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Aging leads to impaired epicutaneous sensitization that causes attenuated allergy and pulmonary inflammation in mice

Short title: Aging, allergy and pulmonary inflammation in mice

Sheila Langier, PhD¹, Shira Benor, MD¹, Laliv Kadar, MD¹, Nir Shani, PhD², Sara Etkin, MSc¹, Moshe Stark, PhD³, Ekaterina Bondar¹, Asaf Aizic, MD⁴ and Shmuel Kivity, MD¹

¹Allergy and Immunology Unit, ²Microsurgery and Plastic Surgery Laboratory of the Plastic Surgery Department, ³Biochemistry Laboratory, and the ⁴Department of Pathology, Tel Aviv Medical Center, Tel Aviv, Israel, affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Correspondence: Shmuel Kivity, MD
Allergy and Immunology Unit
Tel Aviv Medical Center
6 Weizman Street
Tel Aviv 6423906, Israel

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Telephone: +972-3- 6973734

Fax: +972-3-6876972439

Email: allergy@tlvmc.gov.il

Abstract

Aging is associated with altered decreased barrier function in the skin, which can lead to different types of immunoglobulin E (IgE)-mediated sensitization to environmental allergens. Yet, allergen-specific respiratory sensitization among the elderly is not well described. The aim of this study was to investigate the effect of aging on allergic pulmonary inflammation induced by epicutaneous sensitization of mechanically irritated skin in mice. For this purpose, 6-week-, 6-month-, and 18-month-old female BALB/c mice, underwent epicutaneous sensitization with ovalbumin (OVA) or PBS, followed by an inhaled OVA challenge. Blood OVA-specific IgE levels measured after epicutaneous sensitization, as well as, bronchial alveolar lavage fluids (BALF) leucocyte, eosinophil, and cytokine levels measured after OVA inhalation challenge were similar among the 6-week-old (young) and 6-month-old (adult) groups. However, significantly decreased levels of systemic OVA-IgE, and BALF leucocyte, eosinophil and T helper cell type 2 cytokine levels, were measured after OVA inhalation challenge in elderly (18-month-old) mice compared to the other groups of mice. In addition, interleukin-10 (IL-10), a regulatory suppressor cytokine, was more abundant in the BALF of the elderly group after epicutaneous sensitization and inhalation challenge. Our results suggest that elderly mice have a reduced allergic response to induced sensitization with OVA, possibly regulated by increased IL-10 levels.

Keywords: aging, allergy, epicutaneous sensitization, pulmonary inflammation

Introduction

A strong association between allergic sensitization and respiratory allergy has been described in children, adolescents and young adults.^{1, 2} The role of allergen-specific sensitization in the pathophysiology and severity of allergy in older patients is not well understood. It is generally accepted that total immunoglobulin E (IgE) levels decline with increasing age.³ Several cross-sectional studies of randomly selected individuals from the Tucson Epidemiological Study and the National Health and Nutrition Examination Survey, have demonstrated that total serum IgE titers peaks at 20 years of age and is lowest at 70 years of age.³ Similarly, the prevalence of allergen-specific IgE sensitization was found to be higher in younger populations than in older ones. Another study looked at adult inner-city patients with moderate-to-severe persistent asthma and found that 41% of those older than 60 years were sensitized to at least one antigen, compared to 73% sensitized individuals among patients between 18-35 years of age.⁴

It is fairly well established that there is a T helper cell type 2 (Th2) cytokine bias in childhood, while reports on cytokine expression later in life are conflicting. Several investigations of cytokine responses to nonspecific stimulants in aged rodents and elderly adults without atopic diseases, demonstrated a bias towards a Th1 effector response. In contrast, other studies have suggested an association between aging and a Th2 shift, with decreased interferon- γ (IFN- γ) production and higher interleukin-4 (IL-4), IL-5 and IL-10 responses. Sensitization and challenge of 10- and 12-month-old BALB/c mice resulted in a reduced Th2-like response and a significantly decreased bronchial alveolar lavage fluid (BALF) total cell count and eosinophil count compared to antigen-sensitized younger mice.⁵ Despite increased pulmonary inflammation and mucus metaplasia, significantly lower airway hyperresponsiveness (AHR) was demonstrated in older mice compared with younger mice

after antigen sensitization/challenge.⁵ Moreover, antigen sensitization produced a unique cytokine profile in older mice (elevated IFN- γ and IL-5) compared with younger mice (elevated IL-4 and IL-13).⁵

