteen years and now affects 10–15% of Western populations [1]. A current explanation for the increase in allergic disease is offered by the hygiene hypothesis [2]. Western societies have, for the most part, successfully limited major infectious diseases (through sanitary measures and vaccinations) with a possible inadvertent adverse effect [3]. Such decrease in stimulation of the Th1 arm of the immune system due to decrease in infectious stimuli, either bacterial or viral, may have led to skewing of immune responses towards the allergen-specific IgE producing Th2 arm of the immune system. Allergic disorders such as allergic rhinitis and asthma result, in part, when allergen-specific IgE binds to its receptors on the surfaces of mast cells and basophils [4]. Subsequent exposure to allergens in the airways results in the release of histamine and

Abbreviations: MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cells; BPO-KLH, benzylpenicilloyl-keyhole limpet hemocyanin; CMT, chemically modified tetracyclines; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot assay; JNK, c-jun N-terminal kinases; ERK, extracellular signal-regulated kinases; DTH, delayed type hypersensitivity; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; Treg, regulatory T cell; Th, helper T cell; TNF, tumor necrosis factor; FOCIS, Federation of Clinical Immunology Societies.

* Corresponding author at: Department of Medicine, SUNY Downstate Medical Ctr., Rm B6-515, Box 50, 450 Clarkson Ave., Brooklyn, NY 11203, USA. Tel.: +1 718 270 2156; fax: +1 718 270 3289.

E-mail address: Rauno.Joks@downstate.edu (R. Joks).

other mediators that are involved in the eosinophilic inflammation and airway hyperreactivity thought to be characteristic of asthma [4].

1.2. Inner city allergy and asthma—unmet needs

State University of New York Downstate Medical Center, located in Brooklyn, serves a large population of racial and ethnic minorities of low socioeconomic status residing in an urban environment, a population that is disproportionately at high risk for asthma morbidity and mortality [5,6]. Epidemiologic studies suggest that key risk factors contributing to asthma morbidity within the inner city include social demography, the physical environment (indoor and outdoor), and health care access and quality [7]. Death from asthma is two to six times more likely to occur among African Americans and Hispanics than among whites [8,9]. Rates of hospitalization for asthma demonstrate similar variations; rates for African Americans hospitalized are almost triple those for whites [8,9]. For children living in the inner city, asthma tends to be more frequent and severe. Although the number of deaths annually from asthma is low compared to other chronic diseases, the death rate for children aged 5–14 years and young adults aged 15–34 years doubled from 1979–80 to 1993–95 [8,9].

Recent studies at our institution [10,11] have shown a high prevalence of self-reported food allergy. Adult and pediatric patients seen at our institution for non-allergy reasons report that 12.9% and 20.5%, respectively, have food allergy symptoms. Both adult and pediatric groups show a strong correlation between food allergy symptoms and asthma [10,11]. This cohort of asthma patients, vulnerable to increased morbidity and mortality from asthma, has been understudied with respect to new treatments for asthma.

1.3. Current therapies for allergic asthma

The mainstay of treatment of asthma at present is inhaled corticosteroid therapy [12]. While inhaled corticosteroids are effective, rates of adherence are low and they do not seem to modify the course of the disease. All inhaled corticosteroids are absorbed in the lung and might have systemic effects [12]. Ciclesonide is a recently introduced inhaled steroid that is a pro-drug activated by esterases and may be safer with fewer systemic effects [13]. While it has been approved for treatment of allergic rhinitis and asthma, its long term effects are not known.

The only currently available treatment approved specifically for allergic asthma is omalizumab, an anti-IgE monoclonal antibody injected once or twice per month. It is a useful adjunct for allergic asthma [14]. However, omalizumab is an expensive treatment that is not easily obtainable for many patients.

While investigation of inflammatory mediator antagonists is an active area of research, the only ones currently approved and available are the anti-leukotriene agents. As a group these are considered weakly effective and may not contribute much to treatment of severe asthma [15]. Studies of treatment of asthma with anti-TNF biologic agents such as etanercept and infliximab have demonstrated benefit, but are not approved for asthma, are expensive, and may only be beneficial in the treatment of severe asthma [15]. Experimental agents that inhibit phosphodiesterase dampen inflammatory responses in a wide range of cells involved in airway inflammation. In studies done by Bousquet et al. [16], there was decrease in allergen induced responses in asthma after treatment with roflumilast. However, the side effect profile of this class of drugs is limiting and includes both upper and lower gastrointestinal effects and headaches [15].

1.4. Mechanisms of allergic inflammation—controlling IgE

Current therapies, including omalizumab (anti-IgE monoclonal antibody), target cellular and clinical sequelae of IgE after it has been produced and binds to its cellular receptors [15]—i.e. a post-factum approach rather than decreasing IgE responses at the level of IgE production.

While IgE is central to the immune processes resulting in allergic disease such as asthma [17–21], there is, at present, no commercially available drug that decreases human IgE responses at the level of IgE production. As reviewed by Blaser and colleagues [22], there has been an ongoing effort in this area. Promising approaches to decreasing IgE responses include blockage of IL-4 and IL-13, and targeting of GATA-3, the major transcription factor controlling Th2 associated cytokines, with antisense message [22]. Strategies for augmenting natural CD4+CD25+ FoxP3+ regulatory T cells may result in suppression of allergen specific Th2 responses [22].

2. Minocycline as asthma therapy

Our studies of the role of tetracyclines (minocycline, doxycycline, chemically modified tetracyclines [CMT]) in the regulation of human and murine IgE responses began when we discovered that treatment of allergic asthmatic patients with minocycline significantly improved their asthma symptoms, had significant oral steroid sparing effects, and improved their spirometric outcomes [23], findings which indicate the potential usefulness of minocycline as therapy for allergic disease and asthma. Our randomized, double-blind, placebo-controlled, crossover study was conducted in 17 adult patients with moderate and severe persistent asthma with a history of current or recurrent use of oral corticosteroids. The subjects were maintained on their baseline drug regimens for asthma, including inhaled corticosteroids, throughout the study, with the exception of prednisone, the doses of which were self-adjusted by the patients, according to their symptoms [23]. The patients were treated for eight weeks with either minocycline (150 mg po twice daily) or placebo, followed by a four week period in which no treatment was given (wash out). They were then treated for another eight weeks with the alternate therapy. The patients did not have evidence of sinusitis at baseline before commencing treatment. They also did not have *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* infections of the respiratory tract at baseline before each treatment arm. Minocycline compared with placebo treatment was associated with improved patient health, as judged by symptom scores, ~40% reduction in mean daily prednisone use, and improvement in forced vital capacity [23] (Table 1). Interestingly, similar treatment of allergic asthmatic patients with chronic obstructive pulmonary disease (COPD) did not produce these effects (Joks, unpublished studies).

