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Selenoprotein Expression in Macrophages Is Critical for Optimal Clearance of Parasitic Helminth *Nippostrongylus brasiliensis**

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Shakira M. Nelson[‡§1], Ashley E. Shay[‡1], Jamaal L. James[‡], Bradley A. Carlson[¶], Joseph F. Urban, Jr.[‖], and K. Sandeep Prabhu[‡2]

From the [‡]Center for Molecular Immunology and Infectious Disease and Center for Molecular Toxicology and Carcinogenesis, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, Pennsylvania 16802, [§]Division of Cancer Epidemiology and Genetics, NCI, National Institutes of Health, Rockville, Maryland 20850, [¶]Molecular Biology of Selenium Section, Mouse Cancer Genetics Program, NCI, National Institutes of Health, Bethesda, Maryland 20892, and [‖]United States Department of Agriculture, Agriculture Research Service, Beltsville Human Nutrition Research Center, Diet, Genomics, and Immunology Laboratory, Beltsville, Maryland 20705

The plasticity of macrophages is evident in helminthic parasite infections, providing protection from inflammation. Previously we demonstrated that the micronutrient selenium induces a phenotypic switch in macrophage activation from a classically activated (pro-inflammatory; M1/CAM) toward an alternatively activated (anti-inflammatory; M2/AAM) phenotype, where cyclooxygenase (COX)-dependent cyclopentenone prostaglandin J_2 (15d-PGJ_2) plays a key role. Here, we hypothesize that dietary selenium modulates macrophage polarization toward an AAM phenotype to assist in the increasing clearance of adult *Nippostrongylus brasiliensis*, a gastrointestinal nematode parasite. Mice on a selenium-adequate (0.08 ppm) diet significantly augmented intestinal AAM presence while decreasing adult worms and fecal egg production when compared with infection of mice on selenium-deficient (<0.01 ppm) diet. Further increase in dietary selenium to supraphysiological levels (0.4 ppm) had very little or no impact on worm expulsion. Normal adult worm clearance and enhanced AAM marker expression were observed in the selenium-supplemented $Trsp^{fl/fl}Cre^{WT}$ mice that express selenoproteins driven by $tRNA^{Sec}$ (*Trsp*), whereas *N. brasiliensis*-infected $Trsp^{fl/fl}Cre^{LysM}$ selenium-supplemented mice showed a decreased clearance, with lowered intestinal expression of several AAM markers. Inhibition of the COX pathway with indomethacin resulted in delayed worm expulsion in selenium-adequate mice. This was rescued with 15d-PGJ_2, which partially recapitulated the effect of selenium supplementation on fecal egg output in addition to increasing markers of AAMs in the small intestine. Antagonism of PPARγ blocked the effect of selenium. These results suggest that optimal expression of selenoproteins and selenium-dependent production of COX-derived endogenous prostanoids, such as Δ^{12}-PGJ_2 and 15d-PGJ_2, may regulate AAM activation to enhance anti-helminthic parasite responses.

The gastrointestinal nematode parasite *Nippostrongylus brasiliensis*, whose life cycle closely resembles that of human hookworm *Ancylostoma duodenale*, has a short infection cycle, with infective larvae invading through the skin followed by migration to the lungs and small intestine where they mature into adult worms, after which they are cleared from the body (1). In general, gastrointestinal parasites infect over 3.5 billion people worldwide, with severe infections often affecting children in underdeveloped and developing countries, leading to developmental and cognitive impairment. Recent studies have indicated dietary selenium deficiency exacerbates parasite pathogenesis and prolongs infection and disease (2, 3); however, the underlying mechanisms have not been elucidated.

The trace element selenium is a key component in immune responses to helminth infections (4). Selenium is an essential micronutrient that exists in the form of diverse metabolites and selenoproteins within the body (5–8). Selenoproteins exhibit disulfide oxidoreductase, peroxidase, and deiododinase activities in addition to other functions such as regulation of intracellular calcium flux and protein palmitoylation (9). Previous studies have shown that selenium exerts an anti-inflammatory effect by down-regulating the expression of pro-inflammatory mediators (10). Selenoprotein synthesis involves enzymatic incorporation of selenium as the 21st amino acid, selenocysteine (Sec),[3] by a complex process that is driven by *Trsp* that encodes $tRNA^{Sec}$ (8, 11). Targeted deletion of the floxed *Trsp* allele by a tissue/cell-specific promoter-driven Cre recombinase markedly diminished expression of all selenoproteins (12). Substitution of Sec residue with Cys in some selenoproteins has

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[1] Both authors contributed equally.

[2] To whom correspondence should be addressed: Dept. of Veterinary and Biomedical Sciences, 115 Henning Bldg., Pennsylvania State University, University Park, PA 16802. Tel.: 814-863-8976; E-mail: ksprabhu@psu.edu.

[3] The abbreviations used are: Sec, selenocysteine; *Trsp*, $tRNA^{[Sec]}$ gene; COX, cyclooxygenase; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; H-PGDS, hematopoietic prostaglandin D_2 synthase; CAM, classically activated macrophage; AAM, alternatively activated macrophage; AA, arachidonic acid; p.i., post inoculation; qPCR, quantitative real time-PCR; PE, phosphatidylethanolamine; MPO, myeloperoxidase; ANOVA, analysis of variance.

been observed during selenium deficiency, which also markedly reduces their enzymatic activity (13, 14).

Infections with intestinal parasites such as *N. brasiliensis* are characterized by a rapid and biased Th2-type response, producing elevated levels of interleukin-4 (IL-4) and IL-13 (15–18). These cytokines are thought to play a major role in intestinal physiology, causing rapid expulsion of parasites from the intestine (17, 19–22). Interestingly, a robust Th2 response inhibits the generation of a Th1 response, protecting the host from excess inflammation (23–25) as well as priming the intestine for increased infiltration of macrophages, basophils, and eosinophils (17, 26). As one of the most abundant immune cells in the gut mucosa, macrophages play a fundamental role in host defense to helminthic parasites (17, 19, 20, 27).

Based on gene expression patterns, macrophages are often classified to belong to classically activated (CAM; M1) or alternatively activated (AAM; M2) phenotype, which represent two ends of a spectrum with poorly defined intermediate stages (17, 28, 29). As seen in a variety of helminthic parasite infections, AAMs are induced by IL-4 and IL-13 (15, 18, 29). These cells express high levels of Fizz1, Arg1, and Ym1 (15, 30). Of particular interest is the synergistic relationship between selenium and IL-4 to skew macrophage activation toward an AAM-like phenotype, where selenoprotein expression was pivotal (7).

Herbert *et al.* (21) have reported that IL-4 and IL-13 can also induce the expression of Relm-β (resistin-like molecule-β) by goblet cells upon differentiation from intestinal epithelial cells to cause expulsion of *N. brasiliensis* and *Heligmosomoides polygyrus*. Although this report suggests a minimal role for macrophages, recent studies suggest that neutrophils are differentially activated in the context of a Th2 response to prime long-lived macrophages that effect rapid clearance of *N. brasiliensis* (31). Thus, it is clear that macrophages do have a role in optimal clearance of infection. Although the underlying mechanism of AAMs in resistance to *N. brasiliensis* is not completely understood, studies have identified possible pathways involved. In the absence of STAT6, *N. brasiliensis* adult worms are not cleared effectively (24) due to a decrease in mucous secretion (19) and changes to intestinal physiology (17, 32). In fact, STAT6 is well known to facilitate nuclear hormone receptor PPARγ-regulated gene expression in macrophages (33) that also plays a major role in AAM activation and resolution of inflammation (34–36). Along these lines, previous studies from our laboratory have established a significant deficit in selenium-dependent AAM polarization in the absence of PPARγ and STAT6 (7). Although a functional relationship between IL-4, IL-13, and PPARγ has yet to be established in *N. brasiliensis* infection, studies have demonstrated that increased activation of PPARγ via the production of its endogenous ligand in the form of cyclopentenone prostaglandins, Δ^{12}-PGJ_2 and 15d-PGJ_2, through selenium supplementation (37, 38) polarizes macrophages toward an alternative phenotype (7). Interestingly, complete abrogation of the cyclooxygenase (COX)-hematopoietic prostaglandin D_2 synthase (H-PGDS) pathway inhibited endogenous cyclopentenone prostaglandins and consequent polarization of macrophages (7).

Although AAM-dependent mechanisms of helminth clearance have been previously reported, there is limited mechanistic data on the relationship between selenoprotein expression and macrophages during helminth infections. Here we demonstrate that macrophage expression of selenoproteins regulate the arachidonic acid (AA)-COX pathway to effect their polarization toward functional AAMs that are associated with reduced number of adult nematode worms in the small intestine.

