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Dietary lycopene supplementation suppresses Th2 responses and lung eosinophilia in a mouse model of allergic asthma☆

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Abstract

Allergic airways disease (AAD) is associated with an increased influx of eosinophils to the lungs, mucus hypersecretion and Th2 cytokine production. Dietary antioxidant supplementation may alter cytokine responses and thus allergic inflammation. Lycopene is a potent dietary antioxidant. The objective of this study was to investigate the effects of lycopene, on allergic inflammation, in a mouse model of AAD. BALB/c mice receiving lycopene supplement or control were intraperitoneally sensitised and intranasally challenged with ovalbumin (OVA) to induce AAD. The effect of supplementation on inflammatory cell influx into bronchoalveolar lavage fluid, lung tissue and blood, mucus-secreting cell numbers in the airways, draining lymph node OVA-specific cytokine release, serum IgG1 levels and lung function in AAD was assessed. Supplementation reduced eosinophilic infiltrates in the bronchoalveolar lavage fluid, lung tissue and blood, and mucus-secreting cell numbers in the airways. The OVA-specific release of Th2-associated cytokines IL-4 and IL-5 was also reduced. We conclude that supplementation with lycopene reduces allergic inflammation both in the lungs and systemically, by decreasing Th2 cytokine responses. Thus, lycopene supplementation may have a protective effect against asthma.

Keywords: Lycopene; Antioxidants; Asthma; Airway inflammation; Eosinophils; Th2 response

1. Introduction

Asthma is a chronic inflammatory disease that is underpinned by the recruitment and activation of inflammatory cells into the airways. Allergic inflammation in asthma is characterised by increased levels of eosinophils in the lung tissue and blood that is associated with enhanced production of the Th2 cytokines IL-4, IL-5 and IL-13 [1]. Generally, there is a correlation between the number of eosinophils and asthma severity [2]. Inflammatory cells including eosinophils, neutrophils and macrophages release oxidants when activated [3–5], which can overwhelm antioxidant defences, resulting in oxidative stress in the lung [6]. Oxidative stress is increased in the airways of asthmatics [7,8] and causes detrimental effects on airway function [6].

Dietary antioxidants have the capacity to boost host antioxidant defences, thereby suppressing oxidative stress [7,8]. Lycopene is a dietary carotenoid with powerful antioxidant capacity [9]. Some studies have indicated that lycopene may have a protective effect in asthma [10–12]. Lycopene or lycopene-rich supplementation reduced bronchoconstriction and neutrophil influx and elastase activity in the airways of asthmatics [13,14]. It has been suggested that dietary antioxidants have the most potent effects when consumed in combination with the other nutrients as part of whole foods, rather than in isolation [15,16].

Lyc-O-Mato® (LycoRed Natural Products Industries Ltd, Beer Sheva, Israel) is a concentrated whole tomato extract, which provides a rich source of lycopene, as well as other antioxidants, including vitamin E and β-carotene. In this study, daily supplementation with a lycopene-rich supplement, Lyc-O-Mato, was employed to determine the effects of a whole-food approach to dietary antioxidant administration on a mouse model of ovalbumin (OVA)-induced allergic airways disease (AAD).

The hypothesis was that daily supplementation with Lyc-O-Mato prior to, and during, AAD would decrease allergic inflammation and Th2 responses in AAD.

2. Methods and materials

2.1. Mice

Male BALB/c mice (aged 6–8 weeks) were obtained from the Animal Services Unit, The University of Newcastle, Australia. Mice were housed under specific pathogen-free conditions and experiments were performed with approval by the Animal Care and Ethics Committee of The University of Newcastle, Australia. Mice were fed a controlled background diet containing 10% (w/w) lipids (Specialty Feeds, Glen Forrest, Australia; SF05-055) with minimal antioxidants to prevent lipid peroxidation, *ad libitum*.

2.2. Induction of AAD

Allergic (OVA/OVA) groups were sensitized by intraperitoneal injection of OVA [50 μg, combined with 1 mg of Alhydrogel (Commonwealth Serum Laboratories,

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Melbourne, Australia) in 200 μl of 0.9% sterile saline] on Day 0. Control groups (PBS/OVA) were sham sensitised (1 mg of Alhydrogel in 200 μl of 0.9% sterile saline). Mice were challenged by intranasal instillation of OVA (10 μg in 0.9% saline) on Days 12–15. AAD was assessed 24 h after the final OVA challenge. Naïve mice had no intervention.