The mechanisms by which minocycline treatment improved health of allergic asthmatic patients were investigated. We studied the ability of minocycline to suppress ongoing human and murine IgE responses, as well as their ability to suppress in vitro induction of specific memory IgE responses by PBMC of ragweed sensitized allergic asthmatic humans and by spleen and mesenteric lymph node cells of BPO-KLH sensitized mice.

3. Tetracycline mediated suppression of ongoing IgE responses of allergic asthmatic humans, BPO-KLH sensitized mice, and toluene-diisothiocyanate sensitized mice

3.1. Allergic asthmatic humans

We found that when allergic asthmatic patients (serum IgE 505 ± 535 IU/ml) were given minocycline (150–250 mg po BID) as

Table 1
Clinical studies assessing the use of tetracyclines as anti-allergy and asthma drugs.

Identification	Title	Status
ClinicalTrials.gov NCT00456677	Evaluation of the Utility of Minocycline as an Anti-Inflammatory Agent in the Treatment of Asthma	Closed
ClinicalTrials.gov NCT00536042	Open-label Trial of the Use of Minocycline in the Treatment of Asthma	Ongoing
SUNY Downstate IRB 08-097	Evaluation of Minocycline Treatment on Development of Late Phase Cutaneous Responses to Aeroallergens And Recall Antigens	Ongoing
SUNY Downstate IRB 09-032	Effect of Topical Minocycline on Immediate and Late Phase Allergic Skin Responses	Ongoing

add on therapy to standard care for up to ten months, their ongoing serum IgE responses first decreased at 1–2 months (~20%), further decreased at 4–6 months, and were still suppressed at 10 months ($47.6 \pm 14.7\%$) ($p=0.012$) (ELISA) (Fig. 1) [24]. In contrast to the minocycline group, serum IgE levels of the control group (serum IgE > 100–1146 IU/ml) were not suppressed. Important to its potential as an anti-allergy drug, minocycline appears to affect an epsilon specific pathway (i.e. the IgE pathway), because IgM, IgG and IgA responses did not decrease. Further, minocycline did not interfere with delayed type hypersensitivity responses to recall antigens [25]. IgE responses which are inhibited by minocycline also include cutaneous mast cell mediated late phase responses [25].

With respect to the doses of minocycline used in these studies, the regimen selected was that previously used by others to treat patients with rheumatoid arthritis [26]. It is well recognized that minocycline can cause side effects such as nausea or dizziness [26,27]. Therefore, if a subject had either of these symptoms, she/he was advised to decrease the dose by first discontinuing the 50 mg capsule of their 150 mg twice daily dosing. If side effects persisted, the patient discontinued the 100 mg capsule and contin-

ued only with the 50 mg capsule twice daily [24]. This approach optimized dosing and assured potential for maximum benefit with minimal adverse effects. Data presented in Fig. 1 are for patients who completed the ten month clinical trial [24].

3.2. BPO-KLH sensitized mice

It was important to develop a mouse model in which varying regimens of minocycline, doxycycline, and CMTs could be tested for their ability to suppress IgE production and to determine toxicity, etc. which is required for development of new anti-allergy drugs for eventual use in humans. The mouse model also is used to define mechanisms by which tetracycline suppresses production of IgE by B cells. We selected a well established mouse model in which to test the ability of tetracyclines to suppress ongoing (including memory) IgE responses [28,29]. Use of the mouse model allows us to monitor IgE production by B cells in lymphoid organs and related immune responses (other cells/cytokines) required for induction of, and suppression of IgE responses, as well as changing levels of serum IgE.

