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THE ESSENTIAL ROLE OF SELENOPROTEINS ON MACROPHAGE PHENOTYPE SWITCHING IN HELMINTH CLEARANCE

A Dissertation in

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by

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ABSTRACT

Selenium (Se) is an essential micronutrient with anti-inflammatory properties that are fundamental to human health. A vital component of many metabolic pathways, an inverse causal relationship of Se with inflammatory diseases, including cancers, and infections has been suggested. While deficiency is frequently the center of discussion, some studies have emphasized the importance of different Se levels in disease regulation, establishing the true physiological significance of this element. The metabolic action of Se is seen through selenoproteins, which are synthesized by a complex mechanism. With over 25 identified selenoproteins, the health implications of these proteins are seen in their ability to decrease oxidative stress and inflammation. The study presented here aims to understand the anti-inflammatory effects of Se.

Using a combination of *ex-vivo* and *in-vitro* macrophage models, the anti-inflammatory benefits of Se were investigated using a bacterial endotoxin, LPS, and Th-2 cytokine, IL-4, treated murine bone marrow derived macrophages (BMDM). Previous studies have shown that Se supplementation of macrophages led to a decrease in LPS-induced expression of inducible nitric oxide synthase (iNOS), a prototypical marker of classical (M1) macrophage activation. In the current study, IL-4 treated macrophages supplemented with 100 nM Se significantly increased the expression of alternatively activated macrophage (M2) markers, Arg-I, Ym1, and Fizz1. Se treatment also increased the enzymatic activity of Arg-I. Conversely, expression of classically activated macrophage (M1) markers, TNFα, iNOS, and IL1β, were significantly decreased in LPS treated macrophages cultured in 100 nM Se and IL-4, suggesting a synergistic effect of Se and IL-4. Interestingly, studies utilizing $Trsp^{fl/fl}Cre^{LysM}$ KO mice macrophages, characterized by a total loss of selenoprotein expression, or those lacking GPX1, a highly expressed selenoprotein, showed a complete abrogation of Arg-I activity, even under high Se (250nM) conditions, demonstrating the importance of selenoproteins for alternative macrophage activation, leading to attenuated inflammation.

To effectively translate these findings into an *in-vivo* model, a gastrointestinal helminthic parasite model was used. The helminthic parasite *Nippostrongylus brasiliensis (Nb)* is a short-lived murine parasite that migrates from the skin via the lungs to the small intestines, before clearance 14 days after infection. Mice infected with *Nb* and supplemented with Se significantly increased the expression of the M2 markers Arg-I, Ym1, and Fizz1 in the small intestines, while decreasing the presence of intestinal worms and fecal eggs. To implicate macrophage-specific selenoproteins in resolution of the infection, Se supplemented $Trsp^{fl/fl}Cre^{WT}$ and $Trsp^{fl/fl}Cre^{LysM}$ mice were infected and compared. Results showed a complete abrogation of M2 marker expression with a significant increase in intestinal worms and fecal eggs in the Trsp KO mice, even in the presence of Se supplementation. Moreover, inhibition of the COX pathway using indomethacin, a non-specific COX inhibitor, stunted the expression of M2 markers despite high Se levels. Intriguingly, treatment of *Nb*-infected Se deficient mice with 15d-PGJ_2, an anti-inflammatory prostaglandin metabolite of the COX pathway enhanced by dietary Se, partially recapitulated the effect of Se deficiency.

Taken together, the experiments conducted in this dissertation suggest that optimal Se status, in the form of selenoproteins, is critical to shunt macrophage activation towards an M2 phenotype that promotes enhanced clearance of gastrointestinal parasites.

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LIST OF ABBREVIATIONS

Se-selenium

Sec-selenocysteine

SeMet-selenomethionine

H_2Se-selenide

GS-Se-GS- selenodiglutathione

GSH- glutathione

Cys- cysteine

GPx- Glutathione peroxidase

TrxR- thioredoxin reductase

DIO- iodothyronine deiodinases

H_2O_2- hydrogen peroxide

PLA2- phospholipase A2

AA- arachidonic acid

FAD- flavin adenine dinucleotide

NADPH- Nicotinamide adenine dinucleotide phosphate

ER-endoplasmic reticulum

ROS- reactive oxygen species

RONS-reactive oxygen nitrogen species

SECIS- Sec insertion sequence element

SBP2- SECIS-binding protein 2

COX- cyclooxygenase

iNOS-inducible nitric oxide synthase

NFκB- nuclear factor-kappa B

PUFA- polyunsaturated fatty acids

15d-PGJ_2- 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2

PG-prostaglandin

CyPG-cyclopentenone prostaglandin

$\mathbf{\Delta}^{12}$-PGJ_2- $\mathbf{\Delta}^{12}$- prostaglandin J_2

IκB- inhibitor of kappa B

IKK- inhibitor of kappa B kinase

PPAR- Peroxisome proliferator-activated receptors

RXR- retinoid-X receptor

PPRE- PPAR response elements

TNFα– tumor necrosis factor alpha

Arg-I-arginase I

IFNγ-interferon gamma

TLR-toll like receptor

NO-nitric oxide

IL-interleukin

LPS-lipopolysaccharide

STAT-signal transducer and activator of transcription

KO-knockout

WT-wild type

MDSC- myeloid derived suppressor cells

TAM-tumor associated macrophages

IBD-inflammatory bowel disease

BMDM-bone marrow derived macrophage

NSAID- nonsteroidal anti-inflammatory drugs

EPA- eicosapentaenoic acid

DHA- docosahexaenoic acid

Mrc-1- mannose receptor

H-PGDS- hematopoietic prostaglandin D_2 synthase

MSA- methylseleninic acid

Se-D- selenium deficient (0.01ppm selenite diet)

Se-A- selenium adequate (0.1ppm selenite diet)

Se-S- selenium supplemented (0.4ppm selenite diet)

GAPDH-glyceraldehyde 3-phosphate dehydrogenase

ANOVA-analysis of variance

PGC-1β- PPARγ co-activator 1

LP-lamina propria

IEL-intraepithelial lymphocytes

CL-clodronate liposomes

GFP-green fluorescent protein

i.p- intraperitoneal

ACKNOWLEDGEMENTS

I frequently make a joke that I have been at Penn State since Jesus was a boy. And while that is somewhat true, my decision to return to Penn State to obtain my PhD, after being here for 5 years as an undergrad, was a simple decision for me, and one of the best ones I've made. My original desire was to go to medical school, with plans on becoming a radiation oncologist. This type of physician seemed to have a better life than others- (almost) regular hours, very few weekends on call, and a bunch of zero's at the end of ones paycheck. Who wouldn't want that? But when I really started to prepare myself for medical school and taking the MCAT's, my heart wasn't in it. Medical school didn't make me excited about my future. Working as hard as I had seen other work just to get accepted into a school did not seem like a great career choice after all. Luckily my decision to not pursue medical school did not put a dark cloud of worry over my head about my future. I already had a new passion -research.

As an undergrad at Penn State, I worked in two laboratories. The first was a virology lab, which I hated, mostly because I was the only girl surrounded by men who knew more than me and didn't make me forget that. My second lab experience was with Dr. Andrea Mastro. I had a fantastic time here and was able to learn a lot. In fact, it was the support of Dr. Mastro and the other students in the lab that allowed me to see how fulfilling a career in research was. So I really owe a lot of gratitude to Dr. Mastro, as it was her willingness to bring me into her lab and give me my first solo project that propelled me on the path I am on today. I also want to thank Dr. Laurie (Schuman) Moss, who worked with me in Dr. Mastro's lab. She was one of many who encouraged me to come back to grad school. She has also been an adviser and great friend from afar, and her encouragement has been wonderful. My old Penn State family, from my time as an undergrad, has provided me with encouragement, an ear to listen, proof reading, a ride to the airport, scholarships, hot fries from McDonalds, cards on my birthday, encouraging text messages and bible versus, and much more. It would take too much space to list all their names, but I hope when they read this they will understand how valuable they have been in my life. My new Penn State family has done the same. Since coming back for grad school, I have met some people who

have completely changed my life: Dr. Barbara Farmer, Angeline Brown, Dominique Cowart, Cheri Lee, Dr. Stephanie Preston, Pastor Harold MacKenzie, Ms. Vicki, and Candace Lyons. There were times when I was three steps from walking away from grad school and going back to work in DC. But the individuals above, plus others, held me down, kept me going, and were there for me whenever I needed them. I look forward to keeping our friendships going for many years, and hope that I have affected their lives and they have mine.

I cannot write much more and not mention my graduate lab. I have no idea how I was lucky enough to become a part of this lab, but I am so happy and grateful that it happened. Coming back to grad school, I had no idea what I wanted to do. I just knew I loved research and wherever I ended up I would do the best I could. During my first semester, I rotated in Dr. Sandeep Prabhu's lab. I didn't know much about what he did, except that I remember he did collaboration with Dr. Mastro many years ago. So I decided to give his lab a chance. Best. Decision. Ever. I do not think I would be the scientist I am today if I was in any other lab. Looking back on who I was in 2008 until now, I can see the changes within myself. I am more confident about my abilities in learn and teach, I feel prepared and knowledgeable when I speak with others inside and outside my field, and I feel like I have a great future ahead of me that I have been well prepared for. Sandeep is a wonderful adviser who is patient and understanding. He pushes you, but not more than he knows you are capable of. He prepares you for any and every situation, and is there to lend a helping hand if you need it. The lab environment is one of learning and freedom. Not once did I feel I was being micromanaged or held back. He allowed me to become independent, and encouraged further growth. I am truly grateful for all the opportunities afforded to me in his lab. And I will always remember the type of adviser he was and hope to emulate that when my times comes. And a million thank you's to all those who helped me within the lab; Dr. Ryan Arner, Dr. Naveen Kaushal, Dr. Vivek Narayan, Dr. Ujjawal Gandhi, Emily Finch, Stephan Fincham, Ariel Baggett, Denzel Middleton, and all the others. Your help over the years has made me a better scientist and a better person.

Lastly, I want to thank my family. I know for a fact I would not have grown into the person I am without the love and support they have given to me over the years. My parents have been a tremendous support system, always encouraging me and showing me how my decisions have encouraged them and my younger sisters to do greater things in their own lives. Just the other day my mother told me she always wondered what her purpose in life was, but it is clear to her now. It was to have me, so I can go out into the world and cure it of all its problems. Encouraging words such as this have driven me to push myself to be great, and I feel I have done that. My time in grad school was allowed me to do many things and accomplish a great deal more than I ever imagined I was capable. I hope that the long hours, and blood sweat and tears I put into grad school encourage my sisters to pursue their own dreams and goals. I hope I have been an example to them that any and everything is possible, and with hard work and dedication you can achieve a lot. I want to thank my friends, who call daily, send text messages and cards, visit for the weekend, send cookies and other delicious sweets, and have been there for me from day 1-I love you all dearly and cannot express in words how grateful I am to have you in my life. Grad school is not easy, but the rewards at the end far outshine any of the bad things. I am truly grateful to everyone who had touched my life and I hope I am able to make you proud in the future. I thank God for this opportunity, and for providing me with the strength to complete this journey successfully.

Chapter 1

Literature Review

Selenium forms and selenoproteins

Discovered in 1817 by Swedish chemist Jöns Jacob Berzelius, Selenium (Se) is an essential trace element that plays a major role as an antioxidant and chemopreventive agent. A metalloid that belongs to the same group as oxygen, sulfur, and tellurium (1, 2), Se is found in several areas including soil and numerous food sources. Inorganic forms of Se, selenite and selenate, are found in soil where concentrations are derived from seleniferous (Se containing) sources such as sandstone and limestone, or natural sources such as volcanoes (3, 4). Fish, eggs, dairy products, fruits, and vegetables are also important sources of inorganic Se. Interestingly, these food groups also contain large amounts of organic forms of Se, such as selenomethionine (SeMet) and selenocysteine (Sec) (4) (5). These forms of selenium are generally known as selenoamino acids (2). The amount of Se available in these sources greatly depends on the concentration, quality, and species contained within the soil or animal feed. Plant uptake studies reveal selenate to be taken up more rapidly than selenite; however, selenite is better retained (3, 4).

Assimilation of inorganic and organic Se occurs through a series of metabolic interconversions, culminating at the production of selenide (H_2Se), the checkpoint metabolite. While inorganic forms of selenium are reduced directly to H_2Se (Figure. 1), their mechanisms differ significantly. Selenate is directly reduced to selenite, utilizing rigorous, yet to be described, reducing conditions (1, 2, 6). Conversely, through a series of intermediates, which includes selenodiglutathione (GS-Se-GS), selenite is reduced to H_2Se using both a glutathione-glutaredoxin and thioredoxin systems (1, 4, 7). In contrast to inorganic Se species, SeMet and Sec are transformed to selenide through a lyase-reaction dependent pathway. Through the utilization of β-lyase, Sec is transformed directly to selenide (Figure. 1) (1, 4, 6). SeMet is transformed to selenide in one of two ways. The first is through a trans-selenation pathway into Sec via a selenocystathionine intermediate, followed immediately by a β-lyase reaction (Figure. 1) (4). The second method utilizes a γ-lyase reaction, producing methylselenol, followed by demethylation into selenide (1, 2) (Figure. 1).

The γ-lyase reaction occurs via direct cleavage of the C-Se bond at the γ position of SeMet.

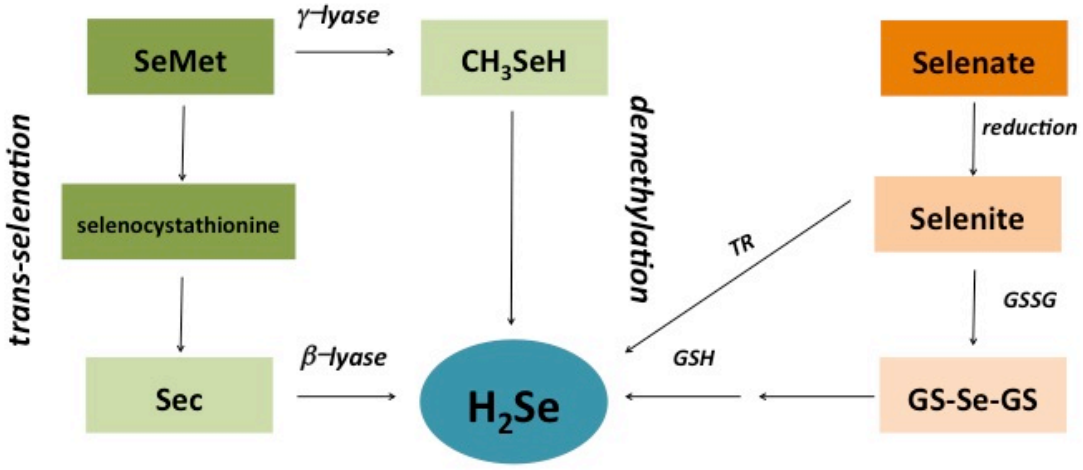

Figure 1: Metabolic pathway of the assimilation of inorganic and organic forms of Se. Inorganic forms of Se, selenite and selenate, are reduced directly to H_2Se through reduction and GSH. Conversely, organic forms of Se utilize a γ-lyase and β-lyase pathway. Sec is converted directly to selenide via β-lyase. SeMet can be converted in two ways: trans-selenation utilizing β-lyase, or demethylation utilizing γ-lyase.

As an intermediate in the interconversions of Se, the importance of selenide lies in its utilization for the biosynthesis of selenoproteins. Found in all lineages of life, selenoproteins are defined as proteins that contain Se in the form of Sec. The selenoamino acid categorized as an organic form of Se, Sec is also commonly known as the 21st amino acid. Similar in structure to cysteine (Cys), Sec replaces the sulfur atom with Se. Glutathione peroxidase (GPx), thioredoxin reductase (TrxR), and iodothyronine deiodinases (DIO) are the three most common classes of selenoproteins and are the most extensively studied.

The first protein shown to incorporate Se in the form of Sec, glutathione peroxidases are known as the major component of antioxidant defense. There are five Sec-containing species of GPx found in humans: GPx1, GPx2, GPx3, GPx4, and GPx6 (7, 8). The original glutathione peroxidase now referred to as cytosolic or cellular, GPx1 is a ubiquitously expressed tetrameric protein with four identical subunits that contain

a Sec residue, metabolizing hydrogen peroxide (H_2O_2) and some organic hydroperoxides (8-11). Mice deficient in GPx1 show no changes in normal development. However, studies have demonstrated an increased susceptibility to H_2O_2-induced oxidative stress, indicating the importance of GPx1 in mediating the protective effects of Se (8). GPx1 has also been shown to metabolize cholesterol and long-chain fatty acid peroxides. It cannot, however, metabolize fatty acid hydroperoxides in phospholipids (9). To be able to carry this out, it must be accompanied by phospholipase A2 (PLA2), the enzyme responsible for the release of fatty acids such, as arachidonic acid, (AA) from phospholipid membranes (9, 12).

Members of the pyridine nucleotide-disulfide oxidoreductase family, three TrxR have been identified in mammals: TrxR1 found in cytosol/nucleus, TrxR2 in mitochondria, and TrxR3, a thioredoxin-glutathione reductase, specific to the testes (7, 8, 13). The structure of TrxR contains both FAD and NADPH binding domains, which take part in reduction and electron transfer. Sitting in a head-to-tail position, the N and C-termini of TrxR contain conserved active site sequences (7, 8)- a redox-active dithiol and a selenothiol active site, respectively, where Sec is located (Figure. 2). Together these form the redox active center. In general, TrxR plays a direct role in reducing the intracellular redox environment through reduction of H_2O_2 and lipid hydroperoxides (14). TrxR1 has been described as essential to cell proliferation, often found in developing tissues. In TrxR1 knockout models, deletion resulted in early embryonic death (8) due to developmental abnormalities and growth inhibition. Embryonic death is also seen in mice with deletions of TrxR2. Within the heart, mutations in TrxR2 lead to the development of cardiomyopathy as well as other cardiac defects (15).

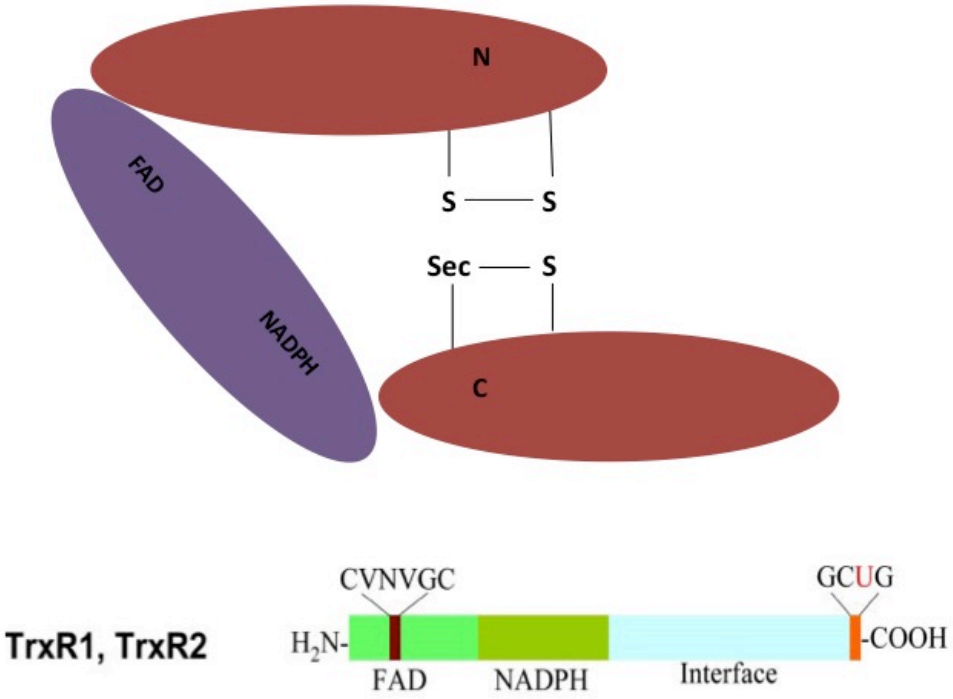

Figure 2: Thioredoxin Reductase structure. TrxR sits in a head-to-tail configuration. The N-termini of TrxR contains an active dithiol. The C-termini contains a selenothiol in its active site. In between the N and C terminus are the FAD and NADPH binding sites (*Adapted and modified from Lu et.al, JBC 2009*).

Iodothyronine deiodinases (DIO) are a third group of selenoproteins whose redox-protective effects are important in thyroid hormone metabolism (4, 8, 16). Through cleavage of iodine-carbon bonds, DIOs catalyze the activation and inactivation of thyroid hormones T4, T3, and rT3 (8, 17). DIO1 and DIO2 work in activation, catalyzing the deiodination of T4 into T3 (7). This conversion supplies a significant portion of circulating T3 in plasma (16), with recent evidence indicating DIO2 may have a greater contribution (8). Interestingly, DIO1 shows a more substantial contribution when there are higher levels of T4 (8). DIO3 catalyzes the deiodination of T4 to rT3, the inactive iodothyronine through an iodine moiety removal (8). The significance of Sec presence in DIOs lies in its catalytic activity, where *in vitro* experiments replacing Sec with Cys in the active site shown a significant decrease in catalytic activity, abrogating deiodinase activity (8). Found mostly in the thyroid, liver, and pituitary, DIO1 knockout mice exhibit no changes in growth, development or reproduction under laboratory conditions, but show abnormal patterns of

circulating hormones and metabolites (16). Expressed most prominently in the pregnant uterus, placenta, and neonatal brain, DIO3 knockout models exhibit significant changes in growth, impaired fertility, and reduced viability (8). These knockout models demonstrate that the function of DIOs in specific tissues not only help maintain plasma levels, but also regulate organ homeostasis and function (7, 18).

In addition to the three common classes of selenoproteins, thirteen others have been identified through various biochemical studies. First reported in 1982, SelP is the second major selenoprotein found in plasma (8), suggested to be a Se transport protein (19). Expressed in several tissues including brain and testes, SelP is unusual in that it contains 10 Sec residues per protein molecule (8). Strikingly, in vitro data has shown SelP can directly reduce lipid hydroperoxides, possibly possessing antioxidant functions. Another selenoprotein, SelW, also has potential antioxidant functions, binding with high affinity to glutathione (GSH). Interestingly, SelW was originally identified as a selenoprotein lost in white muscle disease in cattle and sheep, possibly inheriting its letter designation from this observation. Structure-determination studies show that Sep15 may be a thiol disulfide isomerase, involved in disulfide bond formation in the ER (8). As part of the methionine sulfoxide reductase (Msr) family, SelR catalyzes the reduction of oxidized methionine, which occurs during increases in reactive oxygen species (ROS). SelN is localized to the ER membrane, playing a vital role in muscle development (17). Also found localized to the ER, SelS expression is induced by ER stress, possibly regulating cytokine production in macrophages (8). Distantly related to Sep15, SelM is moderately expressed in heart, lung, and kidney tissues. High expression of SelM is seen in the brain, playing a possible role in Alzheimer's (17). Further studies are required to completely determine its function. Expressed predominately in the heart and skeletal muscles, overexpression studies of SelK lead to decreased intracellular levels of ROS, indicating SelK may perform antioxidant functions (8). A nuclear-localized DNA binding protein, SelH can increase GPx activity and GSH levels. Furthermore, SelI was found to be an ethanolamine phosphotransferase, acting as a mammalian

phospholipid-synthesizing enzyme. The three remaining selenoproteins, SelT, SelV, and SelO require further elucidation to determine their functions.

Although the specific functions and roles selenoproteins play within the human body have been identified, it is necessary to understand the mechanisms behind their biosynthesis.

Selenoprotein biosynthesis

The mechanism of selenoprotein biosynthesis is quite remarkable. In the initial step, selenophosphate is synthesized from selenide via a selenophosphate synthetase (SPS2) (1, 19). Catalyzed by phosphoseryl-tRNA $^{[ser]\,sec}$ kinase, this phosphate is used to phosphorylate the serine on seryl-tRNA $^{[ser]\,sec}$, followed by conversion to Sec-tRNA $^{[ser]\,sec}$ catalyzed by selenocysteine synthase (20, 21). A unique trait of Sec-tRNA $^{[ser]\,sec}$ is that is controls all selenoprotein family expression (8).

Synthesized on its own tRNA, Sec is coded by UGA, which in most circumstances is one of the three translation termination codons. Therefore, it is important to be able to differentiate between the two functions. To do so, there are several components that are required for the synthesis of selenoproteins. Seemingly the most important piece to the biosynthesis puzzle is the Sec insertion sequence element, or SECIS, a *cis*-acting stem loop structure present in the 3' untranslated region of selenoprotein genes. The presence of the SECIS element not only helps increase read-through of the mRNA (8), but prescribes that any UGA sequence within the coding region of the mRNA serve as Sec, dependent upon the distance between the sequence and SECIS element (22). Each SECIS element contains a conserved AAR motif within an apical loop, and a SECIS core quartet containing four non Watson-Crick interacting base pairs, which serves as the main function site (22). The SECIS element can be categorized into two forms, based on the absence (form 1) or presence (form 2) of an additional internal loop formed by an A or G residue that precedes the SECIS core (8, 22) (Figure. 3).

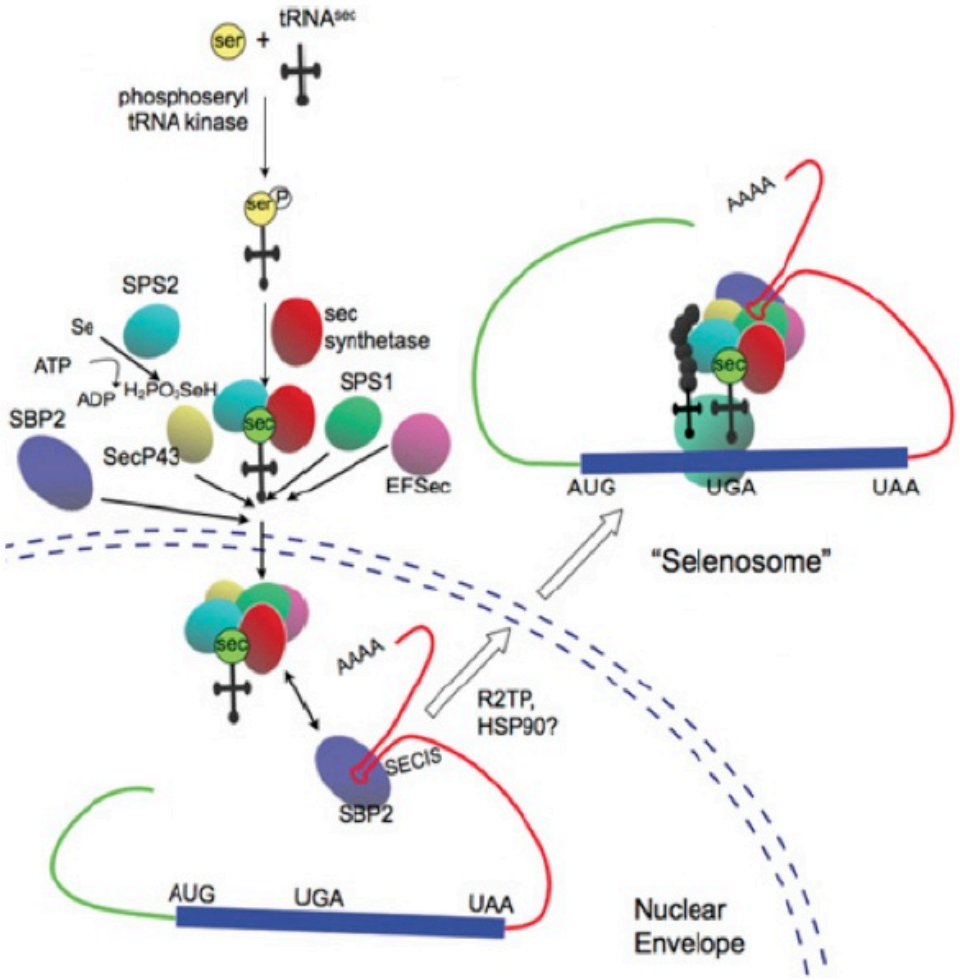

Figure 3: Biosynthesis of selenoproteins. SBP2, eEFSec, SLA, and SecP43 bind to the Sec insertion sequence (SECIS) element located in the 3' UTR of selenoproteins, forming a complex. This complex binds to the UGA codon, inducing translation and the production of selenoproteins (*Adapted and modified from Bellinger et.al, Biochemical Journal, 2009*).

Another important task of the SECIS element is recruitment of the *trans*-acting factor SBP2 (SECIS-binding protein 2), forming a tight SECIS-SBP2 complex (22, 23). Binding directly to SECIS through the core quartet, SBP2 also binds to a ribosomal subunit, prompting some to suggest pre-selection of ribosomes for Sec insertion (20, 22). In addition, SBP2 binds and recruits a second *trans*-acting factor eEFSec, an elongation factor that recruits Sec-tRNA $^{[ser]\,sec}$ for insertion into the growing polypeptide chain (8, 14, 22). Further conveying specificity in selenoprotein biosynthesis, eEFSec is specific only to the insertion of Sec; it is not involved in the insertion of the 20 other amino acids (22). It is interesting to note that most

selenoproteins contain one SECIS element, resulting in the addition of one Sec residue in each protein molecule. The exception to the rule, SelP contains two SECIS elements that decode 10 UGA codons (22). The mechanisms behind this are very complicated, concluding in the incorporation of up to 10 Sec residues in this selenoprotein.

Recent studies have determined additional *trans*-acting co-factors as contributors to selenoprotein biosynthesis. Initially established in E. coli and encoded by the SelA gene (20), a mammalian Sec synthase protein has been identified as SLA (soluble liver antigen). Specifically, SLA functions in mediating Sec incorporation into selenoproteins through formation of a complex with SecP43 (8, 20). SecP43 was first identified as a regulator of Sec synthase-Sec-tRNA $^{[ser]\,sec}$ complex shuttling between the nucleus and cytoplasm (20). Further characterization has shown it to stabilize the eEFSec-SBP2- Sec-tRNA $^{[ser]\,sec}$ complex. Together with SLA, SecP43 enhances selenoprotein expression (20), where depletion of either protein leads to decreased expression (8).

Lastly, the ribosomal protein L30 competitively binds to the SECIS element, displacing SBP2 (8, 20). Binding of L30 happens preferentially in the presence of Mg^{2+}, which induces a kink-turn in the SECIS structure (20). This structural change helps tether the SECIS element to the large ribosomal subunit, directly releasing Sec-tRNA $^{[ser]\,sec}$ into the ribosomal A site (8, 20).

Selenoprotein biosynthesis can only happen with the right concentrations of Se in the body. Countless studies have assessed the proper amount of Se needed to have any measurable effects in the population. While estimates of Se intake needed vary geographically, the overall effects of Se on human health remain the same.

Selenium and Health

Cellular redox and oxidative stress control: Cellular respiration produces ROS, by-products that can cause cellular damage through the oxidation of lipids, proteins, and nucleic acids (24). While accumulation of ROS can be injurious, studies have shown they can also participate in cell signaling and regulation (25). A disruption in the balance between ROS and antioxidants leads to oxidative stress (24). Types of ROS include hydrogen peroxide (H_2O_2), superoxide anion (O_2-•), and the highly reactive hydroxyl radical (•OH) (26, 27). Antioxidants that counter ROS include superoxide dismutase (SOD), catalyze, and GPx.

As a selenoprotein, GPx functions specifically through its reduction of peroxides (i.e. H_2O_2). The GPx catalytic cycle includes GSH as a necessary co-factor, acting as the reducing substrate (24). The mechanism of H_2O_2 reduction begins with the catalytic oxidation of the thiol group in Sec on GPx (24, 28). This reduces H_2O_2 to water and produces selenenic acid, which undergoes immediate reduction by GSH to generate a selenenyl-sufide adduct (24). The adduct then reacts with an additional GSH, and is reduced again (28) (Figure. 4). The reduction and oxidation within the cycle is coupled with the pentose phosphate pathway, which provides NADPH for the reduction of oxidized GSH (28). Although GPx1, GPx2, and GPx3 share substrates, the locations of their activity differ. GPx2 antioxidant abilities are focused in the gastrointestinal tract, quenching any increases in H_2O_2 concentration from bacterial-induced inflammation or natural microbial disturbance (8). Further, the antioxidant abilities of GPx3 are seen best in plasma, where it is the second most abundantly found selenoprotein (29). Here, it effectively reduces substrates that include H_2O_2, and fatty acid hydroperoxides (8). Coincidentally, GPx4 also reduces fatty acid hydroperoxides (29). However, unlike the other GPx proteins, GPx4 directly reduces its substrate by electrons from protein thiols (4). It is interesting to note that extensive research has been done to investigate Se-containing GPx mimics. Ebselen, a well-known GPx mimetic, inhibits ROS induced DNA damage (24, 30). Unfortunately, however, the exact mechanisms of how Ebselen achieves this are still not well understood.

