ORIGINAL ARTICLE Experimental Models of Allergic Disease

Lidocaine–derivative JMF2–1 prevents ovalbumin–induced airway inflammation by regulating the function and survival of T cells

P. C. Olsen[1], T. P. T. Ferreira[1], M. F. Serra[1], F. A. Farias-Filho[1], B. P. Fonseca[2], J. P. B. Viola[2], R. S. B. Cordeiro[1], P. M. R. Silva[1], J. C. S. Costa[3] and M. A. Martins[1]

[1]*Laboratory of Inflammation, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil,* [2]*Division of Cellular Biology, INCA, Rio de Janeiro, Brazil and* [3]*Farmanguinhos, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil*

Clinical & Experimental Allergy

Correspondence:
Marco Aurélio Martins, Laboratory of
Inflammation, Oswaldo Cruz Institute,
Oswaldo Cruz Foundation (FIOCRUZ),
Av. Brazil 4365, Manguinhos CEP.
21045-900, Rio de Janeiro, Brazil.
E-mail: mmartins@ioc.fiocruz.br
Cite this as: P. C. Olsen, T. P. T. Ferreira,
M. F. Serra, F. A. Farias-Filho,
B. P. Fonseca, J. P. B. Viola,
R. S. B. Cordeiro, P. M. R. Silva,
J. C. S. Costa and M. A. Martins, *Clinical
& Experimental Allergy*, 2011 (41)
250–259.

Summary

Background Inhalation of the local anaesthetic lidocaine has been suggested to be beneficial for asthmatics, but airway anaesthesia is unpleasant and may exacerbate bronchoconstriction. Our previous study showed that inhalation of the lidocaine analogue JMF2-1 can elicit the anti-inflammatory properties of lidocaine without anaesthesia. This prompted further research on the mechanism of action and putative therapeutic application of JMF2-1.

Objective We tested the hypothesis that JMF2-1 would prevent allergen-induced lung inflammation and airway hyperresponsiveness (AHR) by modulating T cell function *in vivo* and *in vitro*.

Methods Local and systemic changes in leucocyte levels, cytokine production and lung mechanics were examined in a murine model of lung inflammation. JMF2-1 (0.05–2%) or saline was aerosolized twice a day during the ovalbumin (OVA)-provocation period (19–21 days post-sensitization). Analyses were performed 24 h after the final challenge. Primary cultured lymph node cells were used to assess the effects of JMF2-1 (100–600 μM) at the cellular level.

Results OVA challenge resulted in lung recruitment of $CD4^+$ T cells and eosinophils, increased generation of inflammatory cytokines and AHR to inhaled methacholine within 24 h. These changes were prevented by JMF2-1 nebulization, and occurred in parallel with an increase in the number of apoptotic cells in the lung. JMF2-1 treatment did not alter levels of $CD4^+$ or $CD8^+$ T cells in the thymus or lymph nodes of naïve mice, although it inhibited OVA-induced IL-13 production and the lymphocyte proliferative response *in vitro*. It also induced apoptosis of OVA-activated lymphocytes in a mechanism sensitive to z-VAD, indicating that JMF2-1 mediates caspase-dependent apoptosis.

Conclusion Inhalation of JMF2-1 prevents the cardinal features of asthma by reducing T_H2 cytokine generation and lung eosinophilic inflammatory infiltrates via local inhibition of T cell function and survival. JMF2-1 may represent a novel therapeutic alternative for asthma control with distinct advantages over local anaesthetics.

Keywords apoptosis, asthma, lidocaine, lung inflammation, T cells
Submitted 10 April 2009; revised 18 June 2010; accepted 22 June 2010

Introduction

Asthma is a chronic inflammatory disease of the airways. It is driven by a T_H2 immune response to inhaled allergens, and is closely associated with infiltration of the bronchial mucosa with eosinophils, $CD4^+$ T cells and neutrophils. The major symptoms of asthma are airway obstruction and reversible bronchial hyperresponsiveness (BHR) to non-specific irritants [1]. Anti-inflammatory treatment with inhaled glucocorticoid alone and combined preparations of a corticosteroid and a long-acting β_2-agonist are the most effective therapies for asthma. Most asthmatics respond to these treatments, but some subjects require additional oral glucocorticoids, and the long-term use of these agents has been strongly associated with several adverse effects [2]. In addition, ~5–10% of

asthmatic patients are entirely insensitive to corticosteroids, reinforcing the need for new therapies [3].

The use of local anaesthetics to treat patients with asthma is a new concept [4–6]. This concept has been mainly motivated by findings in which lidocaine was found to inhibit the IL-5-mediated survival of human eosinophils [7]. However, the safety and usefulness of inhaled lidocaine for controlling asthma remain controversial because topical anaesthesia of the airways can lead to some degree of irritation and bronchoconstriction in asthmatics [8–11].

The pharmacological properties of lidocaine analogues synthesized and screened for reduced local anaesthetic activity were recently reported. Changes in the aromatic ring of lidocaine led to the production of JMF2-1 [12, 13]. This analogue can combine reduced local anaesthetic activity with increased anti-spasmodic and anti-inflammatory properties in one molecule. The anti-inflammatory mechanism of JMF2-1 is incompletely understood. We hypothesized that, using a short-term model of allergic inflammation in the mouse lung, inhalation of JMF2-1 would affect the activation of T lymphocytes and the generation of important cytokines. To assess the potential direct effects of JMF2-1 on lymphocytes, we also measured the proliferation, cytokine generation and apoptosis of lymph node cells from BALB/c and DO11.10 ovalbumin (OVA)-specific T cell receptor transgenic mice.

Methods

Animals

Four-week-old (18–20 g) BALB/c and DO11.10 TCR Tg (background on BALB/c) mice were obtained from the Oswaldo Cruz Foundation and the National Institute of Cancer breeding colonies (Rio de Janeiro, Brazil), respectively. They were kept in the animal-housing facilities at a controlled room temperature (22–25 °C) and a 12-h (6 am–6 pm) light–dark cycle. Procedures involving the care and use of laboratory animals were examined and approved by the Animal Ethics Committee of the Oswaldo Cruz Foundation (L-0028/06; CEUA-FIOCRUZ, Rio de Janeiro, Brazil).

Sensitization, challenge and treatment protocol

BALB/c mice were sensitized on day 0 by a dorsal subcutaneous injection (0.2 mL) of a mixture containing OVA (50 μg) and aluminium hydroxide (5 mg) in 0.9% NaCl sterile solution (saline). On day 14, mice were boosted with the solution described above administered via the intraperitoneal (i.p.) route. On the 19th, 20th and 21st day after sensitization, mice were challenged by exposure for 30 min to an aerosol of OVA generated from a 20 mg/mL solution by the use of an air-driven nebulizer (model NS I -210/12,

Indústria de Aparelhos Biomédicos, São Paulo, Brazil) at a flow rate of 10 L/min. In sham-challenged mice, the aerosol of OVA was replaced by its vehicle (saline). Treatment was by exposure for 30 min to an aerosol of JMF2-1 (0.05–2%) (M/V) concomitantly and 8 h after each OVA challenge. Dexamethasone treatment was administered once a day (1 mg/kg, i.p.) 1 h before OVA challenge.