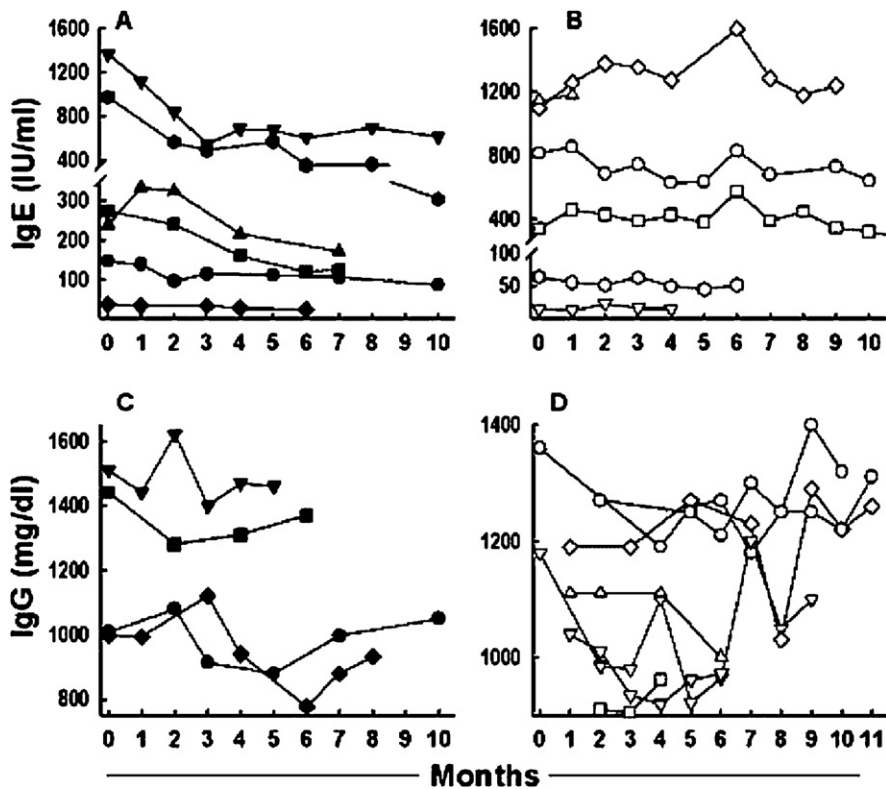


Fig. 1. Effect of oral minocycline treatment on serum immunoglobulin (IgM, IgG, IgE, and IgA) levels of allergic asthmatic humans. Adult allergic asthmatic subjects ($n=6$) were given minocycline (150–250 mg po (per os (by mouth)) BID (bis in die) (twice daily)) as add-on to standard therapy for up to 1 year. Control allergic asthmatic subjects ($n=8$) received standard therapy. Serum IgE and IgM, IgG, IgA levels of all subjects were measured before and monthly after initiation of minocycline treatment (total IgE fluoroenzymeimmunoassay and nephelometry, respectively). IgE levels of minocycline treated subjects are shown in panel A, and controls in panel B; IgG levels of minocycline treated subjects are shown in panel C and controls in panel D. IgM and IgA levels, like IgG levels, did not significantly change in minocycline treated or control groups ($p=ns$) (data not shown). Data for IgE are expressed as IU/ml; for IgG, mg/dl. Joks et al. [24]. Reproduced with permission.

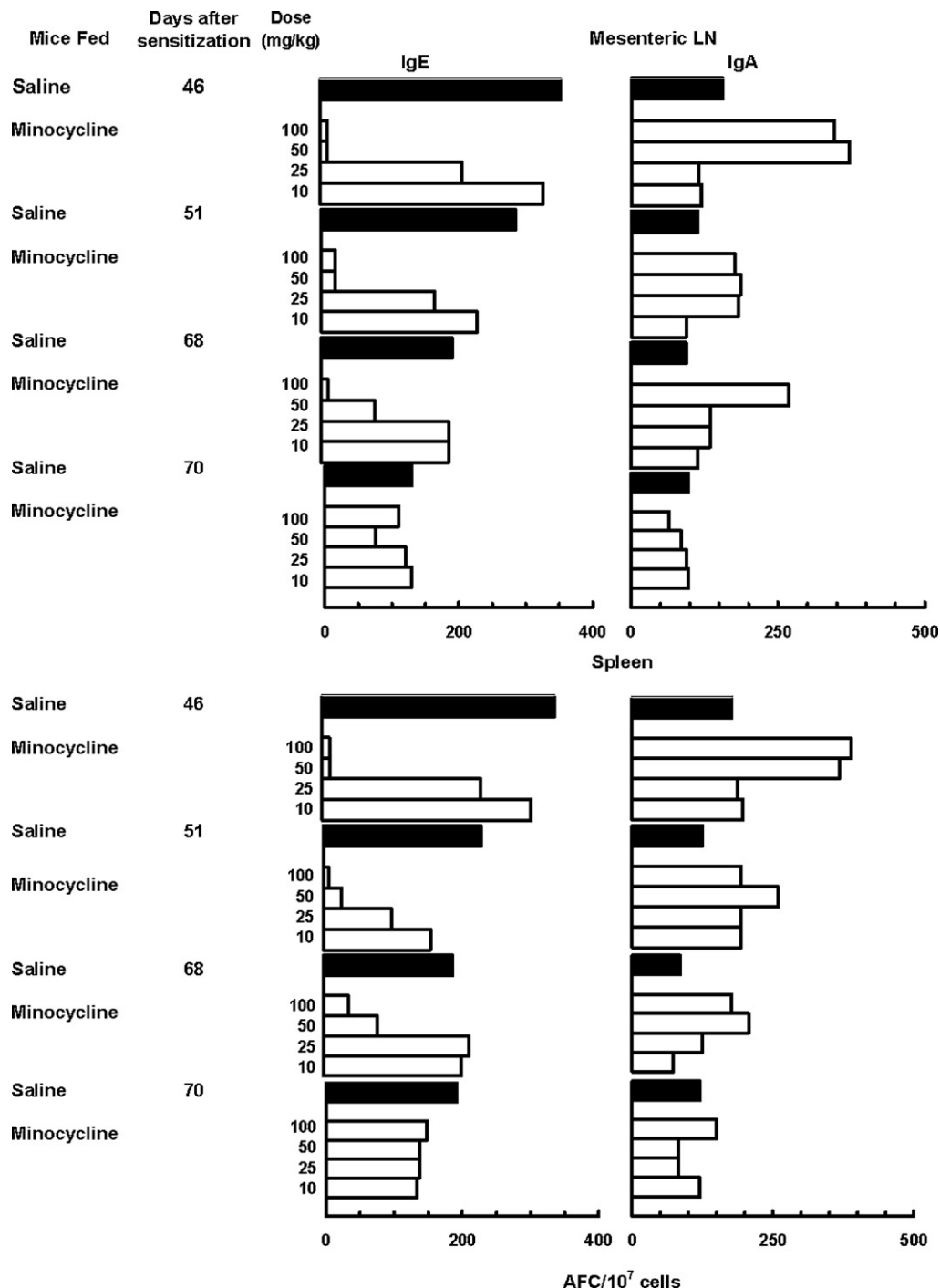


Fig. 2. Effect of oral minocycline and doxycycline given at the peak of the BPO-specific IgE response in vivo on antibody forming cell (AFC) (IgG1, IgE, and IgA) responses in mesenteric lymph nodes and spleen of BPO-KLH sensitized mice (ELISPOT assay). BALB/c mice ($n = 6/\text{group}$) were injected ip with BPO₁₄-KLH (10 μg) in alum (0.2 mL) on days 0, 21 and 43, fed (gavage) with minocycline or doxycycline (10–100 mg/kg) or with saline on day 44, and killed on days 46–70, at which times the numbers of BPO specific IgG₁ (data not shown), IgE and IgA AFC in mesenteric lymph node and spleen were determined ex vivo in ELISPOT assay using BPO-BSA coated plates. Representative experiment, 1 of 3 with similar results. Data are from 6 mice/group, and represent mean values obtained with triplicate wells. Data are expressed as AFC/10⁷ cells. Similar results obtained with doxycycline are not shown. With minocycline (and doxycycline) treatment, IgG₁, like IgA, AFC numbers did not decrease. In all experiments, peak suppression of IgE responses (>95% on day 46) was obtained with minocycline (or doxycycline) at 100 and 50 mg/kg. Joks et al. [24]. Reproduced with permission.