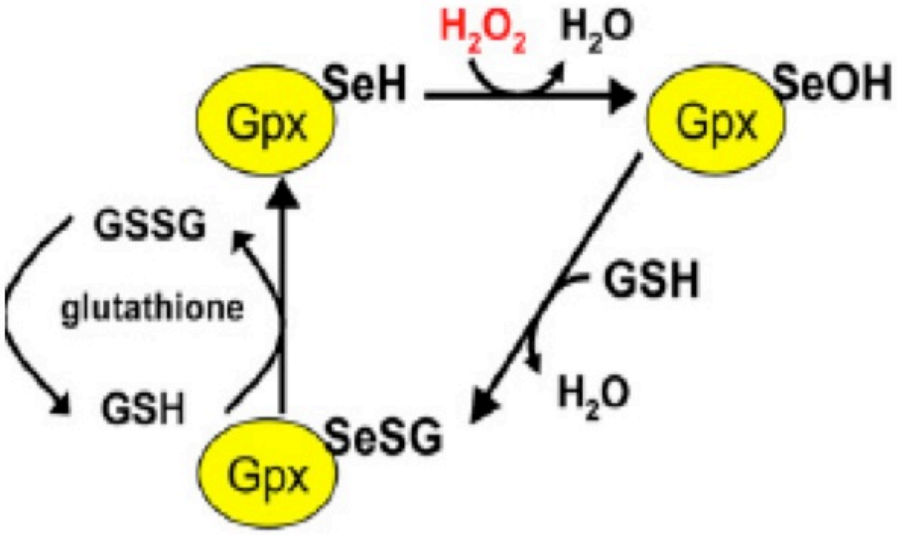

Figure 4: GPX Redox cycle. The catalytic reduction of peroxides, such as H_2O_2, involves the oxidation of thiol group, such as those contained in GPX. Recycling these oxidized peroxidase enzymes requires glutathione (GSH). (*Adapted and modified from Veal et. al, Molecular Cell, 2007*).

Similar to GPx, TrxR is part of a redox system (8), alerted to increased levels of ROS through Sec, which acts as a redox sensor. As its name suggests, TrxR acts upon the protein substrate thioredoxin (Trx), catalyzing the NADPH-dependent reduction of the oxidized substrate (7, 8). This is significant because Trx itself is an important cellular redox regulator, reducing protein disulfide bonds in enzymes and transcription factors critical in DNA synthesis, apoptosis, cellular proliferation and oxidative stress defense (8). The mechanism of TrxR-dependent reduction involves an NADPH to FAD electron transport, reducing the disulfide bond in the N-terminal (7). The N-terminal thiol then transfers the electrons to a selenenylsulfide bond in the C-terminal active site (8), where it will then transfer and reduce the substrate (7, 15) (Figure. 5). In addition to Trx, substrates of TrxR include ebselen, calcium binding protein 1 and 2, and lipid hydroperoxides (7, 29).

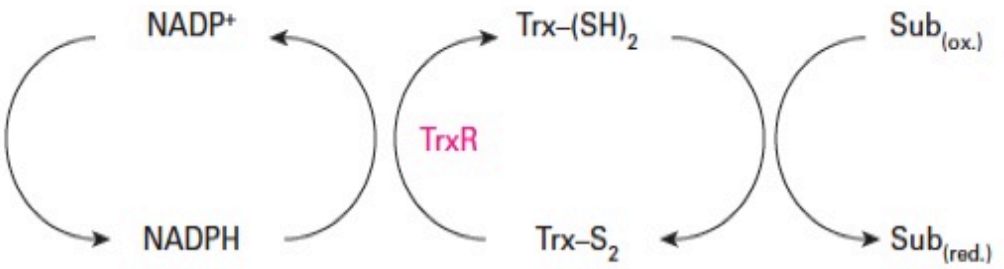

Figure 5: Thioredoxin Reductase system. TrxR relies upon thioredoxin (Trx) and NADPH to catalyze the reduction of an oxidized substrate. Substrates include lipid hydroperoxides and hydrogen peroxide. The mechanism involves an NADPH/FADH electron transport chain (*Adapted and modified from Mustacich et.al, Biochemical Journal, 2000*).

Immune system effects: Levels of ROS produced by immune cells often influence the expression of inflammatory genes (17). To modulate this oxidative burst, cells such a neutrophils and macrophages use Se as a modulator. Expression of pro-inflammatory genes, inducible nitric oxide (iNOS) and cyclooxygenase (COX) 2, have been shown to be significantly up regulated in macrophages cultured in a Se deficient environment (17, 31). Further, this process is dependent upon the transcription factor NFκB, which itself is regulated by the Trx system (8, 31). Moreover, to kill ingested microbes, neutrophils produce superoxide radicals. In a Se deficient environment, neutrophils become defective and less effective at microbial killing (9, 32). Studies have suggested these effects are caused by a loss of GPx1 activity, allowing the produced free radicals to attack and kill the neutrophils themselves (9, 33).

Studies have also shown Se to influence the adaptive immune response (32). T cell proliferation responses to antigen stimulation, and the total percentage of T cells are enhanced by increased Se concentrations (33). In murine T cells, selenoprotein deletion resulted in a decreased production of functional T cells, leading to atrophy of the spleen, lymph nodes and thymus, as well as diminished levels of IL-2R, an important receptor found on the T cell surface (17) (34). In humans, IgG and IgM antibody titers are decreased in the absence of Se (32). Although lymphocytes require ROS for activation, infiltration, and phagocytosis, it is important that their levels are controlled and counteracted by anti-oxidants like Se to prevent the development of cancer and disease (4).

Effects of Se in cancer and disease: Some of the most compelling data demonstrating the effects of Se on cancer and disease are associated with deficiencies. The most well studied Se deficiency disorder is Keshan disease. First identified in China, Keshan is an endemic cardiomyopathy characterized by an enlarged heart muscle, and in some cases necrosis and calcification (35). The etiology of Keshan disease is not the absence of Se, but rather infection by Coxsackie virus (CVB). Normally a harmless virus, Se deficiency can lead CVB to become virulent, going through genomic mutations that results in myocarditis (4, 36, 37). Effective prevention of this disease was established through Se supplementation to individuals, helping to elevate antiviral immunity and prevent changes in the viral genome (4, 38). Studies with other RNA viruses, such as influenza A, have also observed changes to the RNA genome as a result of Se-deficiency (38), raising public health implications (37). Further studies with influenza A and Se deficiency have shown an augmented infiltration of cells into the lungs, suggesting an increase in inflammation due to oxidative stress (38).

Basic and clinical studies in humans have shown mounting evidence of a protective role for Se in various types of cancer (38). The two biggest clinical trials to date are the Nutritional Prevention of Cancer (NPC) Trial and Selenium and Vitamin E Cancer Prevention Trial (SELECT). NPC, carried out by Dr. Larry Clark and his group, was originally designed to study the relationship between Se supplementation and skin cancer risk (14, 39). A total of 1312 patients with a history of basal cell and/or squamous cell skin carcinoma were randomly placed into a placebo or Se (selenized yeast) groups. The results of this study showed that Se treatment did not significantly affect the incidence of skin cancer (14, 17, 36). Secondary endpoint analysis, however, showed Se supplementation to result in a decreased risk of colorectal, prostate, and lung cancers (14, 17). To clarify the role of Se in prostate cancer, researchers developed the SELECT study to examine if Se, Vitamin E, or both could prevent prostate cancer. The results of this study showed prostate cancer incidence did not differ with the use of Vitamin E supplementation (14). With the use

of Se alone or with Vitamin E, the results were null (14). Part of the explanation for the null results were that this study had several design differences from the NPC trial, including the use of selenomethionine as the Se source, which may have increased the baseline status of the study subjects. This, initially, was not taken into consideration (14). For many, these design shortcomings tainted the study results, which indicated that Se or Vitamin E, either alone or in combination, did not prevent the development of prostate cancer (14, 40).

Arachidonic Acid metabolism

Inflammatory influences comprise more than increased levels of ROS or oxidative bursts. Numerous studies have shown that long chain polyunsaturated fatty acids (PUFA) also influence the induction of inflammation (41, 42). One of the most highly studied PUFA is arachidonic acid (AA), a 20 carbon omega-6 fatty acid released from the phospholipid by the enzyme PLA_2. Eicosanoids, including prostaglandins (PGs), are generated from AA that contributes to inflammation, thrombosis, and gastrointestinal secretions, regulate numerous functions (41, 43, 44). The pathways of PG production have been elucidated (Figure. 6), where AA is converted to PGG_2 by cyclooxygenase enzymes COX-1 and COX-2. COX-1 is a constitutively expressed enzyme that acts to maintain homeostasis, while COX-2 is an inducible enzyme (43, 45, 46). In addition to their cyclooxygenase activities, COX1/COX2 contain a heme group with peroxidase activity, which catalyzes the conversion of PGG_2 to PGH_2. Specific synthases catalyze the conversion of PGH_2 into a series of PG including PGD_2, PGE_2, PGI_2, $PGF_2\alpha$, and thromboxane TXA_2 (41, 43, 47). These metabolites are involved in modulating the intensity and duration of inflammation in various ways.

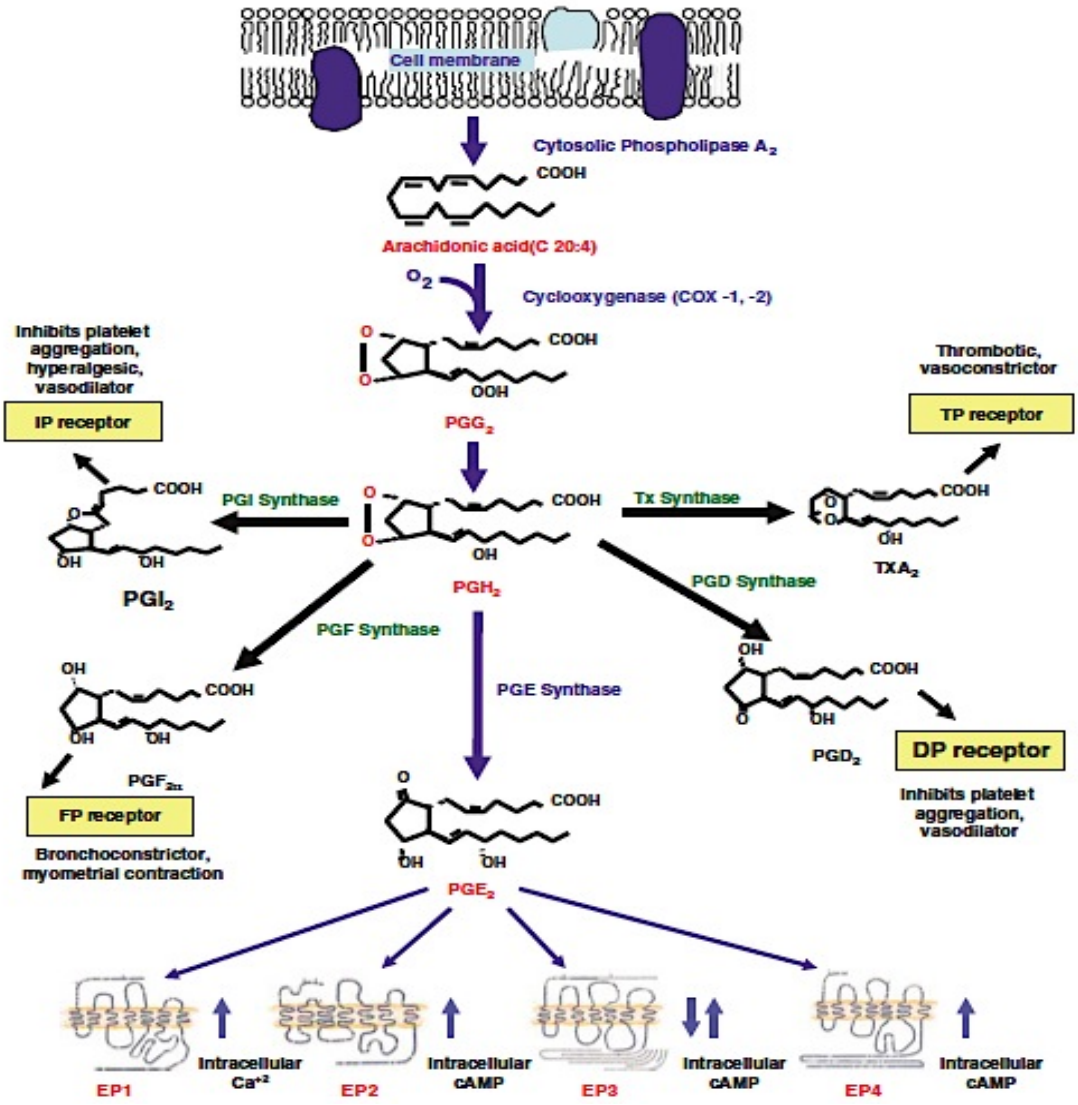

Figure 6: Cyclooxygenase induced conversation of arachidonic acid to prostaglandins. COX1 and COX2 are responsible for the conversion of AA to PGG2. Here, a series of synthases catalyze the conversion of prostaglandin subsets. Each subset has its own receptor, inducing specific effects within the body (*Adapted from Dey et.al, British Journal of Pharmacology, 2006*).

Produced by macrophages and monocytes through mPGES-1, PGE_2 has been identified as pro-inflammatory, although some reports suggest that PGE2 may have anti-inflammatory effects (41-43). In short, PGE_2 binds to one of four EP receptors (EP1-4), inducing the expression of pro-inflammatory genes (43, 45, 48, 49). Studies have also found PGE_2 to differentially regulate the activity of T cells, inhibiting the production of Th1 cytokines while inducing production of Th2 cytokines (43, 50).

However, the effect on Th2 cytokines is likely due to mediation by cAMP, as it has been shown to mimic the effects of PGE_2 (43).

During the resolution phase of inflammation, AA metabolism shifts from PGE_2 production towards PGD_2, the anti-inflammatory PG. These observations have been demonstrated by Gandhi *et.al*, who showed that Se supplementation shunts the AA pathway towards the production of PGD_2 through an enhanced expression of H-PGDS (47). Interestingly, PGD_2 is the precursor of three important metabolites, PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15d-PGJ_2) that belong to the class of cyclopentenone PGs (CyPGs) (44, 47, 51, 52). Not surprising, the production of these CyPGs were enhanced by Se, likely contributing to the anti-inflammatory effects of Se (47, 53). Although no specific synthase has been discovered, 15d-PGJ_2 is produced through double dehydration of PGD_2 (44, 51, 54). Within mast cells and macrophages, the conversion involves spontaneous dehydration of PGD_2 into PGJ_2, followed by an intramolecular rearrangement to $\boldsymbol{\Delta}^{12}$-PGJ_2, ending with a second dehydration to form 15d-PGJ_2 (43, 51) (Figure. 7).

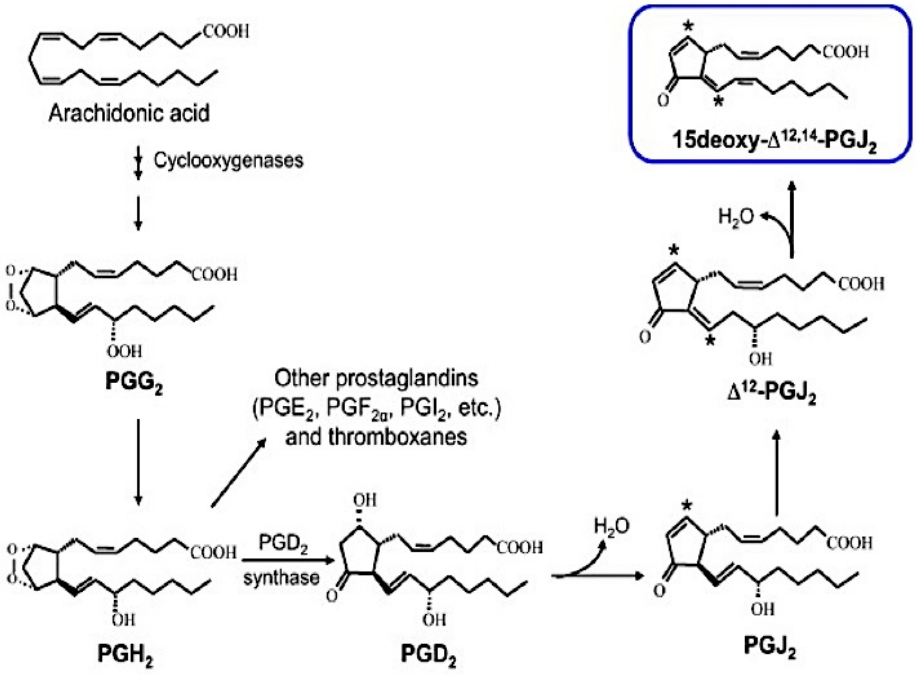

Figure 7: Formation of 15d-PGJ_2 via the arachidonic acid pathway. Formed through spontaneous dehydration of its precursor, PGD2, 15d-PGJ2 has may key activities, including modulation of the NFκB pathway (*Adapted from Surh et.al, Biochemical Pharmacology, 2011*).

One of the key activities of 15d-PGJ_2 is modulation of the redox transcription factor NFκB (44, 51). Normally maintained in the cytoplasm, NFκB subunits (p65 and p50) are released from its IκB inhibitory complex upon phosphorylation from IκB kinase (IKK), which triggers degradation through ubiquitin (51, 55). The NFκB complex is then free to translocate to the nucleus, where it binds to its target genes (56). The ability of 15d-PGJ2 to modulate NFκB involves two mechanisms. One is the direct modification of the cysteine thiol group in NFκB, which is attacked by the cyclopentenone ring of 15d-PGJ_2, which acts as a Michael acceptor (51, 57). This entire process is known as a Michael's addition reaction. The second mechanism covalently binds 15d-PGJ_2 to IKK, blocking the phosphorylation of IκB and subsequent release of NFκB for nuclear translocation (44, 58). Interestingly, 15d-PGJ_2 can exert both of these processes in a Peroxisome proliferator-activated receptors (PPAR)γ-independent fashion (59).

Peroxisome proliferator-activated receptors are a nuclear receptor family that regulates metabolic and cellular processes. Found in many types of cells including macrophages/monocytes, T-cells, fibroblasts, neutrophils and human bone marrow precursors (54), the methods of action of PPAR are well understood. In its inactivated state, PPAR is found in the cytoplasm bound to a co-repressor (44, 60-62). Only binding of a ligand can displace the co-repressor and recruit a co-activator, forming a heterodimer. The most well known co-activator is retinoid-X receptor (RXR) (43, 63). PPAR and RXR binding results in translocation from the cytoplasm to the nucleus, where the heterodimer binds to specific target genes at PPAR response elements (PPRE) in the promoter region (63, 64). Most target genes are associated with inflammation, lipid metabolism, and cell proliferation (44). There are three known PPAR isotypes- PPARβ/δ, PPARα, and PPARγ. Each isotype has a different pattern of expression, playing a role in the specificity of their functions. PPARα is found primarily in heart, liver and muscle where it regulates fatty acid catabolism (65). Less defined in its physiological functions, PPARβ/δ is expressed in most tissues, implicated in lipid homeostasis and wound healing (65-67). Found in immune

cells, and white and brown adipose tissue, the PPARγ isotype is best identified as a soluble receptor for 15d-PGJ_2 (44, 47, 68). Thus, through the activation of PPARγ, 15d-PGJ2 can impart anti-inflammatory effects. For instance, studies of autoimmune diseases in humans suggest PPARγ regulates inflammation in lupus nephritis, inflammatory bowel disease (IBD), and arthritis (44, 69, 70). Colitis and ischemia-reperfusion are additional inflammatory models that use PPARγ and 15d-PGJ_2 as anti-inflammatory agents (43, 71, 72). Furthermore, T cell proliferation and apoptosis are also regulated by the PPARγ/15dPGJ_2 combination (39, 43). In macrophages, secretions of pro-inflammatory cytokines such as IL-6, iNOS, and TNFα are down regulated (44, 65). It has long been known that PPARγ plays a role in regulating these types of cytokines, playing a role in macrophage differentiation and polarization (73).

Macrophages

Characterized as phagocytic cells responsible for eliminating pathogens, macrophages are one of the most important immune effector cells, playing key roles in tissue homeostasis, and antigen presentation (74). Their ability to recognize 'danger' signals through detection of necrosis debris or pathogen associated molecular patterns (PAMPs) happens through toll-like receptors (TLRs), triggering cell activation within tissues (75, 76). In addition to endogenous signal detection, macrophages are also activated in response to cellular microenvironment changes (73, 77), encompassing incredible plasticity to change phenotypes depending on the signals received (75, 76, 78). The conventional macrophage activation phenotypes are classically activated (M1) and alternatively activated (M2) (Figure. 8), with newly discovered intermediary phenotypes (79).

The nomenclature of M1 macrophages is derived from the cytokines responsible for their activation- namely IFNγ and TNFα, which are associated as Th1-type cytokines (73, 77). Intriguingly, both signals have been shown to only prime the macrophage for activation, but not actually activate it (75). Activation occurs in response to exposure to a microbial product, such as lipopolysaccharide (LPS) (75, 78, 80-82). Once activated, M1 macrophages are responsible for microbicidal activity, migrating

to areas of inflammation, where encountered pathogens are phagocytized and destroyed (75, 83). The killing of pathogens is accomplished through production and release of pro-inflammatory molecules such as nitric oxide (NO) and cytokines like interleukin (IL)-1β and IL-12 (84, 85). Nitric oxide is produced from the amino acid L-arginine (L-Arg), a substrate found in macrophage cells. L-Arg is acted upon by the enzyme inducible nitric oxide synthase (iNOS) (83, 84) (Figure. 8). Although M1 macrophages are necessary to fight inflammation and microbes, their persistence is detrimental, often leading to tissue damage and tumor development (85). Studies have shown M1 macrophages to contribute to increased levels of oxidative stress, prompting damage to proteins, DNA, and tissue (79, 86). Therefore, activation of M1 macrophages must be controlled. To counteract these developments, an anti-inflammatory alternative mechanism is employed.

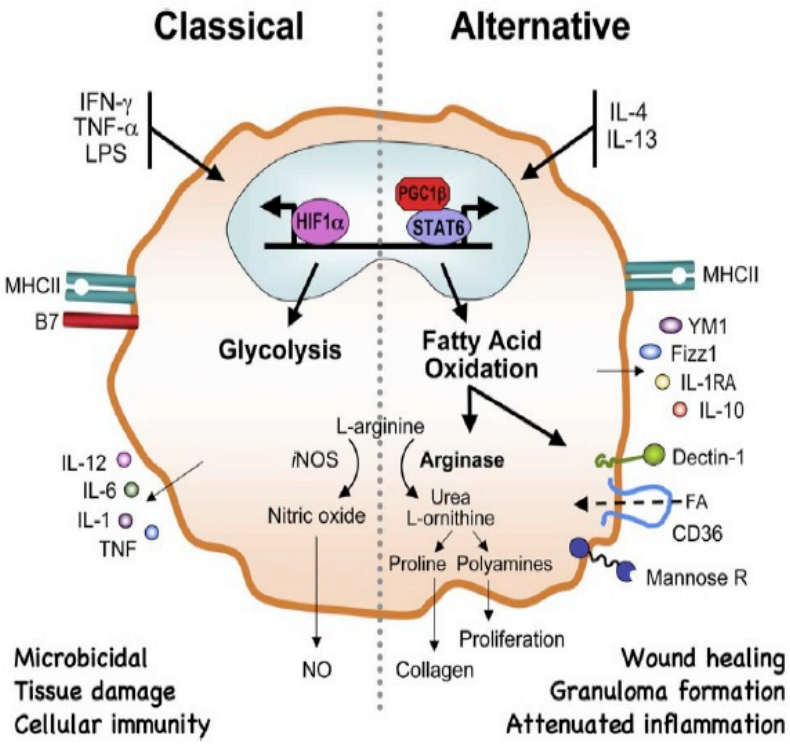

Figure 8: Classical vs. Alternatively activated macrophages. Classical (M1) macrophages are induced by pro-inflammatory stimulants such as IFN-γ and LPS. Products produced by M1 macrophage induce oxidation and inflammation in the body, leading to microbial killing and tissue damage. Conversely, alternative (M2) macrophages are induced by the Th2 produced cytokines IL-4

and IL-13, through a STAT6 specific pathway. The main enzyme used to distinguish M2 macrophages is Arginase, working upon L-Arginine to produce prolines and polyamines, which subsequently leads to wound healing and fibrosis (*Adapted from Vats et.al, Cell Metabolism, 2006*).

Similar to M1 macrophages, alternatively activated M2 macrophages are developed in response to immune signals. The most well known signals of induction are the Th2-produced cytokines IL-4 and IL-13 (64, 80, 87, 88). A vital function of M2 macrophages is the down regulation of production of cytotoxic inflammatory mediators by M1 macrophages (79). A second more intriguing function of M2 macrophages is stimulation of tissue repair, playing a significant role in wound healing (75, 76, 79, 87). Various markers are used to identify M2 macrophages, such as Arg-I, Ym1, Fizz1, and mannose receptor (Mrc-1) (74, 89), where their expression is induced by IL-4 (82, 90-92). Intriguingly, Arg-I seems to be the prototypic marker studied in alternative activation. In a move that illustrates M2 macrophage regulation of M1 macrophages, Arg-I uses L-Arg as its substrate, providing completion of the intracellular L-Arg pool (64, 93, 94) (Figure. 8). In M2 macrophages, L-Arg is converted to urea and L-ornithine. The significance of L-ornithine is its conversion into prolines and polyamines, which contribute to wound healing and fibrosis (79, 84, 87). A very fascinating discovery of M2 macrophages are the multiple subtypes that are based on their mechanism of induction (95)- M2a, M2b, and M2c. M2a macrophages are activated specifically by IL-4 and IL-13, and are the subtype most usually studies in papers (79). The M2b subtype is induced by immune complexes in combination with LPS (79, 95). Finally, the M2c subtype is induced by IL-10 and glucocorticoids, playing a significant role in immunosuppression and tissue remodeling (95). Recently a fourth subtype, M2d, has been discovered, but its inducers and function are not well understood.

In addition to cytokines as immune regulators, M2 macrophage activation is controlled by transcriptional regulators, PPARγ and STAT6 (94, 96). As previously discussed, PPARγ is a nuclear receptor that plays a role in macrophage differentiation (73). Recently, Chawla's group has found PPARγ to be specifically induced in

macrophages stimulated by IL-4 (97), coordinating the metabolic programs that provide the energy for M2 macrophage activation, such as β-oxidation of fatty acids (60, 97). Subsequently, studies have suggested the critical role PPARγ plays in M2 macrophage activation is regulation of *Arg-I* transcription (97), helping in the long-term maintenance of the M2 phenotype. In PPARγ KO mouse models, IL-4 stimulation results in a 40% decrease in *Arg-I* mRNA expression and a 50% decrease in its activity (60). Direct transcriptional regulation was determined through a PPARγ response element (PPRE) on the Arg-I promoter (60, 64). Through various experiments, including an electrophoretic mobility shift assay (EMSA), it was determined that the PPARγ/RXR heterodimer directly binds to the PPRE (97), regulating *Arg-I* transcription. Interestingly, recent studies have shed light on the IL-4 induced expression of PPARδ by M2 macrophages (98). Specifically, PPARδ works to repress pro-inflammatory genes while inducing the expression of M2 markers, such as Arg-I. Surprisingly, this occurs through a synergistic relationship with STAT6 (98).

Activation of STAT6 is well established- cytoplasmic phosphorylation by JAK leads to dimerization and translocation of STAT6 to the nucleus, where it binds to specific gene promoters ((80, 87, 94, 99). In M2 type macrophages, STAT6 activation and phosphorylation is initiated by IL-4 binding to its receptor, IL-4Rα. It has been demonstrated that the cytokine IL-13 also has the ability to activate STAT6, however the intensity of its signaling is not as strong as IL-4 (87, 94). Similar to PPARγ, STAT6 regulates *Arg-I* expression, through an enhancer region located upstream of the *Arg-I* transcription start site (100, 101). The enhancer region was identified through site-directed mutagenesis to be within the IL-4 response element of the *Arg-I* promoter. These experiments located a single STAT6 binding site (100). In addition to STAT6, several other binding sites located a few base pairs downstream were found and discovered to be imperative for *Arg-I* expression. Transcription factors PU.1, C/EBPβ, and its co-factor CBP also bind within the enhancer region, forming a transcriptional complex, inducing *Arg-I* transcription (100, 101) (Figure. 9).

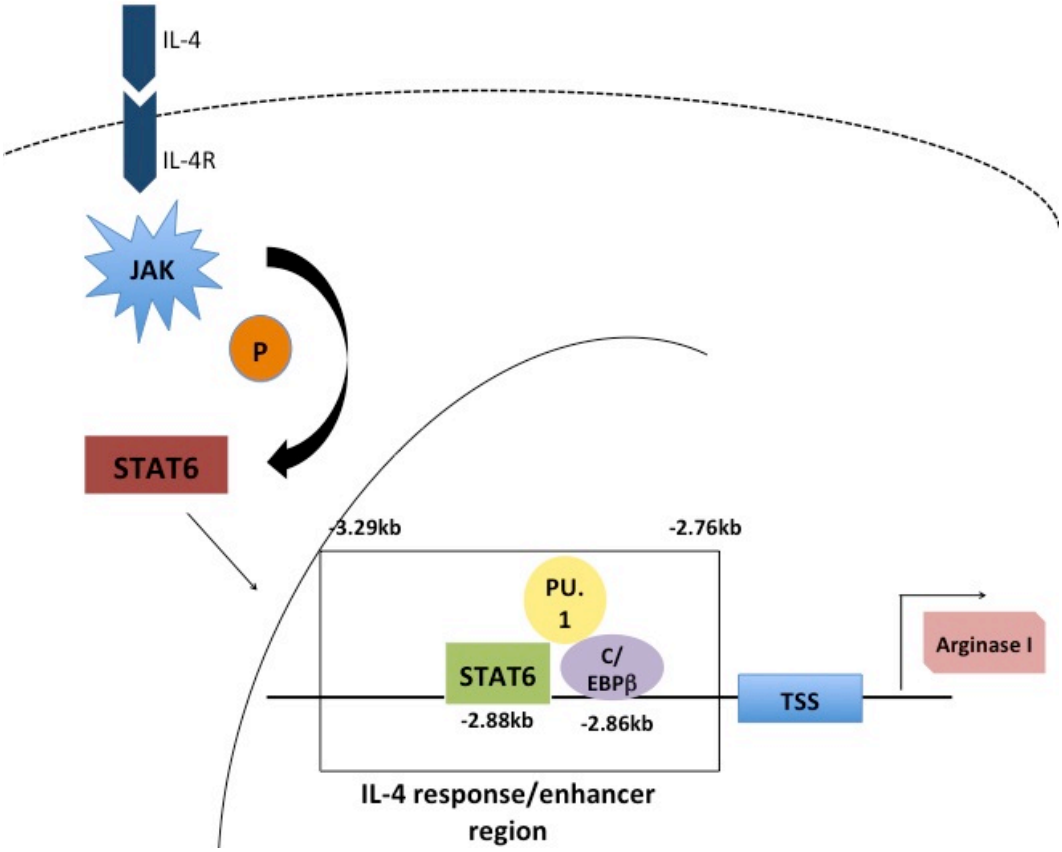

Figure 9: Arginase-I transcription and transcriptional complex. Upon binding of IL-4 to its receptor, STAT6 becomes phosphorylated by JAK, translocating to the nucleus. Here, it binds to its binding site within the IL-4 response region (enhancer element), located ~3kb upstream from the transcription start site on the Arg-I promoter. Additionally, PU.1 and C/EBPβ bind to a site 8bp downstream from STAT6, forming a transcriptional complex.

Although transcriptional regulation is a major factor in M2 macrophage activation, numerous studies have shown epigenetic regulation is also essential for control of activation and gene expression. The importance of epigenetic regulation lies in the ability to regulate cellular differentiation and activation, altering histone modifications associated with gene expression alterations (102), where a histone demethylase, Jmjd3 catalyzes the conversion of H3K27 to its monomethylated form. Histone H3K27 is linked to silencing of gene transcription (103), and studies have shown Jmjd3 to be important in M2 macrophage polarization (102). One of the genes silenced by H3K27 is IRF4, a transcription factor responsible for controlling M2

macrophage polarization in response to parasitic infections (73, 102) (Figure. 10). Knockout studies of *Irf4* have shown M2 marker expression to decrease; however the underlying mechanisms are not well understood (103, 104). Recent studies have shown *Irf4* expression to be induced by IL-4, indicating a possible relationship with STAT6 (73). However, under these circumstances, a role for Jmjd3 has yet to be established (73, 86).