Invasive assessment of respiratory mechanics

Airway responsiveness was assessed as a change in airway function 24 h after the last challenge with aerosolized methacholine in a FinePoint R/C Buxco® Platform (Buxco Electronics, Sharon, CT, USA). Mice were anaesthetized with nembutal (60 mg/kg). Neuromuscular activity was blocked with bromide pancuronium (1 mg/kg). Tracheostomized mice were mechanically ventilated and lung function was assessed. The trachea was cannulated and the cannula was connected to a pneumotachograph. Airflow and transpulmonary pressure were recorded using a Buxco Pulmonary Mechanics Processing System (Buxco Electronics, Wilmington, NC, USA). This instrument was used to calculate lung resistance (R_L) (cm H_2O/mL/s) and dynamic lung compliance (mL/cm H_2O) in each breath cycle. Lung elastance was calculated as the inverse of compliance values. Analogical signals from the computer were digitized using a Buxco Analog/Digital Converter (Buxco Electronics). Mice were allowed to stabilize for 5 min and increasing concentrations of methacholine (3, 9 and 27 mg/mL) were aerosolized for 5 min each. Baseline pulmonary parameters were assessed with aerosolized phosphate-buffered saline (PBS). Expressed results comprised the mean absolute values of the responses of lung resistance and elastance collected during 5 min after the administration of methacholine aerosol.

Leucocyte enumeration in digested lung tissue

BALB/c sensitized mice were killed (sodium pentobarbital, 500 mg/kg, i.p.) 24 h after the final antigen challenge. The lungs were perfused with 20 mL of saline through the heart. The right lung was weighed, chopped and digested with 1 mL of 0.2% collagenase diluted in RPMI media for 40 min at 37 °C to obtain lung leucocytes. Total leucocyte counts were carried out in Neubauer chambers by means of light microscopy after dilution of the samples of lung cells in Türk's (acetic acid, 0.2%) solution. Eosinophils, neutrophils, lymphocytes and macrophages were counted by means of standard morphologic criteria from cytocentrifuge preparations stained with May–Grünwald–Giemsa stain.

Cell recovery from the airway lumen

BALB/c sensitized mice were killed (as described above) 24 h after the final antigen challenge. The trachea was

immediately cannulated. Cell influx into the airway lumen was quantified by counting cells recovered from bronchoalveolar lavage fluid (BALF). BAL was undertaken by flushing the airways with 1 mL of PBS with ethylenediamine tetra-acetic acid (EDTA, 10 mM). BALF was retrieved by gentle aspiration. BALF was centrifuged (239 g, 10 min) and the cell pellet was resuspended in EDTA–PBS. T cells recovered in BALF were distinguished and counted after staining with CD3 [fluorescein isothiocyanate (FITC)] and CD4 [phycoerythrin (PE)] monoclonal antibodies (BD Biosciences PharMingen, San Jose, CA, USA). Cells were analysed by flow cytometry (FACSCalibur; BD Biosciences PharMingen).

Blood cell counts

Blood from the tail vein was obtained immediately before killing 24 h after the final antigen challenge. Total leucocyte counts were carried out in Neubauer chambers using Türk's solution. Differential leucocyte analyses were determined on blood smears stained with May–Grünwald–Giemsa dye under an oil immersion objective and light microscopy.

Detection of cytokines from lung explants

Analyses of the production of cytokines by mice lung explants were undertaken as described previously [14]. Briefly, 24 h after the final challenge, BALB/c sensitized mice were killed and lungs were perfused with saline through the heart. The right lung was chopped into 16 small pieces. Each four pieces were placed per well in a 24-well plate. Explants were cultured for 24 h at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) without serum. Secretion of IL-4, IL-5, IL-13 and IFN-γ in the supernatants of the lung explants was measured with ELISA kits. Reagents from BioSource (Invitrogen, Carlsbad, CA, USA) were used to detect IL-13 and IL-4 and those from DuoSet (R&D Systems, Minneapolis, MN, USA) were used to detect IL-5 and IFN-γ following the instructions of each manufacturer. Results were expressed in picograms of cytokine produced per milligram of lung tissue as reported [14]. At the end of the experiment, after recovering the supernatant for cytokine measurements, lung fragments were dried overnight at 60 °C and weighed using a Mettler Toledo precision balance (Columbus, OH, USA).

Effect of JMF2-1 and dexamethasone on the proliferation of T cells and cytokine generation *in vitro*

Pooled cervical, axial and inguinal lymph node cells (10^6/well) from naïve DO11.10 TCR Tg mice were extracted. The cells obtained were exposed to OVA (0.5 mg/mL) or vehicle in the presence or absence of increasing concentrations of JMF2-1 (100, 300 or 600 μM) or 100 μM of dexamethasone for 72 h at 37 °C and 5% CO_2. Secretion of IL-13 and IFN-γ in the supernatant was measured with ELISA kits from BioSource (Invitrogen) and DuoSet (R&D Systems), respectively. For the measurement of the proliferation of T cells, cells were pulsed with 1 μCi of tritiated methylthymidine (Amersham Corporation, Arlington Heights, IL, USA) for the final 8 h of the culture period. Proliferation was monitored by incorporation of tritiated thymidine in new DNA strands of daughter cells.

Analyses of apoptosis by flow cytometry

Naïve cells obtained from the lymph nodes of DO11.10 TCR Tg male mice (10^6 cells/well) were stimulated with OVA (0.5 mg/mL), whereas cells from BALB/c mice were stimulated with anti-CD3 (10 μg/mL) (BD Biosciences PharMingen). In both cases, cells were treated with JMF2-1 (300 and 600 μM) or dexamethasone (100 μM) for 72 h at 37 °C in an atmosphere of 5% CO_2. DNA fragmentation of retrieved cells was analysed by permeabilizing and staining cells with propidium iodide (PI) as described previously [15–17]. Briefly, cells were stained with PI (75 μM) in the presence of NP-40, a non-ionic detergent (Calbiochem, San Diego, CA, USA), for 15 min. Analyses of DNA content and calculation of the percentage of hypodiploid cells were performed by collecting 20 000 events in a FACSCalibur flow cytometer for Sub-G0 analysis using CellQuest software (BD Biosciences PharMingen).

To assess the putative involvement of caspase in the mechanism of cell death, cells from DO11.10 mice were pre-treated with z-VAD 50 μM (Calbiochem) for 1 h before stimulation with OVA and exposed to JMF2-1 (300 μM) or dexamethasone (100 μM). Treatments lasted for 72 h and apoptosis was analysed as described above.

