

A Role for Dietary Selenium and Selenoproteins in Allergic Airway Inflammation¹

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Asthma is driven by allergic airway inflammation and involves increased levels of oxidative stress. This has led to speculation that antioxidants like selenium (Se) may play important roles in preventing or treating asthma. We fed diets containing low (0.08 parts per million), medium (0.25 parts per million), or high (2.7 parts per million) Se to female C57BL/6 mice and used an established OVA challenge protocol to determine the relationship between Se intake and the development of allergic airway inflammation. Results demonstrated that mice fed medium levels of Se had robust responses to OVA challenge in the lung as measured by lung cytokine levels, airway cellular infiltrate, eosinophilia, serum anti-OVA IgE, airway hyperreactivity, goblet cell hyperplasia, and phosphorylated STAT-6 levels in the lung. In contrast, responses to OVA challenge were less robust in mice fed low or high levels of Se. In particular, mice fed low Se chow showed significantly lower responses compared with mice fed medium Se chow for nearly all readouts. We also found that within the medium Se group the expression of lung glutathione peroxidase-1 and liver selenoprotein P were increased in OVA-challenged mice compared with PBS controls. These data suggest that Se intake and allergic airway inflammation are not related in a simple dose-response manner, which may explain the inconsistent results obtained from previous descriptive studies in humans. Also, our results suggest that certain selenoproteins may be induced in response to Ag challenges within the lung. *The Journal of Immunology*, 2007, 179: 3258–3267.

Asthma is an inflammatory pulmonary disorder that involves increased levels of oxidative stress. Allergic asthma, in particular, is driven by inappropriate Th2 immune responses against otherwise innocuous environmental allergens. This results in the infiltration of inflammatory leukocytes into the lung tissue, airway hyperreactivity (AHR),³ epithelial damage, and tissue remodeling. Infiltrating leukocytes increase the inflammatory process and some of these leukocytes release toxic reactive oxygen species (ROS) into the surrounding tissue, resulting in increased oxidative stress (1–3). ROS such as superoxide radical, hydrogen peroxide, hypochlorous acid, and hydroxyl radical have been associated with many pathogenic features of asthma. Moreover, these ROS can react with nitrite and NO to form reactive nitrogen species, which can cause further damage to lung tissue. Pathological features associated with ROS and reactive nitrogen species include increased lipid peroxidation, increased

airway reactivity and secretions, increased production of chemoattractants, and increased vascular permeability (4, 5). This has led investigators to speculate about the potential roles that various antioxidants may play in preventing or treating asthma.

Selenium (Se) is a particularly potent nutritional antioxidant that is essential for optimal immune function (6). Higher levels of Se enhance the function of cells of both the innate and the adaptive immune system and lead to stronger immune responses against particular pathogens or Ags (7–9). Se is unique among antioxidants in that it exerts its biological effects through its direct incorporation into proteins (selenoproteins) as the amino acid selenocysteine. To date, 25 human selenoproteins have been identified (10). Because many of the selenoprotein family members have only recently been identified, their specific functions have yet to be determined. Some selenoproteins that have been characterized as important antioxidant enzymes include glutathione peroxidase (GPX)-1 and GPX-4, thioredoxin reductase-1 and thioredoxin reductase-2, and selenoprotein P (SelP) (11–13). Roles may be elucidated for other selenoproteins in reducing the oxidative stress or pathology of diseases like asthma as work continues to functionally characterize this important family of proteins.

Extreme Se deficiency is rare, particularly in developed countries where asthma has in recent years been on the rise (14). However, it remains unclear whether moderately low Se intake may affect asthma pathology or whether Se supplementation would be beneficial for preventing or treating asthma or other immunologically based disorders. Some descriptive studies in humans have suggested that lower levels of serum Se are associated with the increased prevalence and severity of asthma (15–17). Other studies have failed to verify such correlations (18, 19). To gain insight into the relationship between dietary Se and the pathogenesis of asthma, we conducted a series of in vivo experiments. In this article we report that dietary Se does not affect allergic airway inflammation in a simple dose-response manner. Rather, mice fed

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³ Abbreviations used in this paper: AHR, airway hyperactivity; BAL, bronchoalveolar lavage; GPX, glutathione peroxidase; log PC₂₀₀, logarithm of the dose of acetylcholine required for a 200% increase in pulmonary resistance above baseline; pSTAT-6, phosphorylated STAT-6; ROS, reactive oxygen species; ppm, parts per million; SelP, selenoprotein P.

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medium Se diets were found to have the highest levels of asthma-related pathology after Ag challenge and those fed low Se chow demonstrated significantly lower responses to OVA challenge. Within the medium Se dietary group, mice challenged with OVA in the lungs were shown to have increased levels of lung GPX-1, but not lung GPX-4, and increased liver SelP. Thus, certain antioxidant selenoproteins may be induced during allergic airway inflammation.

Materials and Methods

Mice and special diets

Female 3-wk-old C57BL/6J mice were purchased from The Jackson Laboratory and maintained on chow purchased from Research Diets containing either 0.08, 0.25, or 2.7 parts per million (ppm) Se. Each lot of chow was independently tested to confirm Se content by inductively coupled plasma mass spectrometry (West Coast Analytical Services). Mice were maintained on the special diets for 8 wk before the induction of allergic airway inflammation as well as throughout the remainder of the protocol. To ensure that the high Se diet was not inducing toxicity, mice were monitored for indicators of general health including food consumption, body weight, coat appearance, and general grooming. In addition, liver tissue sections from these mice were analyzed by an independent pathologist for histological features of liver toxicity in a blinded manner and found to contain no such features. All animal experimental protocols were approved by the University of Hawai'i Institutional Animal Care and Use Committee.

Mouse model of allergic airway inflammation

The mice were sensitized by i.p. injections on days 0 and 12 with 50 μ g of OVA (Sigma-Aldrich) precipitated with 0.5 mg of aluminum hydroxide in 200 μ l of PBS. The mice were then challenged by administering 20 μ g of OVA in 50 μ l of PBS directly into the nostrils using a micropipetter on days 24, 26, and 28 using isoflurane as an anesthetic. Negative control mice were sensitized and challenged with PBS. On day 29, the mice were sacrificed and various tissues were collected for analyses. Pilot experiments were conducted that included control mice sensitized with either alum alone or OVA/alum and challenged with PBS. These mice were found to have levels of lung inflammation similar to those found in mice that received PBS for both sensitization and challenges (P. Hoffmann, unpublished data). Therefore, control mice from the latter group were used for all subsequent experiments.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was collected by injecting and withdrawing 0.8 ml cold PBS. Recovered BAL (70–80%) was centrifuged at $300 \times g$ for 8 min. The fluid was collected for cytokine analyses, the cells were resuspended in 0.5 ml of PBS, and an aliquot was removed for cell concentration using a Coulter particle counter. The remaining cells were adhered to glass slides using cytocentrifugation. Slides were stained using Wright-Giemsa stain and differential cell counts (minimum of 300 total cells) were obtained using a Zeiss upright light microscope.