Experimental Procedures

Mice—Three-week-old C57Bl/6 male mice were purchased from Charles River (Wilmington, MA) or Taconic Laboratories (Hudson, NY). Breeding pairs of IL-4 reporter mice (4Get mice) on a Balb/c background were generated by Dr. Richard M. Locksley (University of California, San Francisco, CA) and generously provided by Dr. Avery August (Cornell University, Ithaca, NY) (39, 40). A transgenic C57Bl/6 line carrying a lysozyme M Cre (Cre^{LysM}) transgene was crossed to a C57Bl/6 mouse with a floxed *Trsp* ($Trsp^{fl/fl}$) allele, both generously provided by Dr. Dolph Hatfield (NIH, Bethesda, MD). These lines were crossed to obtain $Trsp^{fl/fl}Cre^{LysM}$ mice, as previously described (12). Targeted removal of the floxed *Trsp* allele by a Cre recombinase driven by the lysozyme M promoter disabled the expression of all selenoproteins in macrophages, monocytes, and some granulocytes (12). All mice were maintained on selenium-deficient (<0.01 ppm), selenium-adequate (0.08 ppm), or selenium-supplemented diets (0.4 ppm) purchased from Harlan Teklad, Madison, WI, for at least 12 weeks before use in experiments. Selenium in the form of sodium selenite was used in selenium-adequate and selenium-supplemented diets. Studies were preapproved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee at Penn State University.

Genotyping—The extent of *Trsp* deletion was determined by PCR analysis of the floxed region of the gene. Tail snips were taken from all mice. A mixture of 250 μl of lysis buffer and 5 μl of proteinase K (20 mg/ml, New England BioLabs, Ipswich, MA) was added to each tail snip and incubated overnight in a 65 °C water bath. Lysed tail snips were centrifuged at 20,800 × *g* for 5 min at 25 °C. Supernatants were collected and diluted (1:11) with diethyl pyrocarbonate water. PCR was carried out using 0.2 μM concentrations of primers, 2.5 mM $MgCl_2$, 0.2 mM concentrations of each deoxyribonucleotide triphosphate, 1.25 units of GoTaq DNA polymerase (Promega, Madison, WI), GoTaq buffer, and 1 μl of diluted DNA. To detect the transgene, two sets of primers were used as follows: primer set 1, CKNO2 (5′-GCAACGGCAGGTGTCGCTCTGCG-3′) and 8RP (5′-CGTGCTCTCTCCACTGGCTCA-3′) and primer set 2, Cre 8 (5′-CCCAGAAATGCCAGATTACG-3′), Mlys1 (5′-CTTGGGCTGGCCAGAATTTCTC-3′), and Mlys2 (5′-TTACAGTCGGCCAGGCTGAC-3′). The PCR products ($Trsp^{fl/fl}$, 1.1 kb; $Trsp^{fl/fl}Cre^{LysM}$, 700 bp; $Trsp^{fl/fl}Cre^{WT}$, 350 bp) were separated by electrophoresis on a 2% agarose gel and visualized by UV transillumination.

Infection of Mice with N. brasiliensis—Infective third stage larvae (L3) were maintained in a mixture of charcoal and lightly dampened *Sphagnum* moss and stored in plastic Petri dishes

(1). Mice were subcutaneously inoculated with 500 L3 larvae in ~250 μl of PBS after collection from cultures using a modified Baermann's technique (1, 24, 41) and were studied on days 7, 8, 9, 11, and 14 post inoculation (p.i.). The timing of the studies correlated with the maximum effects of the parasite on gut function and coincided with ascending and descending egg production and worm expulsion (1, 41). Fecal egg production was quantified using a modified McMaster technique (42), and adult worms were detected quantitatively by dissecting the intestine (below the stomach to above the cecum) lengthwise and submerging the tissue in a beaker of warm PBS using a tea strainer. The beaker was placed in a 37 °C water bath for 45 min. Remaining worms in the intestine tissue were counted using a microscope. Worms in suspension were counted on a gridded Petri plate.

Treatments—Indomethacin (Cayman Chemicals) was administered to mice in drinking water (containing 0.1% (v/v) ethanol) at a concentration of 0.00325% (w/v) (37) for 2 weeks before *N. brasiliensis* infection until 2 weeks p.i., when the animals were euthanized. As a vehicle control, 0.1% ethanol (v/v) was used. Lipid extraction was performed from the jejunal tissue of indomethacin or vehicle-treated infected mice on day 8 p.i., and LC-MS/MS was performed with multiple reaction monitoring (*m/z* 332.72 to 271.2) to quantify Δ^{12}-PGJ_2 as described earlier (37). Indomethacin inhibited the production of Δ^{12}-PGJ_2 in the jejunum of infected mice day-8 p.i., as indicated by LC-MS/MS (data not shown). 15d-PGJ_2 was administered daily at a concentration of 0.050 mg/kg/day (dissolved in sterile PBS) by intraperitoneal injection (~0.5 ml) for 7 days. PPARγ antagonist, GW9662 (Cayman Chemicals), was administered at 1 mg/kg body weight. GW9662 was dissolved in ethanol and diluted in sterile PBS (to 4% v/v) and intraperitoneally administered to selenium-adequate mice starting a day before infection with 500 larvae and continued each day during the 9-day period. Diluted ethanol in PBS was used as a vehicle control for comparison. The effect of GW9662 treatment on the jejunal expression of PPARγ target genes, *Arg1* and *Mrc1* (*Cd206*), was assessed using quantitative real time-PCR (qPCR) on day-8 p.i. as a measure of its *in vivo* efficacy. 16,16-Dimethylprostaglandin E_2 (Cayman Chemicals) was formulated similarly in 4% (v/v) ethanol in PBS and injected at (10 μg/kg/day) starting simultaneously as infection with 500 larvae. The effect of indomethacin (2.5 μM) or GW9662 (1 μM) on the viability of L3 stage larvae as well as the viability, fecundity, and egg-laying capacity of adult worms were assessed after 12 h of treatment in RPMI 1640 medium containing 10% FBS, 400 IU of penicillin, and 400 μg/ml streptomycin as described earlier (4, 31). ATP levels were measured as an indicator of viability (metabolic activity) using the Promega CellTiter-Glo luminescent cell viability assay as described earlier (4, 31).

qPCR—Total RNA was isolated from 1-mg sections of jejunum using Isol-RNA lysis reagent (5 Prime; Gaithersburg, MD). RNA concentrations were determined by UV spectroscopy. Briefly, 2 μg total RNA was reverse-transcribed into cDNA as previously described (7). TaqMan probes for *Arg1*, *Fizz1*, *Ym1*, *Mrc1* (*Cd206*), *Tnfa*, *Il1β*, *Inos*, and *Il-13* (from Applied Biosystems) were used to quantitate cDNA. As an internal control, a *Gapdh* probe was used to normalize the data. Amplifications were performed using PerfeCTa qPCR SuperMix Master Mix (Quanta Biosciences) in a 7300 Real time PCR system (Applied Biosystems). ΔC_t ($Ct_{Gene} - Ct_{GAPDH}$) was calculated for each sample and used for analysis of transcript abundance with respect to the untreated negative control.

Isolation of Epithelial Layer and Lamina Propria Lymphocytes from Small Intestine Tissue—Lymphocytes from the intestinal intra-epithelial lymphocyte and lamina propria were isolated as described (43). Briefly, small intestines were taken from mice 9 days p.i., and all Peyer's patches were removed. To isolate intra-epithelial lymphocytes, 20 ml of Hanks' buffer (Sigma) containing 1 mM DTT and 5 mM EDTA and one drop of 1 M HCl was added to tissues for 30 min and shaken at 250 rpm at 37 °C. This step was repeated until the supernatant became clear, each time collecting the supernatant and keeping it on ice. After the last wash, tissue pieces were rinsed in RPMI media to remove EDTA. To isolate lymphocytes from the lamina propria, tissues were incubated in 30 ml of RPMI containing 300 mg of collagenase (300 units/ml) (Worthington Biochemical Corp., Lakewood, NJ) and 0.09 g of dispase (Sigma) for 1 h at 250 × *g* at 37 °C. After incubation, the supernatants were filtered using a mesh strainer (Fisher) into a fresh tube and centrifuged at 500 × *g* for 5 min at 4 °C. Lymphocyte pellets were resuspended in 40% Percoll and placed over an 80% Percoll mixture, creating a 40% (v/v)–80% (v/v) gradient. Tubes were centrifuged at 800 × *g* for 20 min at room temperature with the brake off. The lymphocyte interface between the gradients was collected into a new tube, and the cells were rinsed twice in flow buffer (pH 7.2; 50 ml of 10× PBS, 25 ml of FBS, and 2.5 ml sodium azide in a final volume of 500 ml with deionized water). A total of 500,000 cells were used for flow cytometric analysis.