Apoptosis of lymph node (cervical, axial and inguinal) cells and BALF cells from saline- or OVA-challenged mice nebulized or not nebulized with JMF2-1 (1% and 2%) was assessed by double staining with CD4 PE and Annexin V FITC (BioSource, Invitrogen). Samples were analysed by flow cytometry (FACSCalibur). BALF cells were obtained 24 h after the final challenge with OVA and analysed in pools to increase the number of cells per sample. $CD4^+$ cells that were annexin V positive were considered to be early apoptotic T cells.

Detection of apoptotic cells in the lung sections of mice by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling assay

BALB/c sensitized mice were killed 24 h after the final challenge, as described previously, and the lung was perfused with 20 mL of saline through the heart. Lungs were prepared, fixed and stained following the instructions of the manufacturer (R&D Systems). Identification of

apoptotic cells in the lungs was performed using an *in situ* Cell Death Detection Kit (the TACS TdT Kit from R&D Systems). Briefly, paraffin sections were dewaxed, hydrated to water before permeabilization with proteinase K for 15 min at room temperature. Slides were treated with 3% (v/v) H_2O_2 for 5 min before incubation with terminal deoxynucleotidyl transferase in a humidified chamber at 37 °C for 1 h. Biotinylated nucleotides were detected using streptavidin–horseradish peroxidase conjugate followed by the substrate, diaminobenzidine. Cells were counterstained with methyl-green to discriminate viable cells. Twenty fields were randomly chosen for each lung section, and all cells were counted. The percentage of apoptotic cells was determined (number of apoptotic cells/total number of cells counted×100%) from four sections per group. Assays were carried out in a blinded manner. The original magnification was ×1000.

Effects of treatment with JMF2-1 or dexamethasone upon systemic lymphocytes

To assess the potential repercussions of treatment with nebulized JMF2-1 on the lymphocyte content of peripheral lymphoid organs, naïve BALB/c mice were subjected to an aerosol of 2% JMF2-1 or saline for 30 min twice a day for 3 days as described above. Dexamethasone (1 mg/kg, i.p.) was administered once a day for 3 days for comparison. Mice were killed by anaesthesia (sodium pentobarbital, 500 mg/kg, i.p.) 24 h after the final treatment. Thymus as well as cervical, axial and inguinal lymph nodes were extracted. Thymus and pooled viable lymph node cells were counted by exclusion of Trypan blue. T cells were distinguished by staining with CD3 (FITC), CD4 (PE) and CD8 (PE-Cy5) monoclonal antibodies (BD Biosciences PharMingen). Cells were analysed by flow cytometry (FACSCalibur).

The proliferation of lymph node (cervical, axial and inguinal) cells from OVA-challenged mice nebulized or not nebulized with JMF2-1 (1% and 2%) was evaluated 24 h after the final *in vivo* antigen challenge. This was achieved by retrieving cells from the lymph nodes, re-challenging cells with OVA (0.5 mg/mL) *in vitro* for 72 h and permeabilizing and staining cells with PI as described previously [15]. Briefly, cells were stained with PI (75 μM) in the presence of NP-40. Analyses of the DNA content were carried out by collecting 10 000 events for cell-cycle analysis using a FACSCalibur flow cytometer and Cell-Quest software (BD Biosciences PharMingen). Proliferation values were determined by calculating the percentage of cells in phase S+G2 of the cell cycle.

Chemicals

JMF2-1 HCl was synthesized in the Department of Organic Synthesis of Far-Manguinhos (CEUA-FIOCRUZ, Rio de Janeiro, Brazil) from 2-trifluormethyl-aniline. Dexamethasone, OVA (grade-V) and methacholine were purchased from Sigma-Aldrich. Dexamethasone and JMF2-1 were dissolved in sterile saline solution immediately before use.

Statistical analyses

Statistical analyses were carried out with ANOVA followed by the Newman–Keuls–Student test. $P \leqslant 0.05$ (two-tailed test) was considered significant.

Results

Effects of JMF2-1 on allergen-evoked leucocyte changes

Allergen provocation of actively sensitized mice yielded a significant increase in total leucocyte numbers detected in digested lung tissue and blood samples 24 h post-challenge (Fig. 1a and b). In the samples of digested lung tissue, the elevation in leucocyte counts was accounted for by a significant increase in the numbers of macrophages, lymphocytes, eosinophils and neutrophils. These changes were clearly sensitive to treatments with JMF2-1 (1–2%), as well as to dexamethasone (Fig. 1a). In this model, allergen-evoked blood leucocytosis appeared to be associated with increased numbers of lymphocyte, eosinophil and neutrophil leucocyte subtypes, which were also inhibited by treatment with JMF2-1 and dexamethasone (Fig. 1b). The numbers of eosinophils and macrophage were less sensitive to lower concentrations of JMF2-1 (0.05–0.5%).

Effect of JMF2-1 treatment on the allergen-induced accumulation of CD4$^+$ T cells in bronchoalveolar lavage fluid

As expected, allergen provocation was followed by a significant increase in the number of CD4$^+$ T cells in BALF 24 h post-challenge compared with the negative control (saline provocation) (Fig. 2). These results revealed that the accumulation of CD4$^+$ T cells (Fig. 2) was inhibited by treatment with JMF2-1 (2%, nebulized) or dexamethasone (1 mg/kg, i.p.).

Effect of JMF2-1 on airway hyperresponsiveness

Antigen challenge with OVA exacerbated airway resistance and elastance lung response to inhaled methacholine (3–27 mg/mL) as compared with control mice challenged with saline (Fig. 3a and b, respectively). Figure 3 also revealed that treatment with JMF2-1 (0.5–2%, aerosol) or dexamethasone (1 mg/kg, i.p.) abolished the airway hyperresponsiveness (AHR) noted in mice subjected to OVA
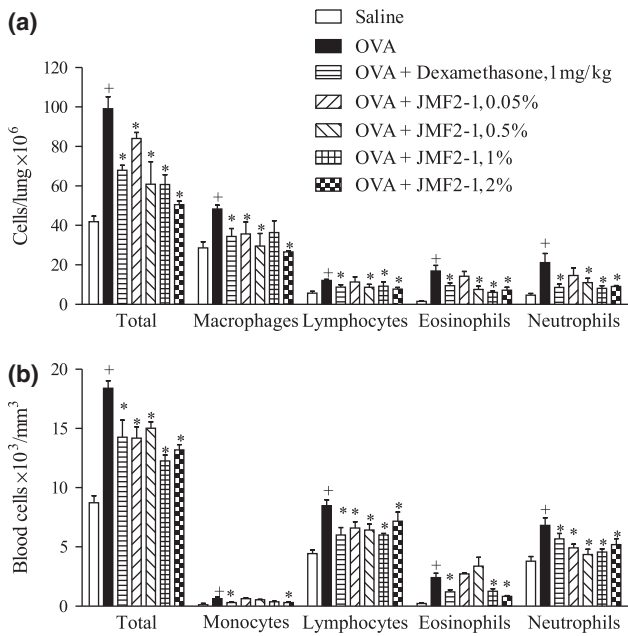
Fig. 1. Effect of treatment with JMF2-1 (0.05–2%, nebulization) or dexamethasone (1 mg/kg, intraperitoneal) on allergen-evoked leucocyte changes (total and differential cell counts) in samples of digested lung tissue (a) and blood (b) of BALB/c mice sensitized and challenged with ovalbumin (OVA). Values are mean±SEM from at least five mice. $^+P<0.05$ as compared with sham-challenged mice. $^*P<0.05$ as compared with OVA-challenged mice.

challenge. Elastance and resistance were not sensitive to a lower concentration of JMF2-1 (0.05%).