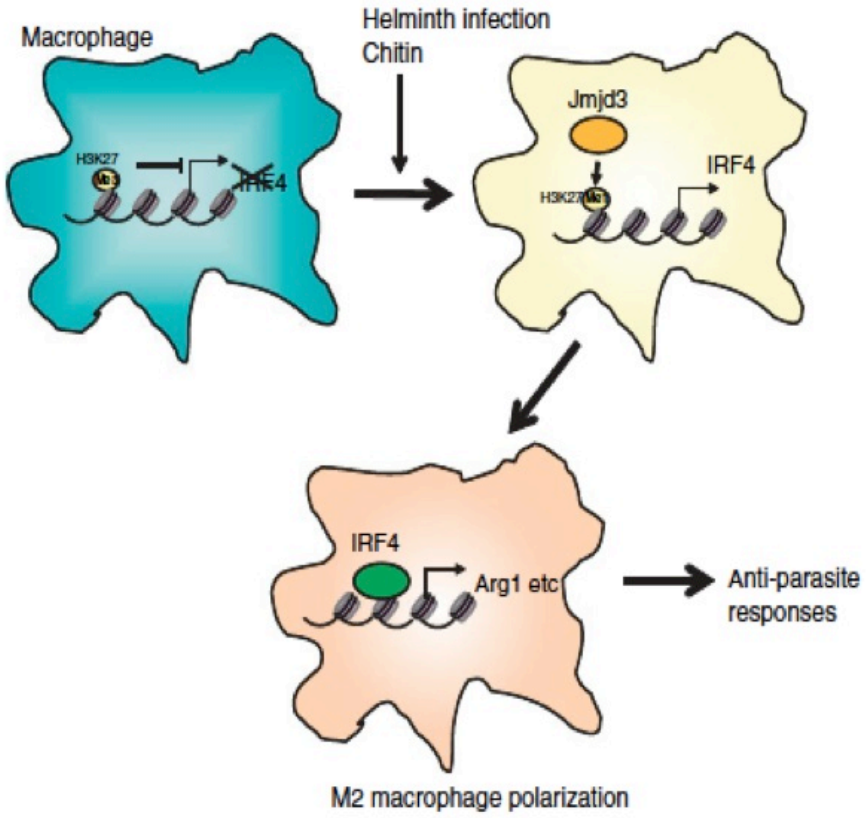

Figure 10: *Irf4* expression via Jmjd3 demethylation of H3K27. Linked to silencing genes, histone H3K27 is converted to is monomethylated form via Jmjd3, a histone demethylase. This leads to Irf4 transcription, a factor responsible for M2 macrophage polarization during parasitic infections (*Adapted from Takeuch et.al, European Journal of Immunology, 2011*).

Role of macrophages in disease: There is overwhelming evidence that certain diseases and infections require the activation of M2 type macrophages for clearance and resolution. In chronic inflammatory diseases, such as asthma, alveolar macrophages depend on Th2 cytokines IL-4, IL-5, and IL-13 to be highly expressed,

inducing the activation of M2 macrophages (38, 94). Studies in mice have shown alveolar macrophages to express M2 type markers, like Arg-I, Fizz1, and Ym-1 (80, 86, 87, 94). However, the exact mechanisms and function of these macrophages in asthma are still debated, as some have identified it as having suppressive functions against pro-inflammatory cytokines, and others have determined M2 macrophages to promote allergic inflammation (86). Studies of insulin resistance show adipose tissues in lean mice contain a majority of M2 macrophages, maintaining insulin sensitivity (64, 97, 98, 105, 106). Conversely, obese mice adipose tissue contains M1 macrophages, releasing pro-inflammatory cytokines and inducing the NFκB pathway, developing insulin resistance and other metabolic disease (60, 98, 107). As wound healing macrophages, M2 phenotype can play a detrimental role to the host through deregulated activity (76, 108, 109). In parasitic diseases such as schistosomiasis, tissue fibrosis creates granulomas around the parasite, as a containment mechanism (94, 110). However, chronic schistosomiasis has been attributed to uncontrolled M2 macrophage secretion of extracellular matrix components (75, 94, 110).

Studies have shown excessive release of ROS from classically activated macrophages can lead to the beginning stages of neoplasia (75, 76, 81, 94, 111). Tumor promotion may also stem from chronic inflammation, contributing to tumor grown, tumor progression, angiogenesis and immunosuppression (73, 85, 112). It is believed that chronic inflammation comes from the enhanced presence of M1 macrophages (76, 80, 81, 86). Intriguingly, accumulating evidence suggests cells with M2 like phenotypes, tumor associated macrophages (TAM) and myeloid derived suppressor cells (MDSC), have also been identified as contributors to tumor promotion (86, 113). Displaying pro-tumoral functions such as survival, proliferation, and dissemination, TAM and MDSC have been characterized by their abilities to inhibit T cell proliferation, enhance angiogenesis, and suppress the immune system in response to tumor growth (86, 114, 115). Studies have suggested TAM and MDSC inhibit both the innate and adaptive immune responses (108, 116, 117). Because of this ability, these suppressor cells must escape immune surveillance, allowing for dysfunctional neoplasia to survive (114, 117). Specifically, MDSC have been characterized as immature

precursors of dendritic cells and macrophages (117). Accumulation and activation of these cells are driven by multiple factors, including VEGF, IL-6, and PGE_2 (117, 118). In a study by Sinha *et.al* (118), it was determined PGE_2 is expressed by tumor-infiltrating macrophages, inducing MDSC differentiation from bone marrow stem cells (118). Moreover, MDSC express all four PGE_2 receptors, where deficiency in EP2 demonstrated reduced tumor growth and less suppressive MDSC (117). Cell surface markers used to identify MDSC are quite heterogeneous, a trait which is owed to MDSC differentiation through different tumor-secreted factors (108, 114). In a study done by Umemura *et.al*, it was determined that MDSC bear characteristics of both M1 and M2 macrophages, challenging the convention that they comprise strictly M2 qualities (86, 116, 119). A few papers have suggested MDSC may be precursors to TAM (76, 86). However, this is has not been completely determined. In contrast to MDSC, it is accepted that TAM have an M2 phenotype, expressing high levels of M2 type markers and low expression of IFN-γ (115). Recruitment and differentiation of TAM to tumor sites occurs through molecules produced by neoplastic cells, such as CCL2, VEGF, and M-CSF (108, 115). Studies have identified NFκB as a regulator of transcriptional programs within TAM (83, 108). Furthermore, it has been determined that through regulation of NFκB, the pro-tumoral functions of TAM can be controlled and maintained (108). In addition, transcriptional control has also been associated with STAT3 expression, leading to inhibition of pro-inflammatory cytokine and chemokine production (83, 108, 120). Moreover, inhibition of STAT3 expression inhibits tumor growth, decreasing accumulation of MDSC (108, 121).

In intestinal tissue, macrophages are the most abundant immune cell, found in both the mucosa and lamina propria (122, 123). Similar to other tissues, the intestines are home to both resident and infiltrating macrophages. Resident macrophages are highly phagocytic with strong activity against bacteria (124). Uniquely, resident macrophages do not respond to stimuli by producing pro-inflammatory cytokines, but rather constitutively produce anti-inflammatory cytokines, such as IL-10 (86, 124). This mechanism is used to protect the intestinal tissue from explicit inflammation and from commensal bacteria crossing the epithelial barrier through a loss of epithelial

integrity (83, 124). Loss of control of responses to intracellular bacteria, viruses and parasites is associated with the development of IBD. Studies have shown the expression of PPARγ in resident macrophages contributes to its ability to prevent local inflammation through suppression of pro-inflammatory genes (60, 122). Interestingly, intestinal macrophage expression of COX-2 has also been associated with production of IL-10, protecting the intestines from inflammation that may lead to IBD (124, 125). In other circumstances, infiltrating macrophages act as inflammatory mediators. Studies have shown some infiltrating macrophages to have M2-like qualities, expressing markers such as Mrc-1, and tissue remodeling factors such as VEGF (124). In some parasitic infections, infiltrating M2 macrophages play a large role in clearance of the infection, while protecting the intestinal environment (126, 127). It is believed that use of M2 macrophages is preferred, as M1 macrophages can exacerbate the infection, leading to possible development of chronic inflammation and prolonged diseases (127).

Eosinophils

Similar to macrophages, eosinophils are immune cells that play a large role in parasitic diseases and allergic responses (128, 129). The development of eosinophils is unique, dictated by the association of three transcription factors- GATA-1, PU.1, and C/EBPβ (130). As discussed previously, the transcription of Arg-I is dependent upon PU.1 and C/EBPβ, but in eosinophils these transcription factors work synergistically to regulate eosinophil lineages (130). Additional factors that affect eosinophil development are cytokines IL-5, IL-3, and GM-CSF (129-131). Studies have shown IL-5 to be the most important cytokine, responsible for selective differentiation and maturation, prolonging survival in different tissues (130). Absence of IL-5 severely reduces eosinophil recruitment to the blood, increasing susceptibility to allergic stimulants and parasitic infections. Moreover, the chemokine eotaxin-1 also plays a role in eosinophil development and recruitment (129, 132, 133). Cooperation between IL-5 and eotaxin-1 has been well studied; however in the absence of IL-5, recruitment of eosinophils does not decrease, suggesting IL-5 to be an expendable cytokine (130).

In studies of parasitic infections, eosinophils have an integrated relationship with Th2 cells and macrophages (132, 134-136). Specifically, the Th2 cytokines IL-4 and IL-13 have been demonstrated to be potent inducers of eosinophil recruitment, working with the transcription factor STAT6 (128-130). In their defense against certain helminth infections, eosinophil levels increase in both the lungs and small intestines in a STAT6 dependent fashion (129, 130). Further, studies have shown eosinophils express IL-13, albeit at lower levels than Th2 cells, contributing to infection regulation ((80, 87, 129, 131). Interestingly, eosinophil recruitment into the gastrointestinal tract is a common feature in numerous other disorders such as IBD, allergic colitis, and reflux disease (130). Here, recruitment through increased levels of eotaxin-1 stimulates the expression of Th2 cytokine production, inducing the differentiation and activation of M2 macrophage leading to the alleviation of gastrointestinal inflammation (130, 131).

Resolution of Inflammation

The immune systems response to inflammation is well documented. However, in an effort to treat inflammation through outside methods, drugs targeting specific inflammatory pathways have been developed, most notably nonsteroidal anti-inflammatory drugs (NSAIDs). The most prominent NSAIDs are ibuprofen, naproxen, and aspirin (designated as the archetypal form) (137). NSAIDs act to inhibit the enzymatic activity of COX-1 and COX-2, thus inhibiting the downstream production of pro- and anti-inflammatory prostaglandins (137, 138). An interesting side effect to NSAID use is an increased induction of intestinal damage. Studies have determined that together, COX-1 and COX-2 maintain intestinal mucosa integrity, and therefore inhibition of both enzymes leave the mucosa open to attack and subsequent damage by commensal bacteria (137, 139, 140) To counteract these effects, specific inhibitors of COX-2 have been developed and tested to measure their effects on the intestinal mucosa. A COX-2 specific inhibitor rofecoxib, and COX-1 specific inhibitor SC-560 were tested to establish their effects on intestinal integrity (137, 141). Compared to a non-specific inhibitor of both COX-1 and COX-2, oral

administration of either inhibitor did not induce intestinal damage (141). However, use of these inhibitors still exhibited decreased mucosal levels of PGE_2 (141), suggesting conventional uses of NSAIDs may not be ideal to decrease inflammation. Additional studies have demonstrated inhibition of COX-1interferes with the gastrointestinal housekeeping functions, leading to the development of gastritis with acid reflux, commonly seen in many consuming NSAIDs (142). These results raise the possibility that specific COX inhibitors, particularly COX-2 inhibitors, may be of better use. Interestingly, the focus of how to reduce inflammation has begun to shift away from passive forms, and more towards active forms of resolution.

The struggle to combat inflammation has shifted to looking outside of conventional anti-inflammatory methods. Specifically, regulation of inflammation has gravitated towards catabasis, or pro-resolution methods, enabling tissues to return to homeostasis. Seminal studies demonstrating the role of pro-resolving metabolites have come from Dr. Charlie Serhan's group, that have identified lipid mediator families, lipoxins, resolvins and protectins, which are part of the active biochemical and metabolic resolution process (143, 144).

Resolvins are enzymatic endogenous chemical mediators, biosynthesized from omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which can form unique derivatives, termed E-series and D-series, respectively (143-146) (Figure. 11). Moreover, resolvins can be produced in a COX2-dependent fashion in the presence of aspirin, forming aspirin-triggered resolvins (143, 144). E-series resolvins, which includes resolvin E1 and E2, reduce inflammation via blocking of neutrophil transmigration. These results contribute to the growing body of evidence that resolvins possess anti-inflammatory and immunoregulatory actions (140, 143). In specific diseases, such as colitis, E series resolvins reduce excessive infiltration of neutrophils (140, 146), leading to a reduction of tissue damage that is usually seen with cell infiltration. This is what categorizes resolvins as 'pro-resolving' and not just anti-inflammatory-controlling the response of phagocytes and clearing debris (139). D-series resolvins are similar to the E-series resolvins in terms of their effects on

inflammation (139, 140), however their control is seen more in neural tissues, skin inflammation, and kidney ischemia-reperfusion injury (145).

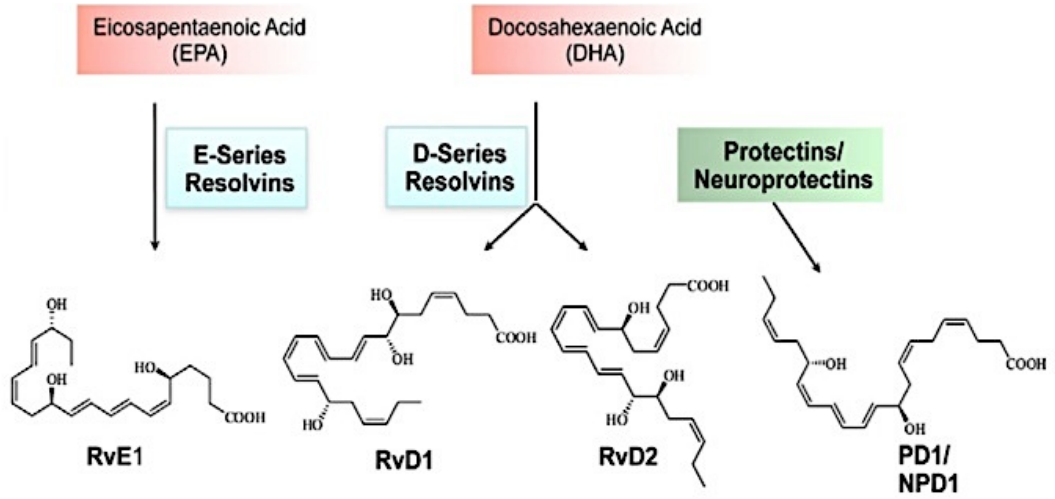

Figure 11: Endogenous chemical mediators biosynthesized from EPA and DHA: Biosynthesized from EPA and DHA, resolvins and protectins reduce inflammation through anti-inflammatory and pro-resolving actions. Discovery of these mediators has lead to targeted therapeutics (*Adapted from Serhan, American Journal of Pathology, 2010*).

Distinguished from resolvins through the presence of a conjugated triene double bond, protectins are also biosynthesized from DHA (140, 143). The role of protectins was established in neural tissues, where it inherited the name neuroprotectin D1 (140, 147). Protectins*, in vivo*, inhibit neutrophil infiltration, similar to activities seen in the resolvins. They have also been revealed to act on glial cells, reducing cytokine production (140, 148, 149). In addition, protectins have been demonstrated to block T cell migration, promote T cell apoptosis, and reduce IFN-γ secretions (143, 150). The discovery of resolvins and protectins has allowed for new pathways of research, developing treatments for diseases and infections that target resolution-based therapeutics.

Helminthic Parasites

Nematoda and platyhelminths are members of two phyla that cause helminthic parasite infections (151, 152). Within these two phyla exist individual species of parasites, including whipworms, hookworms, roundworms, cestodes (tapeworms), trematodes (flukes), and filarial worms (133, 153, 154) (Figure. 12). All species infect specific niches within their hosts, regularly seen in but not limited to gastrointestinal and alveolar tissues. The epidemiology of infection is diverse, infecting upwards of 3 billion people worldwide (155, 156). Although many are infective, helminth parasites very rarely lead to death. In most cases, parasites lead to an increase existence of morbidity, specifically in children living in Sub Saharan African, and certain parts of Asia and Latin America (152, 153, 155, 157, 158). Modes of transmission in these areas are primarily through fecal-oral exposure that are consequences of countries having little to or no regulated sanitation of water or plumbing. In many cases, infected individuals are unaware that they carry the parasite, a consequence that can be linked directly to anti-inflammatory properties of the parasite species itself.

Helminths are master immune regulators with the ability to subvert host immune responses (153, 157, 159). Different levels of immune suppression have been well documented in both human and animal studies. Notably, the ability of the parasite to avoid the immune response is related to its life cycle within its host. The life cycle of helminth parasites vary, where some are short lived, quickly exiting the host, and others are long lived, leading to the development of chronic infectivity (122, 133, 151). An example of chronic infectivity can be seen in infections of the trematode *Schistosoma mansoni* (*S. mansoni*). Adult worms reside within the portal vasculature, preferentially draining to the large intestine, which is their main site of infection. Eggs layed by female worms that travel to the liver and become lodged are an aberration of infection because the life cycle is continued by eggs that migrate through the intestinal wall for passage through the feces (151). These eggs induce an immune response that leads to the development of granulomatous lesions within the liver, where overtime fibrosis can developed, leading to increased portal blood pressure and bleeding, the most common form of death from *S. mansoni* infection

(151, 160, 161). In cases of chronic or short-lived infections, helminths can elicit a Th1 or Th2-type responses. Interestingly, whether it is nematodes, cestodes, or trematodes, the most common response to infection is an elicited Th2-type response.

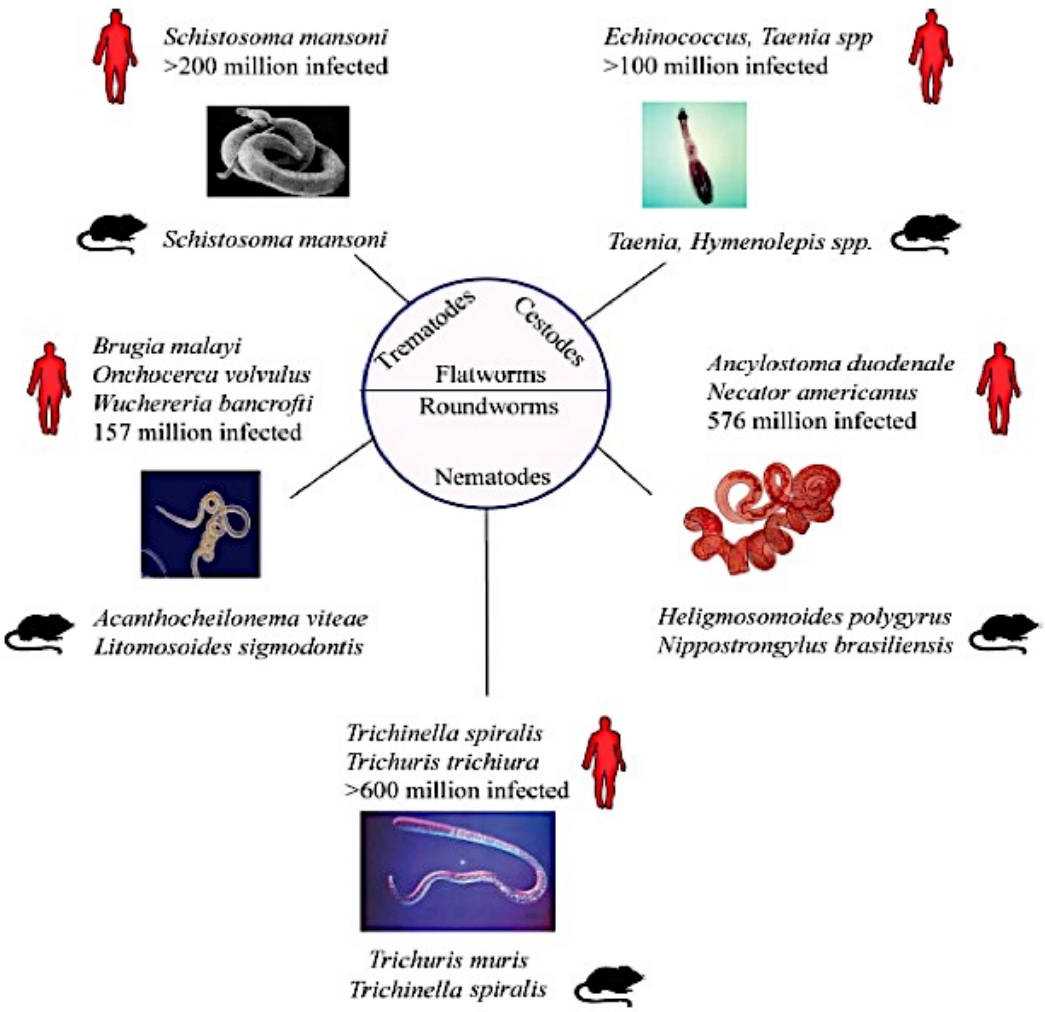

Figure 12: Helminth species infections. Within the Nematoda and Platyhelminths phyla, exists three species of helminths: cestodes, trematodes, and nematodes. Each species has types that can infect both humans or mice (*Adapted from Maizels et.al, Journal of Experimental Medicine, 2009*).

The Th2-type response typically includes the production of cytokines (IL-4, IL-10, IL-5, and IL-13), expansion of immunoglobulin E (IgE), induced goblet cell hyperplasia, and mobilization of effectors cells; mast cells, eosinophils, macrophages, and basophils (122, 126, 133, 153, 154, 159, 162, 163). Many studies have pointed to these effector cells as playing a role in early induction of the cytokines as part of the

innate immune response (127, 133, 164). For example, basophils have been determined to be potent producers of IL-4 in response to parasitic infection (165). In a study conducted by Fallon *et.al* (165), a novel cytokine, IL-25, was shown to be effective in polarizing Th2-type responses in helminthic parasite infections. A member of the IL-17 cytokine family, IL-25 induces development of a population of NBNT (non-B/non-T) c-kit^{+}, FcεR1 cells from mesenteric lymph nodes. These cells have been demonstrated to produce IL-4, IL-5, and IL-13 (165). Based on the studies in IL-25$^{-/-}$ mice, it was confirmed that IL-25 induced NBNT cells, subsequently generating a Th2 response (165). This study further helped establish the importance of the innate immune system in response to helminthic parasite infections, whose cytokine expression helps regulate differentiation of Th2 cells and the induction of adaptive immunity (165).

The slow development of a Th2-type response may be key to a successful infection allowing the parasite time to differentiate and complete its life cycle with the ultimate goal of transmission to the next host (127, 151, 152, 159, 166). The strongest, and most popular inducers of Th2 type responses in mouse models are gastrointestinal nematodes like *Heligmosomoides polygyrus (H. polygyrus)* and *Nippostrongylus brasiliensis (N. brasiliensis)* (126, 152, 153, 167-169). The murine nematode *H. polygyrus* is a trichostrongyle nematode roundworm parasite (153, 159) that has a direct life cycle and is commonly used to study chronic intestinal helminth infection (170, 171). Infective third-stage larvae (L3) are given per mouse and invade and develop in the submucosa of the intestinal duodenum before emerging into the intestinal lumen as adult worms at (122, 153, 160, 170). The adult worms then mate and the females release eggs that are passed in feces with a chronic infection that lasts for weeks to months depending on the genetics of the host. Gause *et.al* (172) showed that adult *H. polygyrus* cleared by an anti-helmintic drug and followed by a secondary "challenge" infection had a much stronger Th2-type response then to a primary infection. This resulted in rapid worm expulsion by 12 days post inoculation (154, 172) triggered by a highly polarized Th2-type host memory response (172). In further experiments, it was determined that a CD4+ T cell response was required early in the

secondary infection for effective parasite clearance (170, 173). These results helped establish the importance of an adaptive immune response in host responses to secondary infections (160, 170, 172). In addition, dietary protein levels also played an important role in chronic parasite responses. Ing's group showed (174) that worm burdens were significantly higher in mice fed low protein diets during secondary *H. polygyrus* infection. Changes in the eosinophil response and IL-4 expression were also lower in low-protein diet group (174). Together, these studies showed the capabilities of a Th2-type response in an *H. polygyrus* infection.

Nippostrongylus brasiliensis is a rat parasite that has been adapted to be studied in mice. The life cycle of *N. brasiliensis* is quite different from that of *H. polygyrus*. To start, *N. brasiliensis* is a short-lived infection, lasting only 14 days before being cleared from the mouse by a spontaneous cure (133, 136, 154, 172). Infective L3 penetrate through the skin or and migrate within 36 hours to the lungs. The parasitic larvae reside in the lungs for 2-3 days and mature to the fourth-stage (L4) stage before being "coughed up" and swallowed, traveling to the small intestines. The L4 mature into the adult (L5) stage, residing in the lumen of the jejunum. By seven days post inoculation the adult worms begin to produce fertilized eggs that are excreted from the mouse in the feces (126, 154, 160, 163, 172, 175) (Figure. 13).

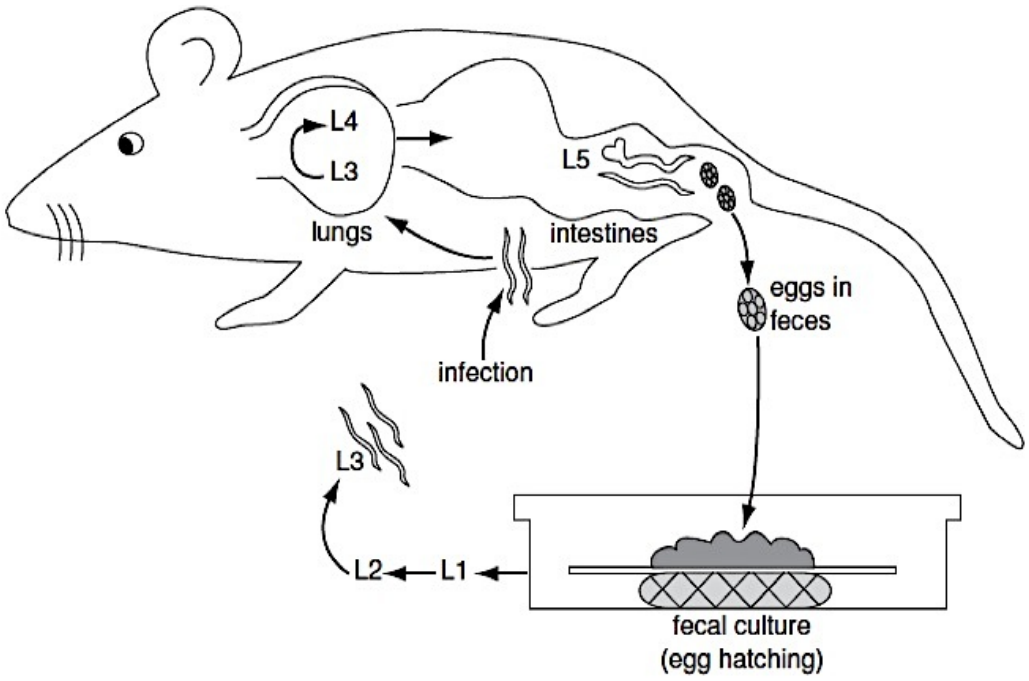

Figure 13: life cycle and progression of N. brasiliensis in mice. Infection with N. brasiliensis lasts for 14 days, and is cleared from the mouse through a self-clearing mechanism. L3 larvae are injected subcutaneously, traveling to the lungs within 2 hours. Larvae mature to the L4 stage after 2-3 days, before leaving the lungs and traveling to the small intestine. Here, adult worms mature to the L5 stage, mate and lay eggs. Eggs are shed in the feces and are seen at 7 days post infection. Adult worms begin to clear from the mice 10 days after infection (*Adapted from Camberis, Current Protocols in Immunology, 2003).*

In a normal immune response, a Th2-type response that is initiated in the small intestine expels worms as early as day 8-10 days post inoculation, with complete clearance by days 12-14 (154, 176, 177). Studies have shown treatment of infected mice with the pro-inflammatory cytokine IL-12 prolonged the infection period, suppressing host-protection against *N. brasiliensis* through induction of a Th1-type response (154). This data suggest a Th1-type response is not conducive to clear this parasitic species, but rather exacerbates the infection (178). As established in *H. polygyrus* infections, *N. brasiliensis* utilize a Th2-type response for parasite clearance. Most notably seen in these infections is the activation and increased presence of M2 macrophage (133, 160, 163, 170, 175). Activation of M2 macrophages in *N. brasiliensis* infections is no different from the activation in other

scenarios where cytokines IL-4 and IL-13 signal through the IL-4Rα to induce activation. Furthermore, these macrophages are characterized by up regulation of Arg-I, Fizz1, Ym-1, and Mrc-1 (133, 163). Together, these traits would best classify these macrophages into the M2a subtype (79, 133).

Further examination of the role M2 macrophages play in *N. brasiliensis* infections has produced remarkable results. Measurement of smooth muscle contractility in the jejunum of infected mice was significantly decreased in the absence of M2 macrophage infiltration (179). Elimination of *Arg-I* activity also played a role in inhibiting smooth muscle contractility, however these effect were not as detrimental as complete macrophage ablation (163, 179). In a separate study, smooth muscle hyper-contractility and mucous secretions were affected by stimulation of proteinase-activated receptor 1 (PAR-1). Normally expressed in the gut, PAR-1 is a seven transmembrane-spanning G protein-coupled receptor whose expression is increased in response to *N. brasiliensis* infections (180). Interestingly, PAR-1 activity and smooth muscle contractility changes were determined to be reliant upon STAT6 signaling (154, 180). Experiments conducted by Urban *et.al* (154) showed that $STAT6^{-/-}$ mice failed to expel *N. brasiliensis* from the intestinal lumen. Moreover, it was determined that the Th2 cytokine IL-13 had a stronger STAT6-dependent role in clearance of *N. brasiliensis* than IL-4 (68, 154, 163). This data should not discount the importance of IL-4 signaling in the general Th2-type response that contributes to development of the Th2 response and facilitates expulsion. Expression of other M2 markers, Ym1 and Fizz1, is also up regulated in *N. brasiliensis* infections. Apart from being used to identify M2 macrophages, these secreted cytokines play a role in wound healing, specifically in the lungs where parasite infections cause hemorrhaging and other tissue destruction (154, 181-184).

Independent of M2 macrophages, there are a plethora of other proteins and mechanisms involved in parasite clearance. A goblet cell-produced protein, Fizz2/Relmβ, is secreted into the intestinal lumen where it mediates mucous production (185), but is not expressed in any of the M2 lineages. Increased mucous

secretions work with smooth muscle contractility to clear the parasite from the intestinal lumen. The expression of Fizz2/Relmβ is regulated by Th2 cytokines IL-4 and IL-13 (185, 186). Herbert *et.al* (186) determined that binding of IL-4 or IL-13 to its receptor induced intestinal epithelial cells to differentiate into goblet cells that expressed Fizz2/Relmβ. Most importantly, Fizz2/Relmβ is not produced as part of a generic response to intestinal pathogens, but its expression is specifically restricted to helminthic parasite infections (185).

Although most of the information discussed here are from mouse models, it is important to point out there are gastrointestinal helminth in other vertebrate species including humans. The hookworms *Necator americanus* and *Ancylostoma duedenale*, are commonly found in humans imbedded within the intestinal tissue and feeding off the blood (187, 188). These hookworms have a free-living first stage larval stage (L1) that feeds on microorganisms within the soil before maturing to the infective L3 stage. From here, the larvae position themselves to be available for skin penetration, usually through the sole of a barefoot person (187). The parasitic larvae then travel to the blood or lymphatic capillaries where feeding and larval development continues (187, 189). Next, through passive transport to the pulmonary circulation, the larvae are coughed up and swallowed, traveling to the gut (187). Here, the larvae molt to the fourth stage of maturation, attaching to the intestinal villi and feeding on blood (187-189). These hookworms down-regulate host immunity to prevent elimination and can survive within its hosts for 5-7 years (190, 191). Interestingly, recent studies have suggested hookworms activate a combination of Th2 and Th1-type cell response (189). However, the exact mechanisms are not well understood. Furthermore, while immune cells such as dendritic cells and eosinophils have been identified as effectors, some studies have suggested the involvement of regulatory T cells (191), and their contribution to the longevity of the hookworm infection.