Lung histology and immunofluorescence

Lung tissue was prepared for histological analyses by fixation in 10% buffered formalin overnight followed by a PBS wash and stepwise dehydration in 60–100% ethanol. The tissue was then incubated in xylene, embedded in paraffin, sectioned, and stained with a standard H&E staining method. In addition, nine sections per mouse were also stained using standard periodic acid-Schiff techniques to visualize mucus and goblet cells in airways. Small airways of comparable size were used to enumerate the mucin-positive cells and data were pooled from all of the slides for calculating the percentage of cells lining the airway that were positive for mucin. For immunofluorescence, lung tissue was frozen in Tissue-Tek OCT compound (Ted Pella) and stored at -80°C until sections were cut using a cryostat and mounted onto glass slides. Immunofluorescence was then conducted by first adding Fc block (BD Pharmingen) at a final concentration of 1/1,000 for 30 min. The sections were then washed and fixed with 4% paraformaldehyde containing 30% sucrose for 15 min at room temperature. After washing away the fixative with PBS, permeabilization solution (0.03% Triton X-100) was added for 10 min and then washed with PBS. The sections were then blocked with 5% normal goat serum followed by incubation with rabbit anti-phosphorylated STAT-6 (pSTAT-6; Upstate/Chemicon) at a final concentration of 1/200 in PBS for 45 min at room temperature. Primary Ab was washed away with PBS and an Alexa Fluor 594-goat anti-rabbit secondary Ab (Invitrogen Life Technologies) was added at a final concen-

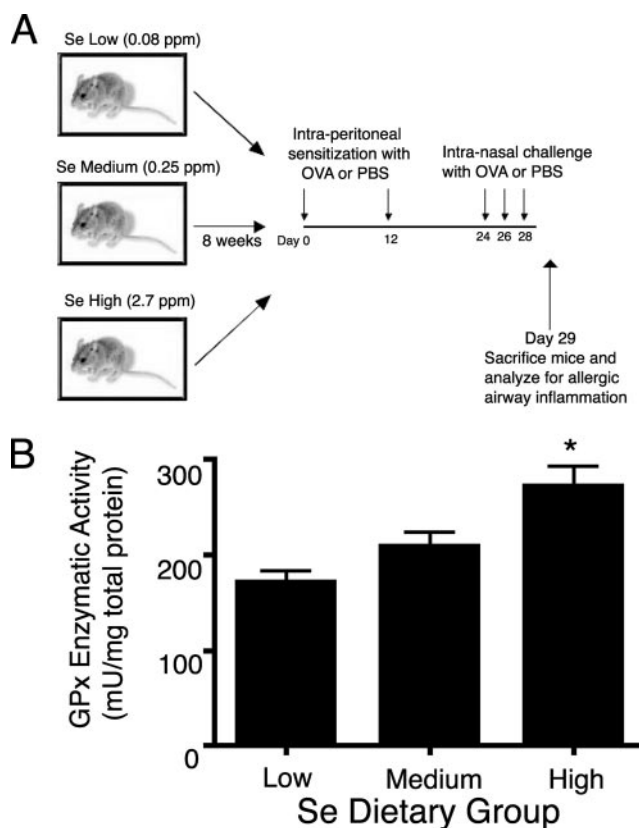


FIGURE 1. Dietary Se intake affects levels of GPX activity in mice. *A*, The experimental protocol used for this study involved feeding weanling mice diets that were either low (0.08 ppm), medium (0.25 ppm), or high (2.7 ppm) in Se. The mice were maintained on the special diets for 8 wk before the induction of allergic airway inflammation as well as throughout the remainder of the protocol. An established protocol involving sensitization and challenges with OVA was used to induce experimental allergic airway inflammation in the mice. Control mice were sensitized and challenged with PBS. *B*, Weanling mice were fed low, medium, or high Se diets for 8 wk and then the levels of GPX enzymatic activity were measured in lysates from erythrocytes and normalized to total protein. Results represent mean $\pm$ SE and statistical significance was determined by a non-parametric Mann-Whitney *U* test. Significant differences were found when comparing means of medium Se to high Se (*, $p = 0.0221$), but not low Se and medium Se. A total of seven mice per group were included.

tration of 1/500 in PBS for 45 min at room temperature. The secondary Ab was washed away with PBS and tissue mounted in a 4',6'-diamidino-2-phenylindole mounting medium (Vector Laboratories). Fluorescence was visualized using an Olympus IX71 microscope with an Olympus U-CMAD3 camera and images were captured by using SlideBook 4.0 software (Intelligent Imaging Innovations). For quantification of fluorescence, a minimum of three sections per mouse and three images per section were captured and analyzed with SlideBook 4.0 for the determination of sum intensity, which gave the sum of all marked voxel intensity values for the red channel for each image. Levels of red fluorescence associated with cells (appearing as strong nuclear staining) were included in the sum intensity, whereas the background red fluorescence in structural features of the tissue were excluded from the sum intensities.

Cytokine profile and IgE measurement

Concentrations of TNF- α , IFN- γ , IL-2, IL-4, and IL-5 were measured in BAL fluid using the mouse Th1/Th2 cytometric bead array kit (BD Biosciences) following the manufacturer's instructions and data were collected using a BD FACSCaliber flow cytometer. Serum anti-OVA IgE was measured using a BD OptEIA set mouse IgE kit (BD Biosciences). In brief, mouse sera were first incubated with Reacti-Bind protein G-coated plates (Pierce) overnight at 4°C to remove IgG. The supernatants from these reactions were then diluted 1/3 with PBS and added in duplicate to wells of a 96-well BD OptEIA plate that had been coated with 20 mg/ml OVA

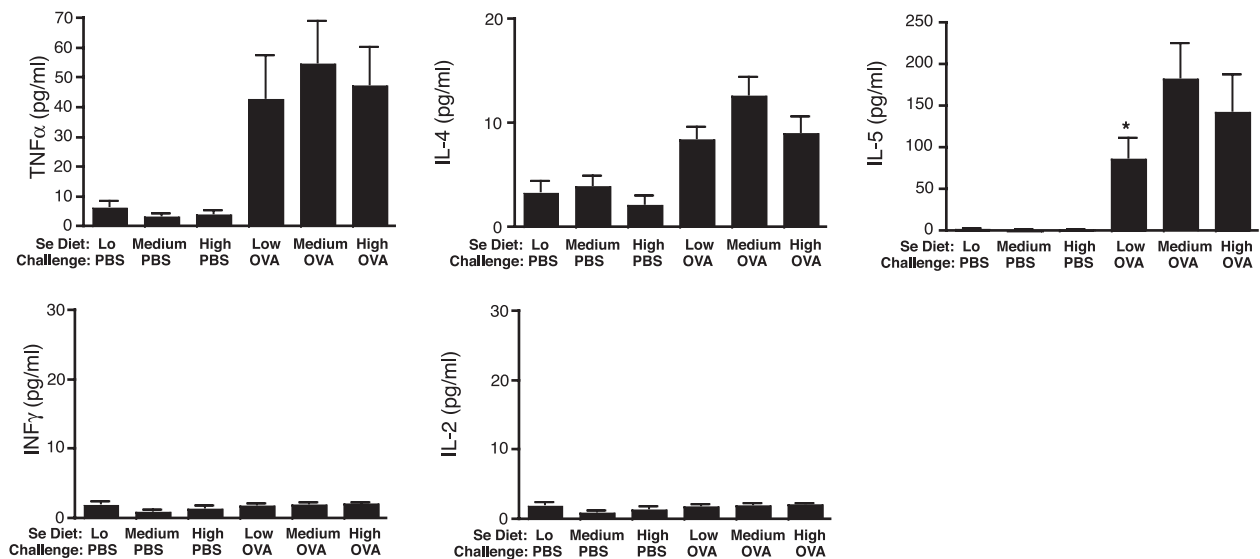


FIGURE 2. The levels of TNF- α , IL-4, and IL-5 are highest in the lungs of OVA-challenged mice fed medium levels of Se. Mice from low, medium, and high dietary Se groups were challenged with PBS (negative controls) or OVA. BAL fluid was analyzed for levels of five different cytokines. The number of mice included in analyses was 18 per OVA-challenged group and six per the PBS control group. Results represent mean $\pm$ SE. Means of groups were compared using a nonparametric Mann-Whitney *U* test and significant differences were found when comparing IL-5 levels in low and medium Se groups (*, $p = 0.0282$).

per the manufacturer's instructions. For standard curves, mouse anti-OVA IgE was used in serial dilutions (Serotec). Colorimetric assay readout was obtained using a SpectraMax 340 (Molecular Devices).