Effect of JMF2-1 or dexamethasone on cytokine production by lung explants

One study showed that lidocaine inhibited cytokine production *in vitro* [18]. We therefore studied the effect of the lidocaine analogue JMF2-1 in the secretion of cytokines in mice lungs. Lung explants from OVA-challenged mice spontaneously produced more IL-4, IL-5, IL-13 and IFN-γ *in vitro* than saline-challenged mice (Fig. 4). Such an increase in the level of IL-4, IL-5 and IL-13 was reduced in mice subjected to treatment with dexamethasone or JMF2-1 (Fig. 4a–c, respectively). The elevation in IFN-γ levels was not sensitive to the concentration of 0.5% JMF2-1, but was significantly inhibited by dexamethasone and high concentrations of JMF2-1 (1% and 2%) (Fig. 4d).

Effect of dexamethasone and JMF2-1 on T cell function

To assess the effect of JMF2-1 or dexamethasone on T lymphocytes, lymph node cells from DO11.10 (TCR Tg) mice were cultured *in vitro*. As illustrated in Fig. 5, OVA challenge significantly increased the production of IL-13 and IFN-γ (a and b, respectively) and the proliferative
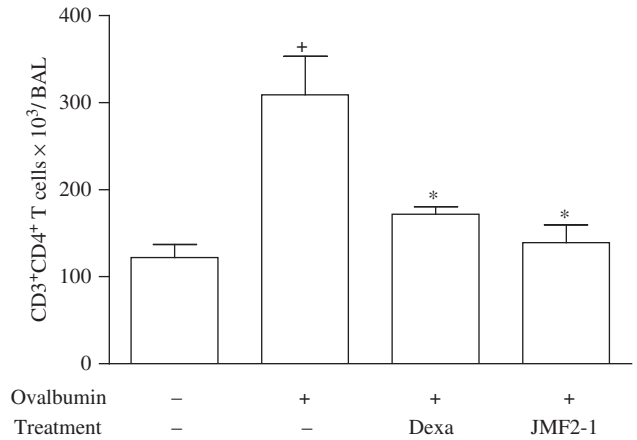

Fig. 2. Effect of JMF2-1 (2%, nebulization) or dexamethasone (1 mg/kg, intraperitoneal) on the accumulation of CD4$^+$ T lymphocytes in bronchoalveolar lavage (BAL) effluents 24 h after ovalbumin (OVA) provocation of sensitized BALB/c mice. Values are mean±SEM from three cell pools, containing effluents from four mice. $^+P<0.05$ as compared with sham-challenged mice. $^*P<0.05$ as compared with OVA-challenged mice.
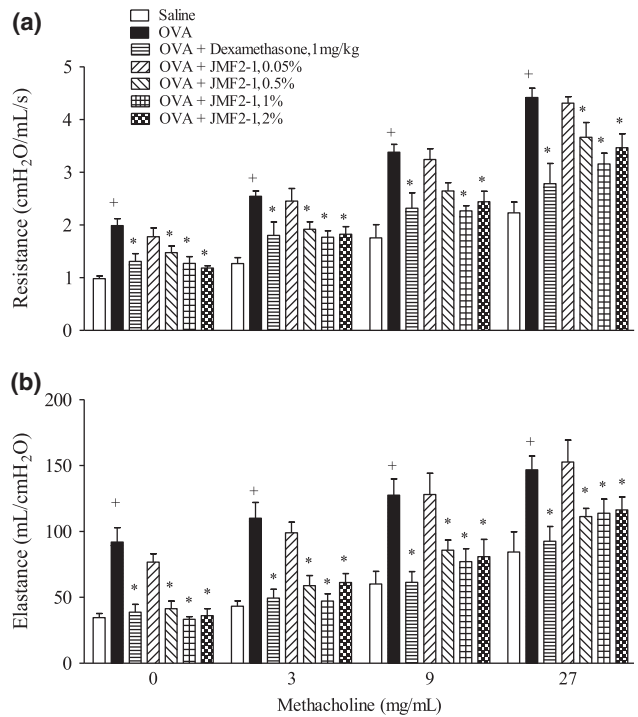

Fig. 3. Effect of JMF2-1 (0.05–2%, nebulization) or dexamethasone (1 mg/kg, intraperitoneal) on methacholine-induced increase in lung resistance (a) and lung elastance (b) in BALB/c mice sensitized and challenged with ovalbumin (OVA). Values are mean±SEM from five mice. $^+P<0.05$ as compared with sham-challenged mice. $^*P<0.05$ as compared with OVA-challenged mice.

response of lymphocytes (c). Exposure to increased concentrations of JMF2-1 (100–600 μM) led to a dose-dependent reduction in the level of IL-13 (Fig. 5a), IFN-γ

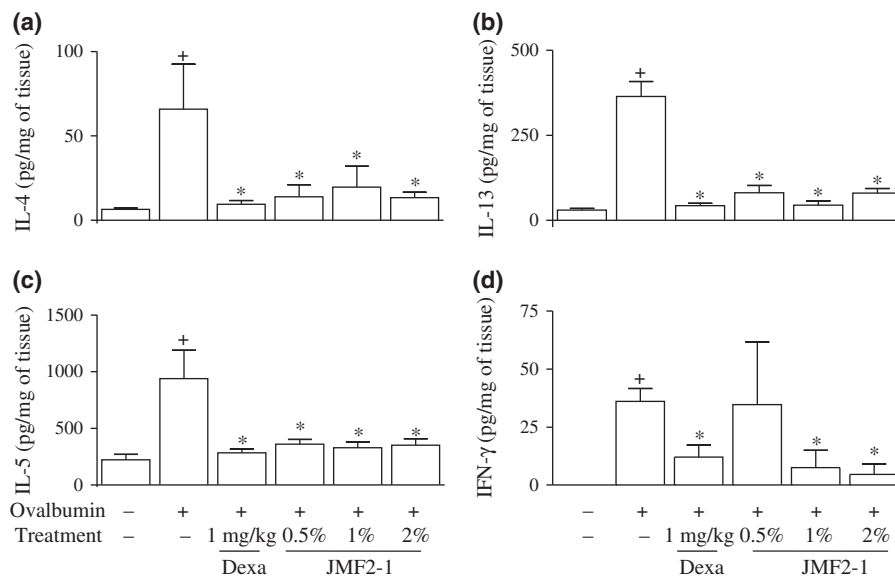**Fig. 4.** Effect of JMF2-1 (0.5%, 1% and 2%, nebulization) or dexamethasone (1 mg/kg, intraperitoneal) on ovalbumin (OVA)-induced generation of IL-4 (a), IL-5 (B), IL-13 (c) and IFN-γ (d) by cultured lung explants. Values are mean±SEM from four mice. $^+P<0.05$ as compared with sham-challenged mice. $^*P<0.05$ as compared with OVA-challenged mice.

production (Fig. 5b) and proliferative response of T cells (Fig. 5c). Figure 5 also reveals that the proliferative response and generation of IL-13 and IFN-γ triggered by OVA were clearly sensitive to treatment with 100 μM dexamethasone.