Helminths and T cells

While it is commonly known that Th2 cells play an enormous role in the adaptive immune response to helminthic parasites, there are other T cell types that have been

identified as important players as well. Within the intestinal intraepithelial lymphocytes, there is a high frequency of γ/δ T cells. Previously, the function of these T cells was unknown, however recent data has brought to light the influence these cells have on gastrointestinal helminth infections. In a study conducted by Adrian Smith's group (192), γ/δ $TCR^{-/-}$ mice were shown to have increased susceptibility to infection with *N. brasiliensis* when compared to wild-type mice. Both adult worm and fecal egg counts were higher in the KO mice starting as early as day 8-post inoculation, suggesting a delay in expulsion. This could be explained by the reduction in goblet cell hyperplasia, and thus decrease mucous production as in the γ/δ $TCR^{-/-}$ mice (192). Furthermore, a decreased production of Th2 cytokines, specifically IL-13, was seen in these mice suggesting that γ/δ T cells play an integral role in epithelial cell integrity during helminthic parasite infections.

In a novel experiment, Panzer *et. al* (193) showed the ability of adoptively transferred Th1 and Th17 cells to convert their expression to that of a Th2 cell, in a *N. brasiliensis* infection. Specifically, Th1 and Th17 cells were generated *in vitro* by appropriate stimulation. IFN-γ and IL-17A producing cells were purified by capture assays and sorted (193). These cells were then adoptively transferred to a recipient mouse, which was infected with *N. brasiliensis* three days later. Analyzing the donor cells, this group found approximately 30-40% of the cells acquired an IL-4 expressing phenotype (193). To investigate the effects in an *in vivo* environment, mice were stimulated to produce Th1 or Th17 cells. These cells were adoptively transferred to recipient mice infected with *N. brasiliensis* (193). Approximately 40-50% of the cells reflected a conversion to a Th2 phenotype. These experiments highlight the plasticity of T cells in an *N. brasiliensis* infection, possibly developing therapeutic approaches against other helminthic parasites and inflammatory diseases.

Nutrient deficiencies

As discussed above, there are a number of therapeutic approaches to combating inflammation, and more specifically, intestinal inflammation. It is interesting to note,

however, that many of these therapeutic approaches fail in nutrient deficient environments. This is due to the role nutrients play in the activation and differentiation of immune cells. While the aspects of Se deficiency have been discussed, there are other trace elements, such as zinc, and vitamins, such as vitamin E and vitamin C, that also play important roles.

The trace element zinc provides protection against reactive oxygen species via superoxide dismutase (194). During a helminth infection, studies have determined zinc deficiency to affect the structure and integrity of the intestinal epithelial tissues, inducing greater penetration of and establishment of worms (195, 196). Studies have also shown changes to intestinal mobility that alters parasite expulsion (195). Furthermore, zinc deficiency affects the production of Th2 responsiveness more so than a Th1 response. Thus, there is less secretion of IL-4, IL-5 and IL-13, all major cytokine players in parasite infections (194, 196). Immune cell recruitment and localization to the intestinal epithelium is also affected by zinc deficiency impairing the ability to initiate an effective immune response (196). In an *H. polygyrus* infection, Scott *et.al* (196) found that zinc deficiency reduced priming of the Th2 response to a secondary challenge infection, providing evidence that deficiency impairs the memory immune response.

Vitamin E and vitamin C have been demonstrated to stimulate (or boost) the immune system via their antioxidant properties. Specifically, vitamin C enhances T cell proliferation and inhibits monocytes producing TNF-α and IL-2, both pro-inflammatory cytokines (197). This data demonstrate that vitamin C regulates the inflammatory response. Therefore, deficiencies in vitamin C may lead to an increased oxidative attack on immune cells, increasing the expression of the oxidant-sensitive transcription factor NFκB, leading to amplified inflammation (197). This may play a detrimental role on intestinal tissues triggering loss of cell integrity and decreased infiltration of neutrophils and monocytes (198). Interestingly, studies have shown vitamin E to induce greater promotion of Th1-type response and suppress any Th2-type response (197). Others have suggested supplementation with vitamin E increases

IL-2 production while decreasing production of IL-4 and IL-13 (197). Conversely, data indicates vitamin E may be inadequately absorbed in gastrointestinal tissues, decreasing the infiltration of immune cells (199, 200). In a study done by Dr. Shea-Donohue's group (199), vitamin E deficiency was determined to have a detrimental impact on immune responses to parasitic helminthes. In this study, vitamin E deficiency yielded a significant increase in numbers of adult *H. polygyrus* worms and fecal eggs during a challenge infection. Further experiments suggested the delayed expulsion was due to decreased smooth muscle contraction (199). Together, this data propose that effective helminth clearance from the intestines is a complex process that requires both immune system modulation and proper nutrient levels. A nutritional factor that plays a tremendous role in regulating immune responses and helminthic parasite infections is Se. This thesis will address the following unanswered questions:

1. How does Se, in the form of selenoproteins, switch macrophage activation from a pro-inflammatory phenotype towards an anti-inflammatory M2 phenotype?

2. What are the specific pathways Se utilizes to mediate these effects?

3. What is the relationship between Se and macrophages during gastrointestinal helminthic parasite infections?

4. Are there novel Se-dependent pathways that are important for clearance of parasites from intestinal tissues?

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Chapter 2

Selenium levels affect the IL-4 induced expression of alternative activation markers in murine macrophages

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[**Contributions**: Shakira Nelson (all figures) performed this study. GPx KO and WT mice (Fig. 18B) were kindly provided by Xingen Lei]

Abstract

Selenium (Se), in the form of selenoproteins, imparts many health benefits with anti-inflammatory properties. Previous studies have shown that Se supplementation of macrophages negatively regulates the LPS dependent production of inducible nitric oxide synthase (*iNOS*), a pro-inflammatory gene. Therefore, we hypothesized that L-arginine, a substrate for iNOS, is acted upon by arginase-I (*Arg-I*), contributing to the resolution of inflammation. We investigated the anti-inflammatory activity of Se using LPS and IL-4 treated C57/BL6 murine bone marrow-derived macrophages (BMDM) from mice fed Se deficient (Se-D) and Se adequate (Se-A) diets. Supplementation with Se (100 nmol/L) of IL-4-treated macrophages significantly increased the expression of alternatively activated macrophage (M2) markers, *Arg-I, Fizz1*, and *Mrc-1*. Se treatment also increased the enzymatic activity of *Arg-I* and surface expression of *Mrc-1*. Conversely, expression of classically activated macrophage (M1) markers, *TNFα*, and *IL-1β,* were significantly decreased in LPS-treated macrophages, that were cultured in Se and IL-4, suggesting a synergistic effect between Se and IL-4. Additionally, *Arg-I* activity was decreased in BMDM harvested from GPx KO mice, when compared to GPx WT mice, further establishing an important role for Se. Furthermore, BMDM treated with inhibitors of PPARγ and STAT6, pivotal transcription factors that mediate the activity of Se and IL-4, respectively, showed complete ablation of Se-dependent expression of M2 markers. In summary these studies suggest that Se supplementation of macrophages produces endogenous activators to mediate the PPARγ-dependent switch from M1 to M2 phenotype in the presence of IL-4, possibly affecting pathways of wound healing and inflammation resolution.

Introduction

Macrophages are key components of the innate immune system that play a pivotal role in pathogen clearance and resolution of inflammation (1). It is well established that macrophages are activated along two distinct pathways; the classical (M1) pathway and the alternative (M2) pathway (2). Known to initiate an inflammatory response, M1 macrophages possess an enhanced phagocytic and anti-microbial phenotype (3-5). Moreover, M1 macrophages become primed to release increased amounts of reactive oxygen and nitrogen species (RONS). Nitric oxide (NO), a known RON, is produced by the enzyme inducible nitric oxide synthase (iNOS) from L-arginine (L-Arg), an available substrate found in macrophage cells (6).

Alternatively activated macrophages are stimulated by the Th2 cytokines, IL-13 and IL-4 (2, 7-9). M2 macrophages function in resolving inflammation while promoting cell proliferation and wound healing (3, 5). The stimulation of M2 macrophages by IL-4 leads to the production of arginase-I (Arg-I) (3, 6). Arg-I acts on L-Arg, the same substrate that is acted upon by iNOS, to produce L-ornithine (L-Orn) and urea, precursors of polyamines and collagen (5, 10). This competition for substrate acts as a way to control the production of NO (10, 11). In addition to *Arg-I*, IL-4 stimulates the expression of other M2 markers, such as *Fizz-1* (found in inflammatory zone-1), and *Mrc-1* (mannose receptor 1) (9, 12, 13), while also playing a role in the activation of STAT6 and PPARγ (4, 5, 7). PPARγ, peroxisome proliferator-activated receptor, a fatty acid sensor that plays a critical role in atherosclerosis and glucose metabolism (14), can be activated by both exogenous and endogenous ligands (14). Cyclopentenone prostaglandins (e.g.15d-PGJ_2) are cyclooxygenases (COX) and hematopoietic prostaglandin D_2 synthase (H-PGDS) catalyzed products of arachidonic acid that are known to function as endogenous ligands of PPARγ (14, 15). In addition, the transcription factor STAT6 has been implicated as an integral participant of many cellular functions, including *Arg-I* expression (5, 16). IL-4 causes dimerization of STAT6 followed by translocation into the nucleus to modulate the expression of anti-inflammatory markers by up-regulating the expression of PGC-1β, a PPARγ co activator (5, 16). Thus, in the presence of IL-4 and 15d-PGJ_2, both the

pathways synergize to drive the expression of M2 pathway markers. Previous studies in our laboratory have demonstrated enhanced activation of PPARγ via the increased production of 15d-PGJ_2 in macrophages that were supplemented with selenium (Se). Selenium, an essential micronutrient, has both anti-inflammatory and cancer chemopreventative properties (17-20), is found in the body in the form of selenoproteins; proteins that contain a selenocysteine (Sec) group covalently bound to Se (17, 19, 21). Selenocysteine, also known as the 21^{st} amino acid, is coded for by the UGA codon and is recognized by a specific tRNA, ^{Sec} tRNA (17, 20). Among the identified selenoproteins, glutathione peroxidases (GPx) and thioredoxin reductases (TrxR) are the two most well characterized and abundantly expressed (15, 17). Recent research has shown an inverse causal relationship between Se deficiency and many diseases and disorders (17, 21). The Se found in foods is in its organic form, with the most common form being L-selenomethionine (SeMet) (22). A commonly used inorganic form is sodium selenite (22). Both forms of Se are metabolized via hydrogen selenide (H_2Se) for incorporation into selenoproteins (22). Due to the lack of lyase expression in cell culture models, Se in most organic forms is not readily bioavailable. To circumvent this major metabolic limitation, methylseleninic acid (MSA), a readily available organoSe source has been used in most cancer prevention studies (23).

Previously we have demonstrated the ability of Se to downregulate the LPS-induced expression of iNOS (24) and other pro-inflammatory genes (15) in macrophages. Here, we demonstrate the ability of Se, in the form of selenoproteins, to effectively switch macrophage activation from M1 towards a M2 phenotype, up-regulating the expression M2 markers, while decreasing the expression of M1 markers. Using murine macrophages, we have examined the effect of Se on Arg-I at the transcriptional, protein expression, and enzymatic activity levels, as well as other M2 markers, following stimulation with LPS or IL-4. We describe specific pathways that are important in mediating the effects of Se. In conclusion, our data shows that optimal Se status is critical for alternative macrophage activation, leading to attenuated expression of pro-inflammatory mediators.

Materials and Methods

Cell culture

Bone marrow-derived macrophages (BMDM) were prepared from 3 mo old C57/Bl6 mice maintained on Se-D (0.01 mg/kg), Se-A diets (0.1 mg/kg), and Se supplemented diets (0.4 mg/kg) (Harlan Teklad custom diets, Madison, WI). All mice, purchased from Charles River Laboratories, were 3 wk old upon arrival to the animal facilities. In accordance with Penn State University IACUC guidelines, all mice were appropriately maintained and ethically treated. Mice were killed using a CO_2 chamber. BMDM cells were collected and cultured as previously described (15). Previous studies have been done to establish the Se status of each group of mice. Total blood and erythrocyte levels were used as part of a standard procedure (15). Additionally, BMDM were prepared from GPx KO mice. The specific procedure used to create the GPx KO mice has been described previously (25). All mice used to collect BMDM were age and sex matched. RAW 264.7 macrophage cells were obtained from ATCC (Manassas, VA) and maintained as previously described (15). Specific concentrations of exogenous Se were as follows: 0 nmol/L in Se deficient cells and 100 nmol/L in Se adequate cells.

Custom diet compositions

All custom diets were purchased from Harlan Teklad (Madison, WI). The three diets; Se -D, Se-A, and Se-S, are all composed of the same base materials: Torula Yeast, 300 g/kg; DL-Methionine, 3 g/kg; Sucrose, 590 g/kg; Corn Oil, 50 g/kg; Mineral Mix (Harlan Teklad product number 80313) (26), 35 g/kg; Calcium Carbonate, 11 g/kg; Vitamin Mix (Harlan Teklad product number 40060) (26), 10 g/kg. The sodium selenite concentrations within each diet are as follows: Se-D diets, 0 g/kg; Se-A diets. 0.4 g/kg; and Se-S diets, 2 g/kg.

Treatments

Upon reaching 80% confluency, macrophages were pre-treated for 2-3 h with synthetic compounds in various combinations: Rosiglitazone (PPARγ agonist; 2 μmol/L), GW9662 (PPARγ antagonist; 1 μmol/L), leflunomide (STAT6 inhibitor;

100 μmol/L), and HQL-79 (H-PGDS inhibitor; 25 μmol/L). Rosiglitazone, GW9662 and leflunomide were purchased from Sigma-Aldrich (St. Louis, MO), while HQL-79 was purchased from Cayman Chemicals (Ann Arbor, MI). Following pre-treatment, the cells were stimulated with 5 μg/L or 10 μg/L of recombinant mouse IL-4 (R&D Systems) for 20 h, or 0.1-1 mg/L LPS (Sigma-Aldrich) for 2-12 h. Rosiglitazone, GW9662 and leflunomide were dissolved in DMSO, and HQL-79 was dissolved in 0.1 mol/L citric acid. Cells were stimulated with DMSO (0.1%, Sigma-Aldrich) and citric acid (0.1 mol/L, Sigma-Aldrich) for 20-23 h as vehicle controls. In addition, organo- Se compounds were added to cells. BMDM's and RAW 264.7 cells were also supplemented with either SeMet (100 nmol/L, Sigma-Aldrich) or MSA (100 nmol/L, Sigma- Aldrich).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP Assays were performed on RAW 264.7 macrophage cells by using the protocol provided by Ghisletti *et.al* as a template (27). Briefly, following stimulation, cells were fixed in 1% formaldehyde for 10 min at room temperature followed by the addition of 0.125 mol/L glycine for 5 min at room temperature to stop the fixation. Cells were washed two times with ice-cold wash buffer (ice-cold PBS with protease inhibitors), collected in 1mL of buffer and counted. A total of $10x10^6$ cells were collected and spun down at 3000*g* at 4°C for 5 min, and resuspended in 350 μl of cell lysis buffer (50 mmol/L Tris-HCl pH 8.0, 10 mmol/L EDTA, 1%SDS with protease inhibitors). Chromatin was sonicated using the Diagenode Bioruptor (power setting high, 20 cycles of 30 seconds on, 60 seconds off) to achieve sheared DNA between 200 and 600bp in length. Sonicated samples were centrifuged at 14,000g for 15 min at 4˚C to remove debris. A volume equivalent to 25 μg of DNA was aliquoted into dilution buffer (0.5% Triton-X 100, 2.2 mM EDTA, 22 mM Tris-HCl pH 8.0, 150 mL NaCl) to a, final volume of 1mL. Samples were pre-cleared to reduce non-specific binding, with 20 μl of protein A/G agarose beads (Santa Cruz Biotechnology) and rocking for 30 min at 4°C. Samples were spun down at 3000g for 5 min at 4°C and transferred to a fresh tube. Primary antibody p-STAT6 (sc-101808; Santa Cruz Biotechnology) and fresh protein A/G agarose beads (Santa Cruz Biotechnology)

were added to each sample and rotated overnight at 4°C. The next day, beads were collected via centrifugation at 3000g for 5 min at 4°C. Beads were washed three times with 500µl low salt wash buffer (0.1% SDS, 1% Triton-X 100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl pH 8.1, 150 mmol/L NaCl) and twice with Tris-EDTA (TE) buffer. Proteins were eluted from the beads using 200µl elution buffer (1% SDS, 0.1M $NaHCO_3$), rotating at room temperature for 1 hr, followed by reverse crosslinking (5M NaCl, 125 µg/mL proteinase K) overnight in a 65 °C water bath. Next day, sample DNA was cleaned and concentrated using a PCR clean up kit (Promega, Madison, WI) and eluted in 30µl water. A total of 5 µl of DNA was used in each qPCR reaction, using PerfeCta qPCR SuperMix SYBR (Quanta Biosciences, Gaithersburg, MD), and analyzed on an Applied Biosystems 7300. STAT6 primers are as follows: sense 5' AAGTGAGGCATTGTTCAGACTTCCTTATGCT 3', anti-sense 5'TGAGGGTGCTGGGCTAACACAGATC 3'.

Nuclear lysate extraction

Cytoplasmic fractions of RAW 264.7 cells were separated from the nuclei by lysing the cells in a buffer containing 10mmol/L HEPES-KOH, pH 7.9, 1.5mmol/L $MgCl_2$, 10mmol/L KCl, 0.1% NP-40, 0.1 mmol/L DTT, 1 mmol/L PMSF, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 µg/ml pepstatin, followed by centrifugation at 10,000g for 10 min at 4°C. The nuclear pellet was then lysed with a buffer containing 20 mmol/L HEPES-KOH, pH 7.9, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, protease inhibitors, and a phosphatase inhibitor cocktail (Research Products International, Mount Prospect, IL). Lysates were sonicated using the Diagenode Bioruptor (power setting high, 5 min of 30 seconds on, 30 seconds off), and centrifuged for 10 min at 10000g for 4°C. A total of 15µg of nuclear protein was run on an SDS-PAGE gel.

Immunoblotting

Whole cell lysates from BMDM's and RAW 264.7 cells were prepared as previously described (15). The following primary antibodies were used to probe the membranes: purified anti-mouse Arg-I (BD Transduction Laboratories), anti-rabbit phospho-

STAT6 (Santa Cruz Biotechnology), anti-rabbit β-Actin (Rockland), anti-rabbit polyclonal GPx1 (Abcam), and anti-mouse monoclonal GAPDH (Fitzgerald; Concord, MA). Near equal loading of protein was confirmed using GAPDH as the control. Chemiluminescent detection by autoradiography was used to visualize bands followed by densitometric evaluation using Image J program (National Institutes of Health).

Arginase assay

Arginase activity, assessed by a colorimetric assay that detects urea production generated by arginase hydrolysis of L-Arg, has been previously described (28). Se-D and Se-A BMDM and RAW264.7 macrophages assayed were cultured as described earlier (15). Optical density at 560 nm was recorded on a Packard plate reader. A urea standard calibration curve (0-1 μmol; $y = 9x10^{-5}x + 0.0007$, $R^2 = 0.99$) was used to calculate the Arg-I activity. Enzyme activity is expressed as μmol of urea produced/mg of protein (28).

Real time PCR

Total RNA from BMDM and RAW 264.7 cells was extracted using Isol-RNA lysis reagent (5 Prime; Gaithersburg, MD). RNA purity and concentrations were determined by agarose gel electrophoresis and UV-spectroscopy, respectively. Precisely, 1 μg total RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcriptase kit, per the manufacturer's instructions (Applied Biosystems, Foster City, CA). For the analysis of M1 and M2 markers, cDNA was analyzed using TaqMan probes for Arg-I, Fizz1,Ym1, IL12, iNOS, macrophage scavenger receptor (MSR), IL-β, TNFα, and Mrc-1. A GAPDH probe was used as an internal control to normalize the data. Amplifications were performed using PerfeCTa qPCR SuperMix Master Mix (Quanta Biosciences) in a 7300 Real time PCR system (Applied Biosystems). ΔCt ($Ct_{Gene} - Ct_{GAPDH}$) was calculated for each sample and used for analysis of transcript abundance with respect to the untreated negative control as described (29).

Flow Cytometry

RAW 264.7 cells were cultured in their appropriate media and prepared for FACS analysis. Cells were washed with ice cold 2 % FBS in PBS. Samples were blocked with purified rat anti-mouse CD16/CD32 Fc Block (BD Pharmingen, San Jose, CA) for 10 min on ice. Without removing the FC block, samples were treated with anti-mouse Mrc-1 antibody (Abcam) for 40 min on ice, followed by anti-mouse IgG1 FITC labeled secondary antibody for 40 min on ice in the dark. Samples were centrifuged, washed twice in 2% FBS, and resuspended in 500 µl of 2% FBS in PBS. Samples were analyzed on the FC500 Benchtop Cytometer using CXP software (Beckman Coulter, Brea, CA).

Statistical analysis

Results are presented as mean ± SEM. Significant differences between two groups were analyzed by Student's *t*-test, using GraphPad Prism©. Significant differences comparing more than two groups were analyzed by ANOVA, with appropriate post hoc testing, using GraphPad Prism©. Results were considered significant at $P < 0.05$. 3-way ANOVA with appropriate testing was used to analyze the interaction between diets, treatment groups, and stimulation (diet x treatment x stimulation). All experiments were performed in triplicate; triplicate indicates BMDM came from three separate mice. RAW 264.7 cells were cultured triplicate. By definition, synergistic relationships were determined by $x + y = \#xy$ ($\# \geq 2$). By definition, additive relationships were determined by $x + y = xy$.

Results

Effect on GPx1 expression in macrophages supplemented with Se.

To determine the most effective Se concentrations to use in our experiments, we used BMDM from mice on Se-D (0.01 mg/kg) and Se-A (0.1 mg/kg) diets, and RAW264.7 macrophages cultured in media containing 0 nmol/L or 100 nmol/L Se. Using cytosolic GPx1 as a marker of Se status, we examined the expression of *GPx1* in BMDM cell lysates treated with LPS or IL-4 in the presence or absence of Se. There was a clear distinction in *GPx1* expression between Se-D and Se-A cell lysates, indicating an increased expression of *GPx1* in BMDM from mice fed Se-A diets (Figure. 14A). Similarly, RAW264.7 cells treated with LPS or IL-4 showed an increase in GPx1 expression in those cultured in the presence of 100 nmol/L Se, but not in those cultured in 0 nmol/L Se (Figure. 14B). Furthermore, the presence of IL-4 or LPS did not significantly affect the expression of *GPx1* in the Se-D or Se-A BMDM or RAW264.7 cells ($P > 0.05$).

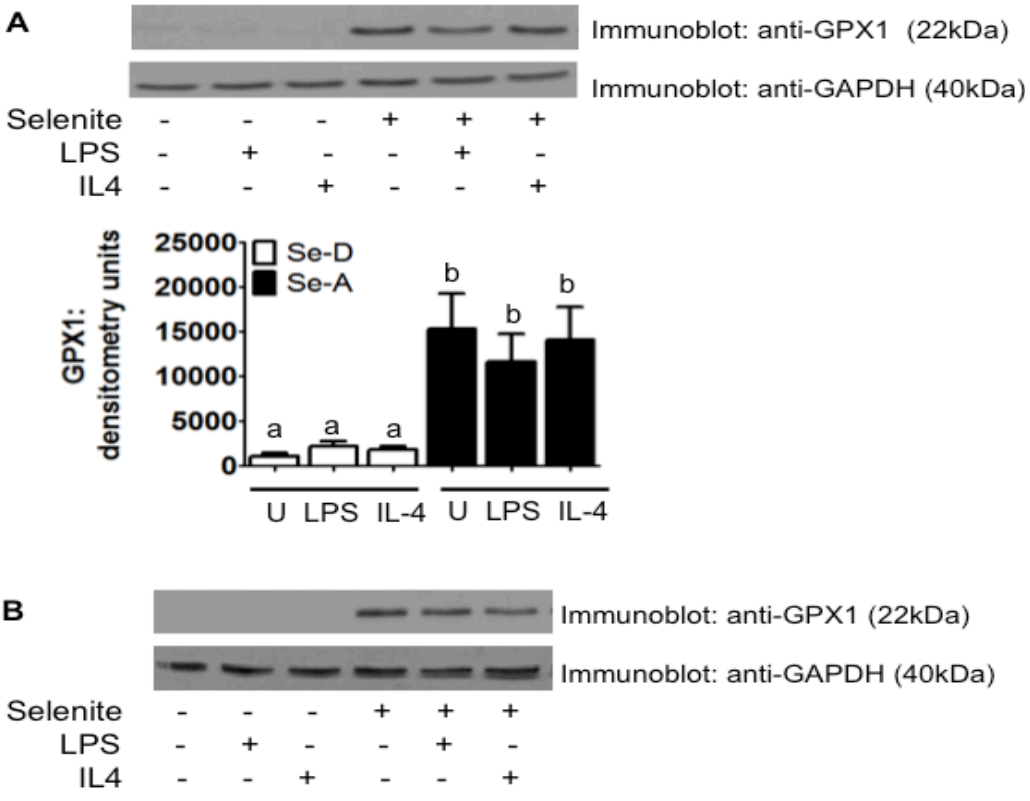

Figure 14: Effect of inorganic selenium on the expression of GPx1 in macrophages. (A). BMDM were isolated from Se-D or Se-A mice. Cells were stimulated with IL-4 (5 mg/L) for 20 h or LPS (1 mg/L) for 12 h. (B). RAW 264.7 macrophages were cultured in the absence or presence of Se for 4 d, and GPx1 expression was examined. Bands were evaluated by densitometry. Values are means ± SEM, n=3. Data was analyzed by ANOVA with tukey post-hoc testing. Within each graph, means without a common letter differ, a>b>c; $P < 0.01$.

Selenium supplementation of macrophages increases Arg-I expression and activity

Given that the BMDM responded to exogenous Se by increasing the *GPx1* expression levels, we examined if supraphysiological (250 nmol/L) levels of exogenous Se would further increase the activity of Arg-I. *Arg-I* activity in BMDM from Se-A mice, treated with IL-4 (20 h) was greater than the activity in BMDM from Se-D mice (students t test analysis; * $P < 0.05$) (Figure 15A). However, there was no significant difference between BMDM from Se-A mice and Se-S mice(data not shown). Thus, given that supraphysiological levels of Se does not necessarily increase the *Arg-I* activity, we examined the modulation of *Arg-I* within mice fed Se-D and Se-A diets. *Arg-I* activity in IL-4 stimulated BMDM was greater than LPS-stimulated and unstimulated BMDM from Se-A mice (Figure. 15B). IL-4 stimulated BMDM showed a significant increase in activity when comparing Se-D to Se-A fed mice ($P < 0.001$) (Figure. 15B). Similar results were obtained in experiments performed with Se-D (0nmol/L Se) and Se-A (100nmol/L Se) RAW 264.7 macrophages ($P < 0.001$) (Figure. 15C). Furthermore, *Arg-I* RT-PCR analysis of the BMDM cDNA indicated a similar pattern; *Arg-I* mRNA expression in BMDM treated with IL-4 from Se-A mice was significantly greater than in Se-D BMDM with IL-4 treatment (Figure. 15D). Western blot analysis of BMDM extracts showed a greater expression of *Arg-I* in IL-4 stimulated BMDM from Se-A mice, when compared to those BMDM from Se-D mice (Figure. 16; compare lanes 3 and 6). Surprisingly, *Arg-I* protein expression was greater in Se-A BMDM than in Se-D BMDM, prior to treatment with LPS or IL-4 (Figure. 16, compare lanes 1 and 4) ($P < 0.05$). Taken together, these data clearly indicate that Se status plays an important role in the expression of Arg-I in macrophages.

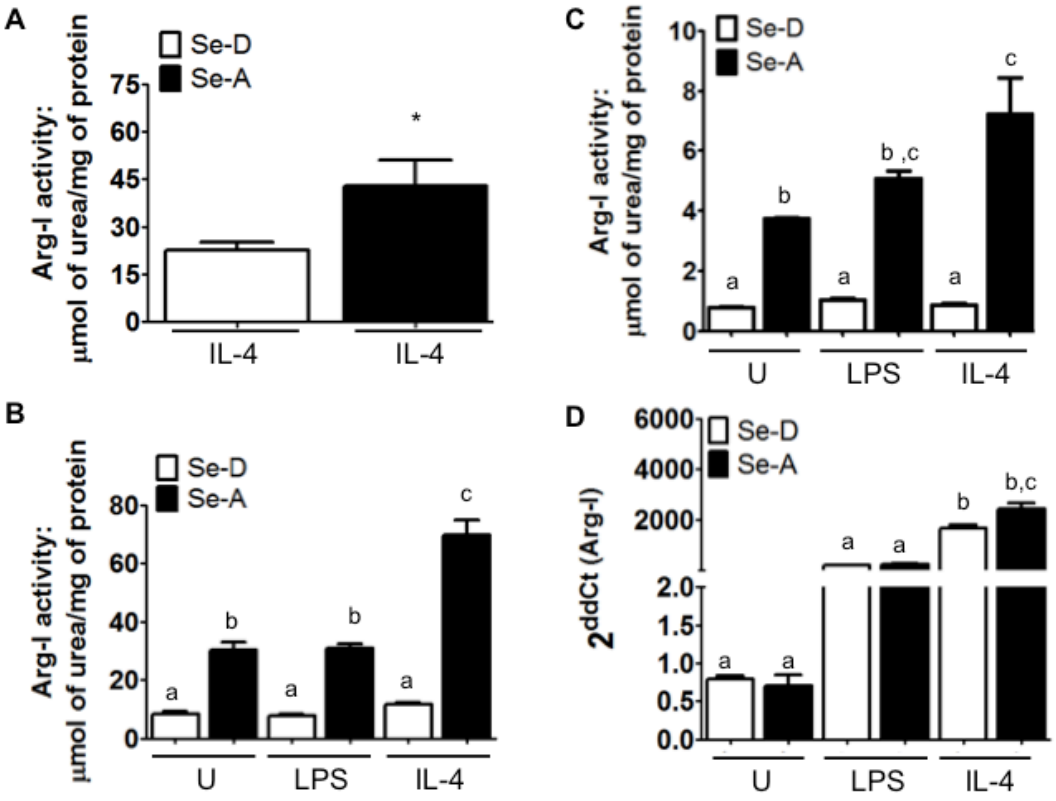

Figure 15: Effect of Se on the expression and activity of Arg-I in BMDM and RAW264.7 macrophages. (A). *Arg-1* activity measured in BMDM from Se-D and Se-A mice, stimulated with 5 mg/L IL-4. (B). BMDM from Se-D and Se-A mice, and (C) RAW 264.7 cells, stimulated with IL-4 (10 mg/L; 20 h) and LPS (1 mg/L; 12 h). (D). *Arg-I* mRNA expression determined in BMDM from Se-D and Se-A mice by real time RT-PCR. Values are means ± SEM, n=3. Within each graph, means without a common letter differ, a>b>c; $P < 0.01$

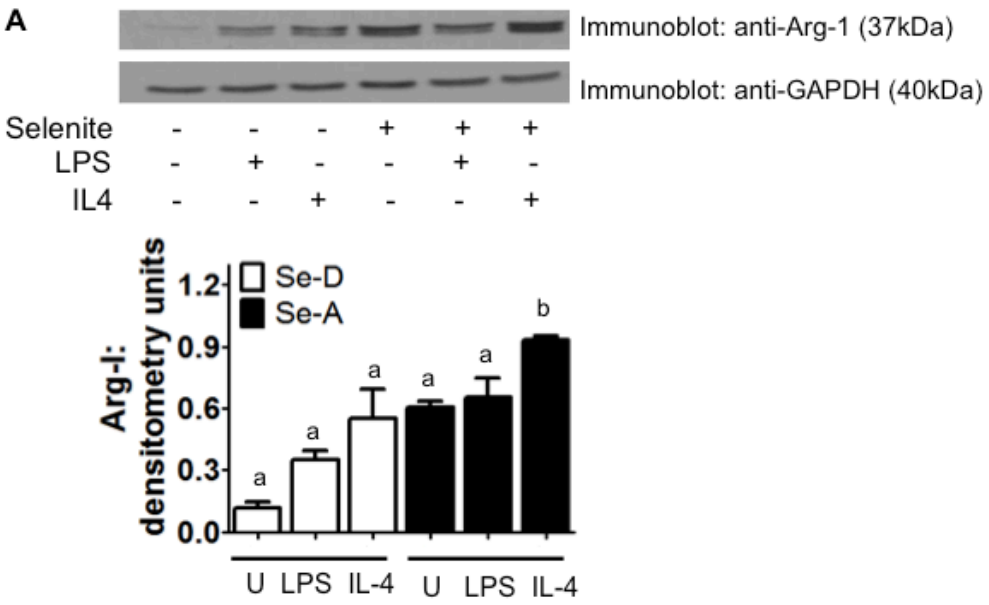

Figure 16: Selenium supplementation of macrophages increases Arg-I expression. BMDM were stimulated with IL-4 (5mg/L; 20 h) and LPS (1mg/L; 12 h) and prepared for western blot analysis. Bands were evaluated by densitometry. Data was analyzed by ANOVA with tukey post-hoc testing. Within each graph, means without a common letter differ, a>b>c; $P < 0.01$.