Flow Cytometry—Cells isolated from the small intestine were washed in 1 ml of flow buffer (pH 7.2) and pelleted by centrifuging at 250 × *g* for 5 min at 4 °C. Pellets were resuspended in 100 μl of flow buffer containing F_c block (BD Biosciences) and stained with the following antibodies: PE-conjugated rabbit anti-mouse CD3, PE-Cy7™-conjugated anti-mouse CD11b (encoding integrin αM, Itgam), PE-conjugated rabbit anti-mouse Siglec-F antibodies (BD Pharmingen), rabbit anti-mouse Fizz1(Retlna1; Relmα) and rabbit anti-mouse Relmβ (Fizz2) (Peprotech, Rocky Hill, NJ), FITC-conjugated rat anti-mouse F4/80 (AbD Serotec, Raleigh, NC), PE-conjugated anti-mouse arginase-1, or FITC-conjugated anti-mouse CCR3 (R&D Systems) for 30 min at 4 °C in the dark. Cells were washed with 1 ml of flow buffer and centrifuged at 250 × *g* for 5 min. Unconjugated primary antibody samples were stained with AF-647 goat anti-rabbit IgG secondary for 30 min at room temperature in the dark. For intracellular staining (Arg-1 or Fizz1), cells were fixed with 2% paraformaldehyde for 20 min and permeabilized for 15 min followed by staining. Stained cells were analyzed on a BD Accuri C6 Benchtop Cytometer using BD Accuri and FlowJo data analysis software programs (FlowJo, LLC, Ashland, OR). All data shown are compared with their respective isotype controls.

Myeloperoxidase (MPO) Assay—Jejunum was homogenized in 50 mM potassium phosphate buffer (pH 6.0) and centrifuged. The pellet was resuspended in 50 mM potassium phosphate buffer containing 50 mM hexadecyltrimethylammonium bro-

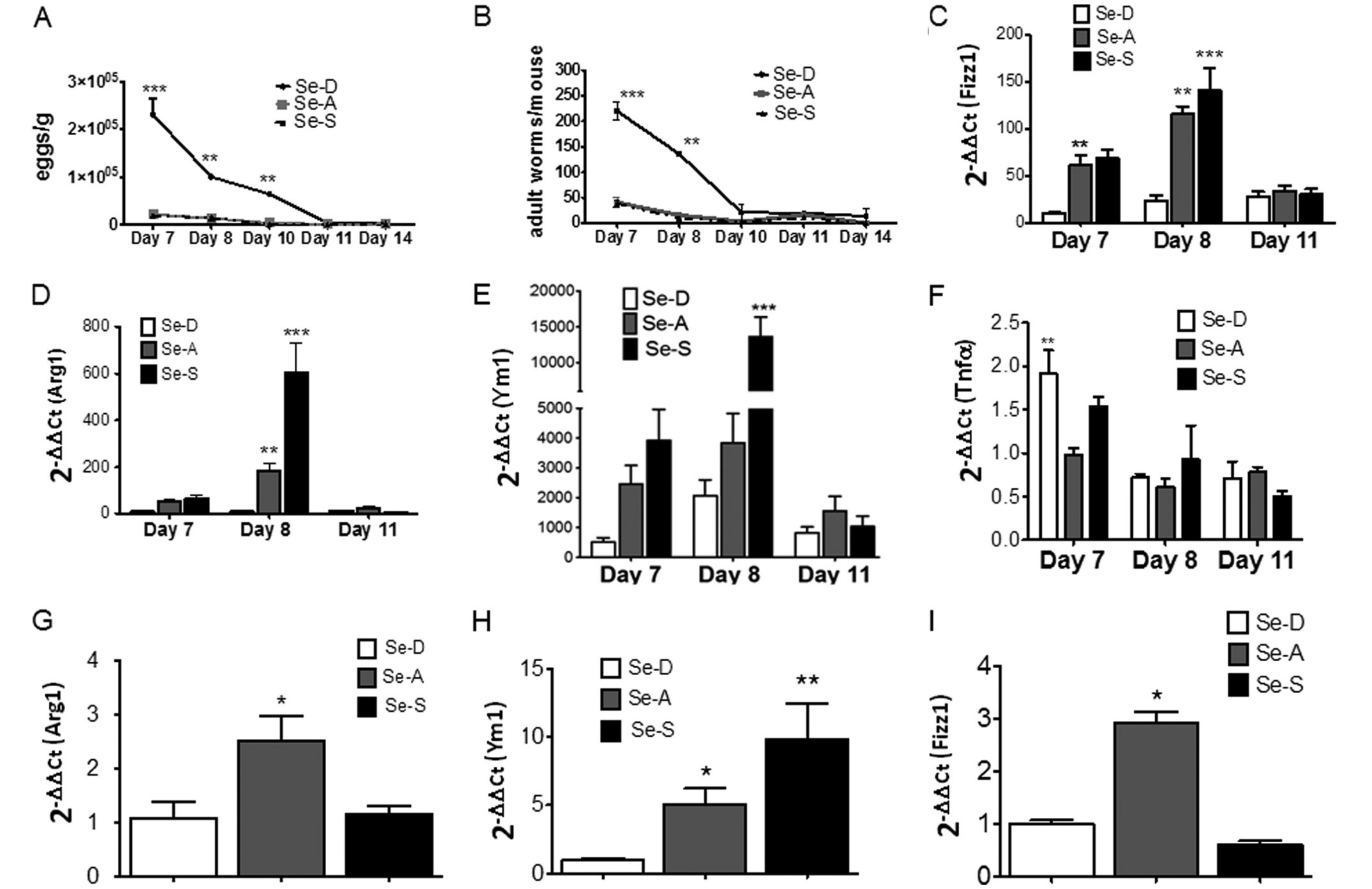

FIGURE 1. **Effect of selenium on adult worm burden and AAM marker expression in *N. brasiliensis*-infected mice.** 500 infective larvae were injected into selenium-deficient (*Se-D*), selenium-adequate (*Se-A*), and selenium-supplemented (*Se-S*) mice. In each *panel*, selenium-deficient mice are statistically compared with selenium-adequate and selenium-supplemented mice within each day. *A*, number of *N. brasiliensis* eggs per gram of feces from each mouse. *B*, number of adult worms per mouse in the whole small intestine (below the stomach to above the cecum) upon extraction. *C–E*, qPCR analysis of the expression of *Fizz1* (*C*), *Arg1* (*D*), and *Ym1* (*E*), and a CAM marker gene *Tnf*α (*F*) in the jejunum. *G–I*, qPCR analysis of the expression of AAM genes *Arg-I* (*G*), *Ym1* (*H*), and *Fizz1* (*I*) in non-infected mice on selenium diets. Selenium-deficient mice are compared with selenium-adequate and selenium-supplemented mice. All data shown are the mean ± S.E., with a total of $n = 9$ mice used per group. *Asterisks* represent differences within days between diets. *, $<$0.05; **, $<$0.01; ***, $<$0.001 and were analyzed using two-way ANOVA.

mide followed by sonication and centrifugation. 50 μl of supernatant was incubated with 1.45 ml of potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. Absorbance was measured at 460 nm every 30 s for 10 min. Activity of MPO was calculated using the change in absorbance over time and the molar extinction coefficient of *o*-dianisidine.

Statistical Analysis—Results are presented as the mean ± S.E. To compare means, groups were analyzed using two-way ANOVA on GraphPad® Prism followed by appropriate post hoc tests. Results were considered significantly different at *p* value ≤0.05. All experiments were performed in triplicate using at least three mice per experiment, for a total of $n = 9$.

Results

Effects of Dietary Selenium on Adult Worm Burden and Fecal Egg Production in N. brasiliensis-infected Mice—To determine the effects of dietary selenium on parasite clearance, mice fed either a selenium-deficient, selenium-adequate or selenium-supplemented diet were inoculated subcutaneously with 500 *N. brasiliensis* third-stage larvae (L3). Fecal eggs were isolated and quantified (1, 24) on days 7, 8, 10, 11, and 14 p.i. Compared with selenium-adequate- and selenium-supplemented mice, selenium-deficient mice had a significant increase in the number of eggs (Fig. 1*A*). There was no significant difference in fecal eggs or number of adult worms between selenium-adequate- and selenium-supplemented mice throughout the infection (Fig. 1, *A* and *B*). However, selenium-deficient mice showed a significantly increased number of worms on days 7 and 8 p.i. (Fig. 1*B*). Worm counts, however, began decreasing after day 8 p.i., supporting previously published data (1, 24).

Selenium Increases Intestinal AAMs in Response to Infection—The selenium-dependent mechanisms underlying increased anti-parasite effects were examined. It has been previously shown that mice utilize a biased Th2 response to clear the *N. brasiliensis* infection from the intestine (15). Moreover, our previous data indicated a synergistic relationship between IL-4 and selenium as a key-contributing factor in the polarization of macrophages toward the AAM phenotype (7). To examine if selenium-dependent changes in AAM polarization were associated with a change in worm burden, we examined the expression of characteristic AAM markers *Arg1*, *Ym1*, and *Fizz1* in the jejunum of *N. brasiliensis*-infected mice as a function of dietary selenium. Jejunal tissue was collected on days 7, 8, and 11 p.i.