Pro-apoptotic effect of JMF2-1 on T cells

Lymph node cells exposed to allergen or anti-CD3 provocation in the presence or absence of treatments were permeabilized, stained with PI and analyzed by flow cytometry. As shown in Fig. 6, there was an obvious decrease in the percentage of apoptotic cells when they were exposed to OVA for 72 h (cells from naïve DO11.10 donors) (a) or to anti-CD3 mAb (cells from BALB/c donors) for 48 h (b). Treatment of OVA- or anti-CD3-activated cells with JMF2-1 (300 μM) or dexamethasone (100 μM) significantly increased the percentage of apoptotic cells.

Studies were conducted under conditions in which the lymph node cells from naïve DO11.10 mice were pretreated with the pan-caspase inhibitor z-VAD (50 μM) then exposed to OVA in the presence or absence of 300 μM JMF2-1 or 100 μM dexamethasone for 72 h *in vitro*. z-VAD equally prevented the pro-apoptotic effect of JMF2-1 and dexamethasone (Fig. 6c).

Pro-apoptotic effect of JMF2-1 in vivo

We assessed the impact of treatment with JMF2-1 on the population of apoptotic cells in the lung by enumerating apoptotic [terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)-

positive] and viable cells in lung sections obtained from mice challenged with OVA. Lungs of sensitized mice subjected to saline challenge showed few apoptotic cells within the peribronchial spaces (Fig. 7a and d). After OVA challenge, numerous viable infiltrating leucocytes (counterstained with methyl-green) and few TUNEL-positive cells (Fig. 7b and d) were observed. Under this condition, JMF2-1 reduced the number of viable cells and increased the percentage of apoptotic cells in the lung (Fig. 7c and d).

We also assessed the apoptosis of T CD4$^+$ lymphocytes in BALF samples. We demonstrated that 2% JMF2-1 increased apoptosis from 2.6±0.5% (mean±SEM, $n = 3$) in OVA-challenged mice to 5.7±0.7% (mean±SEM, $n = 3$) ($P<0.05$).

Effect of treatment with JMF2-1 on the distribution of T cells in lymphoid organs

As illustrated in Fig. 8, no difference in the levels of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells was observed in the thymus (a and b) or peripheral lymph nodes of naïve BALB/c mice subjected to inhalation of JMF2-1 (2%) (c and d) as compared with saline-treated mice. In contrast, an intense reduction in the number of CD3$^+$CD4$^+$ cells and CD3$^+$CD8$^+$ cells was noted in the lymph nodes and the thymus of mice subjected to treatment with dexamethasone (1 mg/kg, i.p.).

We also assessed the capacity of JMF2-1 to alter the viability and functional profile of T cells in peripheral lymph nodes 24 h after allergen provocation. Lymph node cells from OVA-challenged mice increased their
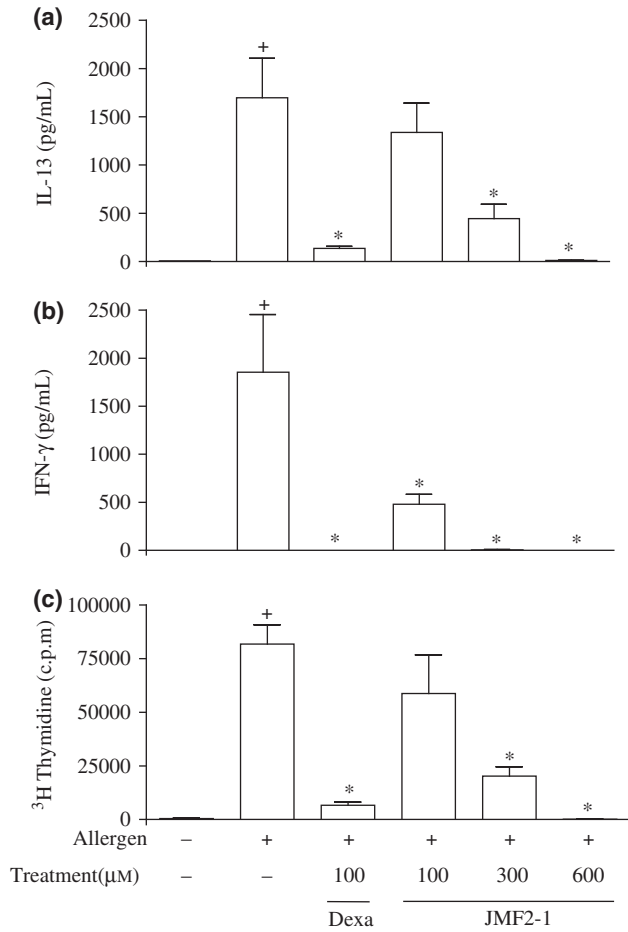
Fig. 5. Effect of *in vitro* treatment with JMF2-1 (100–600 μM) or dexamethasone (100 μM) on ovalbumin (OVA)-evoked IL-13 and IFN-γ production by DO11.10 lymphocytes (a) and (b) and proliferation (c). Values are mean±SEM from three mice (representative of two experiments). $^{+}P < 0.05$ as compared with sham-challenged lymph node cells. $^{*}P < 0.05$ as compared with OVA-challenged lymph node cells.

proliferative response following *ex vivo* exposure to OVA (9.9±0.9%; $n = 6$) as compared with those from sham-challenged mice (6.2±0.3%; $n = 6$) ($P < 0.05$), which was not modified by prior nebulization with JMF2-1 1% (10.6±1.8%; $n = 4$). Nevertheless, as nebulized at 2%, JMF2-1 tended to reduce the proliferative response to recall antigen (7.5±0.9%; $n = 6$) indicating that, at this concentration, it can be absorbed through the airways into the systemic circulation and interfere with antigen-mediated immune responses. Furthermore, neither 1% JMF2-1 (14.7±0.2%; $n = 3$) nor 2% JMF2-1 (17.5±2.4%; $n = 5$) could alter the apoptosis of lymph node T cells as compared with those obtained from untreated OVA-challenged mice (17.6±0.8%; $n = 5$).