Selenium supplementation of macrophages leads to differential modulation of M1 and M2 markers

Having established that Se supplementation significantly increases Arg-I expression, we examined the modulation of three other M2 macrophage markers; *Mrc-1*, *Fizz1*, and *Ym-1* (30, 31). RAW 264.7 macrophages and BMDM were treated with IL-4 (20 h) and LPS (4 h). IL-4 treatment only, but not LPS, increased the expression of *Mrc-1* in Se-A RAW 264.7 cells when compared to Se-D cells ($P < 0.001$) (Figure. 17A). Expression of Fizz-1 and Ym-1 in Se-A BMDM treated with IL-4 was greater than that seen in Se-D BMDM, similarly treated ($P < 0.01$) (Figure. 17B, 17C). Furthermore, flow cytometric analysis of IL-4 treated RAW 264.7 macrophages showed a significantly greater surface expression of *Mrc-1* in Se-A cells than in Se-D cells ($P < 0.05$) (Figure. 17D). These data complement the real time PCR results described above.

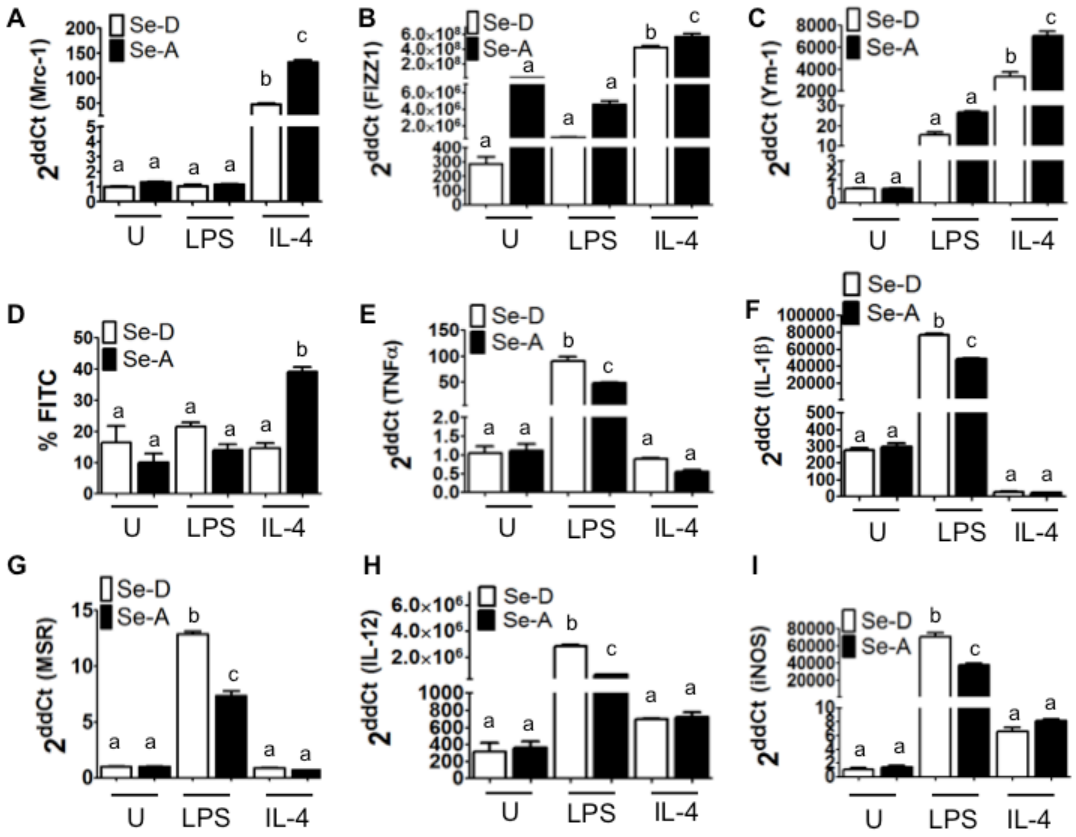

Figure 17: Modulation of expression of M1 and M2 macrophage markers by Se in RAW264.7 macrophage cells and BMDM. (A) RAW 264.7 macrophages and (B-I) BMDM were cultured with and without Se and stimulated with IL-4 (5 mg/L; 20 h) or LPS (0.1 mg/L; 4 h). Real Time RT-PCR expression of (A) *Mrc-1*, (B) *Fizz1*, (C) *Ym1*, (E) *TNFα*, (F) *IL-1β*, (G) *MSR*, (H) *IL-12*, and (I) *iNOS*. (D) RAW 264.7 macrophage cells were cultured with and without Se, followed by stimulation with IL-4 (5mg/L; 20 h) or LPS (0.1mg/L; 4 h). Flow cytometry was used to analyze the expression of Mrc-1. Values are means ± SEM, n=3. Within each graph, means without a common letter differ, a>b>c; $P < 0.01$.

Given that Se status of macrophages determined whether M1 or M2 pathway was activated, we also examined selenium's modulation of an array of M1 markers; *TNFα*, IL-*1β, IL-12, iNOS*, and *MSR*. All well known markers of classical macrophage activation, BMDM from Se-D and Se-A mice were examined. Stimulation of Se-A BMDM with LPS alone showed significantly decreased marker expression when compared to their Se-D counterparts (Figure. 17E-I). Treatment with IL-4 did not induce the expression of the M1 markers, when compared to their Se-D

counterparts ($P > 0.05$). Upon further examination, Mrc-1 and Ym-1 showed a greater than two fold change in transcript expression in Se-A cells treated with IL-4 compared to Se-D cells similarly treated (Figure. 17A, 2C). IL-12 showed similar changes in Se-A BMDM treated with LPS, when compared to Se-D BMDM (Figure. 17G). Conversely, Fizz-1, TNFα, IL-1β, iNOS, and MSR show additive changes in transcript expression in Se-A cells, treated with either IL-4 or LPS, when compared to their Se-D counterparts (Figure. 17B, 17E, 17F, 17H, 17I). These results indicate that Se synergizes with IL-4 to up-regulate the expression of M2 markers, while down regulating the expression of LPS-induced M1 markers, facilitating the switch towards alternative pathway of macrophage activation.

Selenoproteins are required for Arg-I expression

Since Se is incorporated into proteins via a co-translational mechanism as Sec, we examined if Se in the form of selenoproteins were required for the expression of *Arg-I* expression. RAW 264.7 macrophage cells were supplemented with two different organic forms of Se; Se-Met, and MSA. The difference between Se-Met and MSA is that MSA can form selenoproteins, while Se-Met is unable to release Se without γ-lyase, particularly in macrophages. Much like sodium selenite, MSA also significantly increased *Arg-I* activity when compared to Se-D cells ($P < 0.001$; comparisons done using ANOVA with Dunnett's post hoc testing) (Figure. 18A). Interestingly, RAW 264.7 macrophages supplemented with Se-Met failed to show any increase in *Arg-I* activity when compared to Se and MSA supplemented RAW 264.7 cells ($P > 0.05$) (Figure. 18A). Furthermore, IL-4 treatment did not increase the *Arg-I* activity in Se-Met supplemented RAW cells ($P > 0.05$) (Figure. 18A). Western blot analysis of *GPX1* mimicked these results, exhibiting a clear expression in MSA and sodium selenite supplemented cells, while Se-Met cells showed no GPX1 expression (Figure. 19).

To further confirm the effects of selenoproteins on *Arg-I* expression, we utilized a *GPx1* knockout (GPx1 KO) mouse model. BMDM macrophages from WT and GPx1 KO mice were cultured as stated in *Materials and Methods* and treated with LPS (12

h) and IL-4 (20 h). *Arg-I* activity was significantly higher in the WT BMDM treated with IL-4, when compared to the *GPx1* KO cells treated with IL-4 ($P < 0.001$) (Figure. 18B). Interestingly, untreated and LPS-treated BMDM from *GPx*1 KO, compared to *GPx1* WT, mice failed to show an increase in *Arg-I* activity (Figure. 18B). Both the *ex vivo* and *in vivo* results clearly demonstrate a requirement for Se in the form of selenoproteins to increase Arg-I activity.

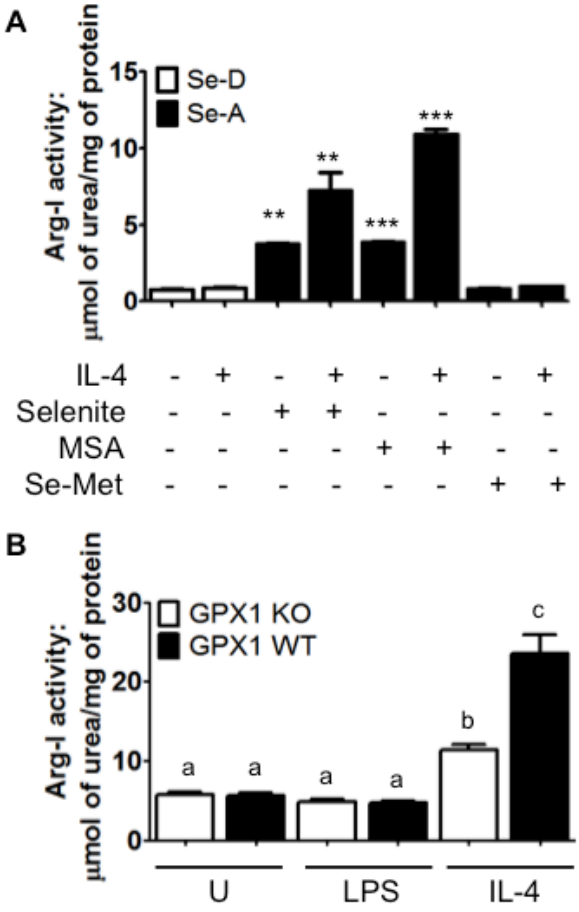

Figure 18: Selenium in the form of selenoproteins is essential for Arg-I expression in RAW 264.7 macrophage cells and BMDM cells. (A). RAW 264.7 cells were cultured with 100 nmol/L MSA or 100 nmol/L Se-Met for 4 d, and stimulated with IL-4 (5 mg/L; 20 h). Values are means ± SEM, n=3. ** $P < 0.01$, *** $P < 0.001$. All means were compared to one control (Se-D RAW 264.7 cells) and analyzed using ANOVA with Dunnett's post hoc testing. (B). BMDM cells isolated from GPx1 KO and GPx1 WT C57bl/6 mice were cultured with 100 nmol/L sodium selenite for 3 d, and stimulated with IL-4 (5 mg/L; 20 h) and LPS (1mg/L; 12 h). Values are means ± SEM, n=3. Within each graph, means without a common letter differ, a>b>c; $P < 0.01$.

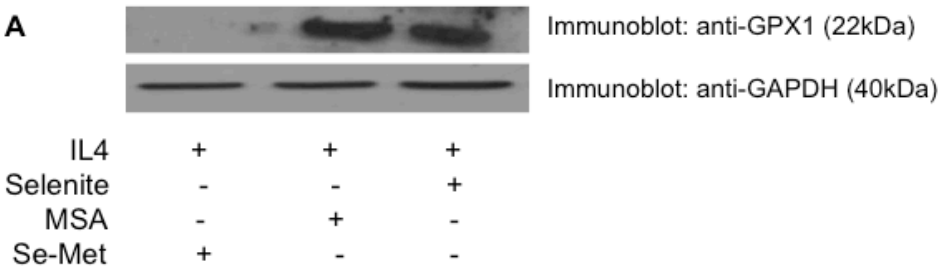

Figure 19: Selenium in the form of selenoproteins is essential for Arg-I expression. Following treatment with sodium selenite, MSA, or Se-Met, RAW 264.7 were stimulated with IL-4 (5mg/L; 20 h) and prepared for western blot analysis. Values are representative data from one experiment, which is indicative of a pattern seen.

PPARγ and STAT6 is essential for the Se-dependent up-regulation of Arg-I

To connect the increased activation of PPARγ to Arg-I expression in the context of Se status, we used rosiglitazone, a synthetic PPARγ agonist, and GW9662, a PPARγ antagonist (32). RAW 264.7 and BMDM were pre-treated with either rosiglitazone or GW9662 for 2 h. Following pre-treatment, cells were stimulated with IL-4 as described earlier. Both rosiglitazone and GW9662 remained on the cells for a total of 22 h. Pretreatment of cells with rosiglitazone at1 μmol/L significantly increased the activity of *Arg-I* in IL-4-treated Se-A RAW264.7 macrophages, when compared to Se-D RAW 264.7 macrophages treated with IL-4 ($P < 0.001$) (Figure. 20A). Moreover, rosiglitazone pretreatment significantly increased *Arg-1* activity in IL-4-treated Se-A RAW 264.7 macrophages when compared to vehicle control IL-4-treated Se-A RAW 264.7 macrophages ($P <0.001$) (Figure. 20A). Pretreatment of BMDM, from Se-A mice, with GW9662 at 1 μmol/L completely blocked the effect of Se, while greatly inhibiting activity in IL-4-treated cells, such that the *Arg-I* activity in Se-A BMDM was not different from that in IL-4 treated Se-D BMDM (Figure. 20B). In contrast to rosiglitazone treatment, GW9662 treatment significantly decreased *Arg-1* activity in IL-4-treated Se-A BMDM, when compared to vehicle control IL-4-treated Se-A BMDM ($P < 0.01$) (Figure. 20B). Furthermore GW9662 treated Se-A RAW264.7 cells significantly inhibited the IL-4 dependent increase in *Mrc-1* expression, when compared to the vehicle control, suggesting a critical role for PPARγ in the regulation of M2 markers by Se ($P < 0.001$) (Figure. 20C).

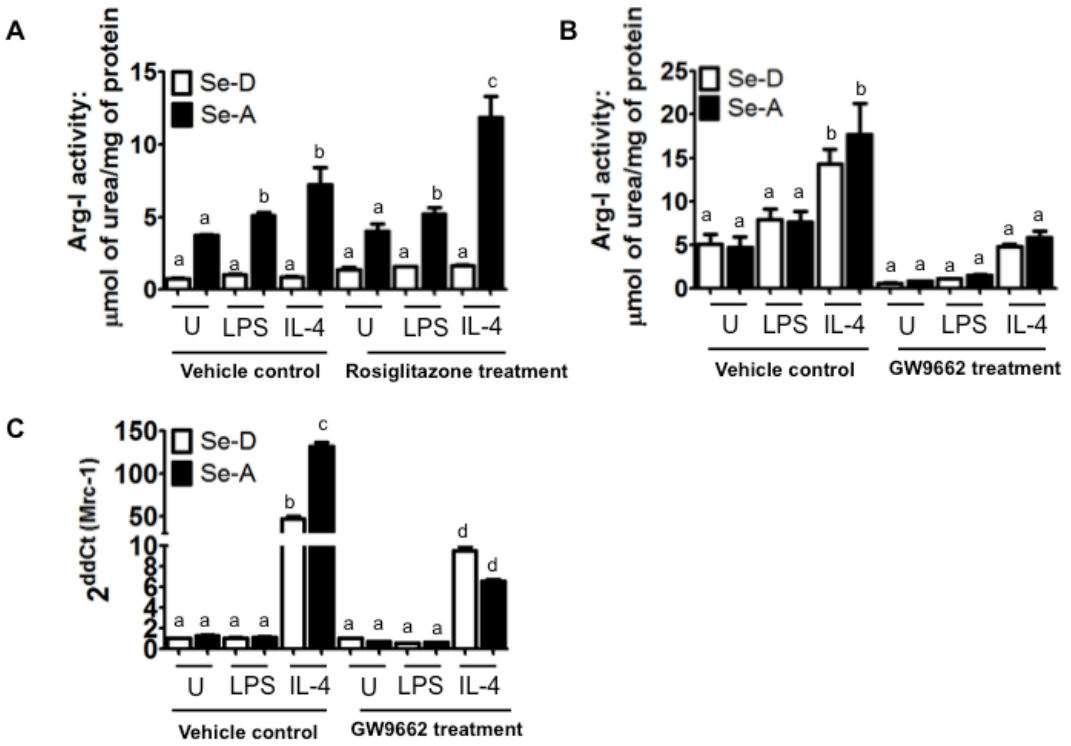

Figure 20: Selenium acts through PPARγ to up-regulate the expression of Arg-I and Mrc-1. (A). RAW 264.7 cells were pre-stimulated with 1µmol/L rosiglitazone for 2 h, followed by IL-4 (5 mg/L; 20 h) and LPS (1 mg/L; 12 h). (B). BMDM isolated from Se-D and Se-A mice were pre-stimulated with 1µmol/L GW9662 for 2 h followed by IL-4 (5 mg/L; 20 h) and LPS (1 mg/L; 12 h).. (C). RAW 264.7 macrophage cells were cultured with and without Se followed by pretreatment with 1µmol/L GW9662 for 2 h prior to stimulation with IL-4 (5 mg/L; 20 h) and LPS (0.1mg/L; 4 h). Values are means ± SEM, n=3. Within each graph, means without a common letter differ, a>b>c; $P < 0.01$.

Interleukin-4 mediates PPARγ-dependent gene expression through a STAT6-dependent mechanism involving enhanced recruitment of PGC-1β, a well-known PPARγ coactivator (5). To examine the role of STAT6, we utilized leflunomide, which inhibits the phosphorylation of STAT6, preventing the nuclear translocation and subsequent binding to cognate sites on the DNA (33). BMDM were pretreated with 100 µmol/L leflunomide for 2 h, followed by IL-4 treatment as described earlier. Consistent with the PPARγ antagonist results, leflunomide treatment significantly inhibited *Arg-I* activity in IL-4 treated Se-A BMDM (compare vehicle control Se-A BMDM to leflunomide treated Se-A BMDM) ($P < 0.001$) (Figure. 21). This data suggests Se up regulates Arg-I expression through a STAT6 dependent pathway.

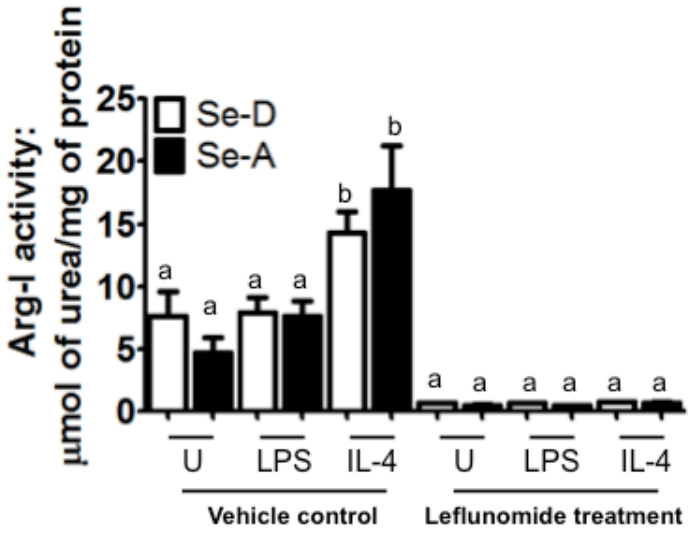

Figure 21: STAT6 pathway inhibitor treatment plays a critical role in the expression of Arg-1 in Se adequate macrophages. BMDM isolated from Se-D and Se-A mice were pretreated with 100 µmol/L leflunomide for 2 h, followed by IL-4 (5 mg/L; 20 h) and LPS (1 mg/L; 12 h). Values are means ± SEM, n=3. Means without a common letter differ, a>b>c; $P < 0.01$.

Se does not increase STAT6 binding to the Arg-I promoter or STAT6 nuclear translocation

In order to determine if the increase in Arg-I expression is due to an enhanced binding of STAT6 to the Arg-I promoter, we used RAW 264.7 macrophages cultured in media containing 0 nmol/L or 100 nmol/L of Se (in the form of selenite), and treated with IL-4 (20 h). Utilizing a ChIP assay, when compared to its Se-D counterpart, adequate levels of Se did not show an increase in binding of STAT6 to the Arg-I promoter (Fig. 6A). Expectedly, treatment with IL-4 significantly increased STAT6 binding ($P < 0.01$) (Figure. 22A), as IL-4 stimulation induces activation of STAT6 (7). However, when compared to Se-D macrophages treated with IL-4, Se-A cells showed no increase in binding (Figure. 22A).

To determine if Se supplementation increases the translocation of STAT6 into the nucleus, nuclear extracts from RAW 264.7 macrophages, treated with and without leflunomide (100 µmol/L) for 2 h prior to IL-4 treatment, were collected. In our non-leflunomide treated lysates (Figure. 22B, first blot), expression of phospho-STAT6 was increased equally in Se-D and Se-A lysates stimulated with IL-4 ($P > 0.5$) (Figure. 22B, first blot). Interestingly, leflunomide treatment completely abrogated

any expression of phospho-STAT6, regardless of Se presence or IL-4 stimulation (Figure. 22B, second blot). In addition, we used β-actin as an internal control to confirm equal protein loading (Figure. 22B, third blot). Together, this data demonstrates Se treatment does not enhance binding to the Arg-I promoter, nor increase translocation of phosph-STAT6 into the nucleus. Moreover, in the presence of IL-4, Se is unable to augment any of the changes seen, suggesting Se may be working through other mechanisms to increase the expression or Arg-I.

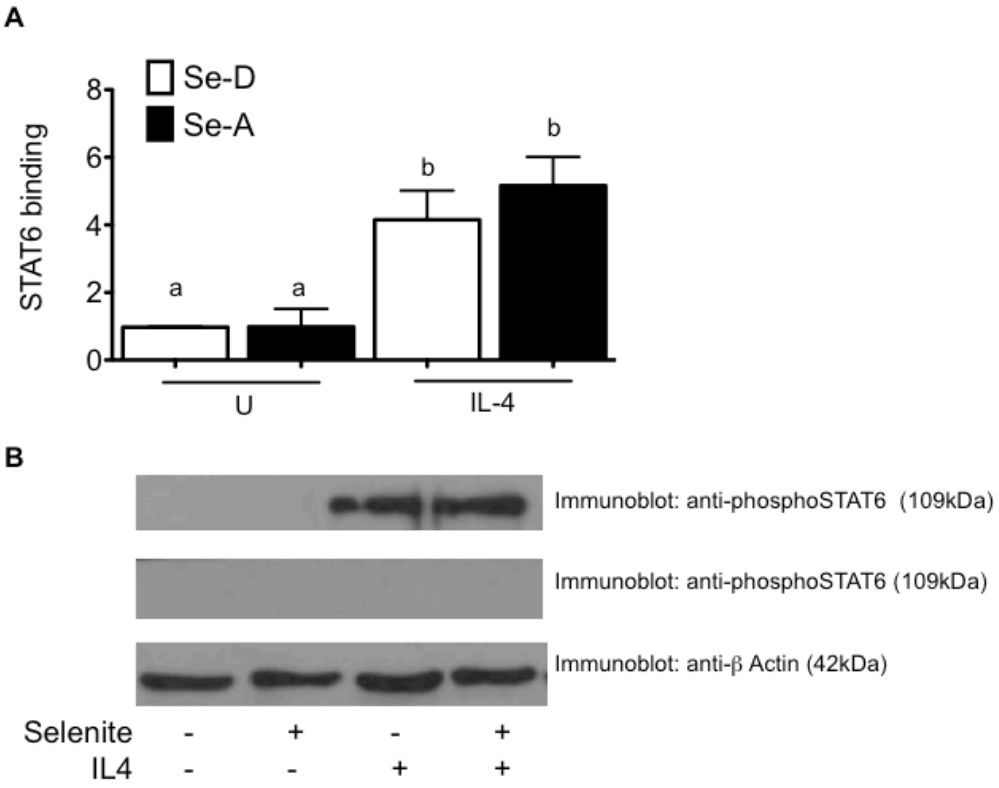

Figure 22: STAT6 binding to Arg-I promoter is unaffected by Se. (A). RAW 264.7 macrophages were cultured in the absence or presence of Se for 4 d, followed by IL-4 (5 mg/L) for 20 h. Lysates were analyzed using a ChIP assay. (B). Following treatment with sodium selenite, RAW 264.7 macrophages were pre-treated with 100 μmol/L leflunomide for 2 h (second blot), followed by IL-4 (5 mg/L; 20 h) (first and second blot). Nuclear extracts were collected and prepared for western blot analysis. Values are means ± SEM, n=3. Means without a common letter differ, a>b>c; $P < 0.01$

Selenium-dependent up-regulation of Arg I expression is mediated via arachidonic acid metabolism by H-PGDS

To provide a mechanistic explanation of the effect of Se on the PPARγ -dependent expression of *Arg-I*, we examined the role of endogenous ligands of PPARγ, particularly 15-deoxy-$\Delta^{12, 14}$- PGJ_2 (15d PGJ_2) formed through an H-PGDS pathway. Se-A BMDM were pre-treated with 25 μmol/L HQL-79 for 2 h, followed by IL-4 and LPS treatments as described earlier. HQL-79 inhibits H-PGDS, a cytosolic enzyme responsible for the synthesis of PGD_2 from PGH_2, an arachidonic acid-derived COX metabolite (34, 35). Treatment of Se-A BMDM with HQL-79 and IL-4 significantly decreased the Se-dependent expression of *Arg-I* (compare vehicle control Se-A BMDM to HQL-79 treated Se-A BMDM) ($P < 0.01$) (Figure. 23). Though IL-4-treated Se-A BMDM showed higher activity than unstimulated and LPS-treated samples, they were still lower than vehicle control treated Se-A BMDM. Taken together, these results suggest that the arachidonic acid pathway plays a critical role in mediating the effect of Se**.**

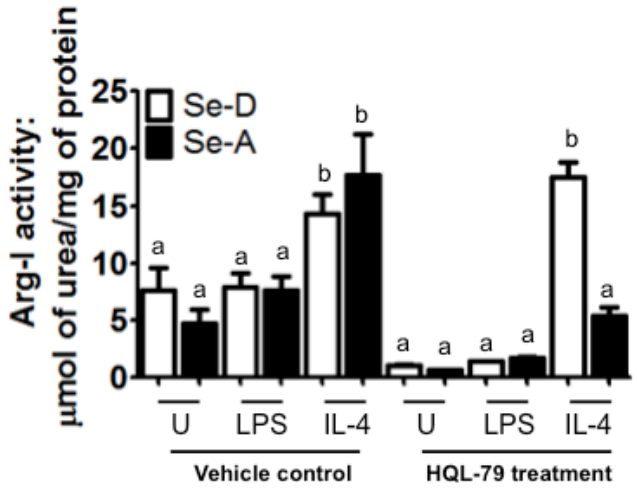

Figure 23: HQL-79 treatment plays a critical role in the expression of Arg-1 in Se adequate macrophages. BMDM isolated from Se-D and Se-A mice were pretreated with 25 μmol/L HQL-79 for 2 h followed by stimulation with IL-4 (5 mg/L; 20 h) and LPS (1 mg/L; 12 h). Values are means ± SEM, n=3. Within each graph, means without a common letter differ, a>b>c; $P < 0.01$.

Discussion

Macrophages are well known effectors that have a significant bearing on the duration, magnitude, and quality of the immune response. Mounting evidence describes a more complex model that involves multiple macrophage phenotypes, which influences immunity not only via their ability to downregulate the production of pro-inflammatory mediators, but also to facilitate pathways of resolution and wound healing. The latter property arises from a subset of macrophages (M2a-c) that express a battery of cytokines and cell surface receptors to promote catabasis responses leading to tissue repair and angiogenesis (2, 36). We present here novel findings that Se status of macrophages is critical to promote the expression of an alternatively activated phenotype that are linked to wound healing and collagen synthesis. Such a switch from M1 towards M2 pathway is further complemented by a substantial decrease in the expression of NF-κB dependent pro-inflammatory genes, such as *TNFα*, *IL-1β*, and *iNOS*, which are markers of the classically activated macrophages (15, 24). Our studies have shown that macrophages cultured with Se produce an endogenous lipid mediator, 15d-PGJ_2, which activates PPARγ-dependent pathways of anti-inflammatory gene expression (15), while repressing expression of pro-inflammatory genes. In addition, we demonstrate the ability of Se to synergize with IL-4-dependent activation of STAT6 to activate PPARγ.

Previously it has been shown that IL-4 can act as a stimulus to drive the expression of many M2 markers, such as *Arg-I*, *Mrc-1*, and *Fizz-1* (9, 30, 31). In Se-A cells, IL-4 treatment significantly increased *Arg-I* at the activity, protein, and transcript levels. *Arg-I* activity increased in both the Se-D and Se-A macrophages. However, the greatest increase in activity levels were seen in Se–A macrophages treated with IL-4 compared to their Se-D counterpart, suggesting a synergistic effect between IL-4 and Se. Further assessment revealed there was no interaction. Additionally, Arg-I activity levels were assessed using IL-13, another Th2 cytokine shown to act as a stimulus of M2 marker expression (9, 30). While IL-13 treatment of Se-A macrophages increased the activity of *Arg-I*, the magnitude of increase was much lower than with IL-4 (data not shown). Seen even in a pro-inflammatory state, Se shifts the L-Arg metabolism

from LPS-induced production of NO via iNOS toward production of L-Orn and polyamines that are important for wound healing. *Arg-I* is a key determinant of M2 macrophage activation that is widely studied as a marker of M2 macrophages in murine macrophages; however, *Arg-I* is not expressed in human macrophages (2). While *Fizz-1* and *Ym-1* are also restricted to the murine system, we examined *Mrc-1*, which is expressed in both murine and human systems (2, 31). As reported earlier, IL-4 stimulation greatly enhanced the expression of *Mrc-1* (9, 31). Upon Se supplementation and IL-4 treatment, our data shows a significant increase in *Mrc-1* transcript levels, when compared to the cells cultured in Se-D media with IL-4. Intriguingly, exogenous addition of Se showed no increased levels of *Mrc-1* expression with LPS, when compared to the Se-D cells cultured in the presence of LPS. A similar pattern was also observed with *Fizz1* expression. Although expression of *Ym-1* was significantly increased by LPS treatment of Se-A macrophages, when compared to the Se -D cells, its levels are far below those seen with IL-4 treatment. While the repression of IL-4 dependent M2 genes by LPS is thought to be an event required for the polarization of macrophages towards M1 pathway, Se supplementation appears to favor the polarization towards a resolution response, which might be essential to prevent the exacerbated activation of pro-inflammatory genes leading to tissue destruction and inflammation. On the other hand, *IL-12*, *iNOS*, *IL-1β* and *TNFα,* all M1 markers, showed the opposite trend upon stimulation with LPS. LPS increased *IL-1β* and *IL-12* transcript expression, while IL-4 had very little to no effects. Interestingly, IL-4 significantly increased transcript expression of *TNFα*. However their expression levels were far below those seen with LPS. Indeed, expressions of all markers were significantly decreased with Se supplementation. Although there were differences between BMDM and RAW264.7 cells, which represent primary and immortalized cultures, respectively, the trend towards increased M2 markers in Se-A cells with IL-4 was near identical. Such a concordance in results further lends credence to the idea that Se status, indeed, does play a pivotal role in the expression of some of the M2 markers.