2.3. Dietary supplementation with Lyc-O-Mato

Treatment groups received a daily dose of Lyc-O-Mato (4 mg in 200 μl of water) commencing 14 days prior to OVA sensitisation. This dose of Lyc-O-Mato delivered contained 0.16 mg lycopene (equivalent to approximately 8 mg/kg per day), 0.006 mg β-carotene and 0.05 mg vitamin E. Mice were anaesthetized with isofluorane, and a 21-gauge ball-ended gavage needle was inserted into the stomach. Nutrients were injected and the gavage needle was removed. Mice were held upright for approximately 5 s after the procedure to prevent aspiration. The Lyc-O-Mato was mixed in water to the correct dosage. Sham-treated OVA/OVA control groups received 200 μl of water by gavage and PBS/OVA, and naïve and controls received no treatment.

2.4. Measurement of body mass

Mice were weighed prior to airways hyperresponsiveness (AHR) testing.

2.5. Collection and analysis of inflammatory cells in blood and bronchoalveolar lavage (BAL) fluid

Blood was collected by cardiac puncture, and smears were prepared and stained with May-Grunwald Giemsa for differential leukocyte counts. The remaining blood was centrifuged and serum was stored at −80°C until analysis.

BAL fluid was obtained by cannulation of the trachea and washing the lumina of the airways with HBSS (2×0.9 ml). Approximately 0.7 ml of the instilled fluid was recovered per wash, pooled and centrifuged (150×*g*, 4°C, 5 min). Recovered cells were treated with erythrocyte lysis buffer and resuspended in HBSS (100 μl). Cell numbers were determined using a standard hemocytometer, and cells were cytocentrifuged and stained with May-Grunwald Giemsa solution. Cell types were enumerated according to morphological criteria, with a minimum of 200 cells counted per slide. Slides were examined in a blinded fashion.

2.6. Assessment of eosinophils and mucus-secreting cells (MSC) in lung tissue

Lung tissue was fixed in phosphate-buffered formalin (10%), sectioned and stained with hematoxylin and eosin for the determination of eosinophils or with Alcian blue/periodic acid-Schiff for enumeration of MSCs. The mean number of eosinophils or MSC per high-powered field (×100) within 100 μm basement membrane was assessed following examination of at least 10 fields. Slides were examined in a blinded fashion.

2.7. Lymph node cell culture and cytokine production

Peribronchial lymph nodes were dispersed through 70 μm nylon mesh, treated with erythrocyte lysis buffer. Cells were cultured (1×10^6 cells/well, 37°C, 5% CO_2) with OVA (200 μg/ml) in 96-well plates in animal cell culture medium (0.1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin/streptomycin, 50 μM 2-ME and 10% FBS in RPMI 1640). Unstimulated wells contained cells and medium only. CD3/CD28 stimulation was used as the positive control; wells were first coated with 0.5 μg anti-CD3, and 0.4 μg/ml anti-CD28 (both from BD Biosciences, North Ryde, Australia) was added to the medium. After 6 days, supernatants were removed and