In the skin, aging is associated with thinning of the epidermis, reduced elastosis, loss of melanocytes associated with increased paleness and lucency of the skin, and a decrease in the immunological barrier function.⁶ Atopic dermatitis (AD)-associated skin inflammations manifest as a relapsing form of skin inflammation, disturbance of the epidermal-barrier function that culminates in dry skin, and IgE-mediated sensitization to environmental allergens.⁷ AD is considered an entry point of the “atopic march”, that involves the progression of an atopic disorder from AD in infants to allergic rhinitis and finally to asthma in children and adults.^{8,9} Epidemiologic data from cross-sectional and longitudinal studies support the sequential development from AD to asthma.^{8, 10, 11} The progression from AD to asthma was also supported by experimental data showing that allergen exposure through the epidermis can initiate systemic allergy.¹² These data suggest a causal link between childhood AD and the later onset of respiratory allergic disorders what have not fully investigated yet. The physical structure and function of the skin might have significant influence on the allergic sensitization in at a later age. The present study was designed to evaluate the effect of aging on epicutaneous respiratory sensitization and allergic pulmonary inflammation in mice.

Results

Antigen-specific IgE levels were reduced in elderly mice compared to middle-aged mice following epicutaneous allergen sensitization

In this study, we first evaluated the effect of epicutaneous ovalbumin (OVA) and Staphylococcus Aureus Enterotoxins (SE) exposure on systemic allergic responses in elderly and young and adult mice.

Young and adult mice did not present statistical differences in most of the parameters investigated in this study, so we elect to combine these two groups (**middle aged**) in the statistical analysis to increase sample size and veracity of the data (see materials and methods section "*Sensitization and challenge*").

OVA-specific IgE levels were detected in the serum of both groups of mice 24 hours after the last antigen exposure, with the elderly mice exhibiting significantly lower levels compared to the middle-aged mice ($P = 0.02$) (Fig. 2). PBS-sensitized mice, of both groups, had very low or undetectable levels of OVA-specific IgE.

Distinct airway inflammation patterns among middle-aged versus elderly mice following epicutaneous sensitization and inhalation challenge

To evaluate the effect of aging on pulmonary inflammation profiles, we first determined total leukocyte numbers and eosinophil percentages in BALF, both of which were significantly lower in the elderly OVA/SE mice compared with the middle-aged mice subjected to the same treatment regimen and challenged with OVA inhalation ($P = 0.008$ and $P = 0.007$, respectively) (Fig. 3A,B). The PBS control of both group showed low to undetectable leukocyte counts and eosinophil percentages.

To correlate BALF leukocyte counts with lung tissue inflammation, lung sections collected following OVA challenge, were stained with H&E. Lung inflammation was scored by degree of perivascular and, peribronchial inflammation and cell infiltration, all of which

are considered typical signs of asthma. The disease score was mild for the elderly OVA/SE mice (Fig. 4A), but moderate to severe for the middle-aged OVA/SE group (Fig. 4B). Mice that underwent sensitization with PBS followed by an OVA challenge did not exhibit any lung inflammation (Fig. 4C). These results demonstrated that aging reduced lung pulmonary inflammation following epicutaneous sensitization with OVA/SE and an OVA challenge in mice.

Reduced TH2-like cytokines and increased regulatory cytokines in elderly mice compared to younger mice following epicutaneous allergen sensitization and inhalation challenge

In an attempt to better characterize the nature of the lung inflammation, cytokine profiles in the BALF of middle-aged versus elderly mice in this model were compared. IL-4 levels in the latter were significantly lower compared to the former group ($P = 0.05$) (Fig. 5). IL-13 levels were decreased among the elderly compared to young group, however adult mice showed low levels of IL-13, similar of those observed in elderly mice. In the other hand, regulatory suppressor cytokine IL-10 release was very significantly increased in the elderly OVA/SE group compared to the middle-aged group ($P = 0.001$) (Fig. 6). These findings suggest that IL-10 was involved in regulating the mild allergic airway inflammation observed in the elderly mice.