The mechanisms for the up-regulation of *Arg-I* by Se are still not well understood. Our earlier work conclusively showed that Se supplemented macrophages produced high amounts of endogenous PPARγ agonist, 15d-PGJ_2, to accompany activation of PPARγ (15). The antagonist, GW9662, inhibited such an activation of PPARγ. However, the PPARg agonist, Rosiglitazone, did not greatly enhance Arg-I activity in the presence of Se. A possible explanation for this is dependency on PPARβ/δ. Use of PPARβ/δ agonists would be useful to examine this. Given that PPARγ activation up-regulated *Arg-I* expression (5) and our results show that the use of HQL-79 (H-PGDS inhibitor) completely blocked the effect of Se-dependent increase in *Arg-I*, these results further confirm the ability of Se to enhance the production of an endogenous ligand that plays a major role in the expression of Arg-I. Curiously, treatment with HQL-79 did not block Arg-1 activity in our Se-D BMDM stimulated with IL-4. A possible explanation for this may be a specific induction of an unidentified transcription factor in BMDM, as HQL-79 treatment in RAW 264.7 cells does not show the same pattern. In addition to PPARγ, the use of leflunomide also indicates a potential cross talk between IL-4 activation of STAT6 and PPARγ, where Se plays a key role as a positive modulator, perhaps by recruiting PGC-1β, a PPARγ coactivator (Figure. 24). Intriguingly, Se does not play a role in increasing binding or localization of STAT6 to the Arg-I promoter. These results create interesting scenarios of how Se can regulate the expression of Arg-I, while having such little effect at the promoter level.

In addition to PPARγ, the ability of Se to increase Arg-I activity was only seen with those selenocompounds that led to the cellular synthesis of selenoproteins. Given that GPx1, an abundant selenoprotein whose expression is increased by bioavailable Se, the use of a genetic GPx1 KO mouse model was pertinent in confirming this observation. Our results conclusively show that the absence of GPx1 significantly decreases *Arg-I* activity, as compared to WT, confirming the need for Se in the form of selenoproteins to effectively modulate the redox status of cells. Furthermore, the addition of IL-4 confirmed the synergistic relationship seen with Se. Needless to say, further examination of the *Arg-I* promoter for the binding of transcription factors and

accessory proteins as a function of Se status are awaited to get a complete picture of the underlying basis of the molecular transition of M1 to M2 phenotypes by specific selenoproteins.

In conclusion, our results clearly demonstrate that Se supplementation shunts macrophage activation from a pro-inflammatory M1 state towards an anti-inflammatory M2 state. In doing so, we speculate that such macrophages become prone to helping cell proliferation and promoting cell growth after insult or injury. This is reminiscent of a recent report where Se status in T-cells was shown to be critical for activation, differentiation, and proliferation (37). Further work to validate these *ex-vivo* studies will be needed to examine the role of such a phenotypic switch in models that are known to trigger highly polarized immune responses associated with increased M2 signatures, such as gastrointestinal helminth models, increasing parasite expulsion and regulating inflammation.

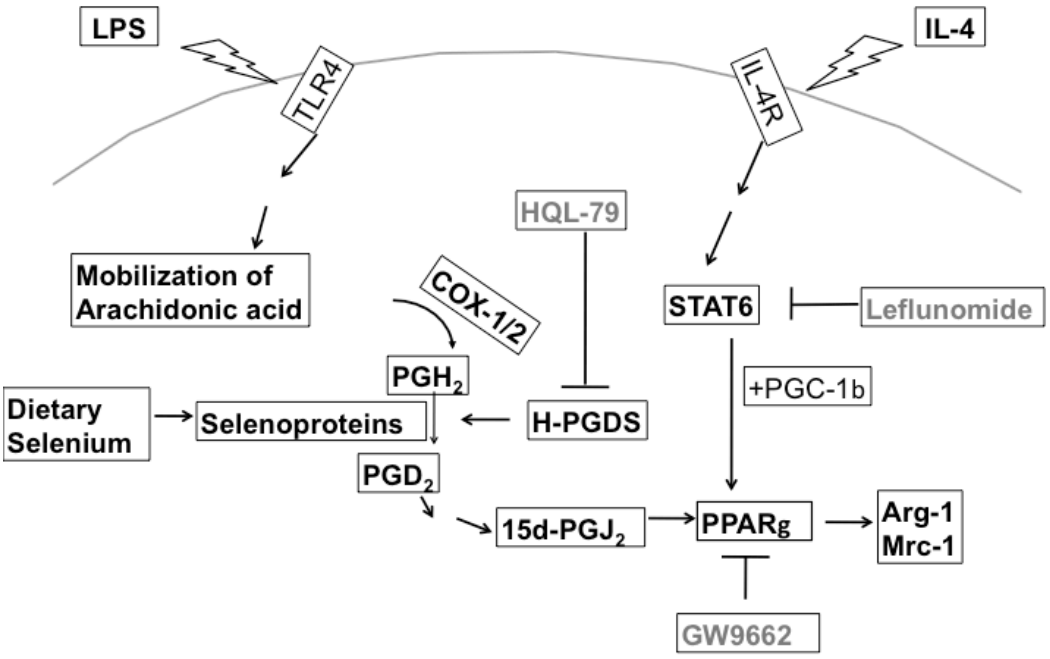

Figure 24: A proposed mechanism underlying Se dependent up-regulation of *Arg-I*, *Mrc-1* and other M2 markers through the modulation of STAT6 and PPARγ -dependent pathways in macrophages.

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Chapter 3

Selenoprotein enhanced expression of M2 macrophages increases clearance of *Nippostrongylus brasiliensis*

Shakira M. Nelson, Jamaal James, Joseph Urban Jr., Bradley Carlson, K. Sandeep Prabhu

[**Contributions**: This study was co-performed by Jamaal James (Fig. 28, 34, 35). $Trsp^{fl/fl}$ Cre^{LysM} mice (Fig. 29, 30) were kindly provided by Bradley Carlson. Joseph Urban provided cultures of *N. brasiliensis* larvae]

Abstract

The plasticity of macrophages is evident in helminthic parasite infections where they play a role in both inflammation and protection. Previously, we demonstrated that Se, in the form of selenoproteins, induced a phenotypic switch in macrophage activation from a pro-inflammatory (M1) towards an anti-inflammatory (M2) phenotype. While some helminthic species induce a pro-inflammatory Th1 response, the gastrointestinal nematode parasite *Nippostrongylus brasiliensis* (*N. brasiliensis*) induces a biased Th2 response that activates M2 macrophages and contributes to parasite clearance. Additional studies have shown Se deficiency inhibits *N. brasiliensis* expulsion. Here we hypothesized that Se modulates macrophage activation towards an M2 phenotype that can decrease the infectivity and increase the clearance of adult *N. brasiliensis* from the intestine. Se supplementation significantly augmented intestinal M2 macrophage infiltration, while decreasing adult worms and fecal eggs. To implicate macrophage-specific selenoproteins in resolution, Se supplemented WT $^{fl/fl}$ mice, compared to Trsp$^{fl/fl}$Cre^{LysM} mice, showed a complete abrogation in M2 marker expression with a significant increase in intestinal worms and fecal eggs. Studies inhibiting the COX pathway using indomethacin displayed delayed worm and egg expulsion, as well as reduced expression of M2 markers despite high Se levels. Treatment of Se deficient mice with 15d-PGJ_2 partially recapitulated the effect of Se supplementation on reduced fecal egg output by *N. brasiliensis* through the COX pathway. These results suggest that optimal Se status in the form of selenoproteins and selenium-dependent production of anti-inflammatory prostaglandins regulate M2 macrophage activation to promote resolution of helminthic parasite infections.

Introduction

Helminths comprise a variety of parasitic worms that trigger common characteristics of infection and disease within its host (1-4). Interestingly, responses to these infections not only share many common features, but also induce a host response that is similar between species (3, 5-9). While helminthic parasites can infect numerous tissues, many invade a specific organ niche and remain there throughout their life cycle, undergoing growth and differentiation into adult stages with the ultimate goal of transmission to the next host (4, 6, 10-12). Helminth infections can be brief, quickly leaving the host, while others are chronic, residing within the host for months to years (3, 6, 13-16). Gastrointestinal nematode parasites such as *Nippostrongylus brasiliensis* have short infection cycles, fully maturing to adult worms within the small intestine before being expelled (9, 17-19). Although a murine parasite, *N. brasiliensis* is closely related to human hookworms (7, 8, 20, 21). Parasites infect over 3.5 billion people worldwide, but do not normally result in death (10, 16). However, morbidity associated with infections can be severe, often affecting children in areas such as South America, tropical Asia, and Sub-Saharan Africa (22-24), leading to developmental and cognitive impairment (16). Previous work has identified nutritional status as an important player in parasite susceptibility and infection (25-28).

The anti-oxidant Selenium (Se) has been identified as a key component in immune responses to helminth infections (9, 23, 24). An essential micronutrient, Se is found in the form of selenoproteins within the body (29-32), acting as both an anti-inflammatory and a chemopreventative agent (33-36). Recent studies have indicated Se deficiency can exacerbate parasite pathogenesis and prolong infection and disease (23, 25, 27). Within the gut environment, Se deficiency decreases responses to enteric parasites, increasing morbidity and inflammation (23, 25). A possible cause of this decreased response is an inhibition of intestinal Th2-mediated immunity (37-39).

Infections with intestinal parasites such as *N. brasiliensis* are characterized by a rapid and biased Th2-type response, producing elevated levels of IL-4 and IL-13 (3, 14, 19,

40-42). These cytokines play a major role in intestinal smooth muscle contractility that contribute to rapid expulsion of parasites from the intestines (13, 14, 19, 43). Interestingly, the Th2 response inhibits the generation of any Th1 response, protecting the host from excess inflammation (4, 9, 37, 41, 44-46), but primes the intestine for increased infiltration of macrophages, basophils, and eosinophils (19, 24, 47, 48). As one of the most abundantly found immune cells in the gut environment, macrophages play a fundamental role in host defense to helminthic parasites (7, 8, 13, 14, 19).

Macrophages have been characterized through two distinct pathways, the classical (M1) and alternative (M2) pathway (19, 49-51). As seen in a variety of helminthic parasite infections, M2 macrophages are induced by IL-4 and IL-13 (3, 42, 52, 53), expressing high levels of Fizz1, Arg-1, and Ym-1 (3, 54, 55). Our lab has previously shown Se to work synergistically with IL-4 to shift macrophage activation towards an M2 phenotype (31). Equally, we have shown Se to inhibit M1 macrophage activation, reducing the production of pro-inflammatory cytokines (35, 56) which can harm the host and exacerbate the infection (3, 8, 37, 57, 58). Immune responses rely on M2 macrophages to increase mucous secretion by intestinal goblet cells that augment helminth clearance (19, 59, 60). Furthermore, the response of M2 macrophages to intestinal helminths has evolved to promote wound healing within the small intestine, enhancing repair and regenerating damaged tissues (4, 8, 17, 61).

While M2-dependent clearance of *N. brasiliensis* is not completely understood, studies have identified the possible pathways involved. In the absence of STAT6, *N. brasiliensis* infections are unable to be cleared effectively (9) due to a decrease in mucous secretions and changes to intestinal physiology (19, 62). In addition, studies have shown the ability of PPARγ to play a major role in M2 macrophage activation (63-66). Although a functional interaction between IL-4, IL-13 and PPARγ has not been established in *N. brasiliensis* infection, an indirect relationship between PPARγ and Th2 cells has been described (10, 64). Our own studies have shown regulation of the PPARγ endogenous agonist 15d-PGJ_2 through Se supplementation (67) that

activates macrophages towards an M2 phenotype (31). Interestingly, complete abrogation of the COX pathways inhibits endogenous production of 15d-PGJ_2 thereby influencing the progression of M2 macrophage development (31, 67).

Although M2 macrophage-dependent mechanisms of helminth clearance have been discussed, there is limited data on the relationship between Se and macrophages during helminth infections. Here we demonstrate that Se affects M2 macrophages to directly lower the infectivity period and increase adult worm clearance. The results demonstrate that selenoproteins in macrophages regulate adult worm fecundity and expulsion, and may play an important role in pathogenesis.

Materials and Methods

Animals

Three-week-old C57Bl/6 male mice were purchased from Charles River Laboratories (Wilmington, MA). Breeding pairs of interleukin-4 (IL-4) reporter mice (4-get mice) on a Balb/c background were generated by Dr. Richard M. Locksley, and generously provided by Dr. Avery August (Cornell University, Ithaca, NY) (39, 68, 69). A transgenic C57Bl/6 line carrying a Lysozyme M Cre transgene was mated with a C57Bl/6 mouse with a floxed TRSP ($Trsp^{fl/fl}$) gene; both generously provided by Dr. Dolph Hatfield (NIH, Bethesda, MD). These lines were mated to obtain TrspM mice, as previously described. (70). All mice were maintained on Se deficient (Se-D, <0.01 ppm), Se adequate diets (Se-A, 0.08 ppm), or Se supplemented diets (Se-S, 0.4 ppm) (Harlan Teklad custom diets, Madison, WI) for 12 weeks. All procedures were performed in accordance with Penn State University IACUC guidelines. All mice were age and sex matched prior to experimental use.

Parasitic infection with *Nippostrongylus brasiliensis*

Infective third stage larvae (L3) were maintained in a mixture of charcoal and lightly dampened sphagnum moss and stored in plastic Petri dishes (17). Mice were subcutaneously infected with 500 L3 in approximately 225µl of PBS after collection from cultures using a modified Baermann's technique (17), and were studied on days seven, eight, nine, 11, and 14 post infection (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 (17, 71). Fecal egg production was quantified using a modified McMaster technique, as previously described (9). Adult worms were detected quantitatively by opening the intestine (below the stomach to above the cecum) lengthwise and submerging in a beaker of warmed PBS using a tea strainer. The beaker was placed in a 37°C water bath for 45 minutes. Remaining worms in the intestine tissue were counted using a microscope. Worms in suspension were counted on a gridded petri plate.

Treatments

The COX inhibitor indomethacin was administered to mice in drinking water at a concentration of 0.00325% (w/v) (67, 72) from two weeks prior to *N. brasiliensis* infection until two weeks p.i., when the animals were euthanized. To deplete infiltrating macrophages, a total of 200 µl of clodronate-loaded (CL) or PBS-control loaded liposomes were injected via retro orbital vein into mice on days zero, one, three, five, seven, and nine p.i. The liposomes were generated as previously described (14). The clodronate was a gift from Roche Diagnostics GmbH. 15d-PGJ2 was exogenously administered daily at a concentration of 0.050 mg/kg/day (dissolved in PBS) intraperitoneally (i.p) for seven days.

Genotyping

The extent of *Trsp* deletion was determined by PCR analysis of the floxed region of the gene. Tail snips were taken from all TrspM mice. A mixture of 250µl lysis buffer and 5µl proteinase K (20mg/ml, New England Biolabs, Ipswich, MA) was added to each tail and incubated overnight in a 65°C water bath. Lysed tails were spun down at 20800x g for 5min at room temperature. Supernatants were collected and diluted (1:11) with DEPC water. PCR was carried out using 0.2 µM of primers, 2.5mM MgCl2, 0.2mM of each deoxyribonucleotide triphosphate, 1.25 units of GoTaq DNA polymerase (Promega, Madison, WI), and 1µl of diluted DNA. To detect the transgene, two sets of primers were used. 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'. Primer set one had a resulting fragment of ~1.1kb in length signifying a homozygous floxed *trsp* gene (Fig. 25A). Primer set two had a resulting fragment of 700bp signifying the LysMCre knockout, while wild type bands had a fragment of 350bp (Fig. 25B). The PCR conditions used were: 5 min at 94°C, 30 sec at 94°C, 60 sec at 58°C, 60 sec at 72°C (30 cycles steps 2-4), and 3 min at 72 °C. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by UV transillumination.

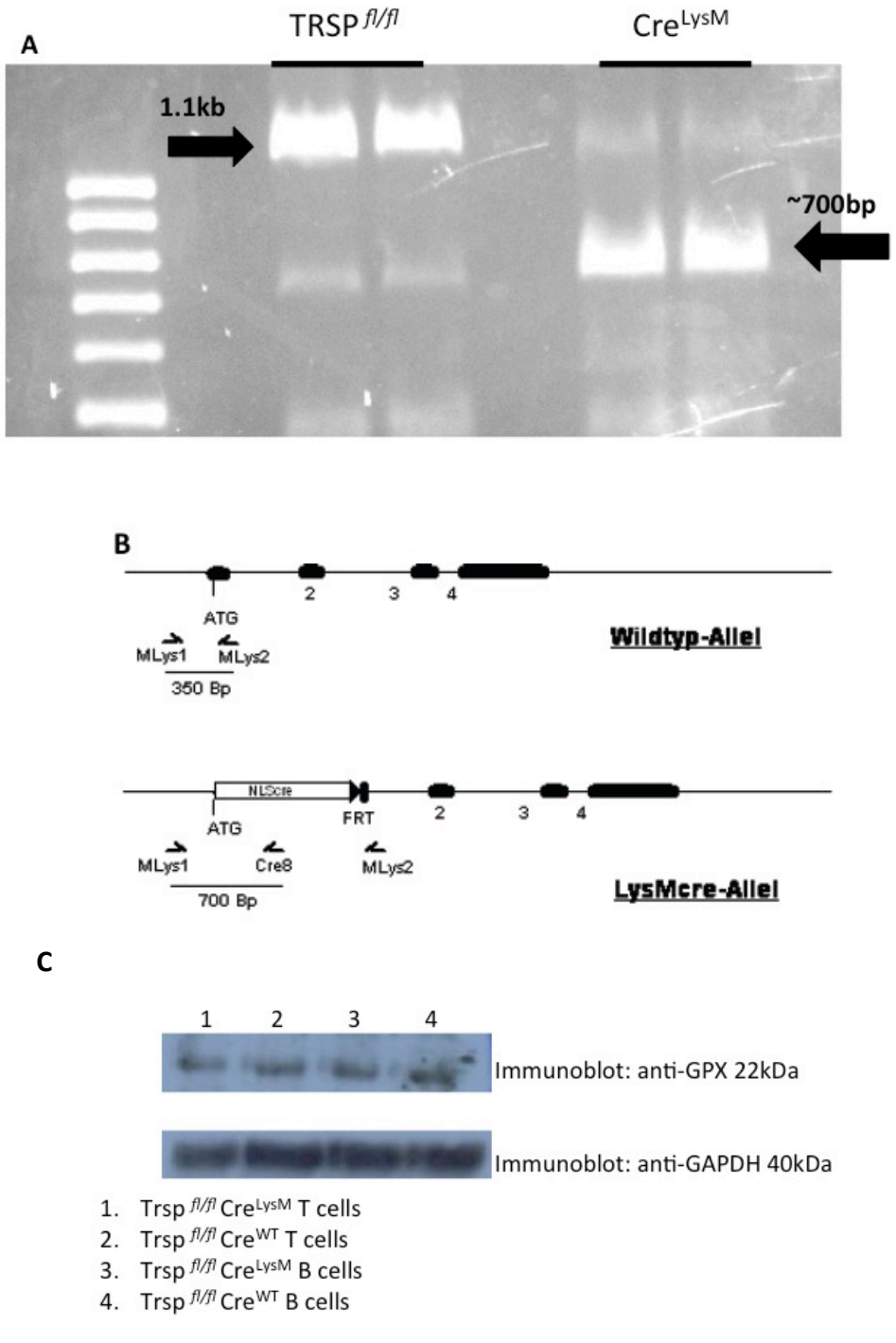


Figure 25: Genotyped mice display Trsp floxed and Cre^{LysM} bands. (A). Genotyping was accomplished through PCR, visualizing products on a 2% agarose gel. Trsp floxed bands were confirmed at 1.1kb, while Trsp Cre^{LysM} bands were confirmed at 700bp. (B). Schematic representation of genotype of wild type and Cre^{LysM} knockout mice. (C). Western blot results visualizing GPx1 in T and B cell lysates, confirming the selenoprotein knockout is only in macrophage cells, and not other immune cells.

Real time PCR

Total RNA was isolated from one mg sections of jejunum using Isol-RNA lysis reagent (5 Prime; Gaithersburg, MD). RNA concentrations were determined by UV-spectroscopy. Briefly, two μg total RNA was reverse transcribed into cDNA as previously described (31). TaqMan probes for Arg-I, Fizz1, Ym1, and IL-13 was used to analyze 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). ΔCt ($Ct_{Gene} - Ct_{GAPDH}$) was calculated for each sample and used for analysis of transcript abundance with respect to the untreated negative control (73).

Isolation of intraepithelial and lamina propria lymphocytes from small intestine tissue

Lymphocytes from the intestinal epithelial layer (IEL) and lamina propria (LP) were isolated (74, 75). Briefly, the small intestines were taken from mice nine days p.i. To isolate IELs, 20 ml of Hanks buffer (Sigma, St. Louis, MO), 20 μl 1M DTT, 400μl 0.25M EDTA, and one drop of 1M HCl was added to tissues for 30 min and shaken at 250RPM at 37 °C. This step was repeated until the supernatant became clear, each time collecting the supernatant and keeping it on ice. Following the last wash, tissue pieces were rinsed in RPMI to remove any remaining remnants of EDTA. To isolate lymphocytes from the LP, tissues were incubated in 30mL RPMI, 300mg collagenase (300U/mL) (Worthington Biochemical Corp, Lakewood, NJ), and 0.09 g dispase (Sigma, St. Louis, MO) for 1 hr at 250RPM at 37°C. Following incubation, the supernatants were filtered using a mesh strainer (Fisher Scientific, Pittsburgh, PA) into a fresh tube and spun down at 500x g for five min at 4°C. Lymphocyte pellets were resuspended in 40% Percoll and placed over a 80% Percoll mixture, creating a 40% (vol/vol), 80% (vol/vol) gradient (74, 75) Tubes were spun at 800 x *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 10X PBS, 25 mL FBS, and 2.5 mL sodium azide, final volume made

to 500 mL with deionized water). A total of 500,000 cells were taken for flow cytometry.

Splenic lymphocyte isolation

Isolated spleens were homogenized in one mL of Hanks buffer and filtered using a mesh strainer (Fisher Scientific, Pittsburgh, PA). Cells were washed in one mL flow buffer and spun down at 500x g for 5 min. Red blood cells were lysed with lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA in 1L deionized water) for three min at room temperature. Cells were washed two times with flow buffer and spun down at 500x g for five min;1 X 10^6 cells were counted for flow cytometry.

Flow cytometry

Cells isolated from the small intestine and spleen were washed in one mL of flow buffer (pH 7.2) and pelleted by centrifuging at 250x g for five min at 4°C. Pellets were resuspended in 100µl flow buffer and stained with the following antibodies: PE conjugated anti-mouse CD3 antibody (BD Pharmingen, San Jose, CA), rabbit anti-mouse RELMα (Fizz1) (Preprotech, Rocky Hill, NJ), rabbit anti-mouse RELMβ (Fizz2) (Preprotech, Rocky Hill, NJ), FITC conjugated rat anti-mouse F4/80 (AbD Serotec, Raleigh, NC), PE conjugated anti-mouse Siglec-F (BD Biosciences, San Jose, CA), FITC conjugated anti-mouse CCR3 (R&D Systems), for 30 min at 4°C in the dark. Next, cells were washed with one mL flow buffer and centrifuged at 250x g for five min. Un-conjugated primary antibody samples were stained with AF-647 goat anti-rabbit IgG secondary for 30 min at room temperature in the dark. Cells were washed with flow buffer, and analyzed on the BD Accuri C6 Benchtop Cytometer using BD Accuri software (Beckman Coulter).

Arginase Assay

Jejunual tissue was homogenized on ice for 30 seconds, in one mL 25mM Tris-HCl, pH 8, containing 0.2 % (v/v) Triton X-100 and protease inhibitors (76). Homogenates were incubated on ice for 30 min with intermittent rocking. Arginase assay was performed on these homogenates as per the protocol (76, 77). Optical density at 560

nm was recorded on a Packard plate reader. A urea standard calibration curve (range 0-1 µmol; $y = 3x10^{-4}x - 0.015$, $R^2 = 0.97$) was used to calculate the Arg-I activity. Enzyme activity is expressed as µmol of urea produced/mg of protein (77).

Statistical analysis

Results are presented as mean ± SEM. Significant differences between groups were analyzed using two-way ANOVA on GraphPad ® Prism, followed by appropriate post-hoc tests. Results were considered significant at $P \leq 0.05$. All experiments were performed in triplicate from three-five mice per group.

Results

Effects of dietary Se on adult worm and fecal egg clearance in Nippostrongylus brasiliensis-*infected mice*

To determine the effects of Se on parasite clearance, fecal eggs were isolated and quantified. Three - four pellets of feces were collected from Se-D, Se-A and Se-S mice subcutaneously infected with 500 L3 (9, 17), on days seven, eight, 10, 11, and 14 p.i. Compared to Se-A and Se-S mice, Se-D mice had a significant increase in the number of eggs (Figure. 26A). Interestingly, there was no significant difference between Se-A and Se-S mice throughout the infection (Figure. 26A). In addition to fecal eggs, the quantity of adult worms counted in the small intestine showed a similar pattern. While Se-A and Se-S mice displayed almost identical numbers of adult worms, Se-D mice showed a significantly larger number of worms on days seven and eight p.i. (Figure. 26B). Worm counts, however, began decreasing at day 10 p.i., supporting previously published data (9, 17).

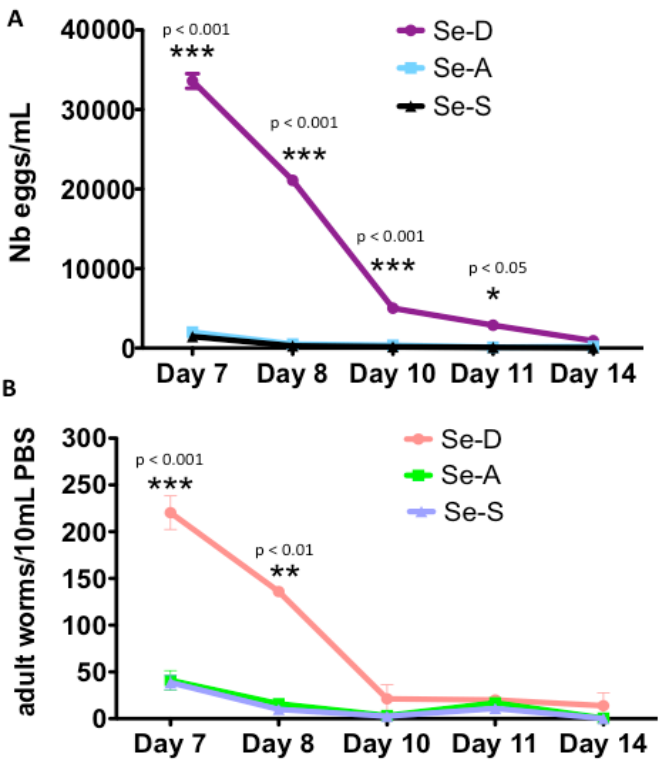


Figure 26: Se concentration affects fecundity and adult worm burden in *N. brasiliensis* infected mice. 500 L3 were injected into Se-D, Se-A, and Se-S mice. (A). two - four fecal pellets were collected per mouse, and eggs were counted. (B). Whole intestine were extracted from each mouse to count adult worms in the lumen. Values are means ± SEM, n=3. Significant differences between groups are represented by stars. * = <0.05, ** = < 0.01, *** = < 0.001, and were analyzed using 2-way ANOVA.

Intestinal M2-like macrophage differentiation is mediated by Se concentration

The Se-dependent mechanisms underlying the increased anti-parasite effects were examined. It has been previously shown that mice utilize a biased Th2 response to clear *N. brasiliensis* from the intestines (3, 14, 38). Moreover, our previous data showed a synergistic effect between the Th2 produced cytokine IL-4 and Se, shunting macrophage activation towards the M2 phenotype (31). To relate the increased M2 polarization to changes in worm burden, we examined transcript expression of M2 markers Arg-I, Ym-1 and Fizz1 in the jejunum, using qRT PCR. Jejunual tissue was collected on days seven, eight, and 11 p.i. (19). Expression of all three M2 genes was significantly higher in Se-A and Se-S mice starting on day seven p.i. and increased further by day eight p.i., but decreased significantly by day 11 p.i (Figure. 27A, B, C). Furthermore, expression of the M2 genes was significantly higher than baseline levels seen in non-infected mice (data not shown).

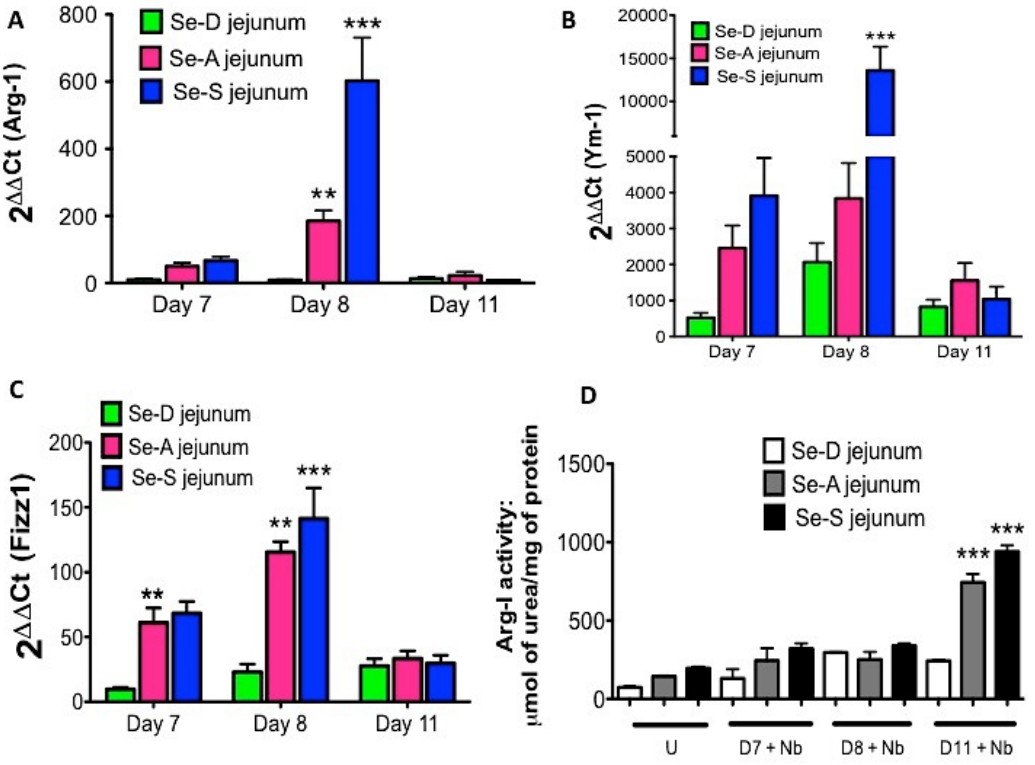


Figure 27: Se-dependent increase in M2 macrophage marker expression in the jejunum. Real Time RT-PCR expression of (A) *Arg-I*, (B) *Ym1*, and (C) *Fizz1*. (D). Arginase activity measured in jejunal tissue from non-infected and infected mice. Values are means ± SEM, n=3. Significant differences between groups are represented by stars. * = <0.05, ** = < 0.01, *** = < 0.001, and were determined using 2-way ANOVA.

Given the increased transcript expression of M2 markers, we next examined the modulation of arginase activity in homogenized jejunal tissue. In comparison to non-infected Se-D, Se-A, and Se-S mice, *N. brasiliensis* infection of Se-A and Se-S mice showed the largest increases in arginase activity on day 11 p.i. suggesting that Se adequate and supplemented diets enhance M2 macrophage differentiation following infection (Figure. 27D). This data also suggests that Se has the ability to increase the differentiation of macrophages towards an M2 phenotype, leading to an increased presence of M2 macrophages in the intestine that is key to increased parasite clearance.