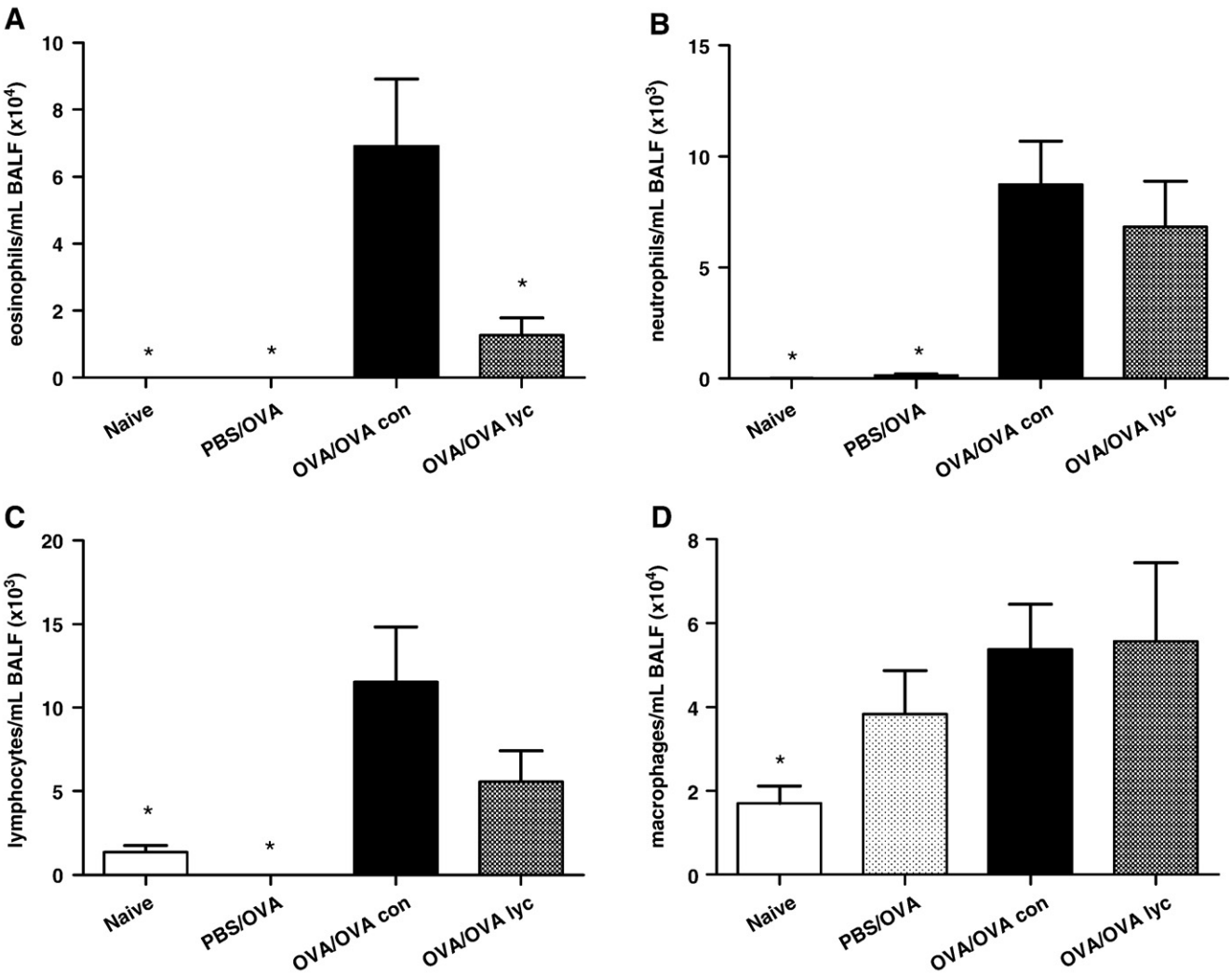

Fig. 1. Effect of Lyc-O-Mato supplementation on leukocyte levels in BALF in AAD. Experimental groups received Lyc-O-Mato supplementation prior to the induction of AAD (OVA/OVA lyc). Eosinophil influx into the airways in AAD was selectively decreased with Lyc-O-Mato supplementation. (A) Eosinophils, (B) neutrophils, (C) lymphocytes and (D) macrophages are expressed as the number of cells per milliliter of BALF. Data represent the mean±S.E.M. for a minimum of six mice per group. *$P<.05$ compared with untreated (OVA/OVA con) controls.

stored at −80°C until analysis. IL-4, IL-5, IFN-γ (BD Biosciences) and IL-13 (R&D Systems, Gymea, Australia) concentrations were measured in supernatants by ELISA, according to the suppliers' recommendations.

2.8. Determination of OVA specific serum IgGs

OVA-specific IgG1 and IgG2a were semiquantified by ELISA. Plates were coated with either OVA (2 μg/well) for sample wells or unlabeled anti-IgG of corresponding isotype for standard wells. After blocking with 3% bovine serum albumin, wells were incubated with serum or standards (mouse IgG1 or IgG2a), followed by detection with streptavidin-HRP anti-IgG1 or anti-IgG2a. Plates were developed with tetramethylbenzidine substrate; the reactions were terminated with 0.3 M H_2SO_4; and OD was determined at 450 nm using a Bio-Rad 680 Microplate reader. All reagents were from BD Biosciences.

2.9. Measurement of AHR

AHR measurements were conducted according to previously described methods [17]. Responses to inhaled β-methacholine are expressed as the percentage change over baseline (saline control).

2.10. Statistical analysis

The significance of differences between experimental groups was analysed using Student's unpaired *t* test for all parameters except lung function tests which were analysed by one-way repeated measures ANOVA. All analyses were performed using GraphPad Prism 4.03 (GraphPad Software, San Diego, CA, USA). Values are reported as mean±S.E.M., with $P<.05$ taken to be significant.