Discussion

We report novel observations related to the anti-inflammatory and immunoregulatory properties of JMF2-1, a lidocaine analogue screened for reduced local anaesthetic activity [12, 13]. Treatment of OVA-challenged mice with nebulized JMF2-1 prevented crucial asthma events, including airway hyper-reactivity, leucocyte infiltration (eosinophils, CD4 T cells), and the production of pro-inflammatory cytokines in lung tissue. In *in vitro* settings, JMF2-1 dose-dependently inhibited antigen-induced T cell proliferation and IL-13 production. Furthermore, T cells exposed to JMF2-1 underwent apoptosis as attested by flow cytometric analyses. This phenomenon was impaired when T cells were treated with the pan-caspase inhibitor z-VAD, and hence it is suggested that JMF2-1 mediates the caspase-dependent apoptosis of lymphocytes. Altogether, these observations indicate that the protective effect of JMF2-1 upon allergen-evoked airway inflammation and bronchial hyper-reactivity may be
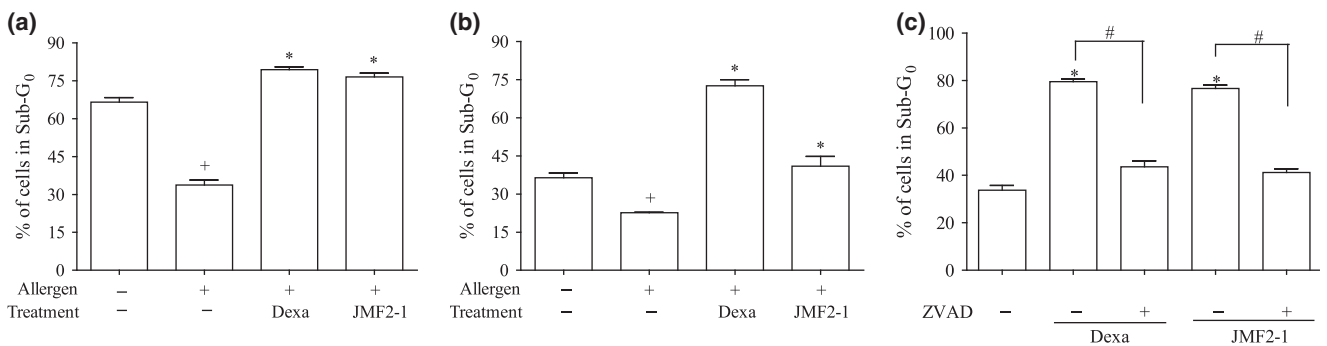

Fig. 6. Effect of dexamethasone (100 μM) or JMF2-1 (300 μM) on the subdiploid DNA (sub-G_0/G_1) content of lymph node cells recovered from DO11.10 transgenic mice (a) and from BALB/c mice (b) subjected to stimulation with ovalbumin (OVA) and anti-CD3, respectively. (c) Shows the effect of z-VAD pre-treatment (50 μM) upon apoptosis of DO11.10 lymph node cells induced by JMF2-1 or dexamethasone. The percentage of cells undergoing DNA fragmentation was determined by propidium iodide staining (flow cytometry) performed within 72 and 48 h after exposure to OVA and anti-CD3, respectively. Values are mean±SEM from three mice (representative of two experiments). $^{+}P < 0.05$ as compared with saline-stimulated lymph node cells. $^{*}P < 0.05$ as compared with OVA or anti-CD3-stimulated but untreated cells. $^{\#}P < 0.05$ as compared with cells not treated with z-VAD.
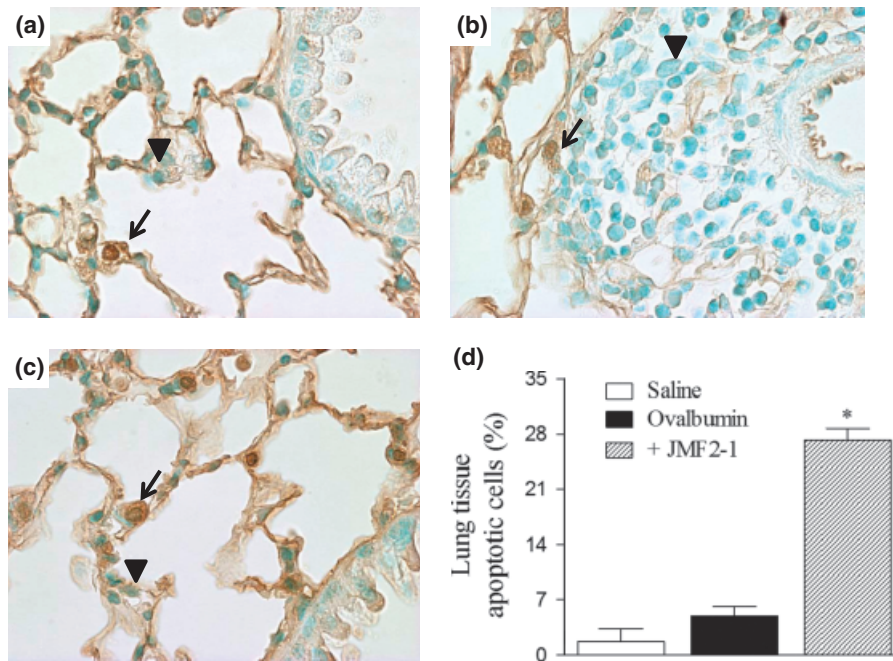
Fig. 7. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling labelling of lungs of allergen-challenged mice treated with JMF2-1. (a) Shows a lung section of a sensitized mouse subjected to saline provocation demonstrating a few apoptotic cells (arrow) in the peribronchial area. (b) Shows a lung section of a sensitized mouse subjected to ovalbumin (OVA) provocation demonstrating a few apoptotic cells (arrow) in the peribronchial area and numerous viable cells counterstained with methyl-green (arrowhead). (c) Shows a lung section of a sensitized mouse subjected to OVA provocation and treatment with JMF2-1 (2%) showing numerous apoptotic cells (arrow) and fewer viable cells counterstained with methyl-green. (d) Shows an apoptotic index reflecting the effect of *in vivo* treatment with JMF2-1 (2%, aerosol) on the number of peribronchial apoptotic cells in sensitized mice subjected to OVA challenge. Values are mean±SEM from at least three mice. *$P < 0.05$ as compared with OVA-challenged untreated mice. Original magnification ×1000.
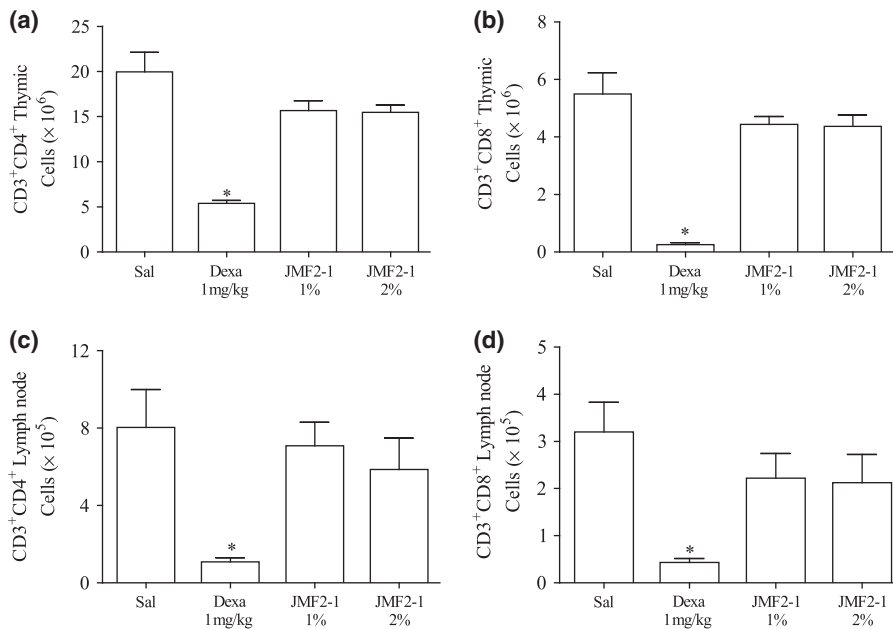