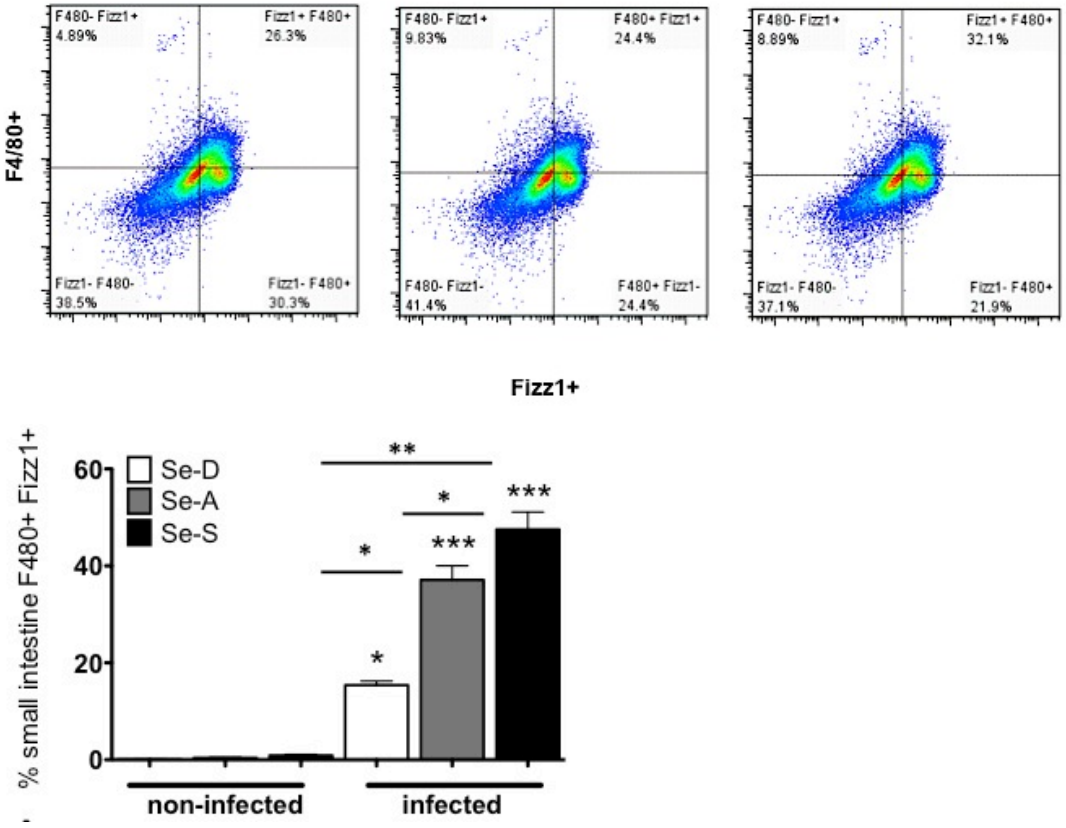
Increases in M2 macrophages in the small intestine is Se-dependent

To verify that increasing Se in the diet increased the number of intestinal M2 macrophages following infection with *N. brasiliensis*, macrophages from the lamina propria (LP) were isolated at nine days p.i. and prepared for flow cytometry. Cells were stained with two macrophage-specific antibodies, F4/80 that is a general macrophage marker, and Fizz 1, which specifically mark M2 type macrophages. When compared to non-infected mice, the *N. brasiliensis*-induced increase in the number of M2 macrophages was Se-dependent (Figure. 28A). These results complement the pattern seen with increases in Fizz1 transcript expression (Figure. 27C).

We examined if Se is able to augment the expression of Fizz 2, a goblet cell marker, independent of M2 macrophages that is seen in response to *N. brasiliensis* infection and is regulated by IL-4 (185, 186). To examine the goblet cell expression of Fizz 2, we isolated intraepithelial lymphocytes (IELs) from the small intestine. As expected, *N. brasiliensis* significantly increased the expression of Fizz 2 compared to non-

infected mice (Figure. 28B). Notably, the expression of Fizz 2 failed to show any significant variations in response to dietary Se (Figure. 28B). These results demonstrate the specificity of Se to induce increases in M2 macrophages, while not effecting the expression of other (non-immune) cells.

A



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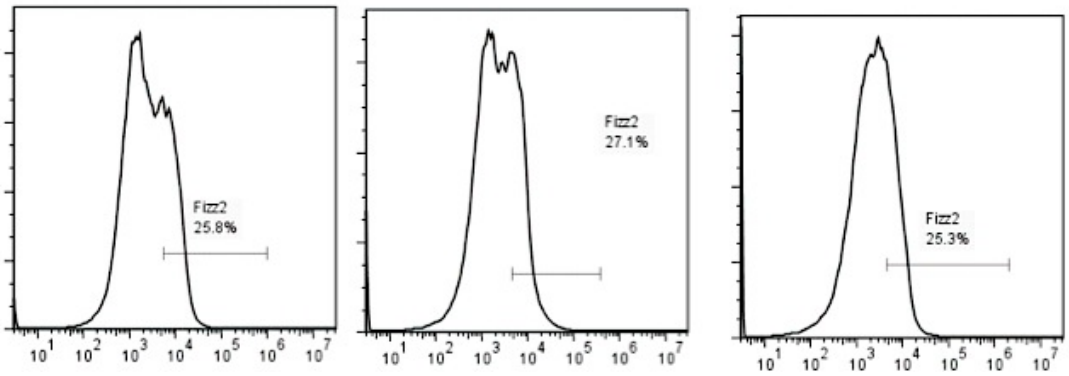


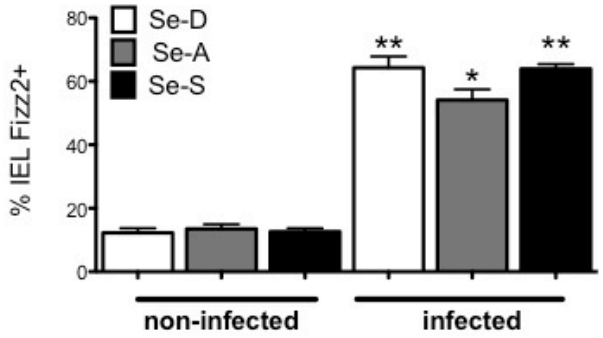


Figure 28: Induction of M2 macrophage by Se supplementation (A) Macrophages isolated from the lamina propria of infected and non-infected mice were stained with F4/80 and Fizz1. (B) IELs were collected and stained with Fizz 2, a non-M2 marker. All cells were analyzed via flow cytometry. Values are means ± SEM, n=3. Stars represent significant differences between groups. * = <0.05, ** = < 0.01, *** = < 0.001, and were determined using 2-way ANOVA with tukey's post hoc testing.

Selenoproteins are required for optimal M2 macrophage-induced parasite clearance

Recently, we have demonstrated the pivotal role of selenoproteins in macrophage phenotype switching (31). To examine how selenoproteins affect parasite clearance, we used mutant mice with a *Trsp* deletion that do not express selenoproteins in macrophages, monocytes, and some granulocytes. To confirm that *Trsp* deletion inhibits M2 phenotype switching, an arginase assay was performed on bone marrow derived macrophages (BMDM) collected from $Trsp^{fl/fl}$ Cre^{LysM} and $Trsp^{fl/fl}$ Cre^{WT} mice maintained on a Se-S diet. Cells were cultured, and treated with 0.1 μg/mL LPS (4h) or 10 ng/μl IL-4 (20h) (31). IL-4 stimulated BMDM from the wild-type (WT) mice showed higher arginase activity than LPS-stimulated and unstimulated BMDM (Figure. 29). Interestingly, the Trsp deletion completely abrogated arginase activity, even in the presence of IL-4 (Figure. 29). These results demonstrated that selenoprotein expression serves as a critical factor in the phenotype switching to M2 type macrophages.

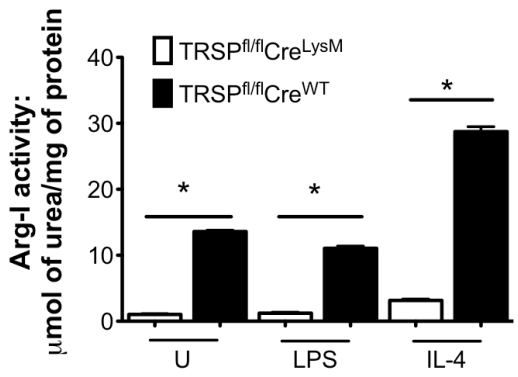


Figure 29: Trsp is required for M2 macrophage phenotype switching. Arginase activity was measured in BMDM cultured in Se-S conditions for 4 d. Values are means ± SEM, n=3. * P < 0.05. All means were compared to one another and analyzed using 2-way ANOVA with tukey's post hoc testing.

Seven days after infection with *N. brasiliensis*, *Trsp* $^{fl/fl}$ *Cre* ^{LysM} mice showed a significant increase in the amount of fecal eggs when compared to the *Trsp* $^{fl/fl}$ *Cre* ^{WT} (Figure. 30A). A similar pattern was also seen in the number of adult worms in the small intestine (Figure. 30B). Both fecal egg and adult worms were diminished by day 11 p.i.

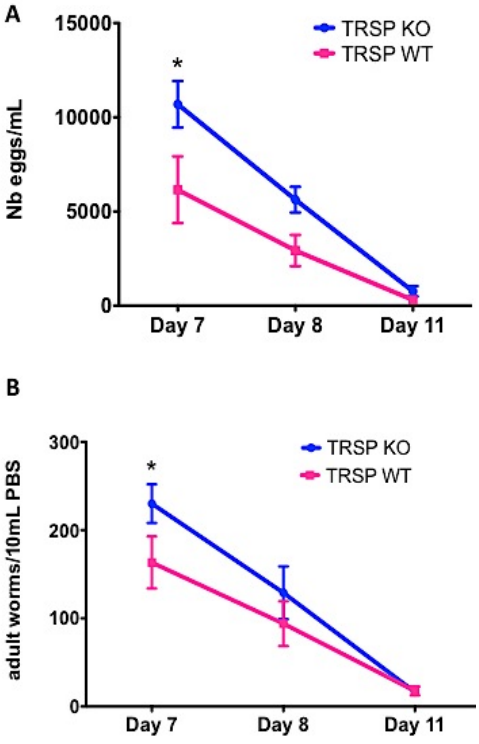
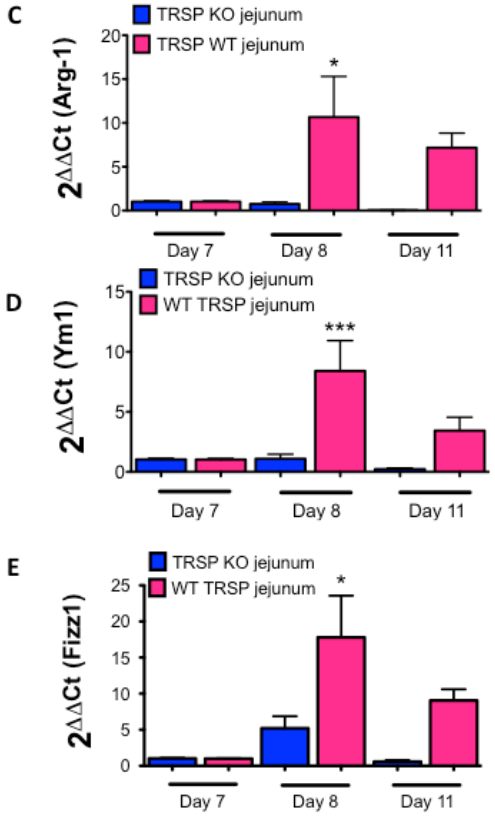


Figure 30: Selenoproteins are required for M2 macrophage marker expression and optimal *N. brasiliensis* clearance. *Trsp* $^{fl/fl}$ *Cre* ^{LysM} and *Trsp* $^{fl/fl}$ *Cre* ^{WT} mice maintained in Se-S conditions, were infected with 500 L3. (A). 2-4 fecal pellets were collected per mouse, and eggs were counted. (B). Whole intestine were extracted from each mouse to count adult worms in the lumen. *Trsp* $^{fl/fl}$ *Cre* ^{LysM} and *Trsp* $^{fl/fl}$ *Cre* ^{WT} mouse jejunum were analyzed with Real Time RT-PCR for expression of (C) *Arg-I*, (D) *Ym1*, and (E) *Fizz1*. Values are means ± SEM, n=3. Stars represent significant differences between groups. * = <0.05, *** = < 0.001. Statistical differences analyzed using ANOVA with post-hoc test.

To determine the effects the *Trsp* deletion has on M2 marker expression, we used RT PCR to examine the modulation of M2 marker gene expression in the jejunum. A complete abrogation of gene expression for Arg-1, Fizz1, and Ym1 was observed in *Trsp*$^{fl/fl}$ *Cre*^{LysM} mice compared to *Trsp*$^{fl/fl}$ *Cre*^{WT} mice at days seven, eight, and 11 p.i. (Figure. 30C, 30D, 30E). Together, these data illustrate the requirement for selenoproteins in optimal clearance of *N. brasiliensis* and the role of M2 macrophages.



Inhibition of the COX pathway reduces M2 macrophage effects

Previous studies have demonstrated an important role for the transcription factor STAT6 in helminth clearance (9, 62). Along these lines, our previous work showed that Se worked through a STAT6 dependent pathway to induce the activation of M2 macrophages (31). In addition to STAT6, our studies identified the nuclear receptor PPARγ to be important in M2 macrophage activation associated with the Se-

dependent production of the endogenous agonist 15d-PGJ_2 (31). To establish if Se works through a PPARγ dependent pathway to modulate *N. brasiliensis* infection, we used the COX pathway inhibitor indomethacin to block the endogenous production of prostaglandins, including 15d-PGJ_2. Indomethacin was administered via drinking water for two weeks prior to infection and for up to two weeks p.i. Inhibition of the COX pathway significantly increased fecal eggs and adult worms on days seven and eight p.i. in Se-A and Se-S mice (Figure. 31A, 31B). Se-D mice given indomethacin had similar patterns of clearance when compared to non-indomethacin-treated mice (Figure. 31C, 31D). Most interesting, however, were the patterns seen in Se-A and Se-S mice on indomethacin. When compared to non-indomethacin-treated mice, COX-inhibition increased fecal eggs (Figure. 31C) and adult worms (Figure. 31D). Although not significant, this data suggest inhibition of the COX pathway mutes the effects of Se on optimal clearance of *N. brasiliensis*.

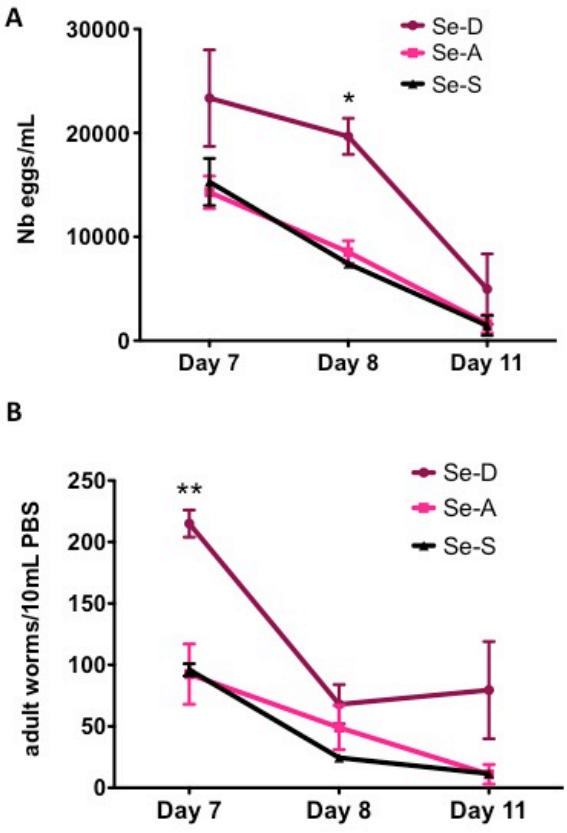
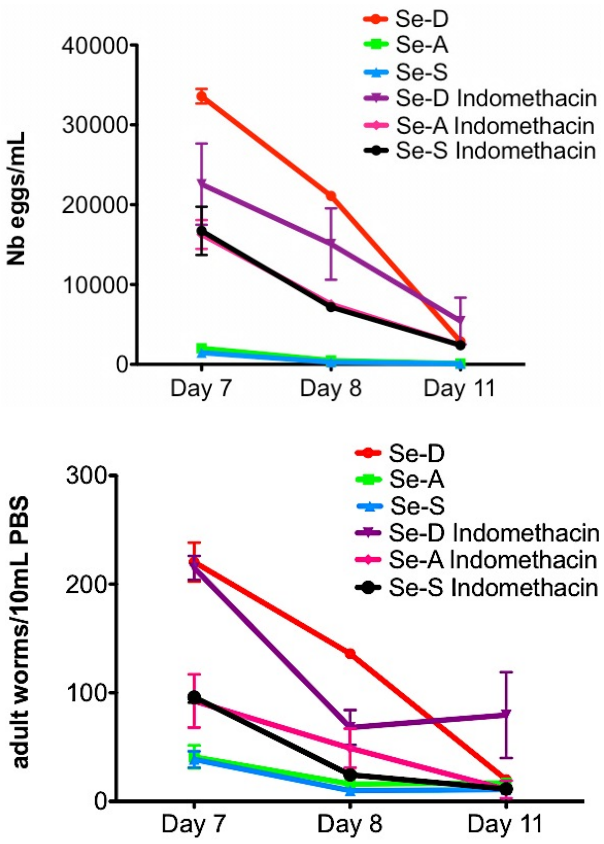


Figure 31: Indomethacin blocks the effects of Se on *N. brasiliensis* clearance. Indomethacin was administered through the drinking water (0.00325% w/v) for two weeks prior to infection, through two weeks p.i. (A) fecal eggs and (B) adult worms were counted on days seven, eight, and 11-p.i. (C) and (D) graphically compared to non-indomethacin treated mice. Values are means ± SEM, n=3. Stars represent significant differences between groups. * = <0.05, ** = < 0.01. Statistical differences analyzed using 2-way ANOVA with Bonferoni post-hoc testing.

C



Furthermore, qRT-PCR analysis of the effects of indomethacin on M2 marker expression in the jejunum of these mice indicated similarities to non-indomethacin treated mice (Figure. 32A, 32B, 32C), where Se fed *N. brasiliensis* infected mice had a significantly higher expression of *Arg-I*, *Fizz1*, and *Ym1* on day seven p.i. compared to Se-D mice. In contrast, expression began to decrease at day eight p.i. in both the Se-D and Se fed mice (Figure. 32A, 32B, 32C). This data demonstrates the importance of the COX pathway in mediating the effects of Se on M2 macrophage expression and optimal parasite clearance.

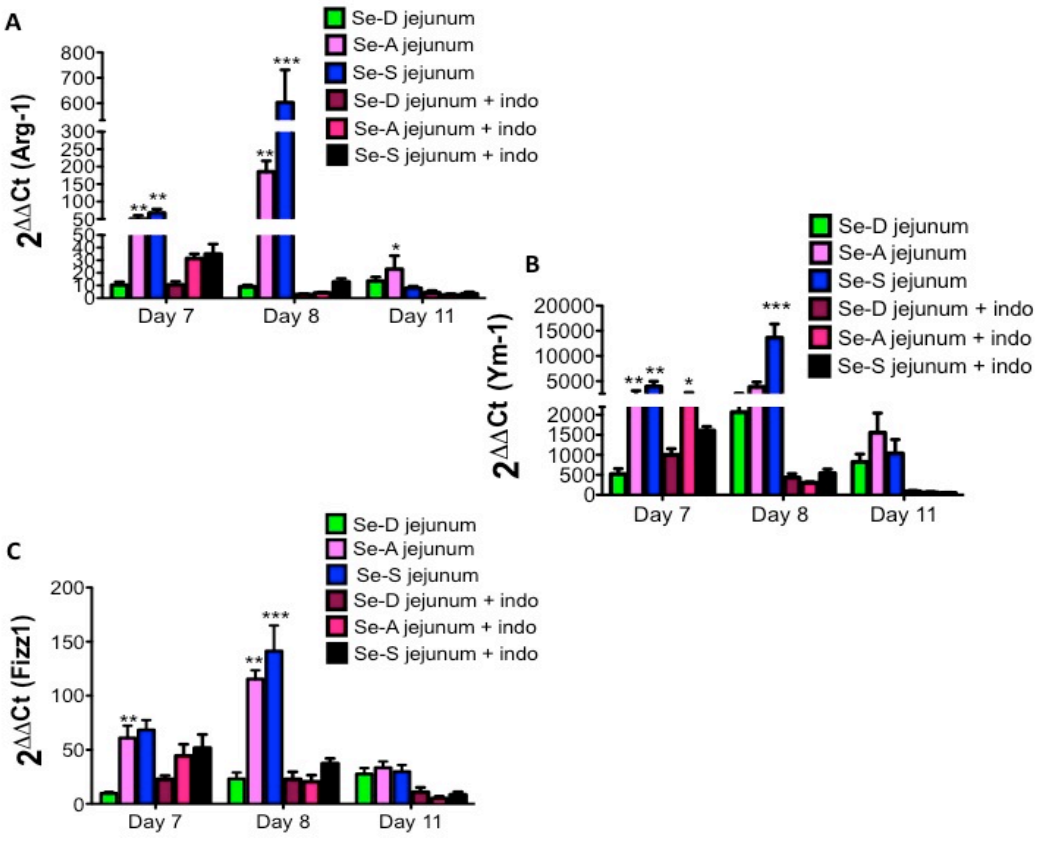


Figure 32: COX-AA pathway inhibition decreases M2 marker expression. Real Time RT-PCR was used to analyze expression of (A) Arg-I, (B) Ym1, and (C) Fizz1 from the jejunum of *N. brasiliensis*-infected mice treated with 0.00325% (w/v) indomethacin for two weeks prior to infection, and two weeks thereafter. Values are means ± SEM, n=3. Stars represent significant differences between groups. * = <0.05, *** = < 0.001. Statistical differences analyzed using 2-way ANOVA with tukey post-hoc testing.

15d-PGJ$_2$ rescues fecal egg shedding in N. brasiliensis infected mice

Given the fact that *N. brasiliensis*-infected mice treated with indomethacin have increased parasite egg shedding, we wanted to determine if this is a result of a decreased production in 15d-PGJ$_2$ to further establish a PPARγ–dependent pathway coupled to Se modulation. Indomethacin treated Se-D mice were administered 15d-PGJ$_2$ i.p. at 0.050 mg/kg/ approximately 12 h before infection with *N. brasiliensis* L3. Mice were continuously given 15d-PGJ$_2$ for a total of seven days. As shown in Figure 33, 15d-PGJ$_2$ treatment reduced fecal egg shedding on days seven and eight p.i. to

levels significantly below those seen in non-J_2 treated mice (Figure. 33). By day 11 p.i, however, egg shedding in 15d-PGJ_2 treated and non-treated mice was nearly absent, corroborating patterns previously seen (Figure. 33) (9, 42). This data further demonstrates the importance of the COX pathway in modulating parasite egg shedding

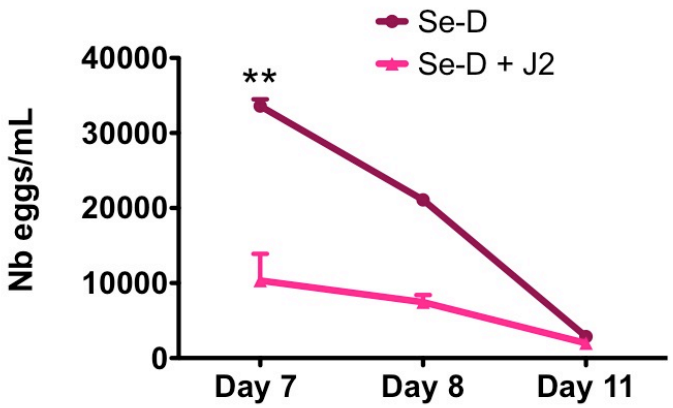


Figure 33: Modulation of fecal egg shedding by 15d-PGJ_2. Mice were administered 0.00325% (w/v) indomethacin in their drinking water for two weeks. 12 h prior to infection with N. brasiliensis L3, mice were injected i.p with 0.050 mg/kg 15d-PGJ_2 continuously for seven days. Fecal eggs were counted using a modified McMaster technique. Values are means ± SEM, n=3. Stars represent significant differences between groups ** = < 0.01. Statistical differences analyzed using 2-way ANOVA with post-hoc testing.

Se affects Th2 cells

The clearance of *N. brasiliensis* is Th2-dependent (13, 14). To determine if Se increases the presence of IL-4 producing Th2 cells in the small intestine to facilitate increases in M2 macrophages, we used flow cytometry to determine the number of Th2 cells in infected mice. Interlukein-4 (IL-4) reporter mice (4-get mice) were injected with 500 L3 subcutaneously. On day nine post-infection, small intestine lymphocytes from the lamina propria were collected from *N. brasiliensis* infected and non-infected mice, and stained with anti-CD3-PE; a GFP tag was already attached to all IL-4 producing cells. Interestingly, only Se-A mice showed a change in Th2 cells in infected mice (Figure. 34A, double positive cells). Although the pattern indicates Se-S mice increase Th2 cells more than Se-D, the increase was not significant (Figure. 34B). Overall, the data suggests the percentage of $CD3^+$ GFP^+ cells that

represent Th2 cell development may play a small role in increased M2 macrophages in the small intestine (Figure. 34B).

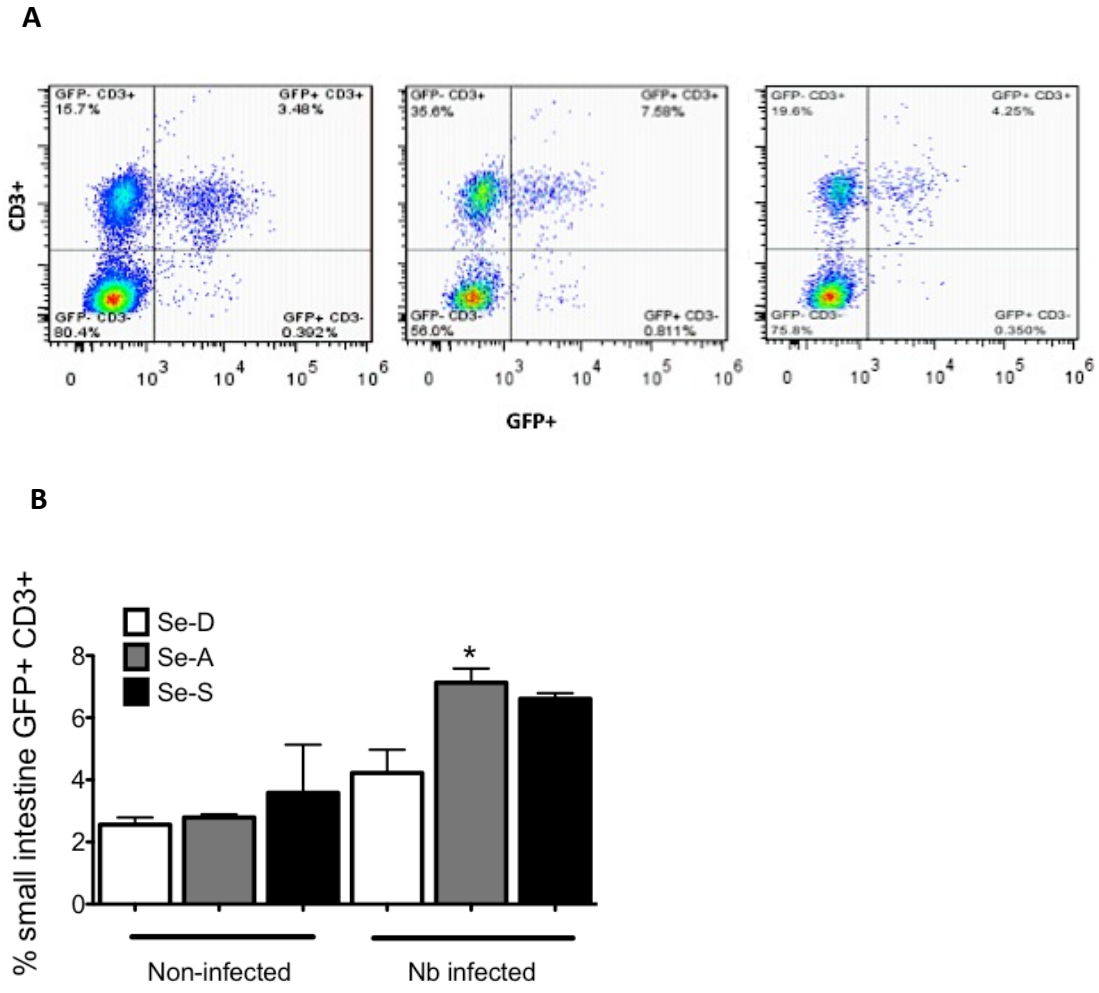


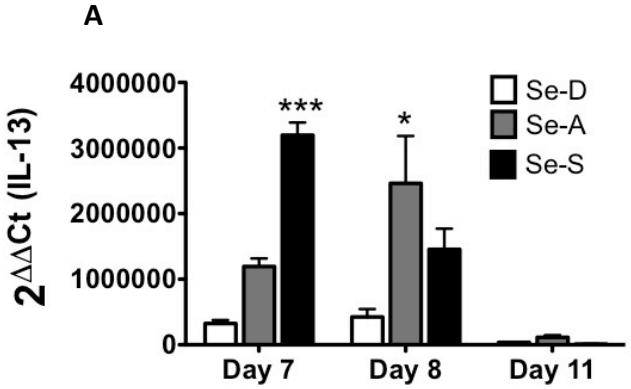
Figure 34: Adequate Se levels affect Th2 cells. Whole small intestines from *N. brasiliensis*-infected 4-get mice were used to isolate T cells. (A). Flow cytometry was used to identify CD3+ GFP+ intestinal Th2 cells. (B). Data was graphed and analyzed by ANOVA with tukey post-hoc testing. Values are means ± SEM, n=3. Stars represent significant differences between groups * = < 0.05.

Se dependent effects on IL-13 and eosinophilia

In addition to IL-4, IL-13 is also produced by Th2 cells and is highly expressed in *N. brasiliensis* infections (3,9) and is important in clearance of the adult worms (3, 19, 42). Given that the number of Th2 cells remains unaltered in Se-D versus Se fed

mice, we sought to determine if Se affects IL-13 cytokine expression. Real time RT-PCR was used to examine expression of IL-13 in jejunal tissue collected on days seven, eight, and 11 p.i. Expression was highest on days seven and eight p.i. in mice fed Se-S and Se-A compared to mice fed a Se-D (Figure. 35A). Furthermore, expression was significantly decreased day 11-p.i.in all Se diets suggesting that IL-13 production is dependent on adult worms in the intestine (Figure. 35A).

Nippostrongylus brasiliensis infection induces intestinal eosinophilia that could contribute to local production of IL-13 (39, 48). To determine if intestinal eosinophilia was Se-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 to non-infected mice, the percentage of Siglec $F^+/CCR3^+$ cells detected in mice fed Se-D, Se-A, and Se-S were significantly increased (Figure. 35B). Interestingly, the percentage of Siglec $F^+/CCR3^+$ cells was not significantly different between mice fed different levels of Se.



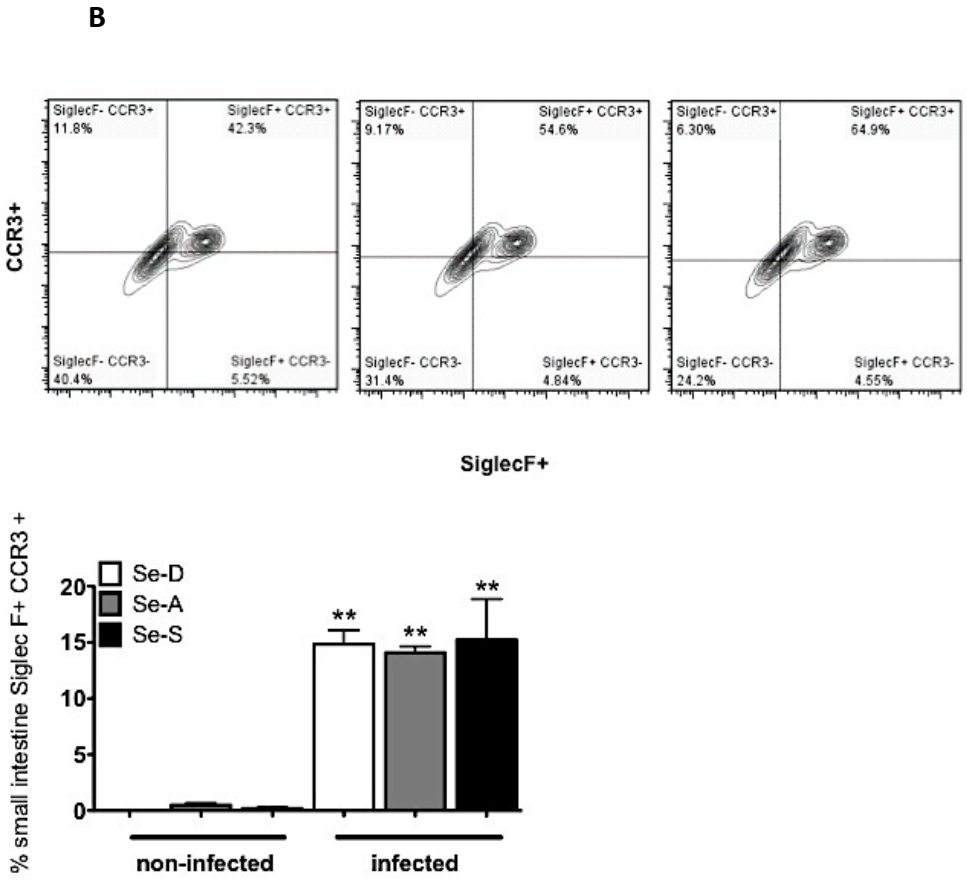


Figure 35: Se affects IL-13 expression and eosinophilia. (A). Real time RT PCR was used to measure transcript expression of IL-13 in jejunal tissue from infected mice. (B) Small intestines from non-infected and infected mice were collected to isolate cells from the lamina propria. Cells were stained for Siglec F and CCR3, and analyzed using flow cytometry. Percentages of double positive cells are graphed. Values are means ± SEM, n=3. Stars represent significant differences between groups * = <0.05 ** = < 0.01. Statistical differences analyzed using 2-way ANOVA with tukey post-hoc testing.