3. Results

3.1. Body mass

There were no differences between the body mass of mice from any group (data not shown).

3.2. Dietary supplementation with Lyc-O-Mato suppresses allergic inflammation

The induction of AAD by sensitisation and challenge with OVA (OVA-con group) resulted in increases in the numbers of eosinophils, neutrophils, lymphocytes and macrophages in the BALF compared to naïve and PBS-sensitised controls (Fig. 1). Dietary supplementation with Lyc-O-Mato (OVA-lyc) resulted in a decrease in the number of eosinophils in the BALF compared to untreated (OVA-con) controls (Fig. 1A) but did not alter neutrophil (Fig. 1B), lymphocyte (Fig. 1C) or macrophage (Fig. 1D) numbers.

The development of AAD (OVA-con) also induced increases in the numbers of eosinophils and MSC in the lung tissue surrounding the airways (Fig. 2A and B) and eosinophils as a percentage of leukocytes in the blood compared to naïve and PBS-sensitised controls (Fig. 3). Lyc-O-Mato supplementation (OVA-lyc) resulted in a decrease in each of these features of allergic inflammation (Figs. 2A, B and 3).

3.3. Dietary supplementation with Lyc-O-Mato suppresses Th2 responses

The development of OVA-induced AAD resulted in an increase in OVA-specific serum IgG1 antibody levels compared to naïve and PBS controls. Lyc-O-Mato supplementation did not alter OVA-specific IgG1 antibody levels (Fig. 4). OVA-specific serum IgG2a values were below the ELISA detection limit for all groups (data not shown).

The development of OVA-induced AAD (OVA-con) resulted in the increased release of OVA-specific IL-4, IL-5 and IL-13, and decreased IFN- γ production from draining lymph node cells compared to naïve

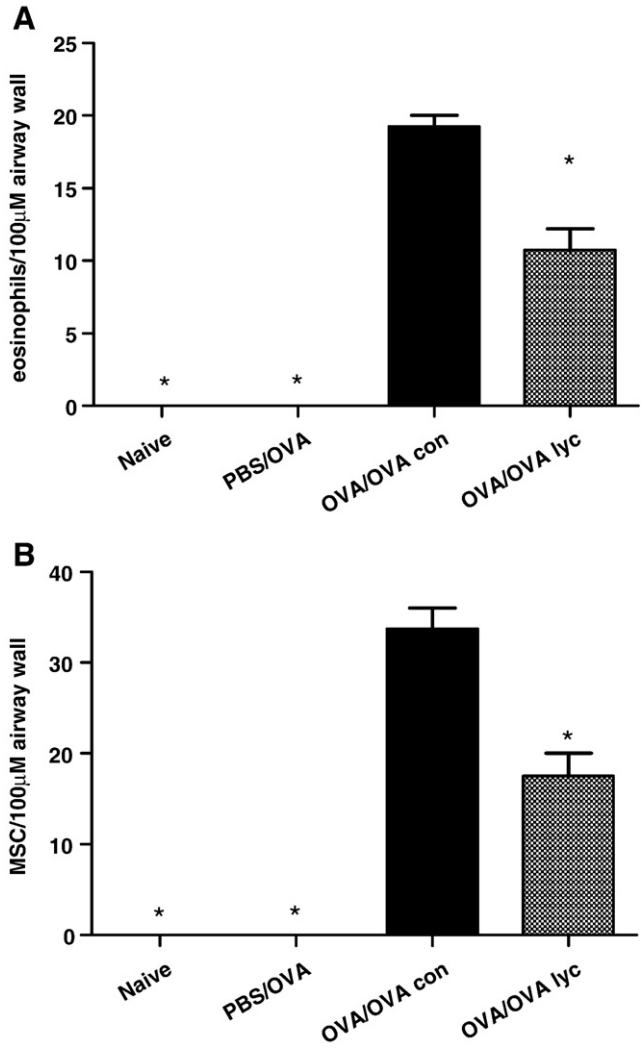

Fig. 2. Histological analysis of lung sections from naïve control (naïve), PBS/OVA control, OVA/OVA control and OVA/OVA Lyc-O-Mato. (A) Eosinophils in airway surrounding tissue were reduced with Lyc-O-Mato, and (B) goblet cell hyperplasia was decreased with the Lyc-O-Mato supplement. Data represent the mean±S.E.M. for a minimum of three mice per group. *$P<.05$ compared with the OVA/OVA control group. Significant differences between groups are indicated.