As in our earlier studies [30], to induce peak IgE responses, we injected BALB/c mice intraperitoneally with 10 μg benzylpenicilloyl₁₄-keyhole limpet hemocyanin (BPO₁₄-KLH) in alum on days 0, 21 and 42 [28,29]. Mice were fed (gavage) with varying doses of minocycline or doxycycline (10–100 mg/kg) on day 44, and killed on days 46–70, at which time the numbers of BPO specific IgE, IgG₁ and IgA antibody forming cells (AFC) in

mesenteric lymph nodes and spleen and serum immunoglobulin levels were determined (ELISPOT assay, ELISA) (Fig. 2) [28,29].

We found that minocycline (and doxycycline) strongly suppressed peak IgE AFC and serum IgE responses (>95%, ~75%, respectively) [24]. Suppression of IgE responses in vivo was dose dependent and lasted 5–7 days, when IgE responses increased without boosting. If animals were then boosted, and then fed again with

minocycline, their IgE responses again decreased (Durkin, unpublished studies). It is important to emphasize that, as in our human studies (see Section 3.1), minocycline (and doxycycline) affect epsilon specific pathways because treatment of mice with these tetracyclines did not suppress responses of other immunoglobulin isotypes (IgG1 or IgA) in lymphoid organs or in serum [24]. Mice tolerated the treatment well and resumed feeding within an hour [24].

3.3. Toluene-diisothiocyanate induced murine model of asthma

Lee et al. [31] reported that doxycycline, as well as other known matrix metalloprotease (MMP) inhibitors, reduced airway hyper-responsiveness, inflammation, and levels of airway MMP-9, which may play a role in airway remodeling in asthma [32], in mice with toluene diisothiocyanate-induced asthma [31].

4. Tetracycline mediated suppression of murine memory IgE responses in vitro

4.1. BPO-specific memory IgE responses by murine spleen and mesenteric lymph node cells

To induce memory IgE responses in vitro, spleen or mesenteric lymph node cells from BPO₁₄-KLH immunized mice (see Section 3.2) were obtained on day 42 (mice were not treated with minocycline in vivo) and cultured for 0–12 days $\pm$ varying concentrations of BPO₂₅-KLH; peak memory IgE responses occurred with BPO₂₅-KLH at 50 ng/ml [24]. We found that inclusion of minocycline (or doxycycline) (100 ng/ml) in culture strongly suppressed induction of memory IgE AFC responses (ELISPOT assay). The suppression obtained was dose dependent [24]. It was IgE isotype specific because BPO-specific memory IgG1 and IgA AFC responses did not decrease [24]. It was transient, lasting 5–7 days.

4.2. LPS-IL-4 induction of IgE responses by murine splenocytes

Bottaro and colleagues [33] demonstrated that doxycycline inhibited IL-4 mediated in vitro induction of IgE production by murine spleen cells. Primary mouse splenocytes (C57Bl/6 or BALB/c mice) were cultured in medium containing LPS/dextran, with or without the addition of IL-4. At day 5, activated B cell blasts were harvested and analyzed for expression of Ig isotypes with flow cytometry. Treatment with 5 μ g/ml doxycycline resulted in a significant decrease in the percentage of IgE⁺ lymphoblasts to 14% of baseline.

5. IgE and related immune responses (cells/cytokines/signal transduction) of allergic asthmatic humans before minocycline treatment

We made several new findings with respect to cells, cytokines and signal transduction required for induction and/or maintenance of ongoing, including memory, IgE responses of allergic asthmatic humans. In Section 7, we will describe the ability of minocycline to suppress cytokines and signal transduction related to suppression of IgE responses.

5.1. Requirement for two distinct T cell subsets: CD4⁺ and CD8⁺ and their cytokines for induction of memory IgE responses by PBMC of ragweed sensitized humans

It is well recognized that CD4⁺ T cells and their cytokines (IL-4, IL-13) are required for induction of human IgE responses in vivo and in vitro (reviewed in Refs. [34,35]). We recently discovered that two

distinct T cell subsets: CD4⁺ and CD8⁺CD60⁺, and their cytokines are required for induction of human ragweed specific memory IgE responses [36]. Smith-Norowitz et al. in our laboratory was the first to demonstrate that CD8⁺CD60⁺ (80–98% CD45RO⁺) T cell numbers were significantly increased in blood of serum IgE⁺ ragweed sensitized humans obtained during ragweed allergy season, compared with serum IgE-negative humans [36]. Their CD8⁺CD60⁺ T cells, like their CD4⁺ T cells, produced IL-4. In addition, the CD8⁺CD60⁺ T cells also produced IL-5, IL-10, IL-12, IFN alpha and IFN gamma, and, sometimes, IL-2, but not IL-6 or IL-13; unfractionated PBMC of the same humans produced the same cytokines [36]. When PBMC from the ragweed sensitized humans are cultured for 10 days with ragweed antigen, memory IgE responses are induced. However, depletion of either CD4⁺ or CD8⁺CD60⁺ T cells before culture, or inclusion of antibodies to either IL-2, IL-4, IL-5, IL-10, IL-12, IFN alpha or IFN gamma in culture abrogated the ability to induce ragweed specific memory IgE responses. The ability to induce memory IgE responses was restored by adding back the depleted subset [36]. Interestingly, although CD8⁺CD60⁺ T cells added back to cultures in low numbers restored ability to induce memory IgE responses by PBMC depleted of these cells, addition of higher numbers (but still low numbers) of these cells to cultures suppressed induction of memory IgE responses, suggesting involvement of a feedback mechanism, and that CD8⁺CD60⁺ T cells may serve as Treg cells [36].