Fig. 8. Effect of JMF2-1 (1% and 2%, nebulization) or dexamethasone (1 mg/kg, intraperitoneal) on the numbers of CD4 and CD8 T lymphocytes recovered from the thymus (a and b, respectively) and peripheral lymph node cells (c and d, respectively) in naïve BALB/c mice. Values are mean±SEM from four mice. *$P < 0.05$ as compared with negative controls (mice nebulized with saline).

accounted for by the down-regulation of T cell survival and the inhibition of T_H2 cytokine production.

T cells play a paramount part in asthma pathogenesis; hence, the present study was undertaken to test the hypothesis that JMF2-1 would prevent allergen-induced lung inflammation and AHR by modulating T cell function. There is an emphasis in the literature on the need to treat the eosinophilic inflammatory changes that appear to contribute to airway hyper-reactivity in asthmatics [3, 19, 20]. Using actively sensitized BALB/c mice, we showed that an allergen-evoked increase in the number of eosinophils, neutrophils, macrophages and lymphocytes in lung tissue (as well as concomitant elevation in the circulating levels of eosinophils and lymphocytes in the blood) was prevented by nebulization with JMF2-1 with an efficacy comparable with that presented by dexamethasone treatment. As expected, assuming a causative association between eosinophilic inflammatory infiltrates and airway hyper-reactivity, treatment with JMF2-1 and dexamethasone also inhibited the methacholine-evoked increases in lung resistance and elastance noted after OVA challenge, reinforcing the idea of beneficial effects of JMF2-1 on airways. A recent study conducted in guinea-pigs raised the possibility that local anaesthesia with lidocaine in the airways improved airway hyper-reactivity by inhibiting neurogenic inflammation [21]. The evidence from the current study, however, is that the protective effect of lidocaine on airway hyper-reactivity is probably unrelated to its ability to cause local anaesthesia because the analogue JMF2-1, despite the lack of anaesthetic activity [13], remained active against allergen-evoked airway hyper-reactivity in mice.

Several very successful asthma treatments in murine models of asthma are related to the ability to reduce the expression of T_H2 cytokines, which are mainly generated by infiltrating CD4 T cells [22–25]. In the present study, we confirmed the findings showing that OVA provocation of sensitized mice enhanced the production of IL-4, IL-5 and IL-13 by lung explants [14]. This is in line with the assumption that the generation of T_H2 cytokines is a feature of the allergic inflammatory process. IFN-γ levels also appeared to be increased in this condition, although at a lower level as compared with T_H2 cytokines. Treatment with dexamethasone or high doses of JMF2-1 (1% and 2%) prevented the production of T_H2 and T_H1 cytokines, whereas 0.5% JMF2-1 selectively prevented the generation of T_H2 cytokines. This is interesting considering the possibility of IFN-γ-producing T_H1 cells having protective effects in asthma [26]. Nebulized JMF2-1, similar to dexamethasone, clearly inhibited allergen-induced influx of CD4 T cells into the airways. Therefore, the simplest explanation would be that JMF2-1 down-regulated the generation of pro-inflammatory cytokines by preventing the accumulation of CD4 T cells in the lung tissue.

We then examined the effect of JMF2-1 on the proliferative response, cytokine production by lymphocytes and the survival of these cells after allergen-specific stimulation *in vitro*. A close correlation between the magnitude of blockade of allergen-induced IL-13 production and the impairment of the proliferative response of OVA-stimulated DO11.10 T cells following treatment with JMF2-1 or dexamethasone was noted. The recognized capacity of the prototype lidocaine to prevent mitogen-induced proliferation and secretion of cytokines from activated T cells is independent of cell toxicity and of interference with mitogen binding on the cell membrane [18, 27, 28], but appears to involve the inhibition of signalling of nuclear factor κB [27]. In the present study, T cells exposed to JMF2-1 or dexamethasone underwent apoptosis as attested by flow cytometric analyses. The pro-apoptotic effect of JMF2-1, similar to that observed with dexamethasone in the present study and others [29], was inhibited when T cells were treated with the pan-caspase inhibitor z-VAD. This indicated that JMF2-1 and dexamethasone mediate the caspase-dependent apoptosis of lymphocytes.

Apoptosis may promote the resolution of inflammation in asthma [30, 31]. Furthermore, several anti-inflammatory drugs (including glucocorticoids) may act through the induction of apoptosis of inflammatory cells [31–33]. We investigated the effect of treatment with JMF2-1 upon the apoptosis ratio in the lung of OVA-challenged mice by *in situ* detection of DNA fragmentation with the TUNEL technique. JMF2-1 significantly increased the ratio of apoptotic cells to viable migrated inflammatory cells in the subepithelium of bronchi 24 h post-challenge. These results demonstrated, for the first time *in situ*, that the inflammatory cells that migrate into lung tissue undergo apoptosis within the subepithelium after aerosolization with JMF2-1. We also detected increased levels of apoptosis in the CD4 T cell population in BALF fluid following JMF2-1 treatment. This adds support to the interpretation that the anti-inflammatory effect of this lidocaine analogue is accounted for by the down-regulation of CD4 T cell survival.

In another set of *in vivo* experiments, we demonstrated that, differently from dexamethasone, JMF2-1 did not alter CD4 or CD8 T cell numbers in peripheral lymphoid organs, such as the thymus and lymph nodes of naïve mice. Moreover, 1% JMF2-1 did not impair the proliferative response to recall antigen in *ex vivo* settings. This suggested that this analogue can inhibit allergen-evoked inflammation and airway hyper-reactivity without causing the systemic down-regulation of T cell proliferation.