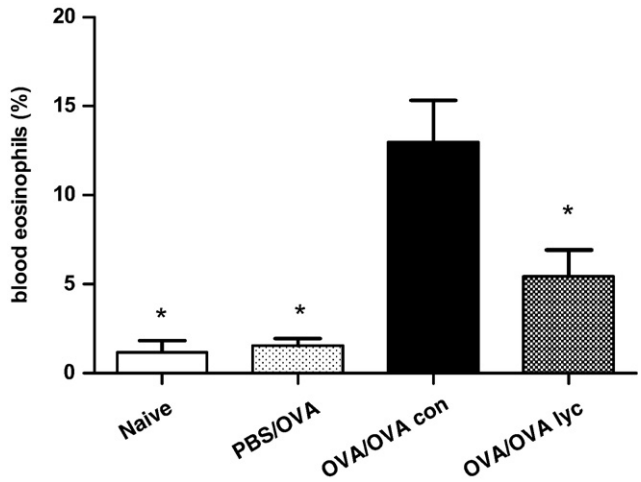

Fig. 3. Blood eosinophils from naïve control (naïve), PBS/OVA control, OVA/OVA control and OVA/OVA Lyc-O-Mato. Eosinophils are expressed as percent of blood leukocytes. Lyc-O-Mato treatment decreased % blood eosinophils. Data represent the mean±S.E.M. for a minimum of six mice per group. *$P<.05$ compared with the OVA/OVA control group. Significant differences between groups are indicated.

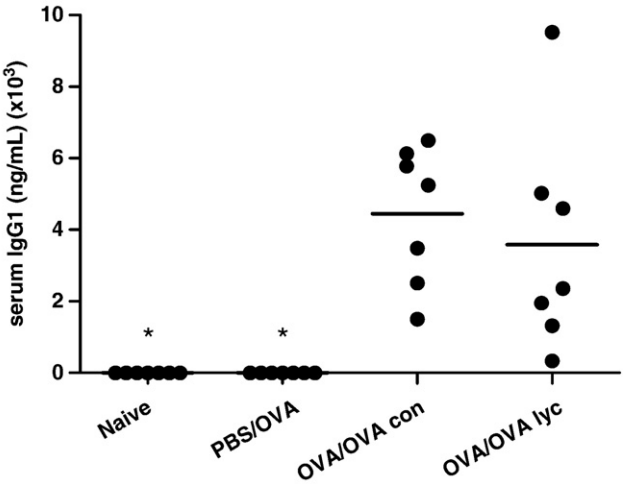

Fig. 4. Serum IgG1 from naïve control (naïve), PBS/OVA control, OVA/OVA control and OVA/OVA Lyc-O-Mato. Each data point represents an individual mouse with a minimum of seven mice per group. Values are expressed as nanograms per milliliter of IgG1 detected in the serum. *P<.05 compared with the OVA/OVA control group. Lyc-O-Mato treatment did not alter serum IgG1.

and PBS-sensitised controls (Fig. 5). Lyc-O-Mato supplementation (OVA-lyc) suppressed the OVA-specific release of Th2 cytokines, IL-4 (Fig. 5A) and IL-5 (Fig. 5B); however, IL-13 (Fig. 5C) and IFN-γ (Fig. 5D) levels were not altered. CD3/28 stimulation (positive control) resulted in strong cytokine responses in each assay (data not shown).

3.4. Dietary supplementation with Lyc-O-Mato does not affect AHR

The development of AAD (OVA-con) resulted in increased airway resistance and decreased dynamic compliance upon aeroallergen challenge with β-methacholine compared to naïve and PBS-sensitised controls (Fig. 6). Lyc-O-Mato supplementation (OVA-lyc) did not alter resistance (Fig. 6A) or compliance (Fig. 6B).

4. Discussion

It has been suggested that dietary change, including a reduced anti-oxidant intake, has contributed to the dramatic increase in asthma rates in recent decades [16]. Here we demonstrate that dietary supplementation with Lyc-O-Mato decreases eosinophilic infiltrates and mucus production in the lungs, airways and systemic