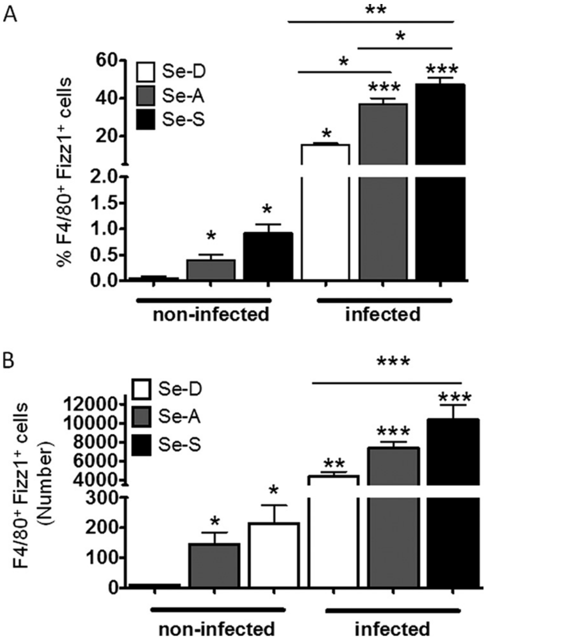

FIGURE 2. **Increase in AAMs in the lamina propria by selenium supplementation (*A* and *B*).** Quantitation of F4/80$^+$Fizz1$^+$ cells in the jejunum of *N. brasiliensis*-infected and uninfected control mice on the three diets. *Bar graph* percentages shown were calculated by averaging cell expression of F4/80 and Fizz1 from three separate flow cytometric experiments. All data shown are compared with isotype controls. Values are the mean ± S.E. with a total of $n = 9$ mice used. Across all groups mice are compared with non-infected selenium-deficient (*Se-D*) mice. Within the infected group, selenium-deficient mice were compared with both selenium-adequate (*Se-A*) and selenium-supplemented (*Se-S*) mice, indicated by the *lines above each diet*. *Asterisks* represent significant differences between groups. *, <0.05; **, <0.01; ***, <0.001 and were determined using two-way ANOVA with Tukey's post hoc testing.

(17). Expression of all three AAM genes was significantly higher in selenium-adequate- and selenium-supplemented mice compared with selenium-deficient mice starting on day 7 p.i. and increased further by day 8 p.i. followed by a significant decrease by day 11 p.i., possibly related to worm clearance (Fig. 1, *C–E*). As expected, the expression of CAM markers, such as *Tnfα* (Fig. 1*F*) and *Ifnγ* and *Il1β* (not shown), was much lower than expression of AAM markers. The expression of all three AAM genes was significantly higher in all three diet groups in the *N. brasiliensis*-infected mice compared with their uninfected counterparts (Fig. 1, *G–I*), where *Arg1* and *Fizz1* increased with the selenium-adequate diet (*versus* selenium-deficient diet) but decreased with the selenium-supplemented diet, whereas *Ym1* increased with selenium-supplemented (*versus* selenium-deficient and selenium-adequate).

To verify that the selenium-dependent increases in expression of AAM genes was associated with increased numbers of intestinal AAMs after infection, cells in the lamina propria were isolated at day 9 p.i. and analyzed by flow cytometry for F4/80$^+$ and Fizz1$^+$-expressing cells, which mark AAMs. Compared with non-infected mice, *N. brasiliensis* infection induced a significant increase in percentage of double positive AAMs in all diet groups (Fig. 2*A*). Moreover, the number of double-positive cells also increased when compared with non-infected cells (Fig. 2*B*). Furthermore, selenium-adequate- and selenium-supplemented mice infected with *N. brasiliensis* led to a significantly greater percentage of double-positive cells when compared with selenium-deficient mice (Fig. 2*A*). Taken together, these results suggest that selenium status increases in the presence of AAMs in the intestine of infected mice.

Selenoproteins Are Required for Optimal AAM-induced Parasite Clearance—Recent studies from our laboratory have indicated a pivotal role for selenoproteins in the polarization of macrophage phenotypes (7). To determine the link between dietary selenium and cellular selenoproteins in parasite clearance, $Trsp^{fl/fl}Cre^{LysM}$ mice were infected with *N. brasiliensis*. $Trsp^{fl/fl}Cre^{LysM}$ mice fed a selenium-supplemented diet showed a significant increase in the amount of fecal eggs when compared with control $Trsp^{fl/fl}Cre^{WT}$ mice at day 7 p.i. (Fig. 3*A*). A similar pattern was also seen in the number of adult worms in the small intestine (Fig. 3*B*). Fecal egg and adult worm burdens were diminished by day 11 p.i. in both strains of mice (Fig. 3, *A* and *B*). To determine the effect of *Trsp* deletion on AAM marker expression, we used qPCR to examine the modulation of AAM marker gene expression in the jejunum. A significant abrogation in the expression of *Arg1*, *Fizz1*, and *Ym1* was observed in $Trsp^{fl/fl}Cre^{LysM}$ mice compared with $Trsp^{fl/fl}Cre^{WT}$ mice at days 7, 8, and 11 p.i. (Fig. 3, *C–E*). Interestingly, expression of CAM markers, *Tnfα*, *Inos*, *Il-1β*, and *Ifnγ* were increased in the $Trsp^{fl/fl}Cre^{LysM}$ mice, particularly on days 7 and 8 post inoculation compared with their $Trsp^{fl/fl}Cre^{WT}$ counterparts (Fig. 3, *F–I*). Taken together, these data illustrate the essential role of selenoproteins in the optimal clearance of *N. brasiliensis*.

Essential Role of the COX Pathway in Selenium-dependent Macrophage Polarization and Helminth Infection—Previous studies from our laboratory have demonstrated a selenium-dependent production of anti-inflammatory prostaglandin Δ^{12}-PGJ_2 and its dehydration product, 15d-PGJ_2, that serve as endogenous ligands for PPARγ in macrophages leading to the increase in AAM markers (38). Along these lines, qPCR analysis of the jejunal tissue on day 8 p.i. indicated a selenium-dependent increase in the expression of *Ptgs2* (COX-2) and *Hpgds* (H-PGDS), two critical enzymes required for the endogenous production of PGD_2-derived cyclopentenone prostaglandins, Δ^{12}-PGJ_2 and 15d-PGJ_2 (Fig. 4*A*). To examine if selenium functions through a COX-dependent pathway to modulate *N. brasiliensis* infection, we used indomethacin, a non-steroidal anti-inflammatory drug that inhibits COX-derived biosynthesis of prostaglandins, including Δ^{12}-PGJ_2 and 15d-PGJ_2. LC-MS/MS analysis of jejunal extracts indicated a 6.6-fold decrease in the endogenous levels of Δ^{12}-PGJ_2 in selenium-adequate mice on day 8 p.i. upon treatment with indomethacin (data not shown). Inhibition of the COX pathway significantly increased fecal eggs and adult worm burden on days 7 and 8 p.i. in selenium-adequate and selenium-supplemented mice compared with infected vehicle-treated mice (Fig. 4, *B* and *C*). However, incubation of L3 stage larvae or adult worms with indomethacin for 12 h had no impact on the viability as seen in the form of ATP levels in addition to not affecting their fecundity (Fig. 4*H*). Together, these results suggest the importance of the COX-H-PGDS pathway in selenium-dependent parasite clearance.

qPCR analysis was used to measure the effects of indomethacin on AAM and CAM marker expression in the jejunum of