Discussion

The results of this study demonstrated that OVA/SE epicutaneous sensitization followed by OVA inhalation challenge induced an attenuated systemic allergic response as well as milder subsequent airway inflammatory response in elderly mice, compared to middle-aged mice. The attenuated response was characterized by reduced serum-specific OVA IgE and decreased airway inflammation, manifested by reduced lung tissue perivascular and

peribronchial inflammation, and by notably reduced BALF leukocyte counts and eosinophil percentages. Furthermore, the elderly antigen-treated mice displayed a distinct TH2 cytokine pattern (decreased IL-4 and IL-13), together with elevated IL-10 levels (suppressor cytokine) in BALF samples.

The pathophysiology of atopic asthma is characterized by elevated serum IgE levels, airway eosinophilia, bronchial hyperresponsiveness, inflammatory cell infiltration and goblet cell metaplasia in the airways. Th-2-type cytokines, IL-4, IL-5, and IL-13 are thought to play a pivotal role in this process.^{13, 14} Th2 deviation in atopic asthmatic patients has been reported to be impacted by several factors, including genetic background, antigen sensitization doses,¹⁵ and the cytokines involved in early T-cell activation.¹⁴ The studies delineating the physiological and immunological abnormalities associated with atopic asthma were based upon data in humans and in animal models of asthma.¹⁶ However, the association between allergen-specific sensitization and asthma pathophysiology and severity in elderly patients, has been studied by only a few groups. Using a mouse model of allergic asthma based on intraperitoneal sensitization with OVA and aluminum hydroxide adjuvant, followed by an OVA inhalation challenge, Hasegawa et al., demonstrated that elderly mice exhibited an attenuated allergic airway inflammation dependent on impaired GATA3, a transcription factors that control TH2 responses.¹⁷ Another study that used in a similar mouse model of allergic asthma, demonstrated that age at the time of initial allergen exposure, dictated subsequent responses in the lung. More specifically, sensitization in elderly mice was associated with reduced airway hyperresponsiveness iARH, eosinophilia, TH2-type cytokines and allergen-specific IgE.¹⁸ In addition, aged brown Norway rats failed to develop eosinophilic bronchial inflammation and ARH after antigen exposure. Using a mouse model of allergic asthma based on OVA and aluminum hydroxide intraperitoneal (i.p.) sensitization

and OVA intratracheal challenge, Busse et al.¹⁸ showed greater OVA-specific IgE, eosinophilia and mucus cell metaplasia in elderly mice, but significantly lower AHR compared to younger mice. Following OVA and aluminum hydroxide i.p. sensitization, followed by intranasal OVA challenge, higher total cell and eosinophil counts, IL-4 cytokine levels and peribronchial collagen deposition were measured in the BALF of the elderly versus young mice.¹⁹ The different modes of antigen introduction during the sensitization and challenge phases and the antigen concentration used in the above-mentioned studies can explain the discrepancies in the reported results. In the current study, we investigated allergic response and airway inflammation in elderly mice by means of epicutaneous sensitization with OVA and SE in irritated skin, followed by OVA inhalation challenge. We believe that this model is close to the natural mode of sensitization, best resembling allergic sensitization in humans.

Irritated or inflamed skin is known as an important route of antigen penetration and sensitization, and AD diseases, hallmarked by chronic skin inflammation and disturbance of epidermal-barrier function that culminate in dry skin present such conditions in humans. Indeed, the conditions is often associated with IgE-mediated sensitization to environmental antigens and is considered the initial step of the “atopic march”, progressing to allergic rhinitis and asthma.^{8, 20} Previous studies have shown that epicutaneous exposure of mice to allergens results in sensitization when the skin was even slightly damaged.^{12, 21, 22}

Williams et al. reported that *Staphylococcus aureus* chronically colonized AD patient skin, producing superantigen enterotoxin B, which correlated with the severity of the disease.²³ Yu et al. demonstrated that mouse epicutaneous exposure to SE-B enhanced allergic lung inflammation.²⁴ We used SE-B as adjuvant to augment allergic sensitization via skin lesions.²⁵