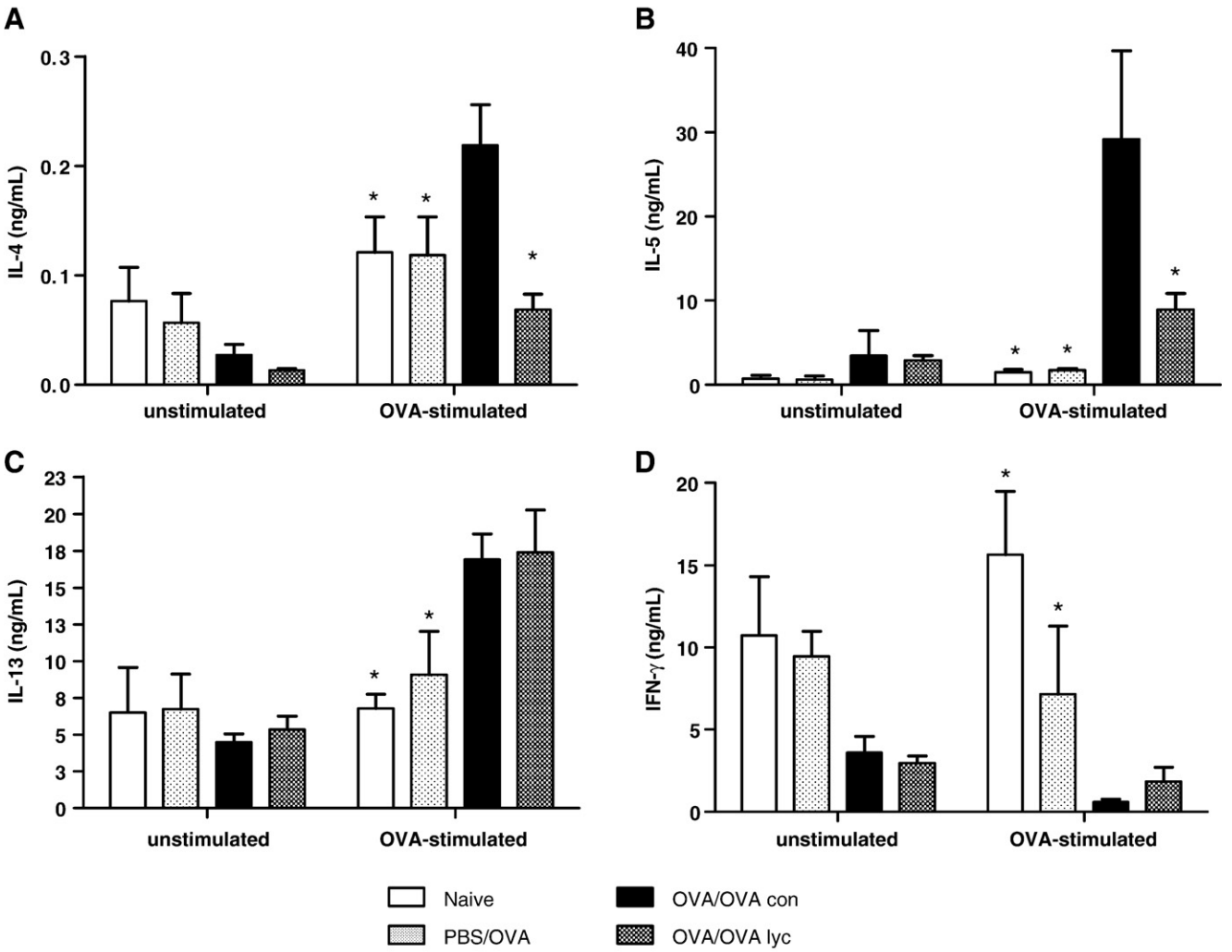

Fig. 5. Lymph node cultures from naïve control (naïve), PBS/OVA control, OVA/OVA control and OVA/OVA Lyc-O-Mato. (A) IL-4 is decreased in the cell culture supernatant from mice given Lyc-O-Mato, (B) IL-5 is reduced after Lyc-O-Mato treatment, (C) IL-13 is unchanged and (D) IFN-γ is not altered after supplementation. Values are expressed as nanograms per milliliter of cell culture supernatant. Data represent the mean±S.E.M. for a minimum of eight mice per group, with two mice pooled per data point. *P<.05 compared with the OVA/OVA control group. Significant differences between groups are indicated.