5.2. Inverse relationship of human CD8⁺CD60⁺ T cells to CD4⁺CD25⁺ Treg cells in human blood

When we studied the distributions of CD8⁺CD60⁺ T cells and CD4⁺CD25⁺ T cells in blood of ragweed sensitized allergic asthmatic patients over a period of >one year, we found that their distributions vary greatly at different times of the year [36]. For example, there is a statistically significant increase in the numbers of CD8⁺CD60⁺CD45RO⁺ T cells in blood of serum IgE⁺ ragweed sensitized allergic asthmatic humans at the peak of the ragweed allergy season, whereas after ragweed allergy season, the numbers of these cells dramatically decrease, and relatively few of the CD8⁺CD60⁺ T cells present in blood outside ragweed allergy season express the memory marker, CD45RO [36]. Interestingly, during ragweed allergy season, when numbers of blood CD8⁺CD60⁺ (CD45RO⁺) are high, numbers of CD4⁺CD25⁺ Treg cells are low or undetectable. In contrast, at other times of the year, i.e. not in ragweed allergy season, when numbers of CD8⁺CD60⁺ T cells are low, and those present express CD45RA rather than CD45RO, the numbers of CD4⁺CD25⁺ T cells are increased [36]. We are studying the ability of CD4⁺CD25⁺ T cells obtained from ragweed sensitized humans throughout the year to suppress memory IgE responses.

5.3. CD4⁺ and CD8⁺ blood T cells of allergic asthmatic humans express increased levels of phosphorylated p38 MAP kinase compared with serum IgE negative nonallergic humans

Adcock et al. [37] demonstrated that the T cell transcription factor, GATA-3, regulates release of IL-4, IL-5 and IL-13 by cells of a human T cell line (HUT-78). In these cells, translocation of GATA-3 to the nucleus and its regulation of IL-4, IL-5 and IL-13 genes depend on phosphorylation of GATA-3 by p38 MAP kinase [37]. Silverberg et al. in our laboratory (Silverberg et al. [49]) recently found that virtually all peripheral blood PBMC (CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, CD14⁺ monocytes) of allergic asthmatic humans and serum IgE negative nonallergic humans express phosphorylated p38 MAP kinase and phosphorylated ERK, with fewer of these cells expressing phosphorylated JNK. Interestingly, we found that CD4⁺ and CD8⁺ T cells, including CD8⁺CD60⁺ T cells, of allergic asthmatic humans, which are required for memory IgE responses (see

Section 5.1), expressed significantly higher levels of phosphorylated p38 MAP kinase, compared with T cells of serum IgE negative nonallergic humans (Silverberg et al. [49]).

5.4. Phosphorylated p38 MAP kinase levels expressed by blood T cells of allergic asthmatic humans are directly associated with their serum IgE levels

In these studies (Silverberg et al. [49]), we also found that the increased levels of phosphorylated p38 MAP kinase expressed by CD4+ and CD8+ T cells, including CD8+CD60+ T cells, which are required for induction of memory IgE responses of allergic asthmatic humans (see Section 5.1), further increase as serum IgE levels increase, suggesting that increased activity of this phosphorylated p38 MAP kinase in CD4+ and CD8+, including CD8+CD60+, T cells is related to or responsible for increases in serum IgE production (Silverberg et al. [49]).

6. Minocycline mediated suppression of ongoing IgE responses and related immune responses (T cell cytokines, signaling molecules) of allergic asthmatic humans in vivo and in vitro

6.1. IgE and cytokine responses

We previously reported that if allergic asthmatic humans were given minocycline treatment for 10 months, their ongoing IgE responses were strongly suppressed (~50%) [24]. After one month of treatment, their IgE responses begin to decrease (~20%); at one week of treatment, no suppression is observed (<10%) (Silverberg, unpublished studies). As described in Section 5.1, PBMC of ragweed sensitized allergic asthmatic humans make IL-2, IL-4, IL-5, IL-10, IL-12, IFN gamma and IFN alpha in ragweed allergy season; they do not make IL-13 or IL-6 [36]. PBMC obtained from other IgE producing allergic asthmatic humans outside of ragweed allergy season, whether or not they are sensitized to ragweed, make IL-4, IL-10, IL-12, IFN alpha, but not IL-2 or IFN gamma (or IL-13 or IL-6). When the latter subjects are given oral minocycline treatment for one week or one month (no suppression of IgE responses, ~20% suppression, respectively), IL-2 and IFN gamma producing cells appeared in blood, with no changes in cells producing the other cytokines (IL-4, IL-10, IL-12, IFN alpha) [38]. If PBMC obtained from the same humans were cultured for 0–12 days in the presence of anti-CD40/rhIL-4, IgE responses were induced, and peaked at day 10. However, inclusion of minocycline in culture abrogated the ability of anti-CD40/rhIL-4 to induce IgE responses and PBMC now made IL-2 and IFN gamma, but no longer made IL-4 or IL-10; the other cytokines were unchanged [38].

6.2. Phosphorylated MAP kinases: p38, ERK, and JNK

We found that virtually all PBMC (T and B lymphocytes, monocytes) of allergic asthmatic and IgE negative nonallergic humans (>98%) expressed the phosphorylated MAP kinases, p38 and ERK; about 50% of cells of these subsets also expressed JNK (flow cytometry) (Silverberg et al. [49]). Minocycline treatment of allergic asthmatic humans for one week had no effect on the numbers of PBMC expressing these markers. However, when levels of expression of the phosphorylated MAP kinases/cell subset were determined (MESF, flow cytometry), an entirely different picture emerged. Minocycline treatment for one week resulted in statistically significant suppression of levels of expression of phosphorylated p38 MAP kinase by CD4+ and CD8+ T cells, but not in their levels of expression of ERK or JNK (Silverberg et al. [49]). There was no statistically significant suppression of levels of expression