Depletion of macrophages abolishes Se induced clearance of N. brasiliensis

To determine if Se-dependent clearance of *N. brasiliensis* was M2 macrophage independent, we depleted macrophages using clodronate-loaded (CL) liposomes (14). On the same day as liposome treatment, mice were infected with 500 L3. Se-A mice treated with CL liposomes significantly increased the number of fecal eggs and adult worms on day seven p.i. to levels seen in CL treated Se-D mice (Figure. 36A, 36B).

Furthermore, PBS containing liposome treated Se-A mice showed fewer adult worms and fecal eggs than that seen in Se-D mice (Figure. 36A, 36B) suggesting that adequate Se in the diet was dependent on functional M2 macrophages to optimize reduced fecundity and enhanced clearance of *N. brasiliensis*.

Treatment of mice with CL also negatively affected the expression of M2 macrophage markers, *Arg-I*, *Ym1* and *Fizz1*. Both Se-D and Se-A mice showed significantly decreased marker levels on days seven -11 p.i. comparable to baseline levels seen in non-infected mice. (Figure. 36C, 36D, 36E). Furthermore, when compared to Se-D mice treated with PBS liposomes, Se-A mice showed a significant increase in expression of all three genes on day eight p.i. (Figure. 36C, 36D, 36E). Together, the data indicate the anti-parasite effects of adequate and supplemented Se in the diet are mediated by M2 macrophages.

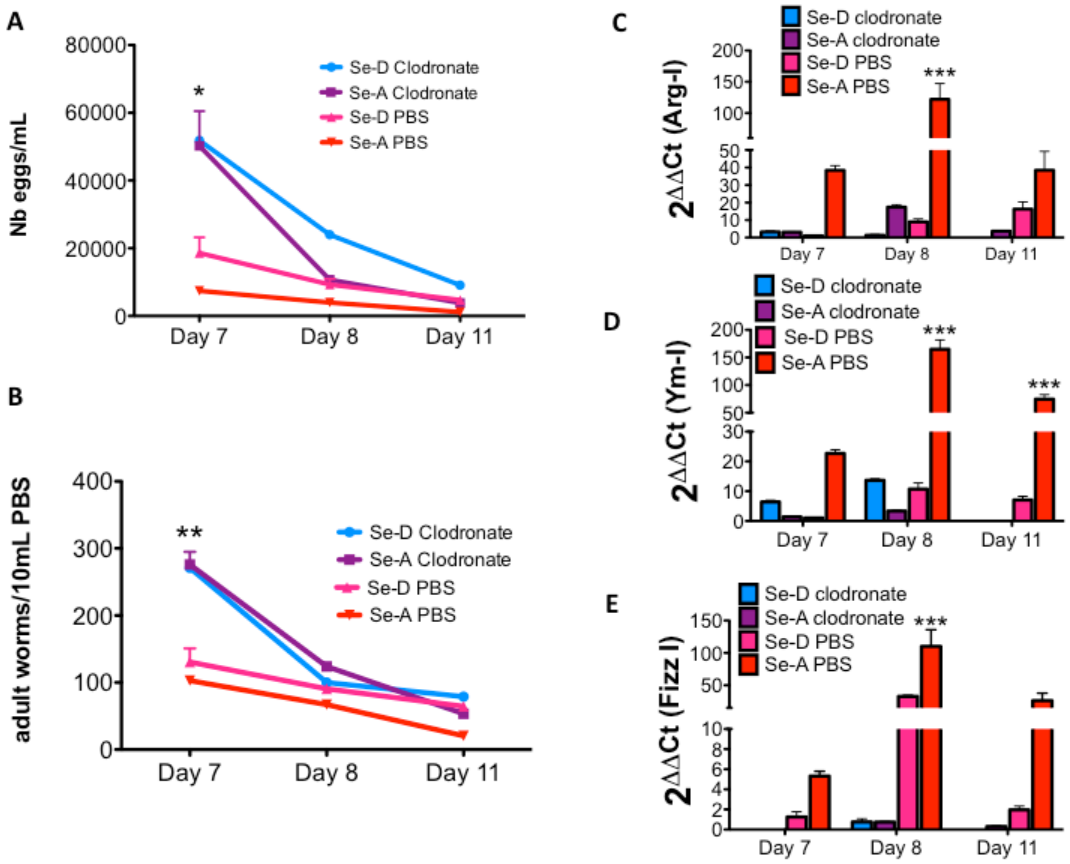


Figure 36: Effects of dietary Se are abrogated in the absence of M2 macrophages. Mice fed Se-D and Se-A diets and infected with *N. brasiliensis* were depleted of macrophages by treatment with clodronate-loaded liposomes. (A) fecal eggs and (B) adult worms were counted. RNA from jejunal tissues were extracted and analyzed using Real Time RT PCR for the expression of (C) *Arg-I*, (D) *Ym1*, and (E) *Fizz1*. Values are means ± SEM, n=3. Stars represent significant differences between groups ** = < 0.01. Statistical differences analyzed using 2-way ANOVA with tukey post-hoc testing.

Discussion

The mechanisms contributing to helminth clearance from intestine are multi-faceted. Studies have demonstrated that enhanced intestinal smooth muscle contractility, epithelial cell permeability, and mucous secretions are Th2-dependent (9, 14, 19). Furthermore, worm clearance from the intestine is abrogated in the absence of the transcription factor STAT6 (9, 19). Moreover, myeloid cells such as macrophages and eosinophils have been identified as major effector cells in Th2-type immune responses to parasitic infections. Interestingly, the gut is one of the richest sources of macrophages (19). The most important characteristic of gut macrophages during helminthic parasite infections is expression of the alternatively activated M2 phenotype induced by the Th2-cytokines IL-4 and IL-13. The gastrointestinal nematode parasite *N. brasiliensis* induces M2 macrophage development in the intestine that contributes to adult worm clearance (17, 19). Previous studies in our laboratory have demonstrated the ability to shunt macrophage activation from a pro-inflammatory M1 phenotype, towards an M2 phenotype through supplementation with the antioxidant micronutrient Se (31). Moreover, this shunting was augmented upon addition of IL-4, demonstrating a synergistic relationship between Se and Th2 cytokines. Other studies have demonstrated the beneficial effects of Se on clearance of gastrointestinal parasites (25). Although these effects correlate with active worm expulsion, there is little information on the relationship between the concentration of dietary Se and macrophage function in a helminth-infected gut.

Clearance of *N. brasiliensis* from the intestines begins as early as day seven p.i, with a reduction in parasite egg shedding. Adult worms are cleared from the intestine soon after, with complete clearance between days 10-14. Compared to Se-D mice, Se-A and Se-S mice showed a significant decrease in parasite eggs present in the feces starting day seven-p.i, and continuing on day eight. Furthermore, Se-A and Se-S mice had decreased numbers of adult worms day seven and eight p.i compared to Se-D mice, but all mice cleared adult worms by day 14 p.i regardless of Se status. We assessed the effects of different concentrations of dietary Se on M2 macrophage marker expression and development during infection with *N. brasiliensis*. In a Se

dependent manner, expression of M2 markers *Arg-1*, *Fizz1*, and *Ym1* significantly increased days seven and eight p.i, corresponding to the patterns seen with clearance of adult worms and changes in fecal egg excretion. Moreover, utilizing flow cytometry, presence of F4/80/Fizz1 double positive macrophages in the small intestine also increased in Se-A and Se-S mice, while single F4/80+ macrophage presence remained the same, regardless of Se status. Remarkably, treatment of *N. brasiliensis*-infected mice with clodronate liposomes completely abrogated expression of M2 marker expression in Se-D and Se-A mice, and blocked adult worm clearance, which is consistent with a previous study (19). Previously it was shown that intestinal smooth muscle contractility is affected by M2 macrophage activity in the intestine during the infection (19), suggesting that Se-dependent effects on M2 macrophage function would alter smooth muscle contractility and contribute to delayed clearance of adult *N. brasiliensis*. Other studies, however, have shown expulsion of the gastrointestinal nematode parasite *H. polygyrus* during a secondary memory response was delayed in Se-D mice despite increased smooth muscle contractility (25). This suggest that Se-dependent activity of M2 macrophages on smooth muscle function during a memory response to *H. polygyrus* may be less critical to a multi-faceted protective immune response against this parasite. Recent evidence has demonstrated a link between dietary Se and Relmβ/Fizz2 production in the intestine (Smith *et al*., in review), during the memory response to *H. polygyrus* that could explain the reduced clearance of adult worms in Se-D mice (25, 60). To support these results, immunofluorescent staining for M2 and general macrophages in the small intestine should be done. Moreover, direct measurement of contractility in different Se diets would help to strengthen our findings and elucidate the exact mechanisms. Furthermore, establishing if other cells express F4/80 may help clarify if macrophages come into the small intestine, and then differentiation towards an M2 phenotype, or if it is specifically M2 macrophages that infiltrate. Ascertaining the number of macrophages vs. M2 type macrophages is important in determining how Se is affecting the presence of these cells. It is of interest to note that in comparison to a normal chow diet, our custom-prepared Se diets revealed no differences in adult

worms or fecal egg counts, suggesting that components in the custom diet do not play a role in the altered adult worm and fecal egg expulsion.

Our previously published data demonstrated the importance of selenoproteins in shunting macrophages activation towards the M2 phenotype, utilizing GPx1 KO mice (31). Here, we have utilized macrophage specific deficiencies in Trsp (*Trsp*$^{fl/fl}$ *Cre*^{LysM}), the Sec tRNA gene responsible for selenoprotein production (70). Compared to WT (*Trsp*$^{fl/fl}$ *Cre*^{WT}) mice, *Trsp*$^{fl/fl}$ *Cre*^{LysM} mice displayed a significant delay in adult worm clearance and fecal egg shedding. Moreover, expression of M2 genes was completely abrogated in *Trsp*$^{fl/fl}$ *Cre*^{LysM} mice. This data demonstrates the importance of selenoproteins to M2 macrophages, and the part these cells play in clearance of *N. brasiliensis*. Intriguingly, the *N. brasiliensis* genome has not been sequenced and expression of selenoproteins by the parasite is unknown. Once this is established, putative selenoproteins can be identified to determine if Se has the ability to directly affect the parasite, decreasing its infectivity. Additionally, we need to demonstrate if there is a decreased presence of M2 macrophages in the small intestine of *Trsp*$^{fl/fl}$ *Cre*^{LysM} mice. This will help to further demonstrate the importance of selenoproteins in clearing *N. brasiliensis* from gut.

While STAT6 has been studied in detail (154), the nuclear receptor PPARγ has also been shown to play a role (63). Our previous data has shown Se supplemented macrophages to shunt the AA-COX pathway towards the anti-inflammatory production of endogenous PPARγ agonist, 15d-PGJ_2 (31, 56, 67). Given that inhibition of the AA-COX pathway through use of indomethacin, a non-specific COX inhibitor, significantly enhanced fecal egg shedding and delayed adult worm clearance in Se-A and Se-S mice, these results support a Se-dependent mechanism of PPARγ-dependent clearance of *N. brasiliensis*. Moreover, Se-D mice given exogenous 15d-PGJ_2 decreased parasite fecal egg shedding, confirming that enhanced production of endogenous ligands could affect M2 macrophage expression. Overall, this data demonstrates the need for further investigations into the mechanistic

implications of daily NSAID intake to gain a clear picture of the relationship between diet and nutrition in helminthic parasite infected individuals.

Past studies have shown clearance of *N. brasiliensis* is Th2-dependent and specifically sensitive to the effects of IL-13. Examining jejunal tissue, our results demonstrated a Se-dependent increase in the expression of IL-13. However, the number of Th2 cells in the small intestine does not change in Se-S mice when compared to Se-D mice. An alternative explanation is that Se induces an increased presence of eosinophils into the small intestine that provide increased production of IL-13. Leukocytes from the lamina propria were isolated and stained for eosinophil cells surface receptors, Siglec F and CCR3. Our results conclusively showed that Se does not enhance the presence of eosinophils into the small intestine. However, an increase in the number of animals used (current n=3) may shift the pattern to reflect a Se effect. This data raises the possibility that Se concentration could enhance production of IL-13 by one or more cell types. Further examination, utilizing techniques such as ELISPOT, may help establish the role of Se in such a process. Interestingly, IL-13 expression decreased significantly in the presence of indomethacin (data not shown), demonstrating a need to examine the relationship between the AA-COX pathway and Th2 cytokine expression. Furthermore, we need to examine the effects of Th2 cells in our $Trsp^{fl/fl}$ Cre^{LysM} mice, establishing the role selenoproteins also play Th2 presence and cytokine expression.

In conclusion, our results clearly demonstrate that dietary Se decreases parasite egg production and increases clearance of adult *N. brasiliensis*. This is achieved through an increased presence of M2 macrophages in the small intestine. Further work is required to establish the exact mechanisms of clearance. Studies examining the role NSAID use plays in triggering macrophage polarization will be needed to elucidate the role of exogenous factors (therapeutic drugs), leading to a greater understanding of the relationship between nutrition and infections.

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Chapter 4

Summary, Implications, and Future Directions

Compared to other nutrients, such as vitamin E, iron, and calcium, our understanding of Se is limited. Numerous reasons have contributed to this, including the ability of Se to be incorporated into over two-dozen proteins, in the form of the 21st amino acid Sec, with unknown functions. Attributes such as this, and other biological activities, make Se a very intriguing nutrient to study.

The expression/biosynthesis of selenoproteins is a very unique mechanism. Able to be formed from both inorganic and organic forms of Se, the identities and functions of all selenoproteins are inadequately understood. Thus, in-depth studies to delineate the physiological significance of each selenoprotein, their various enzymatic activities, and their spatio-temporal expression patterns are essential to fully understand the role Se plays in inflammation, cancer, and other diseases. Although cellular processes of many selenoproteins are known, it is important to establish other processes they may be involved in. For instance, a current health benefit of Se and selenoproteins is known in the context of antioxidant control of oxidative stress, where decreases in either can lead to alterations in redox signaling, increasing the risk of disease or cancer development. Therefore, maintaining optimum levels of Se within the body is critical to achieving cellular homeostasis and optimizing health. Furthermore, genetic variations in selenoprotein genes may change the function of Se. Studies have shown single nucleotide polymorphisms to affect the efficacy of Se. To explore the role these genetic variations may have on human health, anti-oxidant activities, and disease progression, genetic studies must be conducted. Furthermore, analyzing the effects of these polymorphisms on macrophage phenotype expression may also help shed light on the complex nature of these immune cells.

Macrophages are heterogeneous population of cells that reflect their function, plasticity, and versatility. Based on the current literature, macrophage heterogeneity also describes their polarization subsets- classical M1 versus alternative M2. A major component of the infiltrate of inflammation and tumors, macrophages show distinct gene expression signatures that correlate with functional properties that constitute various subtypes in the continuum of M1 and M2 phenotypes. For instance, in

response to bacteria or foreign insult, the body induces a classical immune response, producing pro-inflammatory cytokines from infiltrating leukocytes to induce microbial killing. Intriguingly, such a pro-inflammatory response mechanism has also been affiliated with damage to the tissues that eventually can set the tone for tumor initiation in certain cancers. In contrast to M1 macrophages, M2 macrophages are characterized as predominantly anti-inflammatory, playing a role in resolution and tissue proliferation. Recent studies have identified M2 macrophages as a staple in Th2-type responses, infiltrating different tissues in response to different infections, such as helminthic parasites. Recent investigations have identified novel subsets of M2 macrophages (M2a, M2b, and M2c). Each subset has been identified by their mechanism of induction, but further studies are required to fully elucidate the function of each under different situations. It is important to understand if, like M1 to M2 phenotype switching, M2 subsets could be induced to switch subsets, and the impact of such a transition in disease.

Previous studies have demonstrated the ability of Se, in the form of selenoproteins, to abolish the expression of pro-inflammatory genes, such as iNOS (31). A prototypical M1 marker, iNOS induces the development of NO, a common ROS, from L-Arg. This pathway is inhibited by Se supplementation (31, 53). This discovery, several years ago, opened new avenues to further delineate the role of Se in mitigating inflammation. One question we asked was, did Se have the ability to shunt macrophage activation, from M1 towards the anti-inflammatory M2 phenotype? Within the M2 phenotype, L-Arg is utilized by Arg-I to produce proline and polyamines, important for cell proliferation, fibrosis, and wound healing. Studies described in Chapter 2 demonstrate that the expression of selenoproteins in macrophages aids in skewing the cells towards an M2 phenotype. Moreover, this skewing is enhanced in the presence of the Th2 cytokine IL-4, suggesting a synergistic relationship with Se. The identification of M2 macrophages was achieved through enhanced activity, protein expression, and increased transcript levels of *Arg-I*. Furthermore, increased mRNA levels of *Ym1*, and *Fizz1*, two well-known M2 markers, were seen. Most importantly, the significance of selenoproteins is

emphasized by the observation that expression of Arg-I significantly decreases when these proteins are not expressed, as seen in BMDMs lacking GPx1, one of the most abundant selenoprotein found in the system. One of the main protective effects of GPx1 is to reduce H_2O_2 and alleviate oxidative stress. Therefore, it is not surprising to see that in its absence, the macrophage phenotype still retains its pro-inflammatory characteristics. The significance of selenoproteins is also seen in the form that is utilized. Most of the experiments in Chapter 2 used an inorganic form of Se, sodium selenite. To explore the effects of different forms of Se on macrophage phenotype shunting, two organic forms of Se with prior history of chemoprevention were also used: SeMet and MSA. Surprisingly, results demonstrated MSA to increase *Arg-I* activity in a fashion similar to sodium selenite, while SeMet failed to induce activity. This serves as an example that the biochemical form of Se should be given consideration when planning experiments. Although many forms share the same metabolic fate of biosynthesis into selenoproteins, they do not exert the same effects.

A key aspect of Chapter 2 that needs to be further explored is the physiological relevance of adequate versus supplemented levels of Se. While many papers have explored the effects of Se at deficient, adequate, and supraphysiological levels, the studies presented in Chapter 2 were evaluated using only Se-D and Se-A levels. The reason we chose to explore deficient versus adequate levels is solely based on experimental data showing Se-S levels did not enhance expression of any of the markers we examined. This could simply suggest that in our ex-vivo model, higher levels of Se were not necessary to show the effects of the micronutrient on macrophage phenotype switching. However, these results also point to a need to re-evaluate the levels of nutrients used in *in-vivo* experiments (as in Chapter 3), where multiple cell types are involved, and eventually in clinical studies.

Two transcription factors, PPARγ and STAT6, were examined to assess their effects on M2 activation. Results in Chapter 2 demonstrated that both transcription factors play a significant role in skewing macrophage phenotype and shunting of L-Arg metabolism. The significance of STAT6 was further concluded utilizing a ChIP

assay, investigating if Se enhances STAT6 binding to the *Arg-I* promoter, increasing transcription. Although results showed Se did not enhance binding, further examination is necessary to determine how Se, and the cellular oxidant tone, positively regulates the transcription of *Arg-I* and other M2 genes. There is a possibility of a downstream effect, where Se may enhance the binding of other transcription factors needed for *Arg-I* transcription (see Chapter 1). There may also be STAT6-dependent transcription factors that have yet to be discovered. It is important to elucidate exactly what Se is doing at the promoter level, where discoveries may help in the development of targeted therapeutics. In the presence of Se, inhibitors for both PPARγ and H-PGDS have been shown to decrease Arg-I activity, demonstrating PPARγ and the AA-COX pathway to play a role in macrophage phenotype shunting. Specific PPAR response elements (PPRE) have been identified on the *Arg-I* promoter, suggesting PPARγ responds to Se with an increase in transcription. One possible mechanism of this is through the endogenous production of anti-inflammatory prostaglandin metabolites, $\mathbf{\Delta}^{12}$-PGJ_2 and 15d-PGJ_2. Known to modulate pro-inflammatory transcription factors, such as NFκB, 15d-PGJ_2 is an endogenous ligand for PPARγ, whose expression is significantly increased in the presence of Se. As discussed in Chapter 2, binding of 15d-PGJ_2 to PPARγ increases its activation, and subsequent binding onto the *Arg-I* promoter.

The discoveries in Chapter 2 have shed light on Se and its effects, enriching the field of nutritional affects on immune cell functions and phenotypes. Our focus on macrophage cell activation has broadened the field, helping to initiate new projects that focus on the molecular biology behind nutrients. However, it is necessary to translate these effects into an animal model.

Although macrophages can differentiate into an M1 or M2 phenotype in different diseases, many species of helminths are established in inducing activation, specifically along the M2 pathway. Helminthic parasites are classified into three classes: nematodes, cestodes, and trematodes. A gastrointestinal roundworm, that is a part of the nematode class, *Nippostrongylus brasiliensis* (*N. brasiliensis)* has been

extensively studied in mice, and has been demonstrated to rely on M2 macrophages for clearance. In addition, few studies have focused on the effects of deficiencies in Se and vitamin E. Such studies have indicated *N. brasiliensis* to rely on M2 macrophages for small intestine tissue hypercontractility, allowing for rapid expulsion of the adult worms from the small intestine. The transcription factor STAT6 has also been identified as a major player in parasite clearance (9, 133). Absence of STAT6 in mice abrogated clearance and delayed fecal egg shedding (9). Furthermore, studies examining the effects of clearance in Se and vitamin E deficient models found adult worm clearance to be delayed, even though intestinal motility was still effective (199). While these studies have provided great advances in helminthic parasite clearance mechanisms, it has also left many questions unanswered in our model. One particular question was the relationship between Se and M2 macrophages, and how it affects parasite clearance.

Studies in Chapter 3 closely examined the affects of Se on M2 macrophages in a *N. brasiliensis* model. In Se-A and Se-S mice, adult worm expulsion and fecal egg shedding was augmented in comparison to Se-D mice. Moreover, expression of M2 markers was significantly increased in the days immediately following worm migration to the small intestine. As discussed, previous studies have shown intestinal motility to remain active in Se deficient conditions. Our data suggests there may be enhanced motility as a result of adequate and supplemented Se levels increasing M2 macrophage infiltration. While this is a novel finding, further studies exploring the contractility of the small intestine tissue in the presence of adequate, supplemented, and deficient Se levels need to be examined to be able to elucidate how contractility of the small intestine remains intact in Se-D mice, when M2 macrophage infiltration decreases. It could be possible that contractility is not reliant upon M2 macrophage infiltration alone, but also the interaction of Se with proteins associated with intestinal smooth muscle movement, such as α-smooth muscle actin. Answering questions such as these may strengthen our hypothesis. Mucous secretion also plays a role in adult worm clearance. Future studies examining the expression of mucous related markers,

such as mucin, are needed to determine if Se is increasing mucous production, and therefore increasing parasite clearance.

While contractility and mucous production are critical, forming a clear picture of what happens to the worms during clearance is equally important. As described in Chapter 1, studies have shown complete *N. brasiliensis* clearance to occur 14 days after infection, through self-clearing expulsion. However, it is necessary to study the underlying basis of the self-clearing mechanism. Fecal examination has shown very limited presence of adult worms. The average size of an adult male worm is 4.5mm in length, while a female worm is 6mm in length (71), therefore a fully matured macrophage is unlikely to phagocytize and destroy them. Future studies are needed to explore how exactly adult worms are exiting the intestine in Se-D, Se-A, and Se-S mice. How are Se and M2 macrophages working together to enhance clearance if adult worms are not seen in the feces? One interesting study would be to examine the migration of worms from the lungs to the small intestine. After skin penetration, *N. brasiliensis* larvae travel to the lungs where it matures before traveling to the small intestine. To paint a complete picture of the effects Se has on parasite clearance, it is important to understand if Se has any effect on worm migration.

The presence of fecal eggs is an expected result of gastrointestinal parasite infections. Studies in Chapter 3 have demonstrated delayed egg expulsion under Se deficient conditions. Moreover, in the absence of selenoproteins, egg expulsion is further delayed, signifying how important selenoproteins, and not just Se species, are to helminth clearance. Interestingly, however, very few selenoproteins have been identified in helminthic parasites. In parasitic species that have been shown to express selenoproteins, GPx and TrxR are the best characterized, protecting the helminth from oxidative stress imposed-death by the host. Given what is known about oxidative stress protection by selenoproteins (see Chapter 1), future studies must identify which selenoproteins (in particular the gastrointestinal GPx2) gastrointestinal parasites express. Such identification has the potential to create therapeutic targets. Moreover, if selenoproteins are identified in these parasites, analysis of their

enhanced expression through Se supplementation will be very beneficial given that increasing Se levels could tip the redox balance to negatively impact the worm. Along these lines, future studies should determine if Se-A or Se-S diets change the infectivity of the larvae that hatch from fecal eggs. These results may establish if Se can affect transmission, thus helping reduce infection "outbreaks". Additionally, it is important to examine the effects of Se on the growth of the larvae while they are in the soil. To establish this "growth curve", a future study could culture larvae in soil containing adequate, supplemented, and excessive levels of Se. Infected mice can then be examined to determine if the larvae are able to mature from the L3 to L4, and L4 to L5 stages. This data could help establish a way to decrease infectivity from within the source of the larvae.

Current epidemiologic studies of human gastrointestinal parasites have acknowledged that one of the ways to rid the body of a parasite infection is through the use of anti-helminthic drugs. With over 3.5 billion people worldwide infected with parasites, the development and delivery of drugs can be costly and time consuming. Studies such as ours may have major epidemiological implications, providing a possible alternative. To realize this, future studies need to examine the effects of Se on clearance of human hookworms, *Necator americanus* and *Ancylostoma duedenale.* Because human hookworms have a very different infection cycle, it is imperative to fully understand the ramifications of Se on migration, clearance, and the immune response. Positive results from these studies could be very beneficial, instigating the use of micronutrients in countries that cannot afford the drugs that are available. In addition to costs, prolonged use of anti-helminthic drugs can lead to parasite resistance. In a country that struggles to pay for the medicine it currently uses, a pandemic caused by a resistant parasite is very dangerous. Some studies have suggested combining anti-helminthic therapy with nutritional supplementation, providing a superior therapeutic protocol. While this may be a feasible option, a considerable number of studies must be done *a priori* to determine the mechanisms of how it could work.

Another interesting epidemiologic study to consider is the use of helminths to combat human inflammatory bowel disease (IBD). In two studies done by Dr. Joel Weinstock and his group (169), patients suffering from IBD were given 2500 larvae of *Trichuris sui*s (*T. suis*), a pig whipworm, once every two weeks for a total of 24 weeks. Results demonstrated a 50% rate of improvement, compared to 15% in the placebo group. The positive effect against IBD has been linked to a modulated balance between Th1 and Th2 cytokines. IBD initiates a Th1 type response, increasing pro-inflammatory cytokines. A shift to a biased Th2 response, through the parasites, significantly decreases inflammation. Furthermore, parasite infection increases intestinal mucous production, and thus provides a barrier against inflammation and damage. Currently, there are studies testing efficacy of human hookworms.

While these results seem novel, there are many questions that still remain to be answered. For instance, what is the reaction of these parasites to exogenous nutritional therapy? No studies have been conducted to establish the reaction of these parasites to Se supplementation. Without this knowledge, how would the patients be targeted to use this form of therapy? For example, if *T. suis* succumbed to Se, as in the case with murine gastrointestinal parasites, decreasing infection and enhancing clearance, their use as an IBD therapeutic would be greatly repressed. An additional issue that stems out of this research is the role of COX pathway metabolites as critical mediators in resolution. While our studies are in agreement with the pro-resolving role of cyclopentenone PGs as being important in Se's effect, it would be interesting to see if increased usage of NSAIDs has any impact on worm infestation and clearance. Chapter 3 presents data that suggests inhibition of the AA-COX pathway, through the use of the COX inhibitor indomethacin, hinders Se ability to clear the helminths. This is most likely a result of lost production of the anti-inflammatory prostaglandin 15d-PGJ_2, which when given exogenously, was shown to rescue fecal egg shedding. In an era where many consume NSAIDs on a daily basis, and adequate levels of Se are found in many of the foods we eat, these results make a compelling

case for studies to fully understand the interplay of diet and nutrition with immune modulation of helminth pathogenesis.

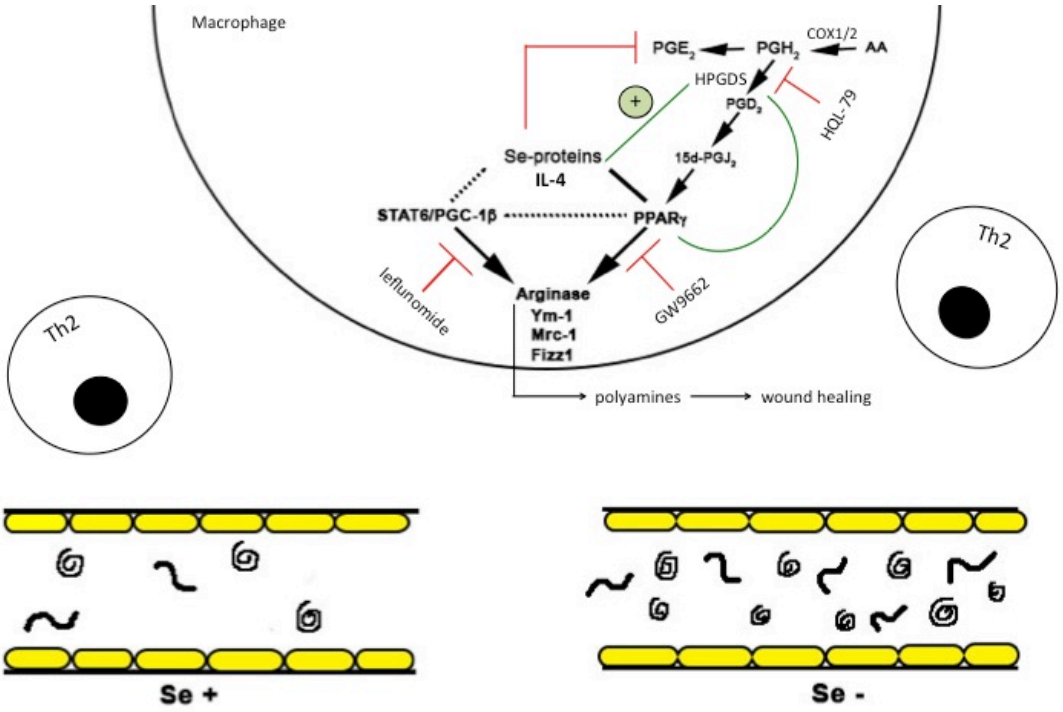

Figure 37: Schematic of overall project. Se presence shift macrophage activation towards an M2 phenotype. Specifically, Se in the form of selenoproteins increases the production of anti-inflammatory prostaglandins, while inhibiting pro-inflammatory prostaglandins. M2 macrophage makers, including Arginase, increase expression in a PPARγ and STAT6 dependent pathways. Moreover, selenoproteins work synergistically with the Th2 produced cytokine, IL-4, to increase expression of the M2 markers. In a *Nippostrongylus brasiliensis* infection model, mice on variable Se diets show a decreased presence of adult worms in the small intestines when compared to mice on Se deficient diets, due to an increased presence of M2 macrophages.

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A. Education

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Penn State University	Bachelors	2001-2006	Microbiology
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B. Positions and Honors.

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C. Publications

- **Nelson, SM**, Lei X, Prabhu KS (2011). Selenium Levels affect the IL-4 Induced Expression of Alternative Activation Markers in Murine Macrophages. *J Nutr,* 141(9):1754-61 July 2011

- Gandhi UH, Kaushal N, Ravindra KC, Hegde S, **Nelson SM**, Narayan V, Vunta H, Paulson RF, Prabhu KS (2011). Selenoprotein-dependent Up-regulation of Hematopoietic Prostaglandin D2 Synthase in Macrophages is Mediated through the Activation of Peroxisome Proliferator-activated Receptor (PPAR) gamma. *J Biol Chem*. 2011 Aug 5; 286 (31): 27471-82

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D. Book Chapters

- Kaushal N, Gandhi UH, **Nelson SM**, Narayan V and Prabhu KS. Selenium and Inflammation. *In Selenium: Its Molecular Biology and Role in Human Health* (2012) 3rd Edition. Springer Publications. Chapter 35, pp 443-456.

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