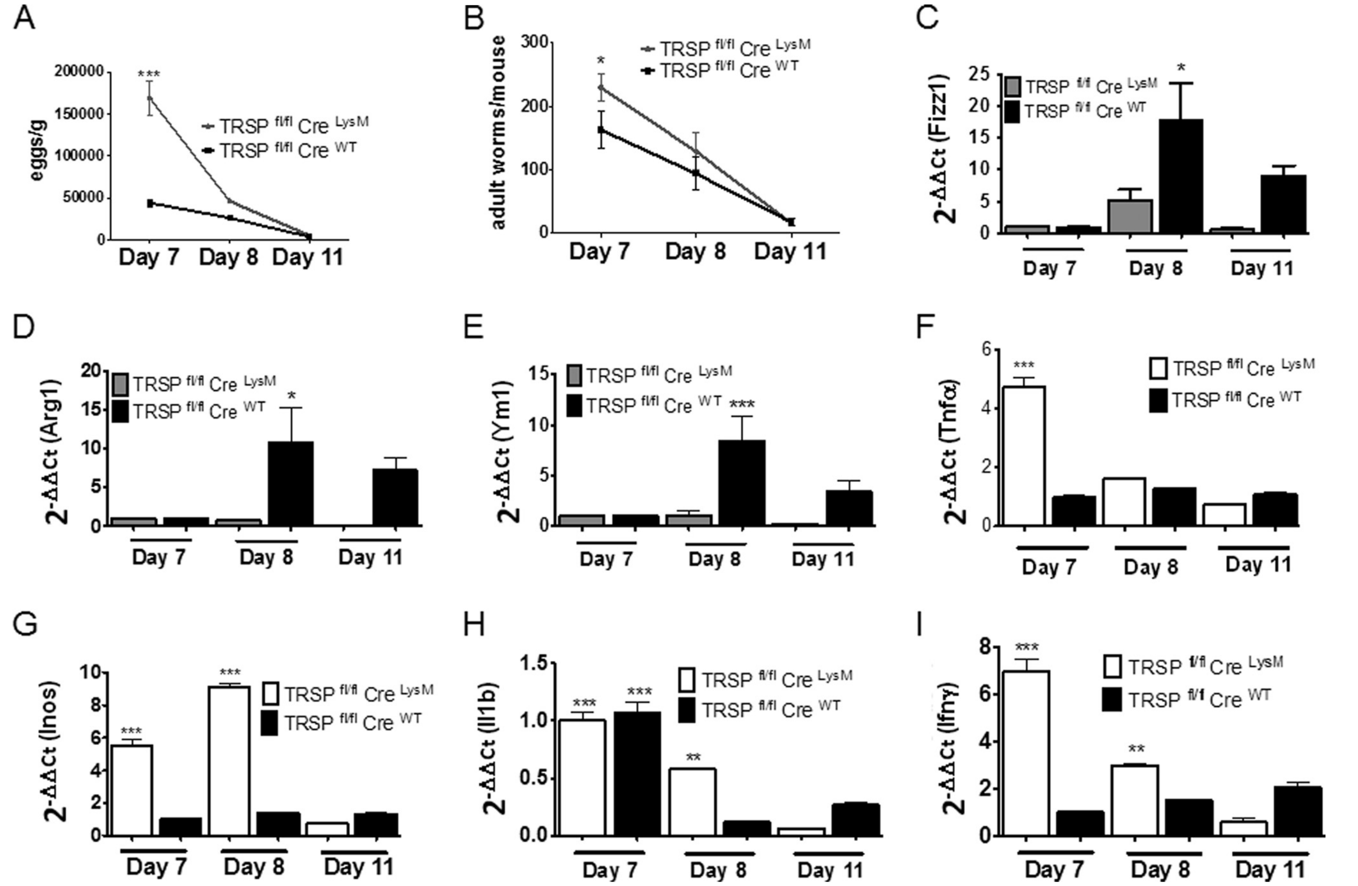

FIGURE 3. **Selenoproteins are required for AAM marker expression and optimal *N. brasiliensis* clearance.** $Trsp^{fl/fl}Cre^{LysM}$ and $Trsp^{fl/fl}Cre^{WT}$ mice maintained on selenium-supplemented (*Se-S*) diets for 8–10 weeks were infected with 500 infective larvae. *A*, number of *N. brasiliensis* eggs per gram of feces from each mouse. *B*, worm burden in the whole small intestine upon extraction to count adult worms in the lumen of $Trsp^{fl/fl}Cre^{LysM}$ and $Trsp^{fl/fl}Cre^{WT}$ mice. *C–E*, qPCR analysis of the expression of *Fizz1* (*C*), *Arg1* (*D*), and *Ym1* (*E*). *F–I*, expression of *Tnfα* (*F*), *Inos* (*G*), *Il1β* (*H*), and *Ifnγ* (*I*) in the jejunum of *N. brasiliensis*-infected $Trsp^{fl/fl}Cre^{LysM}$ and $Trsp^{fl/fl}Cre^{WT}$ mice. Values are the mean ± S.E., with a total of $n = 9$ mice used. Within each day $Trsp^{fl/fl}Cre^{LysM}$ are statistically compared with $Trsp^{fl/fl}Cre^{WT}$ mice. *Asterisks* represent significant differences between groups. *, <0.05; ***, < 0.001. Statistical differences were analyzed using ANOVA with post hoc test.

these mice. The selenium-dependent increases in *Fizz1*, *Arg1*, and *Ym1* expression in *N. brasiliensis*-infected mice on days 7 and 8 p.i. were blocked by treatment with indomethacin (Fig. 4, *D–F*). Conversely, the selenium-dependent inhibition of *Tnfα* expression (Fig. 4*G*) was reversed with indomethacin treatment showing a significant increase in its expression on days 7, 8, and 11 p.i. These data further demonstrate the importance of the COX pathway in mediating the effects of selenium on the expression of AAM markers in the jejunum.

15d-PGJ$_2$ Reduces Fecal Egg Shedding in N. brasiliensis-infected Mice—Based on the above data that demonstrated the selenium induction of macrophage polarization to be dependent on the COX pathway, presumably mediated by 15d-PGJ$_2$-dependent mechanisms, we examined if exogenous treatment of selenium-deficient mice with 15d-PGJ$_2$ would recapitulate the protective effect of selenium. Indomethacin-treated selenium-deficient mice were administered 15d-PGJ$_2$ intraperitoneally (at 0.050 mg/kg/day) ~12 h before infection with *N. brasiliensis*, and the treatment was continued daily with 15d-PGJ$_2$ for a total of 7 days p.i. As shown in Fig. 5*A*, 15d-PGJ$_2$ treatment of selenium-deficient mice reduced fecal egg shedding on days 7 and 8 p.i. to levels below those seen in the untreated selenium-deficient control mice also on indomethacin (Fig. 5*A*). Similar experiments were performed in selenium-adequate mice on indomethacin followed by treatment with 15d-PGJ$_2$ and infection (as above). Flow cytometric analyses of small intestinal tissue on days 7 and 8 p.i. indicated significantly increased CD11b$^+$Arg-1$^+$ cells in 15d-PGJ$_2$-treated groups on both days compared with the PBS control (Fig. 5, *B* and *C*). Furthermore, qPCR of prototypical markers (*Arg1*, *Ym1*, and *Fizz1*) in the small intestine were significantly increased by exogenous 15d-PGJ$_2$ treatment (data not shown). Given that 15d-PGJ$_2$ could partly mediate effects through PPARγ, we tested the role of a PPARγ antagonist, GW9662, in this model. Interestingly GW9662 treatment greatly increased the worm load in the jejunum (on day 8 p.i.) when compared with the vehicle control (Fig. 5*D*). Although treatment of selenium-adequate mice with GW9662 reduced the expression of PPARγ target genes, *Mrc1* and *Arg1* (Fig. 5*E*), *in vitro* studies showed that GW9662 had no effect on the viability of L3 stage larvae or adult worm or even fecundity (Fig. 5*F*). However, treatment of selenium-adequate mice with 16,16-dimethyl-prostaglandin E$_2$ had no affect the clearance of adult worms (data not shown). Together, these data suggest the importance of the COX-H-PGDS pathway in modulating parasite egg shedding, where PPARγ-dependent modulation of AAMs is likely involved.