RNA extraction and real-time quantitative PCR

Tissues were placed in RNeasy lysis solution (Ambion) and RNA was extracted using an RNeasy mini kit and RNase-free DNase I (all from Qiagen). The concentration and purity of extracted RNA were determined using A_{260}/A_{280} measured on an ND-1000 spectrophotometer (NanoDrop Technologies). Synthesis of cDNA was conducted using SuperScript III (Invitrogen Life Technologies) with an oligo(dT) primer with 4 μ g of RNA per 20- μ l reactions. For real-time PCR, 2 μ l of the cDNA was used in 20- μ l reactions using Platinum SYBR Green quantitative PCR SuperMix-UDG (Invitrogen Life Technologies) conducted in a LightCycler 2.0 thermal cycler (Roche Applied Biosystems). The oligonucleotides used for quantitative PCR included β -actin (forward: 5'-TGACAGGATGCA GAAGGAGA-3'; reverse: 5'-CGCTCAGGAGGAGCAATG-3'), hypoxanthine phosphoribosyltransferase (forward: 5'-TCCTCTCAGACCGCT TTT-3'; reverse: 5'-CCTGGTTCATCGCTAATC-3'), SeIP (forward: 5'-CCTGGTTTGCCCTTACTCCTTC-3'; reverse: 5'-TTTGTGTGGTGTG TGTGGTGG-3'), GPX-1 (forward: 5'-ACAGTCCACCGTGTATGCCT TC-3'; reverse: 5'-CTGTTTCATCTTGGCATTCTCC-3'), and GPX-4 (forward: 5'-TCTGTGTAATGGGGACGATG-3'; reverse: 5'-TCTCTA TCACCTGGGGCTCCTC-3'). Cycling conditions were used as suggested in the SYBR Green kit instructions and results were analyzed using Relative Quantification software (Roche).

Protein extraction, Western blots, and GPX activity assays

Protein was extracted from tissue by homogenizing 0.5 g of tissue on ice in 10 ml of CellLytic MT buffer (Sigma-Aldrich) containing 1 mM DTT, 1 $\times$ protease inhibitor mixture (Calbiochem), and 5 mM EDTA. The homogenate was centrifuged at 12,000 rpm and the supernatant was removed and stored at -70°C . A Bradford assay was conducted using a Bradford reagent (Bio-Rad) and 30 μ g of total protein was combined with reduced Laemmli buffer, boiled at 95°C for 10 min, cooled on ice, and loaded into wells of 10–14.5% polyacrylamide gel (Bio-Rad). Protein was transferred to a polyvinylidene difluoride membrane that was blocked for 1 h with 5% BSA and then probed for 1 h with primary Abs including rabbit polyclonal anti-GPX-1 and anti-GPX-4 (Lab Frontier) and mouse monoclonal IgG anti- β -actin (Sigma-Aldrich). The appropriate HRP-conjugated secondary Abs were purchased from Jackson ImmunoResearch Laboratories, incubated with the membranes for 45 min, and detected using ECL Plus (GE Healthcare). For densitometry, digital images of autoradiographic film were captured using Gel Logic 200 and Kodak MI software (Kodak Scientific Imaging Systems). This software was used to measure the mean

intensity from regions of interest that corresponded to bands to be measured. The intensity of the target bands (e.g., GPX-1 band) was normalized to that of the loading control band (e.g., β -actin band) to obtain normalized levels of target proteins.

For GPX assays the protein extracted by using CellLytic MT buffer was found to be comparable to the protein extracted by using nondetergent buffers, so CellLytic MT buffer extracts of lung were used for all experiments. For other experiments involving erythrocyte GPX activity measurements, erythrocytes were separated from whole blood (50 μ l of whole blood plus 150 μ l of 0.04 M EDTA) by centrifugation at 3,000 rpm for 10 min. Erythrocytes were then lysed in 200 μ l of dH₂O and these lysates or lung extract lysates were analyzed for GPX activity using a Bioxytech GPX-340 kit (OxisResearch) according to the manufacturer's instructions.

Flow cytometry

Lungs from which BAL had been collected were removed, washed extensively with PBS, minced, and incubated for 1 h at 37°C in digestion buffer consisting of RPMI 1640 with 10% FBS (Invitrogen Life Technologies), 0.5 mg/ml Liberase CI, and 30 μ g/ml DNase I (both from Roche). After digestion, the lung tissue was forced through a 40- μ m cell strainer, cells were pelleted by centrifugation, and erythrocytes were lysed with RBC lysing buffer (Sigma-Aldrich). The cells were then washed one more time with RPMI 1640 and plated in 200 μ l of complete RPMI 1640 medium in wells of a 96-well plate at a density of 2×10^6 cells per well. The cells were then incubated with Fc block (BD Pharmingen) for 20 min at room temperature followed by three Abs, allophycocyanin-anti-CD4, FITC-anti-CD122, and PE-anti-CD25 (BD Pharmingen), all used at a final concentration of 1/200. After 30 min of incubation at room temperature, cells were washed and analyzed on a FACSCaliber flow cytometer (BD Biosciences). Data were analyzed using CellQuest Pro (BD Biosciences).

Measurement of airway reactivity

Airway reactivity to acetylcholine was measured using a *flexiVent* instrument (SCIREQ) as previously described (20). Briefly, mice were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Trachea were exposed surgically, cannulated, and attached to the *flexiVent* for ventilation followed by an i.p. injection of pancuronium bromide for paralysis and pulmonary mechanics were then measured. Increasing doses of acetylcholine (0, 0.03, 0.1, 0.3, 1.0, and 3.2 μ g/g) were injected (2 μ l/g body weight) via the tail vein at 3-min intervals. Data were used to generate a dose-response curve. The results were reported as the logarithm of the dose of acetylcholine required for a 200% increase in pulmonary resistance above baseline (log PC₂₀₀; microgram per gram). Lower log PC₂₀₀ values represent greater airway reactivity.