Skin renewal of keratinocytes and the epidermis dramatically slows down with age. Aging is associated with dermatologic hallmarks, such as thinning of the epidermis, elastosis, loss of melanocytes, associated with increased paleness and lucency of the skin, and decreased barrier function, which can all contribute to allergic sensitization.²⁶ The results of our study demonstrated an attenuated allergic inflammatory profile among the elderly mice of our model as compared to middle-aged animals. Notably, high IgE levels and the presence of eosinophilia are determinant factors in the diagnosis of allergic asthma in humans. No differences in the serum IgE levels and evidence of eosinophilia in the BALF of the young versus adult mice were noted, yet significant differences between them and the elderly mice were recorded. In addition, less allergic airway inflammation, serum OVA IgE, leukocyte counts in BALF, eosinophil percentages, lung tissue inflammation and TH2 cytokine counts was observed in the elderly mice as compared to the other age groups. Release of IL-10, an immunological regulatory suppressor cytokine, was higher in the BALF of the elderly mice, correlating with the lower extent of airway inflammatory disease observed in these animals. Our results suggest that elderly mice have a diminished allergic response to induced sensitization with OVA. These findings support reports demonstrating that specific allergic sensitivity as well as total IgE levels diminish with age.

Methods and Materials

Mice

Six-week-old (young), 6-month-old (adult) and 18-month-old (elderly) female BALB/c mice were purchased from Harlan Laboratory, Israel. All the mice were maintained in the animal facility at Tel Aviv Medical Center. Laboratory animal care followed standard Israel Ministry of Health guidelines, and institutional approval for animal handling in our animal facilities was granted.

Sensitization and challenge

To establish epicutaneous sensitization, the animal's abdominal fur was removed by shaving. The abdominal skin was tape-stripped three times and immediately exposed to 2 mg/mL OVA (Sigma, Israel) and 100 µg/mL SE-B (Sigma, Israel), or PBS (Biological Industries, Israel) in a final volume of 50 µL. The mice were exposed for 5 consecutive days, followed by 10-day intervals for a total of 15 exposures (Fig. 1). Twenty-four hours after the final exposure, the mice were challenged with allergen on 5 consecutive days, by inhalation of a 4% OVA solution for 40 minutes. SE-B, a well-known triggering factor in the development of atopic dermatitis in humans,²³ was selected as adjuvant to mimic natural exposure to skin inflammation (Figure 1).

No significant difference between the young and adult mice was observed for the two parameters. The analysis of deviation of each group's mean compared to the mean of both groups combined was under 2% for OVA-specific IgE and eosinophil percentage. Thus, the young and adult mice were grouped into a single group that we labeled "middle-aged", to increase sample size and level of veracity.

Bronchoalveolar lavage fluid (BALF)

To collect BALF, the trachea was surgically exposed, cannulated with a syringe, and flushed twice with 1 ml PBS. Cells in the lavage fluid were counted using a hemacytometer, and BALF cell differentials were determined on slide preparations stained with May-Grünwald-Giemsa viewed in 15–20 high-power fields under a light microscope (Leica DM 4000B). The supernatants were stored at -20°C for cytokine analysis.

Measurement of OVA-specific IgE antibodies

Blood samples were obtained from each group of mice via the retro-orbital vein, immediately after the last sensitization time point. The sera were separated and stored at -20°C until analysis. To determine OVA-specific IgE, plates were coated with OVA (100 µg/mL) and then processed with an ELISA kit for IgE detection, according to the manufacturer's instructions (Bethyl Laboratories, Inc., USA). Sera samples were diluted 1:10.

Cytokine quantification

BALF concentrations of IL-4, IL-13 and IL-10 were determined using commercially available ELISA kits (e-bioscience Kit (USA) DuoSet ELISA), as per the manufacturer's instructions. The absorbance was measured at 450 nm by a microplate ELISA reader (Bethyl, CA, USA). Cytokine release is shown as pg/mg of total protein contained in BAL supernatant.. Total protein concentration was quantitated using a Bio Basic Bradford reagent assay (Bio basic Canada Inc, Canada).

Histologic examination of lung tissue

The animals were sacrificed by overdose of ketamine-xylazine and the lungs were surgically removed 24 hours post-OVA challenge. The tissue was fixed in neutral buffered formaldehyde, and 5-µm paraffin sections were stained with hematoxylin and eosin (H&E) for evaluation of the inflammation. The slides were examined in a blinded fashion. Perivascular and peribronchial inflammation were graded using a standard scoring method (0 = normal, 1 = mild, 2 = moderate, 3 = severe).⁵

Statistical analysis

Statistical analyses were performed using the Mann Whitney U test for non-parametric data.

A *P* value smaller than or equal to 0.05 was considered statistically significant. All statistical analyses were performed using Prisma Graph Software (USA).

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Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Epicutaneous sensitization in damaged skin induces allergic asthma in mice.