In conclusion, the present study suggests that the protective effect of JMF2-1 upon allergen-evoked airway inflammation and BHR in actively sensitized mice is (at least in part) accounted for by the down-regulation of T cell survival and the inhibition of T_H2 cytokine production. It demonstrated that JMF2-1 induces caspase-dependent apoptosis of T cells. This probably contributes to

impairment of the accumulation of these cells in the lung mucosa and the subsequent amplification of the local eosinophilic inflammatory response without inducing systemic immunosuppression.

Acknowledgements

References

1 Moore WC. Update in asthma 2007. *Am J Respir Crit Care Med* 2008; **177**:1068–73.

2 Barnes PJ. New drugs for asthma. *Nat Rev* 2004; **3**:831–44.

3 Adcock IM, Ford PA, Bhavsar P, Ahmad T, Chung KF. Steroid resistance in asthma: mechanisms and treatment options. *Curr Allergy Asthma Rep* 2008; **8**:171–8.

4 Hunt LW, Frigas E, Butterfield JH *et al.* Treatment of asthma with nebulized lidocaine: a randomized, placebo-controlled study. *J Allergy Clin Immunol* 2004; **113**:853–9.

5 Hunt LW, Swedlund HA, Gleich GJ. Effect of nebulized lidocaine on severe glucocorticoid-dependent asthma. *Mayo Clin Proc* 1996; **71**:361–8.

6 Saito K, Sato N, Shimono N, Hagiwara K, Kanazawa M, Nagata M. A pregnant woman with severe asthma effectively treated by inhalational lidocaine therapy. *Nihon Kokyuki Gakkai zasshi (J Jpn Resp Soc)* 2006; **44**:828–32.

7 Okada S, Hagan JB, Kato M *et al.* Lidocaine and its analogues inhibit IL-5-mediated survival and activation of human eosinophils. *J Immunol* 1998; **160**:4010–7.

8 Harrison TW, Tattersfield AE. Effect of single doses of inhaled lignocaine on FEV1 and bronchial reactivity in asthma. *Respir Med* 1998; **92**:1359–63.

9 Hirota K, Hashimoto Y, Sato T *et al.* I.v. lidocaine worsens histamine-induced bronchoconstriction in dogs. *Br J Anaesth* 1999; **82**:87–9.

10 Beydon L, Lorino AM, Verra F *et al.* Topical upper airway anaesthesia with lidocaine increases airway resistance by impairing glottic function. *Intensive Care Med* 1995; **21**:920–6.

11 Burches BR Jr, Warner DO. Bronchospasm after intravenous lidocaine. *Anesth Analg* 2008; **107**:1260–2.

12 Costa JC, Neves JS, de Souza MV *et al.* Synthesis and antispasmodic activity of lidocaine derivatives endowed with reduced local anesthetic action. *Bioorg Med Chem Lett* 2008; **18**:1162–6.

13 da Costa JC, Olsen PC, de Azeredo Siqueira R *et al.* JMF2-1, a lidocaine derivative acting on airways spasm and lung allergic inflammation in rats. *J Allergy Clin Immunol* 2007; **119**:219–25.

14 Proust B, Nahori MA, Ruffie C, Lefort J, Vargaftig BB. Persistence of bronchopulmonary hyper-reactivity and eosinophilic lung inflam-

mation after anti-IL-5 or -IL-13 treatment in allergic BALB/c and IL-4Ralpha knockout mice. *Clin Exp Allergy* 2003; **33**:119–31.

15 Robbs BK, Cruz AL, Werneck MB, Mognol GP, Viola JP. Dual roles for NFAT transcription factor genes as oncogenes and tumor suppressors. *Mol Cell Biol* 2008; **28**:7168–81.

16 Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 2006; **1**:1458–61.

17 Migliorati G, Nicoletti I, Pagliacci MC, D'Adamio L, Riccardi C. Interleukin-4 protects double-negative and CD4 single-positive thymocytes from dexamethasone-induced apoptosis. *Blood* 1993; **81**:1352–8.

18 Tanaka A, Minoguchi K, Oda N *et al.* Inhibitory effect of lidocaine on T cells from patients with allergic asthma. *J Allergy Clin Immunol* 2002; **109**:485–90.

19 Holgate ST, Holloway J, Wilson S *et al.* Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities. *J Allergy Clin Immunol* 2006; **117**:496–506; quiz 07.

20 Hogan SP, Rosenberg HF, Moqbel R *et al.* Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy* 2008; **38**:709–50.

21 Muraki M, Iwanaga T, Haraguchi R, Kubo H, Tohda Y. Continued inhalation of lidocaine suppresses antigen-induced airway hyperreactivity and airway inflammation in ovalbumin-sensitized guinea pigs. *Int Immunopharmacol* 2008; **8**:725–31.

22 Stam WB, Van Oosterhout AJ, Nijkamp FP. Pharmacologic modulation of Th1- and Th2-associated lymphokine production. *Life Sci* 1993; **53**:1921–34.

23 Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004; **202**:175–90.

24 Kips JC. Cytokines in asthma. *Eur Respir J* 2001; **34**:24s–33s.

25 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996; **183**:195–201.

26 Li XM, Chopra RK, Chou TY, Schofield BH, Wills-Karp M, Huang SK. Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice. *J Immunol* 1996; **157**:3216–9.

27 Lahat A, Horin SB, Lang A, Fudim E, Picard O, Chowers Y. Lidocaine down-regulates nuclear factor-kappa B signalling and inhibits cytokine production and T cell proliferation. *Clin Exp Immunol* 2008; **152**:320–7.

28 Ferguson RM, Schmidtke JR, Simmons RL. Inhibition of mitogen-induced lymphocyte transformation by local anesthetics. *J Immunol* 1976; **116**:627–34.

29 Ivanov VN, Nikolic-Zugic J. Biochemical and kinetic characterization of the glucocorticoid-induced apoptosis of immature CD4+CD8+ thymocytes. *Int Immunol* 1998; **10**:1807–17.

30 Kodama T, Matsuyama T, Miyata S *et al.* Kinetics of apoptosis in the lung of mice with allergic airway inflammation. *Clin Exp Allergy* 1998; **28**:1435–43.

31 Woolley KL, Gibson PG, Carty K, Wilson AJ, Twaddell SH, Woolley MJ. Eosinophil apoptosis and the resolution of airway inflammation in asthma. *Am J Respir Crit Care Med* 1996; **154**:237–43.

32 McColl KS, He H, Zhong H, Whitacre CM, Berger NA, Distelhorst CW. Apoptosis induction by the glucocorticoid hormone dexamethasone and the calcium-ATPase inhibitor thapsigargin involves Bc1-2 regulated caspase activation. *Mol Cell Endocrinol* 1998; **139**:229–38.

33 Herold MJ, McPherson KG, Reichardt HM. Glucocorticoids in T cell apoptosis and function. *Cell Mol Life Sci* 2006; **63**:60–72.