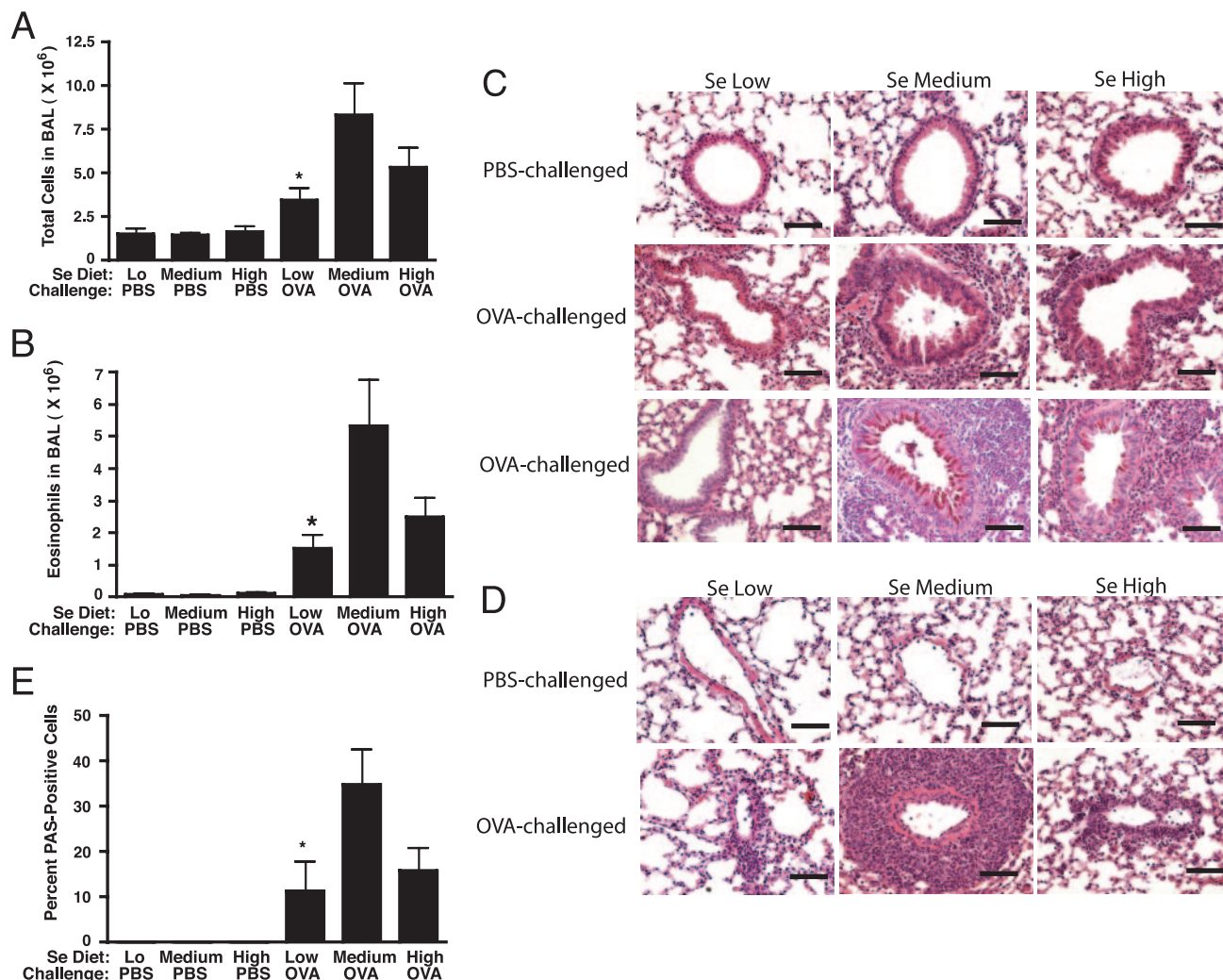


FIGURE 3. Cellular infiltrate and eosinophilia are increased in the lungs of OVA-challenged mice fed medium levels of Se. Mice from low, medium, and high dietary Se groups were challenged with PBS (negative controls) or OVA. *A*, BAL was analyzed for total cell concentration using a Coulter particle counter. *B*, BAL was also analyzed for a number of cells with the morphological features of eosinophils as determined by cytopsin and standard H&E staining. A minimum of 300 total cells per mouse was counted under $\times 20$ power light microscopy. For both *A* and *B*, the number of mice included in the analyses was six for the PBS control group and 14 for the OVA-challenged group. Results represent mean $\pm$ SE. Means were compared using a nonparametric Mann-Whitney *U* test and significant differences were found when comparing low Se to medium Se groups for both total cells (*, $p = 0.0196$) and eosinophils (*, $p = 0.0308$). *C* and *D*, Lung tissue sections were analyzed by standard H&E staining from a minimum of five mice per group for cellular infiltrate surrounding the lower airways (*C*) and vasculature (*D*). The sections shown in the lower panel in *C* were stained with a standard periodic acid-Schiff method and the dark red-purple staining represents the presence of mucus and helps highlight goblet cell hyperplasia. Scale bars represent 250 μ m. *E*, Cells lining small airways were examined for periodic acid-Schiff staining by light microscopy. Results represent mean $\pm$ SE from experiments involving four mice for the PBS group and 6–8 mice for the OVA group. Mann-Whitney *U* test was used to compare means and a significant difference was found between low Se and medium Se groups (*, $p = 0.0462$).

Statistical analyses

All statistical tests were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software). Means were compared using a nonparametric Mann-Whitney *U* test. The means of OVA-challenged mice were compared with their respective PBS controls to determine how well the asthma model worked for each group. Means of each OVA-challenged group were also compared with each other to determine statistical differences due to diets. Statistical significance was considered at $p < 0.05$.

Results

Selenium intake affects *in vivo* glutathione peroxidase levels

We began our investigation into how dietary Se affects the development of allergic airway inflammation by dividing weanling mice into three groups and feeding them chow containing either low (0.08 ppm), medium (0.25 ppm), or high levels (2.7 ppm) of Se (Fig. 1A). The mice were maintained on the special diets for 8 wk

before the induction of allergic airway inflammation as well as throughout the remainder of the protocol. An established sensitization/challenge protocol involving OVA as an Ag was used to induce allergic airway inflammation in these mice (21). Dietary Se concentrations for each group were chosen to reflect the levels of moderately low Se, normal Se, and supplemented levels of Se in humans (22, 23). Before commencing with the allergic airway inflammation protocol, we thought it necessary to confirm that the chows were effective in establishing different levels of selenoproteins, i.e., Se status, in the mice. A reliable method of determining *in vivo* Se status in humans or research animals is the measurement of enzymatic activity of the selenoprotein GPX family members (commonly referred to as GPX activity) in erythrocytes (22, 24). To this end, mice were maintained on the special diets for 8 wk and erythrocyte lysates were analyzed for levels of GPX activity. GPX

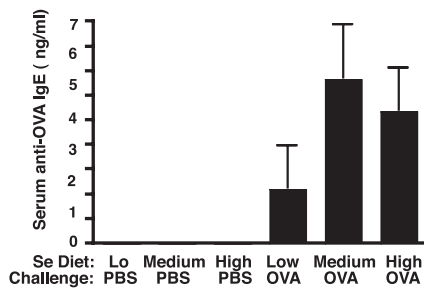


FIGURE 4. Levels of anti-OVA IgE are increased in the lungs of OVA-challenged mice fed medium levels of Se. Mice from low, medium, and high dietary Se groups were challenged with OVA or PBS (negative controls). Serum was analyzed for levels of anti-OVA IgE by enzyme immunoassay. The number of mice included in analyses was eight for the OVA-challenged group and six for the PBS group. Results represent mean $\pm$ SE. Means were compared using a nonparametric Mann-Whitney *U* test and no significant differences were found when comparing OVA-challenged mice from the different dietary groups.

activity was found to increase with increasing levels of dietary Se (Fig. 1B). In particular, significant differences in GPX activity were found between the high Se dietary group compared with the low Se and medium Se groups. Only a trend toward significance was found between the low Se and medium Se groups, reflecting the moderate differences in dietary Se content between these two groups. Overall, these results supported the notion that the different levels of Se used in the diets were having an effect on the levels of selenoprotein expression in these mice.