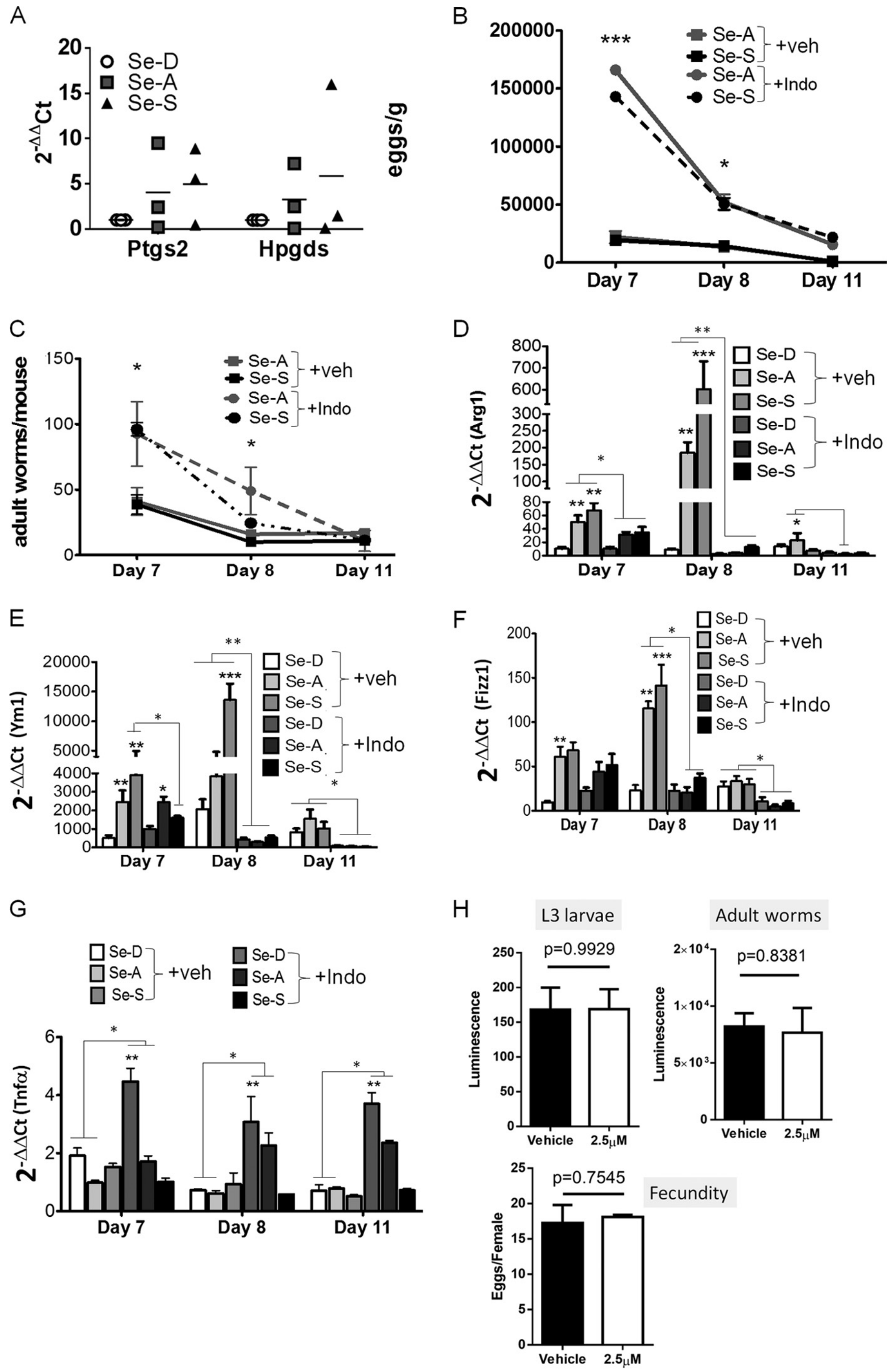

FIGURE 4. **Involvement of the COX pathway in *N. brasiliensis* clearance by selenium and its effect on the expression of AAM and CAM markers.** *A*, expression of COX-2 (*Ptgs2*) and H-PGDS (*Hpgds*) in the jejunum of selenium-deficient (*Se-D*), selenium-adequate (*Se-A*), and selenium-supplemented (*Se-S*) mice on day 8 post-inoculation by qPCR. Values are mean of $n = 3$ independent experiments from each diet group performed in triplicate. *B* and *C*, indomethacin (*Indo*) was administered to selenium-adequate and selenium-supplemented mice through drinking water (0.00325% w/v) for 2 weeks before infection through 2 weeks p.i. Fecal eggs (*B*)and adult worms (*C*) were counted on days 7, 8, and 11 p.i. All data are compared with vehicle-treated mice. Statistical differences comparing selenium-adequate and selenium-supplemented mice within each day were analyzed using two-way ANOVA with Bonferroni (*B* and *C*). qPCR was used to analyze expression of *Arg1* (*D*), *Ym1* (*E*), *Fizz1* (*F*), and *Tnf*α (*G*) from the jejunum of *N. brasiliensis*-infected mice treated with 0.00325% (w/v) indomethacin for 2 weeks before infection and 2 weeks thereafter. Values are the mean ± S.E. with a total of $n = 9$ mice used. Two-way ANOVA with post hoc Bonferroni method was used to control for multiple comparisons between diet groups from vehicle- or indomethacin-treated mice as well as statistical differences comparing vehicle selenium-deficient mice to diet combinations within each day were analyzed using Tukey's post hoc test. *H*, approximately five adult worms isolated from the small intestine of three infected C57BL/6 mice were plated per well in 0.2 ml of RPMI 1640 medium with 10% FBS and antibiotics and incubated overnight with indomethacin (2.5 μM) or vehicle at 37 °C. After incubation, female worms and eggs in the media were counted to assess the effect of indomethacin on egg laying and fecundity. The number of eggs was normalized to the number of females per well. Similarly, 10 larvae were incubated as described above with indomethacin or vehicle. Adult worms and larvae were processed and used for chemiluminescence-based viability assay to detect ATP levels. As a negative control, adult worms or larvae in media were incubated at 80 °C for 5 min and homogenized with reagent after cooling. $n = 3$ per group. Unpaired two-tailed *t* test. *Asterisks* represent significant differences between groups. *, <0.05; **, <0.01.