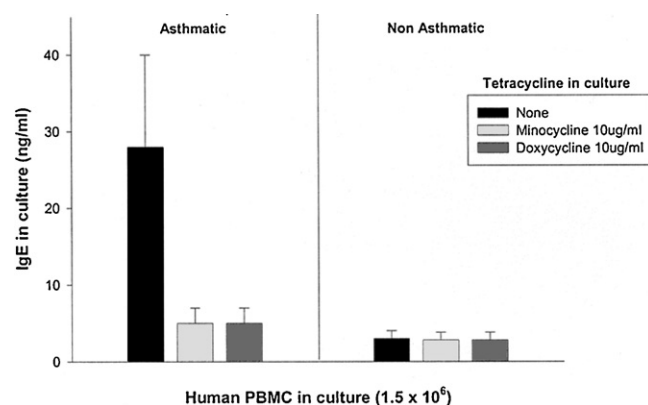


Fig. 3. IgE production in vitro by PBMC from asthmatic and nonasthmatic subjects after tetracycline treatment. Human PBMC ($1.5 \times 10^6 \text{ mL}^{-1}$) were obtained from asthmatic and nonasthmatic subjects and cultured $\pm$ anti-CD40 mAb ($1 \mu\text{g}/\text{mL}$), $\pm$ rhIL-4 ($200 \text{ U}/\text{mL}$), in the presence or absence of minocycline ($10 \mu\text{g}/\text{mL}$) or doxycycline ($10 \mu\text{g}/\text{mL}$) for 10 days. High levels of IgE obtained on day 10 in the absence of tetracyclines were strongly suppressed when tetracyclines were included in cultures of PBMC from asthmatic patients. There was no change in IgE levels when tetracyclines were included in cultures of PBMC from nonasthmatic subjects. Results are from 12 experiments, repeated three times, using PBMC obtained from asthmatic subjects ($n=5$) and nonasthmatic subjects ($n=7$). Data are expressed as $\text{ng}/\text{mL} \pm$ standard error (SE).

Smith-Norowitz et al. [40]. Reproduced with permission.

of the phosphorylated MAP kinases p38, ERK or JNK by B cells or monocytes.

To further investigate the role of T cells in minocycline mediated suppression of IgE responses in vitro, we used anti-CD40/rhIL-4 to stimulate IgE production directly by B cells [39], enabling us to deplete T cell subsets before culture and study the effect of their depletion on minocycline mediated suppression. The effect of minocycline on T cell suppression cannot be studied using ragweed specific memory IgE responses because induction of these responses requires both CD4+ and CD8+ T cells (see Section 5.1). Before the allergic asthmatic subjects were treated with minocycline, we obtained their PBMC and cultured these cells for 10 days in the presence of anti-CD40 and rhIL-4 $\pm$ minocycline. Without minocycline, IgE responses were first induced on day 6, reached peak levels on day 10, and remained high on day 12 (ELISA) (see also [39]). We found that inclusion of minocycline (or doxycycline) in culture prevented induction of IgE responses (>98%) (isotype specificity not tested) (Fig. 3) [40]. We also found that if either CD4+ or CD8+, including CD8+CD60+, T cells were depleted from PBMC before culture, minocycline and doxycycline no longer suppressed induction of IgE responses; IgE responses in these cultures resembled those of cultures that did not contain the tetracycline (Durkin, unpublished studies). We conclude that both CD4+ and CD8+ T are required for minocycline mediated suppression of anti-CD40/rhIL-4 induced IgE responses by PBMC of allergic asthmatic humans.

6.3. Effect of minocycline on phosphorylated MAP kinases: p38, ERK, and JNK

Inclusion of minocycline in cultures in which anti-CD40/rhIL-4 induced peak IgE responses on day 10 not only entirely suppressed induction of IgE responses, but also significantly suppressed expression of phosphorylated p38 MAP kinase by CD4+ and CD8+ T cells (Silverberg et al. [49]). Again, as in vivo, minocycline had no significant effects on their levels of expression of the phosphorylated MAP kinases, ERK or JNK. Further, minocycline had no significant effects on levels of expression of phosphorylated MAP kinases p38, ERK or JNK by B cells or monocytes (Silverberg et al. [49]).

7. IgE and related immune responses of BPO-KLH sensitized mice (cells, cytokines) before and after minocycline treatment

7.1. Requirement for two distinct T cell subsets: CD4+ and CD8+ (formerly Thy 1+ Asialo GM 1 ganglioside negative and Thy 1+ Asialo GM 1 ganglioside+, respectively) for induction of memory IgE responses by spleen cells of BPO-KLH sensitized mice

The requirement for two distinct T cell subsets and their cytokines for induction of memory IgE responses, discussed above for humans (see Section 5.1) had previously been demonstrated by Herrick et al. in our laboratory [41] in BALB/c mice which were immunized by injecting 10 µg BPO-KLH in alum intraperitoneally on days 0, 24, and 42. BPO specific IgE, as well as IgG1 and IgA AFC responses in spleen and mesenteric lymph node were determined on days 44–70 (ELISPOT assay); serum IgE and IgM, IgG and IgA levels also were determined (ELISA). To induce memory IgE responses, spleen or mesenteric lymph node cells were obtained on these days, and then cultured for 0–10 days in the presence or absence of BPO-KLH. Herrick found that Thy 1+ Asialo Gm1 ganglioside negative and Thy 1+ Asialo Gm1 ganglioside+ (now known to be CD4+ and CD8+ T cells, respectively) were required for in vitro induction of hapten specific memory IgE responses. In these studies, she also demonstrated that IL-4, IL-10, IFN alpha and IFN gamma all were required for induction of memory IgE responses (other cytokines not studied), a finding that exactly mimicks the more recent findings of Smith-Norowitz et al. in humans [36]. It should be noted that like Asialo Gm1 ganglioside, CD60 expressed by human CD8+ T cells required for induction of memory IgE responses, also is a ganglioside. The significance of the gangliosides, albeit different gangliosides, expressed by CD8+ T cells required for induction of murine and human memory IgE responses, if any, is unknown. Asialo Gm 1 ganglioside is not expressed by human T cells.

8. Effect of tetracyclines and CMT on mast cell activity and cutaneous late phase responses

8.1. Inhibition of mast cell activation and proapoptotic effects of CMTs on mast cell responses in vitro

Sandler et al. [42] investigated the in vitro effects of CMT-3 or COL-3, and doxycycline on functions of mast cells and demonstrated suppression of 48/80 induced histamine release from rat serosal mast cells to less than 50% that of control. Production of TNF-α and IL-8 by HMC-1, a human mast cell line, was significantly decreased with suppression of cytokine mRNA production. Further, CMT-3 significantly suppressed protein kinase C (PKC) activity in these cells. In separate studies, Sandler et al. [43] investigated the antiproliferative effects of four different CMTs (CMT-1, CMT-3, CMT-8 and CMT-308) on mast cells in vitro and demonstrated induction of apoptosis including induction of caspases and down-regulation of antiapoptotic Bcl-2 protein.