Inflammatory and Th2 cytokines evaluation

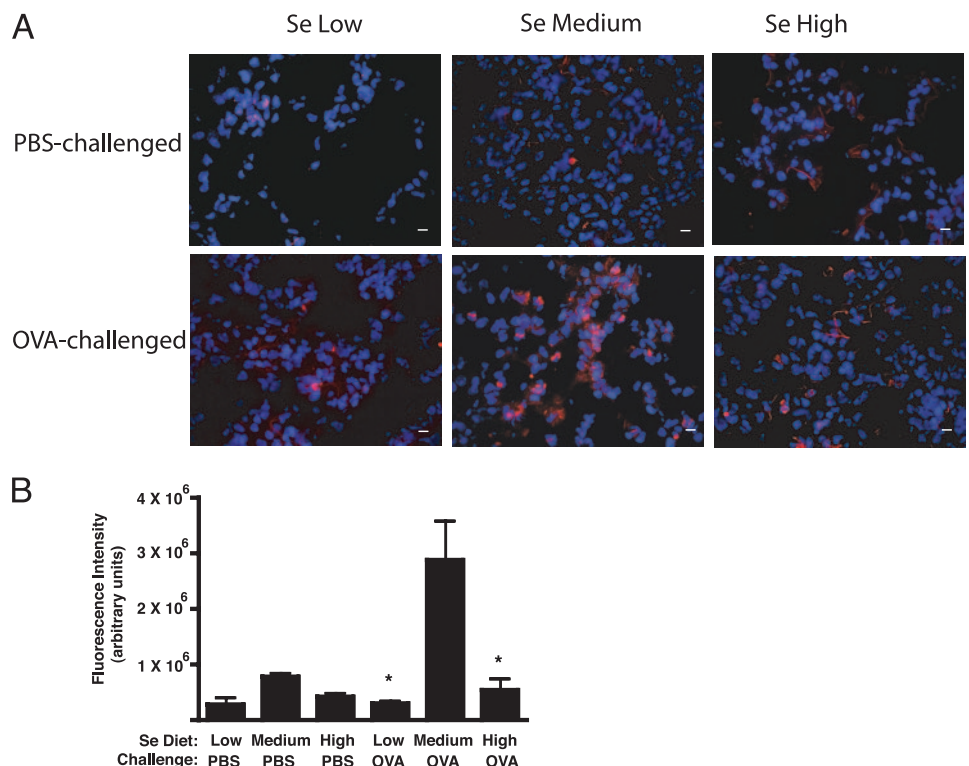
To analyze the development of allergic airway inflammation in mice from the different dietary groups, the levels of five cytokines were measured in the BAL fluid from OVA-challenged mice and PBS controls. The cytokines included inflammatory (TNF- α), Th2 (IL-4 and IL-5), and Th1 (IFN- γ and IL-2) types. Mice fed me-

dium levels of Se responded to OVA challenge by secreting the highest levels of TNF- α , IL-4, and IL-5 in the airways (Fig. 2). Low levels of Se intake resulted in the lowest levels of cytokines, with IL-5 levels significantly lower than those in OVA-challenged mice fed medium Se diets. Although mice fed high Se diets did not have responses as robust as those in mice fed medium Se diets, the differences were not statistically significant. Th1-type cytokines were not detected at significant levels in BAL fluid from any of the mice, which is consistent with results obtained by others using this mouse model of allergic airway inflammation (21, 25).

Cellular infiltrate, eosinophilia, and goblet cell hyperplasia evaluation

Another pathological feature of allergic airway inflammation is the infiltration of leukocytes into the lower airway tissues. These cells include lymphocytes, monocytes, neutrophils, and the particularly damaging eosinophils. To determine levels of cellular infiltrate in the lungs of mice challenged with either PBS or OVA, a particle counter was used to enumerate the cells present in the BAL of these mice. Mice from the medium Se dietary group exhibited highest BAL cell counts (Fig. 3A). Mice fed low Se diets were significantly lower than those fed medium Se diets, but mice in high Se group were not significantly different from either the medium or low Se group. To further assess the cellular infiltrate in these mice, histology was performed on lung tissue sections and a qualitative assessment of small airways and vasculature in H&E-stained tissue sections was conducted. Representative images from each dietary group are shown in Fig. 3, C and D, which illustrate that the OVA challenge produced the highest levels of cellular infiltrate in tissue surrounding both small airways and vasculature for mice fed medium Se diets. In addition, goblet cell hyperplasia was elicited to the highest degree in the airways from OVA-challenged mice of the medium Se dietary group (Fig. 3, C and E). Once again, mice fed low Se diets had significantly lower levels of goblet cell hyperplasia than those fed medium Se diets, but mice in the high Se diet were not significantly different from either group.

FIGURE 5. Levels of pSTAT-6 are increased in lungs of OVA-challenged mice fed medium levels of Se. Mice from low, medium, and high dietary Se groups were challenged with OVA or PBS (negative controls). Lung tissue sections were analyzed for levels of pSTAT-6 by immunofluorescent microscopy. A, Representative images from each experimental and control group are shown demonstrating pSTAT-6 positive nuclear staining within cells (red) and all nucleated cells stained with 4',6'-diamidino-2-phenylindole (blue). Scale bars represent 10 μ m. B, Levels of pSTAT-6 nuclear staining were quantified using SlideBook software from a minimum of four different images per mouse. Results represent mean $\pm$ SE from experiments involving a total of four mice per group. Means were compared using a nonparametric Mann-Whitney *U* test and significant differences were found between low Se and medium Se (*, $p = 0.0159$) and medium Se and high Se (*, $p = 0.0357$).



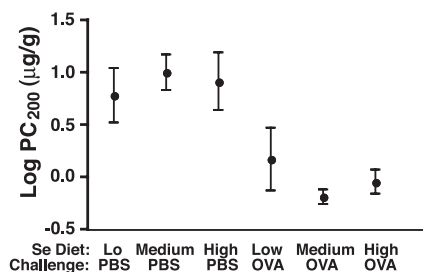


FIGURE 6. AHR is increased highest in OVA-challenged mice fed medium levels of Se. An invasive measurement of AHR was made in response to increasing doses of i.v. challenges with acetylcholine. Lower values of log PC₂₀₀ indicate increasing AHR. Results represent mean $\pm$ SE from experiments involving 5–9 mice per group. Means were compared using a nonparametric Mann-Whitney *U* test and no significant differences were found when comparing OVA-challenged mice from the different dietary groups.

Levels of eosinophilia were also determined for the mice in our study by analyzing cytospin preparations of BAL cells for the percentage of total cells that were eosinophils, which was then used to determine the total eosinophils in the BAL. Results again showed that mice fed medium Se diets had the strongest response to OVA challenge (Fig. 3B). Mice fed low Se diets had significantly lower

eosinophilia compared with mice fed medium Se diets, but mice in the high Se diet were not significantly different from either group.

Serum anti-OVA IgE evaluation

A crucial component of allergic immune responses is the production of allergen-specific IgE. Therefore, levels of anti-OVA IgE were measured in sera from the OVA-challenged mice and PBS controls. The results demonstrated that medium Se mice had the strongest responses to the OVA challenge, but the lower responses in the low Se group were not significant. Again, the high Se group showed responses lower than those of the medium Se group, but not at statistically significant levels (Fig. 4).