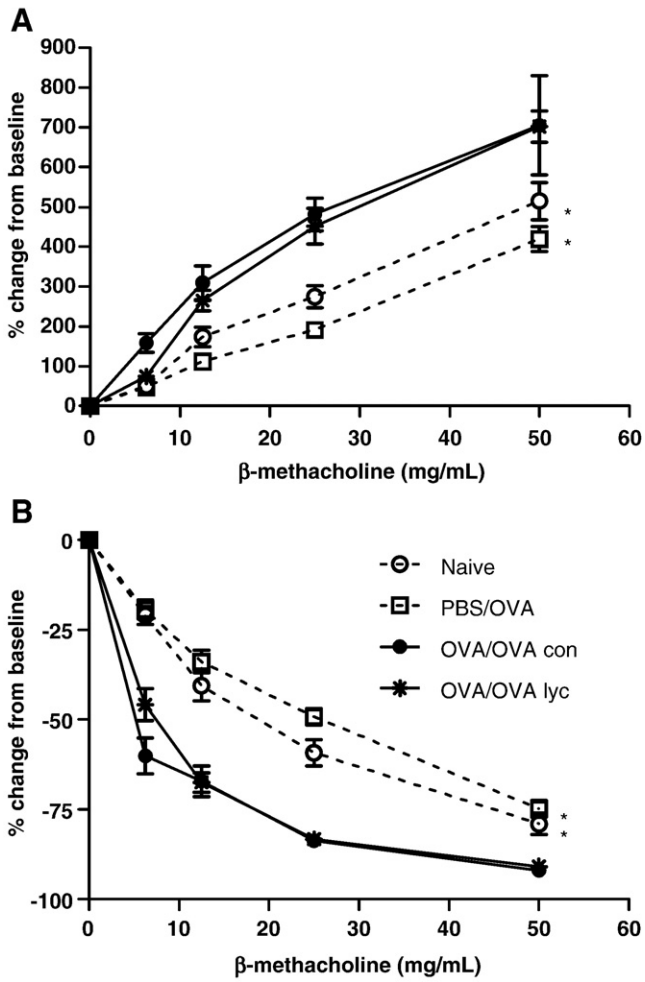

Fig. 6. Measurement of airways reactivity to β-methacholine in naïve control (naïve), PBS/OVA control, OVA/OVA control and OVA/OVA Lyc-O-Mato mice. (A) Lung resistance and (B) dynamic compliance are expressed as a percentage of the baseline reactivity to saline in the absence of cholinergic stimuli. Data represent the mean±S.E.M. for a minimum of six mice per group. *P<.05 compared with the OVA/OVA control group. Lyc-O-Mato supplementation did not alter lung resistance or dynamic compliance compared to OVA/OVA control.

eosinophil recruitment, as well as reduces Th2 cytokine responses from draining lymph node cells.

Nutrition intervention studies in humans are often limited by poor patient compliance to the treatment. To this end, we used experimental mice to enable the daily delivery of a specific dose of supplement. Mice were fed a controlled background diet *ad libitum* as it has been demonstrated that the composition of the background diet can alter *in vivo* incorporation of nutritional products, such as omega-3 PUFA [18]. The gavage delivery method used here was designed to replicate a human consuming a specific-dose capsule daily. Similarly, the dose of supplement administered was calculated to reflect a realistic human daily dose. The metabolic rate of the mice was calculated to be approximately 20 times faster than a human metabolic rate so the supplements were administered at a 20 times greater dosage, on a milligram-per-kilogram basis. Thus, the administered amounts equate to a human dose of approximately 50 mg/day Lyc-O-Mato, which is a suitable therapeutic dose [14].

Lyc-O-Mato supplementation resulted in a sevenfold (approx) reduction in eosinophils in the BALF, a twofold (approx) decrease in eosinophils in the lung tissue surrounding the airways and a twofold (approx) reduction in the percent of blood eosinophils compared to other leukocytes. Goblet cell hyperplasia was also decreased twofold (approx) in the airways. However, there was no change in the numbers of the other leukocytes examined, including neutrophils, lymphocytes and macrophages. It is possible that the reduction in eosinophils observed in this study is a result of decreased IL-5-dependent eosinophil recruitment to the lungs, since Lyc-O-Mato supplementation also suppressed T-cell IL-5 release by a factor of ~3. IL-5 is the major cytokine that mediates eosinophil expansion, priming, recruitment and survival [1]. The reduction in lung eosinophils observed may also be the result of decreased eosinophil expansion from the bone marrow and priming, as eosinophil numbers in the blood were also reduced. OVA-specific IL-4 release from draining lymph nodes was also reduced with Lyc-O-Mato supplementation. IL-4 is essential for the early expansion phase of the allergic response and acts in synergy with IL-5 to promote eosinophilic inflammation [1]. Therefore, the suppression of IL-4 may also contribute to the decreased lung eosinophils. IL-4 also promotes IgE isotype switching and up-regulation of MHC-II on B-cells, which are associated with Th2 allergic responses [19]. However, Lyc-O-Mato supplementation did not affect antigen-specific IgG1 levels. In spite of the suppression of allergic inflammation, supplementation did not alter lung function. This may be because IL-13 release from the draining lymph nodes was unaffected. The importance of IL-13 to airway function has previously been demonstrated, and IL-13 can induce AHR in mice [20,21].