8.2. Minocycline-mediated inhibition of mast cell mediated cutaneous late phase responses of allergic humans

As reported by Erstein [25], oral minocycline treatment results in a significant suppression of mast cell mediated late phase swelling at 30 h in adults with allergic respiratory disease induced by cutaneous allergen challenge with epicutaneous scratch test, with no effect on classic late phase memory responses to recall antigen. As compared to baseline responses, there is a 72.3% decrease in mean wheal diameter at 30 h with 1 week of minocycline treatment

with no significant change in induration induced with *C. albicans* (Table 1).

9. Summary

We demonstrated that tetracycline (minocycline) treatment of adult asthma is of clinical benefit, with oral steroid sparing effects. While it has pleiotropic anti-inflammatory effects [44–48], we demonstrated that it decreases IgE production in an isotype specific manner. Minocycline-mediated decreases in IgE responses are associated with suppression of phosphorylated p38 MAP kinase by T lymphocytes of allergic asthmatic humans. These anti-allergy effects of minocycline are independent of its antibiotic effects as IgE and p38 MAP kinase suppression occurred in culture systems devoid of bacteria. Other IgE responses which are also inhibited by minocycline include cutaneous mast cell mediated late phase responses.

10. Why tetracyclines and CMT would be advantageous as drugs to treat allergic disease

There are no current allergy/asthma therapies that inhibit IgE production. Further, there are no currently clinically available p38, ERK, or JNK MAP kinase inhibitors. Development of tetracyclines and chemically modified tetracyclines as regulators of these signal pathways in lymphocytes such as CD4+ T cells, as well as other cell types of humans and animals, introduces many novel mechanistic and therapeutic options for treatment of diseases with chronic and other inflammation, including asthma, allergic rhinoconjunctivitis, food allergy, and allergic skin disorders. This is a novel capability for an inexpensive, established oral therapy with a known side effect profile.

References

- [1] Kay AB. Allergy and allergic diseases. *N Engl J Med* 2001;344:30–7.
- [2] Strachan DP. Hay fever, hygiene and household size. *BMJ* 1989;299:1259.
- [3] Bach J-F. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347:911–20.
- [4] Lemanske RF, Busse WW. Asthma: factors underlying inception, exacerbation, and disease progression. *J Allergy Clin Immunol* 2006;117:S456–461.
- [5] American Lung Assoc. Trends in asthma morbidity and mortality. Epidemiology and Statistics Unit. Research and Program Services Division; February 2010.
- [6] Grant EN, Alp H, Weiss KB. The relation of socioeconomic factors and racial/ethnic differences in US asthma mortality. *Am J Public Health* 2000;90(12):1923–5.
- [7] Grant EN, Alp H, Weiss KB. The challenge of inner-city asthma. *Curr Opin Pulm Med* 1999;5(1):27–34.
- [8] Mannino DM, Homa DM, Pertowski CA, Ashikawa A, Nixon LL, Johnson CA, et al. Surveillance for asthma—United States, 1960–1995. *MMWR CDC Surveill Summ* 1998;47(1):1–27.
- [9] Busse WW, Mitchel A. Addressing issues of asthma in inner-city children. *J Allergy Clin Immunol* 2007;119(1):43–9.
- [10] Karanickolas R, Kumar S, Katayeva I, Joks R. Self reported food allergy prevalence in an immigrant minority adult population in Brooklyn. *Ann Allergy Asthma Immunol* 2010;105(5):A105.
- [11] Kumar S, Shams P, Nalluri P, Moallem J, Joks R. Self reported prevalence of food allergy symptoms in an inner city minority general pediatric population. *J Allergy Clin Immunol* 2010;123(2):AB217.
- [12] Expert panel report 3. Guidelines for the diagnosis and management of asthma. Available online at www.nhlbi.nih.gov/guidelines/asthma/epr3/index.htm [last accessed 31.08.07].
- [13] Reynolds NA. Ciclesonide. *Drugs* 2004;64:511–9.
- [14] Strunk RC, Bloomberg GR. Omalizumab for asthma. *N Engl J Med* 2006;354:2689–95.
- [15] Barnes PJ. New therapies for asthma. *Trends Mol Med* 2006;12:515–20.
- [16] Bousquet J, Aubier M, Sastre J, Izquierdo JL, Adler LM, Hofbauer P, et al. Comparison of roflumilast, an oral anti-inflammatory, with beclomethasone dipropionate in the treatment of persistent asthma. *Allergy* 2006;61:72–8.
- [17] Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE level and skin-test reactivity to allergens. *N Engl J Med* 1989;320:271–7.
- [18] Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD, et al. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 1991;325:1067–71.