Abdominal mouse skin was tape-stripped, exposed to ovalbumin (OVA) and *Staphylococcus aureus* enterotoxins (SE) or PBS, and then challenged with OVA inhalation, as described in Methods. Blood and BALF (bronchial alveolar lavage fluid) and lung tissue were obtained from the animals for results analysis.

Fig. 2. Lower levels of antigen-specific immunoglobulin E (IgE) in elderly versus younger mice. The skin of young and adult (6 weeks and 6 months) and elderly (18 months) mice was tape-stripped, exposed to ovalbumin and *Staphylococcus aureus* enterotoxins (OVA+SE) as described in Methods. Serum OVA-specific IgE levels were determined in all groups 24 hours after final OVA+SE sensitization. * $P = 0.02$. (8 animals in the middle aged -young-adult) group and 3 animals in the elderly group). Standard bar in our figures relate to standard deviation.

Fig. 3. Reduced leukocyte and eosinophil infiltration in the bronchial alveolar lavage fluid (BALF) of elderly versus young and adult mice. BALF samples were collected 24 hours after OVA challenge, as described in Methods (*Sensitization and challenge* Section). Total cell count (A) and eosinophil percentage (B) were analyzed in the different age groups. * $P = 0.008$, ** $P = 0.007$ ($n = 7$ animals per group). Standard bar in our figures relate to standard deviation.

Fig. 4. Association of advanced age with lung inflammation severity. Lung sections were obtained 24h after OVA inhalation challenge. Samples were staining with H&E and analyzed under an optical microscope x200. (A) PBS (inflammation score zero), (B) OVA/SE in middle-aged mice (inflammation score 2-3) and (C) OVA/SE in elderly mice (inflammation score 1). There were two representative histologic samples per group. Data are representative of two-four animals per group.

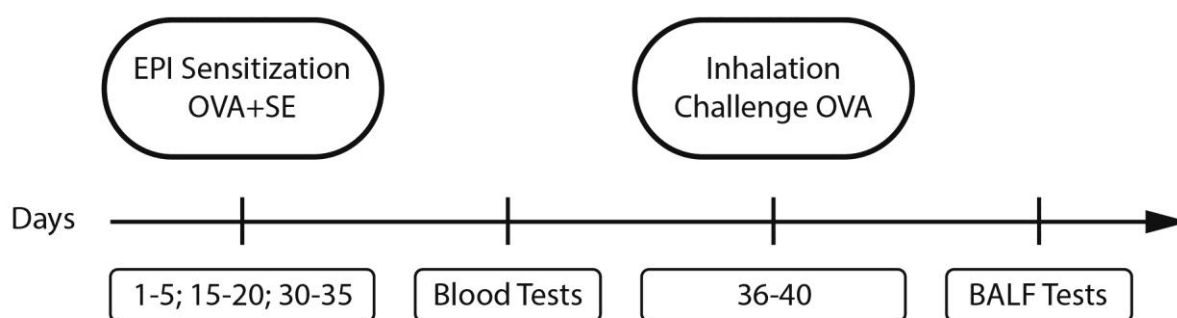
Fig. 5. T helper cell type 2 (TH2)-like cytokine release in the bronchial alveolar lavage fluids (BALFs) of young, adult and elderly mice. BALF supernatant was collected 24 hours after challenge with ovalbumin. Interleukin-4 (IL-4) levels in BALF supernatants were determined by ELISA. Young : 6 weeks, adult: 6 months; elderly: 18 months. * $P = 0.05$ (animals per group: interleukin-4 (IL-4) Young-adult, $n = 7$, elderly $n = 2$; IL-13 young $n = 4$, adult $n = 3$ and elderly $n = 2$). Cytokine release is shown as pg/mg of total protein contained in BAL supernatant. Standard bar in our figures relate to standard deviation.