Expression of phosphorylated STAT6 in lung tissue

Th2 immune responses that drive allergic airway inflammation depend on signaling pathways that drive the differentiation of Th cells toward the Th2 phenotype, and a key step in this process is the phosphorylation of STAT-6 (26, 27). Therefore, we analyzed lung tissue sections from OVA-challenged mice and PBS controls from the different Se dietary groups using immunofluorescence microscopy to determine the levels of pSTAT-6. Our results indicated that medium Se mice challenged with OVA had significantly higher levels of pSTAT-6 in lung tissues compared with OVA-challenged mice fed either low or high Se diets (Fig. 5). These data

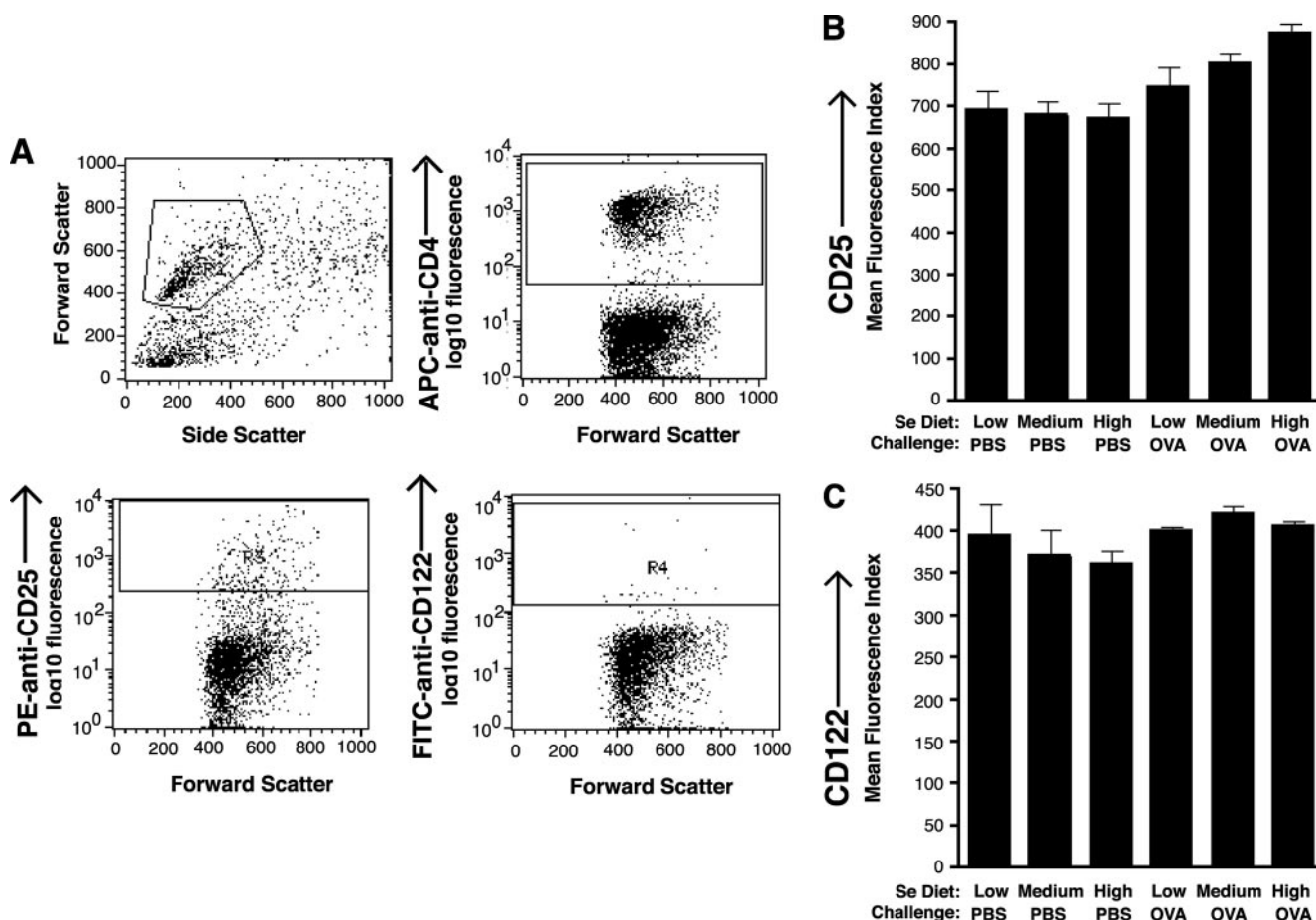


FIGURE 7. Levels of CD25 on CD4⁺CD25⁺ T cells increase with increasing levels of Se intake in OVA-challenged mice. Lung tissue was digested and cells analyzed by flow cytometry. A, Cells with forward and side scatter properties consistent with lymphocytes were analyzed for CD4 expression. Those cells positive for CD4 expression were then analyzed for fluorescence intensity of staining for CD25 or CD122. Dot plots in this figure correspond to an OVA-challenged mouse fed medium Se chow, but the same instrument settings and gates were used for all samples. APC, Allophycocyanin. B and C, The mean fluorescence intensity was graphed for CD25 (B) and CD122 (C) for each group of mice and the data represent mean $\pm$ SE from experiments involving six mice per group. This graph represents results from two independent experiments.

Table I. Levels of GPX-1 and GPX-4 mRNA, protein, and activity in lung tissue

Experiment No.	Mouse No.	Mouse sensitized and challenged with:	TNF- α detected in BAL fluid (pg/ml)	IL-5 detected in BAL fluid (pg/ml)	Ratio of lung GPX-1 mRNA levels compared to PBS control ^a	Ratio of lung GPX-4 mRNA levels compared to PBS control ^a	Ratio of lung GPX-1 protein levels compared to PBS control ^b	Ratio of lung GPX-4 protein levels compared to PBS control ^b	GPX activity in lung tissue (mU/mg total protein)
1	1	PBS	3.90	0.80	1.00	1.00	1.00	1.00	142.00
	2	OVA	253.00	321.80	2.73	1.26	3.01	1.00	121.00
	3	OVA	29.75	120.85	3.40	0.91	2.39	1.34	82.00
2	1	PBS	2.03	0.00	1.00	1.00	1.00	1.00	119.00
	2	OVA	241.63	505.53	4.19	0.83	3.02	1.51	84.00
	3	OVA	72.80	32.00	2.56	1.04	4.11	0.77	126.00
	4	OVA	148.87	434.93	5.38	1.16	3.54	0.86	101.00
3	1	PBS	3.55	0.00	1.00	1.00	1.00	1.00	103.00
	2	OVA	43.50	144.25	1.90	1.15	1.54	0.84	129.00
	3	OVA	8.40	57.00	1.29	1.22	1.05	0.75	118.00
	4	OVA	22.05	209.35	0.99	1.58	1.46	0.43	96.00

^a Ratios reflect those of target (GPX-1 or GPX-4) mRNA signal normalized to housekeeping gene (hypoxanthine phosphoribosyltransferase) signal for each OVA mouse compared to PBS mouse for that experiment.

^b Ratios reflect those of target (GPX-1 or GPX-4) normalized to loading control (β -actin) for each OVA mouse compared to PBS mouse for that experiment.

support the notion that Se medium diets led to the strongest Th2-type immune responses resulting in the highest levels of susceptibility to allergic airway inflammation.

AHR evaluation

Asthma is physiologically characterized by AHR, which is defined as exaggerated airflow obstruction in response to bronchoconstrictors. To determine levels of AHR in the mice included in this study, we used an established invasive technique that measures the resistance of airflow in the lungs in response to i.v. challenge with acetylcholine (20). As shown in Fig. 6, the patterns of AHR in the dietary groups are similar to those found for the readouts of allergic inflammation in that the mice fed medium Se diets showed the highest levels of reactivity compared with their PBS controls. However, the differences between the dietary groups were not significant.

Expression of CD25 on CD4⁺ T cells from OVA-challenged lung tissue increases with increasing levels of selenium intake

To begin to investigate the possible mechanisms by which dietary Se may affect the responses to OVA challenge within this mouse model, we focused on IL-2R expression. The reasoning for our focus on the IL-2R lies in the fact that increased levels of Se in humans have been demonstrated to enhance the expression of both the α (CD25) and β plus γ (CD122/CD132) subunits of the IL-2R, which resulted in a greater number of high affinity IL-2R per cell and enhanced proliferation and differentiation of T cells (28, 29). In addition, IL-2/IL-2R interactions play key roles in the proliferation and differentiation of CD4⁺ T cells during immune responses

such as those that occur during allergic asthma (30). Therefore, we measured the level of both CD25 and CD122 in CD4⁺ T cells in the lung tissue of mice used in this model. Our initial analyses indicated that the percentage of CD4⁺ T cells that were either CD25 or CD122 were not influenced by Se intake (P. Hoffmann, unpublished data). However, when we analyzed the CD4⁺CD25⁺ or CD4⁺CD122⁺ cell populations for levels of CD25 or CD122, respectively, we obtained interesting results. As shown in Fig. 7, the levels of CD25 on CD4⁺CD25⁺ T cells showed a trend toward increasing along with the increasing levels of dietary Se in mice challenged with OVA. There seemed to be no influence of Se intake levels on CD25 levels on these cells in lungs from PBS-challenged mice, suggesting that OVA challenge was necessary for the increased CD25 expression to occur. The levels of CD122 on CD4⁺CD122⁺ T cells were not different between mice of different dietary Se groups, regardless of whether they were challenged with PBS or OVA.