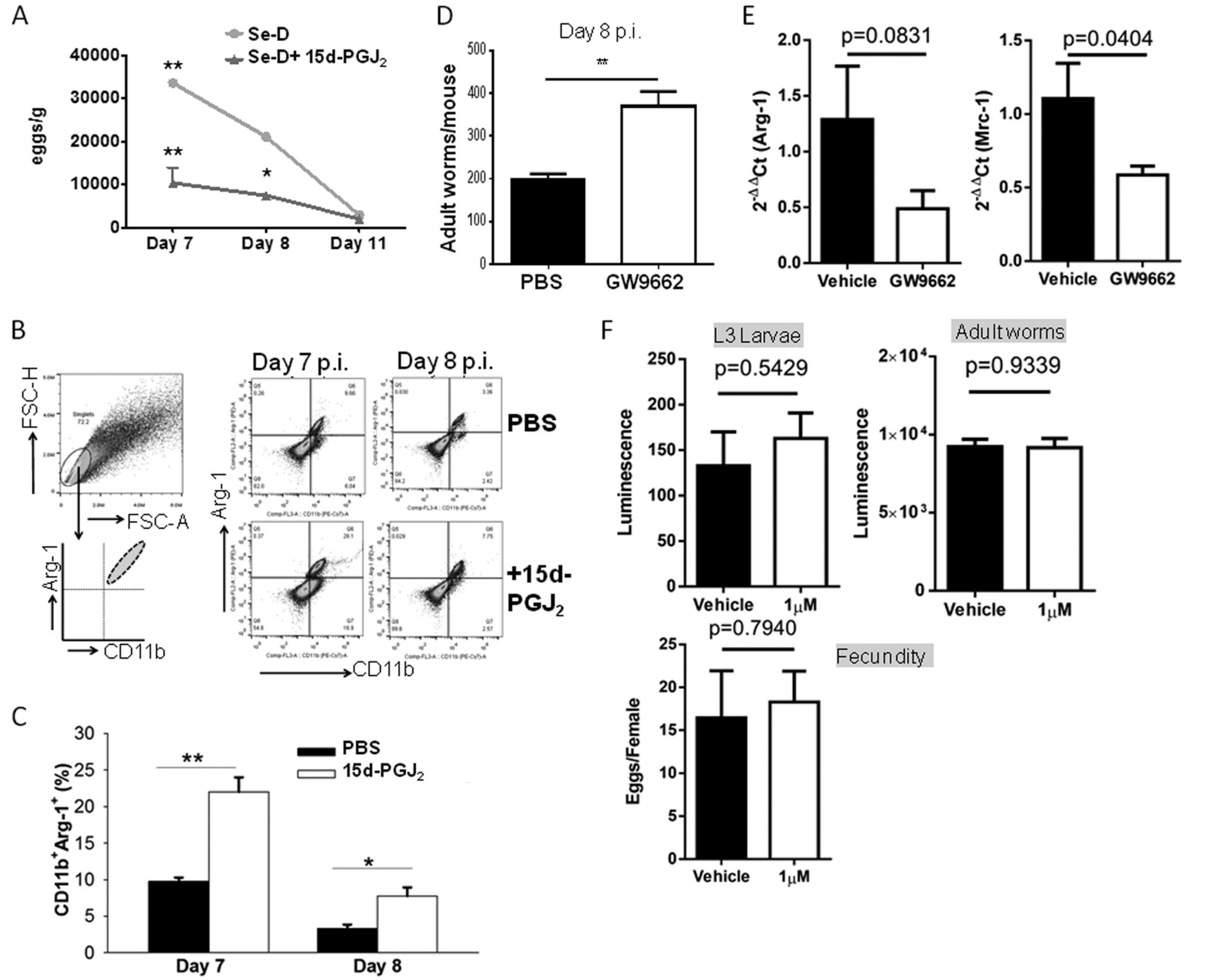

FIGURE 5. **Effect of 15d-PGJ$_2$ and GW9662 on selenium-dependent adult worm clearance in *N. brasiliensis*-infected mice.** *A*, fecal eggs were counted in selenium-deficient mice treated with 0.00325% (w/v) indomethacin for 2 weeks before infection and 2 weeks thereafter. 12 h before infection with *N. brasiliensis*, selenium-deficient (*Se-D*) mice were injected intraperitoneally with 0.050 mg/kg 15d-PGJ$_2$ or sterile PBS once daily for 7 days. Values are the mean ± S.E. of $n = 4$ per group. *Asterisks* represent significant differences between the selenium-deficient and selenium-deficient with 15d-PGJ$_2$ groups. *, <0.05; **, < 0.01; ***, < 0.001. Statistical differences were analyzed using two-way ANOVA with Tukey's post hoc testing. *B* and *C*, indomethacin-treated mice on selenium-adequate (*Se-A*) diet (as above) received sterile PBS or 15d-PGJ$_2$ injections (0.05 mg/kg/day) starting 12 h before infection. Single cell suspensions from the small intestine were prepared on days 7 and 8 p.i., and cells were stained for CD11b (PE-Cy7) and Arg-1 (PE). Gating strategy and representative flow cytometry plots are shown in *panels B* and *C*, respectively. FSC-H and FSC-A represent forward scatter-height and forward scatter-area, respectively. *D*, mice on selenium-adequate diet received either vehicle (ethanol in sterile PBS; 4% v/v) or GW9662 (formulated in 1 mg/kg/day) in vehicle injections starting 1 day before inoculation that were continued to up to 8 days p.i. The number of adult worms per mouse was counted in the whole small intestine (below the stomach to above the cecum). $n = 4$ per group. Unpaired two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$. *E*, expression of *Arg1* and *Mrc1* in the jejunum of selenium-adequate mice treated with GW9662 or vehicle on day 8 p.i. as above. $n = 4$ per group. Unpaired two-tailed *t* test. *F*, effect of GW9662 on the viability of L3 stage larvae, adult worms, and fecundity of adult worms. L3 stage larvae and adult worms were treated with GW9662 or vehicle for 12 h followed by an ATP assay in the larvae and adult worms and number of eggs per female worm. Boiled larvae and worms were used as negative controls. Data shown is $n = 3$ per group. Unpaired two-tailed *t* test.

Selenium Affects Th2 Cells—It is known that the clearance of *N. brasiliensis* is Th2-dependent (19, 20). To determine if selenium increases the presence of IL-4 producing Th2 cells in the small intestine to facilitate a type 2 response, we used flow cytometry to determine the number of CD3$^+$IL-4-producing Th2 cells. IL-4 GFP reporter mice (IL-4/GFP-enhanced transcript, 4Get, knock-in mice) on selenium-deficient, selenium-adequate, and selenium-supplemented diets were injected with 500 L3 larvae subcutaneously as described earlier. On day 8 p.i., CD3$^+$GFP$^+$ lymphocytes from the lamina propria of small intestine were collected from *N. brasiliensis*-infected and non-infected mice. Interestingly, increase in dietary selenium levels led to a corresponding increase in CD3$^+$IL-4 producing (GFP$^+$) cells in the small intestine (Fig. 6*A*). However, only selenium-adequate mice showed a statistically significant increase in CD3$^+$GFP$^+$ cells upon infection compared with their corresponding selenium-deficient control mice.

Selenium Status Affects IL-13 Expression—In addition to IL-4, IL-13 is also highly expressed in *N. brasiliensis* infection (15, 24) and is important in the clearance of adult worms (15,

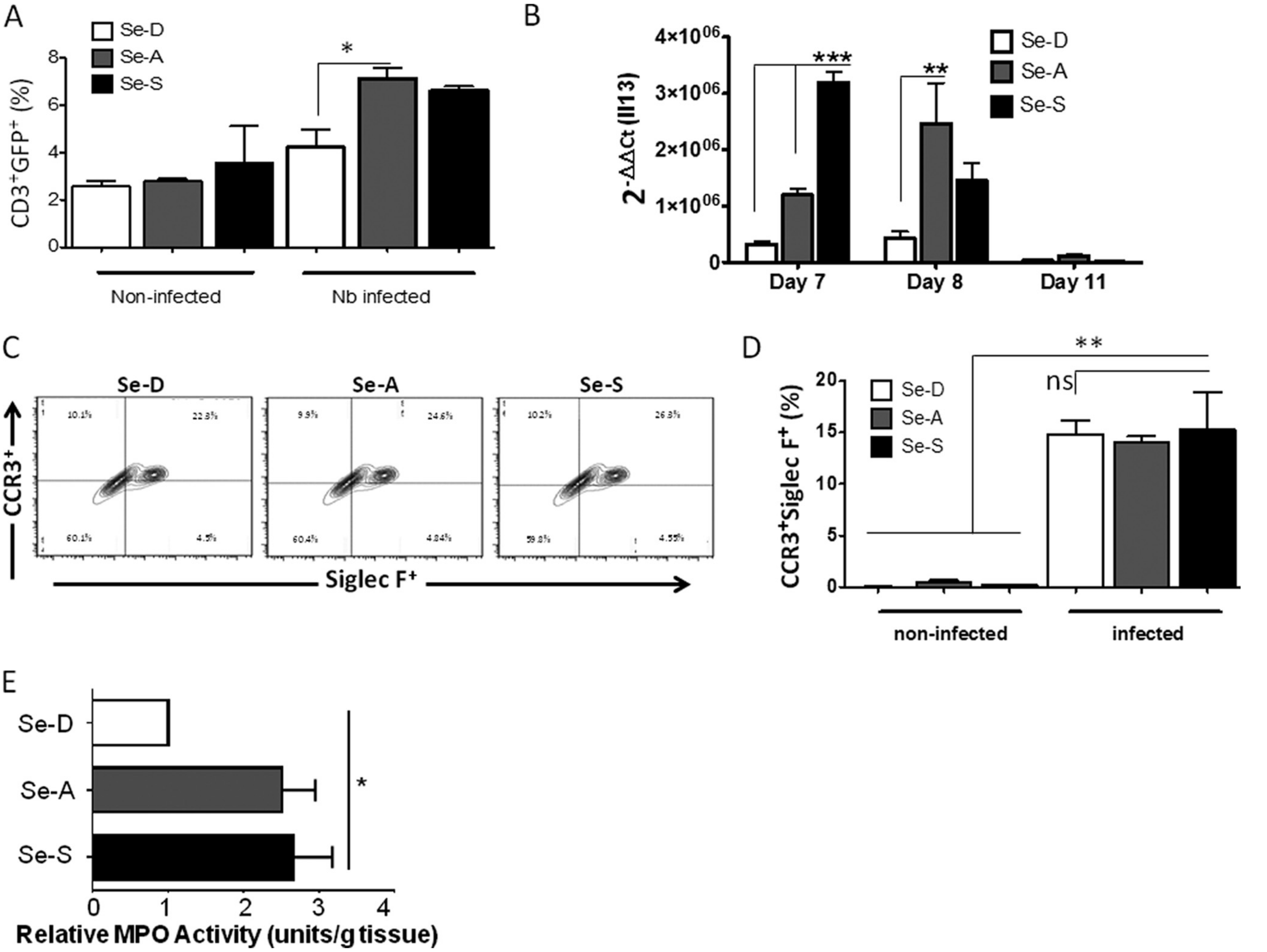

FIGURE 6. **Effect of dietary selenium on the expression of IL-4 and IL-13.** *A*, quantitation of the $CD3^+GFP^+$ T cells in uninfected 4Get mice were compared with *N. brasiliensis*-infected mice on the three diets on day 8 p.i. selenium-deficient (*Se-D*) mice were compared with selenium-adequate (*Se-A*) and selenium-supplemented (*Se-S*) mice within each infection group. *B*, selenium-dependent increase in the expression of IL-13 in the jejunal tissue from *N. brasiliensis*-infected C57BL/6 mice on selenium-deficient, selenium-adequate, and selenium-supplemented diets. Selenium-deficient mice were compared with selenium-adequate and selenium-supplemented mice within each day. *C*, representative scatter plots showing the $SiglecF^+CCR3^+$ cells in the lamina propria isolated from the small intestines of *N. brasiliensis*-infected C57BL/6 mice day 8 p.i. *D*, comparison of $SiglecF^+CCR3^+$ cells in the lamina propria of uninfected and *N. brasiliensis*-infected C57BL/6 mice on selenium-deficient, selenium-adequate, and selenium-supplemented diets day 8 p.i. Values are the mean ± S.E., with a total of $n = 9$ mice used. Selenium-deficient, selenium-adequate, and selenium-supplemented were compared with each other within each infection group. *Asterisks* represent significant differences between groups. *, <0.05; **, <0.01. Statistical differences were analyzed using two-way ANOVA with Tukey's post hoc testing. *E*, MPO activity in *N. brasiliensis*-infected jejunum of mice maintained on selenium-deficient, selenium-adequate, and selenium-supplemented diets. 500 infective larvae were inoculated into selenium-deficient, selenium-adequate, and selenium-supplemented mice, and jejunal tissue on day 8 was used for the assay. Values are the mean ± S.E. of $n = 3$ mice per group and are calculated relative to selenium-deficient group. *, $p < 0.05$ analyzed by one-way ANOVA.