Another study by Lee et al. [22], which used an OVA-induced murine asthma model, also demonstrated that administration of lycopene significantly inhibited the infiltration of inflammatory leukocytes, including eosinophils, neutrophils, macrophages and lymphocytes, into BAL fluid. This was associated with the suppression of the Th2 transcription factor GATA-3, the cytokine IL-4 and the activity of eosinophil peroxidase and matrix metalloproteinase-9 in BAL. However, in contrast to our data, which showed no effect of lycopene supplementation on airways reactivity to methacholine, these authors detected a reduction in AHR to methacholine [22]. It is likely that this difference is due to the different experimental designs employed. Lee et al. [22] administered lycopene *via* intraperitoneal injection. In our study, administration of Lyc-O-Mato was performed by gavage directly into the stomach. The dose that we used, which was calculated to reflect a therapeutic dose in humans, was similar to the lowest dose used by Lee et al. [22]. Thus if the uptake of lycopene from the stomach was not efficient, the availability of circulating lycopene in our study may have been much lower than that achieved by Lee et al. [22]. This may account for the inconsistencies observed in the lung function data.

Lyc-O-Mato contains high levels of the antioxidant lycopene. Lycopene has anti-inflammatory effects in the airways, via a reduction in neutrophil influx and activation in adults with asthma [14]. Although neutrophils were not significantly reduced in our study, the mouse model utilised does not have a substantial neutrophil component and is characterised by a dominant eosinophil response. Therefore, alterations to eosinophil numbers are of more relevance to this experimental system. Indeed, in the study by Lee et al. [22], a reduction in airway neutrophil numbers was observed.

The protective effects of Lyc-O-Mato may result from the anti-inflammatory properties of lycopene. Nevertheless, it cannot be concluded that the effects on inflammation observed in this study are the result of lycopene in isolation, as Lyc-O-Mato is an extract of tomato which also contains low doses of other potent dietary antioxidants, including vitamin E and β-carotene. Vitamin E is present in lipid membranes and extracellular lung fluid where it converts oxygen and lipid peroxyl radicals to less reactive forms. Vitamin E has been reported to be inversely associated with asthma risk in adults [11,23] and children [24–26], although nonassociations have also been reported [27]. A negative association has been reported between

intakes of vitamin E-rich foods and serum IgE and atopy [28]. Additionally, vitamin E has been shown to promote a Th1 phenotype in mice [29] and reduce lipopolysaccharide (LPS)-induced nitric oxide (NO) release from rat alveolar macrophages [30]. The potential benefit of β-carotene in a model of allergic inflammation is less clear. β-Carotene has been shown to either have no effect [31–36] or lead to an increase [34,36,37] in circulating leukocyte and/or natural killer (NK) cell numbers. However, β-carotene has been shown to enhance NK cell function [38–40] and inhibit NF-κB activity in LPS-exposed cells [41]. It is known that synergistic effects between multiple components in whole foods can be more powerful than individual nutrients in isolation [15,16]. Therefore, it is likely that the benefits observed with the Lyc-O-Mato supplement are due to interactions between the many compounds present in this supplement, including vitamin E and β-carotene, as well as lycopene.

Supporting the results of the present study, lycopene consumption has been shown to have anti-inflammatory properties in a rat model of colitis [42]. Also, the administration of lycopene decreased IL-8 and TNF-α secretion as well as increased the phagocytic activity of pulmonary alveolar macrophages in an LPS-induced lung injury model in rats [43]. Similarly, the *in vitro* culture of murine dendritic cells (DCs) with lycopene inhibited DC maturation, allostimulatory capacity and LPS-induced IL-12 production by directly inhibiting the mitogen-activated protein kinase and nuclear factor-κB (NF-κB) pathways [44].

In conclusion, this study provides evidence that dietary supplementation with lycopene prior to and during the onset of AAD may be of clinical benefit in reducing eosinophilic infiltrates both in the lungs and systemically. Additionally, supplementation with this antioxidant has the capacity to decrease mucous in the lungs and reduce Th2 cytokine responses.

Acknowledgments

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