Fig. 6. Regulatory interleukin-10 (IL-10) release in bronchial alveolar lavage fluids (BALFs) of middle-aged and elderly mice. BALF supernatant was collected 24 hours after OVA challenge. IL-10 in the BALF supernatant was analyzed by ELISA. Young : 6 weeks, adult: 6

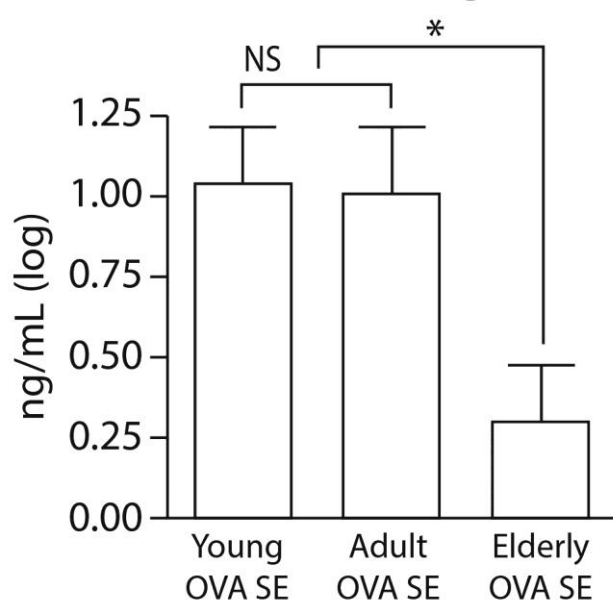
months;; elderly: 18 months. * $P = 0.001$ (young-adult animals $n = 7$ and elderly animals $n =$

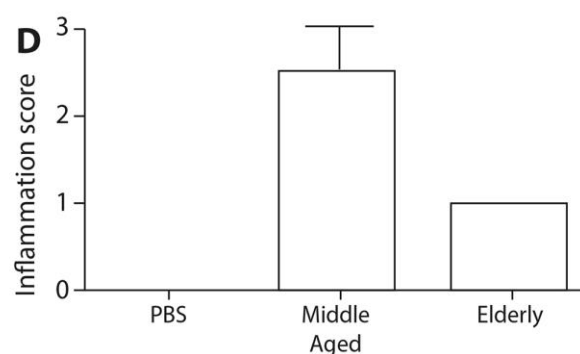
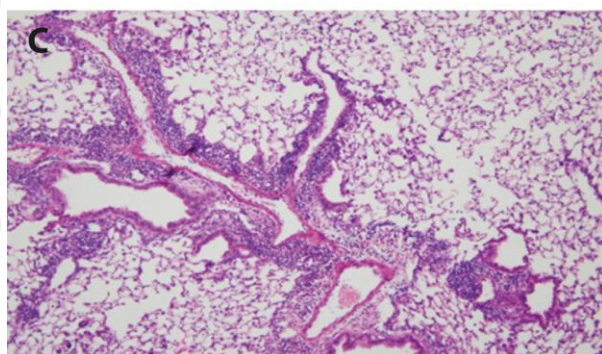
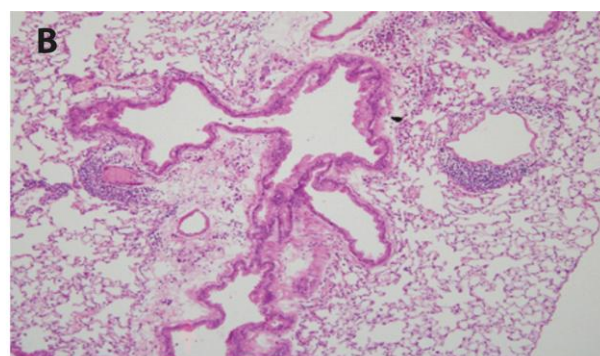
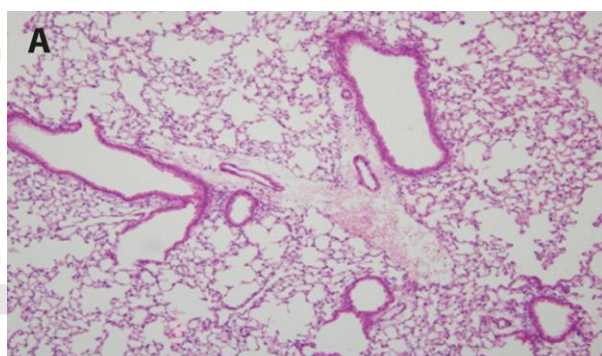
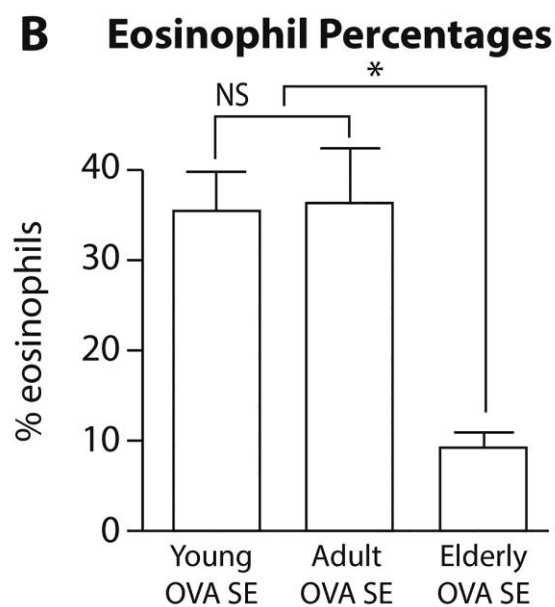
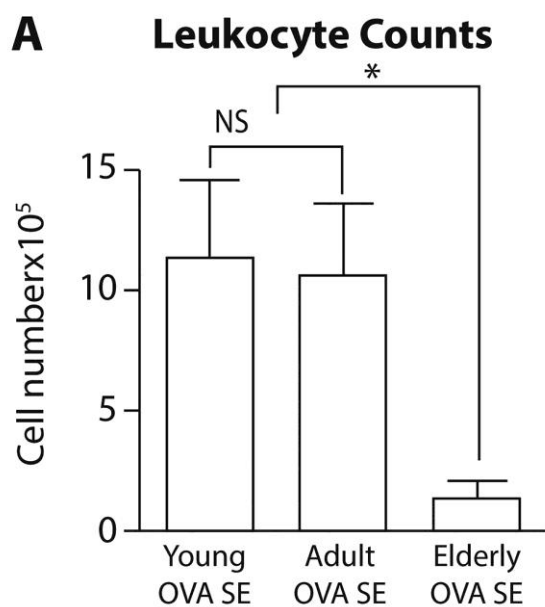
4). Cytokine release is shown pg/mg of total protein contained in BAL supernatant). Standard bar in our figures relate to standard deviation.