17, 31). We determined if expression of *Il13* in *N. brasiliensis*-infected mice was selenium-dependent. qPCR was used to examine expression of *Il13* in jejunal tissue collected on days 7, 8, and 11 p.i. Expression of *Il13* was highest on days 7 and 8 p.i. in mice fed selenium-supplemented and selenium-adequate diets, respectively, compared with mice fed selenium-deficient diet, decreasing on day 11 p.i. in all three groups (Fig. 6*B*). These data strongly suggested that selenium status was an important factor in the regulation of IL-13 production in the gut in response to infection (Fig. 6*B*). *N. brasiliensis* infection is known to induce intestinal eosinophilia that could contribute to local production of IL-13 (44). To determine if intestinal eosinophilia was selenium-dependent, leukocytes were isolated from the lamina propria of *N. brasiliensis*-infected mice and examined by flow cytometry. Cells were stained for surface Siglec F and CCR3 to detect the presence of eosinophils. Compared with uninfected mice, the percentage of Siglec-F^+/$CCR3^+$ cells detected in *N. brasiliensis*-infected mice fed selenium-deficient, selenium-adequate, and selenium-supplemented diets were significantly increased (Fig. 6*C*). However, the percentage of Siglec F^+/$CCR3^+$ cells was not significantly different between mice fed different levels of selenium (Fig. 6*D*). Further analysis of the jejunal extracts on day 8 p.i. was associated with an increase in MPO activity in selenium-adequate and selenium-supplemented mice when compared with the selenium-deficient mice, suggesting the role of neutrophils in worm clearance (Fig. 6*E*).

Discussion

Studies have identified altered intestinal smooth muscle contractility, development of AAMs, and IL-4Rα- and STAT6-dependent Th2 cell polarization as effectors against gastrointestinal infections (1, 17, 20, 24). Although the beneficial effects of selenium on the clearance of gastrointestinal parasites have been reported (2, 4), there is little information on the mechanistic relationship that ties selenium status of the host in a helminth-infected gut.

Clearance of adult *N. brasiliensis* from the intestine between days 7 and 8 p.i. was associated with a reduction in parasite egg shedding in selenium-adequate- and selenium-supplemented mice. We assessed the effects of increasing concentrations of dietary selenium on the expression of macrophage polarization markers in the jejunum during infection with *N. brasiliensis*. Expression of *Fizz1* and *Ym1* increased on days 7 and 8 p.i., whereas expression of *Arg1* increased 7 days p.i. with a significantly high expression on day 8 p.i. Our data demonstrate that all three markers are associated with increasing levels of selenium in the diet, corroborating the relationship between dietary selenium and optimal worm clearance. Previous studies have demonstrated delays in worm expulsion in *N. brasiliensis*-infected mice that lack IL-4Rα on non-bone marrow-derived cells (45). This suggests that selenium-dependent effects may be more important to the pathway the larvae take to the small intestines without affecting the kinetics of worm expulsion itself, but the effects on adult worm fecundity in the intestine would argue for a local selenium-dependent mechanism.

Previous studies have shown the expulsion of a related gastrointestinal nematode parasite (*H. polygyrus bakeri*) during a secondary memory response was delayed in selenium-deficient-fed mice despite increased smooth muscle contractility (2). This suggests that the effect of selenium on smooth muscle function during a memory response in nematode infection may be absent or less critical to result in a multifaceted protective immune response against the nematode. Recent evidence has demonstrated a link between dietary selenium and Relmβ/Fizz2 expression in the intestine (4) during the memory response to *H. polygyrus bakeri* that could explain the reduced clearance of adult worms in selenium-deficient mice (2, 21). Even though the differences in host responses vary with helminths, further studies are necessary to directly implicate the role of AAMs in the small intestine in helminth clearance. It is also important to determine if selenium status affects infiltration and/or development of AAMs in the small intestine. In addition, direct measurement of smooth muscle contractility as a function of selenium concentration would help in elucidating the underlying mechanisms.

To address whether the selenoproteome as a whole had an effect on pathogenesis and AAM polarization, we utilized a macrophage-specific deletion of the *Trsp* allele (*Trsp*$^{fl/fl}$*Cre*^{LysM}) (12). Compared with WT mice, *Trsp*$^{fl/fl}$*Cre*^{LysM} mice displayed a significant delay in adult worm clearance despite being fed diets supplemented with selenium (0.4 ppm). These data demonstrate that the ability to increase selenoprotein expression in monocytes/macrophages via dietary supplementation with selenium can be potentially harnessed to impact host-pathogen interaction. Comparative proteomic analysis of infective larval (L3) and adult worm stages of *N. brasiliensis* indicated the expression of a group of antioxidant enzymes, including the protein disulfide oxidoreductase (most likely a thioredoxin reductase), protein disulfide isomerase, peroxiredoxin, superoxide dismutase (Cu/Zn), and thioredoxin-like proteins (46). Thus, it appears that the larvae and/or adult worms may be well positioned to maintain infectivity and fecundity even under selenium-deficient conditions. However, systematic knockdown studies could provide further evidence once the complete genome sequence of *N. brasiliensis* becomes available.

Although worm clearance from the intestine requires STAT6 (17, 24), the nuclear receptor PPARγ has also been shown to play a role in clearance (34). Infection of selenium-adequate and selenium-supplemented mice increased the expression of COX-2 and H-PGDS complementing our previous data that demonstrated the ability of selenium to shunt the AA-COX pathway from pro-inflammatory PGE_2 and thromboxane A_2 toward anti-inflammatory and endogenous PPARγ agonist, 15d-PGJ_2 (7, 37, 38), in macrophages. Inhibition of the COX pathway by indomethacin significantly delayed adult worm clearance in selenium-adequate and selenium-supplemented mice, whereas GW9662 decreased the expression of *Mrc1* and *Arg1*, two downstream PPARγ target genes (47, 48), increased adult worm burden in selenium-adequate mice. *In vitro* treatment of L3 stage larvae and adult worms with indomethacin or GW9662 had no impact on the viability of the L3 stage larvae or viability and fecundity of adult worm *per se*. Taken together these results suggest that the selenium status of the host is a key factor in the clearance of *N. brasiliensis* that involves PPARγ. Similarly, administration of 15d-PGJ_2 (0.050 mg/kg) to selenium-deficient mice treated with indomethacin significantly decreased fecal egg shedding from days 7 to 11 p.i., whereas 16,16-dimethyl-prostaglandin E_2 had no effect. In agreement with the qPCR results of expression of prototypical AAM markers, 15d-PGJ_2 treatment also increased CD11b$^+$ Arg-1$^+$ cells in the small intestine, suggesting that selenium effects are mediated in part through the endogenously produced prostanoids, such as 15d-PGJ_2, to modulate AAM expression. More importantly, the role of COX-derived metabolites in helminth clearance also begs an important question regarding the likely role of nonsteroidal anti-inflammatory drugs as a potential confounder in selenium-dependent anti-helminth-protective mechanisms, which is currently unknown.

Previous studies have shown clearance of *N. brasiliensis* to be sensitive to the effects of IL-13 (15, 18, 24). Interestingly, selenium-dependent increase in the expression of IL-13 in the jejunum of infected mice perhaps serves as a key mediator of helminth clearance. This is likely because IL-13 has also been reported to increase the endogenous production of 15d-PGJ_2 in macrophages (49). Thus, it is possible that selenoprotein expression is critical in the IL-13-dependent induction of 15d-PGJ_2 by macrophages. An additional question that is equally important is the source of IL-13. Based on our data (Fig. 6*E*), it appears that neutrophils, in addition to ILC2 cells (16), could serve as a potential source of IL-13, which has been demonstrated recently (31), but the role of selenium in this process is intriguing and needs to be further examined.

In conclusion, our results suggest that increases in dietary selenium decreases parasite egg production (fecundity) and lower numbers of adult *N. brasiliensis* in the intestine. This is likely achieved through an increased activity of selenoprotein expressing AAMs in the small intestine. Further studies are required to establish the exact mechanisms of clearance, particularly the role of selenoproteins in innate immune cells, such as neutrophils. The role of nonsteroidal anti-inflammatory drugs and PPARγ agonists in macrophage polarization needs to be elucidated to examine if exogenous factors (therapeutic drugs) impact host-pathogen interactions. Little is known about the dynamics of these therapies in gastrointestinal helminth infections, and a better understanding of these processes may help develop more effective regimens to cure such infections.

Author Contributions—S. M. N. and K. S. P. conceived and coordinated the study and wrote the manuscript. S. M. N. designed, performed, and analyzed the data shown in Figs. 1, 2, 3, 4, 5, and 6. A. E. S. contributed to the preparation of the manuscript, examined gene expression in Figs. 1 and 3, and performed and analyzed the data shown in Fig. 4*A*, Fig. 5, *B–F*, and Fig. 6*E* and LC-MS/MS analysis of Δ^{12}-PGJ_2. J. L. J. provided technical assistance with Fig. 2. B. A. C. provided mice and contributed to the preparation of the manuscript. J. F. U. provided L3 helminth larvae and contributed to the preparation of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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