Thus, our results suggested that the pattern of CD25 expression was not the same as the immune responses in the OVA-challenged dietary group. That is, CD25 increased with increasing levels of Se intake, whereas immune responses were highest in the medium Se group. However, the effects of dietary Se on CD25 expression may still be related to the mechanism of how dietary Se affects allergic airway inflammation, and this is further examined in *Discussion*.

Expression of GPX-1 is increased in lung tissue in response to OVA challenge

We next conducted a series of experiments within the medium Se group to determine how allergic airway inflammation affected the

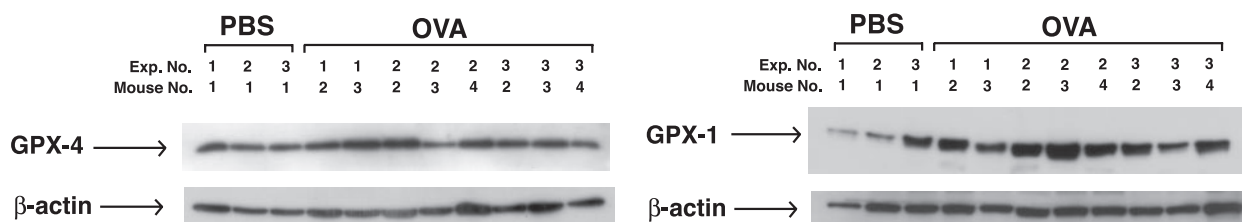


FIGURE 8. Levels of GPX-1, but not GPX-4, are increased in lung tissue from mice challenged with OVA compared with PBS controls. Three independent experiments (Exp.) were conducted involving one PBS-challenged mouse (negative control) and two or three OVA-challenged mice. Protein extracted from lung tissue was then analyzed by Western blotting. Equivalent loading was determined by using anti- β -actin and densitometry performed as described in *Materials and Methods*.

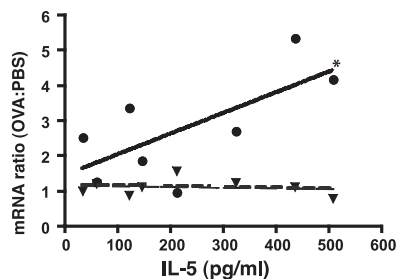


FIGURE 9. Levels of GPX-1 mRNA levels increase relative to controls in a manner that correlates to IL-5 levels in the lung. Values from Table I were used to plot the ratio of mRNA levels (OVA:PBS) vs levels of IL-5 measured in the lung. For each mouse, the level of IL-5 was plotted vs its corresponding mRNA ratio for either GPX-1 (circles) or for GPX-4 (triangles). Linear regression analyses were used to place a best-fit curve for GPX-1 (solid line through circles) and GPX-4 (dashed line through triangles). Pearson's correlation was used to test statistically significant correlation between IL-5 levels and GPX-1 mRNA levels (*, $p = 0.0336$) or GPX-4 levels.

expression levels of certain selenoproteins. The reasoning for this was that allergic airway inflammation involves oxidative stress and several selenoproteins have been demonstrated to possess important antioxidant and redox functions (31–33). Members of the GPX family, particularly cellular GPX (GPX-1) and phospholipid GPX (GPX-4), are important for detoxifying peroxides within cells. Thus, we asked the question of whether the levels of these two selenoprotein antioxidant enzymes were affected by OVA challenge. In three independent experiments, we compared OVA-challenged mice to PBS controls for the expression levels of GPX-1 and GPX-4 in lung tissue of the medium Se mice. Levels of these two selenoproteins were measured in terms of mRNA, protein, and enzymatic activity levels. Ratios for mRNA were determined (OVA:PBS mice) for three independent experiments and results are summarized in Table I. Results demonstrated that GPX-1 mRNA, more than GPX-4 mRNA, was consistently increased in OVA-challenged mice compared with PBS controls. Levels of GPX-1 mRNA were an average of 2.81 times higher in OVA-challenged mice (range = 0.99 to 5.38) compared with PBS controls. In contrast, GPX-4 levels were equivalent when comparing OVA-challenged mice to PBS controls (mean ratio, 1.15; range, 0.83–1.58). Protein levels for these two selenoproteins were determined by Western blotting (Table I and Fig. 8). The GPX-1 protein was consistently increased in response to OVA challenge

with an average of 2.24 times the protein in lung tissue from OVA-challenged mice compared with PBS controls (range, 1.05–4.11). In contrast, the GPX-4 protein in lungs from OVA-challenged mice was equivalent to that of PBS controls (mean ratio, 0.94; range, 0.43–1.51). The GPX enzymatic activity was not increased in OVA-challenged mice compared with PBS controls. However, the results of this assay are expressed in enzymatic units per the mass of total protein and the OVA-challenged mice were shown to have significantly higher levels of cellular infiltrate (Fig. 3). Thus, it is possible that both the GPX activity and total protein increased proportionately in lung tissue during OVA challenge.

The format of Table I also allows an analysis of each mouse to be made in terms of how the different biomarkers for inflammation (TNF- α) and Th2 allergic immune responses (IL-5) in the lung compared with the different readouts for GPX-1 and GPX-4 levels. Overall, higher GPX-1 mRNA levels most consistently corresponded to higher levels of TNF- α and IL-5. For example, mice no. 2 and 4 from experiment no. 2 demonstrated the highest levels of these two cytokines and also the highest levels of GPX-1 mRNA. Levels of the GPX-1 protein were also high in these mice. In contrast, these mice did not show high levels of GPX-4 expression at either the level of mRNA or protein. The correlation between GPX-1 mRNA levels in the OVA-challenged mice and IL-5 is shown in Fig. 9.

SeIP mRNA levels in liver tissue are increased in response to OVA challenge

SeIP is a particularly interesting selenoprotein in that it is a heavily glycosylated, secreted protein found in abundant amounts in plasma, the source of which is predominantly the liver. SeIP is believed to function in large part as a transporter protein that delivers Se to tissues for the new synthesis of selenoproteins (34, 35). It has been reported that serum SeIP levels were lower in patients with the inflammatory bowel disease Crohn's disease (36). Therefore, we thought it important to investigate the level of SeIP produced in the livers of mice in which experimental allergic airway inflammation was induced. As shown in Table II, the levels of SeIP mRNA were on average 2.12 times higher in liver tissue from OVA-challenged mice compared with PBS controls (range, 1.34–2.82). SeIP mRNA levels were consistently increased in the OVA-challenged mice compared with controls, suggesting that oxidative stress in the lung led to increased transcription of the SeIP gene. The availability of a quality commercial Ab against mouse SeIP

Table II. *Levels of SeIP mRNA in liver tissue*

Experiment No.	Mouse No.	Mouse sensitized and challenged with:	TNF- α detected in BAL fluid (pg/ml)	IL-5 detected in BAL fluid (pg/ml)	Ratio of liver SeIP mRNA levels compared to PBS control ^a
1	1	PBS	3.90	0.80	1.00
	2	OVA	253.00	321.80	2.09
	3	OVA	29.75	120.85	2.82
2	1	PBS	2.03	0.00	1.00
	2	OVA	241.63	505.53	1.34
	3	OVA	72.80	32.00	1.35
	4	OVA	148.87	434.93	2.64
3	1	PBS	3.55	0.00	1.00
	2	OVA	43.50	144.25	2.77
	3	OVA	8.40	57.00	2.25
	4	OVA	22.05	209.35	1.70

^a Ratios reflect those of target (GPX-1 or GPX-4) mRNA signal normalized to a housekeeping gene (hypoxanthine phosphoribosyltransferase) signal for each OVA mouse compared to a PBS mouse for that experiment.

will allow a determination of whether the Selp protein is also increased.