- [19] Reischl IG, Corvaia N, Effenberger F, Wolffwiniski B, Kromer E, Mudde GC, et al. Function and regulation of Fc Epsilon R1 expression on monocytes from non-atopic donors. *Clin Exp Allergy* 1996;26:630–41.
- [20] Yamaguchi M, Lantz CS, Oettgen HC, Katona IM, Fleming T, Miyajima I, et al. IgE enhances mouse mast cell Fc epsilon R1 expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. *J Exp Med* 1997;185:663–72.
- [21] MacGlashan DW, Bochner BS, Adelman DC, Jardieu PM, Togias A, Lichtenstein LM, et al. Serum IgE level drives basophil and mast cell IgE receptor display. *Int Arch Allergy Immunol* 1997;113:45–7.
- [22] Blaser K, editor. T cell regulation in allergy, asthma and atopic skin diseases. *Chem Immunol Allergy* 2008; vol. 94. Basel: Karger. p. 83–92.
- [23] Daoud A, Gloria CJ, Taningco G, Hammerschlag MR, Weiss S, Gelling M, et al. Minocycline treatment results in reduced oral steroid requirements in adult asthma. *Allergy Asthma Proc* 2008;29(3):286–94.
- [24] Joks R, Smith-Norowitz T, Nowakowski M, Bluth MH, Durkin HG. Tetracycline mediated IgE isotype specific suppression of ongoing human and murine IgE responses in vivo and in vitro induction of murine memory IgE responses. *Int Immunol* 2010;22(4):281–8.
- [25] Erstein DP, Stanek A, Dapul G, Shams R, Solomon H, Durkin HG, et al. Minocycline therapy inhibits development of mast cell mediated cutaneous late phase responses. *J Allergy Clin Immunol* 2009;123(2):180.
- [26] Breedveld FC, Dijkmans BAC, Mattie H. Minocycline treatment for rheumatoid arthritis: an open dose finding study. *J Rheumatol* 1990;17:43–6.
- [27] Tilley BC, Alarcon GS, Heyse SP, Trentham DE, Neuner R, Kaplan DA, et al. Minocycline in rheumatoid arthritis. A 48 week, double-blind, placebo-controlled trial. The MIRA Trial Group. *Ann Intern Med* 1995;122:81–9.
- [28] Auci DL, Chice SM, Dukor P, Durkin HG. Control of IgE responses. IV. Isotype specific suppression of peak BPO-specific IgE antibody forming cell responses and of BPO specific IgE in serum of BPO-KLH sensitized mice. *Int Arch Allergy* 1993;101:167–76.
- [29] Auci DL, Kleiner GI, Chice SM, Athanassiades TJ, Dukor P, Durkin HG. Control of IgE responses. V. Oral administration of a synthetic derivative of the inner bacterial cell wall (SDZ 280.636) to sensitized mice induces isotype specific suppression of peak hapten specific antibody forming cell responses, serum IgE levels and immediate hypersensitivity responses. *Immunol Invest* 1998;27:105–20.
- [30] Smith MC, Heusser C, Wagner K, Blasier K, Durkin HG. An in vitro murine model of a penicillin specific IgE, anamnestic response. *J Immunol Methods* 1990;131:55.
- [31] Lee KS, Jin SM, Kim SS, Lee YC. Doxycycline reduces airway inflammation and hyperresponsiveness in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* 2004;113:902–9.
- [32] Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998;102:783–8.
- [33] Kuzin II, Snyder JE, Ugine GD, Wu D, Lee S, Bushnell T, et al. Tetracycline inhibits activated B cell function. *Int Immunol* 2001;12:921–31.
- [34] Zhu J, Paul WE. Peripheral CD4 T cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev* 2010;238(1):247–62.
- [35] Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood* 2008;112:1557–69.
- [36] Smith-Norowitz TA, Bluth MH, Chice SM, Nowakowski M, Norowitz KB, Liao K, et al. Two distinct T cell subsets, CD4+ and CD8+CD60+, and their cytokines are required for in vitro induction of human ragweed specific memory IgE responses. *J Immunol* 2008;181(7):4761–9.
- [37] Maneechotesuwan K, Xin Y, Ito K, Jazrawi E, Lee K-Y, Usmani OS, et al. Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *J Immunol* 2007;178:2491–8.
- [38] Smith-Norowitz TA, Joks R, Silverberg JI, Bluth MH, Norowitz KB, Chice S, et al. Effect of minocycline on IgE responses and cytokines in vivo and in vitro. *Int Immunol* 2010;22(Suppl. 1 Pt 1):i138–41, doi:10.1093/intimm/dxq 108.
- [39] Jabara HH, Fu SM, Geha RS, Vercelli D. CD40 and IgE: synergism between anti-CD40 mAb and IL-4 in the induction of IgE synthesis by highly purified human B cells. *J Exp Med* 1990;172:1861–4.
- [40] Smith-Norowitz TA, Bluth MH, Drew H, Norowitz KB, Chice S, Shah VN, et al. Effect of minocycline and doxycycline on IgE responses. *Ann Allergy Asthma Immunol* 2002;89:172–9.
- [41] Herrick CA, Carucci JA, Kleiner GI, Durkin HG. Regulation of hapten-specific memory IgE responses induced in vitro. Requirement for both Thy-1+ AsGM1+ and Thy-1+ AsGM1– cells and for IFN-alpha and IL-4. *J Immunol* 1993;151(7):3440–9.
- [42] Sandler C, Ekoskoski E, Lindstedt KA, Vainio PJ, Finel M, Sorsa T, et al. Chemically modified tetracycline (CMT)-3 inhibits histamine release and cytokine production in mast cells: possible involvement of protein kinase C. *Inflamm Res* 2005;54(July (7)):304–12.
- [43] Sandler C, Nurmi K, Lindstedt KA, Sorsa T, Golub LM, Kovanen PT, et al. Chemically modified tetracyclines induce apoptosis in cultured mast cells. *Int Immunopharmacol* 2005;5(October (11)):1611–21.
- [44] Miyachi Y, Yoshioka A, Imamura S, Niwa Y. Effect of antibiotics on the generation of reactive oxygen species. *J Invest Dermatol* 1986;86:449–53.
- [45] Liu J, Kuszynski CA, Baxter BT. Doxycycline induces Fas/Fas ligand-mediated apoptosis in Jurkat T lymphocytes. *Biochem Biophys Res Commun* 1999;260:562–7.
- [46] Thong YH, Ferrante A. Inhibition of mitogen induced human lymphocyte proliferative responses by tetracycline analogue. *Clin Exp Immunol* 1979;35:443–6.
- [47] Akamatsu H, Niwa Y, Kurokawa I, Masuda R, Nishijima S, Asada Y. Effects of sub-minimal inhibitory concentrations of minocycline on neutrophil chemotactic factor production in comedonal bacteria, neutrophil phagocytosis and oxygen metabolism. *Arch Dermatol Res* 1991;283:524–8.
- [48] Milano S, Arcoleo F, D'Agostino P, Cillari E. Intraperitoneal injection of tetracyclines protects mice from lethal endotoxemia downregulating inducible nitric oxide synthase in various organs and cytokine and nitrate secretion in blood. *Antimicrob Agents Chemother* 1997;41:117–21.
- [49] Silverberg JI, Durkin HG, Joks R. Phosphorylated p38 MAPK expression by blood lymphocytes of allergic asthmatic humans correlates with their serum IgE levels, and phosphorylated ERK correlates with IgG. Inhibition of phosphorylated p38 MAPK expression by T cells is associated with suppression of IgE responses. FOCIS Late Breaking Abstract (Oral Presentation), Washington, DC, June 2011.