Schematic Protocol

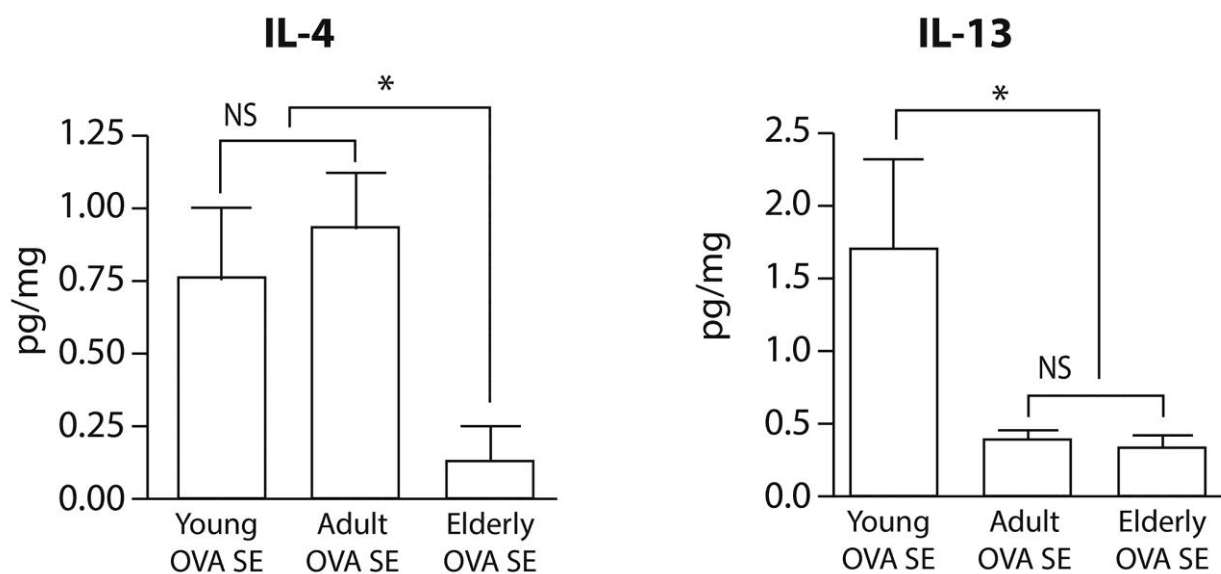


OVA - Specific IgE





TH2 like Cytokine



Regulatory Cytokine IL-10