Discussion

The concept of using Se supplementation for preventing or treating asthma has been largely based on the notion that lower Se intake leads to higher susceptibility to, or severity of, asthma. Results from descriptive studies in humans have failed to firmly establish how Se intake affects asthma. Although some data have suggested that lower levels of Se correlate with increased prevalence and/or severity of asthma (15–17), other studies failed to confirm these results (18, 19). Our results may help explain these conflicting results in that Se intake may not be related to the inflammation and the Th2 immune response that occur during allergic asthma in a simple dose-response manner. The data obtained using a defined mouse model and presented herein suggest that a diet containing adequate (medium) levels of Se lead to the highest susceptibility to induced allergic airway inflammation. The mice from the medium Se dietary group displayed the strongest responses to OVA challenge in terms of cytokine levels and cellular infiltrate in the airways, eosinophilia, serum anti-OVA IgE, goblet cell hyperplasia, AHR, and pSTAT-6 levels in the lung. For nearly all readouts, the low Se dietary group was significantly lower than the medium Se group, while the high Se group was not significantly different from either the low or the medium Se group. One exception was the Th2 marker pSTAT-6, which was significantly lower in both the low and the high Se group compared with the medium Se group.

The results of this study were obtained by conducting several independent experiments in which mice from all three dietary groups and both PBS and OVA sensitizations/challenges were included. The cumulative data suggested a pattern of airway inflammation responses, i.e., the strongest responses were in the medium Se group followed by high Se and low Se groups, which was found consistently from experiment to experiment. It is important to mention that when comparing medium and high Se groups challenged with OVA, statistical differences were usually not found. We found a high degree of biological variability in many of the readouts that limited our ability to clearly state that the medium Se group was statistically higher in inflammatory and Th2 responses compared with the high Se group. However, we determined that the levels of immune responses for the medium Se group were, in all cases, different from those of their PBS controls with a higher degree of significance. Thus, despite the biological variability involved with this model, a consistent pattern emerged with medium Se resulting in robust airway inflammatory responses.

To our knowledge, this is the first study to use a mouse model of allergic airway inflammation to determine the relationship between dietary Se and asthma. Our results suggest that Se-replete diets, the type that most closely reflect Se intake in developed countries like the United States, lead to the highest susceptibility to allergens. Low Se intake did not increase susceptibility, as has been suggested to occur in humans, but decreased susceptibility to allergen challenge. Low Se intake has been shown to lead to lower immune responses against infectious diseases and tumors (6). In vitro studies have demonstrated that T cells from both humans and mice with lower Se status have a lower capacity to respond to stimulation with Ag or mitogen, to differentiate into cytotoxic lymphocytes, and to destroy tumor cells (7, 9). Indeed, our data suggest that moderately low Se status in the mice causes lower expression of the high affinity chain of IL-2R (CD25) on lung T cells and lower immune responses to allergens. Altogether, our data do not support the notion that moderately the low Se intake corresponding to nutritionally imbalanced diets increases susceptibility to asthma. It would be important to determine how switching diets

influences the immune responses. In particular, does switching the mice from medium to high Se lower susceptibility to airway inflammation? If so, at what dose and duration are these effects seen? These and other related questions are currently under investigation.

At the other end of the spectrum, Se supplementation has been suggested as a prevention or treatment measure for asthma. In this sense, Se is suggested to influence or limit immune responses to allergens differently than to viral or cancer immunogens, for which it is immunostimulatory. Data obtained using the mouse model in our study support this notion. Our data suggest that Se is particularly influential on immune responses at the level of Th2 signaling. Our results could not be explained by Se toxicity in the supplemented mice because these mice showed no general features of Se toxicity or signs of adverse effects on liver or immune tissues. In fact, the Se supplementation resulted in increased levels of CD25 on the surface of lung T cells in mice challenged with OVA. Even more interesting is that increased CD25 expression was only found in the mice that received OVA challenge. High Se status may lower immune responses against the OVA challenge not because of low activation or proliferation of T cells but because of altered differentiation. High dietary Se may skew Th cell differentiation toward the Th1 phenotype and away from the Th2 phenotype that drives allergic airway inflammation and asthma. Alternatively, high Se may lead to an abundance of CD4⁺/CD25^{high} T regulatory T cells (Th3 cells), which limit the response to OVA. Studies are underway in our laboratory to determine how increased Se leads to increased level of CD25 on T cells, whether these cells are T regulatory and/or Th2 cells, and how this may in turn influence immune responses to OVA as well as other immunogens. Further investigation is warranted into whether Se supplementation may influence immune responses to allergens by skewing the balance between Th1/Th2 differentiation, the balance between T effector/T regulatory cell populations, or a combination of these effects.

Levels of the antioxidant enzyme GPX-1, but not of GPX-4, were more clearly influenced by the levels of allergic airway inflammation occurring in the mice. GPX-1 functions to detoxify peroxides in the cell (31) and its increased expression during asthma emphasizes the important role it plays in balancing the oxidative stress that occurs during allergic airway inflammation. GPX-4 also acts as a crucial intracellular antioxidant, protecting lipid membranes (plasma membranes and mitochondrial membranes) from oxidative damage (13). When comparing GPX-1 and GPX-4 knockout mice, the genetic loss of GPX-4 is clearly more damaging. GPX-4 knockout is lethal at an early embryonic stage and heterozygous cells are more sensitive to oxidative stress (37), whereas the GPX-1 knockout mice show no obvious phenotype under normal conditions (38). With this in mind, our results showing that GPX-4 expression levels are not affected by the induction of allergic inflammation support the notion that cells require abundant basal amounts of this enzyme to remain viable and that these levels are not substantially altered under conditions of oxidative stress. In contrast, GPX-1 appears to be more inducible under such conditions. This selenoprotein may serve to limit the damage occurring in the lungs during allergic airway inflammation. In this sense, Se supplementation may help not only to reduce the extent to which the Th2 immune responses develop and drive asthma but may lower the oxidative stress that occurs in the lungs during asthma episodes as well.

Overall, our results provide important insight into the relationship between Se intake and the inflammation and immune responses that occur during asthma. Diets that include adequate levels of Se may actually cause the highest levels of susceptibility to

asthma in humans. Lower Se intake due to malnutrition or imbalanced nutritional content has been shown to lead to higher susceptibility to infectious diseases, cancer, and other health disorders. However, our data suggest that allergic airway inflammation and asthma are not among these disorders. Se supplementation is similar to moderately low Se intake in our study in that it lowered susceptibility to allergic airway inflammation. However, Se supplementation has the added benefit of protecting against infectious diseases and cancer. Thus, our study supports the notion that Se supplementation should be further evaluated as a potential preventive measure for asthma as long as the levels of the individual exceed those that reflect the medium Se mice in our study.

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Disclosures

The authors have no financial conflict of interest.

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